Divalent cation tolerance protein binds to β-secretase and inhibits the processing of amyloid precursor protein

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Abstract
The deposition of amyloid-beta is a pathological hallmark of Alzheimer’s disease. Amyloid-beta is derived from amyloid precursor protein through sequential proteolytic cleavages by β-secretase (beta-site amyloid precursor protein-cleaving enzyme 1) and γ-secretase. To further elucidate the roles of beta-site amyloid precursor protein-cleaving enzyme 1 in the development of Alzheimer’s disease, a yeast two-hybrid system was used to screen a human embryonic brain cDNA library for proteins directly interacting with the intracellular domain of beta-site amyloid precursor protein-cleaving enzyme 1. A potential beta-site amyloid precursor protein-cleaving enzyme 1-interacting protein identified from the positive clones was divalent cation tolerance protein. Immunoprecipitation studies in the neuroblastoma cell line N2a showed that exogenous divalent cation tolerance protein interacts with endogenous beta-site amyloid precursor protein-cleaving enzyme 1. The overexpression of divalent cation tolerance protein did not affect beta-site amyloid precursor protein-cleaving enzyme 1 protein levels, but led to increased amyloid precursor protein levels in N2a/APP695 cells, with a concomitant reduction in the processing product amyloid precursor protein C-terminal fragment, indicating that divalent cation tolerance protein inhibits the processing of amyloid precursor protein. Our experimental findings suggest that divalent cation tolerance protein negatively regulates the function of beta-site amyloid precursor protein-cleaving enzyme 1. Thus, divalent cation tolerance protein could play a protective role in Alzheimer’s disease.

Key Words
neural regeneration; brain injury; neurodegenerative diseases; Alzheimer’s disease; amyloid-beta; β-secretase; amyloid precursor protein; beta-site amyloid precursor protein-cleaving enzyme 1; interaction; amyloid precursor protein C-terminal fragment; western blot; yeast two-hybridization; grants-supported paper; neuroregeneration

Research Highlights
(1) Beta-site amyloid precursor protein-cleaving enzyme 1 is a critical enzyme in the processing of amyloid beta precursor protein and participates in the production of amyloid beta during the development of Alzheimer’s disease. In this study, divalent cation tolerance protein was identified as a novel interaction partner for beta-site amyloid precursor protein-cleaving enzyme 1 using the yeast two-hybrid screening system.
(2) Divalent cation tolerance protein associates with endogenous beta-site amyloid precursor protein-cleaving enzyme 1 in the neuroblastoma cell line N2a. The overexpression of divalent cation tolerance protein did not affect the protein levels of beta-site amyloid precursor protein-cleaving enzyme 1; however, it led to increased levels of amyloid beta precursor protein with a concomitant...
INTRODUCTION

Alzheimer’s disease is a progressive neurodegenerative disorder that affects approximately 10% of the population aged 65 years and over, and up to 50% of those 85 years and over[1-2]. In both sporadic and familial forms of Alzheimer’s disease, one of the main pathological features is the presence of senile plaques comprising amyloid beta-peptide aggregates[3-5]. Amyloid beta-peptide is formed through the amyloidogenic pathway in which amyloid precursor protein is sequentially cleaved by β-secretase and γ-secretase[6-7]. The β-secretase cleaves amyloid precursor protein within the extracellular domain at residue Asp1 or Glu11, thereby generating the extracellular N-terminal soluble amyloid precursor protein-β and the membrane-bound C-terminal fragment. The latter is further processed by the γ-secretase to give rise to the amyloid precursor protein intracellular domain and amyloid beta-peptide (40, 42). Alternatively, amyloid precursor protein can be cleaved at an ectodomain site closer to the membrane amyloid beta-peptide aa 17 site (Aβ17) by α-secretase to generate soluble amyloid precursor protein alpha (SAPPα) and the transmembrane C-terminal fragment alpha (CTFα), which is further cleaved by γ-secretase to the p83 fragment, thereby preventing the formation of amyloid beta-peptide (40, 42)[6, 8].

