RESEARCH ARTICLE

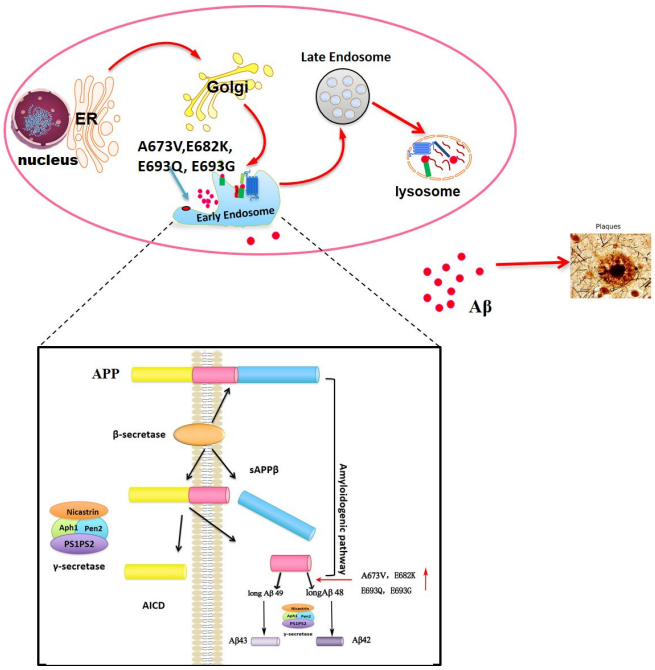
Mutations of beta-amyloid precursor protein alter the consequence of Alzheimer's disease pathogenesis

Nuo-Min Li[1,#], Ke-Fu Liu[1,#], Yun-Jie Qiu[1], Huan-Huan Zhang[1], Hiroshi Nakanishi[2], Hong Qing[1,*]
1 School of Life Science, Beijing Institute of Technology, Beijing, China
2 Department of Aging Science and Pharmacology, Faculty of Dental Science, Kyushu University, Fukuoka, Japan

Funding: *This study was funded by the National Natural Science Foundation of China, No. 81671268 (to HQ); and partially supported by a grant from the Ministry of Science and Technology of China, No. 2013YQ03059514 (to HQ); and a grant from Key Laboratory for Neurodegenerative Disease of Ministry of Education of China, No. 2015SJBX05 (to HQ), 2015SJZS01 (to HQ).*

Graphical Abstract

Amyloid precursor protein (APP) mutations effect on long amyloid beta (Aβ) cleavage pathway and location on cellular parts



***Correspondence to:**
Hong Qing, MD,
hqing@bit.edu.cn.

#These authors contributed equally to this study.

orcid:
0000-0003-0216-4044
(Hong Qing)

doi: *10.4103/1673-5374.247469*

Received: *March 27, 2018*
Accepted: *July 21, 2018*

Abstract

Alzheimer's disease is pathologically defined by accumulation of extracellular amyloid-β (Aβ). Approximately 25 mutations in β-amyloid precursor protein (APP) are pathogenic and cause autosomal dominant Alzheimer's disease. To date, the mechanism underlying the effect of APP mutation on Aβ generation is unclear. Therefore, investigating the mechanism of APP mutation on Alzheimer's disease may help understanding of disease pathogenesis. Thus, APP mutations (A673T, A673V, E682K, E693G, and E693Q) were transiently co-transfected into human embryonic kidney cells. Western blot assay was used to detect expression levels of APP, beta-secretase 1, and presenilin 1 in cells. Enzyme-linked immunosorbent assay was performed to determine $A\beta_{1-40}$ and $A\beta_{1-42}$ levels. Liquid chromatography-tandem mass chromatography was used to examine VVIAT, FLF, ITL, VIV, IAT, VIT, TVI, and VVIA peptide levels. Immunofluorescence staining was performed to measure APP and early endosome antigen 1 immunoreactivity. Our results show that the protective A673T mutation decreases $A\beta_{42}/A\beta_{40}$ rate by downregulating IAT and upregulating VVIA levels. Pathogenic A673V, E682K, and E693Q mutations promote $A\beta_{42}/A\beta_{40}$ rate by increasing levels of CTF99, $A\beta_{42}$, $A\beta_{40}$, and IAT, and decreasing VVIA levels. Pathogenic E693G mutation shows no significant change in $A\beta_{42}/A\beta_{40}$ ratio because of inhibition of γ-secretase activity. APP mutations can change location from the cell surface to early endosomes. Our findings confirm that certain APP mutations accelerate Aβ generation by affecting the long Aβ cleavage pathway and increasing $A\beta_{42/40}$ rate, thereby resulting in Alzheimer's disease.

Key Words: *nerve regeneration; Alzheimer's disease; β-amyloid precursor protein; amyloid β; APP mutations; liquid chromatography-tandem mass chromatography; cellular localization; long Aβ; neural regeneration*

Chinese Library Classification No. *R459.1; R363; R749*

Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders, and pathologically defined by accumulation of extracellular amyloid-beta (Aβ) peptide and aggregation of intracellular hyperphosphorylated tau protein in the hippocampus and cerebral cortex (Chu, 2012; Soldano and Hassan, 2014; Ossenkoppele et al., 2015). The β-amyloid precursor protein (APP) can be cleaved in two different pathways, amyloidogenic or non-amyloidogenic, and involves three different secretases: α-secretase, β-secretase, and γ-secretase (Roberts et al., 1994; Zhou et al., 2011; Lin and van der Wel, 2014). Under normal physiological conditions, APP can be cleaved by α- and γ-secretases (Epis et al., 2012; Postina, 2012; Wu et al., 2017). However, during amyloidogenic processing, APP is sequentially cleaved by β- and γ-secretases to mainly generate $Aβ_{38}$, $Aβ_{40}$, and $Aβ_{42}$ fragments (Okochi et al., 2013). At present, more than 30 coding mutations in the APP gene have been found. Approximately 25 of these coding mutations are pathogenic and most cause Alzheimer's disease (St George-Hyslop, 2000). APP mutations can either increase Aβ accumulation or augment the rate of $Aβ_{42}/Aβ_{40}$ formation (Bertram et al., 2010; Bertram and Tanzi, 2012). Altogether, these findings provide strong support for the amyloid hypothesis of AD. Some reports have shown that the β C-terminal fragment of APP (β-CTF) can be cleaved by γ-secretase at an ε-site into longer fragments, specifically, $Aβ_{49}$ and $Aβ_{48}$. In turn, these long fragments can be cleaved by stepwise cleavage of every three amino acid residues at the C terminus (Qi-Takahara et al., 2005). These findings support the hypothesis that Aβ is cleaved through two Aβ product lines: $Aβ_{49–46–43–40}$ and $Aβ_{48–45–42–38}$, associated generation of specific peptides ITL-VIV-IAT and VIT-TVI-VVIA (He et al., 2010; Fernandez et al., 2016). During this process, specific peptides are generated.

