Up-regulation of miR-335 and miR-674-3p in the rostral ventrolateral medulla contributes to stress-induced hypertension

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Funding information
The Chinese National Natural Science Foundation, Grant/Award Number: 31100838, 31571171, 31671151 and 32071111; The Shanghai Natural Science Foundation, Grant/Award Number: 15ZR1414900; The Zhejiang Chinese Medical University Research Funding, Grant/Award Number: 111100E017/005/007/002; Zhejiang Chinese Medical University

Abstract
The rostral ventrolateral medulla (RVLM) is known as the vasomotor center that plays a crucial role in mediating the development of stress-induced hypertension (SIH). MicroRNAs (miRNAs) are involved in many different biological processes and diseases. However, studies that evaluated the roles of miRNAs in the RVLM during SIH do not exist. Here, we performed RNA sequencing to explore the genome-wide miRNA profiles in RVLM in an SIH rat model established by administering electric foot-shocks and noises. The function of miRNAs in blood pressure regulation was determined in vivo via the intra-RVLM microinjection of the agomir or antagomir. Furthermore, the underlying mechanisms of miRNAs on SIH were investigated through in vitro and in vivo experiments, like gain-of-function. We discovered 786 miRNA transcripts among which 4 were differentially expressed. The over-expression of miR-335 and miR-674-3p in RVLM dramatically increased the heart rate (HR), arterial blood pressure (ABP), systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) levels of normotensive rats, whereas the knockdown of miR-335 and miR-674-3p in RVLM markedly reduced the HR, ABP, SBP, DBP, and MAP levels of SIH rats. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation revealed that miR-335 and miR-674-3p participated in regulating the development of SIH from different aspects, like apoptosis-multiple species pathway. Sphk1, whose expression was markedly decreased in SIH, was identified as a novel target of miR-335. MiR-335 over-expression substantially reduced the expression of Sphk1 and promoted neural apoptosis, and its inhibition had opposite effects. Re-introduction of Sphk1 dramatically abrogated the apoptosis induced by miR-335. This study provides the first systematic dissection of the RVLM miRNA landscape in SIH. MiR-335 and miR-674-3p act as SIH promoters, and the identified miR-335/Sphk1/apoptosis axis represents one of the possible mechanisms. These miRNAs can be exploited as potential targets for the molecular-based therapy of SIH.
INTRODUCTION

Hypertension, which is mainly characterized by the persistent elevation of blood pressure (systolic blood pressure [SBP] ≥140 mm Hg and/or diastolic blood pressure [DBP] ≥90 mm Hg), is a major risk factor for various noncommunicable diseases, including heart attacks, aneurysms, strokes, kidney damage, and blindness (Cuffman et al., 2020; Mills et al., 2020; Unger et al., 2020). The World Health Organization estimated that more than 1 billion people suffer from hypertension globally (World Health Organization, 2020). Its high prevalence imposes a tremendous burden on patients, families, and societies. Several modifiable factors, such as lack of physical activity, are associated with an increased risk of hypertension (Mills et al., 2020). Continued exposure to stressors, such as high-intensity work and long periods of sleeplessness, is also involved in the pathophysiology of hypertension (Liu et al., 2017; Wu et al., 2020). This condition is often referred to as the stress-induced hypertension (SIH). Stress enhances sympathetic activity, which in turn contributes to SIH development (Imumorin et al., 2005).

Rostral ventrolateral medulla (RVLM) is located in a small region of the brainstem reticular formation and controls sympathetic activity by receiving and integrating all internal and external inputs and is thus considered as a major node of the blood pressure regulation network (Barnes & McDougal, 2018). Chronic stress increases angiotensin II level, which in turn up-regulates the TLR4/MYD88/NF-κB signaling in the RVLM that has a major role in sympathetic activity and SIH development (Yang et al., 2020). Numerous other studies have also suggested that the stress-induced increase in sympathetic vasoconstriction drive in the RVLM promotes the overactivation of the sympathetic system and exacerbates SIH (Du et al., 2017; Zhang et al., 2020). However, all these explorations are limited to the alteration level in the expression of related genes. The complex regulatory mechanisms of gene expression in the RVLM underlying sympathetic activation and SIH development remain elusive.