The gene encoding β-secretase has been identified as beta-site amyloid precursor protein-cleaving enzyme 1[9-11]. It is a type I transmembrane aspartic protease, which is mostly localized in acidic subcellular compartments such as the trans-Golgi network and endosomes[12]. Beta-site amyloid precursor protein-cleaving enzyme 1 is highly expressed in neurons of the central nervous system. The critical role of beta-site amyloid precursor protein-cleaving enzyme 1 in amyloid pathology was demonstrated as the presence of alleviated amyloid beta-peptide production, amyloid plaque formation and neurological abnormalities when transgenic mice carrying human amyloid precursor protein mutations were mated to beta-site amyloid precursor protein-cleaving enzyme1/α mice[13-16]. In addition to amyloid precursor protein, multiple substrates have been identified for beta-site amyloid precursor protein-cleaving enzyme 1, all of which are membrane proteins[9]. For instance, beta-site amyloid precursor protein-cleaving enzyme 1 promotes sialytransferase secretion[17], which plays an important role in immune cell development. Moreover, P-selectin glycoprotein ligand 1, which is an important regulator of the inflammatory response[18], also interacts with beta-site amyloid precursor protein-cleaving enzyme 1[19]. Furthermore, the voltage-gated sodium channel β2 subunit[20-21] and the neuregulins NRG1 and NRG3[22] were identified as substrates for beta-site amyloid precursor protein-cleaving enzyme 1, and defects in neuronal activity and myelination are observed in beta-site amyloid precursor protein-cleaving enzyme1/α mice, which is a result of abnormal processing of these proteins. These studies indicate that beta-site amyloid precursor protein-cleaving enzyme 1 plays a critical role in numerous cellular processes, resulting from its ability to cleave a variety of different membrane proteins on the external surface of cells.

While the functions of the extracellular domain of beta-site amyloid precursor protein-cleaving enzyme 1 are increasingly well-known, that of the intracellular domain remains unclear. At present, only a few proteins that interact with this domain have been identified. The reticulons RTN3 and RTN4B were found to interact with beta-site amyloid precursor protein-cleaving enzyme 1 through its cytosolic domain, and were shown to increase the endoplasmic reticulum retention of beta-site amyloid precursor protein-cleaving enzyme 1 and decrease amyloid precursor protein cleavage and the production of amyloid beta-peptide[23-24]. Indeed, when transgenic mice overexpressing RTN3 are crossed with a mouse model of Alzheimer’s disease, the double-transgenic offspring exhibit reduced formation of amyloid plaques[25]. However, transgenic mice overexpressing RTN3 display RTN3 aggregates and impaired neurological functioning[25-26]. These studies highlight that proteins interacting with the intracellular domain of beta-site amyloid precursor protein-cleaving enzyme 1 may have important regulatory functions.
In this study, we screened a human fetal brain cDNA library using the yeast two-hybrid system, and identified divalent cation tolerance protein as a protein interacting with the intracellular domain of beta-site amyloid precursor protein-cleaving enzyme 1. This interaction was confirmed by co-immunoprecipitation experiments in the mammalian cell line N2a. When overexpressed, divalent cation tolerance protein reduced amyloid precursor protein processing, suggesting that it regulates beta-site amyloid precursor protein-cleaving enzyme 1-mediated processing of amyloid precursor protein.

RESULTS

Yeast two-hybrid screening for proteins interacting with the C-terminal domain of beta-site amyloid precursor protein-cleaving enzyme 1
To identify proteins interacting with the cytoplasmic domain of beta-site amyloid precursor protein-cleaving enzyme 1, a bait plasmid for the yeast two-hybrid system was constructed by inserting a fragment encoding the last 29 amino acids (473–501) of human beta-site amyloid precursor protein-cleaving enzyme 1 into the pGBK7-Gal4 BD-myc vector. The bait plasmid was transformed into yeast AH109, and the expression of the myctagged protein was assessed by western blot assay (Figure 1A). As expected, a 20 kDa protein was detected in yeast transformed with the vector alone (lane 1), and a 23 kDa fusion protein was detected in yeast transformed with pGBK7-Gal4 BD-myc-beta-site amyloid precursor protein-cleaving enzyme 1-C29 (lane 2), indicating that the bait protein was correctly expressed.

The bait plasmid was used to screen a human fetal brain library. After excluding redundant clones based on their size and restriction enzyme digestion patterns, 97 independent positive cDNA clones were obtained in the first round of screening. These clones were subjected to a second round of screening by growing the transformants on SD/Adr−/His−/Leu−/Trp−/X-Gal plates. Overall, 65 positive colonies were identified and sequenced. Clone B3 contained a cDNA fragment with 100% identity with nucleotides 621–1031 (encoding amino acids 1–137) of the longest transcript from the human divalent cation tolerance protein gene (NM_015921.2, encoding 156 amino acids).

