

# Electroacupuncture Reduces Scopolamine-Induced Amnesia Via Mediating the miR-210/SIN3A and miR-183/SIN3A Signaling Pathway

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## Research article

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# Abstract

**Background** The expression of SIN3A is closely correlated with EA treatment efficacy of SIA, but its underlying mechanisms remain to be further explored.

**Methods** Quantitative real-time PCR was performed to analyze the expression of candidate miRNAs and SIN3A mRNA in a rat model of SIA. Western blot was carried out to evaluate the differential expression of SIN3A proteins under distinct circumstances. Luciferase assay was used to explore the inhibitory role of certain miRNAs in SIN3A expression. NOR test was performed to assess the memorial ability of SIA rats undergoing EA treatment. Immunohistochemistry was carried out to evaluate the expression of SIN3A in the hippocampus of SIA rats.

**Results** Rno-miR-183-5p, rno-miR-34c-3p and rno-miR-210-3p were significantly up-regulated in SIA rats treated with EA. In addition, rno-miR-183-5p and rno-miR-210-3p exerted an inhibitory effect on SIN3A expression. EA treatment of SIA rats effectively restored the normal expression of rno-miR-183-5p, rno-miR-210-3p and SIN3A. The NOR test also confirmed the effect of EA treatment on the improvement of memory in SIA rats.

**Conclusion** In this study, an animal model of SIA was treated with EA to investigate its therapeutic effect. Moreover, our work presented solid evidence on the regulatory pathway of miR-183/SIN3A and miR-210/SIN3A in the pathogenesis of SIA.

## Introduction

Scopolamine is a strong amnestic compound with the ability to block the activity of muscarinic acetylcholine receptors to impair the memory and learning functions in mammals [1]. In fact, various amnesia models established by induction using scopolamine have been commonly used to screen potential drugs with the ability to enhance the memory [2].

Electroacupuncture (EA) was initially used in European countries such as Italy and France in the 19th century [3]. At this moment, EA has become a popular type of traditional Chinese medicines in the treatment of a wide range of medical conditions [4]. The electro stimulations of anatomical positions generated by EA can affect various physiological processes, such as angiogenesis, tissue regeneration and neuron activation, which may be promoted by the increased levels of expression of some genes in the nervous system induced by EA [5, 6].

MicroRNA (miRNA) belongs to a class of short non-coding RNA transcripts that can modulate the expression of target genes by either translational suppression or mRNA degradation. Since miRNAs can sustain repeated freeze-thaw cycles, they are superior to protein biomarkers in the diagnosis of many medical disorders. For example, one miRNA, miRNA-210, can increase the expression of VEGF to enhance the proliferation of vascular cells [7–10].

Highly expressed in non-neuronal as well as neuronal cells, SIN3A contains different domains, such as a paired helix domain, several core elements, a domain for HDAC interaction, as well as a highly conserved domain. Via the domain for HDAC interaction, SIN3A can bind to a core complex consisted of a RBAP46/48 protein with the ability to bind to histone, a SAP18/20 protein with the ability to stabilize the complex, as well as an HDAC2 enzyme [11]. In fact, the silencing of SIN3A in rats can rescue the impaired memory functions caused by the exposure to scopolamine. Furthermore, the silencing of SIN3A in rat hippocampus can also reduce the binding affinity of SIN3A to the promoter of IEG genes in neuron cells. In addition, the silencing of SIN3A expression in rats with amnesia elevated the level of H3K9 acetylation as well as the level of H3K14 acetylation in the promoter of IEG genes in neuron cells, thus restoring the memory functions in these rats [12].

It has been reported that EA treatment continuously increased the expression of several candidate miRNAs including rno-miR-183-5p, rno-miR-34c-3p, rno-miR-210-3p, rno-miR-758-5p, rno-miR-568, and rno-miR-196c-3p [13]. Meanwhile, SIN3A silencing was found to mitigate SIA [12]. In this study, we set up an animal model of SIA and treated it with or without EA to investigate the effect of EA on SIA.

## Materials And Methods

### Animal grouping and treatment

In this study, 20 Wistar male rats were acquired from the Center of Laboratory Animals at our institute. All rats were about 8 weeks in age and weighed 250 to 300 g. The animal protocols of this study have been approved by the Institutional Animal Ethics Committee. After 7 days of adaptation, the rats were divided into 3 groups, i.e., 1. SHAM group; 2. SIA group; and 3. SIA + EA group. All rats were placed in individual cages at  $22 \pm 1$  °C during the experiment. The establishment of the SIA model and corresponding EA treatment will be described below.

### Establishment Of An Sia Rat Model

Scopolamine hydrobromide (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% saline and then injected into rats via intraperitoneal injection at a dosage of 3 mg/kg. The injection was carried out once a day for seven days consecutively. The rats in the SHAM group were given the injection of 0.9% saline at the same dosage and the same frequency. After the 7 days of consecutive injection were finished, all rats were euthanized by CO<sub>2</sub> suffocation to collect their brain tissues for subsequent analyses.

### Ea Treatment

The treatment with EA was carried out immediately after the SIA rats gained full conscience after the induction of anesthesia. To carry out the EA treatment, the site of acupuncture, Renzhong, which is located at the junction beneath the nasal septum along the cleft lip midline, as well as the site of

Neiguan, which is located at the junction between the flexor carpi radialis and palmaris longus, were subjected to electro stimulation delivered via a 0.25 mm × 30 mm single use needle. During the EA procedure, the frequency of electro stimulation was set to 2 Hz, while the intensity of the electro stimulation was set to 3.0 mA. The EA stimulation was applied for 1 min in 12 h intervals until the rats were sacrificed.

## Novel Object Recognition (nor) Test

The NOR test was carried out to assess recognition memory according to a procedure described in (Singh and Thakur, 2014).

