

miR-132 regulates the expression of synaptic proteins in APP/PS1 transgenic mice through C1q

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Abstract

Cognitive impairment in Alzheimer's disease (AD) is usually accompanied by synaptic loss in both the hippocampus and neocortex. In the early stage of AD, amyloid β ($A\beta$)-induced synapse changes is the main reason, while in the later stage, the accumulation of Tau protein promotes synapse degeneration as the key factor leading to dementia. MicroRNA (miRNA) is closely related to the expression changes of many AD-related genes. One of the most abundant brain-enriched miRNAs is miR-132, which has been shown to regulate both neuron morphogenesis and plasticity. It has been reported that miR-132 is significantly reduced in the brains of Alzheimer's patients. Genetic deletion of miR-132 in mice promotes $A\beta$ deposition, leading to impaired memory and enhanced Tau pathology, but how the miRNA-mediated gene expression dysregulation contributes to AD pathology remains unclear. Here we found the possible downstream target of miR-132 by *in silico* analysis, namely C1q. C1q is the primary protein of classical complement cascade, which is highly expressed in the synaptic regions of the central nervous system in Alzheimer's patients. However, it is not clear whether miR-132 plays a role in AD through regulating C1q. To address this question, the APP/PS1 transgenic mice were transfected with miR-132 and given C1 inhibitors. Behavior tests were conducted to assess memory and cognitive abilities seven days after administration. In addition, we analyzed the expression of PSD95, Synapsin-1 and phosphorylated (p)-Synapsin. We found that the expression levels of the synaptic proteins treated with miR-132 or C1INH were significantly increased compared with the AD group. Further RT-qPCR result suggested that miR-132 might regulate C1q expression in AD.

Introduction

Alzheimer's disease (AD) is a degenerative disorder in the central nervous system (CNS) characterized by progressive cognitive deficit and behavioral impairment. Pathological changes include amyloid plaque, neurofibrillary tangles, and neuronal reduction, which are closely related to the morphological and functional abnormality of neurons and glial cells. It is generally accepted that the combined presence of amyloid β ($A\beta$) and tau protein plays a key role in the AD pathogenesis, causing synaptic dysfunction and cognitive decline.¹ However, studies have shown that regional-specific synaptic loss may be more associated with cognitive decline in AD compared with amyloid plaque, neurofibrillary tangles, and neuronal reduction.^{2,3} Therefore, in the search for understanding the disease mechanism, research is moving increasingly into the role of synaptic proteins at different phases of AD.

Multiple lines of evidence implicate that microRNAs (miRNAs) modulate essential physiological processes, including neuronal differentiation, plasticity and regeneration, and fine-tune post-transcriptional gene expressions.⁴ miRNAs also control innate immune responses and acquired immune responses in mammalian immune systems.⁵ They are abundantly expressed in the CNS and play an important role in neural cell development, differentiation and synaptic plasticity.⁶ Indeed, aberrations in the expression of miRNAs in the CNS result in a variety of neurodegenerative disorders, particularly including Parkinson's and Alzheimer's diseases.^{7,8} Along these lines, it has been reported that miR-132 is a positive regulator of synaptic activity and plasticity, whose expression level is significantly increased in the early AD stage (Braak II phase),⁹ and significantly decreased in the later stage,¹⁰⁻¹² presenting the mode of "increased at first and then decreased". Interestingly, knockout of miR-132 gene in AD transgenic mice results in impaired memory, increased $A\beta$ expression, accumulated amyloid plaques, and hyperphosphorylation of tau protein.¹³ Furthermore, deletion of the miR-132 locus might reduce dendritic spine density and impair the integration of new neurons.^{14,15} Nevertheless, the miRNA-mediated downstream gene dysregulation in AD-induced aberrant synapse formation remains largely unknown.

In the present study, we found that miR-132 has a 3'UTR binding site for C1q mRNA through *in silico* analysis. C1q belongs to the classical complement cas-

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cade proteins, components of the innate immune system, which mediate developmental synaptic pruning, a key process in establishing precise synaptic circuits.¹⁶ Recent study shows that C1q was increased and involved in synaptic loss before apparent plaque deposition.¹⁷ When inhibited, the number of phagocytic microglia reduced and early synaptic loss was observed as well.^{16,18} In addition, the synaptic expression of AD patients in different areas of the brain, including cerebral cortex, hippocampus and dentate gyrus, decreased significantly, and the expression of C1q increased significantly. However, it is not clear whether miR-132 exerts its effect through regulating C1q expression in this process. To address this, we examined the effect of miR-132 on the expression of synaptic proteins in the APP/PS1 transgenic mice and further assessed whether it involved its possible downstream target C1q. The effect of their changes on learning and memory was also detected by Morris water maze test. Our results showed that the expression levels of the synaptic proteins in the temporal cortex of mice treated with miR-132 or the C1q inhibitor C1INH were significantly increased compared with the AD group.

Materials and Methods

Construction and validation of miR-132-3p lentivirus

The lentivirus-delivered miR-132-3p overexpression vector and miR-scramble control were designed and synthesized by Wanlei Biotech (Shenyang, China). The ability of the lentivirus vectors to express the gene of interest was evaluated by sequencing. The final titers used for the experiments were 1×10^8 Tu/mL.

