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Amyloid precursor-like protein 2 C-terminal fragments upregulate S100A9 gene and protein expression in BV2 cells

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Abstract

The murine microglial cell line BV2 has neuroprotective effects, but is toxic to neurons by secreting inflammatory cytokines, and is an important target in the treatment of nerve inflammation and neurodegenerative diseases. In the present study, we observed the effects of transfecting three amyloid precursor-like protein 2 (APLP2) C-terminal fragments (CTFs; C57, C50 and C31) in the pEGFP-N1 vector on S100A9 expression in BV2 cells. Reverse transcription-PCR, western blot assay and immunocytochemistry revealed that S100A9 protein and mRNA expression was greater in BV2 cells after CTF transfection than after mock transfection with an empty vector. Furthermore, transfection of full-length APLP2-751 resulted in low levels of S100A9 protein expression. Our results show that APLP2-CTFs upregulate S100A9 protein and mRNA expression in BV2 cells, and identify a novel pathway involved in neuronal injury and apoptosis, and repair and protection in Alzheimer's disease.

Key Words: *nerve regeneration; neurodegeneration; Alzheimer's disease; APLP2; S100A9; C-terminal fragments; amyloid precursor protein; BV2 cells; γ-secretase; NSFC grant; neural regeneration*

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Introduction

Various hypotheses exist for the pathogenesis of Alzheimer's disease, including cholinergic system dysfunction, aberrant inflammatory or immune responses, gene mutations, oxidative stress, excitotoxicity, apoptosis, and defective metabolism of amyloid precursor protein (APP) (Selkoe et al., 2001; Chang et al., 2005; Walker et al., 2006; Ma et al., 2012; Dong and Chai, 2013). Among these, the APP hypothesis has received much attention (Liu et al., 2002; Scheinfeld et al., 2002; Chang et al., 2006). C-terminal fragments of APP may play a contributing role in the pathogenesis of Alzheimer's disease (Cao and Sudhof, 2001; Kimberly et al., 2001; Minopoli et al., 2001). Amyloid precursor-like protein (APLP) 2 C-terminal fragments (CTFs) were found to translocate to the nucleus and form a ternary complex with the nuclear adaptor protein Fe65 and the transcription factor CP2/LSF/LBP1, subsequently inducing glycogen synthase kinase-3β expression, tau phosphorylation and apoptosis (Ferrer et al., 2002; Liu et al., 2002; Kim et al., 2003; Xu et al., 2007). APP is a highly conserved gene, and APP, APLP1 and APLP2 have been identified in mammals. APP contains an amyloid-beta region, but none of its homologues contain this domain, although their amino acid sequence, domain structure and protein organization are similar to APP (Gu et al., 2001; Galvan et al., 2002; Scheinfeld et al., 2002).

APLP2 matures through the same unusual secretory/cleavage pathway as APP, and sheds its extracellular domain in multiple cell culture systems (Zimmer et al., 2005). APLP2 is cleaved by γ-secretase, ε-secretase and caspases to generate the CTFs C57, C50 and C31, all of which contribute to the pathology of Alzheimer's disease (Gu et al., 2001; Xu et al., 2001; Galvan et al., 2002).

S100A9 is an inflammation-associated calcium-binding protein belonging to the S100 family (Abe et al., 1999; Gebhardt al., 2006; Zhang et al., 2012). Neurological diseases such as cerebral ischemia, traumatic brain injury (Postler et al., 1977) and Alzheimer's disease (Shepherd et al., 2006; Chang et al., 2012; Wang et al., 2014) are associated with altered expression of S100 family members (Zimmer et al., 2005). S100A9 expression was recently found to be increased within neuritic plaques and reactive glia, and was proposed to participate in the inflammation of Alzheimer's disease (Shepherd et al., 2006; Zhang et al., 2012; Kim et al., 2014). However, the detailed molecular mechanisms of these patho-

logical events remain unknown (Kummer et al., 2006; Lee et al., 2012). The *S100A9* gene is significantly upregulated in the brains of animal models of Alzheimer's disease, namely Tg2576 and CT-Tg mice, as well as in patients with Alzheimer's disease (Tae-Young et al., 2010; Chang et al., 2012; Kim et al., 2014). Murine BV2 microglial cells are central nervous system immune cells that protect brain tissue function by phagocytosing neuronal pathogens and harmful particles. However, reactive microglia also release inflammatory factors such as S100A9, and are an important target in the treatment of neuroinflammation and neurodegenerative diseases. In the present study, we used BV2 cells to investigate the effects of APLP2-CTFs on the expression of the S100A9 gene, to elucidate this pathogenic pathway in Alzheimer's disease.

