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

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 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 neuronal apoptosis. However, treatment with a miR-324-3p inhibitor had the opposite effect. 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 negative regulators of gene expression at that posttranscriptional level. miRNAs therefore considered promising targets for developing novel gene therapies. 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 to 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 nervous 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 et al., 2020).

Adenosine A1 receptor expression via GATA2

Materials and Methods

Results

Discussion

References

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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.

| 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 (157–165.7) | 0.435 |
| Weight (kg)       | 65.75 ±11.45 | 57.84 ±7.82     | 0.138   |
| BMI (kg/m²)       | 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) 2015-0021). 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 a 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), penicillin/streptomycin (1% v/v, Thermo Fisher Scientific). The cell suspensions were then seeded into six-well plates (1 × 10⁶ 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-1712, 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) CO₂ at 37°C. The medium was changed every 2 days. When 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/REo), the cells were divided into 11 groups, as shown in Table 2.

| Group                  | PC12 cells |
|------------------------|------------|
| CON                    | PC12 cells |
| Mimic                  | PC12 cells |
| Mimic NC               | PC12 cells |
| Inhibitor              | PC12 cells |
| Inhibitor NC           | PC12 cells |
| OGD/RE                 | PC12 cells |
| OGD/RE mimic           | PC12 cells |
| OGD/RE mimic NC        | PC12 cells |
| OGD/RE inhibitor       | PC12 cells |
| OGD/RE inhibitor NC    | PC12 cells |
| OGD/RE inhibitor + GATA2 sRNA | PC12 cells |

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 sterile water.
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), adenine A1 receptor (A1R), BCL2, and BAX mRNA expression in PC12 cells, primary cortical neurons were transfected with 0.16 μg of GATA2-WT or GATA2-MUT target plasmid in 10 μl DEMEM. miRNA was synthesized from 3 μg RNA using an miRNAFirst Synthesis Kit (Shangdong, Shanghai, China). The qRT-PCR reaction was carried out using an Applied Biosystems QuantoStatio 5 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) and a SYBR® Premix Ex Taq™ Kit (RR820A, Takara, Beijing, China). A standard qRT-PCR protocol (95°C for 30 s, followed by 40 cycles of 94°C for 10 s, 60°C for 10 s, and 65°C for 5 s) was performed using a Cx96 Touch Real Time PCR Detection System (Bio-Rad). Translation levels were quantified using the 2^-ΔΔCt method. All primers used for these experiments are shown in Table 3.

Western blot analysis

After 24 hours of reperfusion, the rats were anesthetized 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 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 a mirNAFirst Synthesis Kit (Shangdong, Shanghai, China). The qRT-PCR reaction was carried out using an Applied Biosystems QuantoStatio 5 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) and a SYBR® Premix Ex Taq Kit (RR820A, Takara, Beijing, China). A standard qRT-PCR protocol (95°C for 30 s, followed by 40 cycles of 94°C for 10 s, 60°C for 10 s, and 65°C for 5 s) was performed using a Cx96 Touch Real Time PCR Detection System (Bio-Rad). Translation levels were quantified using the 2^-ΔΔCt method. All primers used for these experiments are shown in Table 3.

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 were decreased in AIS patients and in vivo and in vitro 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). 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](image1.png)
**Figure 1** | miR-324-3p levels in AIS patients and 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 rats 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 mean ± SEM. The experiments were repeated three times. *P < 0.05, vs. control; **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](image2.png)
**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) BCL2 and BAX protein levels were analyzed by Western blot. BCL2 and BAX protein levels were analyzed by Western blot; n = 3 per group. (H–I) Bcl-2 and Bax protein levels were analyzed by western blot; n = 3 per group. Bars represent mean ± 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 A1R upstream transcription factors in humans, rats, and mice and identified two transcription factors, YY1 and 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 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, bioinformatic 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-324-3p 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](image-url) | 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 neurons were analyzed by qRT-PCR; n = 3 per group. (E, F) A1R protein levels in primary cortical neurons were analyzed by western blot analysis. n = 3 per group. (G, H) A1R mRNA levels in PC12 cells were analyzed via qRT-PCR. n = 3 per group. (I, J) A1R protein levels in PC12 cells were analyzed via western blot analysis. n = 4 per group. (K, L) A1R protein levels in PC12 cells 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](image-url) | 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.
miR-324-3p agomir potentiates ischemic brain injury in rats

Next, we tested the effects of miR-324-3p agomir 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 increased miR-324-3p expression (P < 0.05; Figure 6B). 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 mRNA and 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 revealed that miR-324-3p could regulate A1R expression levels in cerebellar ischemia. Luciferase activity tests revealed that transfection with an miR-324-3p mimic markedly reduced the luciferase activity. Additionally, we confirmed that the miR-324-3p agomir significantly increased miR-324-3p expression (P < 0.05; Figure 7A–E). Moreover, treatment with the miR-324-3p agomir significantly decreased A1R mRNA levels (P < 0.05; Figure 7A–G). We concluded from these findings that miR-324-3p regulates A1R and GATA2, 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 over-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 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 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 revealed that miR-324-3p could regulate A1R expression levels in cerebellar ischemia. Luciferase activity tests revealed that transfection with an miR-324-3p mimic markedly reduced the luciferase activity. Additionally, we confirmed that the miR-324-3p agomir significantly increased miR-324-3p expression (P < 0.05; Figure 7A–E). Moreover, treatment with the miR-324-3p agomir significantly decreased A1R mRNA levels (P < 0.05; Figure 7A–G). We concluded from these findings that miR-324-3p regulates A1R and GATA2, and that the miR-324-3p agomir stimulates brain damage, in a rat MCAO model of ischemic stroke.

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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 Nemeyi 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 (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) A1R and GATA2 protein expression levels were analyzed by western blot analysis; n = 3 per group. (I–K) A1R and 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 1.5/24 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.
cascades, leading to neuronal dysfunction and degeneration (Mayor and Tymiak, 2018). In vivo experiments have shown that clearing extracellular Na+ can completely eliminate the rapid neuronal swelling caused by glutamate, while clearing Ca2+ has no effect. However, removing extracellular Ca2+ can eliminate the delayed neuronal death caused by glutamate, and this effect can be enhanced by increasing the Ca2+ concentration in the medium (Zhang et al., 2018). The initial Ca2+ influx will cause a secondary toxic Ca2+ overload in cells, which is closely related to neuronal death (Mayor and Tymiak, et al., 1993). Therefore, Ca2+ 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 ischemia/infarction (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 neurones, which are more representative of human physiology. Second, we only used a 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 neuronal damage caused by ischemia by reducing synaptic volume, decreasing neurological deficit, and inhibiting apoptosis through increasing adenosine A1R via GATA2. These findings suggest that miR-324-3p could be a therapeutic target for cerebral ischemia.

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Conflicts of interest: The authors declare no conflict of interest.

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