Animals and grouping

Twelve adults male C57BL/6 mice and thirty-six APPswe/PS1dE9 (APP/PS1) transgenic mice were purchased from Liaoning Changsheng biotechnology Co., Ltd (Shenyang, China). The mice were 10 months old (25–30 g) and individually housed under a 12 h light-dark cycle (temperature: $25 \pm 1^\circ\text{C}$; humidity: $50 \pm 10\%$) with *ad libitum* access to water and food. The AD mice were randomly divided into three groups: AD group (n=12); AD+C1INH group (n=12); AD+miR-132 overexpression group (n=12), and the C57 mice served as the normal control group (n = 12). The experiments were in accordance with the guidelines for the Care and Use of Laboratory Animals from the National Institutes of Health, and were approved by the Animal Experimental Committee of China Medical University.

Stereotaxic intracranial injection

The lentivirus vector and the C1q inhibitor C1INH were microinjected into the right lateral ventricle of AD mice. Briefly, the mice were anesthetized with an intraperitoneal injection of 4% chloral hydrate (0.5 mL/100 g body weight) and mounted into a stereotaxic apparatus with a mouse adaptor (RWD Life Science Co., Ltd., Shenzhen, China). The skull was opened using a dental drill, and 2 μL lentivirus vectors were injected into the right lateral ventricle (AP: -2.0 mm, R: -1.5 mm, DV: -3.0 mm, according to bregma) and delivered for 5 min until the entire vector was administered. After another 2–3 min the needle was slowly withdrew. The mice in the control group was subjected to identical surgery. The GFP fluorescence was randomly observed 7 d after administration to verify whether miR-132 was successfully delivered throughout the brain.

Morris water maze behavior test

Seven days after the stereotaxic intracranial injection, Morris water maze (MWM) test was performed as described in our previous study.¹⁹ The MWM consisted of a black circular pool (120 cm in diameter,

divided into four quadrants) filled 2/3 with water ($23 \pm 1^\circ\text{C}$) and additional Titanium dioxide powder. A platform was submerged approximately 2 cm below the water surface during the spatial learning trials. Mice were initially placed into a quadrant (excluding the one with the platform). The time that the mice found the platform was recorded (evacuation latency). If they could not find the escape platform in 60 s, the experimenters would gently guide the mice onto the platform and allowed them to stay there for 20 s. All mice were subjected to an MWM test for 5 consecutive days with 4 acquisition trials per day. On day 6, a probe test was carried out to evaluate spatial memory, where the platform was removed and they were placed at a new random position. The times of mice crossing the platform were calculated. When the MWM test was over, the mice were sacrificed for the following procedure.

Western blot analysis

The mice were anesthetized with an intraperitoneal injection of 4% chloral hydrate (0.5 mL/100g body weight) and immediately perfused transcardially with 0.9% NaCl. The temporal cortex was taken out and homogenized in lysis buffer for 30 min (Beyotime, Shanghai, China), and were homogenized for 20 min and centrifuged at 500 g for 30 min at 4°C . Protein extracts were processed with the following Western blot method. Briefly, Equal amounts of protein (50 mg) were resolved on a 4–12% Bis-Tris gel before transferred onto a polyvinylidene difluoride membrane, and blocked for 1 h at room temperature, in 5% skim milk powder solution. Next, membranes were incubated at 4°C with primary antibodies against PSD95 (1:500, Abcam, Cambridge, MA, USA), Synapsin-1 (1:500, Abcam), and p-Synapsin (1:500, Arigo Biolaboratories Corp., Shanghai, China) and GAPDH (1:5000, Abcam). After washing, the membranes were incubated with goat anti-rabbit IgG (1:5000, Proteintech Group, Inc., Rosemont, IL, USA) secondary antibody for 1 h at 37°C . Blots were imaged with a chemiluminescence imaging system (Bio-Rad, Hercules, CA, USA) and measured with Image J v1.80 software (NIH, Bethesda, MD, USA).

Immunofluorescence analysis

After anesthesia, mice were immediately perfused transcardially with 4% paraformaldehyde (PFA). The brains were fixed in 4% PFA for 24 h after perfusion, and then soaked in 15%, 25% and 30% sucrose for 24 h, 24 h and 48 h. Next, they were immersed in OCT compound and frozen at -80°C . After rinsing with PBST for three times, sections were incubated in blocking

solution containing 0.2% Triton X and 5% goat serum for 40 min. Then they were incubated with primary antibody for the detection of PSD95 (1:200, Abcam), Synapsin-1 (1:200, Abcam), and p-Synapsin (1:200, Arigo) at 4°C overnight. Rabbit IgG monoclonal Isotype Control (1:200, Abcam) served as negative control. On the second day, sections were incubated in Goat Anti-Rabbit IgG (H&L) Cy3 secondary antibody (1:500; Abcam) for 2 h, followed by incubation with DAPI (1 $\mu\text{g}/\text{mL}$; Boster Bio, Wuhan, China) for 5 min. Images were captured using a fluorescence microscope (BX51, Olympus, Japan) and the immunopositive cells was analyzed by ImageJ software (version 1.52, NIH, USA) to calculate the calibrated total fluorescence with this formula: Integrated density - (Area \times Mean background fluorescence), and then normalized to achieve fold changes (% of control).