Materials and Methods

Materials

APLP2-CTFs and the BV2 cell line were kindly provided by Professor Yoo-hun Suh, Department of Pharmacology, College of Medicine, Seoul National University, South Korea.

Plasmid extract and purification

Escherichia coli (DH5a; BS-3236, Biomedal, Beijing, China) with recombinant plasmid pEGFP-N1-APLP2-CTFs (C57, C50, C31) and EGFP-N1 empty vector (Clontech, Mountain View, CA, USA) were cultured in medium containing 30 μg/mL kanamycin (Kan^+). A single colony of each APLP2-CTF was selected into 10 mL Luria-Bertani liquid medium with Kan^+, and mixed overnight on a rotary shaker at 230 r/min and 37°C. The colonies were then transferred to 200 mL of Luria-Bertani liquid medium to grow. The plasmids were extracted using the Plasmid Maxi Kit (12163; QIAGEN, Venlo, Netherlands) and the concentration and purity were measured in an ultraviolet spectrophotometer (CKX41; Olympus, Tokyo, Japan).

BV2 cell culture

Murine microglial BV2 cells (Department of Pharmacology, Seoul National University, South Korea) were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc., Grand Island, NY, USA) in 6-well plates, supplemented with 5% fetal bovine serum (Corning, Steuben Country, NY, USA) and penicillin/streptomycin (100 U/mL/100 μg/mL) at 37°C and 5% CO_2 for 3 days (Munsie et al., 2011; Lorena et al., 2006).

Plasmid transfection

At this point, the culture was divided into four groups: a mock transfection group (pEGFP-N1 empty vector); an APLP2-C57 transfection group; an APLP2-C50 transfection group; and an APLP2-CT31 transfection group. Each group was transfected with 5 μg plasmid for 48 hours and observed 24 hours post-transfection. BV2 cell cultures were maintained in 24-well plates. In addition, to inhibit CTF generation, BV2 cells were transfected with full-length APLP2-751 and incubated with an inhibitor of γ-secretase, N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT, 2 μmol/L; Sigma-Aldrich, St Louis, MO, USA). All transfection procedures were carried out using 5 μL Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) in 1 mL DMEM, according to the manufacturer's instructions. Cells were observed under a fluorescence microscope (Olympus, Tokyo, USA).

Western blot assay

Samples were harvested in radioimmunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.15 mol/L NaCl, and 0.05 mol/L Tris-HCl; pH 7.2) with protease inhibitors (Complete mini; Roche, Indianapolis, IN, USA) at 4°C. The harvested proteins from cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was blocked in Tris-buffered saline containing 4% non-fat milk powder (Lise et al., 2012) for 1 hour at room temperature. The proteins were visualized using activated rabbit anti-S100A9 monoclonal antibody overnight (1:1,000; Sino Biological Inc.) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L) for 1 hour at room temperature (1:800; Beyotime Institute of Biotechnology, Haimen, Jiangsu Province, China). The membrane was scanned in an ultraviolet spectrophotometer (Olympus) and the gray values of the target protein bands were compared against those of β-tubulin (mouse, 1:1,000; Sino Biological Inc.; 1 hour at room temperature) using Quantity One software (Bio Rad, Hercules, CA, USA).

