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Downregulation of miR-33 Has Protective Effect Against $A\beta_{25-35}$ -Induced Injury in SH-SH-SY5Y Cells

Authors' Contribution:

Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1 **Xiaoping Wang**

ABCDEF 1 **Xiaoja Li**

ACDF 1 **Bin Huang**

ABEF 1 **Lili Yang**

BCD 1 **Kai Chen**

AEF 2 **Dongdong Zhao**

ABCD 1 **Xiangdong Luo**

BCDEF 3 **Yingji Wang**

1 Department of Neurology, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, Chengdu, Sichuan, P.R. China
2 Department of Neurosurgery, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, Chengdu, Sichuan, P.R. China
3 Department of Geriatric Endocrinology, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, Chengdu, Sichuan, P.R. China

Corresponding Author: Xiaoja Li, e-mail: xiaojie1910@163.com

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Background: Alzheimer disease (AD) is a significant health issue for the elderly, and there are at present no clinically effective anti-AD agents. Prevention of $A\beta$ -induced neurotoxicity is proposed as a possible modality for treatment of AD. miR-33 has been proven to promote $A\beta$ secretion and impair $A\beta$ clearance in neural cells. The present study assessed whether miR-33 is involved in AD pathology.

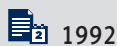
Material/Methods: miR-33 level was detected by qRT-PCR. The Akt/mTOR pathway was analyzed by Western blot analysis. Neuron inflammation and oxidative stress were measured using commercial detection kits. Flow cytometry and Western blot assay were conducted to assess cell apoptosis, and Western blot assay was used to assess synaptic protein levels.

Results: miR-33 expression level was markedly upregulated in SH-SY5Y cells treated with $A\beta_{25-35}$. miR-33 knockdown suppressed inflammation, oxidative stress, and cell apoptosis. In addition, miR-33 knockdown improved synaptic plasticity, and the protective effect of miR-33 knockdown was discovered through suppressing activation of the Akt/mTOR signaling pathway.

Conclusions: Taken together, these findings suggest that miR-33 knockdown protects against $A\beta_{25-35}$ -induced inflammation, oxidative stress, apoptosis, and synaptic damage by suppressing activation of the Akt/mTOR pathway.

MeSH Keywords: **Alzheimer Disease • Autophagy • Neurology**

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Background

Alzheimer disease (AD), characterized by memory loss and progressive cognitive dysfunction, is a common chronic neurodegenerative disease [1]. With the growing elderly population, the incidence of AD is increasing rapidly [2]. It is estimated that more than 47 million people worldwide have AD, making it a major global public health and social problem [3]. Thus, further in-depth study of AD is of great importance to medical research.

Senile plaques (SP), which are associated with excessive aggregation of amyloid-beta (A β) peptides, are the major manifestation of AD [4]. A β peptides accumulation is the primary driver of AD. It has been reported that aggregated and accumulated A β peptides can bring about hyperphosphorylation of tau protein and neurofibrillary tangles [5]. Evidence suggests that multiple factors, including apoptosis, inflammation, and oxidative stress, contribute to AD [6].

Autophagy is an essential degradation pathway in preventing protein misfolding or aggregation in mammalian cells [7]. Several studies have shown that defects in autophagy are major contributors to the etiology of AD [8]. Autophagy plays a vital role in generation and metabolism of A β , assembling of tau, and homeostasis and survival of neurons [7,9]. A substantial body of evidence demonstrates that the Akt/mTOR signaling pathway plays an important role in cell autophagy [10].

Synaptic plasticity is the basis for encoding and storing information in learning and memory. Alterations of synaptic plasticity can be observed in AD [11]. It is now widely accepted that neuronal autophagy is particularly important for synapse plasticity [12]. Normal function of autophagy is essential for the maintenance of local axon homeostasis under stress conditions [13]. The loss of synapse-related proteins, including PSD, NMDA receptor, and synapsin-1, is related to the severity of dementia in AD [14]. Pin1 is a peptidyl-prolyl isomerase, and the loss of Pin1 activity can result in aberrant synaptic structure and exacerbate synaptic degeneration [15].

MicroRNAs (miRNAs) are a class of non-coding RNAs with 18–22 nucleotides [16]. miRNAs play versatile biological functions through targeting the 3'-untranslated region (3'UTR) of their target mRNAs [17]. Recently, miRNAs have emerged as new potential therapeutic targets for neurodegenerative diseases [18]. Evidence supports that miR-33 is involved in many kinds of diseases [19]. Work by Kim et al. suggests that overexpression of miR-33 can promote A β secretion and impair A β clearance in neural cells, representing a potential therapeutic target for AD [20]. A study by Ouimet et al. found that miR-33 can inhibit apoptotic cell clearance through an autophagy-dependent mechanism [21]. Moreover, the miR-33-AMPK

axis can lead to BNIP3-dependent autophagy through the AKT/mTOR signal pathway [22].

Thus, the present study investigated whether miR-33 can affect apoptosis of neural cells and synaptic plasticity through the AKT/mTOR signal pathway.

