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Supplemental Information

miR-204-3p/Nox4 Mediates Memory Deficits in a Mouse Model of Alzheimer's Disease

Wenyuan Tao, Linjie Yu, Shu Shu, Ying Liu, Zi Zhuang, Siyi Xu, Xinyu Bao, Yue Gu, Fang Cai, Weihong Song, Yun Xu, and Xiaolei Zhu

Supplemental Figure

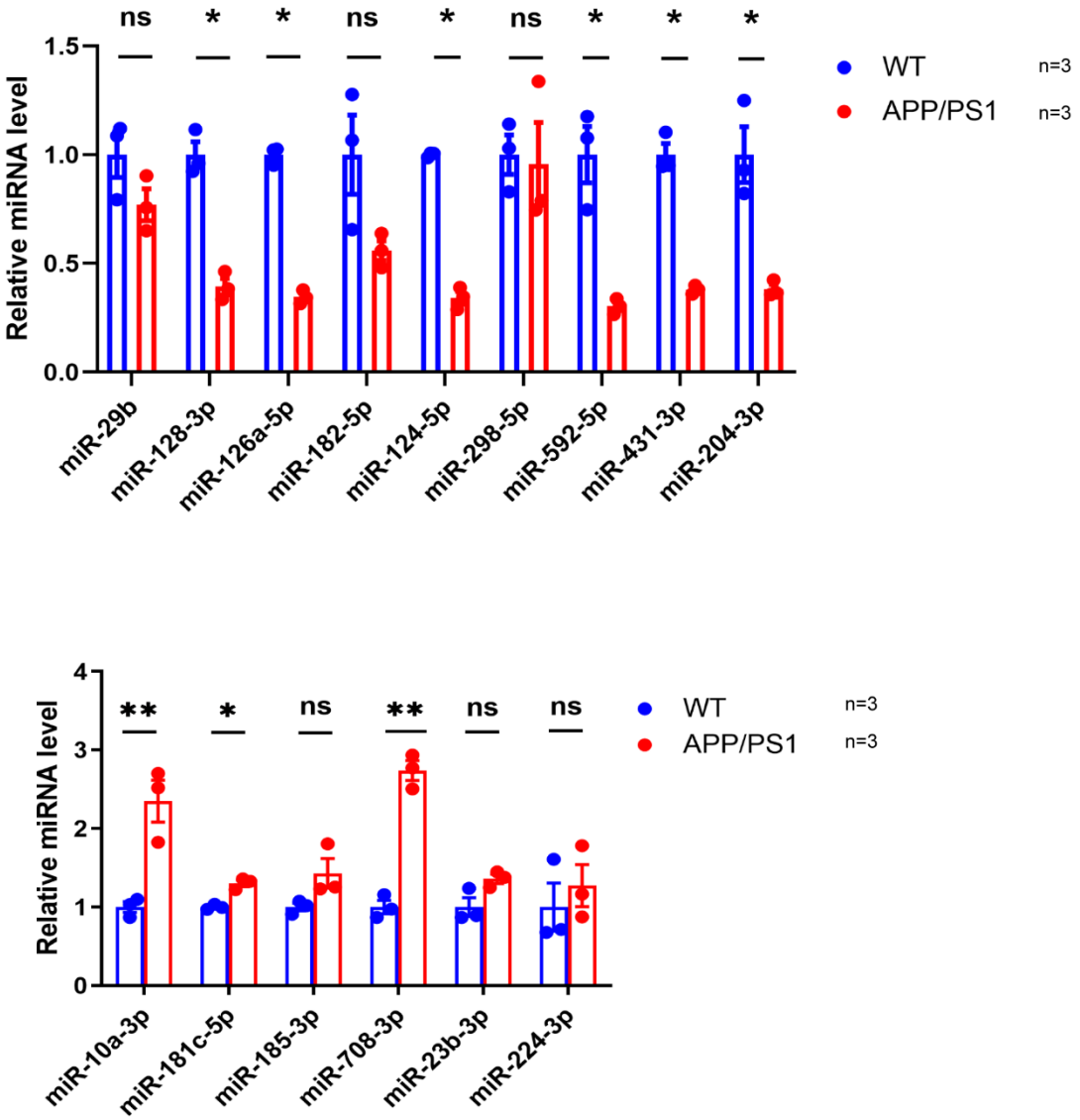

Figure S1. Differently expressed miRNAs in the hippocampus of 6-month-old APP/PS1 mice.
The relative expression of miR-29b, miR-128-3p, miR-126a-5p, miR-182-5p, miR-124-5p, miR-298-5p, miR-592-5p, miR-431-3p, miR-204-3p, miR-10a-3p, miR181c-5p, miR-185-3p, miR-708-3p, miR-23b-3p