To exclude the possibility of an auto-transactivation effect in the B3 clone, we quantified the beta-galactosidase activity of samples transformed with pACT2-B3 or control vector only and those co-transformed with the bait plasmid. The pGBK7-beta-site amyloid precursor protein-cleaving enzyme-C29 bait plasmid and pACT2-B3 individually showed little activation of beta-galactosidase activity, whereas the co-expression of the two produced a high level of beta-galactosidase activity (Figure 1B), indicating a positive interaction between beta-site amyloid precursor protein-cleaving enzyme 1-C29 and divalent cation tolerance protein (amino acids 1–137).

Divalent cation tolerance protein interacted with beta-site amyloid precursor protein-cleaving enzyme 1 in mammalian cells
To test whether the full-length divalent cation tolerance protein interacts with full-length beta-site amyloid precursor protein-cleaving enzyme 1 in mammalian cells, we performed co-immunoprecipitation experiments in human embryonic kidney 293T cells. Plasmid pcDNA3.1 beta-site amyloid precursor protein-cleaving enzyme 1-HA was co-transfected with either control vector pCMV or pCMV-myc-divalent cation tolerance protein in human embryonic kidney 293T cells, and myc-tagged beta-site amyloid precursor protein-cleaving enzyme 1 was immunoprecipitated with anti-myc antibody. As shown in

Figure 1 Identification of a protein that interacts with the C-terminal domain of beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1).
(A) Western blot detection of the expression of the bait protein from yeast cells transformed with control vector pGBK7-Gal4 BD-myc or bait vector pGBK7-Gal4 BD-myc-BACE1-C28. The molecular weights of the detected proteins are indicated.
(B) Quantification of beta-galactosidase activity verified the interaction of positive clone B3 with the bait construct expressing BACE1-C28 fragment. Yeast cells were co-transformed with the indicated plasmids, and the levels of beta-galactosidase activity were plotted based on three independent experiments.
Figure 2A lane 4, beta-site amyloid precursor protein-cleaving enzyme 1-HA protein was clearly detected in anti-myc immunoprecipitates, indicating that it associates with myc-divalent cation tolerance protein.

Figure 2  Divalent cation tolerance protein (CUTA) interacts with beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1) in mammalian cells.
(A) Exogenously transfected myc-CUTA interacts with BACE1-HA. Human embryonic kidney 293T cells were co-transfected with pcDNA3.1 BACE1-HA and either empty vector pCMV or pCMV-myc-CUTA, and myc-CUTA protein was immunoprecipitated with anti-myc antibody, followed by western blot detection of BACE1-HA with anti-HA antibody (top panel). The expression of myc-CUTA and BACE1-HA proteins in cell extracts were detected by western blotting (bottom panel).

(B) Endogenous BACE1 interacts with exogenously transfected myc-CUTA. N2a/APP695 cells were transfected with either empty vector pCMV or pCMV-myc-CUTA, and endogenous BACE1 was immunoprecipitated with RU689 antibody against BACE1, followed by western blot detection of myc-CUTA with anti-myc antibody (top panel). The expression of endogenous BACE1-HA in cell extracts was detected by western blot analysis (bottom panel).

Next, we tested whether divalent cation tolerance protein interacts with endogenous beta-site amyloid precursor protein-cleaving enzyme 1 by using the mouse neuroblastoma cell line N2a/APP695, which stably expresses amyloid precursor protein, APP695, containing the Swedish mutation, which results in enhanced processing of amyloid precursor protein into amyloid beta peptide[^27]. Endogenous beta-site amyloid precursor protein-cleaving enzyme 1 was detected in this cell line (Figure 2B, bottom). Immunoprecipitation with a specific beta-site amyloid precursor protein-cleaving enzyme 1 antibody was carried out in extracts from N2a/APP695 cells transfected with either control vector pCMV or pCMV-myc-divalent cation tolerance protein. Myc-divalent cation tolerance protein was co-immunoprecipitated with endogenous beta-site amyloid precursor protein-cleaving enzyme 1 (Figure 2B, top), indicating that divalent cation tolerance protein is able to interact with endogenous beta-site amyloid precursor protein-cleaving enzyme 1.

**Overexpression of divalent cation tolerance protein increased levels of full-length amyloid precursor protein and reduced levels of amyloid precursor protein C-terminal cleavage product**

To study the functional significance of the interaction of divalent cation tolerance protein with beta-site amyloid precursor protein-cleaving enzyme 1, we explored whether divalent cation tolerance protein affects the expression level of beta-site amyloid precursor protein-cleaving enzyme 1 and amyloid precursor protein were assessed by western blot analysis. As shown in Figure 3A, beta-site amyloid precursor protein-cleaving enzyme 1 protein levels were not affected by divalent cation tolerance protein overexpression.

