Overexpression of C9orf72 exacerbates Aβ_{25-35}-induced oxidative stress and apoptosis in PC12 cells

Jing Chen¹, Mingming Zhang², Hongying Bai¹, Peiyu Shi¹, Meng Du¹, Shijie Zhang²*, Jiyu Lou¹*

¹ The Second Affiliated Hospital of Zhengzhou University, Zhengzhou, China,
² School of Life Sciences, Zhengzhou University, Zhengzhou, China,
*Email: jiyu_lou@126.com; shijie-zhang@zzu.edu.cn

Alzheimer’s disease (AD) is the most common neurodegenerative disease and is manifested by memory loss and spatial disorientation. There is currently no effective treatment for AD. Abnormalities of the chromosome 9 open reading frame 72 (C9orf72) gene have been associated with various neurodegenerative diseases. However, its intrinsic roles in AD remain to be elucidated. Here we found that Aβ_{25-35} increased the expression of C9orf72 in PC12 cells at both mRNA and protein levels. In Aβ_{25-35}-treated PC12 cells, C9orf72 overexpression induced an abnormally condensed and fragmented nucleus and apoptosis, as well as significantly enhanced reactive oxygen species (ROS) levels. Mechanistically, an Aβ_{25-35}-induced decrease of superoxide dismutase activity was augmented by C9orf72 overexpression, which in contrast increased malondialdehyde content. Consistently, further apoptotic analysis revealed significant downregulation of Bcl-2 and Bcl-XL expression and enhanced cleavage of caspase-3 with Aβ_{25-35} treatment, all of which were exacerbated by C9orf72 overexpression. In addition, tau phosphorylation, another hallmark of AD pathology, was induced by Aβ_{25-35} and was remarkably enhanced by C9orf72 overexpression. Our data indicate that C9orf72 plays important roles in intracellular ROS signaling and Aβ_{25-35}-induced neuronal apoptosis in AD. These findings provide insights into C9orf72 function in the pathogenesis of many related neurodegenerative diseases and provide a basis for potential therapeutic interventions.

Key words: Alzheimer’s disease, C9orf72 overexpression, apoptosis, oxidative stress response, ROS, β-amyloid

INTRODUCTION

Dementia has become a major challenge to modern societies, due to the drastic increase in the number of patients and the enormous costs to community health systems. There are more than 50 million people living with dementia worldwide at present, and the number is expected to double every twenty years. Alzheimer’s disease (AD) is the most common type of dementia in the elderly population and comprises 60% to 70% of cases. Generally, AD patients initially exhibit short-term memory dysfunction, impaired judgement, disorientation (including easily getting lost), mood swings, language difficulties, and minor personality changes, but eventually develop a total loss of memory and a change in personality (Martins et al., 2018). The risk of developing AD increases with age. The average life expectancy of AD patients following diagnosis is only three to nine years.

The characteristic neuropathological features of AD are intracellular neurofibrillary tangles, composed primarily of hyperphosphorylated forms of the microtubule-associated protein Tau, and extracellular amyloid plaques, deposits of aggregated and fibrillated amyloid-beta (Aβ) peptides (LaFerla and Oddo, 2005; Martins et al., 2018). The formation of soluble neurotoxic oligomers of Aβ may underlie AD patho-
genesis, and amyloid plaques represent the major source of neurotoxic forms of Aβ oligomers (Cohen et al., 2013). In addition to age, family history, diet and other environmental and genetic factors have been shown to play an important role in the pathogenesis of AD (Davignon et al., 2010). Several genes associated with the accumulation of amyloid deposits, neuronal degeneration, cognitive alterations, and episodic memory were screened and identified, including amyloid precursor protein (APP), PSEN1, and PSEN2, which are associated with familial Alzheimer’s disease (FAD), as well as APOE4, which is associated with sporadic Alzheimer’s disease (SAD) (Mullan et al., 1992; Corder et al., 1993; Cruts et al., 2012). However, additional risk proteins and loci need to be identified in order to further understand the genetic complexity of AD.