## Rna Isolation And Real-time Pcr

Samples of collected tissues and cultured cells were pulverized and then lysed using a QIAzol lysis buffer (Qiagen, Valencia, CA). Then, total RNA content was isolated from each sample by using a miRNeasy mini assay kit (Qiagen, Valencia, CA) according to manual instructions. Then, 2 µg of RNA in each sample was reversely transcribed into cDNA by using a Superscript II assay kit for reverse transcription (Invitrogen, Carlsbad, CA). Finally, real time PCR was carried out by using a Prism 7900 HT real time PCR system (Applied Biosystems, Foster City, CA) in conjunction with a SYBR green master mix (Applied Biosystems, Foster City, CA) following the instructions provided by the manufacturer. The relative expression of rno-miR-183-5p, rno-miR-34c-3p, rno-miR-210-3p, rno-miR-758-5p, rno-miR-568, rno-miR-196c-3p, and SIN3A mRNA was quantified using the  $2^{-\Delta\Delta CT}$  method.

## Cell Culture And Transfection

SH-SY5H cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Ham's F-12 medium added with 5% fetal bovine serum, 100 µg/ml streptomycin, as well as 100 U/ml penicillin. The cell culture was carried out in a 37 °C humidified incubator containing 95% air and 5% CO<sub>2</sub>. When cells were 80% confluent, they were transfected with one of 6 candidate miRNAs, i.e., rno-miR-183-5p (A), rno-miR-34c-3p (B), rno-miR-210-3p (C), rno-miR-758-5p (D), rno-miR-568 (E), or rno-miR-196c-3p (F), for 48 h before the mRNA and protein expression of SIN3A was detected. The transfection was carried out using Lipofectamine 2000 purchased from Invitrogen (Carlsbad, CA) following a routine transfection protocol.

## Vector Construction, Mutagenesis And Luciferase Assay

To clarify the regulatory relationship between SIN3A and rno-miR-183-5p, rno-miR-34c-3p, rno-miR-210-3p, rno-miR-758-5p, rno-miR-568 or rno-miR-196c-3p, the 3'UTR of SIN3A containing the binding sites for above miRNAs were respectively cloned into different pcDNA luciferase vectors (Promega, Madison, WI)

to produce vectors for wide type SIN3A 3'UTR. Then, site-directed mutagenesis was carried out using a Quick Change II site-directed mutagenesis assay kit (Stratagene, San Diego, CA) to generate mutant 3'UTR of SIN3A harboring the mutated binding sites for above miRNAs, respectively, which were also cloned into different pcDNA luciferase vectors to produce vectors for mutant SIN3A 3'UTR. In the next step, SH-SY5H cells were co-transfected with each of rno-miR-183-5p, rno-miR-34c-3p, rno-miR-210-3p, rno-miR-758-5p, rno-miR-568 or rno-miR-196c-3p in conjunction with wild type or mutant 3'UTR of SIN3A using Lipofectamine 2000. At 48 h post transfection, the luciferase activity of transfected cells was assayed using a Bright Glo luciferase kit (Promega, Madison, WI) following the instructions provided by the manufacturer.

## Western Blot Analyses

Samples of collected tissues and cultured cells were prepared into a homogenate and centrifuged to collect the protein supernatant. Then, after quantifying protein concentrations by using a BCA assay kit (Pierce Biotechnology, Rockford, IL), an appropriate amount of sample protein was resolved by 10% denaturing SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA), which were then blocked at room temperature for 1 h by using an Odyssey blocking reagent (Li-Cor, Lincoln, NE), incubated overnight in a 4 °C fridge with anti-SIN3A primary antibodies, and further incubation with appropriate secondary antibodies. The relative protein expression was analyzed by using an Odyssey imaging system (Li-Cor, Lincoln, NE).

## Immunohistochemistry Assay

Samples of collected rat brain tissues were sliced into 4 µm sections, fixed using PBS containing 4% paraformaldehyde, and quenched with 3% H<sub>2</sub>O<sub>2</sub> to remove the activity of endogenous peroxidase. Then, after being blocked for 1 h in 5% BSA, the sections were treated overnight in a 4 °C fridge with anti-SIN3A primary antibodies (1:4000 dilution, Abcam, Cambridge, MA). After washing and further incubation with biotin-labeled secondary antibodies (1:1000 dilution, Abcam, Cambridge, MA), the slides were incubated with HRP-tagged avidin/streptavidin and counterstained with DAPI before the positive expression of SIN3A was analyzed underneath a microscope.

## Statistical analysis

All experimental results were shown in means ± SD. The comparison of different groups was carried out using Student's *t* test. A difference was deemed statistically significant if its probability was < 0.05, i.e., *P* < 0.05. All statistical analyses were done using SPSS 21.0 (IBM, Chicago, IL) and Prism 6.0 (GraphPad, San Diego, CA).

## Results

### **Up-regulation of mo-miR-183-5p, mo-miR-34c-3p and mo-miR-210-3p in rats treated with EA.**

The expression of a group of miRNA candidates was evaluated in rats undergoing EA treatment. Among these miRNAs, the expression of rno-miR-183-5p (Fig. 1A), rno-miR-34c-3p (Fig. 1B) and rno-miR-210-3p (Fig. 1C) was significantly elevated in rats subjected to EA treatment. No obvious difference was observed for the expression of rno-miR-758-5p (Fig. 1D), rno-miR-568 (Fig. 1E) and rno-miR-196c-3p (Fig. 1F) between the two groups.

### **Rno-miR-183-5p and mo-miR-210-3p inhibited the luciferase activity of wild type SIN3A through binding to its 3' UTR.**

SIN3A expression is closely correlated with SIA. To further explore the relationship between miRNAs and SIN3A, luciferase vectors containing wild type and mutant SIN3A 3' UTR were established and co-transfected with candidate miRNAs to SH-SY5H cells. Rno-miR-183-5p (Fig. 2A) and rno-miR-210-3p (Fig. 2C) repressed the luciferase activity of wild type SIN3A, but had no inhibitory effect on mutant SIN3A. Rno-miR-34c-3p (Fig. 2B), rno-miR-758-5p (Fig. 2D), rno-miR-568 (Fig. 2E) and rno-miR-196c-3p (Fig. 2F) showed no effect in suppressing the luciferase activity of either wild type or mutant SIN3A vectors. These results demonstrated that rno-miR-183-5p and rno-miR-210-3p were able to inhibit the expression of SIN3A through targeting to the 3' UTR of SIN3A.