Jonsson et al., (2012) found that A673T can protect against AD and cognitive decline in mild cognitive impairment. Data have also demonstrated that an alanine-to-valine substitution at position 673 (A673V) of APP may be recessively inherited. Further, A673V can significantly increase the rate of $Aβ_{42}/Aβ_{40}$ formation (Giaccone et al., 2010; Messa et al., 2014). The E693Q mutation (Dutch) substitution is located at position 22 in Aβ. This mutation commonly occurs during intracerebral hemorrhage and may enhance Aβ protofibril formation compared with APP-wild-type (WT) (Kumar-Singh et al., 2002; Nicholson et al., 2012; Knight et al., 2015). The E693K mutation (Italian family) is also located at position 22 in Aβ and has similar clinical features as E693Q (Nilsberth et al., 2001a; Bugiani et al., 2010). However, the mechanism of APP mutation on Aβ generation is still unclear. Therefore, investigating the mechanism of APP mutation in AD may provide better understanding of disease pathogenesis.

Here, we have integrated multiple methods to investigate four different APP physiological mutations plus a protective mutation that affects Aβ production through APP processing. First, enzyme-linked immunosorbent assay (ELISA) and western blot assay were performed to detect CTFs of APP and its subsequent product, Aβ. We then quantitatively analyzed specific peptides produced by the long Aβ cleavage pathway using a multiple reaction monitoring model. Finally, we used immunofluorescence to determine APP distribution in cell lines. These studies may help improve understanding of the underlying mechanisms of APP mutation on onset of AD.

Materials and Methods

DNA construction

Wild-type human APP695 (APP-WT) and beta-secretase 1 (BACE1)-myc-his plasmids were obtained from the Weihong Song Lab (University of British Columbia, Vancouver, Canada). Mutants of APP (specifically, A673T, A673V, E682K, E693G, and E693Q) were amplified by overlapping extension polymerase chain reaction (PCR) from pcDNA4.1/APP695 with appropriate primers (**Table 1**). PCR fragments were then subcloned into pcDNA4.1 vector with *Hin*dIII and *Xba*I.

Table 1 Primers for overlapping extension PCR from pcDNA4.1/APP695

Primer	Sequence	Product size (bp)
A673T	Forward: 5′-GAA GTG AAG ATG GAT ACA GAA TTC CGA CAT GAC-3′	306
	Reverse: 5′-GTC ATG TCG GAA TTC TGT ATC CAT CTT CAC TTC-3′	1794
A673V	Forward: 5′-GAA GTG AAG ATG GAT GTA GAA TTC CGA CAT GAC-3′	306
	Reverse: 5′-GTC ATG TCG GAA TTC TAC ATC CAT CTT CAC TTC-3′	1794
E693Q	Forward: 5′-GGT GTT CTT TGC ACA AGA TGT GGG TTC AAA C-3′	516
	Reverse: 5′-GTT TGA ACC CAC ATC TTG TGC AAA GAA CAC C-3′	1584
E682K	Forward: 5′-CCG ACA TGA CTC AGG ATA CAA AGT TAC TCA TCA A-3′	279
	Reverse: 5′-TTG ATG ATG AAC TTT GTA TCC TGA GTC ATG TCG G-3′	1821
E693G	Forward: 5′-GGT GTT CTT TGC AGG AGA TGT GGG TTC AAA C-3′	516
	Reverse: 5′-GTT TGA ACC CAC ATC TCC TGC AAA GAA CAC C-3’	1584

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were obtained from the Weihong Song Lab, and maintained in Dulbecco's modified Eagle's media (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). Cells were transiently transfected with recombinant plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Western blot assay

HEK293 cells were co-transfected with APP-WT or mutant and BACE1 plasmids for 48 hours. Transfected cells were then collected and lysed in lysis buffer (Applygen, Beijing,

China) containing protease inhibitor cocktail (Roche, Basel, Switzerland) and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF; Sigma-Aldrich, Saint Louis, MO, USA), followed by sonication. Supernatants were collected after centrifugation at 14,000 × *g* for 10 minutes at 4°C. Equal amounts of protein were mixed with 4× loading buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 16% Tris-tricine (Li et al., 2006; Schagger, 2006) or 10% Tris-glycine gels. Proteins were separated and transferred to polyvinylidene fluoride membranes (0.22 μm; Millipore, Billerica, MA, USA). After blocking with 5% milk, membranes were incubated overnight at 4°C with rabbit polyclonal C20 antibody (anti-C-terminus of human APP, 1:10,000; gift form Professor Weihong Song), mouse monoclonal BACE1 antibody (1:1000; R&D, Minneapolis, MN, USA), or rat monoclonal presenilin 1 antibody (anti-presenilin 1 N-terminus, 1:500; Millipore). Membranes were incubated with appropriate secondary antibody (goat anti-mouse/anti-rat/anti-rabbit, 1:8000; ZSGB-BIO, Beijing, China) at room temperature for 2 hours. Specific blots were detected using an enhanced chemiluminescence system (Thermo Fisher Scientific, Boston, MA, USA) and quantified with Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

ELISA

HEK293 cells were co-transfected with APP-WT or mutant and BACE1 plasmids (4 μg). Protease inhibitors (Protease inhibitor Cocktail Tablets, Roche) and 1 mM AEBSF were added 44 hours after transfection. Four hours later, conditioned media were collected. Secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in culture media were measured using sandwich ELISA kits (Invitrogen) according to the manufacturer's instruction.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

HEK293 cells were cultured to a confluency of ~70%. They were then transiently co-transfected with APP-WT or mutant and BACE1 plasmids. Protease inhibitors and 1 mM AEBSF were added 44 hours after transfection. Four hours later, cells were collected and immediately boiled for 2 minutes. Boiled samples were then sonicated for 3 minutes and centrifuged at 4°C. Supernatants were vacuumized and dissolved with liquid phase before LC-MS/MS analysis (Okochi et al., 2013).

A triple quadrupole mass spectrometer (6460; Agilent Technologies, Santa Clara, CA, USA) equipped with Agilent Jet Stream Technology accompanied by ultra-performance LC (Agilent 1260) was used to identify and quantify APP cleavage peptides. Peptides were separated using C18 columns (300 Å, 5 μM particle size, 50 mm ID × 150 mm bed length) and quantified by the multiple reaction monitoring mode, as described previously (Takami et al., 2009). The parameters are listed in **Table 2**.

