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Research Article

Silencing miRNA-324-3p protects against cerebral ischemic injury via regulation of the GATA2/A1R axis

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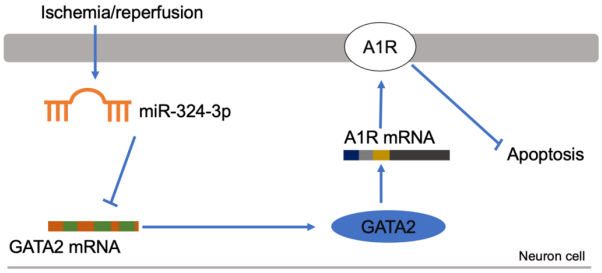
From the Contents

Graphical Abstract *Silencing miRNA-324-3p alleviates ischemic brain damage by increasing adenosine A1 receptor expression via GATA2*



Abstract

Previous studies have suggested that miR-324-3p is related to the pathophysiology of cerebral ischemia, but the mechanism underlying this relationship is unclear. In this study, we found that miR-324-3p expression was decreased in patients with acute ischemic stroke and in *in vitro* and *in vivo* models of ischemic stroke. miR-324-3p agomir potentiated ischemic brain damage in rats subjected to middle cerebral artery occlusion, as indicated by increased infarct volumes and cell apoptosis rates and greater neurological deficits. In a PC12 cell oxygen-glucose deprivation/reoxygenation model, a miR-324-3p mimic decreased cell viability and expression of the anti-apoptotic protein BCL2 and increased expression of the pro-apoptotic protein BAX and rates of cell apoptosis, whereas treatment with a miR-324-3p inhibitor had the opposite effects. Silencing miR-324-3p increased adenosine A1 receptor (A1R) expression through regulation of GATA binding protein 2 (GATA2). These findings suggest that silencing miR-324-3p reduces ischemic brain damage via the GATA2/A1R axis.

Key Words: acute ischemic stroke; adenosine A1 receptor; apoptosis; cerebral ischemia-reperfusion injury; cortical neurons; GATA2; middle cerebral artery occlusion; miR-324-3p; oxygen-glucose deprivation/reoxygenation; PC12 cells

Introduction

Stroke is a serious and common condition that is characterized by high incidence and high rates of morbidity and mortality (Wang et al., 2017). Unfortunately, the ischemia that occurs during stroke is often followed by rapid progression of brain injury, and few therapeutic options are available to halt this progression (Yin et al., 2010). To address the increasing disease burden and lack of treatment options, there is an urgent need to explore the pathogenesis of stroke and find novel potential therapeutic targets (Wang et al., 2011).

Adenosine, an endogenous neuroprotectant, is found throughout the human body and is especially highly expressed in the central nervous system (Bortolotto et al., 2015). Under physiological conditions, adenosine is present in low concentrations both inside and outside of cells, but its levels increase dramatically in response to stress. An increase in plasma adenosine levels has been observed in patients who experience transient ischemic attack or stroke, even a few days after the incident (Laghi Pasini et al., 2000). Generally, the functions of adenosine are mediated by its receptors, which are primarily the A1, A2A, A2B, and A3 receptors (A1R, A2AR, A2BR, and A3R; Cunha, 2001). The vast majority of adenosine A1 receptors (A1Rs) are expressed in the central nervous system, in areas such as the hippocampus, cerebral cortex, cerebellum, spinal cord, and other tissues, where they function as inhibited G-protein-coupled receptors (Bai et al., 2017). Adenosine A1R has analgesic and neuroprotective effects because of the roles of this ligand-receptor interaction in reducing inflammation, promoting the generation of antioxidants, and inhibiting apoptosis (Dai et al., 2017). Adenosine A1R has also been shown to have endogenous neuroprotective functions (Zhang et al., 2020). Researchers have found that electroacupuncture prevents the cognitive impairment induced by cerebral ischemia-reperfusion via adenosine A1R in rats (Shi et al., 2021). Overall, A1R plays a protective role in the brain. However, the mechanism of stroke is complex, and intervention targeting a single gene is prone to failure. miRNAs regulate multiple genes, and are therefore considered promising targets for developing novel gene therapies. To date, there have been no reports of A1R regulation by miRNAs.

MicroRNAs are a class of non-coding RNAs that are approximately 18 to 24 nucleotides in length (Bai et al., 2018). They bind target mRNAs through complementary base pairing, which suppresses or destabilizes the mRNA, thereby exerting posttranscriptional regulation of gene expression (Luo et al., 2017). Bioinformatics analysis estimates that miRNAs regulate more than 60% of genes in mammalian genomes. miRNAs take part in most biological pathways and cellular activities, such as cell proliferation and differentiation, metabolism, apoptosis, cell development, and cell signal transduction (Yang et al., 2017). It has been reported that miRNAs play a role in apoptosis in cerebral ischemia-reperfusion injury (Li et al., 2017). miRNAs are considered a promising potential treatment option for many diseases, including stroke. Generally miRNAs bind to the 3′-untranslated region (3′-UTR) of an mRNA to negatively regulate gene expression at that posttranscriptional level. miRNAs are important molecules in pathophysiological processes (Sorensen et al., 2014). miRNAs are typically produced at high levels in the central nerve system and play determining roles in cerebral diseases, including stroke (Lee et al., 2010). Ni et al. (2015) found that let-7c-5p is important in cerebral ischemia, as up-regulation of this miRNA relieved ischemic neuroinflammation and reduced cerebral ischemic damage. miRNA microarray analysis has shown that the expression levels of specific miRNAs, such as miR-324-3p, vary dramatically during stroke compared with normal conditions (Jeyaseelan

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et al., 2008). Dharap et al. (2009) reported that miR-324-3p expression was increased at different time points after cerebral ischemia/reperfusion in spontaneously hypertensive rats. These studies suggest that miR-324-3p is related to the pathophysiology of cerebral ischemia; however the underlying mechanisms remain unclear. Here, we investigated whether the adenosine A1R can be regulated by miR-324-3p.

The aim of this study was to identify the roles of miR-324-3p in ischemic brain damage and to explore the underlying molecular mechanisms of these effects.

Materials and Methods

In vivo experiments

Animals

Animal care and experimentation were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Wenzhou Medical University on April 27, 2017 (approval No. wydw2017-0104).

Seventy-four Sprague-Dawley male rats (weight 280–320 g, age 10–12 weeks) were purchased from the Beijing Vital River Lab Animal Center, China (license No. SCXK (Jing) 2019-0001). They were housed five per cage in a controlled animal facility that was kept at a constant temperature of 23 ± 1°C and a relative humidity of 55 ± 5% with water and food *ad libitum*.

The rats were randomly divided into seven groups using the random number table method: (1) sham group (n = 20); (2) middle cerebral artery occlusion (MCAO) 1.5/24 group (1.5 hours of ischemia followed by 24 hours of reperfusion, n = 15); (3) MCAO 1.5/72 group (1.5 hours of ischemia followed by 72 hours of reperfusion, n = 3); (4) MCAO agomir group (MCAO followed by miR-324-3p agomir, n = 12); (5) MCAO NC group (MCAO followed by NC, n = 12); (6) agomir group (MCAO followed by agomir, n = 6); (7) agomir NC group (MCAO followed by agomir NC, n = 6). Rats received left lateral ventricle injection of miR-324-3p agomir, agomir NC, or NC (0.125 nmol dissolved in 5 µL PBS; RiboBio, Guangzhou, China) 3 days prior to MCAO.

MCAO models

As previously described (Huang et al., 2020), MCAO performed using intraluminal filament technology was employed to generate focal cerebral ischemia. The rats were anesthetized by intraperitoneal injection of a 3% sodium pentobarbital solution (30 mg/kg body weight; MilliporeSigma, Burlington, MA, USA). After making a 3-cm long carotid midline incision, the left common carotid artery, internal carotid artery, and external carotid artery were separated. The carotid artery was tied with a slip knot made with 6-0 silk suture, and the distal end and small branches of the external carotid artery were ligated. An arterial clamp was used to temporarily clamp the internal carotid artery. A small incision was made at the distal end of the external carotid artery, and the thread plug was inserted from its stump along the internal carotid artery until resistance was felt, at approximately 18–20 mm of depth. After 90 minutes of ischemia, the plug was withdrawn, and the wound was sutured. Animals showing a cerebral blood flow reduction of at least 75% of the baseline level and 70% cerebral blood flow recovery, as determined using a Pericam PSI NR system (Perimed, Järfälla, Sweden), were considered to have successfully undergone MCAO and were used for further experiments. In the sham group, the blood vessels were separated, but the plug was not inserted. A heating pad was used to keep the rats' body temperature at 37.0–37.5°C (measured rectally) until they recovered from the anesthesia.