### **Rno-miR-183-5p and mo-miR-210-3p mimics suppressed the expression of SIN3A in SH-SY5H cells.**

The mimics of rno-miR-183-5p and rno-miR-210-3p were transfected into SH-SY5H cells to evaluate their inhibitory effect on SIN3A expression. In accordance with the results obtained from the luciferase assays, rno-miR-183-5p (Fig. 3A) and rno-miR-210-3p (Fig. 3C) mimics apparently repressed the mRNA and protein expression of SIN3A in SH-SY5H cells. Rno-miR-34c-3p (Fig. 3B), rno-miR-758-5p (Fig. 3D), rno-miR-568 (Fig. 3E) and rno-miR-196c-3p (Fig. 3F) showed no effect on SIN3A expression.

### **EA treatment improved the memory consolidation of SIA rats.**

Scopolamine treatment was performed in rats to induce amnesia, following with EA treatment. NOR test was carried out to assess the discrimination index (DI) of SIA rats. The time spent with novel objects and the DI in SIA rats were dramatically decreased as compared with those in the SHAM control, while the treatment with EA effectively restored the time spent with novel objects (Fig. 4A) and the DI (Fig. 4B) of SIA rats closer to the normal levels.

### **EA treatment restored the dysregulated expression of mo-miR-183-5p, mo-miR-210-3p and SIN3A in SIA rats.**

In rats suffering from scopolamine-induced amnesia, the expression of rno-miR-183-5p and rno-miR-210-3p was notably decreased, while the EA treatment remarkably recovered the down-regulated expression of rno-miR-183-5p (Fig. 5A) and rno-miR-210-3p (Fig. 5B) in SIA rats. However, the mRNA and protein

expression of SIN3A was obviously elevated in SIA rats, while the EA treatment remarkably attenuated the abnormal mRNA (Fig. 6A) and protein (Fig. 6B) levels of SIN3A.

### **EA treatment reduced the expression of SIN3A in the hippocampus of SIA rats.**

As amnesia is closely related to the hippocampus of brain, we further performed IHC on the hippocampus tissues of rat brain to analyze their expression of SIN3A. As shown in Fig. 7, IHC analysis revealed that SIN3A expression was apparently increased in SIA rats, while the treatment with EA down-regulated the expression of SIN3A in the brain of SIA rats. Furthermore, qPCR and Western blot analysis confirmed the up-regulated expression of SIN3A in the hippocampus of SIA rats, while the treatment with EA down-regulated the expression of SIN3A in SIA rats.

## **Discussion**

The dosing of scopolamine can induce the impairment of cognitive functions in experimental animals by changing the levels of oxidative stress in the brain of rats [14]. In addition, several compounds derived from plants, including DM, show promising efficacy in augmenting cognitive behaviors by blocking the functions of scopolamine. As a result, the above mentioned compounds have been tested in animal models to treat memory loss [15]. Interestingly, EA therapies can rescue long-term impairments of memory functions in rats suffering from dementia by protecting the integrity of neuron cells while reducing the expression levels of Noxa, bax, and P53 in rat hippocampus [16, 17]. These results suggested that several signaling pathways may be involved in the role of EA therapies in the treatment of vascular dementia [18]. In this study, we compared the expression of 6 candidate miRNAs in rats to evaluate the effect of EA on their expression. We found that rno-miR-183-5p, rno-miR-34c-3p and rno-miR-210-3p in rats treated with EA were significantly up-regulated. In addition, we performed an NOR test on rats suffering from SIA. We found that EA treatment improved the level of memory consolidation in SIA rats. Furthermore, we analyzed the expression of rno-miR-183-5p, rno-miR-210-3p and SIN3A in SIA rats, and showed that the EA treatment effectively prevented dysregulated expression of rno-miR-183-5p, rno-miR-210-3p and SIN3A in SIA rats. Growing evidence suggests that the treatment by EA can alleviate neuronal disorders caused by neurophathy [19, 20]. Jing et al demonstrated that the treatment by EA can apparently enhance memory as well as learning capacities. In two recent publications, Xue et al demonstrated that the treatment by EA can apparently enhance the ability of long term memory and learning in mice suffering from the AD disease [21–23]. Zhao et al demonstrated that the treatment by EA can reduce the time required to complete face recognition while decreasing the levels of psychological stress [24].

A miRNA, miR-210, may able to regulate the process of cell apoptosis as well as cell proliferation by directly reducing the expression level of SIN3A. In addition, the regulatory effect of miR-210 inhibitors on the process of cell apoptosis as well as cell proliferation could be blocked by silencing the mRNA expression of SIN3A, suggesting that miR-210 exerts its effects by regulating the expression of SIN3A. In addition, a cluster of miRNAs containing miR-183, miR-96, and miR-182 can enhance the ability of brain

learning by regulating the expression levels of a memory suppressor, i.e., protein phosphatase 1 (PP1) [25]. While the biogenesis process of multiple miRNAs highly expressed in neuron cells can be regulated by miR-183, miR-96, and miR-182, these miRNAs mainly exert their effects by elevating the expression levels of PP1 in the nuclear domain of cells [26]. In this study, we transfected SH-SY5H cells with miRNA mimics to evaluate their effect on SIN3A expression. Rno-miR-183-5p and rno-miR-210-3p mimics suppressed the expression of SIN3A in SH-SY5H cells. Moreover, we performed luciferase assay to explore the inhibitory effect of candidate miRNAs on SIN3A. Rno-miR-183-5p and rno-miR-210-3p inhibited the luciferase activity of SIN3A through binding to its 3' UTR.