Real-time RT-PCR (RT-qPCR) analysis

Total RNA from mice temporal cortex was extracted using Total RNA Isolation Kit (Vazyme Biotech, Nanjing, China) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized by a HiScript II Q RT Super Mix for qPCR (+gDNA wiper) Kit and miRNA 1st Strand cDNA Synthesis Kit (Vazyme Biotech), and RT-qPCR was performed using ChamQ Universal SYBR qPCR Master Mix and miRNA Universal SYBR qPCR Master Mix (Vazyme Biotech). Relative miR-132 levels were calculated following a delta delta Ct method, and Ct represents the cycle threshold. The primer sequence of mir-132: 5'—CGCG-TAACAGTCTACAGCCA—3' (forward); 5'—AGTGCAGGGTCCGAGGTATT—3'(reverse); C1q1: 5'—GCACGGCCACC-TATACCAC—3' (forward); 5'—CACGTCGTCAAACCTTGAGCA—3' (reverse); GAPDH: 5'—GGTGAAG-GTCGGTGAACC—3' (forward); 5'—CTCGCTCCTGGAAAGATGGTG—3'(reverse); U6: 5'—CTCGCTTCG-GCAGCACA—3'(forward); 5'—AACGCTTCAACGAATTTGCGT—3'(reverse).

Statistical analysis

Statistical analyses and calculation of p values were performed using Prism software (version 5.0, GraphPad Software Inc., USA). Student's *t*-test was used for pairwise comparisons, and one-way ANOVA followed by Tukey test was used for multiple comparisons. The escape latencies were analyzed by repeated measures ANOVA with *post-hoc* Holm-Sidak test. A value of $P < 0.05$ was considered to indicate statistical significance.

Results

miR-132 was highly expressed in the temporal cortex following transfection of miRNA-132

To detect the transfection efficiency of miR-132 delivered in the lateral ventricle, we first examined the intensity of GFP fluorescence, for the virus carries a GFP reporter, and all the successfully transfected cells expressed GFP (Figure 1A). The control group received LV-null. Moreover, real-time RT-PCR results showed that the expression of miR-132 in the temporal cortex was significantly increased compared with the AD group ($P < 0.05$) (Figure 1B).

Intracerebral injection of miR-132 improved the cognitive performance of APP/PS1 mice

To determine whether the injection of miR-132 mimic can ameliorate the memory deficit in APP/PS1 mice, we performed behavioral tests with Morris water maze. During training, spatial learning was assessed by the amount of elapsed time before the animal climbs onto the platform to escape the water (escape latency). The training time was composed of 1-min periods over 5 days. The escape latency and the track distance of the AD+C1INH and AD+Mir-132 rats were significantly decreased on days 2–5 compared with the AD group (Figure 2 A,B). In the probe trial, we

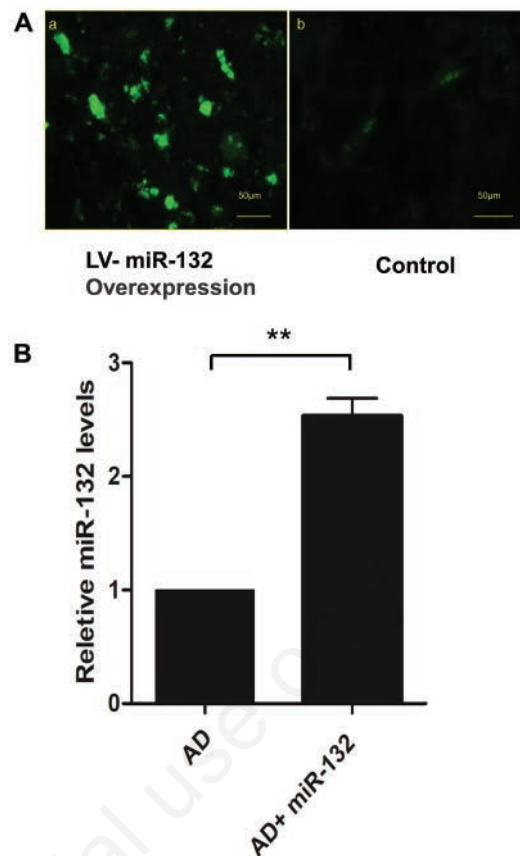


Figure 1. miR-132 was highly expressed in the temporal cortex following transfection of miRNA-132 (A). GFP-miR-132 was observed 14 days after intracerebroventricular injection of lentivirus (a); the control group received LV-null (b). Expression of miR-132 was analyzed by RT-qPCR (B). Data represent the mean \pm SD. ** $P < 0.01$ compared with AD group.