Detection of infarct volume and neurological deficits

At 24 hours after MCAO, the rat brains were quickly removed. The brain samples were frozen at −20°C for 5 minutes and sliced into 2-mm-thick coronal sections with a scalpel blade designed for cutting brain tissue (RWD, Shenzhen, China). The slices were stained at 37°C with 2% (v/v) 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) for 15 minutes, then scanned with a camera, and the infarct area was delineated using ImageJ (ver.1.52, Bethesda, MD, USA; Schneider et al., 2012). The infarct volume (%) was calculated according to the following formula: cumulative infarct size on the affected side/whole brain tissue area × 100.

Rat neurological deficits were evaluated 24 hours after MCAO using a modified neurobehavioral scoring system and scored from 0 to 4 as follows: 0 (normal), without visible neurological deficits; 1 (mild), unable to extend the right forepaw; 2 (moderate), spinning in the contralateral direction; 3 (severe), toppling to the right; 4 (very severe), inability to walk autonomously and loss of consciousness.

Detection of plasma miR-324-3p levels

The research protocol was approved by the Ethics Committee of The First Affiliated Hospital of Wenzhou Medical University (approval No. 2020-142) and conducted in accordance with the *Declaration of Helsinki* (1964), as revised in 2013. The participants or their legally authorized representatives provided written informed consent to participate in the study.

Patients with acute ischemic stroke (AIS) were recruited from The First Affiliated Hospital of Wenzhou Medical University from January 2018 to December 2019. The severity of patients' neurological deficits within 24 hours after stroke was assessed by experienced neurologists according to National Institute of Health Stoke Scale criteria (Mak et al., 2021). Inclusion criteria: ischemic stroke diagnosed by magnetic resonance imaging or computed tomography (Bai et al., 2018). Exclusion criteria: intracranial hemorrhage, transient ischemic attack, disturbance of consciousness or language expression, or dementia (Hou et al., 2020). The average time of blood collection was 11.0 ± 9.1 hours after stroke onset. Plasma miR-324-3p levels were detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Individuals who had not experienced stroke were recruited from patients undergoing an annual medical examination at the hospital to serve as the control group. **Table 1** shows the demographic and clinical characteristics of the 18 stroke patients and 13 non-stroke controls who participated in this study.

Table 1 | **Baseline characteristics of the included participant**

Variable	AIS (n = 18)	Control (n = 13)	P-value
Sex			0.355
Male	10 (55.6)	5 (38.5)	
Female	8 (44.4)	8 (61.5)	
Age (yr)	65 (54–76)	58 (53–67)	0.002
Height (cm)	165.4 (159.5–171.3)	163.5 (157–167)	0.435
Weight (kg)	65.75±13.45	57.87±8.72	0.138
BMI (kg/m^2)	24.08±4.88	21.71±2.67	0.109
Smoking	6 (33.3)	3 (23.1)	0.361
Drinking	4 (22.2)	3 (23.1)	0.361
Hypertension	13 (72.2)	3 (23.1)	0.018
Diabetes	5 (27.8)	1 (7.7)	0.169

Data are expressed as number (percentage), mean ± SD or median (interquartile range) as appropriate. AIS: Acute ischemic stroke; BMI: body mass index.

In vitro experiments

Cell culture

Cortical neurons were extracted from fetal rats from five pregnant dams at embryonic day 16–18 (Wenzhou Medical University [license No. SYXK (Zhe) 2015-0009]). The cortical tissues were digested with trypsin containing 0.25% ethylenediaminetetraacetic acid (EDTA; Thermo Fisher Scientific, Waltham, MA, USA), mixed thoroughly by pipetting up and down a few times, and filtered through a cell strainer with the pore diameter of 100 µm (Biologix, Jinan, Shandong, China). The cells were then centrifuged at 300 × g for 5 minutes at room temperature and resuspended in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific) containing fetal bovine serum (10% v/v, Thermo Fisher Scientific) and penicillin/streptomycin (1% v/v, Thermo Fisher Scientific). The cell suspensions were then seeded into six-well plates (1×10^6 cells/mL), and 2 hours later the culture medium was replaced with Neurobasal Medium (Thermo Fisher Scientific) containing 0.5 mM L-glutamate, 2% (v/v) B27 serum-free supplement, and 0.5% (v/v) penicillin/streptomycin.

PC12 cells (Cat# CRL-1721, RRID: CVCL_0481) were obtained from American Type Culture Collection (ATCC), Shanghai, China and cultured in DMEM (Thermo Fisher Scientific) containing heat-inactivated fetal bovine serum (10%), penicillin (100 U/ mL), and streptomycin (100 mg/mL) in a humidified environment with 5% (v/v) CO_2 at 37°C. The medium was changed every 2 days. Then PC12 cells were then transferred to culture plates at the appropriate density for individual experiments and incubated for 24 hours.

To investigate the effects of miR-324-3p on damage induced to PC12 cells by oxygen-glucose deprivation/reoxygenation (OGD/REP), the cells were divided into 11 groups, as shown in **Table 2**.

Table 2 | **Cell grouping in the *in vitro* experiment**

Groups	PC12 cells
CON	5 µL sterile water
Mimic	50 nM miR-324-3p mimic
Mimic NC	50 nM miR-324-3p mimic NC
Inhibitor	100 nM miR-324-3p inhibitor
Inhibitor NC	100 nM miR-324-3p inhibitor NC
OGD/REP	OGD/REP
OGD/REP mimic	50 nM miR-324-3p mimic+OGD/REP
OGD/REP mimic NC	50 nM miR-324-3p mimic NC+OGD/REP
OGD/REP inhibitor	100 nM miR-324-3p inhibitor+OGD/REP
OGD/REP inhibitor NC	100 nM miR-324-3p inhibitor NC+OGD/REP
OGD/REP inhibitor + GATA2 siRNA	100 nM miR-324-3p inhibitor NC+OGD/REP+20 µM GATA2 siRNA

GATA2: GATA binding protein 2; NC: normal control; OGD/REP: oxygen-glucose deprivation/reoxygenation.

PC12 cells were seeded into 6-well plates, then transfected with 50 nM of an miR-324-3p mimic or a control (RiboBio, Guangzhou, Guangdong Province, China), and 100 nM of an miR-324-3p inhibitor or a control (RiboBio), using Lipofectamine 2000 (RiboBio) according to the manufacturer's instructions. The cells were then washed twice with PBS and cultured in 900 µL minimum essential medium. The following tubes were used for the next steps: tube A contained 50 nM miR-324-3p mimic/control or 100 nM miR-324-3p inhibitor/control mixed with 50 µL OPTI-MEM for 5 minutes, and tube B contained 5

μL Lipofectamine 2000 mixed with 50 μL OPTI-MEM for 5 minutes. Tubes A and B were then combined in tube C and mixed for 15 minutes, after which the mixture was added to the corresponding cells. The cells were cultured for 6 hours, and then the cell dye was discarded and replaced with DMEM. The control group was treated with an equal amount of sterile water. One day later, the cells were subjected to OGD/REP and analyzed by cell counting kit-8 assay, flow cytometry, qRT-PCR, and western blot analysis.

Glucose-oxygen deprivation/reoxygenation
The primary cortical neurons and PC12 cells were transferred into a sealed chamber (Thermo Fisher Scientific) containing N_2 (95%, v/v) and CO_2 (5%, v/v), and cultivated in glucose-free DMEM (Thermo Fisher Scientific). Three hours later, the glucose-free medium was replaced with high-glucose medium, and the cells were restored to normal incubation conditions (95% air and 5% CO_2) to be reoxygenated. The PC12 cells were reoxygenated for 24 hours, and the primary cortical neurons were reoxygenated for 0, 12, 24, or 48 hours.

Cell Counting Kit-8 assay
To detect cell viability, PC12 cells were seeded into 96-well plates at a concentration of 2×10^4 cells per well in a volume of 100 μL. Next, 10 μL of Cell Counting Kit-8 (CCK8) solution (Beyotime, Shanghai, China) was added per well, and the plates were incubated for 2 hours. The light absorption at 450 nm was measured on a microplate reader (Thermo Fisher Scientific).