Noncoding RNAs (ncRNAs), which are transcribed from DNA and lack protein-coding capability, are well known to be emerging key regulators in gene expression at the transcriptional, post-transcriptional, and translational levels (Mattick & Makunin, 2006). MicroRNAs (miRNAs) are typically 19–25 nucleotides long and are a subset of ncRNAs that either mediate the degradation of target mRNAs or simply inhibit their translation. (Cai et al., 2009; Lu & Rothenberg, 2018). An increasing number of reports have shown that miRNA contributes to many pathological and physiological processes (Ali Syeda et al., 2020; Cakmak & Demir, 2020; Jużwik et al., 2019; Bhaskaran & Mohan, 2014). Several miRNAs, such as miRNA-21, miRNA-145, miRNA-4516, and miRNA-181a, have been proven to be associated with hypertension (Biancardi & Sharma, 2020; Li et al., 2018; Ozkan et al., 2019). However, few researchers have looked into the miRNA profiles of the RVLM and their effects on sympathetic activation and blood pressure. DeCicco et al. described the in silico miRNA expression analysis of the RVLM of spontaneous hypertensive rats (DeCicco et al., 2015). At present, this is the only case in this field, and our understanding of it is severely limited. Moreover, DeCicco et al.’s study was mainly descriptive in nature and did not focus on SIH. Studies on the effects of miRNA network changes in the RVLM in sympathetic nervous excitement and SIH do not exist.

This study presents a transcriptome-wide overview of abnormally expressed miRNAs in the RVLM of an SIH rat model established by applying intermittent electric foot-shocks and noise stress. This model exhibits high levels of blood pressure, heart rate (HR), and renal sympathetic nerve activity (RSNA) and is generally accepted as an ideal subject for studies on SIH (Du et al., 2017; Zhang et al., 2020). In vivo analysis was performed to clarify the functions of these differentially expressed miRNAs in the control of sympathetic activation and blood pressure through the intra-RVLM micro-injection of miRNA agonist or antagonism. Furthermore, the potential molecular mechanisms underlying their functions were elucidated via in vivo and in vitro experiments. To the best of the authors’ knowledge, this research is the first to provide systematic insights into functional miRNA signatures in the RVLM of an SIH rat model. The identified miRNAs might be potential therapeutic targets for SIH.

MATERIALS AND METHODS

Animals

A total of 120 male Sprague–Dawley rats (RRID:MGI:5651135) weighing 230–260 g and aged 7 weeks old were obtained from the Animal Resources Center, Shanghai Medical College of Fudan University, Shanghai, China. The rats were maintained in cages (one rat per cage) with a standard environment (23°C ± 1°C, 55% ± 5% humidity, and a 12 h light/dark cycle) in the specified pathogen-free (SPF) facility of Shanghai University. They were...
given chow food and water ad libitum. The timeline of each experiment along with the number of rats used in each experiment is shown in Figure 1. Animals in the SIH group were placed in a cage (22 × 22 × 28 cm) with a grid floor and administered intermittent electric foot-shocks (35–80 V for a duration of 50 ms with an interval of 2–30 s). Noise (88–98 dB) made by a buzzer was given synchronously as a conditioned stimulus. The rats were subjected to stress stimulation for 2 h twice a day (9–11 am and 3–5 pm) for 15 consecutive days. The normotensive (control) rats were placed in cages for the same period, but they were not subjected to foot-shocks or noises. No exclusion criteria were pre-determined. No randomization was performed to allocate subjects in the study. In all cases, the RVLM tissues were collected after animals had been killed through cervical dislocation under inhalational anesthesia with isoflurane (isoflurane, a nonflammable liquid administered by vaporizing, is a general inhalation anesthetic drug). Briefly, place the rat in the induction chamber and adjust the oxygen flowmeter to 0.8–1.5 L/min and the isoflurane vaporizer to 3–5%. All animal studies were approved by the Shanghai University Animal Care Ethics Committee and conducted in accordance with the international guidelines on the ethical use of animals (National Research Council, 2011). The ethics approval number is SYXK (HU) 2019–0020. The number of animals used per group was indicated in the figure legends. The study was not pre-registered. The study was exploratory.