Table 2 Parameters of specific peptides and internal standard substances

Peptides	Precursor ions (m/z)	Product ions (m/z)
VVIAT	502.7	199.2
FLF	425.7	261.2
ITL	345.8	215.2
VIV	329.8	185.2
IAT	303.7	185
VIT	331.8	185.1
TVI	331.8	173.1
VVIA	401.2	171.1

Immunofluorescence staining

HEK293 cell lines were cultured overnight in confocal dishes and transfected with APP-WT or mutant (2 μg) for 48 hours, fixed for 15 minutes in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100 in PBS. Cells were then blocked with blocking solution (5% normal goat serum in PBS) for 1 hour and incubated overnight at 4°C with primary antibody in blocking solution: mouse anti-APP antibody (1:500; Millipore) or rabbit anti-early endosome antigen 1 (EEA1) (1:200; Abcam, Cambridge, UK). After washing with PBS, cells were incubated for 1 hour at room temperature with fluorescent secondary antibodies: Alexa Fluor 488-goat anti-mouse IgG or TRITC-goat anti-rabbit IgG (1:200; Abcam), and then washed with PBS. Hoechst 33258 was used to stain the nucleus. Images were obtained by laser scanning confocal microscope (TCS SP5; Leica, Heidelberg, Germany).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 software (Prism, La Jolla, CA, USA). Each experiment was performed with at least three independent sets of samples. Data are presented as the mean ± SD. Comparisons of multiple groups were performed using one-way analysis of variance.

Results

Effect of APP mutants on CTF generation

To examine the effect of APP mutants on CTF production, five different APP mutations were chosen (A673T, A673V, E682K, E693G, and E693Q) and transiently transfected with BACE1 plasmid into HEK293 cells. Equal levels of BACE1 expression were used to verify transfection efficiency by western blot assay (**Figure 1A** and **C**). Transfected cell accumulated presenilin 1 (**Figure 1A** and **E**), which is a component of the active part of γ-secretase. We also examined levels of APP and CTF production, especially CTF99. Expression of the five APP mutants led to a robust decrease of APP protein levels (**Figure 1B** and **D**), suggesting that these mutations accelerate proteolytic cleavage. Moreover, CTF99 levels in cells with mutant A673V, E682K, or E693G were markedly higher compared with APP-WT cells. Further, CTF83 levels showed an increasing trend in cells with mutant A673T or E693G. These findings demonstrate that the A673T mutation tends to be cleaved by α- rather than β-secretase to produce CTF83. Consequently, this establish-

es the A673T mutation and makes APP less favorable for cleavage by BACE1. To quantify these changes, we calculated CTF99/CTF83 ratio. We found that protective variant, A673T, showed distinctively lower C99/C83 levels compared with APP-WT. Interestingly, the other mutations showed a higher C99/C83 ratio, suggesting these mutations are pathogenic and increase β-cleavage of APP. The only exception was E693G, which showed no change in C99/C83 ratio.

APP mutations influence production of Aβ variants

Amyloid plaques are formed in AD brain by secreted Aβ peptides generated through sequential cleavage of APP (Sun et al., 2018a). Thus, in parallel to examination of accumulation of APP-CTFs in soluble lysates, we also examined secreted Aβ peptides by ELISA. Our results show that APP mutations change $A\beta_{42}/A\beta_{40}$ ratio by affecting Aβ variants. Three mutants (A673V, E682K, and E693Q) increased $A\beta_{42}$ levels in conditioned medium. Among them, A673V and E682K visibly increased $A\beta_{40}$ levels (**Figure 2**). Furthermore, mutant E693G and A673T resulted in reduced $A\beta_{42}$ and $A\beta_{40}$ levels relative to APP-WT. Altogether, $A\beta_{42}/A\beta_{40}$ ratio revealed that different APP mutations change Aβ variants in distinct ways. A673T caused a 3.2-fold decrease in $A\beta_{42}/A\beta_{40}$ ratio. A673V and E682K markedly increased $A\beta_{42}/A\beta_{40}$ ratio. E693G and E693Q showed opposing results despite substitution at the same site. Specifically, E693G decreased $A\beta_{42}$ and $A\beta_{40}$ levels (although $A\beta_{42}/A\beta_{40}$ ratio was not significantly altered). Meanwhile, $A\beta_{42}$ levels were markedly increased in cells with E693Q compared with E693G. Additionally, $A\beta_{40}$ levels were not changed with E693Q; therefore $A\beta_{42}/A\beta_{40}$ ratio was increased. This indicates that APP mutations might not only be involved in CTF production but also Aβ cleavage.

APP mutants differ in specific Aβ cleavage processes

From our above findings, we have shown a distinct impact of APP mutations on generated Aβ variants. This is a novel finding, and we felt it important to examine the impact of these substitutions on Aβ cleavage itself. Previous studies have shown two distinct lines of Aβ cleavage, in which one produces $A\beta_{49}$, $A\beta_{46}$, $A\beta_{43}$, and $A\beta_{40}$ peptides and the other produces $A\beta_{48}$, $A\beta_{45}$, $A\beta_{42}$, and $A\beta_{38}$ peptides (He et al., 2010). Based on this, we examined tripeptide and tetrapeptide production by stepwise cleavage of long $A\beta_{49}$ and $A\beta_{48}$. Our results show distinct levels of VVIA/42–38 peptides relative to total small peptides, suggesting that APP mutants affect selective γ-secretase cleavage of long Aβ. Accordingly, A673T and E693G promote relative VVIA production rate. In contrast, A673V, E682K, and E693Q decreased relative VVIA production rate compared with APP-WT (**Figure 3A**). Further, A673V and E682K increased relative rate of IAT peptides, which was reduced with A673T and showed no significant change with E693G and E693Q (**Figure 3B**). During $A\beta_{48-38}$ and $A\beta_{49-40}$ formation, our data showed that apart from the E693G mutant, different mutations did not affect sequential cleavage of $A\beta_{48}$ to $A\beta_{42}$ or $A\beta_{49}$ to $A\beta_{43}$ but