Flow cytometry assay
A flow cytometry assay was performed to assess the degree of cell apoptosis. PC12 cells were collected and stained using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Franklin Lakes, NJ, USA) according to the manufacturer's instructions. First, the cells were resuspended in 500 μL 1× binding buffer, then 5 μL Annexin V-FITC and 5 μL propidium iodide (PI) were added to the cells, and the cells were vortexed gently. The cells were analyzed using a FACS Canto™ II (BD) flow cytometer within 1 hour after incubation in the dark at room temperature for 15 minutes. At least 10,000 cells were analyzed for each treatment group. The double-staining technique used in this assay can distinguish intact cells (AV^-/PI^-), cells in early apoptosis/apoptosis (AV^+/PI^-), cells in late apoptosis/necrosis (AV^+/PI^+), and necrotic cells (AV^-/PI^+). The results from the apoptosis assay were analyzed with BD FACS Diva software (BD).

Quantitative reverse transcription-polymerase chain reaction
Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to analyze miR-324-3p, GATA binding protein 2 (GATA2), adenosine A1 receptor (A1R), BCL2, and BAX mRNA expression in PC12 cells, primary neuron cells, cerebral infarct regions from rats, or human blood. Total RNA was extracted with Trizol reagent according to the manufacturer's instructions (Thermo Fisher Scientific; Wang et al., 2021). cDNA was transcribed from 2 μg of RNA using a TaqMan MiRNA Reverse Transcription Kit (Applied Biosystems, Shanghai, China). miRNA was synthesized from 3 μg RNA using an miRNAFirst Strand cDNA Synthesis kit (Shenggong, Shanghai, China). The qRT-PCR reaction was carried out using an Applied Biosystems QuantStudio 5 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) and a SYBR® Premix Ex TaqII Kit (RR820A, Takara, Beijing, China). A standard qRT-PCR protocol (95°C for 30 seconds, followed by 39 cycles of 95°C for 5 seconds, 60°C for 30 seconds, and 65°C for 5 seconds) was performed using a Cfx96 Touch Real Time PCR Detection System (Bio-Rad). Transcription levels were quantified using with the $2^{-\Delta\Delta CT}$ method. All primers used for these experiments are shown in **Table 3**.

Western blot analysis
After 24 hours of reperfusion, the rats were anaesthetized with an intraperitoneal injection of 3% sodium pentobarbital solution (30 mg/kg body weight). Total protein was extracted from the ischemic penumbra of brain tissues, PC12 cells, or primary cortical neurons using RIPA lysis buffer (Solarbio, Beijing, China), and the protein concentration was evaluated using a BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein were separated on a 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (Solarbio, Beijing, China) and then transferred to a polyvinylidene difluoride membrane (1620177, Bio-Rad). The membrane was blocked for 1 hour with 5% skim milk (BD, Shanghai, China) in TBST at room temperature and then incubated with primary antibody overnight at 4°C. On the second day, the membrane was washed three times with TBST and then incubated with a secondary antibody conjugated with horseradish peroxidase (HRP) for 1 hour at room temperature, followed by three washes with TBST. Finally, the membranes were visualized with an enhanced chemiluminescence system (Beyotime, Wenzhou, China). Band intensity was quantified using Image Lab software (Bio-Rad).

The antibodies used in this assay were: rabbit anti-GATA2 antibody (1:1000, Abcam, Cambridge, MA, USA, Cat# ab109241), rabbit anti-A1R antibody (1:1000, Cat# ab82477, Abcam), rabbit anti-BCL2 antibody (1:1000, Affinity, Shanghai, China, Cat# AF6139), rabbit anti-BAX antibody (1:5000, Abcam, Cat# ab32503), rabbit anti-β-actin polyclonal antibody (1:1000, Bioworld Technology, Shanghai, China, Cat# AP0060), and goat anti-rabbit IgG-HRP (1:5000, Biosharp Life Sciences, Shanghai, China, Cat# BL003A).

Luciferase reporter assay
A luciferase assay was performed to detect the presence of GATA2 and miR-324-3p binding sites. Human embryonic kidney 293T cells (RRID:CVCL_0063) purchased from Shanghai Institutes for Biological Sciences (Cat# SCSP-502, Shanghai, China) were seeded into 96-well plates at a concentration of 3×10^5 cells/mL (100 μL per well). When the cell density reached 70%, the cells were transfected with 0.16 μg of GATA2-WT or GATA2-MUT target plasmid in 10 μL DMEM (Invitrogen, Shanghai, China) and 5 pmol of miR-324-3p MIMIC or MIMIC NC (Hanheng, Shanghai, China), using 0.3 μL of transfection reagent (Hanheng, concentration 0.8 mg/mL). After 48 hours of incubation, the cells were lysed using Renilla Luciferase Reporter Gene Assay Cell Lysis Buffer (RG129S, Beyotime), and the relative luciferase activity was detected using a Promega Dual-Luciferase™ Reporter (DLR™) Assay System (Thermo Fisher Scientific) as per the manufacturer's instructions.

Table 3 | The primer sequences used in quantitative reverse transcription-polymerase chain reaction in this study

Gene (accession number)	Primer sequence (5'–3')
In rats:	
miR-324-3P (100314181)	F: ACC CAC TGC CCC AGG TGC
	R: ATC CAG TGC AGG GTC CGA GG
	RT primer: GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC AGG GG
U6 (reference gene)	F: cgcgcgcgAGA GAA GAT TAG CAT GG
	R: ATC CAG TGC AGG GTC CGA GG
	RT primer: GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC AGG GG
A1R (29290)	F: CCT ACA TCT CGG CCT TCC AG
	R: GTG CCT GGT TCA CCT TCA CA
GATA2 (25159)	F: AAG GAT GGC GTC AAG TATC AAG TGT C
	R: AGC TGA GGC AGG CAC ATA GGA G
BCL2 (24224)	F: CAC GGT GGT GGA GGA ACT CT
	R: CAC GGT GGT GGA GGA ACT CT
BAX (24887)	F: GGC GAT GAA CTG GAC AAC AA
	R: GGC GAT GAA CTG GAC AAC AA
GAPDH (24383)	F: GAC ATG CCG CCT GGA GAA AC
	R: AGC CCA GGA TGC CCT TTA GT
In humans:	
miR-324-3P (442898)	F: CCC ACT GCC CCA GGT GC
	R: CCC ACT GCC CCA GGT GC
	RT primer: 5'CCC ACT GCC CCA GGT GC
U6 (reference gene)	F: AGA GAA GAT TAG CAT GGC CCC TG
	R: AGA GAA GAT TAG CAT GGC CCC TG
	RT primer: GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA AAA TA

A1R: Adenosine A1 receptor; GATA2: GATA binding protein 2.

Target prediction
GATA2 and miR-324-3p target genes and potential binding sites were predicted in humans, rats, mice, and rhesus monkeys by TargetScan (Lewis et al., 2005). Transcription factors were predicted using PROMO (Farré et al., 2003), and other sequence patterns were detected using the ALGGEN server (PROMO and MALGEN. Nucleic Acids Res 31:3651-3653). Transcription factors were identified by screening miRNA downstream target genes and adenosine-A1R upstream transcription factors in humans, rats, and mice.

Statistical analysis
No statistical methods were used to predetermine sample sizes; however, the number of animals included in each group in our study is similar to that reported in a previous publication (Chen et al., 2020). Neurological deficits were evaluated by blinded assessment. The flow cytometry assay and qRT-PCR results were analyzed by blinded assessors. Data were analyzed using GraphPad Prism 7 (GraphPad, San Diego, CA, USA, www.graphpad.com). No animals or data points were excluded from the analysis. Except for the neurological scores, all data are expressed as mean ± SEM. Student's *t*-test was employed to evaluate statistical significant differences between two groups. Differences among more than two groups were analyzed by one-way analysis of variance followed by a multiple comparisons test with Bonferroni correction. The neurological scores are expressed as medians (range) and were analyzed by non-parametric Kruskal-Wallis *H* test followed by the Nemenyi test. Pearson correlation analysis was applied to assess the relationship between plasma miR-324-3p levels and AIS patient NIHSS scores. *P* values of < 0.05 were considered to be statistically significant.

Results

miR-324-3p levels are decreased in AIS patients and in *in vitro* and *in vivo* models of ischemic stroke
miRNA microarray analysis has shown that miR-324-3p expression levels are markedly different in ischemic stroke in rats (Jeyaseelan et al., 2008). However, the role of miR-324-3p in stroke is still unclear. To confirm that miR-324-3p is involved in stroke, we examined the expression levels of miR-324-3p in AIS patients. We found that miR-324-3p levels were significantly reduced in AIS patients compared with controls ($P = 0.0435$; **Figure 1A**). Plasma miR-324-3p levels were negatively correlated with NIHSS scores in AIS patients ($P = 0.0358$; **Figure 1B**). Consistent with this, we found that the amount of miR-324-3p expressed in the infarct region of rats subjected to MCAO and 24 hours of reperfusion was significantly reduced compared with that

observed in the sham group (P = 0.0002; **Figure 1C**). Similarly, miR-324-3p levels in primary cortical neurons and PC12 cells subjected to OGD/REP were significantly down-regulated compared with those in the control group (P < 0.05; **Figure 1D** and **E**). These *in vitro*, *in vivo*, and clinical results suggest that miR-324-3p levels are decreased after AIS.