and miR-224-3p in the hippocampus of APP/PS1 mice and WT mice were detected by qPCR. * $p<0.05$, ** $p<0.01$, ns, no significance.

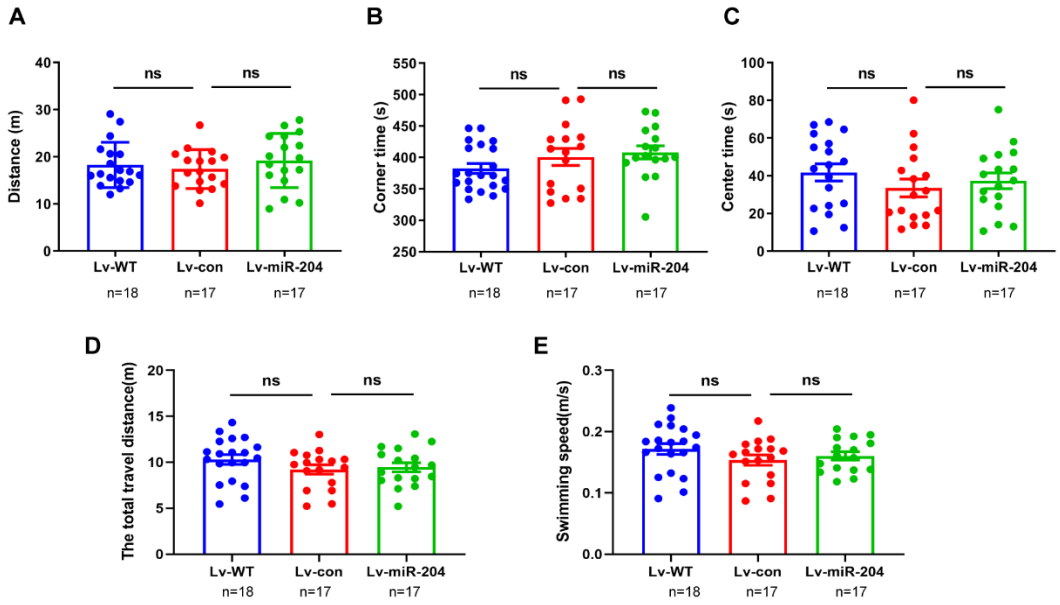

Figure S2. MiR-204-3p overexpression didn't affect the motor performance and exploration behavior of APP/PS1 mice.

In the Open field test, total distance traveled (A) and time spent in the corner (B) and center (C) area were recorded. n=17-18. In the MWM test, total travel distance (D) and swimming speed (E) were analyzed in probe trial. n=17-18. ns, no significance.