The chromosome 9 opening reading frame 72 (C9orf72) gene, located on chromosome 9p21 in the human genome, was demonstrated to be involved in the homeostatic cellular pathways of the central nervous system. It encodes 11 exons and contains specific GGGGCC (G4C2) base pairs, which exist between non-coding exons 1a and 1b (Moens et al., 2017). Advantages in large-scale genetics and genomics have revealed that the hexanucleotide repeat expansion of (G4C2)n in C9orf72 is linked to the neurodegenerative diseases amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (DeJesus-Hernandez et al., 2011; Renton et al., 2011). ALS is the most common motor neuron disease and is characterized by the progressive loss of upper and lower motor neurons (Taylor et al., 2016). FTD is characterized by degeneration of the frontal and temporal lobes of the brain and is the second most common cause of early-onset dementia after AD (Rademakers et al., 2012). Loss-of-function and two separate gain-of-function (accumulation of toxic RNA foci and repeat-associated non-ATG initiated translation) mechanisms have been proposed to explain the causal relationship between C9orf72 hexanucleotide expansions and ALS/FTD (Mori et al., 2013; Kwon et al., 2014).

There are many shared pathological and clinical characteristics of FTD, SLA, and AD, including behavioral symptoms, tau-positive pathology, and C9orf72 repeat expansions (Kohli et al., 2013). Given the clinical and pathologic overlap between familial FTD and AD, increasing evidence suggests that C9orf72 hexanucleotide repeat expansion and its natural functions may also contribute to AD (Majounie et al., 2012; Rollinson et al., 2012). Thus far, C9orf72 has been shown to be involved in a series of homeostatic cellular pathways compromising the pathogenesis of common neurodegenerative diseases, such as actin dynamics, regulation of membrane trafficking, and autophagy (Nassif et al., 2017). However, the relationship between C9orf72 and the risk of AD remains contended.

Here we report increased levels of C9orf72 gene expression in Aβ25-35-induced PC12 cells. Overexpression of C9orf72 in cultured cells led to increased intracellular ROS level, and ultimately induced cellular apoptosis through regulation of Bcl-2/caspase signaling. Our findings suggest that upregulation of C9orf72 may contribute to the development of AD, suggesting a potential target for therapeutic intervention.

METHODS

Reagents

Amyloid beta-peptide (25-35) (Aβ25-35), PMSF, antibody against tau and phospho-tau (phosphorylated tau at the site Ser396) were purchased from Sigma-Aldrich (USA). Dulbecco’s modified Eagle medium (DMEM) and trypsin were obtained from Corning (USA). RIPA lysate buffer, the BCA protein assay kit, the ROS detection kit, and phosphatase inhibitor were purchased from the Beyotime Institute of Biotechnology (China). Antibodies against Bcl-2 and Bcl-xL were purchased from Proteintech (USA). Antibodies against cleaved caspase-3 and caspase-3 were obtained from Cell Signaling Technology (USA). Antibodies against β-tubulin was purchased from MultiSciences (China). The Annexin V-PE/7-AAD apoptosis detection kit was purchased from Yeasen Biotech Co., Ltd. (China). The kits for detection of malondialdehyde (MDA) and superoxide dismutase (SOD) were purchased from the Nanjing Jiancheng Bioengineering Institute (China).

The dissolution method of Aβ25-35

Aβ25-35 was dissolved in sterile double-distilled water to prepare a 1 mM mother liquor. After dissolution, it was placed in a 37°C incubator and aged for 72 h. After aging, it was diluted with PBS and stored at -20°C.