Altered miR-324-3p expression regulates the damage to PC12 cells induced by OGD/REP

To verify the effects of miR-324-3p expression on the damage to PC12 cells induced by OGD/REP, PC12 cells were transfected with miR-324-3p mimic to overexpress miR-324-3p or with miR-324-3p inhibitor to suppress miR-324-3p expression. PC12 cell viability was not affected by either the miR-324-3p mimic or the inhibitor (**Figure 2A**). Furthermore, miR-324-3p expression was markedly increased compared with the control group after transfection with the miR-324-3p mimic and decreased after transfection with the inhibitor (P < 0.05; **Figure 2B**). To clarify the role of miR-324-3p in ischemic stroke, PC12 cells were transfected with the miR-324-3p mimic or inhibitor prior to OGD/REP. As expected, miR-324-3p expression increased markedly in the mimic group but decreased in the group transfected with the inhibitor (**Figure 2C**). We found that transfection with the miR-324-3p mimic significantly decreased the viability of PC12 cells compared with the control cells, whereas transfection with the miR-324-3p inhibitor had the opposite effect (P < 0.05; **Figure 2D**). qRT-PCR and western blot analyses of apoptosis-related proteins indicated that OGD/REP treatment markedly increased levels of the pro-apoptotic factor BAX and decreased levels of the anti-apoptotic factor BCL2 in PC12 cells compared with the control cells (P < 0.05), while miR-324-3p overexpression significantly increased BAX levels and decreased BCL2 levels after OGD/REP compared with the control cells (P < 0.05; **Figure 2E–I**), suggesting that miR-324-3p overexpression substantially increases the effects of ischemic stroke in an *in vitro* model. In addition, we found that BAX levels in PC12 cells were markedly reduced by transfections with the miR-324-3p inhibitor, whereas the BCL2 levels rose significantly compared with the control cells (P < 0.05; **Figure 2E–I**). In addition, flow cytometry analysis suggested that transfection with the miR-324-3p inhibitor reduced apoptosis in PC12 cells subjected to OGD/REP compared with the OGD/REP inhibitor NC group (P < 0.05). Furthermore, miR-324-3p overexpression significantly increased PC12 cell death after OGD/REP 24 hours after transfection with the miRNA compared with the miR-324-3p mimic NC group (P < 0.01; **Figure 2J** and **K**). These data indicate that altered miR-324-3p expression regulates OGD/REP-induced cell damage in PC12 cells.

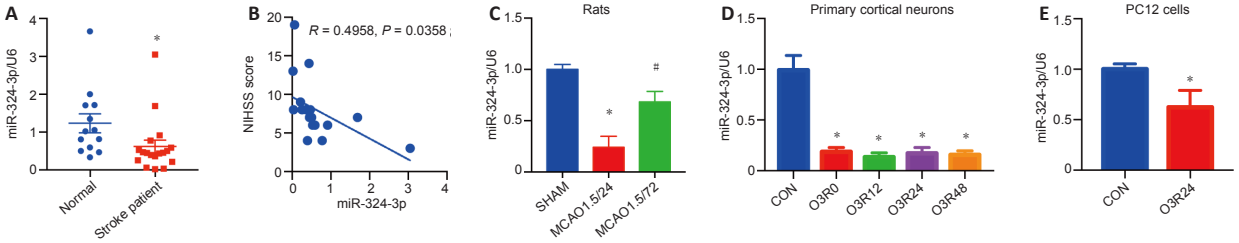

Figure 1 | **miR-324-3p levels in AIS patients and in *in vivo* and *in vitro* models of ischemic stroke.**
(A) miR-324-3p levels in AIS patients and in age- and gender-matched non-stroke controls, as detected by quantitative reverse transcription-polymerase chain reaction. (B) Correlation between miR-324-3p and NIHSS scores in AIS patients (Pearson correlation analysis). (C) miR-324-3p levels in the ischemic cerebral cortex of MCAO-treated rat at 24 and 72 hours after cerebral ischemia and reperfusion; n = 5 per group. MCAO 1.5/24 group: 1.5 hours of ischemia and 24 hours of reperfusion; MCAO 1.5/72 group: 1.5 hours of ischemia and 72 hours of reperfusion. (D) miR-324-3p expression levels in primary cortical neurons subjected to OGD/REP; n = 6 per group. After 3 hours of glucose oxygen deprivation, the primary cortical neurons were reoxygenated for 0, 12, 24, or 48 hours (O3R0, O3R12, O3R24, and O3R48, respectively). (E) miR-324-3p expression levels in PC12 cells subjected to OGD/REP; n = 3 per group. Bars represent means ± SEM. The experiments were repeated three times. *P < 0.05, *vs.* normal, sham, or CON; #P < 0.05, *vs.* MCAO 1.5/24 (Student's t-test for A, C, and E; one-way analysis of variance followed by Bonferroni correction for D). AIS: Acute ischemic stroke; CON: control; MCAO: middle cerebral artery occlusion; NIHSS: National Institute of Health stroke scale; OGD/REP: glucose oxygen deprivation/reoxygenation; SEM: standard error of the mean.