Next, we tested whether amyloid precursor protein was affected by divalent cation tolerance protein. Remarkably, the levels of full-length amyloid precursor protein increased significantly by divalent cation tolerance protein overexpression (Figure 3B, top panel). Significantly, the levels of amyloid precursor protein C-terminal fragment were greatly decreased by divalent cation tolerance protein overexpression in a dose-dependent manner, indicating that divalent cation tolerance protein reduces amyloid precursor protein processing.

**DISCUSSION**

Beta-site amyloid precursor protein-cleaving enzyme 1 is the rate-limiting enzyme in the production of amyloid beta-peptide, and its expression and/or activity is directly related to the accumulation of amyloid plaques and deficits in neurological functioning[^9]. In this study, we identified a novel interaction between divalent cation tolerance protein and the intracellular domain of beta-site amyloid precursor protein-cleaving enzyme 1. We also demonstrated that divalent cation tolerance protein reduces the processing of amyloid precursor protein and increases accumulation of the processing intermediate amyloid precursor protein C-terminal fragment.
Nevertheless, the close association of divalent cation tolerance protein with acetylcholinesterase in independent studies suggested that divalent cation tolerance protein may be involved in regulating the biological function of acetylcholinesterase. A recent study showed that divalent cation tolerance protein is localized in the endoplasmic reticulum, and despite that a direct interaction was not observed between divalent cation tolerance protein and acetylcholinesterase, overexpression of full-length divalent cation tolerance protein reduced cellular as well as secreted levels of acetylcholinesterase. It is possible that divalent cation tolerance protein functions in the endoplasmic reticulum for the disposal of acetylcholinesterase, as suggested by the finding that while the overexpression of acetylcholinesterase resulted in endoplasmic reticulum stress and the unfolded protein response (signified by the increased levels of BiP), the co-expression of divalent cation tolerance protein alleviated this stress.

In contrast to acetylcholinesterase, the previously identified divalent cation tolerance protein interacting protein, a direct association between beta-site amyloid precursor protein-cleaving enzyme 1 and divalent cation tolerance protein was observed both in the yeast two-hybrid system and in mammalian cells by co-immunoprecipitation. Moreover, the overexpression of divalent cation tolerance protein did not affect the cellular levels of beta-site amyloid precursor protein-cleaving enzyme 1. Remarkably, the overexpression of divalent cation tolerance protein inhibited the processing of amyloid precursor protein in a dose-dependent manner, as shown by the increased levels of full-length amyloid precursor protein and the concomitant decrease in the processing product amyloid precursor protein C-terminal fragment.

The current study suggests that cleavage of amyloid precursor protein by beta-site amyloid precursor protein-cleaving enzyme 1 is modulated by divalent cation tolerance protein. How does divalent cation tolerance protein affect this process? The active site of beta-site amyloid precursor protein-cleaving enzyme 1 is located in its extracellular domain, whereas divalent cation tolerance protein was found to interact with beta-site amyloid precursor protein-cleaving enzyme 1’s C-terminal tail. Divalent cation tolerance protein may affect the enzymatic activity of beta-site amyloid precursor protein-cleaving enzyme 1 by inducing a conformational change in the protein upon association. Alternatively, it may affect beta-site amyloid precursor protein-cleaving enzyme 1 processing and/or transport in the endoplasmic reticulum.

Beta-site amyloid precursor protein-cleaving enzyme 1 is...
a protease of the pepsin family, and optimal enzymatic activity requires a low pH. It is synthesized as a zymogen in the endoplasmic reticulum and subsequently undergoes processing, including cleavage of the pro-domain, glycosylation on four Asn groups in its ectodomain, and S-palmitoylation on four Cys residues at the junction of the transmembrane and intracellular domains. The fully processed beta-site amyloid precursor protein-cleaving enzyme 1 is transported into the trans-Golgi network. Although some beta-site amyloid precursor protein-cleaving enzyme 1 is located on the plasma membrane, the majority of the protease is localized in acidic compartments, such as endosomes and the trans-Golgi network, with its enzymatic domain exposed to the lumen. Hence, the cleavage of amyloid precursor protein mostly occurs in these compartments.