As a large sized scaffold protein, SIN3A contains several domains of paired amphipathic helix as well as a HDAC interaction domain. The paired amphipathic helix domains in SIN3A are responsible for recognizing as well as binding to certain transcriptional factors including MAD and MAX. On the other hand, the HDAC interaction domain can dock HDAC1 proteins as well as HDAC2 proteins to SIN3A, so as to repress the expression of target genes of SIN3A [27]. In addition, the treatment using scopolamine can decrease the levels of acetylation in the promoters of H3K9 as well as H3K14 genes, while the silencing of SIN3A expression elevated the levels of acetylation in the promoters of H3K9 as well as H3K14 genes [28]. In addition, the silencing of SIN3A expression can also alter the levels of acetylation in the promoter of H4K12 gene to deregulate the expression of multiple genes involved in memory consolidation. Similarly, it was reported by Fischer et al that the induced acetylation of the H3K9 gene by environmental factors can restore the consolidation of spatial memory [12, 29].

## Conclusion

Here, we demonstrate that EC alleviates scopolamine-induced amnesia by regulating the miR-210/SIN3A and miR-183/SIN3A signaling pathways, i.e., by increasing the expression of miR-210/miR-183 while reducing the expression of SIN3A to restore memory functions in SIA rats.

## Declarations

### Conflict of interest

None

### Funding

The authors received no specific funding for this work.

### Data Availability

All relevant data are within the paper.

### Acknowledgements

None

## Author contributions

Huimin Hu and Zhengwen Yu conceived and designed the study. Fan Ye performed literature review, wrote the manuscript, produced the figures and compiled the table. Shiming Tian arranged the data and drew the figures. Huimin Hu and Zhengwen Yu supervised and handled correspondence. All authors read and approved the final manuscript.

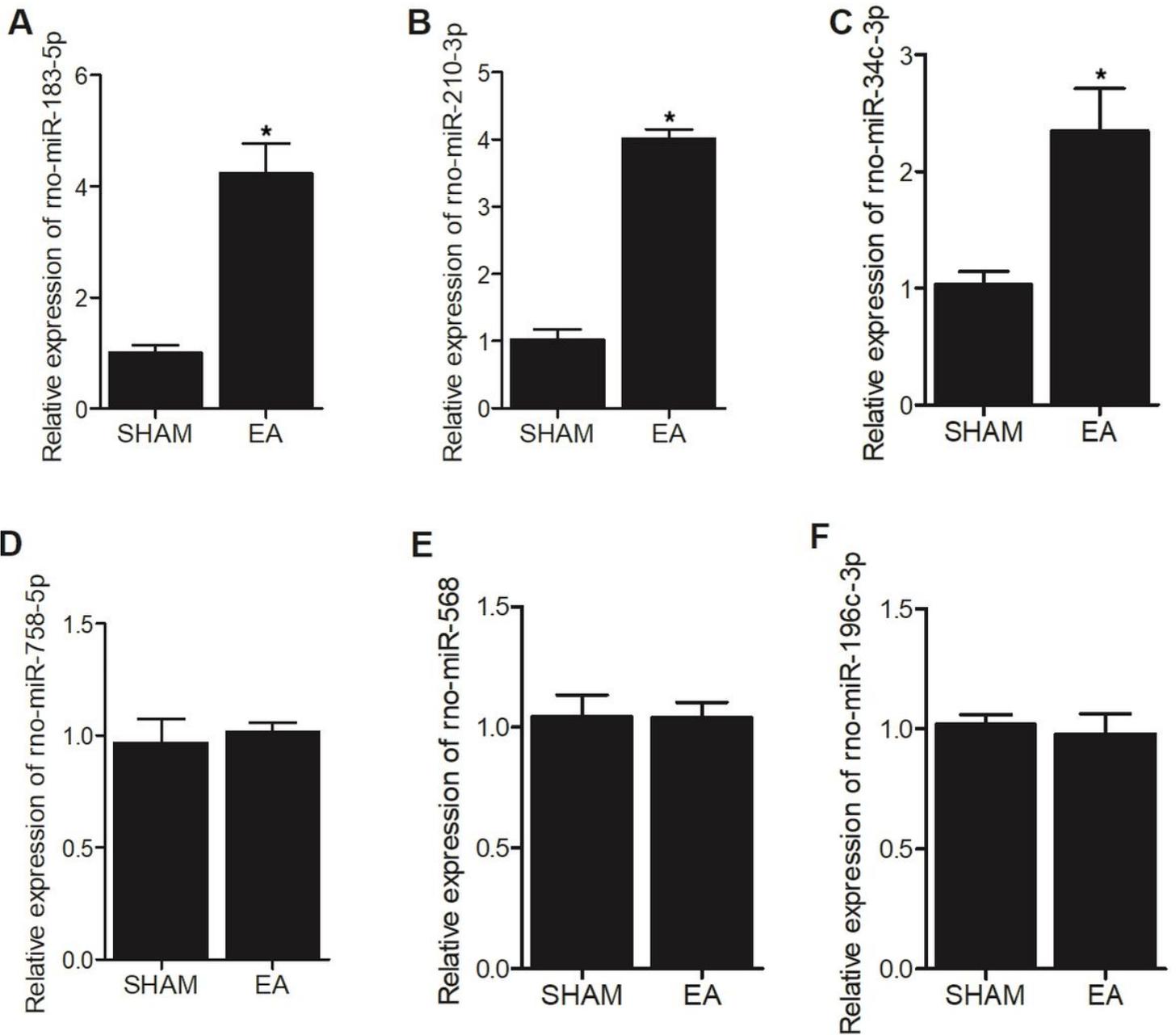
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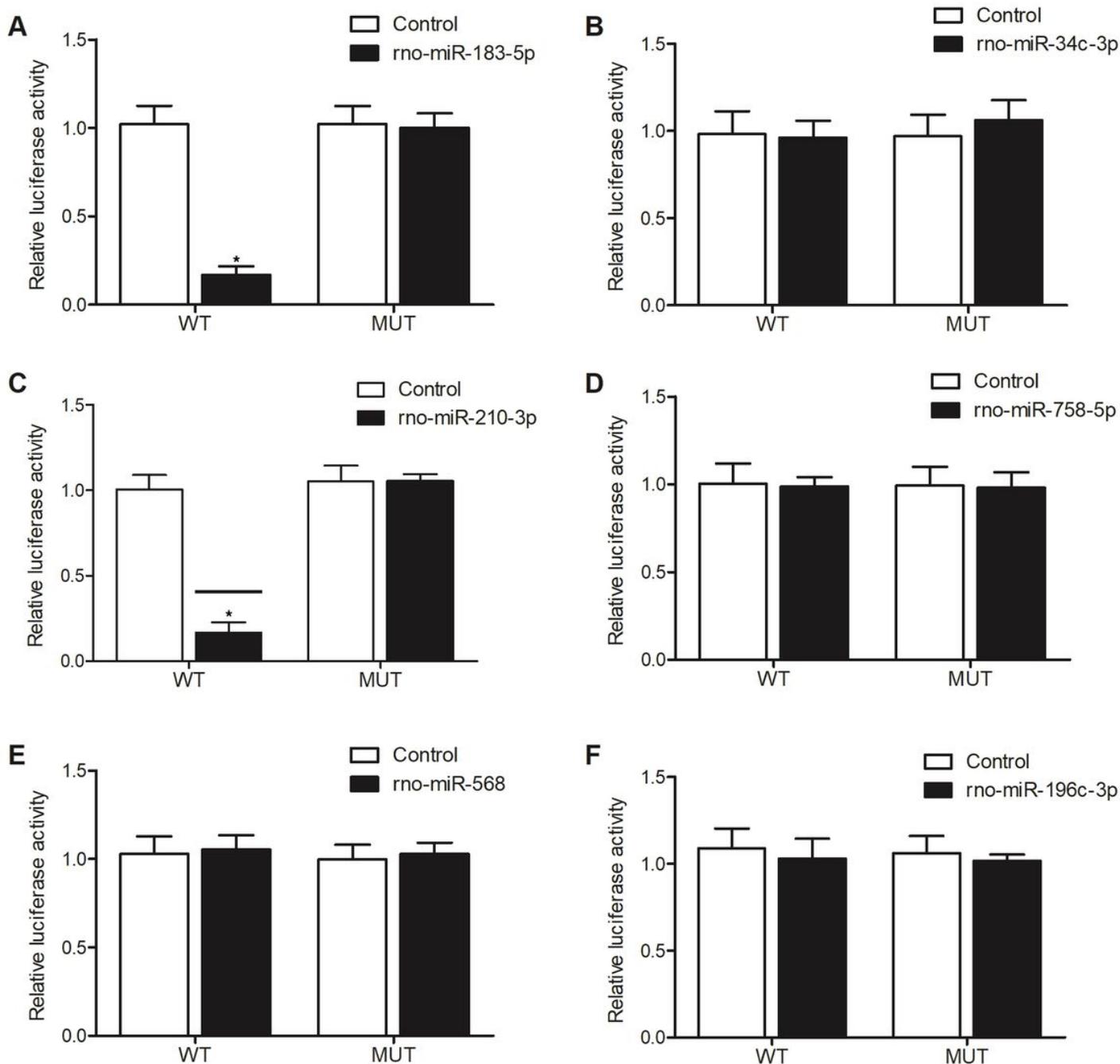
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## Figures