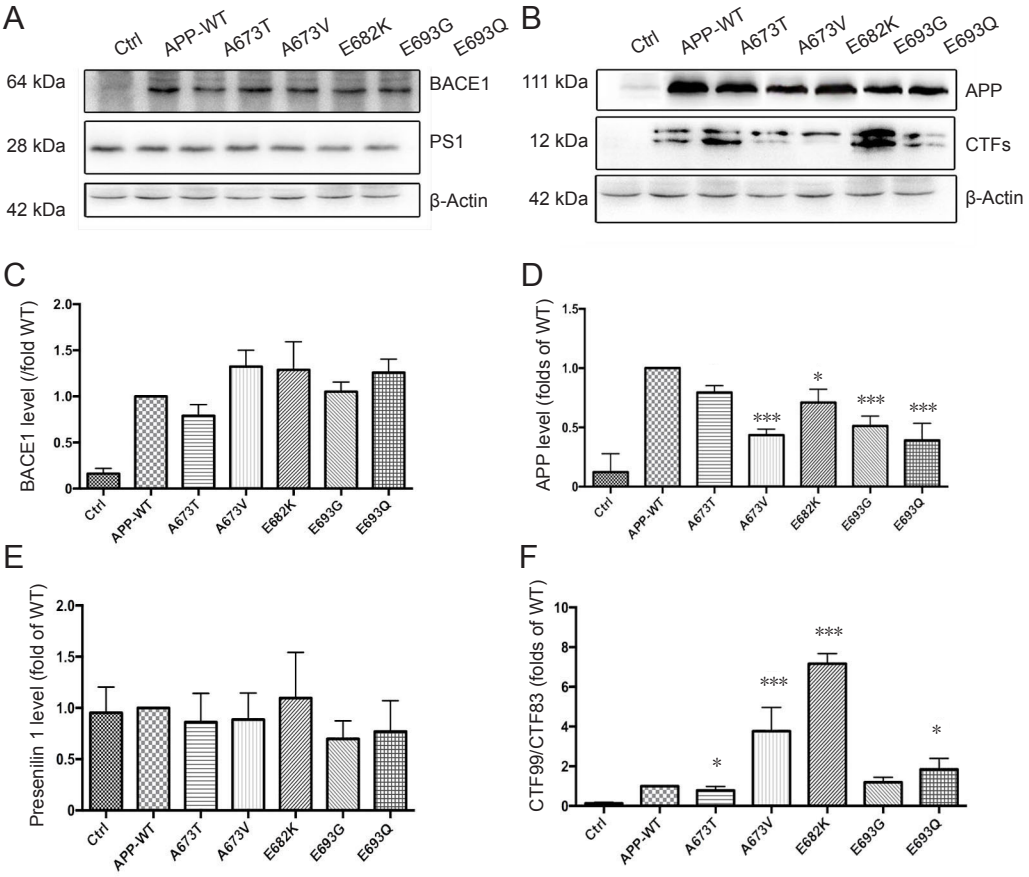


Figure 1 APP mutations affect the APP cleavage pathway.

Expression levels of APP, CTFs, BACE1, and presenilin1 (PS1) in HEK293 cells expressing vehicle, APP-WT, APP mutants A693T, A693V, E682K, E693Q, or E693G plasmids. (A) Western blot assay of cell lysates from HEK293 cell line transfected with APP mutations and BACE1. (B) APP and APP-CTFs in cell lysates. (C–F) Quantification results of BACE1 (C), APP (D), presenilin 1 (E), and CTF99/CTF83 (F). CTFs include CTF99 and CTF83. Data are expressed as the mean ± SD (one-way analysis of variance). $*P < 0.05$, $***P < 0.01$, *vs.* APP-WT group. APP: β-Amyloid precursor protein; WT: wild-type; HEK: human embryonic kidney; CTFs: C-terminal fragments; BACE1: beta-secretase 1.

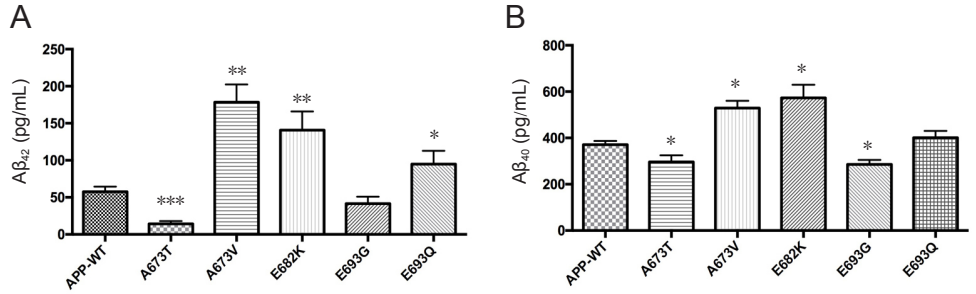


Figure 2 Effect of APP mutations on production of Aβ variants (enzyme-linked immunosorbent assay).

HEK293 cells were transfected with APP-WT, or APP mutants (A693T, A693V, E682K, E693Q, or E693G) plasmids. (A) Secreted $A\beta_{42}$ in conditioned medium was quantified by enzyme-linked immunosorbent assay. APP-A673T *vs.* APP-WT ($P = 0.0006$), APP-A673V *vs.* APP-WT ($P = 0.0011$), APP-E682K *vs.* APP-WT ($P = 0.005$), APP-E693G *vs.* APP-WT ($P = 0.07$), and APP-E693Q *vs.* APP-WT ($P = 0.028$). (B) Secreted $A\beta_{40}$ in conditioned medium was quantified by enzyme linked immunosorbent assay. APP-A673T *vs.* APP-WT ($P = 0.017$), APP-A673V *vs.* APP-WT ($P = 0.002$), APP-E682K *vs.* APP-WT ($P = 0.004$), APP-E693G *vs.* APP-WT ($P = 0.004$), and APP-E693Q *vs.* APP-WT ($P = 0.18$). Data are expressed as the mean ± SD (one-way analysis of variance). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, *vs.* APP-WT group. APP: β-Amyloid precursor protein; Aβ: amyloid-beta; WT: wild-type; HEK: human embryonic kidney.