Material and Methods

Cell culture

The human neuroblastoma cell line SH-SH-SY5Y was obtained from ATCC and maintained in DMEM basal culture medium supplemented with 10% FBS and 1% penicillin/streptomycin in a 5% CO₂ incubator at 37°C. To study the role of miR-33 in neurotoxicity, SH-SH-SY5Y cells (80–90% confluence) were pretreated with A β _{25–35} at a concentration of 20 μ M/L for 24 h and then transfected with miR-33 NC or miR-33 siRNA using Lipofactamine2000 (Invitrogen, Carlsbad, CA, U.S.A.).

qRT-PCR

QRT-PCR was performed to assess miR-33 mRNA level after treatment or transfection. The experiments were conducted according to routine protocols. Total RNA from SH-SH-SY5Y cells were extracted using TRIzol[®] reagent (Invitrogen, USA) and equal amounts RNA were transcribed into cDNA using a cDNA synthesis kit (Takara, Japan). qPCR was carried out using a SYBR Premix Ex Taq kit (Takara, Japan) in a Step One Plus[™] real-time PCR system (Applied Biosystems, Foster City, CA). The PCR was performed under the following reaction conditions: pre-denaturation at 95°C for 30 s, 35 cycles of 95°C for 10 s, and 60°C for 60 s. The 2^{- $\Delta\Delta$ Ct} method was used to calculate the relative expression of each gene, with GAPDH as an internal control. Each experiment was performed in triplicate and each value represents the average of 3 independent experiments.

Western blot assay

Total cellular proteins were harvested using RIPA lysis buffer (Beyotime, CN) supplemented with the proteinase inhibitor and phosphatase inhibitor, and the soluble protein concentration was quantified with a BCA assay kit (Beyotime, CN). Equal protein samples were separated by polyacrylamide gel electrophoresis, then the proteins were transferred onto PVDF membranes. The membranes were treated with the following primary antibodies overnight at 4°C: p-AKT, AKT, p-mTOR, mTOR, beclin1, Atg5, P62, Bcl-2, Bax, cleaved caspase3, caspase3, and GAPDH. On the next day, PVDF membranes were treated with HRP-conjugated secondary antibodies. The target proteins were normalized to GAPDH in an enhanced chemiluminescence system.

Immunofluorescence analysis

Immunofluorescence staining was performed to evaluate autophagy in SH-SH-SY5Y cells. After different treatments, cells were fixed with 4% paraformaldehyde. Then, the cells were permeabilized with 0.2% Triton X-100 and blocked with 1% BSA for 20 min at room temperature. The cells were then incubated overnight with anti-LC3II primary antibody at 4°C. After rinsing 3 times with PBS, corresponding secondary antibody was used for incubation. DAPI was used to stain nuclei. Cells were observed and photographed under a confocal microscope.

Enzyme-linked immunosorbent assay

Briefly, SH-SH-SY5Y cells at a density of 1×10^5 were subjected to the indicated treatments. Untreated cells were used as