**Figure 1**

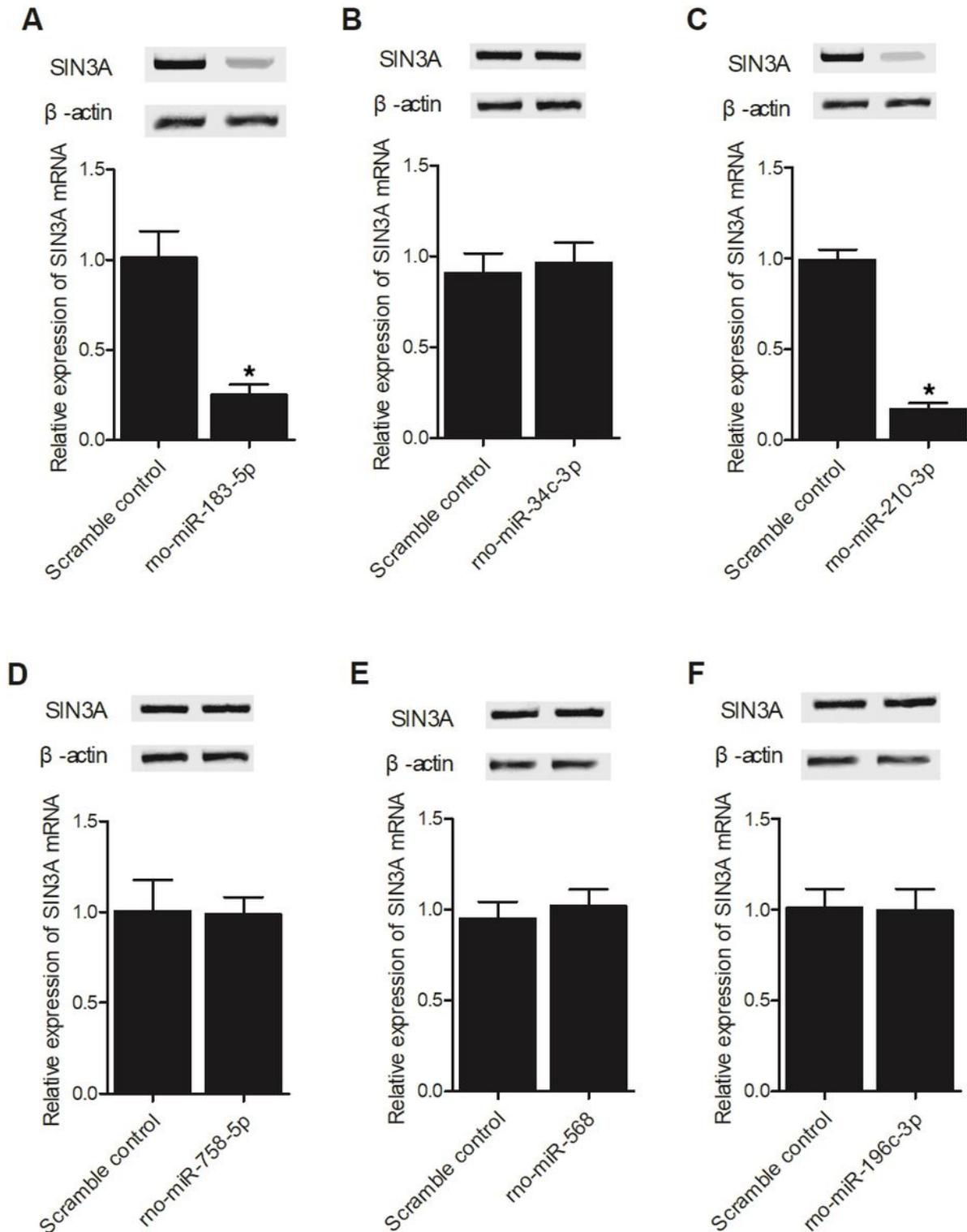
Rno-miR-183-5p, rno-miR-34c-3p and rno-miR-210-3p expression was enhanced in rats treated with EA. A: Increased expression of rno-miR-183-5p in rats treated with EA (\* P value < 0.05, vs sham group). B: Increased expression of rno-miR-34c-3p in rats treated with EA(\* P value < 0.05, vs sham group). C: Increased expression of rno-miR-210-3p in rats treated with EA (\* P value < 0.05, vs sham group). D: Rno-miR-758-5p expression remained unchanged in rats treated with EA. E: Rno-miR-568 expression remained unchanged in rats treated with EA. F: Rno-miR-196c-3p expression remained unchanged in rats treated with EA.