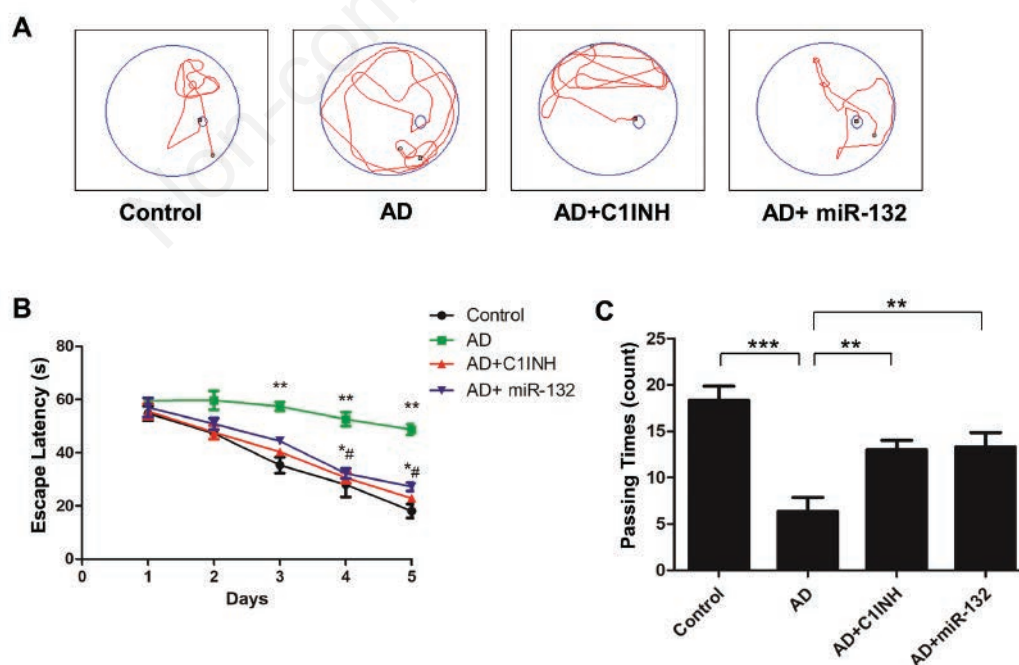


Figure 2. Representative trajectory of each group in place navigation test (A). The escape latency of the four groups in place navigation test (B). ** $P < 0.01$ compared with control group; * $P < 0.05$, # $P < 0.05$ compared with AD group. Comparison of the crossing times of the 4 groups in space probe trial across the original platform (C). ** $P < 0.05$, *** $P < 0.001$ compared with AD group.

observed the times of mice crossing the platform. Among them, the time of AD+C1INH and AD+ Mir-132 groups crossing the original platform was significantly increased (Figure 2C).

PSD95, Synapsin-1 and p-Synapsin expression levels in the temporal cortex of mice were detected by Western blot

The expression changes in synapse-related proteins were measured in order to investigate whether miR-132 and C1INH expressions affected synaptic plasticity, as AD transgenic models showed reduction of PSD-95 and Synapsin-1 in dendrites of different brain regions. We found that either miR-132 overexpression or C1INH administration resulted in a significant increase in the expression of PSD95, Synapsin-1 and p-

Synapsin in the temporal cortex (Figure 3 A-D). The ratio of p-Synapsin over Synapsin-1 in these two groups also increased significantly (Figure 3E). In contrast, the expression of PSD95, Synapsin-1 and p-Synapsin in the AD group revealed decreased.

Immunofluorescent staining results

Representative graphs of immunofluorescent staining are shown in Figures 4A, 5A and 6A. PSD95, Synapsin-1 and p-Synapsin were observed on the cytomembrane. Quantitative analysis showed that the fluorescence intensity of PSD95, Synapsin-1 and p-Synapsin in the AD group was significantly lower than that of the control group. The fluorescence intensity in the C1INH and miR-132 groups was increased

compared with the AD group (Figures 4B, 5B and 6B).

Expression of C1q after transfection of miRNA-132

Based on the role of miRNAs in translational repression, inhibition of miR-132-3p via miRNA sponge principle could increase the expression of target gene. Bioinformatics analysis identified that the 3'-UTR of the C1q mRNA contained a highly conserved miR-132-3p target sequence (Figure 7A). To validate whether C1q is genuinely regulated by miR-132-3p *in vivo*, we performed RT-qPCR test. The result confirmed that the mRNA levels of C1q in the temporal cortex were increased in the AD group, and significantly decreased by overexpression of miR-132-3p (Figure 7B).