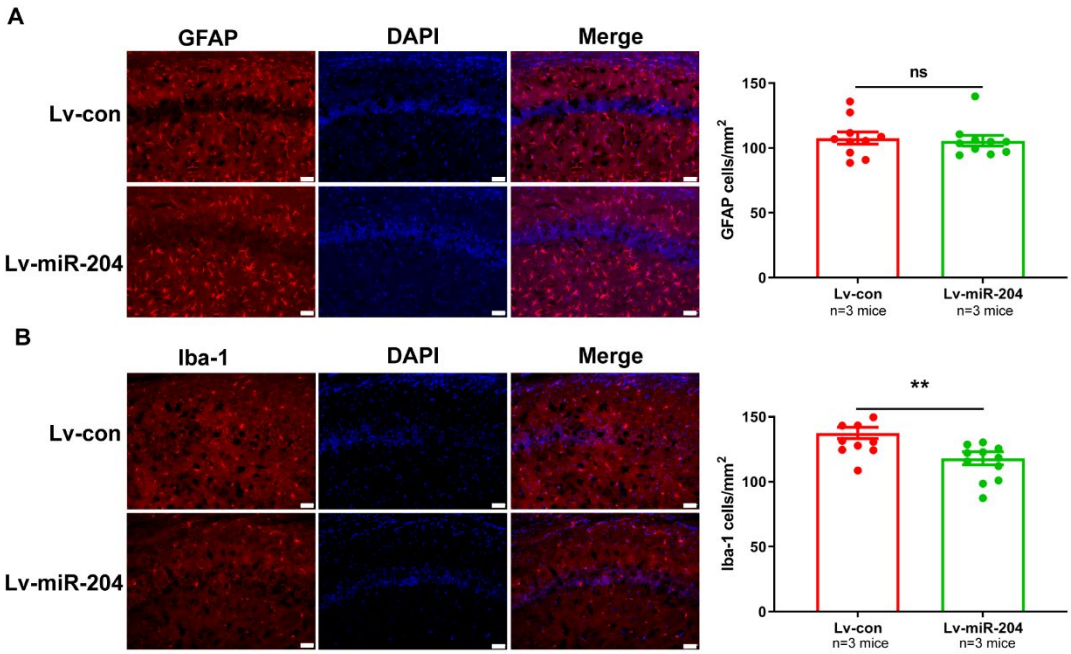

Figure S3. MiR-204-3p overexpression decreased microglia activation in the hippocampus of APP/PS1 mice.

(A) The level of GFAP was determined by immunofluorescence in the hippocampus of Lv-miR-204 treated APP/PS1 mice (left panel) and quantification of signal intensities was shown (right panel). n = 10 slices from 3 mice each group. (B) The level of Iba-1 was determined by immunofluorescence in the hippocampus of Lv-miR-204 treated APP/PS1 mice (left panel) and quantification of signal intensities was shown (right panel). n = 9-11 slices from 3 mice each group. Scale bar = 50 μm. ** $p<0.01$, ns, no significance.

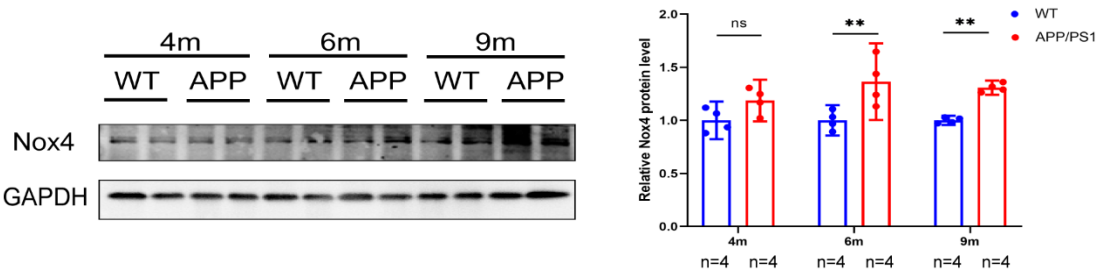

Figure S4. Nox4 was increased in the hippocampus of 6-month-old and 9-month-old APP/PS1 mice
The protein level of Nox4 was measured in the hippocampus of APP/PS1 mice by western blotting and quantification of the intensities normalized to GAPDH was shown. n=4. ** $p<0.01$, ns, no significance.