**Figure 2**

Rno-miR-183-5p and rno-miR-210-3p inhibited the luciferase activity of wild type SIN3A through binding to its 3' UTR. A: The luciferase activity of wild type SIN3A was inhibited by rno-miR-183-5p (\* P value < 0.05, vs control group). B: Rno-miR-34c-3p showed no inhibitory effect on the luciferase activities of wild type and mutant SIN3A. C: The luciferase activity of wild type SIN3A was inhibited by rno-miR-210-3p (\* P value < 0.05, vs control group). D: Rno-miR-758-5p showed no inhibitory effect on the luciferase activities of wild type and mutant SIN3A. E: Rno-miR-568 showed no inhibitory effect on the luciferase activities of

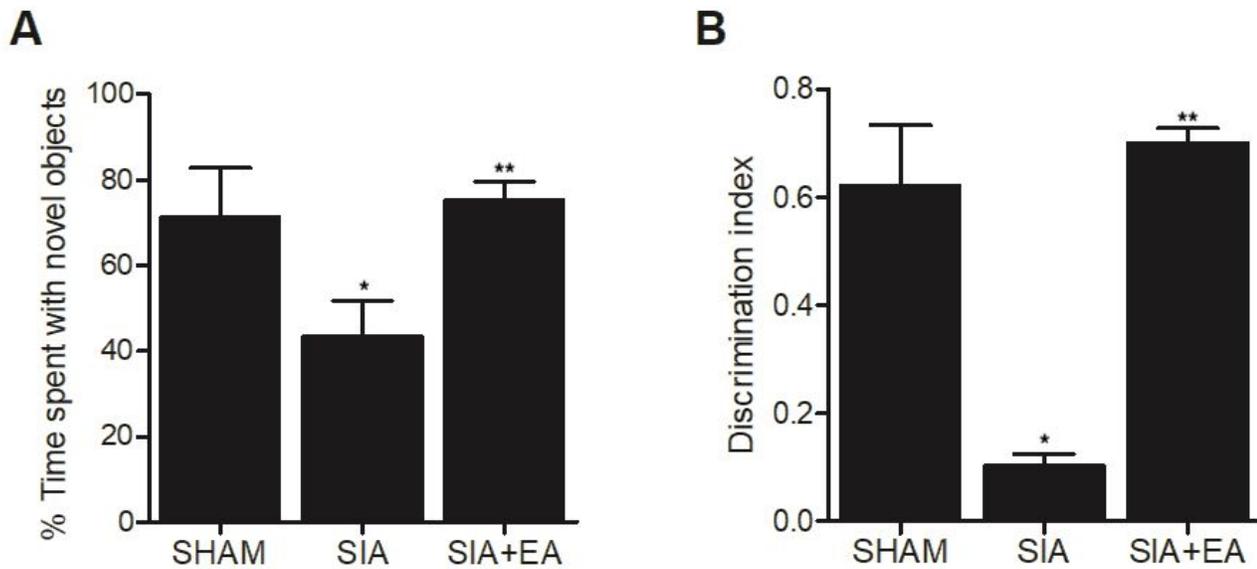
wild type and mutant SIN3A. F: Rno-miR-196c-3p showed no inhibitory effect on the luciferase activities of wild type and mutant SIN3A.