Cell culture

PC12 cells were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (China). The PC12 cells were cultured in DMEM containing 10% horse serum, 2.5% fetal bovine serum, and 1% penicillin/streptomycin at 37°C in the humidified atmosphere of a 5% CO2 incubator.
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**Generation of C9orf72 overexpression constructs**

The full-length C9ORF72 gene was amplified using a polymerase chain reaction and cloned into the pLVX-IRES-ZsGreen vector at the BamHI and EcoRI restriction enzyme sites. The resulting fusion construct was confirmed by sequencing analysis.

**Cell viability assay**

Cell viability was detected by the MTT assay. The PC12 cells were seeded in poly-L-lysine-coated 96-well microplates at a density of 1×10⁴ cell/well. After 24 h or 48 h, 20 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added into each well at a final concentration of 0.5 mg/ml. The medium was removed after another 4 h of culture and 150 μl DMSO was added subsequently. The absorbance at a wavelength of 490 nm was measured with an enzyme-linked immunosorbent instrument (SpectraMax i3, Molecular Devices, USA).

**Apoptosis assay**

1×10⁶ cells were harvested and resuspended in 100 μl annexin V binding buffer. The cells were then co-stained with fluorescein isothiocyanate-annexin V and 7AAD according to the manufacturer’s protocol (BD Pharmingen). Stained cells were analyzed with FACS Canto II (BD Pharmingen), and the data analysis was performed by FlowJo and BD FACSDiva software.

**Intracellular ROS and oxidant/antioxidant system measurement**

The ROS level was detected by 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). The cells were incubated with a 10 μM DCFH-DA solution for 30 min at room temperature. After three washes with PBS, the cells were resuspended and the fluorescence intensity was measured using flow cytometry at the excitation wavelength of 485 nm and an emission wavelength of 550 nm. SOD activity and MDA contents were measured according to the directions of the assay kit.

**Quantitative RT-PCR assays**

All qRT-PCR reactions were performed with the LightCycler 480 II (Roche, Switzerland) using Power SYBR Green PCR Master Mix (Applied Biosystems by Life Technologies, USA). The primers were obtained from the Harvard primer bank. The PCR primers used for qRT-PCR assays were C9ORF72: 5′TTGGCCGGCTACCTTGGTTAC 3′—forward primer and 5′CATTTCAAGTCTCGAGAAGAA 3′—reverse primer; BCL2: 5′CCATTGGTTACCTGCAGCTTC 3′—forward primer and 5′GGAAGGAAAGATGCCAGG 3′—reverse primer; BCL2-XL: 5′TCCCTAAACCACGCTCCTGG 3′—forward primer and 5′AGGCCACACACATCAAAAACC 3′—reverse primer.

**RESULTS**

Aβ<sub>25‑35</sub> induced PC12 cell apoptosis in a dose-dependent manner

Aggregation and deposition of Aβ, a noted pathological characteristic of AD, has been well accepted as a potent activator of neuronal cell death (LaFerla and Oddo, 2005). To establish an AD cell model, PC12 cells were exposed to different concentrations of Aβ<sub>25‑35</sub> (10 μM, 20 μM, 40 μM) for 48 h. The MTT assay was used to detect cell viability and the results revealed that cell viability decreased in a dose-dependent manner in the presence of Aβ<sub>25‑35</sub> compared with the control group (Fig. 1A). To determine whether the reduction in cell survival was caused by apoptosis, we carried out flow cytometry by annexin V staining. The representative profiles of annexin V staining are shown in Fig. 1B. Quantitative analysis revealed that the apoptosis ratio also increased in an Aβ<sub>25‑35</sub> dose-dependent manner compared with the control group (Fig. 1C). Because the
increase in apoptosis generally paralleled the decrease in cell number, these findings imply that apoptosis contributed to the decreased cell survival induced by Aβ treatment.

**Treatment of Aβ_{25-35} increased C9orf72 expression in PC12 cells**

In order to speculate on the functions of C9orf72, we first evaluated expression levels in tissues and cell lines based on the Human Protein Atlas database (https://www.proteinatlas.org/). C9orf72 expression was highest in the brain and in nerve-related cell lines, implying that C9orf72 may be involved in physiological and pathological functions of neurological disorders.