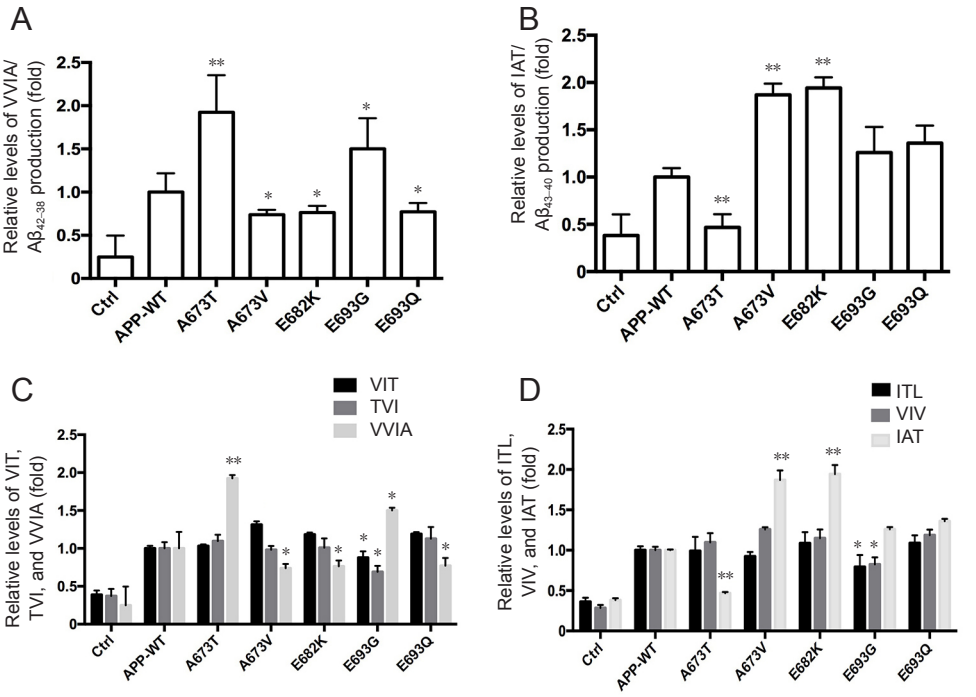


Figure 3 Effect of APP-WT and APP mutations on long Aβ cleavage by LC-MS/MS.
HEK293 cells were transfected with BACE1 and APP-WT or APP mutants (A673T, A673V, E682K, E693G, and E693Q). (A) Relative VVIA levels in cell lysates. (B) Relative IAT levels in cell lysates. (C) Relative VIT, TVI, and VVIA levels in cell lysates during $A\beta_{48-38}$ formation. Results are expressed as a ratio of APP-WT. (D) Relative ITL, VIV, and IAT levels in cell lysates during $A\beta_{49-40}$ formation. Results are expressed as a ratio of APP-WT. Data are expressed as mean ± SD, and analyzed by one-way analysis of variance. $*P < 0.05$, $**P < 0.01$, *vs.* APP-WT group. APP: β-Amyloid precursor protein; Aβ: amyloid-beta; WT: wild-type; LC-MS/MS: liquid chromatography-tandem mass spectrometer; HEK: human embryonic kidney; BACE1, beta-secretase 1.

mainly affected cleavage of $A\beta_{42}$ to $A\beta_{38}$ and $A\beta_{43}$ to $A\beta_{40}$ in distinct ways (**Figure 3C** and **D**). E693G markedly inhibited $A\beta_{48-38}$ and $A\beta_{49-40}$ cleavage pathways. LC-MS/MS results of A673T, A673V, E682K, and E693Q were consistent with our ELISA results. However, LC-MS/MS results were slightly different from our ELISA data for E693G. Under physiological conditions, APP can be cleaved by α-secretase and form CTF83, which can also be cleaved by γ-secretase at the same site as CTF99 and generate small specific peptides. Therefore, we speculated that CTF83 cleavage for this mutant is mostly attributable to the level of small peptide detection, which is reflected in our western blot assay results (**Figure 1B**).

APP mutations cause APP protein localization in different cellular regions and affect APP processing

Based on our data, we have shown that four pathogenic mutants and one protective variant (A673T) have practically distinct effects on APP cleavage and CTF production. Specifically, APP is processed through two lines to separately produce CTF83 by α-secretase and CTF99 by β-secretase. α-Secretase is particularly enriched at the cell surface and competes during APP processing with BACE1, which is rich in the trans-Golgi network and endosomes (Zhang and Song, 2013). Therefore, cellular location of APP protein is associated with cleavage processing and CTF specificity. As shown in **Figure 4**, the majority of APP-WT protein localized to cell membranes. In comparison, APP with A673T mutation was uniformly distributed in the cytoplasm and plasma membrane, in which APP tends to proceed along α-cleavage within the non-amyloidogenic pathway, consistent with our western blot and LC-MS/MS data. A673T reduced APP cleavage and CTF99 generation, as well as Aβ.