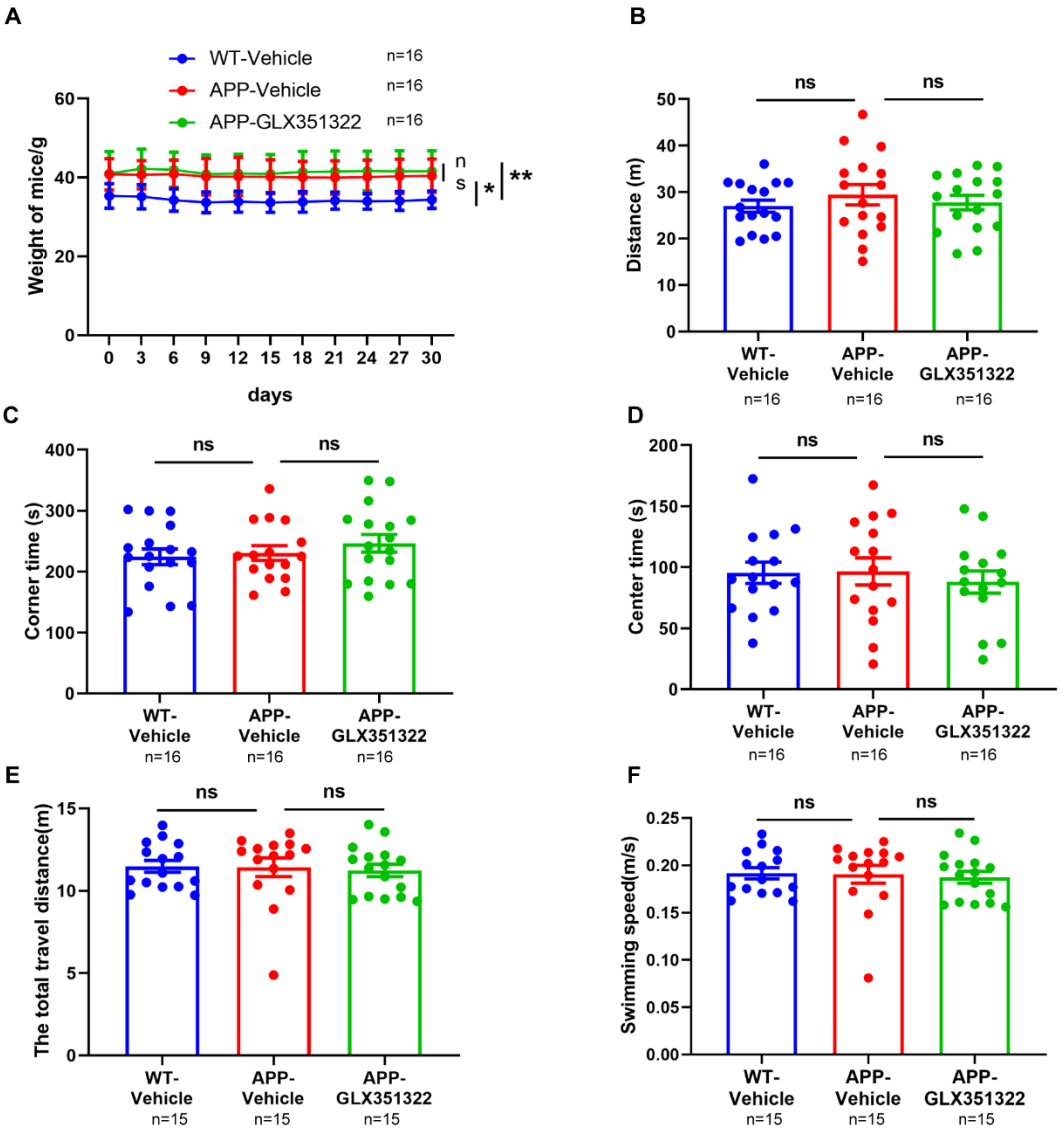

Figure S5. GLX351322 treatment at the dosage of 5 mg/kg/day didn't affect the body weight, the motor performance and exploration behavior of APP/PS1 mice.

(A) The body weights of mice were recorded after GLX351322 treatment. n=15-16. In the Open field test, total distance traveled (B) and time spent in the corner (C) and center (D) area were recorded. In the MWM test, total travel distance (E) and swimming speed (F) were analyzed in probe trial. n=15-16

* $p<0.05$, ** $p<0.01$, ns, no significance.