### Measurement of blood pressure, HR, and RSNA

Blood pressure was monitored and recorded via the femoral arterial cannula method (Du et al., 2017) with a polygraph (Model SMUP-A, Department of Physiology and Pathophysiology, Shanghai Medical College of Fudan University, Shanghai, China) under inhalational anesthesia with isoflurane (the rats were placed in an induction chamber; the oxygen flowmeter and the isoflurane vaporizer were adjusted to 0.8–1.5 L/min and 3%–5%). HR was calculated automatically by a computer in accordance with the blood pressure phasic wave. Throughout the measurement, the body temperature was continuously monitored by a rectal thermometer and kept at 37.5°C ± 0.5°C by using a temperature controller (H-KWDY-III, Quanshui Experimental Instrument). RSNA was evaluated as previously described (Wu et al., 2018). Briefly, we performed incision surgery on the left flank to identify and isolate the renal sympathetic nerve under inhalational anesthesia as above. The nerve was immediately placed on a pair of silver recording electrodes (Teflon 786 500, A-M System). Kwik-Sil gel (World Precision Instruments) was used to cover the exposed nerve and electrodes. A grass P55C preamplifier was utilized to amplify and filter nerve activity. The signal was integrated by a PowerLab system (AD Instruments, RRID:SCR_001620). After the measurement, the animals were killed through cervical dislocation under isoflurane anesthesia.
For cDNA synthesis and amplification. Afterward, 140–160 bp PCR SuperScript II reverse transcriptase, and PCR primer were then used ligated to 3′-adapter and 5′-adapter by T4 RNA ligase. RT primer, total RNA per sample was utilized as the input. The total RNA was subjected to BLAST with the pre-miRNAs of rat and other species in miRBase 22.0; the mapped pre-miRNAs were not further mapped to rat genome, but the reads were mapped to the rat genome. The extended genome sequences from the genomic loci may form hairpins (rat_novel miRNAs). In gp4, reads were mapped to rat genome but not to the pre-miRNAs of rat and other species in miRBase 22.0. The extended genome sequences from the genomic loci may form hairpins (rat_novel miRNAs). MiRNA expression levels were estimated by reads per million mapped reads (RPM). The formula was: the number of reads mapped to a miRNA/total number of mapped reads from given library × 106. Differential expression analysis was performed using the DESeq2. The p value was adjusted using the Benjamini-Hochberg method. MiRNAs with RPM >100 (at least in one of the SH and control groups) and p < 0.05 were described as differentially expressed between the two groups.

2.3 | Total RNA extraction and qualification

TRizol® reagent (Invitrogen, Cat.No.15596026) was used to extract total RNA from RVLM in accordance with the manufacturer’s instructions. RNA purity was examined using a Nanophotometer-NP80 (IMPLEN, Cat.No.CA10770-492). RNA integrity was assessed by using an Agilent Bioanalyzer 2100 system (Agilent Technologies, RRID:SCR_018043). In general, the RNA integrity number (RIN) should be 8. Qubit® 3.0 Fluorometer (Invitrogen, Cat.No.Q33216) and 1% agarose gels were applied to measure RNA concentration and degradation, respectively.