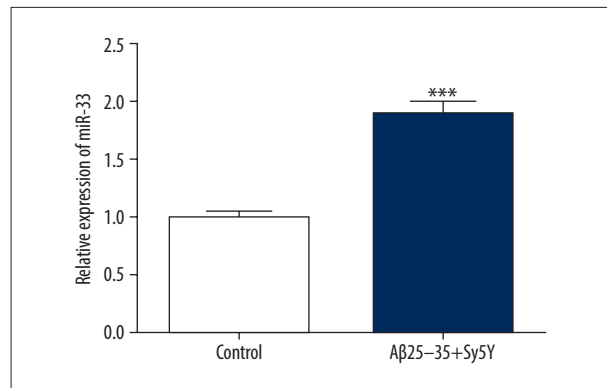
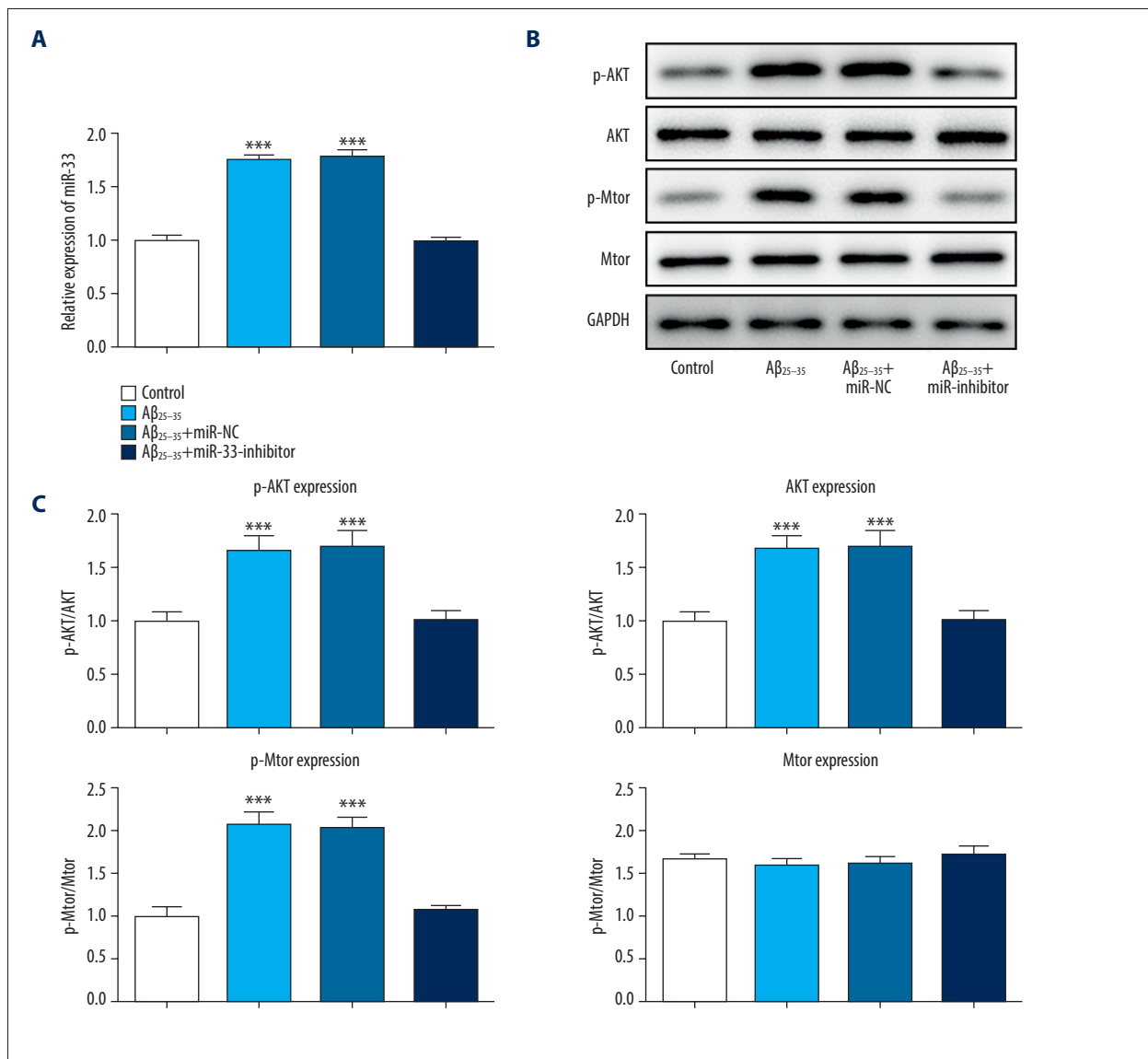


Figure 1. miR-33 is upregulated in Aβ₂₅₋₃₅-induced SH-SY5Y cells. miR-33 mRNA level was determined using qRT-PCR assay. *** P<0.001 vs. control group.