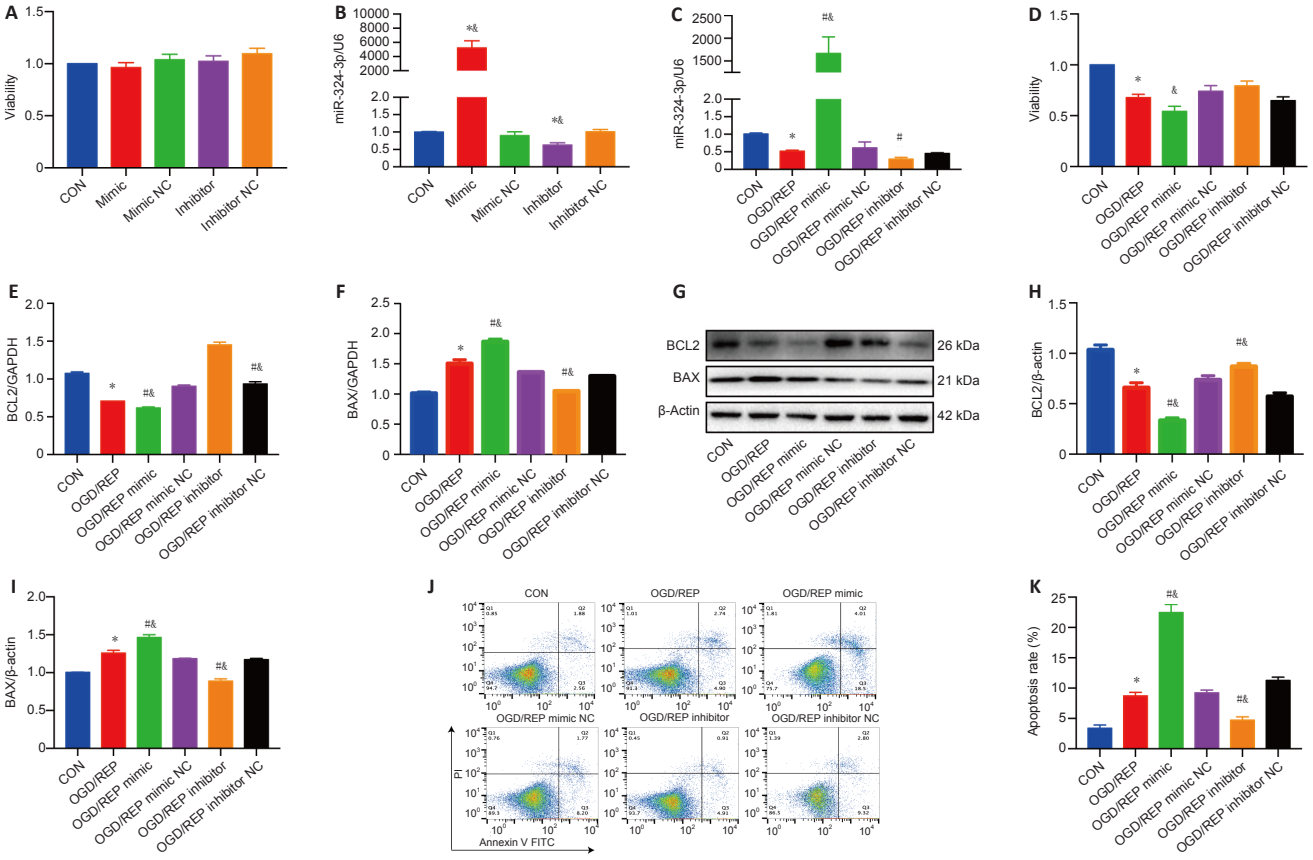

Figure 2 | **Altered miR-324-3p expression regulates OGD/REP-induced cell damage in PC12 cells.**
(A) Cell viability as detected by cell counting kit-8 assay. The percentage of viable PC12 cells is shown; n = 7 per group. (B) The levels of miR-324-3p were analyzed via qRT-PCR. n = 5. (C) miR-324-3p levels were analyzed via qRT-PCR; n = 3 per group. (D) Percentage of viable PC12 cells; n = 6 per group. (E, F) BCL2 and BAX mRNA expression levels were analyzed by qRT-PCR. n = 3 per group. (G–I) BCL2 and BAX protein levels were analyzed by qRT-PCR; n = 3 per group. (J, K) Apoptosis was assessed by flow cytometry, and representative images are shown; n = 3 per group. The percentage of apoptotic cells is also shown. Bars represent means ± SEM. The experiments were repeated three times. *P < 0.05, *vs.* CON; #P < 0.05, *vs.* OGD/REP; &P < 0.05, *vs.* mimic NC or inhibitor NC (one-way analysis of variance followed by Bonferroni correction). BAX: BCL2-associated X; BCL2: cell lymphoma-2; CON: control; FITC: fluorescein isothiocyanate; NC: negative control; OGD/REP: glucose oxygen deprivation/reoxygenation; PI: propidium iodide; SEM: standard error of the mean; qRT-PCR: quantitative reverse transcription-polymerase chain reaction.

miR-324-3p regulates A1R

We next investigated whether A1R expression is important for the neuroprotective effects mediated by miR-324-3p. We found that A1R mRNA expression was increased after OGD/REP in primary cortical neurons and PC12 cells compared with the control group (**Figure 3A** and **D**). In addition, A1R protein expression was significantly increased after OGD/REP in primary cortical neurons and PC12 cells compared with the control group (**Figure 3B–F**). Furthermore, A1R expression was significantly down-regulated in PC12 cells transfected with the miR-324-3p mimic and was up-regulated in PC12 cells transfected with the miR-324-3p inhibitor compared with the OGD/REP group (**Figure 3G–I**). Finally, miR-324-3p inhibition increased A1R mRNA and protein levels in response to OGD/REP, and miR-324-3p overexpression decreased A1R mRNA and protein levels in response to OGD/REP in PC12 cells (**Figure 3J–L**). These data indicate that miR-324-3p regulates A1R expression after cerebral ischemia.

miR-324-3p regulates A1R expression through GATA2

TargetScan and PROMO were used to screen for miRNA downstream target genes and adenosine-A1R upstream transcription factors in humans, rats, and rats, and predicted that miR-324-3p regulates two transcription factors: *yin-yang* 1 (YY1) and GATA2 (**Figure 4A**). However, the 3′UTR of YY1 does not exist in the germline gene sequences of rats and mice, so it is impossible for mir-324-3p to directly regulate YY1. However, bioinformatics analysis identified a highly possible miR-324-3p seed region in the 3′UTR of GATA2 mRNA in human and rat (**Figure 4B**). After being subjected to OGD/REP, the levels of GATA2 mRNA and protein in primary cortical neurons and PC12 increased (**Figure 5A–F**). GATA2 mRNA and protein expression levels in PC12 cells were down-regulated by the miR-324-3p mimic and up-regulated by the miR-324-3p inhibitor (**Figure 5G–I**). In addition, miR-324-3p inhibition increased GATA2 mRNA and protein levels after OGD/REP, and miR-324-3p overexpression decreased GATA2 mRNA and protein levels after OGD/REP (**Figure 5J–L**). These results suggest that miR-324-3p promotes GATA2 mRNA degradation to negatively regulate GATA2 protein levels. However, it remained uncertain whether miR-324-3p inhibits GATA2 transcription or translation through direct recognition of the GATA2 mRNA 3′UTR. To test this, we constructed a plasmid fusing a luciferase reporter to the 3′UTR fragment of GATA2 mRNA containing the presumed miR-324-2p binding site. The luciferase activity observed in cells transfected with this reporter test was decreased significantly by cotransfection with miR-324-3p mimic compared with the control group ($P < 0.05$; **Figure 5M**). Treatment with a GATA2 siRNA reversed the effect of the miR-324-3p inhibitor, which increased A1R protein levels (**Figure 5N**). These data indicated that A1R expression is regulated by miR-324-3p through GATA2.

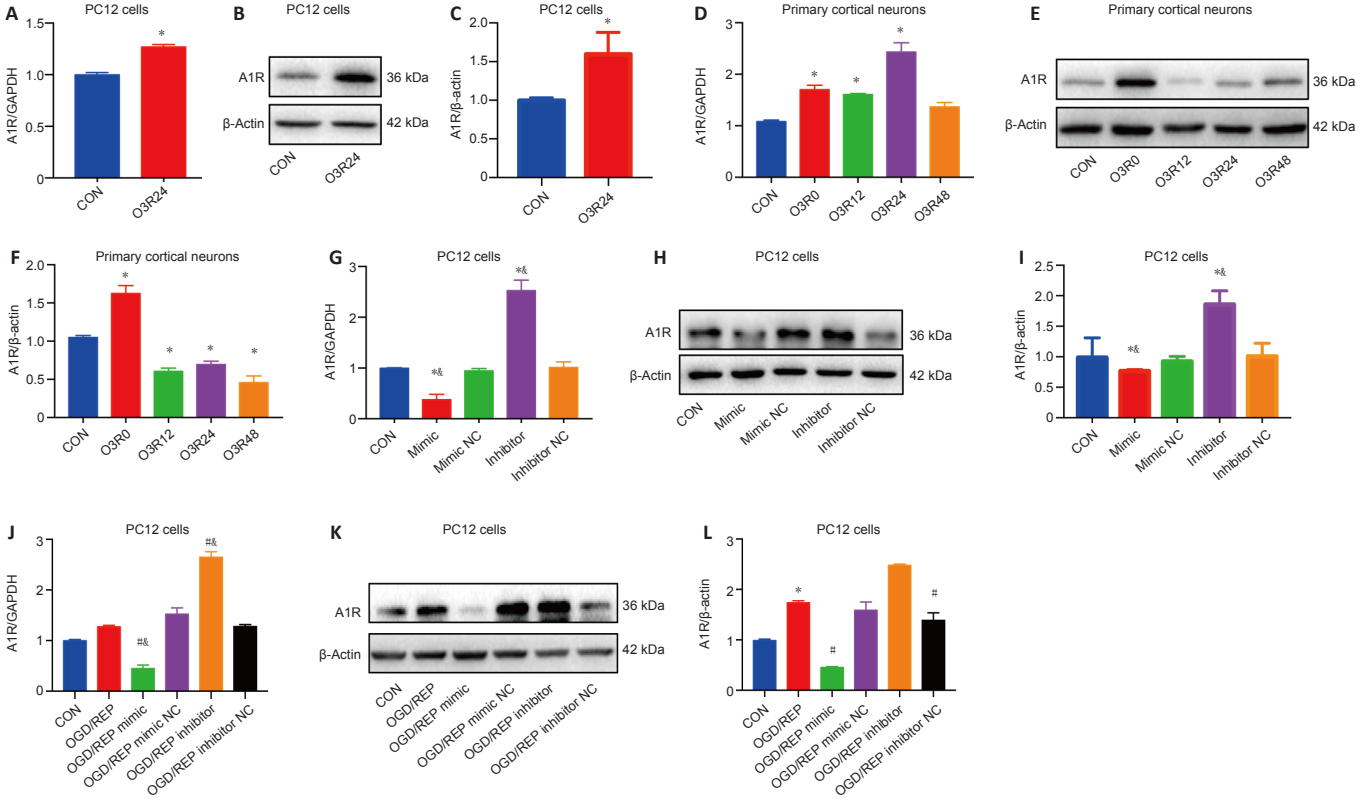

Figure 3 | **miR-324-3p regulates A1R in PC12 cells.**
(A) A1R mRNA levels in PC12 cells were analyzed by qRT-PCR; $n = 3$ per group. (B, C) A1R protein levels in PC12 cells were analyzed by western blot analysis. $n = 6$ per group. (D) A1R mRNA levels in primary cortical neuron cells were analyzed by qRT-PCR. $n = 3$ per group. (E, F) A1R protein levels in primary cortical neuron cells were analyzed by western blot analysis. $n = 3$ per group. (G) A1R mRNA levels in PC12 cells were analyzed by qRT-PCR. $n = 3$ per group. (H, I) A1R protein levels in PC12 cells were analyzed via western blot analysis. $n = 4$ per group. (J) A1R mRNA levels in PC12 cells were analyzed by qRT-PCR; $n = 3$ per group. (K, L) A1R protein levels in PC12 were analyzed by western blot analysis. $n = 3$ per group. Bars represent means ± SEM. The experiments were repeated three times. $*P < 0.05$, *vs.* CON; $\#P < 0.05$, *vs.* OGD/REP; $\&P < 0.05$, *vs.* mimic NC or inhibitor NC (Student's *t*-test for A and C; one-way analysis of variance followed by Bonferroni correction for D, F, G, I, J, and L). A1R: Adenosine A1 receptor; CON: control; NC: negative control; OGD/REP: glucose oxygen deprivation/reoxygenation; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; SEM: standard error of the mean.