Prior to this work, another group of beta-site amyloid precursor protein-cleaving enzyme 1-associated proteins were identified. These proteins, termed reticulons, include RTN3 and RTN4B. Similar to divergent cation tolerance protein, they were shown to reduce the enzymatic cleavage of amyloid precursor protein and the production of amyloid beta-peptide. Detailed studies of subcellular localization showed that RTN3 inhibits the enzymatic cleavage of amyloid precursor protein by increasing the endoplasmic reticulum retention of beta-site amyloid precursor protein-cleaving enzyme 1. Similar to the reticulons, most divergent cation tolerance protein is localized in the endoplasmic reticulum, and it is possible that divergent cation tolerance protein may retain beta-site amyloid precursor protein-cleaving enzyme 1 in the endoplasmic reticulum in a similar manner. Alternatively, divergent cation tolerance protein may interfere with beta-site amyloid precursor protein-cleaving enzyme 1 processing, such as S-palmitoylation, which has been shown to affect the accumulation of beta-site amyloid precursor protein-cleaving enzyme 1 into lipid rafts. However, the biological consequence of this partitioning is controversial, as both an enhanced beta-site amyloid precursor protein-cleaving enzyme 1 cleavage of amyloid precursor protein and a lack of effect have been reported.

Based on these properties of divergent cation tolerance protein, one may envision that the overexpression of divergent cation tolerance protein may protect against Alzheimer’s disease. First, our current study shows that divergent cation tolerance protein inhibits the processing of amyloid precursor protein. Therefore, it should delay the formation of amyloid beta-peptide plaques. Furthermore, its function in reducing the levels of acetylcholinesterase may be advantageous as well, as the inhibition of acetylcholinesterase by small molecule inhibitors has been shown to improve the mental functions of Alzheimer’s disease patients and/or delay the progression of dementia. Future experiments with transgenic mouse overexpressing divergent cation tolerance protein should provide definitive answers on the therapeutic potential of divergent cation tolerance protein in the treatment of Alzheimer’s disease.

MATERIALS AND METHODS

Design
A randomized, controlled, molecular biological and in vitro cell culture experiment.

Time and setting
The experiments were performed in the School of Life Sciences, Xiamen University, China, from March 2006 to July 2007.

Materials
Yeast two-hybrid system
Plasmids pGBK7T7, pACT2, pCMV-myc, pCMV5.0-HA, pGAD7T7, as well as human fetal brain cDNA library, and yeast strain AH109 were provided by the MATCHMAKER GAL4 Two-Hybrid System 3 kit (Clontech Laboratories Inc., San Francisco, CA, USA).

Cells and reagents
N2a and N2a/APP695 cells were from our laboratory stocks. Human embryonic kidney 293T cell line was obtained from Molecular Cell Laboratory of Tsinghua University, Beijing, China. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were from Hyclone (Waltham, MA, USA); nitrocellulose membranes were from Pall Inc. (Ann Arbor, MI, USA); western blot reagents were from Pierce Inc. (Rockford, IL, USA); X-gal was from Clontech Laboratories Inc. Mouse anti-myc, anti-HA and RNase A were from Sigma (St. Louis, MO, USA); goat anti-mouse HRP conjugated secondary antibody was from Pierce (Rockford, IL, USA). Restriction enzymes, T4 DNA ligase and Pyrobest LA DNA polymerase were from Takara (Biotechnology Dalian Co., Liaoning Province, China); DNA and protein markers were from Promega (Madison, WI, USA); pE-beta-site amyloid precursor protein-cleaving enzyme 1 plasmid was provided by...
Sanford-Burnham Medical Research Institute (San Diego, CA, USA). PCR primers were synthesized by Sangon Inc., Shanghai, China.

Methods

Yeast two-hybrid screen
MATCHMAKER GAL4 Two-Hybrid System 3 with a human fetal brain cDNA library was from Clontech Laboratories Inc. To construct the bait protein, the cDNA corresponding to the intracellular domain of human beta-site amyloid precursor protein-cleaving enzyme 1 (amino acids 473–501; NP_036236.1) was amplified from pE-beta-site amyloid precursor protein-cleaving enzyme 1 (gift from Sanford-Burnham Medical Research institute, San Diego, CA, USA) by PCR with primers 5'-GGC GAA TTC TGC CTC ATG GTG TGT CAG-3 and 5'-CGC CTGCAAG TTA TCA CTT CAG CAG GGA GAT GTC ATC-3. The PCR product was digested with EcoRI and PstI, and subcloned into pGBK7-myc to generate pGBK7-Gal4 BD-myc-beta-site amyloid precursor protein-cleaving enzyme 1-C29. The library was screened according to the manufacturer's instructions.