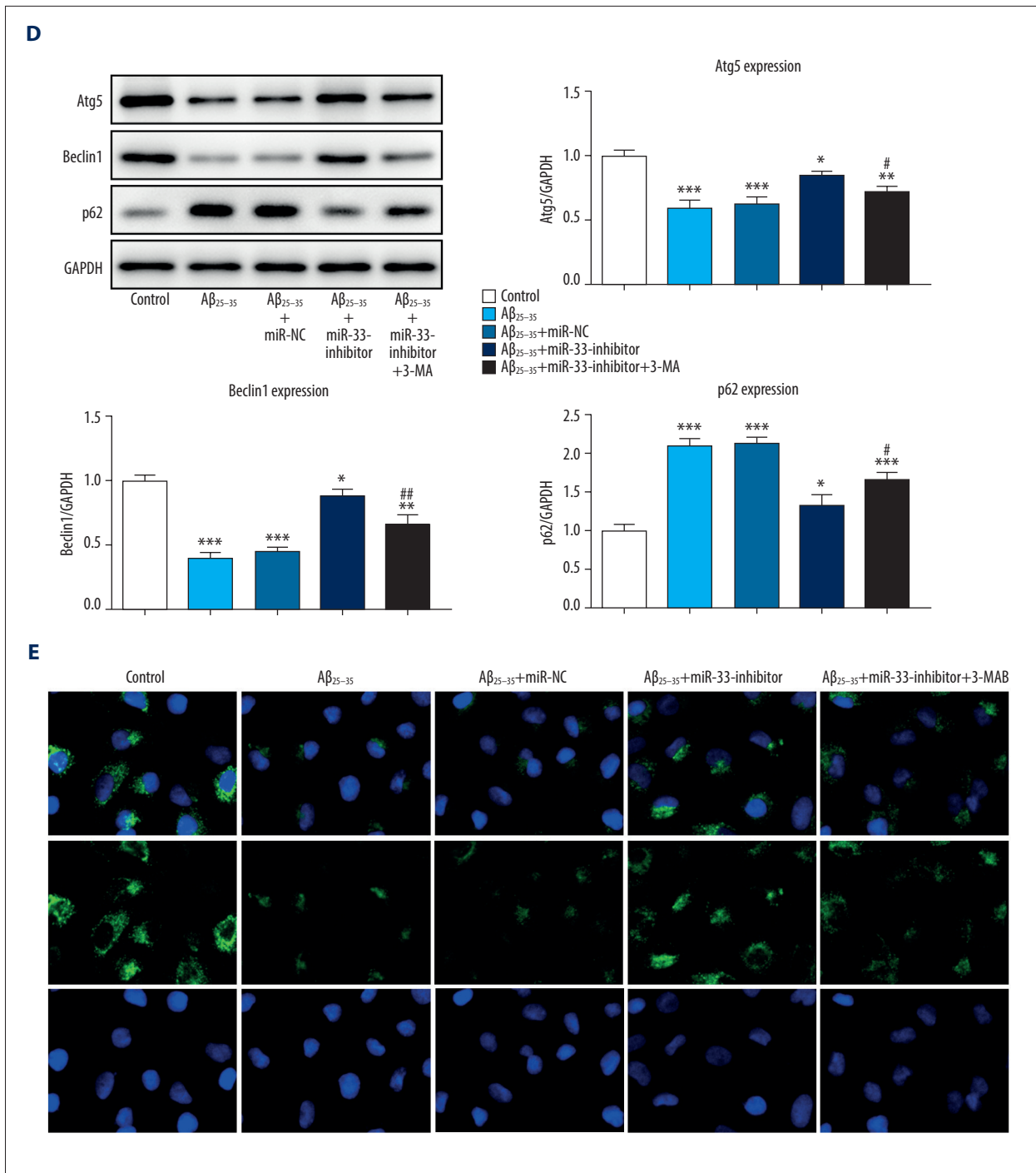


Figure 2. miR-33 knockdown promotes autophagy by suppressing Akt/mTOR pathway. **(A)** miR-33 level was determined using qRT-PCR assay. **(B, C)** The levels of Akt, p-Akt, mTOR, and p-mTOR protein were measured by Western blot assay. **(D)** Atg5, Beclin1, and p62 protein levels were measured by Western blot assay. **(E)** Immunofluorescence staining revealed the expression of LC3II. * P<0.05, *** P<0.001 vs. control group, # P<0.05, ## P<0.01 vs. Aβ₂₅₋₃₅ group.