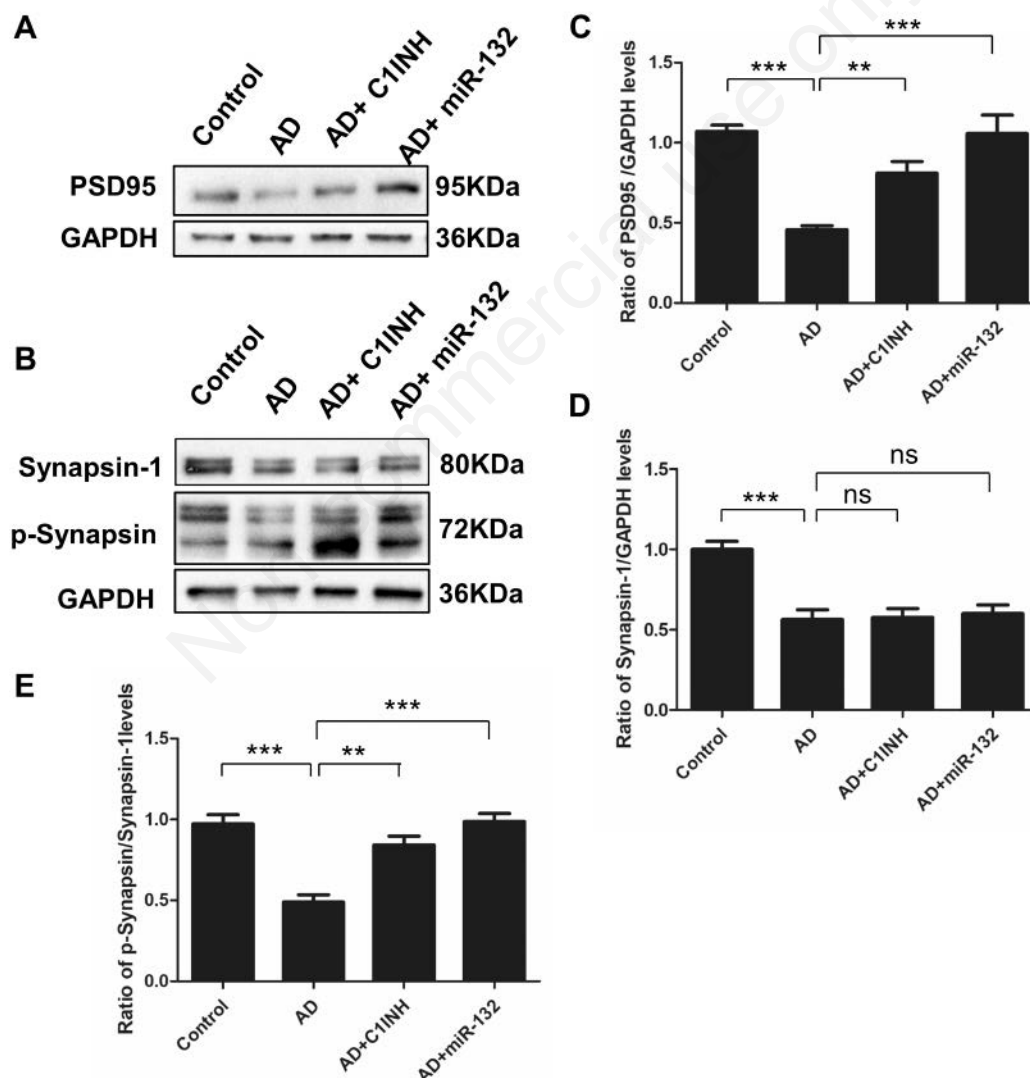


Figure 3. Fourteen days after intracerebroventricular injection of miR-132 lentivirus or C1q inhibitor, the expression of PSD95, Synapsin-1 and p-Synapsin in the temporal cortex was analyzed by western blotting (A and B). Data represent the mean±SD of four independent experiments. Comparison of PSD95 proteins in the four groups (C); Comparison of Synapsin-1 proteins in the four groups (D); The ratio of p-Synapsin over Synapsin-1 (E). **P<0.01, ***P<0.001 compared with AD group.

Discussion

In the present study, we first verified the transfection effect of lentivirus-delivered overexpression of miR-132-3p injected in the lateral ventricle by immunofluorescence analysis in an independent experiment. Further real-time RT-PCR results confirmed that the expression of miR-132 in the temporal cortex was significantly increased, indicating successful transfection. We also

showed that miR-132 mimic could ameliorate the memory deficit in APP/PS1 mice with Morris water maze test, which was partly in agreement with the previous study which indicated that down-regulation of miR-132 exhibited altered learning and probe phases in Barnes maze test.²⁰ Indeed, increasing evidence has shown that aberrant expression of miRNAs is involved in neurodegenerative diseases.⁹ Therefore, further screening of these miRNAs is inevitably

required to determine which miRNAs and downstream molecules are usually involved during disease development.⁹

MiR-132, one of the most functional miRNAs in the brain, is a highly conserved miRNA transcribed from human chromosome 17 by the transcription factor CREB.²¹ In mice, the miR-132/212 gene cluster is transcribed from the first intron of a non-coding transcript, AK006051, on Chromosome 11.²² miR-132 has been