2.4 | MiRNA library construction and sequencing

Six sequencing libraries were constructed, that is, three for normotensive rats (control) and another three for SIH rats, by using TruSeq Small RNA Sample Prep Kit (Illumina, Cat.No.RS-200-0012) in accordance with the manufacturer’s protocol. In brief, 1 μg of total RNA per sample was utilized as the input. The total RNA was ligated to 3′-adapter and 5′-adapter by T4 RNA ligase. RT primer, SuperScript II reverse transcriptase, and PCR primer were then used for cDNA synthesis and amplification. Afterward, 140–160 bp PCR amplified fragments (corresponding to ~18–30 nt small RNAs) were isolated and purified by using 6% polyacrylamide gels. The constructed libraries were validated by the Agilent Bioanalyzer 2100 system (Agilent Technologies, RRID:SCR_018043), after which the samples were used for cluster generation. Finally, the libraries were sequenced at the LC-BIO (Hangzhou, China) on an Illumina HiSeq 2500 Platform (RRID:SCR_016383), and 50 bp single-end reads were generated.

2.5 | Sequencing data analysis and identification of differentially expressed miRNAs

Raw data were subjected to standardized analysis (Liu et al., 2021; Ma et al., 2018). Adapter dimers, low-quality reads, junk reads, common RNA families (mRNA, rRNA, tRNA, snRNA, and snoRNA), and repeats were removed by using the AC1G101-miR program (LC Sciences). The 18–26 nt length unique sequences were then subjected to BLAST with the pre-miRNAs of rat and other species in miRBase 22.0 and with the rat genome to detect known and rat_novel miRNAs. According to the BLAST results, these miRNA sequences were classified into four groups: gp1a, gp1b, gp2a, and gp4. In gp1a, reads were mapped to the pre-miRNAs of rat in miRBase 22.0 and the mapped pre-miRNAs were further mapped to rat genome (known miRNAs). In gp1b, reads were mapped to the pre-miRNAs of other species in miRBase 22.0 and the mapped pre-miRNAs were further mapped to rat genome (rat_novel miRNAs). In gp2a, reads were mapped to the pre-miRNAs of other species in miRBase 22.0; the mapped pre-miRNAs were not further mapped to rat genome, but the reads were mapped to the rat genome. The extended genome sequences from the genomic loci may form hairpins (rat_novel miRNAs). In gp4, reads were mapped to rat genome but not to the pre-miRNAs of rat and other species in miRBase 22.0. The extended genome sequences from the genomic loci may form hairpins (rat_novel miRNAs). MiRNA expression levels were estimated by reads per million mapped reads (RPM). The formula was: the number of reads mapped to a miRNA/total number of mapped reads from given library × 106. Differential expression analysis was performed using the DESeq2. The p value was adjusted using the Benjamini-Hochberg method. MiRNAs with RPM >100 (at least in one of the SH and control groups) and p < 0.05 were described as differentially expressed between the two groups.

2.6 | RT-qPCR assay

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed by using the Hieff® qPCR SYBR Green Master Mix (Yeasen, Cat.No.11195ES03) and CFX96 Touch Real-Time PCR Detection System (Bio-Rad, RRID:SCR_018064). The specific quantitative primers are listed in Table 1. The reaction mixture contained 0.4 μl of each primer (10 μM), 7.2 μl of H2O, 2 μl of cDNA, and 10 μl of Hieff® qPCR SYBR Green Master Mix. U6 was used as an internal control gene in miRNA RT-qPCR assay. The relative mRNA expression of Sphk1 was normalized to that of Gapdh.

2.7 | Intra-RVLM microinjection

The agomir and antagonomir of the miRNAs (miR-335, miR-674-3p, miR-183-5p, and miR-25-5p) and the negative oligonucleotide (negative control, NC) were synthesized by GenePharma (Table S1). They were diluted to 1 nmol/μl following the manufacturer’s instructions and microinjected into the bilateral RVLM (located 0.6–1.0 mm ahead of the most rostral rootlet of the hypoglossal nerve, 1.6–2.0 mm lateral to the midline, and 0.5–0.8 mm below the ventral surface) of normotensive (control) and SIH rats by using the stereotaxic apparatus of the brain (69 100, RWD Life Science, Shenzhen, China) under anesthesia with pentobarbital sodium (50 mg/kg, i.p.). Because the purpose of the microinjection was to examine the effects of miRNAs on blood pressure, no analgesics were used after stereotactic surgery to prevent skewing the results. All the injections were conducted between 9 am and 10 am.