Reverse transcription (RT)-PCR

Trizol reagent (Invitrogen) was used to isolate 2 mg total RNA for cDNA synthesis, which was carried out using AccuPower RT PreMix (Bioneer, Korea). We measured the concentration and purity of RNA by ultraviolet spectrophotometry (CKX41; Olympus). The abundance of transcripts in cDNA samples was measured by RT-PCR (Waters Company, Changchun, Jilin Province, China). The primer sequences are as follows:

Primer	Gene sequence	Product size (bp)
S100A9	Forward 5′- CAG CAT AAC CAC CAT CAT CG-3′	362
	Reverse 5′-GTC CTG GTT TGT GTC CAG GT-3′;	
β-Actin	Forward 5′-CCA GAT CAT GTT TGA GAC CT-3′	206
	Reverse 5′-GTT GCC AAT AGT GAT GAC CT-3′	

The PCR reactions were subjected to 40 amplification cycles of 95°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. PCR products were analyzed on 1.5% agarose gel, and the gels were visualized by staining with ethidium bromide. All absorbance values were normalized to β-actin.

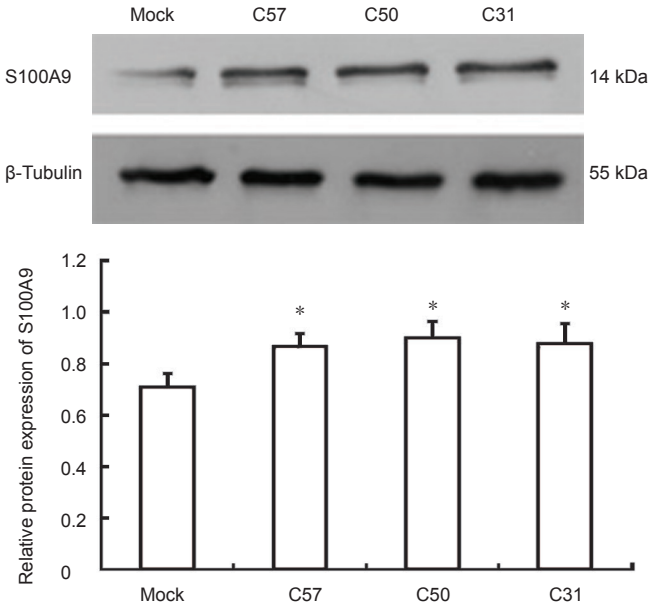

Figure 1 Effects of transfection of BV2 cells with APLP2-CTFs (C57, C50, C31) on S100A9 protein expression (western blot assay).
Expression levels of S100A9 were significantly greater in the APLP2-CTF-transfected groups than in the mock transfected group. Data are expressed as the gray value ratios of S100A9/β-tubulin, and presented as the mean ± SD from three experiments for each group. *$P < 0.05$, *vs.* mock transfected group (one-way analysis of variance and Dunnett's *t*-test). APLP2-CTFs: Amyloid precursor-like protein 2 C-terminal fragments.

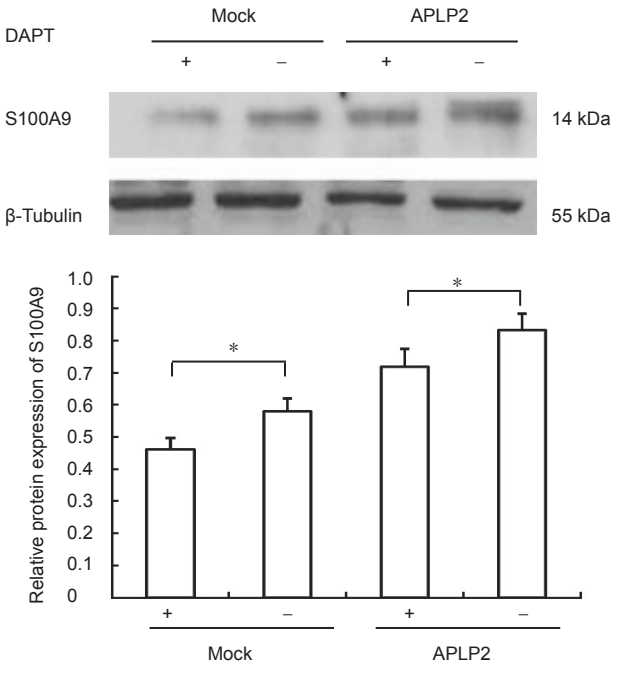

Figure 4 Effects of transfection with APLP2-751 (full-length) on S100A9 protein expression in BV2 cells.
S100A9 levels were not higher in the DAPT-treated group than in the untreated groups in both the mock and the APLP2 groups. Data are expressed as the gray ratios of S100A9/β-tubulin and presented as mean ± SD of three experiments for each group. *$P < 0.05$ (paired *t*-test). APLP2-CTFs: Amyloid precursor-like protein 2 C-terminal fragments; DAPT: N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester; "+", DAPT;" –", vehicle.