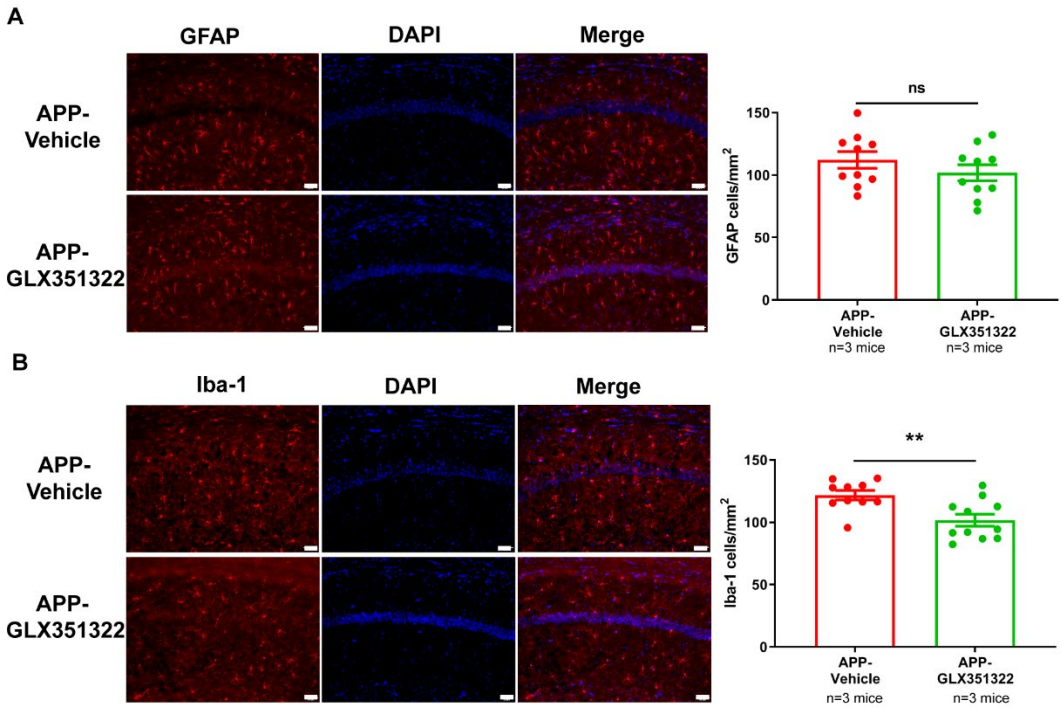

Figure S6. GLX351322 treatment decreased microglia activation in the hippocampus of APP/PS1 mice.

(A) The level of GFAP was determined by immunofluorescence in the hippocampus of GLX351322 treated APP/PS1 mice (left panel) and quantification of signal intensities was shown (right panel). n=10 slices from 3 mice each group. (B) The level of Iba-1 was determined by immunofluorescence in the hippocampus of GLX351322 treated APP/PS1 mice (left panel) and quantification of signal intensities was shown (right panel). n=10-11 slices from 3 mice each group. Scale bar = 50 μm. ** $p<0.01$, ns, no significance.

Supplemental Methods and Materials.

Behavior tests

Open field tests were performed to assess motor function and anxiety. Mice were put in an open field box (48 cm × 48 cm ×36 cm). The box was divided into 16 squares of equal area. The four corner squares were defined as corner area and the four middle squares were defined as center area. The open field area was cleaned with 75% ethanol wipes during each testing. Total distance traveled and time spent in the center area and corner area were measured and recorded by ANY-maze software (Stoelting, USA).

The mice were habituated in a 30 × 30 cm box with nontransparent 45 cm high walls for 30 min 24 h prior to the NOR tests. On the training phase, the mice were presented with two novel objects (object 1 and object 2) and allowed to explore freely for 10 min. On the testing phase, one of the objects (object 2) was replaced with a novel object. The mice were allowed to explore the two objects for 5 min. The exploring procedure was recorded using a visual tracking system and the discrimination index was calculated by the time spent exploring the novel object divided by the total time.

The contextual and cued Fear condition tests were conducted using a conditioning chamber (XR-XC404, Shanghai Softmaze Information Technology Co. Ltd.). In the training phase, the mice were placed in the chamber for 3 min followed by a tone-shock pairing, and kept to remain in the chamber for another 30 s after shock. In the testing phase, the contextual-dependent test was measured 24 h after training, and the mice were allowed to stay in the same chamber for 5 min. Freezing was defined as "absence of movement except for respiration" and the freezing time was recorded using a tracking system. The mice were then taken into a completely different new chamber 2 h later for 3 min, and the training tone was delivered to examine cued fear conditioning.