2.8 | GO and KEGG survey

The TargetScan (Agarwal et al., 2015) and miRanda (Enright et al., 2004) programs were used to search for potential target genes. The pairing score for TargetScan was set as ≥50, and the parameter of miRanda was set as free energy (ΔG) ≤ −10 kcal/mol. Gene Ontology (GO) annotation was performed on the target genes of miR-335 and miR-674-3p with GOseq R package (Young et al., 2010). GO terms
with \( p < 0.01 \) were recognized as significantly enriched. KOBAS (v 2.0) software (Xie et al., 2011) was used to enrich the target genes of miR-335 and miR-674-3p in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. KEGG pathways with \( p < 0.05 \) were identified as statistically significant.

### 2.9 | Cell transfection

Rat neuroblastoma B104 cell line (RRID:CVCL_0154) was purchased from the Shanghai Xuanya Biotechnology Co., Ltd, of which it has been thoroughly tested and authenticated by the supplier. B104 is not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee (ICLAC). The cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Cat.No.10741574) supplemented with 10% fetal bovine serum (Gibco, Cat.No.11573397) in 5% \( \text{CO}_2 \) atmosphere at 37°C. Not more than 15 passages per aliquot were performed. At 24 h prior to transfection assay, the cells were seeded into a six-well plate at a density of \( 4 \times 10^4 \) cells/per well and allowed to grow to 70%-80% confluence. pEX-3-Sphk1 plasmid was constructed to over-express Sphk1 (GenePharma). The cells were transfected with either miR-335 mimics (GenePharma, Table S1) or scrambled oligonucleotide (mimics NC, GenePharma, Table S1) or co-transfected with miR-335 mimics and pEX-3-Sphk1 by using the lipofectamine™ 8000 transfection reagent (Beyotime, Cat.No.C0533) following the manufacturer’s instructions. After 48 h of transfection, the cells were harvested for subsequent experiments.

### 2.10 | Dual-luciferase reporter assay

B104 cells were seeded into a 96-well plate at a density of \( 3 \times 10^4 \) cells/per well. After 24 h, the cells reached approximately 70% confluence and were co-transfected with pmirGLO dual-luciferase miRNA target expression vector containing wild-type (WT) or mutant (MUT) 3’ untranslated region (3’ UTR) sequences of Sphk1 (Genecreate, Wuhan, China) and miR-335 mimics (GenePharma, Table S1) or scrambled oligonucleotide (mimics NC, GenePharma, Table S1) by using the lipofectamine™ 8000 transfection reagent (Beyotime, Cat.No.C0533) following the manufacturer’s instructions. After 48 h of transfection, luciferase activity was measured using the dual-luciferase® reporter assay system (Yeasen, Cat.