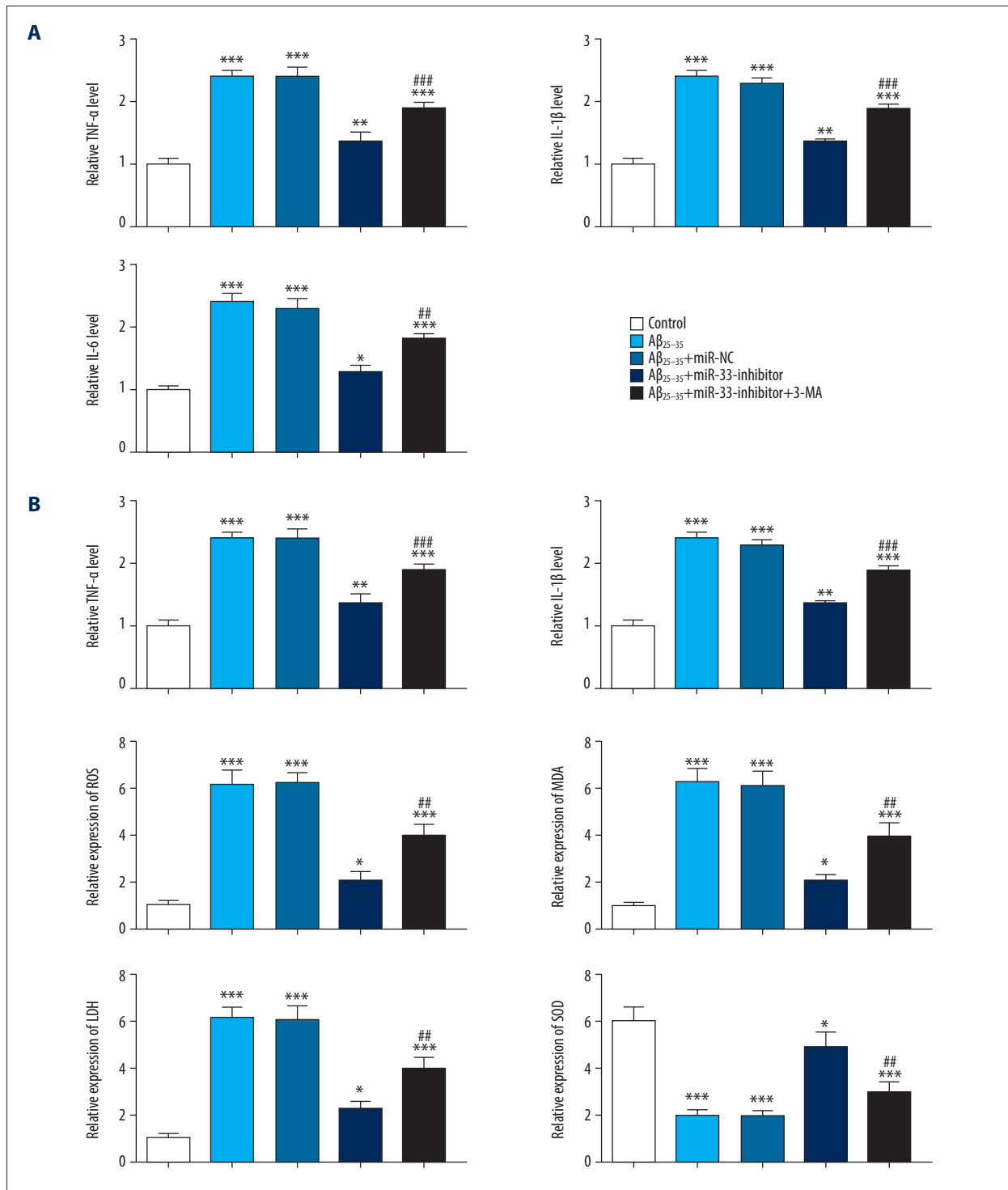


Figure 3. miR-33 knockdown inhibits inflammation and oxidative stress. **(A)** TNF- α , IL-1 β , and IL-6 protein levels were measured by ELISA. **(B)** ROS was assessed by DCFH-DA staining; Changes in MDA content and SOD and LDH activities were measured by commercial detection kits. * P<0.05, ** P<0.01, *** P<0.001 vs. control group, # P<0.01, ### P<0.001 vs. A β ₂₅₋₃₅ group.

the control. The concentrations of the inflammatory cytokines TNF- α , IL-1 β , and IL-6 in cell supernatant collected from each group were detected using an Enzyme-Linked Immunosorbent Assay kit (R&D Systems, USA) following the manufacturer's instructions. Absorbance at 450 nm was recorded in a microplate reader (Thermo Fisher Scientific, USA). ELISA was repeated 3 times.

Determination of intracellular ROS level

An ROS Assay Kit (Beyotime, China) was used to detect the ROS levels. After being washed with D-Hank's solution, cells at the density of 5×10^4 cells/ml were then incubated with DCFH-DA for 30 min at 37°C. Afterwards, cells were washed again with D-Hank's solution and then analyzed using flow cytometry.

Measurement of MDA, LDH, and SOD levels

Cells were collected and lysed. The supernatant was collected by centrifugation at 3000rpm for 15 min at 4°C. The MDA levels and the activities of LDH and SOD were determined using corresponding commercial detection kits (Nanjing JianCheng Institute of Biological Engineering, China) following the manufacturer's recommendations.

Apoptosis analysis assay

The annexin-V fluorescein isothiocyanate (FITC) apoptosis detection kit (Beyotime Inst., China) was used to detect cell apoptosis following the manufacturer's instructions. After indicated treatments, cells were harvested and re-suspended in cold binding buffer. Cells were double-stained with annexin-V FITC (10 μ L) and propidium iodide (PI) (5 μ L) for 15 min in the dark following the manufacturer's protocols. Subsequently, cell apoptosis was analyzed using a flow cytometer.

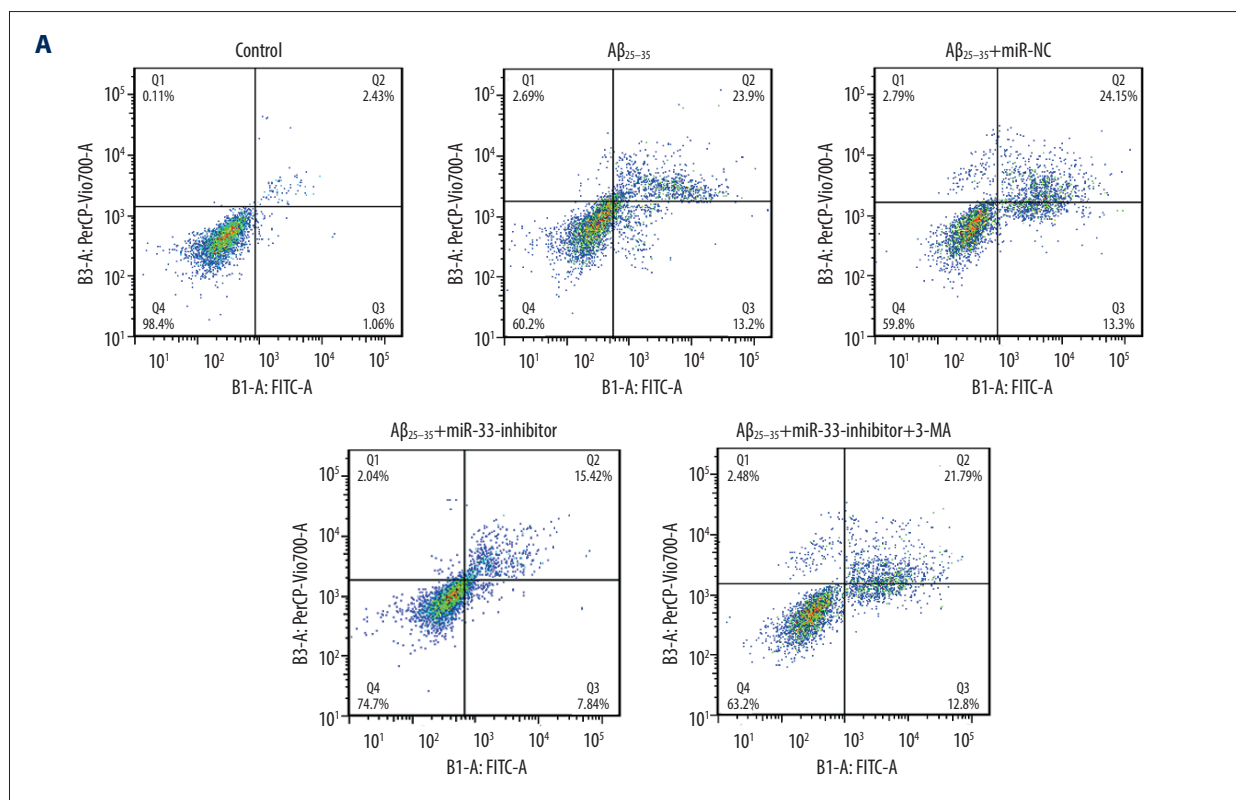
Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). Each group underwent at least 3 independent experiments, and all data are represented as the mean \pm SD. The comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. A statistically significant difference was defined as $p < 0.05$.