We then tested whether Aβ_{25-35} is able to induce the expression of C9orf72. Quantitative real-time PCR (qRT-PCR) showed that the mRNA levels of C9orf72 were prominently upregulated in the presence of 20 µM and 40 µM Aβ_{25-35} (Fig. 2A). Compared with the control group, 20 µM and 40 µM Aβ_{25-35} led to a 1.58- and 4.36-fold increase in the transcriptional level of C9orf72, respectively. The expression patterns at the protein level were further confirmed by Western blotting analysis (Fig. 2B). Quantitative analysis showed the protein was elevated by 1.35- and 2.33-fold when treated with 20 µM and 40 µM Aβ_{25-35}, respectively (Fig. 2C).
These results suggest a strong correlation of C9orf72 expression with amyloid pathology in AD.

**Overexpression of C9orf72 aggravated Aβ25-35-induced apoptosis in PC12 cells**

We then examined whether C9orf72 overexpression in Aβ25-35-treated PC12 cells leads to an increase in the malignant potential. We firstly generated the overexpression construct of C9ORF72 cDNA. Lentivirus containing the C9orf72 overexpression construct or control construct was packaged and the PC12 cells were infected, followed by continuous screening. The overexpression efficiency was examined 48 h later by Western blotting. As shown in Fig. 3A and 3B, a more than four-fold overexpression efficiency was achieved with C9orf72 lentiviral transfection compared with control vector.

To investigate the role of C9orf72 expression in Aβ-induced cytotoxicity, we treated PC12 cells with Aβ25-35 with or without C9orf72 expression. As a result, Aβ25-35 treatment induced notable cytotoxicity, as demonstrated by the apoptosis rate increase to 8.9% compared with the control group (Fig. 3C, D). When the cells overexpressing C9orf72 were exposed to 40 µM Aβ25-35, the apoptosis rate significantly increased (Fig. 3C, D). We next examined the cellular morphology in those treated cells. As shown in Fig. 3E, Aβ25-35-treated PC12 cells exhibited significant morphological alterations, indicated by smaller and irregular cell morphology. Overexpression of C9orf72 exacerbated the morphological alterations, with displayed karyopyknosis, chromatin condensation, and apoptotic bodies (Fig. 3E). Meanwhile, the C9orf72 overexpressing PC12 cells that were not treated with Aβ25-35 exhibited a similar apoptosis rate and cell morphology to the control group (Fig. 3C-E). These results suggest that increased C9orf72 expression can increase the apoptosis in PC12 cells that is induced by Aβ25-35.

**Overexpression of C9orf72 exacerbated the Aβ25-35-induced redox system imbalance in PC12 cells**

Oxidative stress plays a critical role in AD, shown to cause damage to cellular components both *in vitro* and *in vivo* (Smith et al., 1991). Thus, we chose to investigate ROS production using flow cytometry (Fig. 4A). Overexpression of C9orf72 in PC12 cells without Aβ25-35 treatment did not significantly affect ROS production. However, similar to previous reports (Wang and Xu, 2019), cells treated with Aβ25-35 showed increased ROS accumulation compared with the control group (Fig. 4A, B). Consistently, the ROS production in
C9orf27-overexpressing cells remarkably increased, compared with the control cells and Aβ25-35-treated cells (Fig. 4A, B). To determine what accounted for the ROS production, we next measured levels of a key antioxidative enzyme, SOD, as well as MDA, an index of lipid peroxidation, in these groups. As expected, treatment with Aβ25-35 reduced the activity of SOD (Fig. 4C) and increased MDA levels (Fig. 4D), and both results were exacerbated by overexpression of C9orf72. These results indicate that C9orf72 overexpression worsened
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Aβ-stimulated accumulation of intracellular ROS by disrupting the balance of oxidase and anti-oxidative activities.