| Name | GenBank accession no. | Primer sequence (5’-3’) | Annealing temperature (°C) | The size of production (bp) |
|------|----------------------|-------------------------|----------------------------|-----------------------------|
| miR-335 | CM0269771: 59357769–59357866 | F: CGCGTCAAGACAAATACAACAAGAA<br>R: AGTGCAGGATCCAGGTATT | 60°C | 62 bp |
| miR-674-3p | NR_032290.1 | F: CGCGCAGAGCTCCATCT<br>R: AGTGCAGGATCCAGGTATT | 60°C | 59 bp |
| miR-183-5p | NR_031901.1 | F: CGCGTATGACACTGGTAGAA<br>R: AGTGCAGGATCCAGGTATT | 60°C | 64 bp |
| miR-25-5p | NR_031829.1 | F: GCGCGAGACACGGGCA<br>R: AGTGCAGGATCCAGGTATT | 60°C | 57 bp |
| miR-676 | NR_128705.1 | F: GCGCGGTCTGAGCTGTTG<br>R: AGTGCAGGATCCAGGTATT | 60°C | 59 bp |
| miR-18 | NR_037314.1 | F: CGCGTATTGGGCTAATGCTA<br>R: AGTGCAGGATCCAGGTATT | 60°C | 62 bp |
| miR-29a-3p | NR_031836.1 | F: GCGCTAGCACCACCTGAAA<br>R: AGTGCAGGATCCAGGTATT | 60°C | 60 bp |
| miR-3068-5p | NR_106672.1 | F: CGCGTGAGTTCATGCAAAGT<br>R: AGTGCAGGATCCAGGTATT | 60°C | 61 bp |
| miR-135a-5p | NR_031881.1 | F: CGCGTATGCTTCTTTATTATCCT<br>R: AGTGCAGGATCCAGGTATT | 60°C | 60 bp |
| miR-218a-5p | NR_031931.1 | F: GCGCGTTTTGTCATCTAA<br>R: AGTGCAGGATCCAGGTATT | 60°C | 62 bp |
| miR-411-3p | NR_032275.1 | F: GCGCGTATTGTAACAGCGGTCC<br>R: AGTGCAGGATCCAGGTATT | 60°C | 61 bp |
| U6 | K10784.1 | F: GCTTCGGCAGCACATACTAAT<br>R: CGCTTCAGAATTTCGCTGTCAT | 60°C | 94 bp |
| Sphk1 | NM_133386.3 | F: GCCTTGCCCACCTCTCCTGA<br>R: GCTGTACCGCCCCACAC | 60°C | 124 bp |
| Gapdh | NM_017008.4 | F: GTGCGGCTGAAAGGGATTTG<br>R: TCCCATTCTCAGCCTTGTAC | 60°C | 181 bp |
No.11402ES60). The results were normalized to the renilla luciferase activity.

2.11 Western blot

Total protein was extracted from RVLM tissue or B104 cell by using radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Cat.No.P0013B), and the concentration was measured by bicinchoninic acid (BCA) assay (Beyotime, Cat.No.P0012). Protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into the polyvinylidene fluoride (PVDF) membrane (Millipore, Cat.No.IPVH00010). After being blocked for 1 h at 24 °C by QuickBlock™ Western (Beyotime, Cat.No.P0252), the membranes were incubated at 4°C overnight with primary antibodies including mouse monoclonal SPHK1 antibody (1:500, Santa Cruz, Cat.No.sc-365401, RRID:AB_10859210), mouse monoclonal Caspase 3 antibody (1:500, Santa Cruz, Cat.No.sc-56053, RRID:AB_781826), rabbit monoclonal BAX antibody (1:1000, Abways, Cat.No.CY5059), mouse monoclonal BCL2 antibody (1:500, Santa Cruz, Cat.No.sc-7382, RRID:AB_626736), and mouse monoclonal HRP-Conjugated GAPDH antibody (1:50, Santa Cruz, Cat.NO.sc-365401, RRID:AB_10859210), the membranes were incubated at 4°C overnight blocked for 1 h at 24°C and then incubated with proteinase K solution (20 μg/ml) for 5 min at 24°C. After pretreatment, the sections were incubated with 1 x equilibration buffer for 15 min at 24°C, followed by incubation with TdT enzyme solution for 1 h at 37°C in the dark and with 4′,6-diamidino-2-phenylindole (DAPI) solution for 30 min in the dark. Finally, signals were detected by the confocal laser scanning microscope (LSM880, ZEISS, Oberkochen, Germany) to detect RVLM apoptosis following the manufacturers’ instructions. Frozen RVLM sections were fixed with 4% paraformaldehyde for 12 h, and placed in 20% sucrose at 4°C until the tissues sunk and then in 30% sucrose at 4°C until the tissues sunk. Frozen coronal sections containing the RVLM with thickness of 20 μm were cut using a cryostat (HM525, Microm). The RVLM sections were washed three times for 5 min each in PBS and then incubated with 0.3% Triton X-100 for 10 min, followed incubation with 5% goat serum for 30 min to block non-specific proteins. The sections were incubated with the mouse monoclonal Caspase 3 antibody (1:50, Santa Cruz, Cat.No.sc-56053, RRID:AB_781826) and rabbit monoclonal NeuN antibody (1:600, Abcam, Cat.No.ab177487, RRID:AB_2532109) overnight at 4°C, washed three times with PBS at 24°C for 10 min each, and incubated with FITC, goat anti-mouse IgG (1:800, Abbkine, Cat.No.A22110), and Alexa Fluor®594-conjugated goat anti-rabbit IgG (1:800, Abcam, Cat.No.ab150080, RRID:AB_2650602) for 2 h at 24°C. The confocal laser scanning microscope (LSM880, ZEISS, Oberkochen, Germany) was used to monitor the fluorescence signals.