**Figure 3**

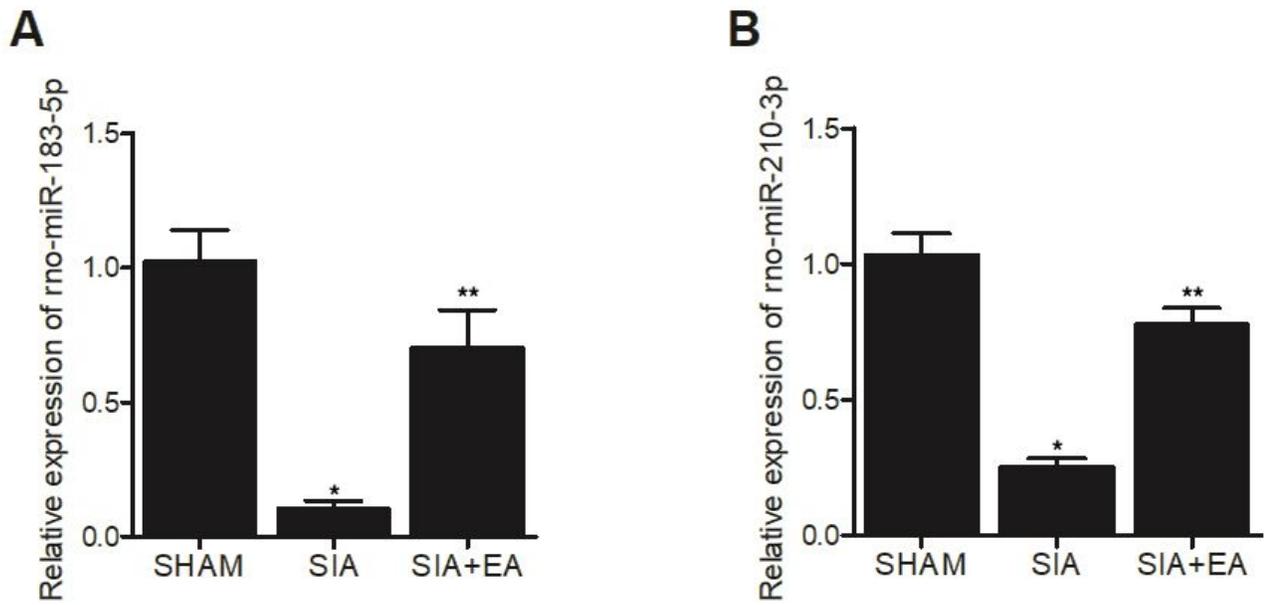
Rno-miR-183-5p and rno-miR-210-3p mimics suppressed the expression of SIN3A in SH-SY5H cells. A: The expression of SIN3A was inhibited by rno-miR-183-5p in SH-SY5H cells (\* P value < 0.05, vs. scramble control group). B: Rno-miR-34c-3p showed no repressive effect on SIN3A expression in SH-SY5H cells. C:

The expression of SIN3A was inhibited by rno-miR-210-3p in SH-SY5H cells (\* P value < 0.05, vs. scramble control group). D: Rno-miR-758-5p showed no repressive effect on SIN3A expression in SH-SY5H cells. E: Rno-miR-568 showed no repressive effect on SIN3A expression in SH-SY5H cells. F: Rno-miR-196c-3p showed no repressive effect on SIN3A expression in SH-SY5H cells.



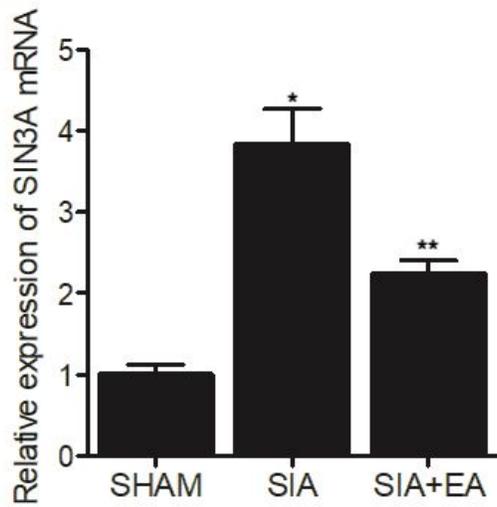
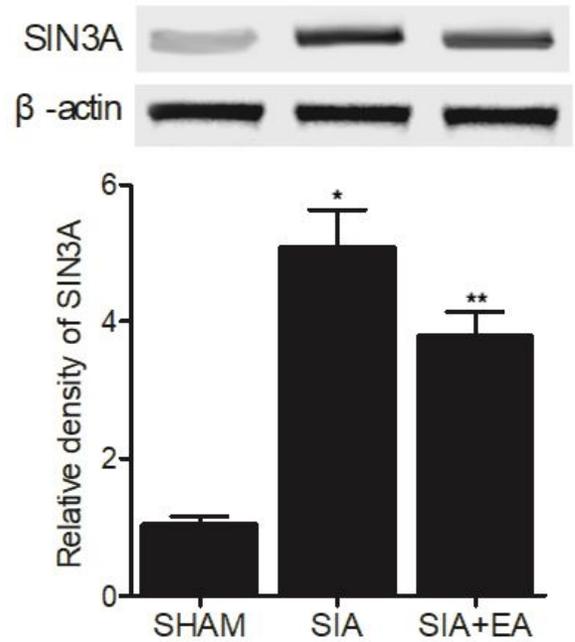
**Figure 4**

EA treatment improved memory consolidation in SIA rats. A: EA treatment restored the time spent with novel objects in SIA rats (\* P value < 0.05, vs sham group; \*\* P value < 0.05, vs. SIA group). B: EA treatment restored the discrimination index in SIA rats (\* P value < 0.05, vs sham group; \*\* P value < 0.05, vs. SIA group).



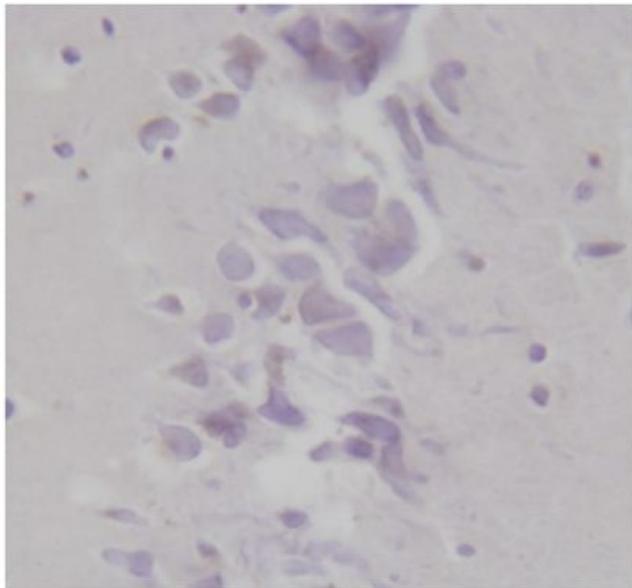
**Figure 5**

EA treatment restored down-regulated rno-miR-183-5p and rno-miR-210-3p in SIA rats. A: Rno-miR-183-5p expression in SIA rats was elevated by EA treatment (\* P value < 0.05, vs sham group; \*\* P value < 0.05, vs. SIA group). B: Rno-miR-210-3p expression in SIA rats was elevated by EA treatment (\* P value < 0.05, vs sham group; \*\* P value < 0.05, vs. SIA group).