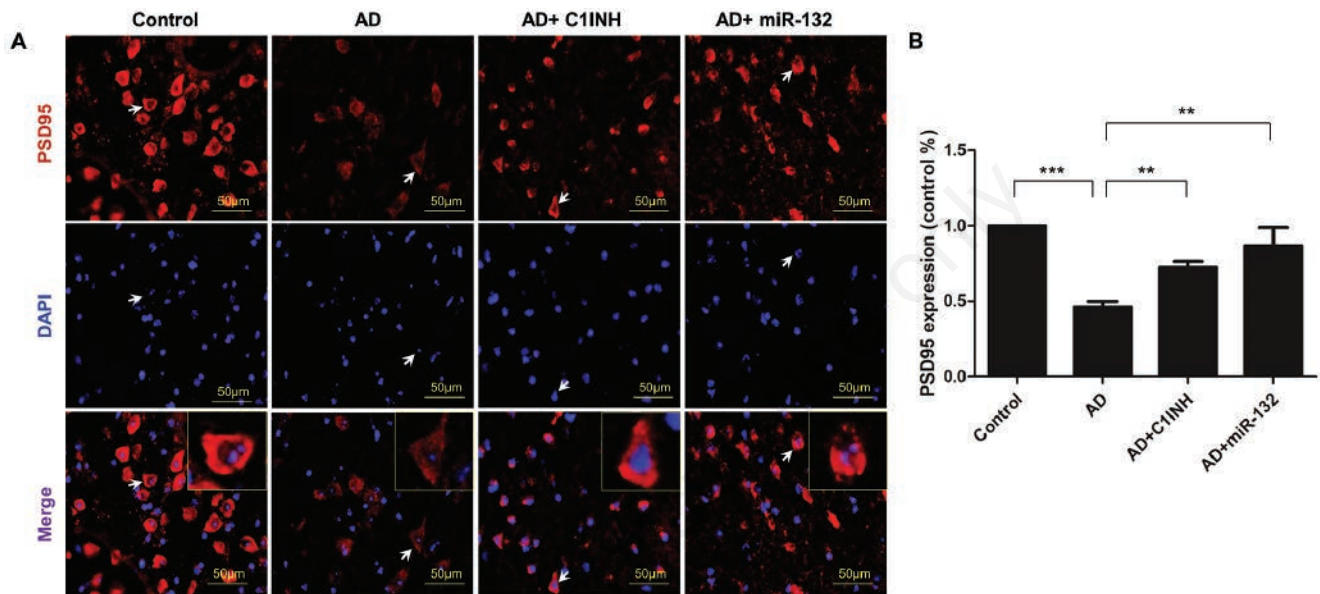


Figure 4. Representative images depicting the immunoreactivity of PSD95 in the temporal cortex (A) and the quantitative analysis of PSD95 expression (B). ** $P < 0.01$, *** $P < 0.001$ compared with AD group.

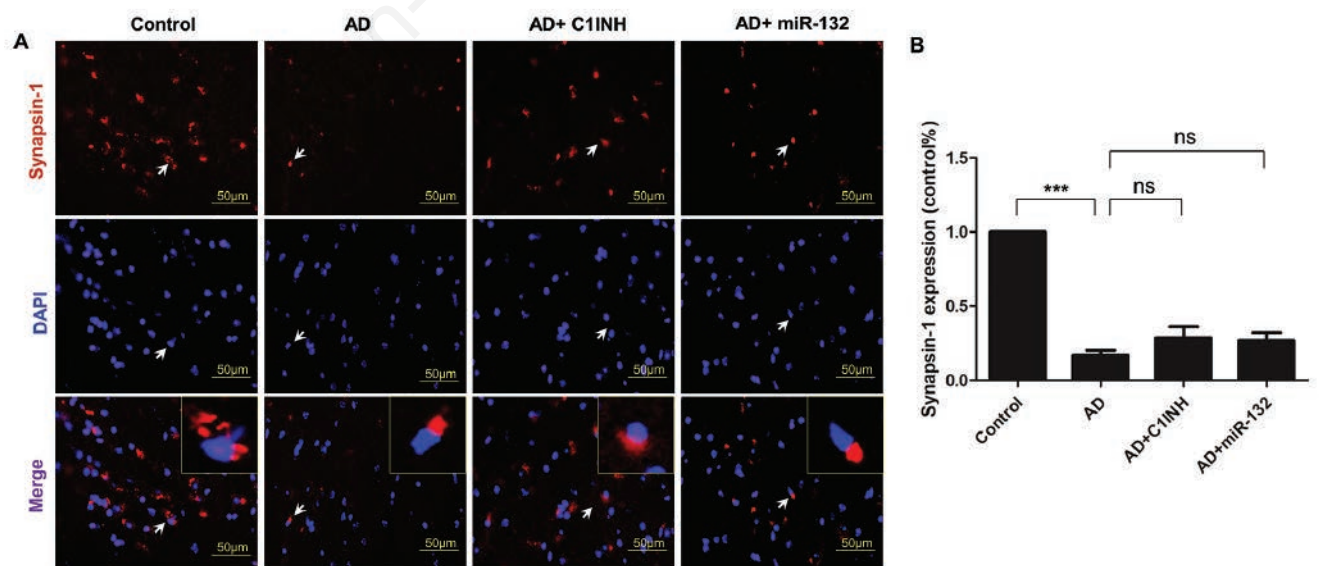


Figure 5. Representative images depicting the immunoreactivity of Synapsin-1 in the temporal cortex (A) and the quantitative analysis of Synapsin-1 expression (B). *** $P < 0.001$ compared with AD group.