The MWM tests were performed to access spatial memory functions. Briefly, during the acquisition trial, the mice were trained within 60 s for 5 consecutive days to find the platform hidden 1 cm under water, and the latency was recorded using ANY-maze software (Stoelting, USA). During the probe trial, the platform was removed and the mice were allowed to swim for 1 min. Then the number of platform crossings, the latency to find the target quadrant, the time spent in target quadrant, swimming speed and total distance were recorded.

Golgi staining

Golgi staining was performed according to the manufacturer's instructions of a fast Golgi staining kit (FD Neurotechnologies, Columbia, USA). The brain tissues of mice were soaked in the mixture of solution A and B for 2 weeks at room temperature keeping away from light and then were transferred to solution C for 6 days. The brains tissues were cut into 100 μm slices with cryostat microtome (Leica, Wetzlar, Germany) and the sections were stained according the manufacturer's instructions. Images were captured with an inverted microscope (Olympus X73, Tokyo, Japan) under a 100 objective. The second-order dendritic branches numbers of spines per 10 μm of dendrite length and the percentage of mushroom spines per 10 μm in CA1 region were counted.

Cell Counting Kit-8 (CCK-8)

A 1:10 diluted CCK-8 solution (CCK-8) (Dojindo, Kumamoto, Japan) was added to primary cortical neurons for 4 h at 37°C. The absorbance was recorded at 450 nm according to the manufacturer’s instructions. Cell survival rates were presented as the percentage of live cells compared to untreated cells.

Western blotting

The protein was separated by SDS-PAGE and transferred to PVDF membrane. The membranes were blocked in 5% nonfat milk and incubated overnight at 4°C with the following primary antibodies: anti-APP (1:200; Abcam, Cambridge, MA, USA), anti-ADAM10 (1:1000; Millipore, Boston, MA, USA), anti-ADAM17 (1:1000; Millipore, Boston, MA, USA), anti-BACE1 (1:1000; Millipore, Boston, MA, USA), anti-presenilin 1 (1:1000; Cell Signaling Technologies, Danvers, MA, USA), anti-Nox4 (1:1000; Abcam, Cambridge, MA, USA) anti-p22phox (1:1000; Santa Cruz, Santa Cruz, CA, USA), anti-NMDAR2A (1:1000; Abcam, Cambridge, MA, USA), anti-NMDAR2B (1:1000; Abcam, Cambridge, MA, USA), anti-NMDAR1 (1:1000; Abcam, Cambridge, MA, USA), anti-GluA1 (1:1000; Abcam, Cambridge, MA, USA), anti-GluA2 (1:2000; Abcam, Cambridge, MA, USA), anti-PSD-95 (1:1000; Abcam, Cambridge, MA, USA), anti-synapsin (1:50000; Abcam, Cambridge, MA, USA) anti-GAPDH (1:5000, Bioworld, Louis Park, MN, USA) and anti-β-actin (1:2000, Bioworld, Louis Park, MN, USA). After washed with 0.1% Tween 20/Tris-buffered saline for 30 min, the membranes were incubated with secondary antibodies at room temperature for 2 h. The protein signals were visualized using an ECL kit (Millipore, Boston, MA, USA), and the intensities were quantified using Image J software.

Immunofluorescence

The mice were perfused with cold saline followed by 4% paraformaldehyde (PFA), and brains were rapidly removed and cryosectioned. Brain sections were blocked with 2% BSA for 2 h at room temperature and incubated overnight at 4°C with the following primary antibodies：anti-Aβ (6E10, 1:500; Biolegend, San Diego, CA, USA), anti-Nox4 (1:500, Abcam, Cambridge, MA, USA), anti-GFAP (1:500, Cell Signaling Technologies, Danvers, MA, USA), anti-Iba1 (1:500, Abcam, Cambridge, MA, USA) and anti-NeuN (1:200, Millipore, Boston, MA, USA). After washed with PBS for 30 min, the sections were incubated with secondary antibody for 2 h at room temperature. The cell nuclei was stained using DAPI reagent (1:1000, Bioworld, Louis Park, MN, USA). Images were captured with an inverted fluorescence microscope. All qualitative immune staining analyses were conducted using Image J software (National Institutes of Health, USA).