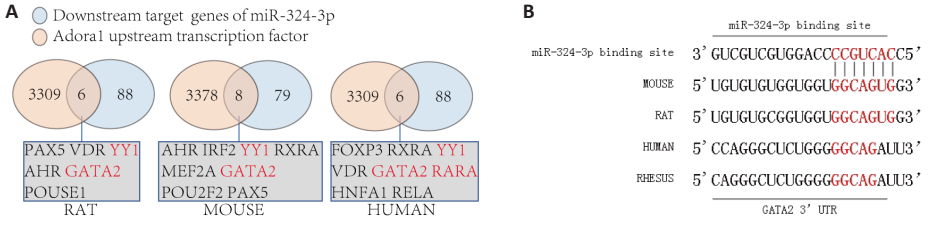

Figure 4 | **Bioinformatics analysis predicts that miR-324-3p regulates GATA2.**
(A) TargetScan and PROMO were used to screen for putative miR-324-3p downstream target genes and A1R upstream transcription factors in humans, rats, and mice and identified two transcription factors, YY1 and GATA2. (B) Predict the binding site of miR-324-3p in the GATA2 mRNA 3′-UTR. Red indicates complementary bases. GATA2: GATA binding protein 2; UTR: untranslated region; YY1: *yin-yang* 1.

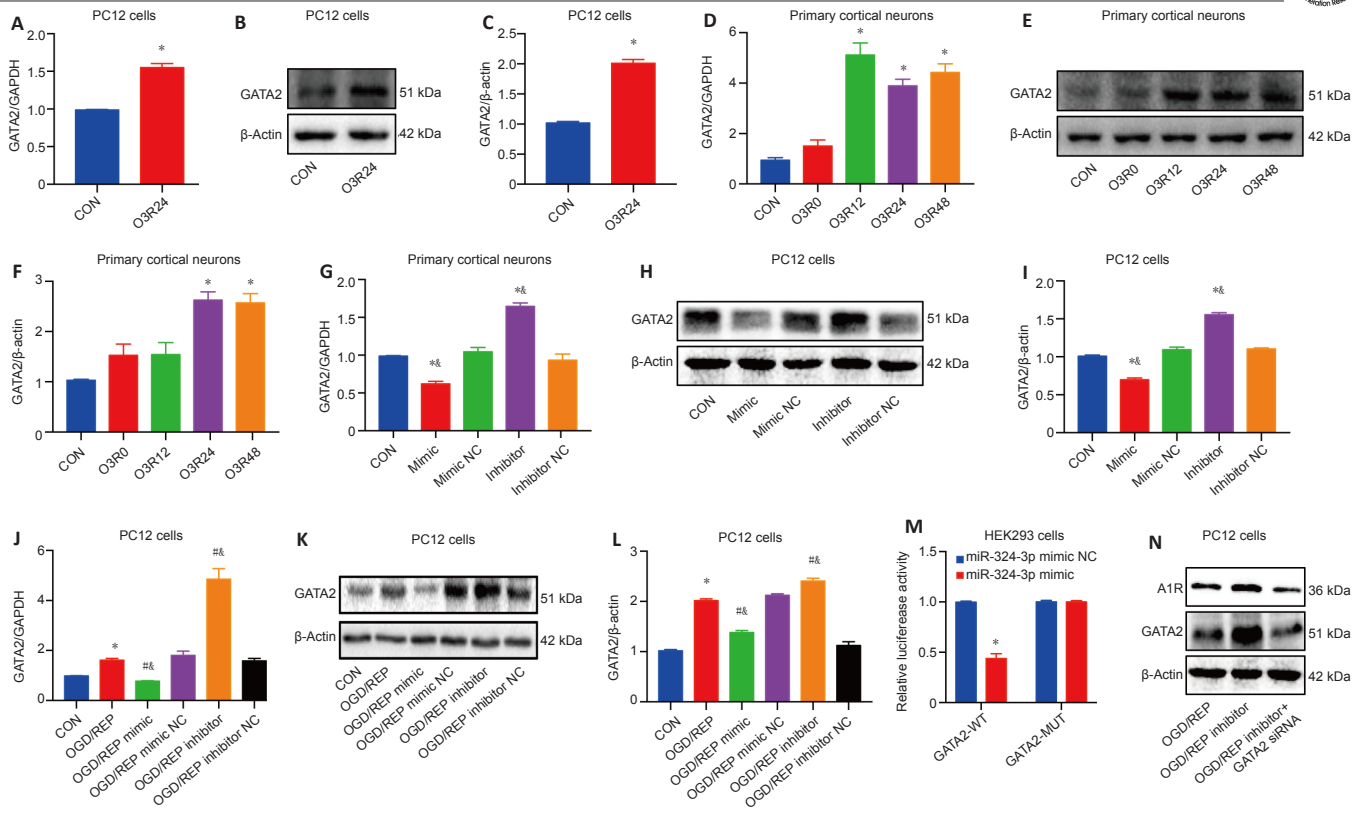

Figure 5 | **miR-324-3p regulates A1R expression through GATA2 in PC12 cells, primary cortical neurons, and 293T cells.**
(A) GATA2 mRNA expression levels in PC12 cells were analyzed by qRT-PCR. *n* = 3 per group. (B, C) GATA2 protein levels in PC12 were analyzed by western blot analysis. *n* = 3 per group. (D) GATA2 mRNA levels in primary cortical neuron cells were analyzed by qRT-PCR. *n* = 3 per group. (E, F) GATA2 protein levels in primary cortical neuron cells were analyzed by western blot analysis. *n* = 3 per group. (G) GATA2 mRNA levels in primary cortical neuron cells were analyzed by qRT-PCR. *n* = 3 per group. (H, I) GATA2 protein levels in primary cortical neuron cells were analyzed via western blot analysis. *n* = 3 per group. (J) GATA2 mRNA levels in primary cortical neuron cells were analyzed via qRT-PCR. *n* = 3 per group. (K, L) GATA2 protein levels in primary cortical neuron cells were analyzed by western blot analysis. *n* = 3 per group. (M) Fluorescence intensity results from the luciferase reporter assay in 293T cells. (N) GATA2 and A1R protein levels in PC12 cells were analyzed by western blot analysis. *n* = 3 per group. Bars = means ± SEM. The experiments were repeated three times. **P* < 0.05, *vs.* CON; #*P* < 0.05, *vs.* OGD/REP; &*P* < 0.05, *vs.* mimic NC or inhibitor NC (Student's *t*-test for A and C; one-way analysis of variance followed by Bonferroni correction for D, F, G, I, J, L, and M). A1R: Adenosina A1 receptor; BAX: BCL2-associated X; BCL2: cell lymphoma-2; GATA2: GATA binding protein 2; MUT: mutation; NC: negative control; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; SEM: standard error of the mean; UTR: untranslated region; WT: wild-type.