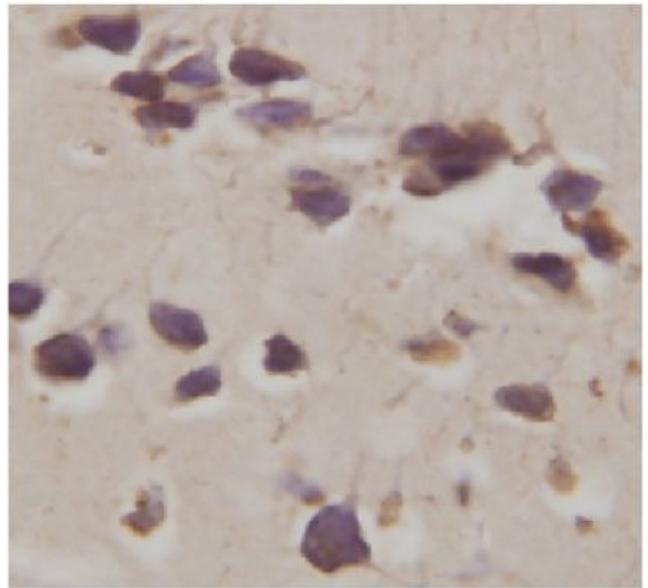
**A****B****Figure 6**

EA treatment reduced SIN3A expression in SIA rats. A: Up-regulated SIN3A mRNA expression in SIA rats was attenuated by EA treatment (\* P value < 0.05, vs. sham group; \*\* P value < 0.05, vs. SIA group). B: Up-regulated SIN3A protein expression in SIA rats was attenuated by EA treatment (\* P value < 0.05, vs. sham group; \*\* P value < 0.05, vs. SIA group).

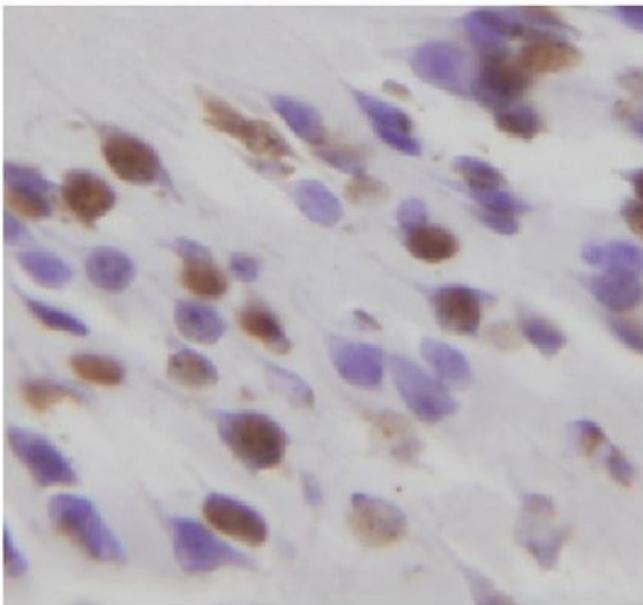
SHAM



SIA

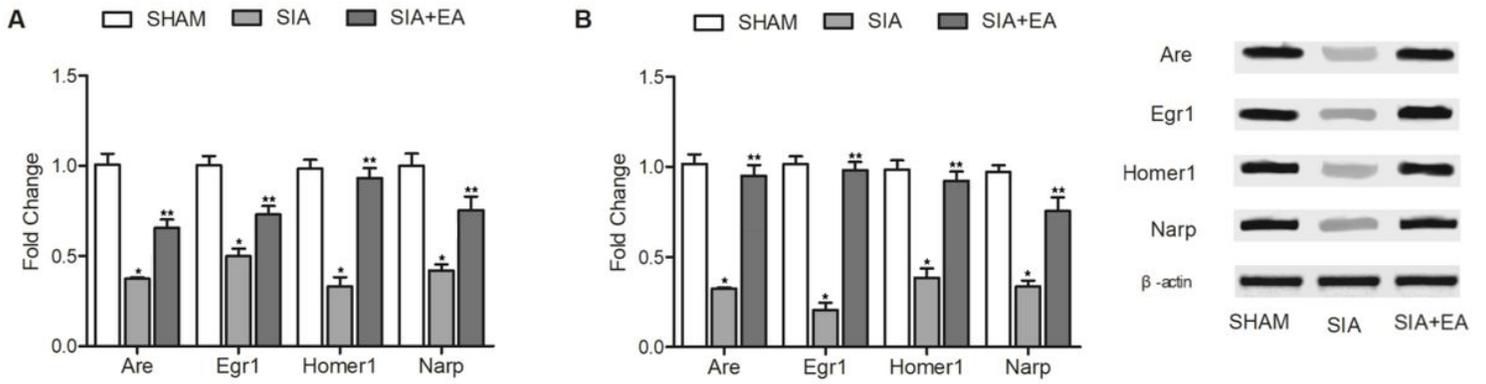


SIA+EA



**Figure 7**

EA treatment reduced SIN3A expression in the hippocampus of SIA rats. Immunohistochemistry analysis showed that the up-regulated expression of SIN3A in the hippocampus of SIA rats was inhibited by EA treatment.



**Figure 8**

SIN3A mRNA and protein expression in the hippocampus of SIA rats was suppressed by EA treatment. A: Overexpression of SIN3A mRNA expression in the hippocampus of SIA rats was attenuated by EA treatment (\* P value < 0.05, vs. sham group; \*\* P value < 0.05, vs. SIA group). B: Overexpression of SIN3A protein expression in the hippocampus of SIA rats was attenuated by EA treatment (\* P value < 0.05, vs. sham group; \*\* P value < 0.05, vs. SIA group).