revealed to play an important role in modulating dendritic morphology and plasticity of synaptic networks. It has been reported that miR-132 was down-regulated in post-mortem AD brain occurring even before abnormal aggregation and accumulation of A β and tau protein and neuronal loss.^{23,24} It

was induced in cultured neurons by neuronal activity and addition of neurotrophins, and was found to have a specific downstream target p250GAP.²⁵ Besides, several target genes and signaling molecules might be regulated by miR-132 neuroprotective functions. Through online TargetScan anal-

ysis, we correlated the down-regulated miR-132 with an up-regulated target in the CNS of the Alzheimer's disease.²⁶ Indeed, recent study showed that C1q, the key initiating protein of the classical complement cascade, was upregulated before overt plaque deposition and closely related to

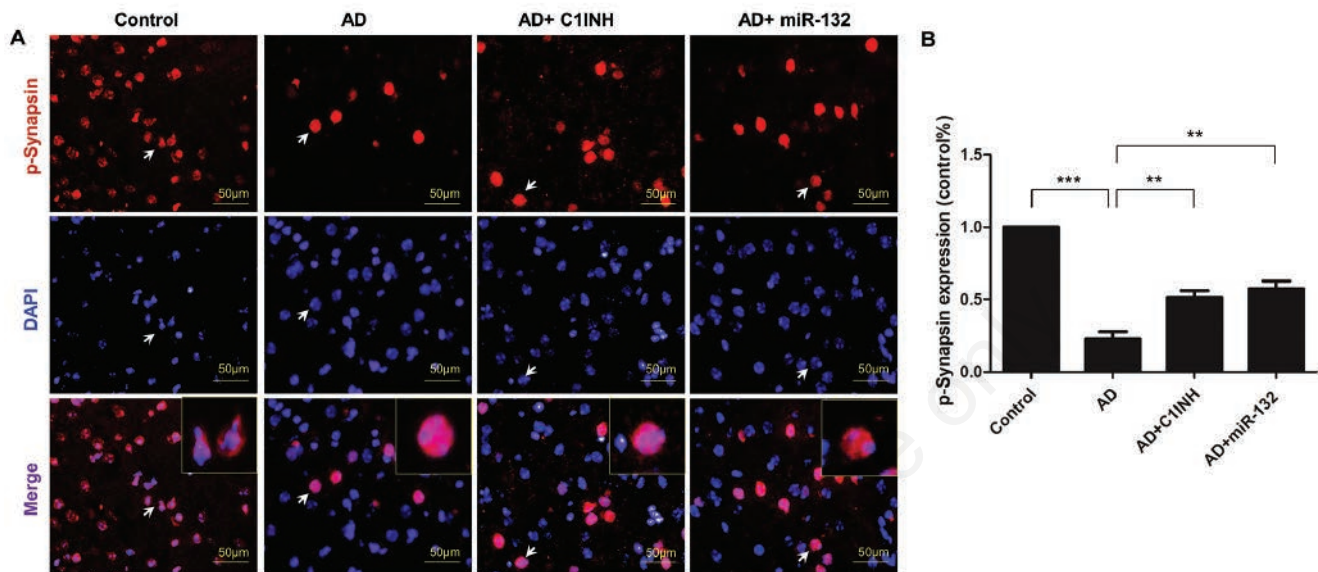


Figure 6. Representative images depicting the immunoreactivity of p-Synapsin in the temporal cortex (A) and the quantitative analysis of p-Synapsin expression (B). ** $P < 0.01$, *** $P < 0.001$ compared with AD group.