miR-324-3p agomir potentiates ischemic brain injury in rats

Next, we tested the effects of miR-324-3p on ischemic stroke in rats. To do this, we injected rats in the left lateral ventricle with either a miR-324-3p agomir or a control agomir (NC) 3 days before MCAO and found that miR-324-3p levels rose significantly in miR-324-3p agomir-treated rats compared with those in the NC rats ($P < 0.05$; **Figure 6A**). Compared with the NC agomir, the miR-324-3p agomir aggravated brain injury ($P < 0.05$). Rats receiving the miR-324-3p agomir displayed larger infarct volumes and worse behavioral scores compared with those of the rats that received NC agomir ($P < 0.05$; **Figure 6B–D**). Compared with the NC agomir, the miR-324-3p agomir significantly increased BAX protein levels ($P < 0.05$; **Figure 6G** and **I**) and decreased BCL2 protein levels ($P < 0.05$; **Figure 6G** and **H**), but the miR-324-3p agomir did not alter BCL2 and BAX mRNA levels (**Figure 6E** and **F**). Next, we assessed adenosine A1R and GATA2 expression in the MCAO model rats. Adenosine A1R and GATA2 mRNA and protein levels were significantly increased in the MCAO model rats compared with the sham group ($P < 0.05$; **Figure 7A–E**). Compared with the NC agomir-treated group, qRT-PCR analysis confirmed that treatment with the miR-324-3p agomir increased miR-324-3p expression ($P < 0.05$; **Figure 7F**). Furthermore, treatment with the miR-324-3p agomir significantly decreased A1R and GATA2 mRNA levels and decreased GATA2 protein levels ($P < 0.05$, *vs.* mimic NC or inhibitor NC), but did not alter A1R protein levels (**Figure 7G–K**). Compared with the NC agomir group, treatment with the miR-324-3p agomir decreased A1R and GATA2 mRNA and protein levels in the MCAO model rats ($P < 0.05$; **Figure 7L–N**). We concluded from these findings that mir-324-3p regulates GATA2 and A1R, and that the miR-324-3p agomir stimulates brain damage, in a rat MCAO model of ischemic stroke.

Discussion

Our study provides new insight into the function of miR-324-3p, which we confirmed plays a role in stroke by binding GATA2 and subsequently targeting A1R. Specific down-regulation of miR-324-3p may be used for clinical treatment of cerebral ischemia in the future.

We found that miR-324-3p expression was down-regulated in AIS patients, as well as in *in vitro* and *in vivo* models of ischemic stroke. We also found that an miR-324-3p agomir potentiates ischemic brain injury in rats subjected to MCAO, as indicated by an increase in infarct volumes as well as apoptosis. We screened several databases to identify probable targets of miR-324-3p to further explore the biological pathway in which the miR-324-3p functions in PC12 cells. Mechanistically, our results suggest that the miR-324-3p-mediated damage is associated with the GATA2/A1R axis. We found that A1R and GATA2 protein levels were increased by OGD/REP in primary cortical neurons and PC12 cells, as well as in an *in vivo* MCAO model. In addition, A1R and GATA2 mRNA levels also increased. First, as expected, the western blot and qRT-PCR analyses revealed that miR-324-3p could regulate A1R expression levels in cerebral ischemia. Luciferase activity tests revealed that transfection with an miR-324-3p mimic markedly reduced the bioactivity of a luciferase reporter construct that included the GATA2 mRNA 3'UTR sequence containing the putative miR-324-3p binding site. To the best of our knowledge, this is the first report of the function of miR-324-3p/GATA2/A1R in ischemic stroke. Our results provide a novel target for treating ischemic stroke.

miR-324-3p is a commonly expressed miRNA that regulates tumorigenesis. The role of miR-324-3p in non-cancer diseases has been receiving increasing attention recently. For example, miR-324-3p protects against coxsackievirus B3-induced viral myocarditis (Liu et al., 2021). In addition, Dex can attenuate the pulmonary inflammatory response by increasing lncH19 expression and reducing miR-324-3p expression. miR-324-3p may have both beneficial and detrimental effects in human disease. In the current study we found that silencing miR-324-3p had neuroprotective effects in the context of ischemic injury. Thus, miR-324-3p is detrimental in the setting of ischemic injury. There is an apparent contradiction in our results in that miR-324-3p expression was decreased in acute ischemic stroke (AIS) patients and in *in vitro* and *in vivo* models of ischemic stroke, and silencing miR-324-3p had a neuroprotective effect. One possible explanation for this observation is that miR-324-3p is down-regulated as a protective measure in the setting of ischemic stroke, and that further silencing of miR-324-3p enhanced this protective effect.

There are six GATA transcription factors, GATA1–6, and GATA2, 3, 4, and 6 are expressed in the central nervous system during the development of vertebrates (Lentjes et al., 2016). GATA2 has a zinc finger structure that it uses to bind target genes, thereby regulating their expression, and is widely expressed in brain, liver, heart muscle, and kidney (Chan et al., 2012; Lahti et al., 2016). Mammoto et al. (2009) showed that GATA2 is closely associated with vascular regeneration after hypoxia in vascular endothelial cells. Chan et al. (2012) found that the down-regulation of miR-200b increased GATA2 expression, which in turn reduced VEGFR2 expression, which helps to restore blood vessels in wounded skin. Shobatake et al. (2018) showed that POMC and CART mRNA expression in human neuronal cells is up-regulated in response to intermittent hypoxia through the transcription factors GATA2 and GATA3. GATA2 has not been reported to play a role in cerebral ischemia. Our study shows that GATA2 plays a protective role in the ischemic brain by regulating AIR.

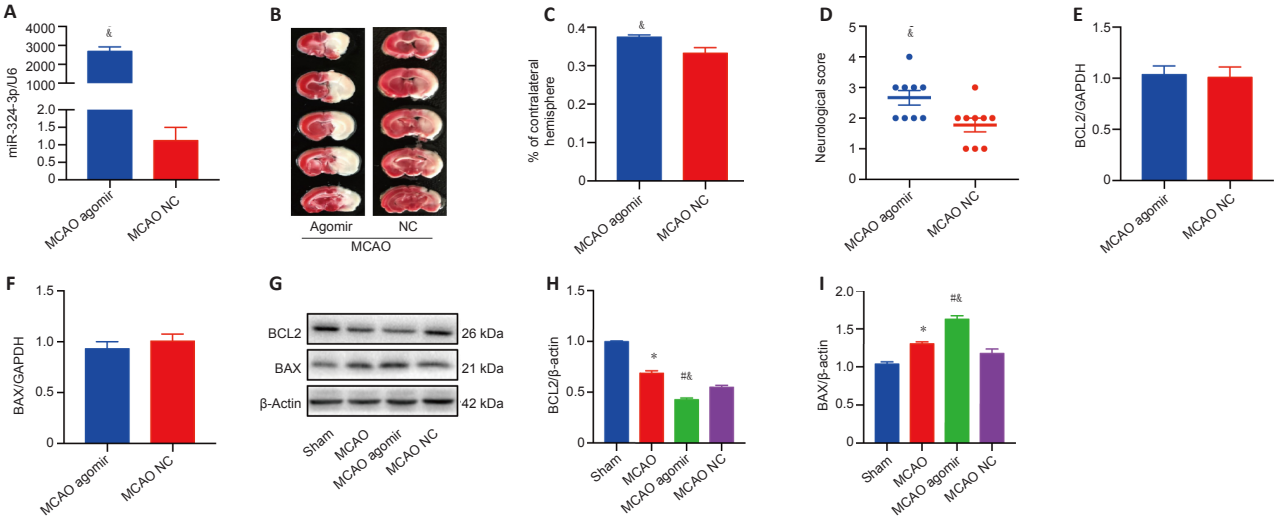

Figure 6 | **miR-324-3p agomir potentiates ischemic brain injury in rats.**
(A) miR-324-3p mRNA levels in the ischemic penumbra of brain tissues were analyzed via qRT-PCR. *n* = 3 per group. (B, C) Quantitative analysis of brain infarct volume. Normal tissue is red and infarcted area is white. *n* = 6 per group. (D) Quantitative analysis of neurological deficits using a modified neurobehavioral scoring system. Higher scores represent greater neurological deficit; *n* = 9 per group. (E) BCL2 mRNA levels were analyzed by quantitative reverse transcription-polymerase chain reaction. *n* = 3 per group. (F) Bax mRNA levels were analyzed by qRT-PCR. *n* = 3 per group. (G–I) BCL2 and BAX protein levels were analyzed by western blot analysis. *n* = 3 per group. The experiments were repeated three times.*$P < 0.05$, *vs*. sham; #$P < 0.05$, *vs*. MCAO; &$P < 0.05$, *vs.* NC. Except for neurological scores, data are expressed as the mean ± SEM and were analyzed with Student's t-test for A, C, E, and F. For H and I, the data were analyzed with one-way analysis of variance followed by Bonferroni correction for. Neurological scores were analyzed with the non-parametric Kruskal-Wallis H test followed by the Nemenyi test for D. BAX: BCL2-associated X; BCL2: cell lymphoma-2; MCAO: middle cerebral artery occlusion.