Luciferase activity assay

The relative luciferase activity was examined by using the Promega Bright-N-Glo system (Promega, USA). MiR-204 overexpression construct and control vector was obtained from Shanghai Genchem (China). The 3'UTR of *Nox4* was amplified using the following primers: forward 5'- CCG CTC GAG AAC CTT AGG AGA CTA CTG GGG ACT TT -3', reverse 5'-TCC GAA GAT CTC CAT CAA AAT TCG TGA TTT AAG ATT T -3'. The polymerase chain reaction (PCR) product was cloned into the pGL3-CMV-LUC-MCS vector (Shanghai Genomeditech, China) and verified by sequencing to create the recombinant plasmid pGL3-*Nox4*-3'UTR. The mutation was created by PCR using the following primers: Fragment 1 forward 5'- CCG CTC GAG AAC CTT AGG AGA CTA CTG GGG ACT TT -3', reverse 5'- CAG TTT CAA CCT GGG AGT GTT TGC CTG GAG CCT AT -3'; Fragment 2 forward 5'- AGG CAA ACA CTC CCA GGT TGA AAC TGT AGC ACA AA -3', reverse 5'- TCC GAA GAT CTC CAT CAA AAT TCG TGA TTT AAG ATT T -3'. The PCR products were mixed as the template and amplified with PCR to generated the mutant 3'UTR of *Nox4* (pGL3-*Nox4*-3'UTR mutant), which was verified by sequencing. N2a cells were transfected with miR-204-3p or control vector and wild type or

mutant *Nox4* 3'-UTR plasmid with phRL-CMV Renilla for 48 h, and relative luciferase activity was examined.

Sequences of primers for quantitative RT-PCR

Primers	Sequence 5′-3′	
	Forward	Reverse
P22phox	CCTCCACTTCCTGTTGTCGG	TCACTCGGCTTCTTTCGGAC
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
mmu-miR-29b	GGGGTAGCACCATTTGAAA	CAGTGCGTGTCGTGGAGT
mmu-miR-128-3p	ACATATACTCACAGTGAACCG	CAGTGCGTGTCGTGGAGT3’
mmu-miR-126a-5p	GGGGGCATTATTACTTTTGG	CAGTGCGTGTCGTGGAGT
mmu-miR-181c-5p	GGGGCATTCAACCTGTCG	CAGTGCGTGTCGTGGAGT
mmu-miR-124-5p	GCGTGTTCACAGCGGA	CAGTGCGTGTCGTGGAGT
mmu-miR-23b-3p	GCATCACATTGCCAGGG	CAGTGCGTGTCGTGGAGT
mmu-miR-592-5p	GGGGATTGTGTCAATATGCG	CAGTGCGTGTCGTGGAGT
mmu-miR-204-3p	GCTGGGAAGGCAAAGG	CAGTGCGTGTCGTGGAGT
mmu-miR-431-3p	GGGACAGGTCGTCTTGCAG	CAGTGCGTGTCGTGGAGT
mmu-miR-10a-3p	GGGCAAATTCGTATCTAGG	CAGTGCGTGTCGTGGAGT
mmu-miR-182-5p	GGATTTGGCAATGGTAGAACTC	CAGTGCGTGTCGTGGAGT
mmu-miR-185-3p	GCAGGGGCTGGCTTTCC	CAGTGCGTGTCGTGGAGT
mmu-miR-708-3p	GGGGCAACTAGACTGTGAGC	CAGTGCGTGTCGTGGAGT
mmu-miR-298-5p	GAAGGCAGAGGAGGGCT	CAGTGCGTGTCGTGGAGT
mmu-miR-224-3p	CCCAAATGGTGCCCTAGTG	CAGTGCGTGTCGTGGAGT