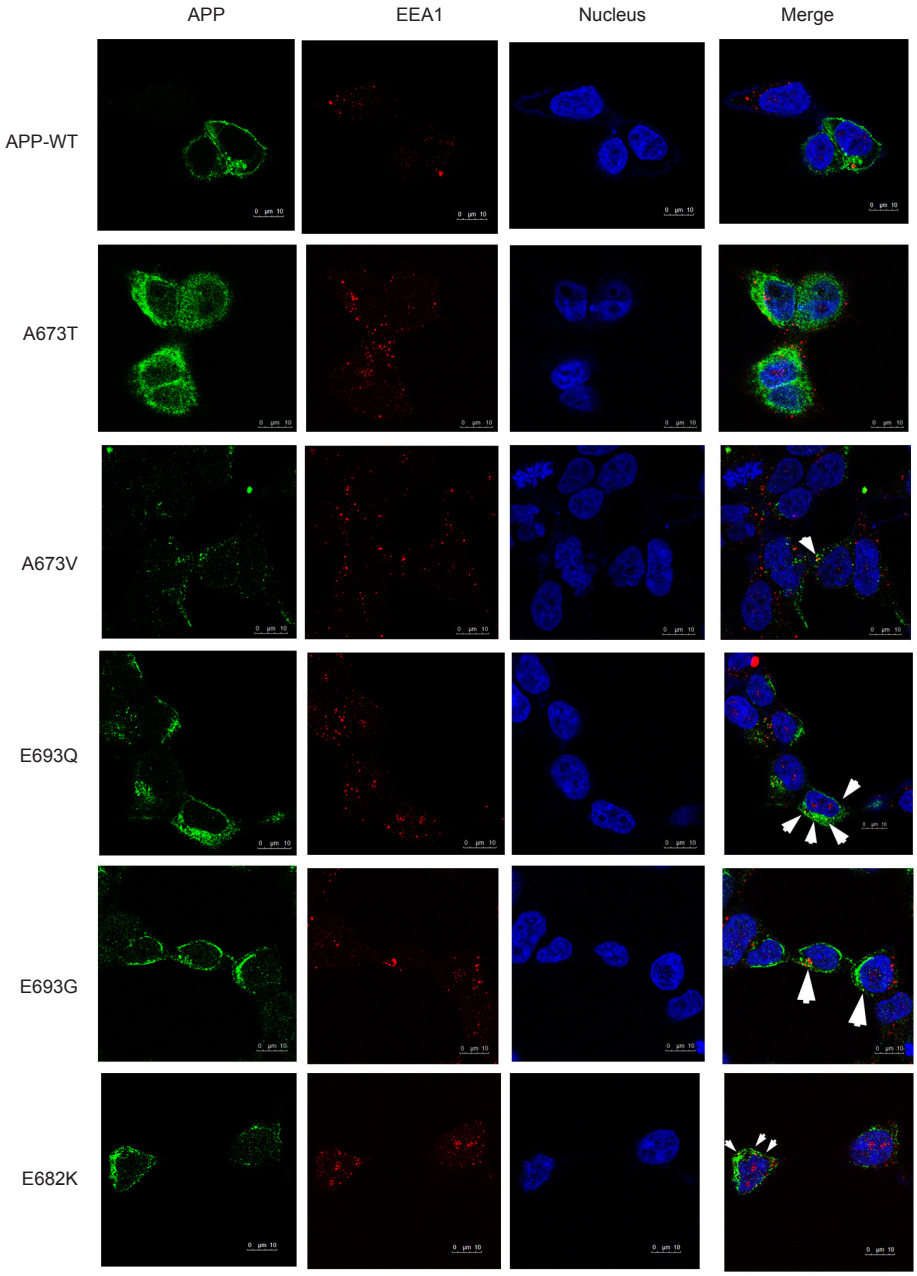


Figure 4 Expression of APP in the HEK293 cell line.
HEK293 cells transfected with BACE1 and APP-WT or APP mutants (A673T, A673V, E682K, E693G, and E693Q). Double immunofluorescence of APP/APP mutations and EEA1 in HEK293 cells. APP or APP mutants are shown in green (Alexa Fluor 488) (arrows) and EEA1 in red (TRITC). The nucleus was stained with Hoechst 33258. Scale bars: 10 μm. APP: β-Amyloid precursor protein; WT: wild-type; HEK: human embryonic kidney; BACE1: beta-secretase 1; EEA1: early endosome antigen 1.

E693G was mainly distributed on the cell membrane and only slightly localized with EEA1. EEA1 is a marker for early nucleosomes, which are important locations for APP cleavage (Sun et al., 2018b). This may explain why E693G has no significant influence on rate of $A\beta_{42}/A\beta_{40}$. E682K was not distributed in cell membranes or early endosomes, which suggests that E682K can promote migration of APP to early endosomes and affect generation of Aβ. As shown in **Figure 3**, we found that fluorescence intensity of E673V was lowest. Our data indicate that A673V can markedly promote the APP cleavage pathway, which is consistent with our previous western blot results. In general, APP mutations localized with a marker of early endosomes (EEA1), in which more APP was cleaved to produce Aβ prior to BACE1 cleavage.

Discussion

APP is a critical factor in AD. To date, more than 30 pathogenic mutations have been identified. Among them, the A673T mutation is the only one that protects against AD (Jonsson et al., 2012; Kokawa et al., 2015; Martiskainen et al., 2017; Lehtonen et al., 2018). Previous studies have shown that A673T can decrease β-processing and produce a less fibrogenic Aβ fragment (Benilova et al., 2014). In contrast, mutation of this residue to valine (A673V) is pathogenic (Di Fede et al., 2009; Giaccone et al., 2010). The APP Swedish mutation can shift APP cleavage, making this APP mutation a better BACE1 substrate and consequently promoting BACE1 cleavage and Aβ production (Citron et al., 1992; Rabe et al., 2011). Further, the Nilsberth group found

that the E693G mutation increases Aβ protofibril formation (Nilsberth et al., 2001b). However, its exact role in familial Alzheimer's disease is still unclear.

In our experiments, five APP mutations were chosen to determine their effect on the APP cleavage pathway. The five mutations were located in the Aβ area. A673T as a protective mutation showed a different physiological role than A673V, despite the presence of both mutations at the same site. Both mutants were adjacent to the β-secretase site. The mutation, E682K, was adjacent to the β-secretase site. The mutation site, E693G/E693Q, was close to the γ-secretase site. Based on western blot assay, ELISA, and LC-MS/MS data, we found the following. (1) The protective mutation, A673T, decreases CTF99 levels but increases CTF83 levels by inhibiting BACE1 activity. This shifts the cleavage site from a β-secretase to α-secretase site. A673T has no impact on selectivity of $Aβ_{48–38}$ and $Aβ_{49–40}$ by γ-secretase. A673T changed $Aβ_{42}/Aβ_{40}$ ratio by elevating cleavage of VVIA and reducing cleavage of IAT. (2) A673V showed opposing results to A673T, and it increased CTF99, $Aβ_{42}$, and $Aβ_{40}$ levels and $Aβ_{42}/Aβ_{40}$ ratio and caused higher $Aβ_{48}$ levels. LC–MS/MS data also showed that A673V changed $Aβ_{42}/Aβ_{40}$ ratio by reducing VVIA cleavage and increasing IAT cleavage. (3) E682K showed similar results as A673V but did not affect γ-secretase selectivity of $Aβ_{48–38}$ and $Aβ_{49–40}$. (4) Interestingly, E693G and E693Q did not show similar results, although they are substitutions at the same site. C99 and C83 levels were elevated and $Aβ_{42}$ and $Aβ_{40}$ markedly decreased by inhibition of the $Aβ_{48–42}$ and $Aβ_{49–40}$ cleavage pathways with E693G mutation. Taken together, these results indicate that E693G inhibits activity of γ-secretase. Additionally, E693G can also induce AD, because Aβ with E693G mutation forms a higher rate and larger quantity of protofibrils compared with Aβ from APP-WT (Nilsberth et al., 2001b; van der Kant and Goldstein, 2015). (5) With mutant E693Q, C99 and C83 levels declined. However, C99/C83 ratio and $Aβ_{42}/ Aβ_{40}$ ratio were slightly elevated.

It is widely believed that AD is caused by a shift in balance between two pathways (non-amyloidogenic and amyloidogenic), which leads to increased Aβ generation (Wang et al., 2012; Triaca and Calissano, 2016). Some researchers have reported that non-amyloidogenic processing occurs mostly at the cell surface, while amyloidogenic processing occurs within intracellular compartments (Fragkouli et al., 2011; Soldano and Hassan, 2014). Immunofluorescence data suggest that APP-WT is largely located at the cell surface. APP-WT with A673T mutation is located at both the cell surface and body, but we did not observe A673T localization with the early endosome marker, EEA1. With A673V, E682K, E693G, and E693Q mutations, we found APP largely intracellular, and more importantly, could localize with EEA1. This means that these APP mutations promote APP transport to early endosomes and interact with BACE1 to generate Aβ.

In future studies, we will use primary cultured neurons to investigate the effect of APP mutations on APP hydrolysis, which occurs in the Golgi apparatus, early endosome, late endosome, lysosomes, and proteasome. Further verification of our results will provide a basis for the pathogenesis of Alzheimer's disease.