2.13 TUNEL staining assay

TdT-mediated dUTP-biotin nick end labeling (TUNEL) apoptosis detection kit (Alexa Fluor 488, Yeasen, Cat.No.40307ES20) was used to detect RVLM apoptosis following the manufacturers’ instructions. Frozen RVLM sections were fixed with 4% paraformaldehyde for 30 min at 24°C and then incubated with proteinase K solution (20 μg/ml) for 5 min at 24°C. After pretreatment, the sections were incubated with 1 x equilibration buffer for 15 min at 24°C, followed by incubation with TdT enzyme solution for 1 h at 37°C in the dark and with 4′,6-diamidino-2-phenylindole (DAPI) solution for 30 min in the dark. Finally, signals were detected by the confocal laser scanning microscope (LSM880, ZEISS).

2.14 CCK-8 assay

Cell viability was detected using cell counting kit-8 (CCK-8, Bimake, Cat.No.B34304). The B104 cells were seeded into 96-well plates and transfected with miR-335 mimics or scrambled oligonucleotide (mimics NC) or co-transfected with miR-335 mimics and pEX-3-Sphk1 for 48, 72, and 96 h. CCK-8 working solution (10 μl) was then added into each well and incubated together for 1 h. Finally, the optical density was tested at 450 nm using a LabServ™ K3 microplate reader (Thermo, Cat.No.117123001).

2.15 Statistical analysis

Investigators were blinded to group allocation during data collection and analysis. Statistical analysis was performed by using Graphpad Prism software (v 9.1, GraphPad Software). Samples sizes used were similar to those used in the field (Du et al., 2017; Wu et al., 2018) and were calculated using the formula \( n = 2 \times \frac{SD^2}{\text{power index}} + \text{delta}^2 \) (Kondak et al., 2022). Based on experience over the years, an SD/delta of 0.4 was expected as the goal of the study. The power index value (alpha = 0.05, two-sided; beta = 0.2; power index = 0.8) was taken from the book Intuitive Biostatistics (Oxford University Press, 1995). No test for outliers was used. The Shapiro–Wilk test was used to evaluate the normality of the data. The two-tailed unpaired Student’s t-test was carried out for the comparison of two datasets. The one-way ANOVA with subsequent post hoc Bonferroni test were conducted for comparison of multiple datasets. Data were expressed as the
3 | RESULTS

3.1 | Altered expression profiles of miRNAs in the RVLM of the SIH rats

Changes in the HR, arterial blood pressure (ABP), SBP, DBP, mean arterial pressure (MAP), and RSNA of the two groups were recorded simultaneously after exposure to chronic stresses (electric foot-shocks and buzzer noises) for 15 successive days (Figure 2a). Compared with the normotensive (control) group, the SIH group exhibited increased HR and dramatically higher ABP, SBP, MAP, and MAP, and RSNA levels (Figure 2b, **p < 0.01, ***p < 0.001). These data revealed that the SIH rats had a significant increase in HR, blood pressure, and RSNA, which are the core clinical features observed in patients with SIH. Therefore, the rat model of SIH was established successfully.