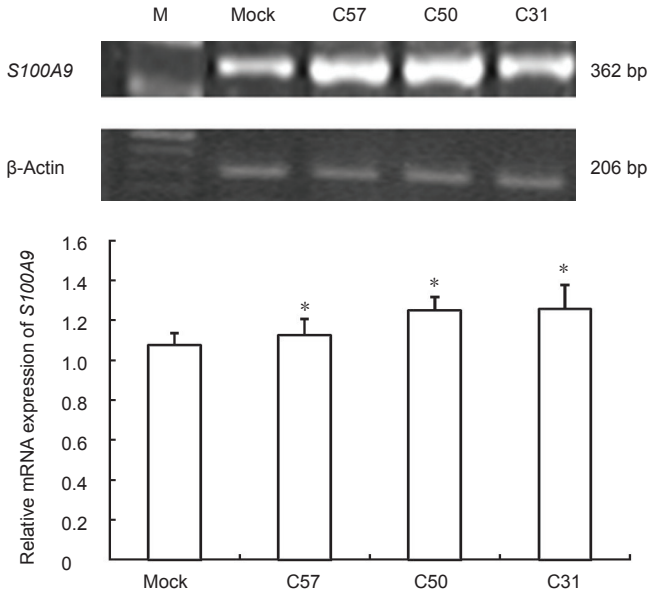

Figure 3 Effects of transfection with APLP2-CTFs (C57, C50, C31) on *S100A9* mRNA expression in BV2 cells (reverse transcription-PCR).
*$P < 0.05$, *vs.* mock transfected group. Data are expressed as the mean ± SD from three experiments for each group (one-way analysis of variance and Dunnett's *t*-test). APLP2-CTFs, Amyloid precursor-like protein 2 C-terminal fragments. M: Marker.

Immunocytochemistry

BV2 cells grown on a glass coverslip were transfected with APLP2-CTFs for 48 hours. Cells were fixed in 4% paraformaldehyde for 1 hour at 4°C and then for 30 minutes at room temperature. After quenching in 50 mmol/L NH_4Cl in phosphate-buffered saline (PBS) for 10 minutes, the cells were washed in PBS and permeabilized for 30 minutes at room temperature in permeabilization buffer (PBS containing 0.1% Triton X-100 and 1 mg/mL bovine serum albumin). The following steps were performed at room temperature in permeabilization buffer. Cells were incubated with rabbit anti-S100A9 antibody (1:1,000; Sino Biological Inc.) for 1 hour at room temperature, washed three times, and incubated with Alexa Fluor 555-labeled goat anti-rabbit IgG (H+L; Institute of Biotechnology; 1:800 dilution) for 1 hour at room temperature. After three washes in permeabilization buffer and one wash in PBS, the cells were mounted on microscope slides in mounting medium (DAKO, Copenhagen, Denmark) containing DAPI (1:50; Invitrogen) for nuclear staining. Cells were observed under a laser scanning confocal microscope (Leica TCS SP5, Leica Microsystems, provided by the Experimental Center of Preventive Medicine, Medical College, Yanbian University, China). Data were analyzed using STED deconvolution software (LAS AF; Leica, Germany).