**Overexpression of C9orf72 inhibited the expression of Bcl-2 and Bcl-xL**

It is well known that B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-xL) act as primary anti-apoptotic proteins by preventing the cytochrome c release from mitochondria. To verify whether the increased apoptosis was associated with these genes, we first evaluated the transcriptional expression of the genes. Exposure of PC12 cells to 40 µM Aβ25-35 for 48 h prominently decreased the expression of Bcl-2 and Bcl-xL compared with the control group (Fig. 5A, B). Both of the above effects were further reduced by overexpression of C9orf72 (Fig. 5A, B). In line with this, the protein levels of Bcl-2 and Bcl-xL also decreased in
Aβ25-35-treated PC12 cells and were further diminished in C9orf72-overexpressing PC12 cells (Fig. 5C, D).

**Overexpression of C9orf72 increased the caspase-3 activity**

Caspase-3, the most classic and predominant caspase, is involved in the cleavage of Aβ 4A precursor protein, which is also associated with neuronal death in AD (Gervais et al., 1999). Western blotting analysis demonstrated that the levels of cleaved caspase-3 significantly increased with Aβ25-35 stimulation, while there were no clear changes in total caspase-3 (Fig. 6A). Quantitative analysis showed that treatment with Aβ25-35 increased the levels of cleaved caspase-3 560% in PC12 cells compared with the levels in the control cells (Fig. 6B). Furthermore, overexpression of C9orf72
resulted in enhanced levels of cleaved caspase-3 (up to 1000% of control) (Fig. 6A, B), suggesting that apoptosis induced by C9orf72 overexpression is associated with the Bcl-2/caspase signaling pathway.

**Overexpression of C9orf72 increased tau hyperphosphorylation**

The accumulation of hyperphosphorylated microtubule-associated protein tau in damaged brain regions has been established as a major hallmark of AD in patients (Grundke-Iqbal et al., 1986; Iqbal et al., 2016). In our study, tau phosphorylation was evaluated by Western blotting analysis and the results showed that treatment with Aβ$_{25-35}$ increased tau phosphorylation levels compared with the control group (Fig. 6A, C). In addition, overexpression of C9orf72 enhanced the Aβ$_{25-35}$-evoled phospho-tau levels in PC12 cells. As shown in Fig. 6C, C9orf72 overexpression increased the phosphorylation of tau by more than 50%, compared with the levels observed in Aβ$_{25-35}$-induced PC12 cells infected with lentivirus containing empty vector.

**DISCUSSION**

C9orf72 is associated with various neurodegenerative diseases such as ALS, FTD, and AD. Most studies focus on the harm and pathogenesis of hexanucleotide repeat expansion of (G4C2)$_n$ in C9orf72, and suggest a synergy of loss-of-function and gain-of-function...
to explain the underlying pathogenic mechanisms (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Moens et al., 2017). However, very few studies have focused on the intrinsic cellular functions of C9orf72.

Excessive accumulation of Aβ peptides in the brain has been proposed as a primary influence driving AD pathogenesis (Seubert et al., 1992). Aβ is a heterogeneous mixture of small peptides 36-43 amino acids in length derived from APP (Trambauer et al., 2017). Treatment of neuronal PC12 cells, derived from rat pheochromocytoma, with the major segment of Aβ, Aβ25-35, is a well-established model to study the pathophysiology of AD. In the present study, Aβ25-35 induced cytotoxicity in PC12 cells, indicated by enhanced apoptosis as measured by MTT assay and flow cytometry with annexin V staining. Oxidative stress is one major factor that induces cellular damage and subsequently apoptosis in various neurodegenerative disorders (Naoi et al., 2009). Aβ25-35 induces cytotoxicity, at least in part, through the overproduction and accumulation of superoxide, in the mitochondria, and intracellular ROS. We also found that Aβ25-35 inhibited the expression of Bcl-2 and Bcl-xL, two primary anti-apoptotic proteins that prevent cytochrome c release from mitochondria, and increased the levels of cleaved caspase-3, the executioner caspase that initiates cellular apoptosis. These results suggest that the cell death caused by Aβ25-35 likely involves the mitochondrial apoptosis pathway.