To conclude, mutations in APP (except A673T) accelerate Aβ generation by affecting long Aβ cleavage, resulting in altered $Aβ_{42/40}$ in familial Alzheimer's disease and ultimately causing dementia. Among these methods, our results are consistent with each other. Our results are useful for in-depth understanding of the mechanism of APP mutations. Moreover, these methods can be used to investigate other mutations.

Author contributions: *Study conception and design: HQ and HN; experiment implement: NML, KFL, YJQ and HHZ; paper writing: NML; paper editing: HQ. All authors read and approved the manuscript.*
Conflicts of interest: *None declared.*
Financial support: *This study was funded by the National Natural Science Foundation of China, No. 81671268; and partially supported by a grant from the Ministry of Science and Technology of China, No. 2013YQ03059514; and a grant from Key Laboratory for Neurodegenerative Disease of Ministry of Education of China, No. 2015SJBX05, 2015SJZS01. The funding bodies played no role in the study design, collection, analysis and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication.*
Copyright license agreement: *The Copyright License Agreement has been signed by all authors before publication.*
Data sharing statement: *Datasets analyzed during the current study are available from the corresponding author on reasonable request.*
Plagiarism check: *Checked twice by iThenticate.*
Peer review: *Externally peer reviewed.*
Open access statement: *This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-Non-Commercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.*
Open peer reviewer: *Xiao-Li Min, Kunming Medical University, China.*
Additional file: *Open peer review report 1.*

References

Benilova I, Gallardo R, Ungureanu AA, Castillo Cano V, Snellinx A, Ramakers M, Bartic C, Rousseau F, Schymkowitz J, De Strooper B (2014) The Alzheimer disease protective mutation A2T modulates kinetic and thermodynamic properties of amyloid-beta (Abeta) aggregation. J Biol Chem 289:30977-30989.

Bertram L, Tanzi RE (2012) The genetics of Alzheimer's disease. Prog Mol Biol Transl Sci 107:79-100.

Bertram L, Lill CM, Tanzi RE (2010) The genetics of Alzheimer disease: back to the future. Neuron 68:270-281.

Bugiani O, Giaccone G, Rossi G, Mangieri M, Capobianco R, Morbin M, Mazzoleni G, Cupidi C, Marcon G, Giovagnoli A, Bizzi A, Di Fede G, Puoti G, Carella F, Salmaggi A, Romorini A, Patruno GM, Magoni M, Padovani A, Tagliavini F (2010) Hereditary cerebral hemorrhage with amyloidosis associated with the E693K mutation of APP. Arch Neurol 67:987-995.

Chu LW (2012) Alzheimer's disease: early diagnosis and treatment. Hong Kong Med J 18:228-237.

Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, Seubert P, Vigo-Pelfrey C, Lieberburg I, Selkoe DJ (1992) Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature 360:672-674.

Di Fede G, Catania M, Morbin M, Rossi G, Suardi S, Mazzoleni G, Merlin M, Giovagnoli AR, Prioni S, Erbetta A, Falcone C, Gobbi M, Colombo L, Bastone A, Beeg M, Manzoni C, Francescucci B, Spagnoli A, Cantu L, Del Favero E, et al. (2009) A recessive mutation in the APP gene with dominant-negative effect on amyloidogenesis. Science 323:1473-1477.

Epis R, Marcello E, Gardoni F, Di Luca M (2012) Alpha, beta-and gamma-secretases in Alzheimer's disease. Front Biosci (Schol Ed) 4:1126-1150.

Fernandez MA, Biette KM, Dolios G, Seth D, Wang R, Wolfe MS (2016) Transmembrane substrate determinants for gamma-secretase processing of APP CTFbeta. Biochemistry 55:5675-5688.

Fragkouli A, Tzinia AK, Charalampopoulos I, Gravanis A, Tsilibary EC (2011) Matrix metalloproteinase-9 participates in NGF-induced alpha-secretase cleavage of amyloid-beta protein precursor in PC12 cells. J Alzheimers Dis 24:705-719.

Giaccone G, Morbin M, Moda F, Botta M, Mazzoleni G, Uggetti A, Catania M, Moro ML, Redaelli V, Spagnoli A, Rossi RS, Salmona M, Di Fede G, Tagliavini F (2010) Neuropathology of the recessive A673V APP mutation: Alzheimer disease with distinctive features. Acta Neuropathol 120:803-812.

He G, Luo W, Li P, Remmers C, Netzer WJ, Hendrick J, Bettayeb K, Flajolet M, Gorelick F, Wennogle LP, Greengard P (2010) Gamma-secretase activating protein is a therapeutic target for Alzheimer's disease. Nature 467:95-98.

Jonsson T, Atwal JK, Steinberg S, Snaedal J, Jonsson PV, Bjornsson S, Stefansson H, Sulem P, Gudbjartsson D, Maloney J, Hoyte K, Gustafson A, Liu Y, Lu Y, Bhangale T, Graham RR, Huttenlocher J, Bjornsdottir G, Andreassen OA, Jonsson EG, et al. (2012) A mutation in APP protects against Alzheimer's disease and age-related cognitive decline. Nature 488:96-99.

Knight EM, Williams HN, Stevens AC, Kim SH, Kottwitz JC, Morant AD, Steele JW, Klein WL, Yanagisawa K, Boyd RE, Lockhart DJ, Sjoberg ER, Ehrlich ME, Wustman BA, Gandy S (2015) Evidence that small molecule enhancement of beta-hexosaminidase activity corrects the behavioral phenotype in Dutch APP(E693Q) mice through reduction of ganglioside-bound Abeta. Mol Psychiatry 20:109-117.

Kokawa A, Ishihara S, Fujiwara H, Nobuhara M, Iwata M, Ihara Y, Funamoto S (2015) The A673T mutation in the amyloid precursor protein reduces the production of beta-amyloid protein from its beta-carboxyl terminal fragment in cells. Acta Neuropathol Commun 3:66.

Kumar-Singh S, Julliams A, Nuydens R, Ceuterick C, Labeur C, Serneels S, Vennekens K, Van Osta P, Geerts H, De Strooper B, Van Broeckhoven C (2002) In vitro studies of Flemish, Dutch, and wild-type beta-amyloid provide evidence for two-staged neurotoxicity. Neurobiol Dis 11:330-340.

Lehtonen Š, Hoytylainen I, Voutilainen J, Sonninen TM, Kuusisto J, Laakso M, Hamalainen RH, Oksanen M, Koistinaho J (2018) Generation of a human induced pluripotent stem cell line from a patient with a rare A673T variant in amyloid precursor protein gene that reduces the risk for Alzheimer's disease. Stem Cell Res 30:96-99.