Statistical analysis

All data were expressed as the mean ± SD of at least three independent experiments. Paired *t*-tests, or one-way analysis of variance followed by Dunnett's *post-hoc t*-test, were per-

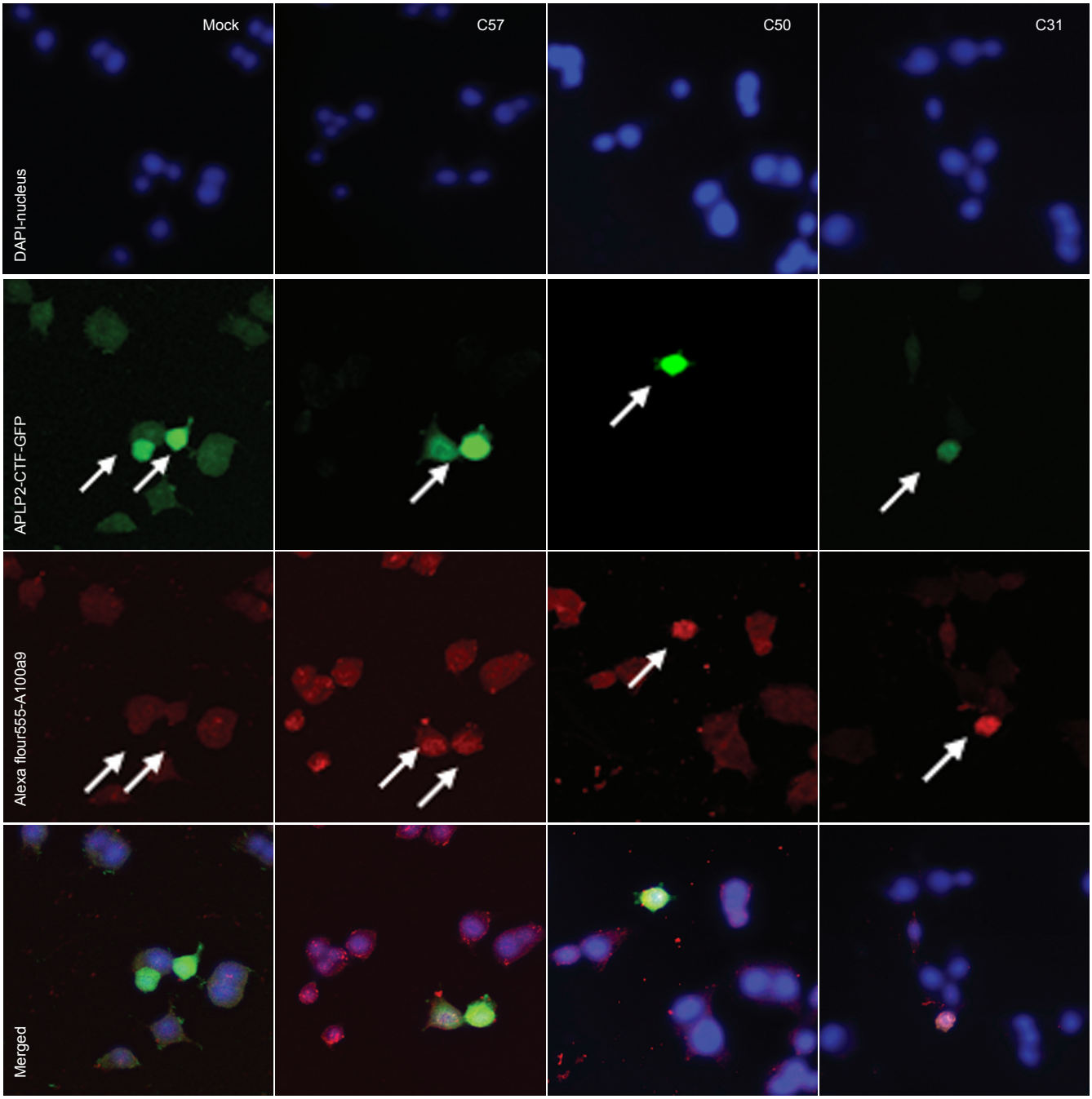

Figure 2 Effects of transfection with APLP2-CTFs (C57, C50, C31) on S100A9 protein expression in BV2 cells (immunocytochemical staining; laser scanning confocal microscope, × 100).
S100A9-positive cells (arrows) were visualized with Alexa Flour 555-conjugated secondary antibody (red). Nuclei were stained with DAPI (blue). Among successfully transfected cells (green), S100A9 protein expression was notably stronger in all three APLP2-CTF-transfected groups than in the mock transfected group.

formed using SPSS 13.0 software (SPSS, Chicago, IL, USA) to study the relationship between the different variables. Values of $P < 0.05$ were considered statistically significant.