Results

miR-33 is upregulated in A β_{25-35} -treated SH-SY5Y cells

To investigate the effect of miR-33 in AD, qRT-PCR assay was first used to assess miR-33 expression. Data in Figure 1 revealed



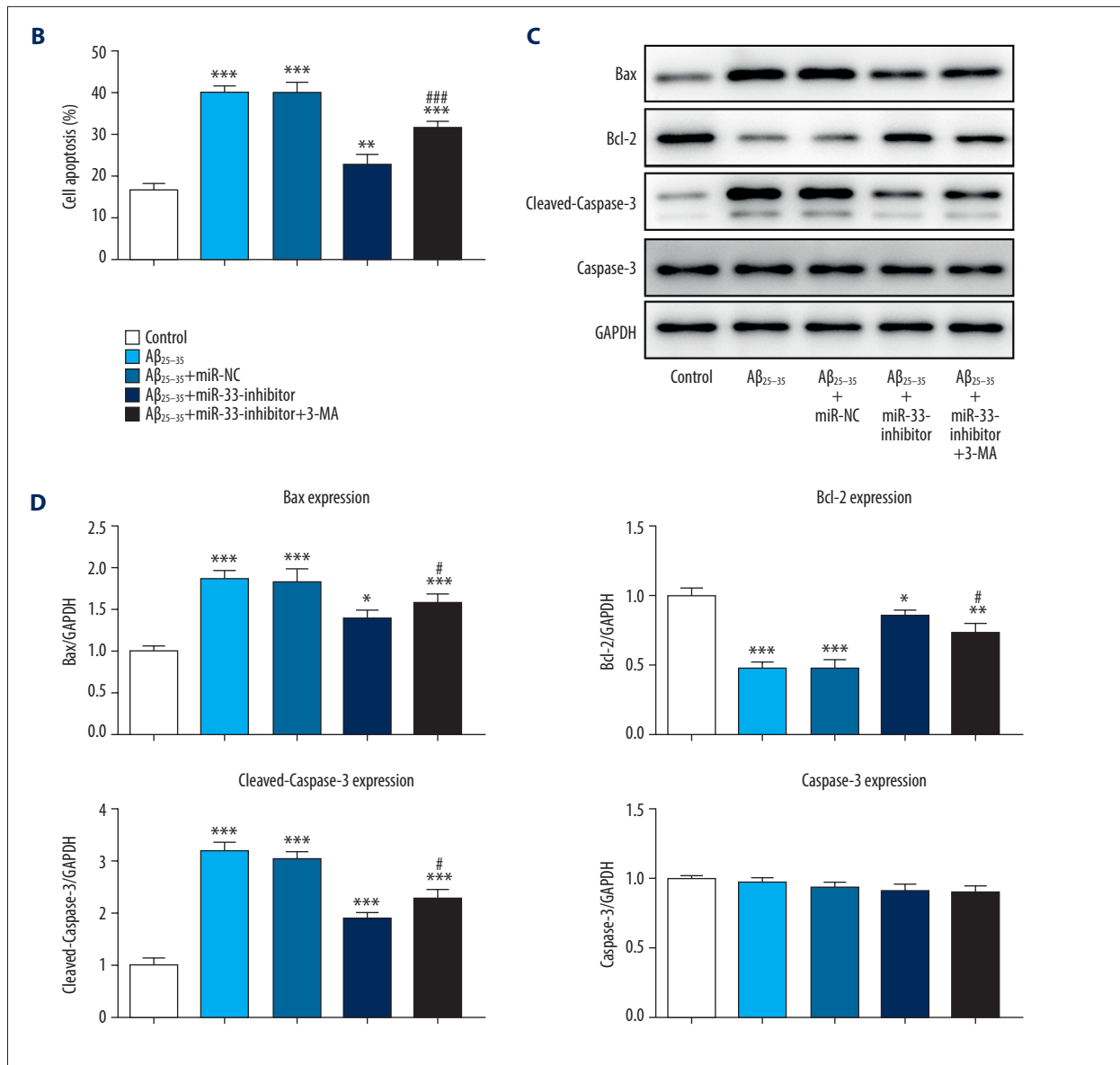


Figure 4. miR-33 knockdown suppresses cell apoptosis. (A, B) Cell apoptosis was determined using flow cytometry assay. (C, D) Bcl-2, Bax, cleaved caspase 3, and caspase 3 protein levels were measured by Western blot. * P<0.05, ** P<0.01, *** P<0.001 vs. control group, # P<0.05, vs. Aβ₂₅₋₃₅ group.