Li Y, Zhou W, Tong Y, He G, Song W (2006) Control of APP processing and Abeta generation level by BACE1 enzymatic activity and transcription. FASEB J 20:285-292.

Lin HK, van der Wel PC (2014) How amyloid precursor protein protects itself from cleavage. Structure 22:361-362.

Martiskainen H, Herukka SK, Stančáková A, Paananen J, Soininen H, Kuusisto J, Laakso M, Hiltunen M (2017) Decreased plasma beta-amyloid in the Alzheimer's disease APP A673T variant carriers. Ann Neurol 82:128-132.

Messa M, Colombo L, del Favero E, Cantu L, Stoilova T, Cagnotto A, Rossi A, Morbin M, Di Fede G, Tagliavini F, Salmona M (2014) The peculiar role of the A2V mutation in amyloid-beta (Abeta) 1-42 molecular assembly. J Biol Chem 289:24143-24152.

Nicholson AM, Wold LA, Walsh DM, Ferreira A (2012) β-Amyloid carrying the Dutch mutation has diverse effects on calpain-mediated toxicity in hippocampal neurons. Mol Med 18:178-185.

Nilsberth C, Westlind-Danielsson A, Eckman CB, Condron MM, Axelman K, Forsell C, Stenh C, Luthman J, Teplow DB, Younkin SG, Naslund J, Lannfelt L (2001a) The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced Abeta protofibril formation. Nat Neurosci 4:887-893.

Nilsberth C, Westlind-Danielsson A, Eckman CB, Condron MM, Axelman K, Forsell C, Stenh C, Luthman J, Teplow DB, Younkin SG, Näslund J, Lannfelt L (2001b) The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced Abeta protofibril formation. Nat Neurosci 4:887-893.

Okochi M, Tagami S, Yanagida K, Takami M, Kodama TS, Mori K, Nakayama T, Ihara Y, Takeda M (2013) gamma-secretase modulators and presenilin 1 mutants act differently on presenilin/gamma-secretase function to cleave Abeta42 and Abeta43. Cell Rep 3:42-51.

Ossenkoppele R, Pijnenburg YA, Perry DC, Cohn-Sheehy BI, Scheltens NM, Vogel JW, Kramer JH, van der Vlies AE, La Joie R, Rosen HJ, van der Flier WM, Grinberg LT, Rozemuller AJ, Huang EJ, van Berckel BN, Miller BL, Barkhof F, Jagust WJ, Scheltens P, Seeley WW, et al. (2015) The behavioural/dysexecutive variant of Alzheimer's disease: clinical, neuroimaging and pathological features. Brain 138:2732-2749.

Postina R (2012) Activation of alpha-secretase cleavage. J Neurochem 120 Suppl 1:46-54.

Qi-Takahara Y, Morishima-Kawashima M, Tanimura Y, Dolios G, Hirotani N, Horikoshi Y, Kametani F, Maeda M, Saido TC, Wang R, Ihara Y (2005) Longer forms of amyloid beta protein: implications for the mechanism of intramembrane cleavage by gamma-secretase. J Neurosci 25:436-445.

Rabe S, Reichwald J, Ammaturo D, de Strooper B, Saftig P, Neumann U, Staufenbiel M (2011) The Swedish APP mutation alters the effect of genetically reduced BACE1 expression on the APP processing. J Neurochem 119:231-239.

Roberts SB, Ripellino JA, Ingalls KM, Robakis NK, Felsenstein KM (1994) Non-amyloidogenic cleavage of the beta-amyloid precursor protein by an integral membrane metalloendopeptidase. J Biol Chem 269:3111-3116.

Schagger H (2006) Tricine-SDS-PAGE. Nat Protoc 1:16-22.

Soldano A, Hassan BA (2014) Beyond pathology: APP, brain development and Alzheimer's disease. Curr Opin Neurobiol 27:61-67.

St George-Hyslop PH (2000) Molecular genetics of Alzheimer's disease. Biol Psychiatry 47:183-199.

Sun BL, Li WW, Zhu C, Jin WS, Zeng F, Liu YH, Bu XL, Zhu J, Yao XQ, Wang YJ (2018a) Clinical research on Alzheimer's disease: progress and perspectives. Neurosci Bull doi: 10.1007/s12264-018-0249-z.

Sun Z, Xie Y, Chen Y, Yang Q, Quan Z, Dai R, Qing H (2018b) Rab21, a novel PS1 interactor, regulates γ-secretase activity via PS1 subcellular distribution. Mol Neurobiol 55:3841-3855.

Takami M, Nagashima Y, Sano Y, Ishihara S, Morishima-Kawashima M, Funamoto S, Ihara Y (2009) gamma-Secretase: successive tripeptide and tetrapeptide release from the transmembrane domain of beta-carboxyl terminal fragment. J Neurosci 29:13042-13052.

Triaca V, Calissano P (2016) Impairment of the nerve growth factor pathway driving amyloid accumulation in cholinergic neurons: the incipit of the Alzheimer's disease story? Neural Regen Res 11:1553-1556.

van der Kant R, Goldstein LS (2015) Cellular functions of the amyloid precursor protein from development to dementia. Dev Cell 32:502-515.

Wang Y, Tang XC, Zhang HY (2012) Huperzine A alleviates synaptic deficits and modulates amyloidogenic and nonamyloidogenic pathways in APPswe/PS1dE9 transgenic mice. J Neurosci Res 90:508-517.

Wu Q, Sun JX, Song XH, Wang J, Xiong CQ, Teng FX, Gao CX (2017) Blocking beta 2-adrenergic receptor inhibits dendrite ramification in a mouse model of Alzheimer's disease. Neural Regen Res 12:1499-1506.

Zhang X, Song W (2013) The role of APP and BACE1 trafficking in APP processing and amyloid-beta generation. Alzheimers Res Ther 5:46.

Zhou L, Brouwers N, Benilova I, Vandersteen A, Mercken M, Van Laere K, Van Damme P, Demedts D, Van Leuven F, Sleegers K, Broersen K, Van Broeckhoven C, Vandenberghe R, De Strooper B (2011) Amyloid precursor protein mutation E682K at the alternative β-secretase cleavage β'-site increases Aβ generation. EMBO Mol Med 3:291-302.

P-Reviewer: Min XL; C-Editor: Zhao M; S-Editors: Yu J, Li CH; L-Editors: James R, Maxwell R, Qiu Y, Song LP; T-Editor: Liu XL