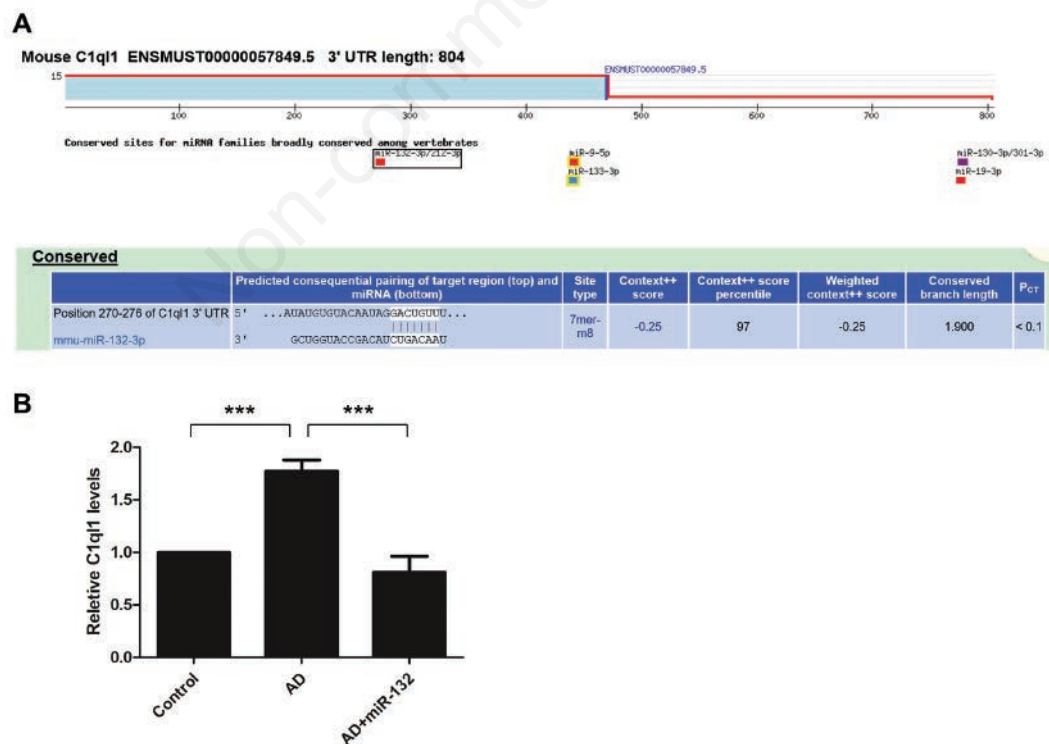


Figure 7. The 3'-UTR of the C1q mRNA was predicted to have a binding site with miR-132-3P through bioinformatics analysis (A). Expression of C1q in the temporal cortex was analyzed by RT-qPCR (B). Data represent the mean \pm SD of three independent experiments. *** $P < 0.001$ compared with AD group.

synapse modulation, and downregulation of C1q rescued early synapse loss.¹⁷ It is reported that C1q may have a potential detrimental effect on neuronal integrity *via* initiating an inflammatory response.²⁷ Zhang *et al.* found that complement C1 inhibitor was reduced in plasma biomarker analysis in AD patients.²⁸ In addition, C1q mRNA was shown to be upregulated in the neurons of AD patients, and dysregulated in other neurodegenerative diseases as well.^{29,30} Thus, it is interesting to know whether miR-132 regulates C1q expression. In this study, we found that inhibition of C1q with its inhibitor C1INH has a positive regulatory effect on cerebral synapsis proteins in AD mice. Then we speculated that C1q might be the possible downstream target of miR-132 by *in silico* analysis and thus C1q might be involved in this neuroprotection function of miR-132. Next through RT-PCR test, we confirmed that the mRNA levels of C1q in the temporal cortex were significantly decreased by overexpression of miR-132-3p, suggesting interaction between miR-132-3p and its validated target C1q through regulating synaptogenesis.

In recent years, synaptic plasticity deficits have become increasingly recognized as a cause of memory impairment and central players in the synapse deterioration characteristic of AD pathogenesis.³¹ The accumulation and deposition of A β in the brain is involved in the morphological and functional changes of synapses by disrupting related signaling pathways, thereby diminishing synapses.³¹ Damage to synaptic structure and plasticity in either hippocampus or cerebral cortex correlates with the severity of the neuropathy and memory deficits present in AD patients.³² Synapse-related proteins are important components in maintaining synaptic morphology and function. Their loss will result in the inevitable deterioration of neurological functions such as cognitive impairment. In AD, synaptic loss is closely related to the severity of dementia, and is mainly caused by mutant proteins in the presynaptic membrane or the postsynaptic densities (PSD), including Synapsin-1, PSD-95, SAP97, Synaptophysin, the NMDA receptor and AMPA receptor. The latter two are essential for glutamatergic synaptic transmission and play key roles in the maintenance of LTP and LTD,³³ exhibiting reduced surface levels and total levels in AD brains.³⁴ Although the involvement of various synaptic proteins in AD has been established, their roles are versatile and remain to be further verified. Here we focused on PSD-95, Synapsin-1 and its phosphorylation p-Synapsin, which are closely related to the onset of AD.

PSD-95, which is highly concentrated

at the PSD domain, has been characterized as one of the most abundant scaffolding proteins in the excitatory neurons and plays a key role in synaptic plasticity. Changes in PSD-95 levels result in alteration of synapse number, and therefore synaptic PSD-95 reduction *in vivo* indicates dendritic spine elimination.³⁵ Many consistent results demonstrated that overexpression of PSD-95 significantly increased the amplitude of AMPAR-mediated EPSCs in hippocampal neurons, while AMPAR EPSCs were decreased by knockdown of PSD-95 through RNA interference.³⁶

Synapsin-1 is one of the major phosphoproteins which regulate neurotransmitter release in the nervous system. The function of synapsin-1 is exerted through phosphorylation, though both phosphorylated and non-phosphorylated Synapsin-1 modulates neuronal development. The phosphorylation and de-phosphorylation cycle is important for neurotransmitter release from synaptic vesicle.³⁷ The present study found that the expression of PSD95, Synapsin-1 and p-Synapsin in the AD group was decreased significantly. Meanwhile, either miR-132 overexpression or C1INH administration resulted in a significant increase in the expression of PSD95, Synapsin-1 and p-Synapsin in the temporal cortex, and the ratio of p-Synapsin over Synapsin-1 in these two groups also increased significantly. Similarly, miR-132 loss resulted in increased ERK1/2 activity and elevated TAU phosphorylation in an AD mouse model.³⁸

Our findings, together with those from other groups, suggest a fundamental role of miR-132 in AD, and emphasize the downstream target C1q that might be of potential clinical interest for AD therapy. In particular, this study demonstrates that administration of C1q inhibitor C1INH exerts therapeutic effect in APP/PS1 mice. This effect can largely be attributed to the protective effect of miR-132 on synapses. In the light of this evidence, our study identifies C1INH as a novel synapto-protective candidate drug for repurposing potential in the clinical treatment of AD.

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