that the miR-33 level was markedly increased in Aβ₂₅₋₃₅-treated SH-SY5Y cells.

miR-33 knockdown promotes autophagy by suppressing Akt/mTOR pathway

To test the effects of miR-33 on the Akt/mTOR pathway, we transfected SH-SY5Y cells with miR-33 inhibitor and analyzed the Akt/mTOR pathway by Western blot assay. As shown in Figure 2A, SH-SY5Y cells were transfected with miR-33 inhibitor and showed significantly decreased miR-33 mRNA expression compared with the control group. The phosphorylation levels

of Akt/mTOR were visibly increased in Aβ₂₅₋₃₅-treated SH-SY5Y cells. However, miR-33 inhibitor down-regulated p-Akt/mTOR expression (Figure 2B, 2C). As expected, silencing miR-33 inhibited p62 and promoted Atg5 and Beclin1 expression, while the level of autophagy in the Aβ₂₅₋₃₅+miR-33 inhibitor group was reversed after treatment with 3-MA (autophagy inhibitor) (Figure 2D). The LC3II level was also measured by immunofluorescence assay. LC3II levels were significantly increased by miR-33 inhibitor at Aβ₂₅₋₃₅-stimulated conditions Figure 2E, and this effect was abolished by 3-MA.

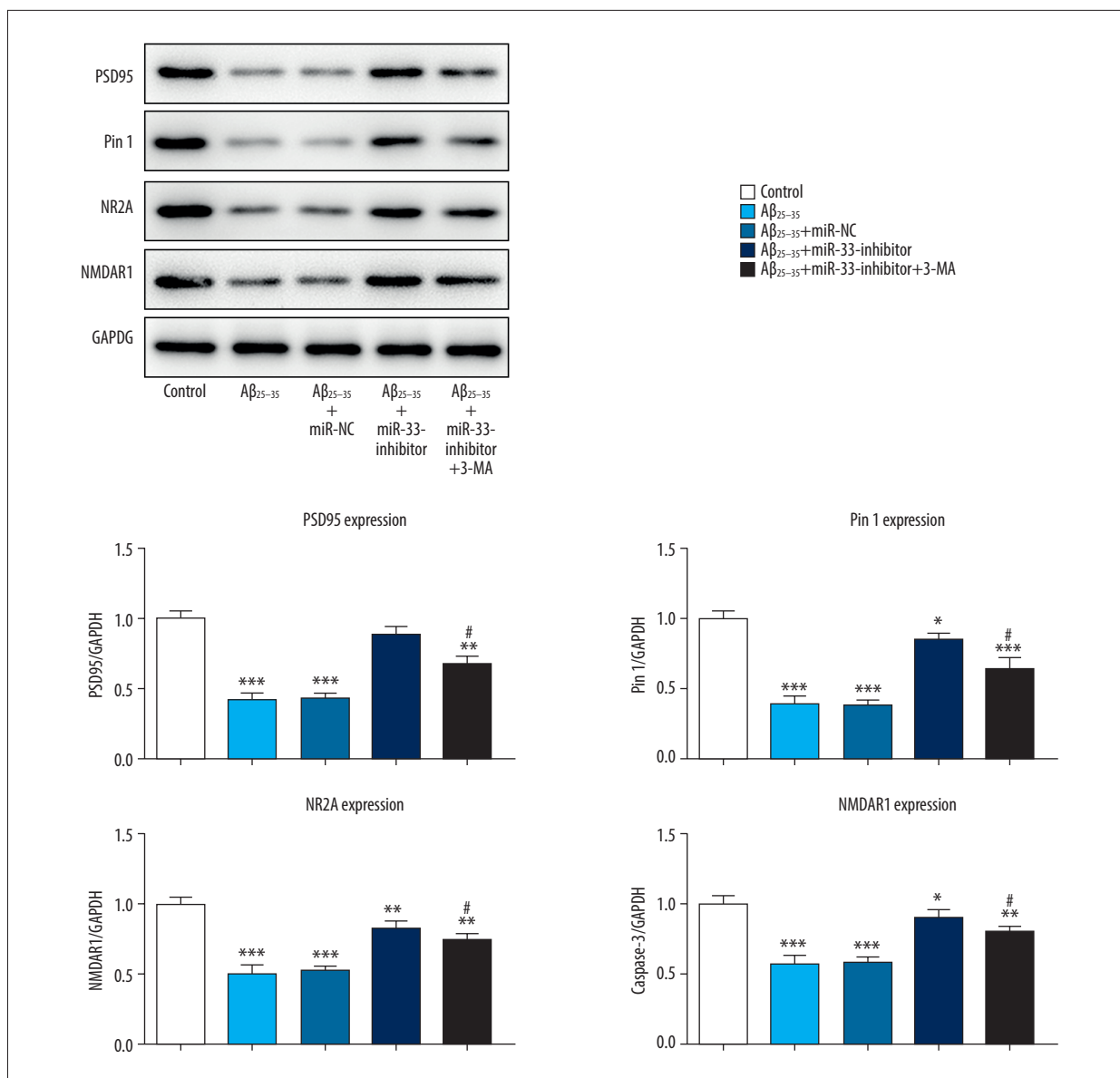


Figure 5. miR-33 knockdown improves synaptic plasticity by activating autophagy. PSD95, Pin1, NR2A, and NMDAR1 levels were detected by Western blot. * P<0.05, ** P<0.01, *** P<0.001 vs. control group, # P<0.05, vs. Aβ₂₅₋₃₅ group.