Quantitative analysis of beta-galactosidase activity
Plasmids from positive colonies were co-transformed with beta-galactosidase reporter into yeast AH109 and inoculated into SD/Leu-/Trp-liquid medium at 30°C overnight, and then cultured in Yeast Extract Peptone Dextrose Medium (YPD) to an absorbance value at 600 nm of 1.0–1.5. The yeast was washed three times in Z Buffer (16.1 g/L Na2HPO4•7H2O, 5.50 g/L NaH2PO4•H2O, 0.75 g/L KCl, 0.246 g/L MgSO4•7H2O; pH 7.0), then frozen and thawed repeatedly three times in liquid nitrogen. After adding the substrate ortho-nitrophenyl-beta-galactoside (ONPG), the yeast lysates were incubated at 30°C for the color reaction, which was terminated by adding Na2CO3 to a final concentration of 0.3 M. The samples were centrifuged, and the absorbance values at 420 nm of the supernatants were measured to calculate the beta-galactosidase activity. Each experiment was repeated three times.

Cell culture
Human embryonic kidney 293T cells were obtained from Molecular Cell Laboratory, Tsinghua University, China. Mouse neuroblastoma N2a cell line and N2a/APP695, the clone stably transfected with human amyloid precursor protein cDNA with the Swedish mutation[27], were gifts from the Sanford-Burnham Institute, Orlando, FL, USA. The cells were maintained in 10% FBS in DMEM in a 5% CO2/37°C incubator.

Western blot analysis
The samples were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride or nitrocellulose membranes. The blotted membrane was blocked with 5% skim milk in Tris-Tween buffered saline (TTBS; 20 mM Tris-Cl, pH 7.5, 0.15 M sodium chloride, 0.1% Tween-20) for 30 minutes. After washing with TTBS three times for 15 minutes each, the membrane was incubated overnight with primary antibody diluted in TTBS with 1% bovine serum albumin (BSA) at 4°C. The membrane was washed three times for 15 minutes each time and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody at room temperature for 1 hour, followed by three 15-minute washes, and then processed using the enhanced chemiluminescence (ECL) detection system (Millipore Corporation, Billerica, MA, USA) according to the manufacturer’s instructions.

The primary antibodies used were as follows: rabbit RU689 (anti-beta-site amyloid precursor protein-cleaving enzyme 1, 1:1 000) and RU369 (anti-amyloid precursor protein C-terminal fragment, 1:1 000), obtained from the laboratory of Dr. Huaxi Xu at the Sanford-Burnham Medical Research Institute; anti-myc (1:1 000), anti-HA (1:1 000) and anti-a-tubulin (1:1 000), from Sigma; and anti-amyloid precursor protein 6E10 (1:3 000), from Calbiochem (Merck Inc, Darmstadt, Germany). The secondary antibodies were horseradish peroxidase-conjugated anti-rabbit or anti-mouse (1:5 000) IgG (Millipore).

Construction of expression vectors and immunoprecipitation
The coding region of the divalent cation tolerance protein gene was amplified by reverse transcription-PCR with primers 5'-TTA GAA TTC GGA TGG CCT CTG GAA GCC CT-3' and 5'-CGC CTG CTC AGA CTT GCA ACC ATG GCC CAA GCC CTG-3' and 5'-GCC TCT GAG GTA GTC TAT GAG TCG ACG TAT GAG GGA GAT GTC ATC-3' using the pE-beta-site amyloid precursor protein-cleaving enzyme 1 plasmid as a template, and was subcloned between the EcoRI and XbaI sites of pcDNA3.1-HA.

The pCMV-myc-divalent cation tolerance protein and pcDNA3.1-beta-site amyloid precursor protein-cleaving...
enzyme 1-HA plasmids were co-transfected into human embryonic kidney 293T and N2a/APP695 cells, respectively, using the calcium phosphate method. The cells were collected 36 hours later, resuspended in TNE buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) with protease inhibitors, sonicated for 30 minutes at 4°C, and centrifuged at 10,000 x g for 15 minutes. The supernatant was pre-cleared with Protein-A Sepharose, incubated with specific antibody and Protein-A Sepharose overnight at 4°C, centrifuged at 10,000 x g for 10 seconds, and washed with TNE buffer four times. The precipitated material was analyzed by western blot assay.

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Author contributions: Runzhong Liu was responsible for the study design and supervision, and participated in data processing and accuracy analysis. Haibo Hou, Xuelian Yi, Shanwen Wu and Huan Zeng participated in experiments and data collection. All authors approved the final version of the manuscript.

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