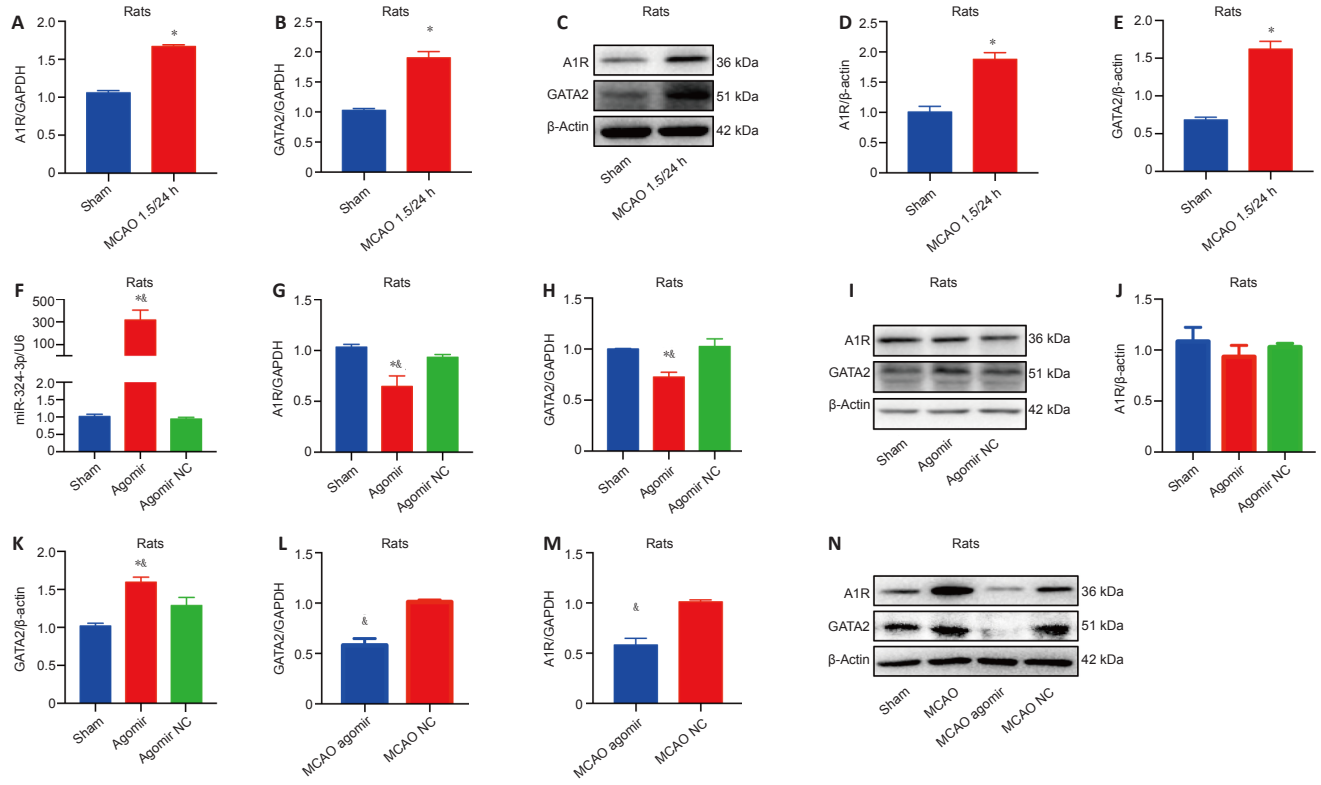

Figure 7 | **miR-324-3p regulates GATA2 and A1R in the ischemic penumbra of rat brains.**
(A, B) A1R (A) and GATA2 mRNA (B) expression levels were analyzed by qRT-PCR; *n* = 6 per group. (C–E) A1R and GATA2 protein expression levels were analyzed by western blot analysis; *n* = 6 per group. (F–H) miR-324-3p, A1R, and GATA2 mRNA levels were analyzed by qRT-PCR; *n* = 3 per group. (I–K) A1R, GATA2 protein levels in the ischemic penumbra of the brain were analyzed by western blot analysis; *n* = 3 per group. (L, M) A1R and GATA2 mRNA levels were analyzed by qRT-PCR; *n* = 3 per group. (N) A1R, GATA2 protein levels in the ischemic penumbra of the brain were analyzed by western blot analysis; *n* = 3 per group. Bars represent means ± SEM. The experiments were repeated three times. *$P < 0.05$, *vs.* sham; &$P < 0.05$, *vs*. NC (Student's *t*-test for A, B, D, E, L, and M; one-way analysis of variance followed by Bonferroni correction for F–H, J, and K). A1R: Adenosine A1 receptor; GATA2: GATA binding protein 2; MCAO: middle cerebral artery occlusion; MCAO 1.5/2.4 h: 1.5 hours of ischemia and 24 hours reperfusion; NC: negative control; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; SEM: standard error of the mean.

A1R is a 326-amino acid glycoprotein with a relative molecular weight of 36,600 Da. Adenosine A1R has the maximum affinity for adenosine out of all known adenosine receptors. Adenosine A1R is expressed at the highest levels in the central nervous system, and is commonly found in the thalamus, cerebral cortex, hippocampus, basal forebrain, medulla oblongata, hypothalamus, and cerebellum. Activation of adenosine A1R opens the potassium channel and increases potassium outflow, resulting in membrane hyperpolarization and thereby decreasing excitability and protecting neurons (Liu et al., 2019). Paeoniflorin has been shown to ameliorate ischemia-induced neuronal damage *in vitro* through trans-activating epidermal growth factor receptor mediated by adenosine A1R (Zhong et al., 2015). Adenosine A1R antagonists can weaken the rapid tolerance that is observed in response to focal cerebral ischemia, which may be mediated by activating adenosine A1R (Nakamura et al., 2002). In summary, adenosine A1R plays a role in protecting against the effects of cerebral ischemia.

After stroke, glutamate, which is a neurotoxic excitatory neurotransmitter, plays a crucial role in ischemia through excitotoxic mechanisms (Wen et al., 2021). Excessive release of glutamate leads to excessive activation of postsynaptic glutamate receptors, which ultimately triggers downstream

cascades, leading to neuronal dysfunction and degeneration (Mayor and Tymianski, 2018). *In vivo* experiments have shown that clearing extracellular Na^+ can completely eliminate the rapid neuronal swelling caused by glutamate, while clearing Ca^{2+} has no effect. However, removing extracellular Ca^{2+} can eliminate the delayed neuronal death caused by glutamate, and this effect can be enhanced by increasing the Ca^{2+} concentration in the medium (Zhang et al., 2018). The initial Ca^{2+} influx will cause a secondary toxic Ca^{2+} overload in cells, which is closely related to neuronal death (Tymianski et al., 1993). Therefore, Ca^{2+} is a key factor involved in glutamate neurotoxicity. Activation of A1Rs, which represses excitatory synaptic transmission, as shown *in vitro* and *in vivo*, is one of the primary adaptive mechanisms adopted by cells in response to hypoxia/ischemia (Corsi et al., 2000). It has been shown that cordycepin decreases the impairment of long-period potentiation and the neuronal loss triggered by cerebral ischemia, as well as excitotoxicity in the CA1 region of the hippocampus, and that adenosine A1R is essential for these effects (Dong et al., 2019). Although our findings show that adenosine A1R exerts a neuroprotective effect during ischemia, the utilization of selective A1R agonists is impeded by unwanted peripheral side-effects such as bradycardia, hypotension, and sedation (Fredholm et al., 2005).

There were some limitations in this study. First, we used PC12 cells to demonstrate the role of miR-324-3p instead of using primary cortical neurons, which are more representative of human physiology. Second, we only used an miR-324-3p agomir to demonstrate the effects of miR-324-3p on ischemic stroke. Third, we did not definitively show that the transcription factor GATA2 directly binds the A1R promoter region to promote A1R transcription.

In conclusion, the results from our study show that silencing miR-324-3p decreases the brain damage caused by ischemia by reducing infarct volume, decreasing neurological deficit, and inhibiting apoptosis through increasing adenosine A1R via GATA2. These findings suggest that miR-324-3p could be as therapeutic target for cerebral ischemia.

Author contributions: *Conceptualization: AQZ and QXD; methodology: AQZ and LW; software technique: YSZ; data validation: SSH and XLW; formal analysis: YCM; investigation, BBZ; data analysis: QMY, SDC; writing—original draft preparation: AQZ and YXW; writing—review and editing: YXW and HFF; study supervision: JLW and RYY; funding acquisition: JLW. All authors approved the final manuscript.*
Conflicts of interest: *The authors declare no conflict of interest.*

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