miR-33 knockdown inhibits inflammation and oxidative stress

To investigate the role of miR-33 in neuron inflammation and oxidative stress during AD, miR-33 inhibitor was used to evaluate the effect of miR-33 knockdown on inflammation and oxidative stress. Inflammation and oxidative stress were significantly enhanced by Aβ₂₅₋₃₅ (Figure 3A, 3B). Moreover, downregulation of miR-33 reduced Aβ₂₅₋₃₅ induced inflammation and oxidative stress. However, the cell inflammation and oxidative stress were evidently enhanced in the Aβ₂₅₋₃₅+miR-33 inhibitor+3-MA group compared with the Aβ₂₅₋₃₅+miR-33 inhibitor group. Taken together, these findings indicate that downregulation of miR-33

attenuates Aβ₂₅₋₃₅-induced neuronal inflammation and oxidative stress by inhibiting the Akt/mTOR pathway.

miR-33 knockdown suppresses cell apoptosis

To analyze the contribution of miR-33 knockdown to apoptosis, flow cytometry and Western blot assay were conducted. The results demonstrated that, in comparison with control group, Aβ₂₅₋₃₅ obviously promoted SH-SY5Y cell apoptosis, and silencing miR-33 suppressed apoptosis (Figure 4A, 4B). Furthermore, compared with the Aβ₂₅₋₃₅ group, miR-33 inhibitor drastically promoted Bcl-2 and inhibited Bax and cleaved caspase 3 expression (Figure 4C, 4D). On the contrary, the effect

of miR-33 knockdown on cell apoptosis was strikingly reversed by 3-MA treatment.

miR-33 knockdown improves synaptic plasticity by activating autophagy

To clarify whether downregulation of miR-33 was correlated with the improvement in synaptic plasticity, we investigated synaptic protein levels by Western blot assay. PSD95, Pin1, NR2A, and NMDAR1 levels were decreased by A β _{25–35} in SH-SY5Y cells (Figure 5). However, miR-33 interference increased the PSD95, Pin1, NR2A, and NMDAR1 levels. In addition, the result showed that 3-MA treatment inhibited the increase of synaptic protein expression.

Discussion

With the growth of the elderly population, the incidence rate of AD is increasing year by year [23]. There is a great need for novel strategies for early diagnosis and treatment of AD [24]. miRNAs can potentially serve as biomarkers and therapeutic targets for many psycho-neurologic disorders [25]. A previous study showed that miR-33 was a potential therapeutic target [20], but the mechanism is unclear.

At present, it is well established that A β has toxic effects on neurons in AD [26]. A β _{25–35} is a shorter toxic fragment with high cytotoxicity and enhanced neurotoxicity, and it has been widely used to establish AD models *in vitro* [27]. A recent study proved that miR-33 regulates secretion and clearance of A β in neural cells [20]. Similarly, we also found high expression of miR-33 in an A β _{25–35}-induced AD model *in vitro*. Autophagy is a lysosome degradation process that can remove and re-purpose damaged cytoplasmic contents and aggregated proteins to maintain cell homeostasis [7]. A report showed that mTOR reduced A β levels in neurons and improved A β -induced

cognitive deficits by regulating autophagy [28], indicating that the Akt/mTOR signal pathway might play a neuroprotective role in AD progression. The present findings suggest that miR-33 interference strikingly inhibits activation of the Akt/mTOR signal pathway and upregulates autophagy. Oxidative stress, inflammation, and the endogenous caspase apoptosis pathway have been implicated in A β neurotoxicity [6,26]. In this study, we uncovered the role of miR-33 in apoptosis, inflammation, and oxidative stress of neurons. The results indicated that downregulation of miR-33 caused a decrease in oxidative stress, inflammation, and apoptosis, while these trends were reversed by treatment with 3-MA (an autophagy inhibitor). Recently, synaptic plasticity deficits have emerged as the main cause of memory impairment in AD. Accumulated A β protein results in synapse deterioration [11]. The present study found that the expression of PSD95, Pin1, NR1, and NR2A in the A β _{25–35} group was significantly decreased, and miR-33 interference caused a significant increase in synapse-related proteins through regulating autophagy.

Conclusions

miR-33 interference can attenuate A β -induced neuronal injury, possibly via regulating autophagy through the Akt/mTOR signal pathway. Our study suggests miR-33 as a potential clinical research focus and a novel strategy for AD therapy.

Availability of data and materials

The analyzed datasets generated during the present study are available from the corresponding author on reasonable request.

Conflict of interest

None.

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