Results

APLP2-CTFs induced upregulation of S100A9 protein expression

BV2 cells were transfected with APLP2-CTFs for 48 hours. Western blot assay revealed that expression of S100A9 was significantly greater in all three groups of APLP2-CTF-transfected cells than in the mock transfected group ($P < 0.05$; **Figure 1**).

Immunocytochemical staining confirmed that S100A9 was induced by APLP2-CTFs (**Figure 2**).

APLP2-CTFs induced upregulation of S100A9 mRNA

RT-PCR showed that S100A9 expression was significantly greater in all three APLP2-CTF-transfected groups than in

the mock transfected group ($P < 0.05$; **Figure 3**).

DAPT treatment induced downregulation of S100A9 protein levels

To determine whether endogenously generated APLP2-CTFs affect S100A9 expression, we investigated the effect of DAPT, an inhibitor of γ-secretase, on S100A9 levels after transfection with full-length APLP2-751. We found that S100A9 expression was lower in the DAPT treatment group than in the vehicle-treated control group ($P < 0.05$), indicating that the generation of CTFs is critical for S100A9 protein expression (**Figure 4**).

Discussion

Alzheimer's disease is characterized by age-dependent formation of amyloid-beta-containing senile plaques and intracellular neurofibrillary tangles (Han et al., 2013; Wang et al., 2013). Induction of the *S100A9* gene occurs in the brains of patients with Alzheimer's disease and in animal models of the disease. S100A9 expression is increased in senile plaques and active gliocytes, suggesting that S100A9 is involved in the pathogenesis of Alzheimer's disease (Zimmer et al., 2005; Shepherd et al., 2006; van Lent et al., 2008). In the present study, we found that transfecting BV2 cells with APLP2-CTFs increased S100A9 protein and mRNA expression.

Previous studies showed that *S100A9* gene silencing significantly reduces Alzheimer's disease pathology, including the number of plaques, and improves the learning ability of Tg2576 mice. Also, treatment with amyloid-beta (1–42) or C105 and transfection with APP-C50 or C99 can elevate *S100A9* mRNA expression (Tae-Young et al., 2010). Our results demonstrate that APLP2-CTF has similar toxic effects to those of APP-CTF and amyloid beta on the inflammatory responses of glial cells.

The amyloid precursor protein cleaved by β-secretase and γ-secretase generates amyloid beta and CTFs of different sizes (Xing et al., 2012; Liu et al., 2013). APP-CFTs play a contributing role in Alzheimer's disease pathogenesis (Cao and Sudhof, 2001; Kimberly et al., 2001; Minopoli et al., 2001; Kim et al., 2003). APLP2-CTFs translocate to the nucleus and form a ternary complex with Fe65 and CP2/LSF/LBP1, and induce the expression of glycogen synthase kinase-3β, tau phosphorylation and apoptosis (Xu et al., 2007).

We also examined changes in S100A9 levels in BV2 cells expressing full-length APLP2-751 in the presence or absence of the γ-secretase inhibitor, DAPT, to investigate the effects of endogenously generated APLP2-CTFs on S100A9 expression. In the DAPT-treated group, S100A9 levels were not significantly greater than those in the control group, suggesting that generation of APLP2-CTFs is essential for the induction of S100A9 gene expression in BV2 cells.

The results of the present study indicate that APLP2-CTF co-contributes to the pathology of Alzheimer's disease with APP-CTF and amyloid beta. The underlying molecular mechanisms need to be further investigated.

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Author contributions: *Li GZ and Xu YJ participated in conception and design of the study, and analysis and interpretation of the data. Li GZ, Chen H and Cheng L were in charge of data collection. Chen H, Li GZ, Zhao RJ and Xu YJ wrote the manuscript or provided critical revision of the manuscript for intellectual content. Zhao JC participated in statistical expertise. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

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