In this study, we unexpectedly found that Aβ25-35 induced the upregulation of C9orf72 expression both at the mRNA and protein levels in PC12 cells. In humans, the C9orf72 gene, located on the short arm of chromosome 9 open reading frame 72, encodes two different isoforms (a 481 amino acid isoform of approximately 50 kDa and a 222 amino acid isoform of 25 kDa), due to alternative splicing. In the present study, we detected a 55 kDa band with Western blotting analysis, which represents the longer isoform.

A few studies have examined the expression of C9orf72 in human tissue and cell lines using a variety of commercial antibodies (Renton et al., 2011; Gijselinck et al., 2012). The protein is abundantly expressed in the cerebral cortical neurons and in some specialized neurons in the brain and spinal cord that control movement. The C9orf72 protein usually locates at the tip of the neuron at the presynaptic terminal, essential for neurotransmission between neurons. Using mouse models, researchers found that expression levels of nuclear and cytoplasmic C9orf72 isoforms changed significantly during development, labeling as puncta throughout neurons, and subsequently extending beyond the microtubule cytoskeleton into actin-rich structures such as filopodia and growth cones (Atkinson et al., 2015). The characteristic expression profile and cellular localization of C9orf72 suggests important roles for endogenous C9orf72 protein in the maintenance of intracellular physiological functions and cellular homeostasis. However, there are few studies that compare expression of C9orf72 between AD and non-AD patients.

The GGGGCC hexanucleotide repeat expansion in intron 1 of the C9orf72 gene has been used as the most common genetic cause of both ALS and FTD (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Subsequently, expansions have been found in other neurodegenerative disorders, such as AD (Majounie et al., 2012; Rollinson et al., 2012). While, to date, pathological roles of C9orf72 hexanucleotide expansion-associated disease, such as protein haploinsufficiency, dipeptide repeat protein expression, and RNA foci accumulation have been intensively investigated, less attention has been directed to investigating its intrinsic function. Recent studies on C9orf72 showed that it is involved in actin dynamics, regulation of membrane trafficking, and macroautophagy (Nassif et al., 2017). Our data demonstrated that overexpression of C9orf72 significantly aggravated apoptosis in an in vitro AD model. Further experiments showed that overexpression of C9orf72 led to a decrease in anti-apoptotic proteins, Bcl-2 and Bcl-xL, and a significant accumulation of cleaved caspase-3. As the major trigger of cellular apoptosis, caspase-3 is the predominant caspase involved in the cleavage of Aβ 4A precursor protein, which is associated with neuronal death in AD (Gervais et al., 1999). Apoptosis and autophagy are two well-controlled biological processes contributing to the maintenance of cellular and tissue homeostasis. Despite marked differences between these two classical pathways, accumulating evidence reveals that apoptosis and autophagy have extensive crosstalk (cooperate or antagonize), and thus influence cell fate decision (Gump and Thorburn, 2011; Wang et al., 2019). Among other factors, caspases play important roles in directing these conversations between autophagy and apoptosis. Additional studies are required to shed light on the relationship between apoptosis and autophagy driven by C9orf72.

**CONCLUSION**

Our finding that treatment of Aβ25-35 increases the transcriptional and translational levels of C9orf72 in PC12 cells reveals potential roles for C9orf72 in AD progression. We found that overexpression of C9orf72 significantly aggravated Aβ25-35-induced cell apoptosis. Moreover, we identified pathways by which C9orf72 regulates apoptosis induced by Aβ. Our findings provide novel insights into C9orf72 functions and have im-
plications for understanding the pathomechanism in AD patients with abnormal C9orf72 expression.

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