Based on miRNA-Seq data, a total of 710,924,899 raw reads, in which 350,270,144 were for the normotensive (control) rats and 360,654,757 for the SIH rats, were generated. After filtering out adapter dimers, low-quality reads, junk reads, <18 bp reads, >26 bp reads, common RNA families (mRNA, rRNA, tRNA, snRNA, and snoRNA), and repeats, a total of 30,353,449 valid reads (13,842,699 for the control rats and 16,510,750 for the SIH rats) were obtained. Most valid reads were 22, 23, 24, and 21 nt in length for both groups (Figure 2c). The valid reads were mapped to the pre-miRNAs of rat and other species in miRBase 22.0 and to the rat genome. One mismatch inside the sequence (seed sequence excepted) and length variation at the 3’end were allowed to the pre-miRNAs of rat and other species in miRBase 22.0 and snoRNA), and repeats, a total of 30,353,449 valid reads (13,842,699 for the control rats and 16,510,750 for the SIH rats) were obtained. After filtering out adapter dimers, low-quality reads, junk reads, <18 bp reads, >26 bp reads, common RNA families (mRNA, rRNA, tRNA, snRNA, and snoRNA), and repeats, a total of 30,353,449 valid reads (13,842,699 for the control rats and 16,510,750 for the SIH rats) were obtained. Most valid reads were 22, 23, 24, and 21 nt in length for both groups (Figure 2c). The valid reads were mapped to the pre-miRNAs of rat and other species in miRBase 22.0 and to the rat genome. One mismatch inside the sequence (seed sequence excepted) and length variation at the 3’end were allowed to the alignments. Comparison results identified 786 miRNAs (504 known miRNAs and 282 rat_novel miRNAs) that were used for subsequent analyses.

With the RPM >100 and p < 0.05 as the significance threshold, 11 miRNAs with differential expression in the normotensive (control) and SIH rats were identified, and their expression was further validated by RT-qPCR to confirm the changes detected in the miRNA sequencing experiments. All the 11 miRNAs were detected in the RVLM of control and SIH rats, of which 4 showed differential expression and had results consistent with the miRNA sequencing data (Figure 2d, Table 2, *p < 0.05, **p < 0.01). In addition, seven miRNAs did not show differential expression (Figure 2d). Some of the results were inconsistent with the miRNA sequencing data possibly because of the biological differences between samples. The four differentially expressed miRNAs (miR-335, miR-674-3p, and miR-183-5p were up-regulated, whereas miR-25-5p was down-regulated in the SIH rats) identified through miRNA sequencing and RT-qPCR were used for principal component analysis (PCA) and cluster analysis. The three replicates of the SIH group were clustered together, and the same situation was observed in the control group (Figure 2e, f).

3.2 | Over-expression of miR-335 and miR-674-3p in the RVLM increased the HR, ABP, SBP, DBP, and MAP of normotensive rats

MI-335 agomir, miR-674-3p agomir, miR-183-5p agomir, miR-25-5p antagonir, NC agomir, and NC antagonir were microinjected into the RVLM of the normotensive (control) rats. After 48 h of microinjection, the expression levels of miR-335, miR-674-3p, and miR-183-5p in the agomir group increased by approximately 1000-, 1500-, and 800-fold, respectively, relative to those in the control and NC agomir groups (Figure S1, ***p < 0.001), whereas the expression of miR-25-5p in the antagonir group dropped to approximately 30% compared with that in the control and NC antagonir groups (Figure S1, **p < 0.001). The levels of HR, ABP, SBP, MAP, and MAP of the normotensive rats were remarkably enhanced by miR-335 and miR-674-3p over-expression (Figure 3a, b, *p < 0.05, **p < 0.01) but were not affected by miR-183-5p over-expression and miR-25-5p knockdown (Figure 3c, d).

3.3 | Inhibition of miR-335 and miR-674-3p in the RVLM reduced the HR, ABP, SBP, DBP, and MAP of SIH rats

The RVLMs of the SIH rats were microinjected with miR-335 antagonir, miR-674-3p antagonir, miR-183-5p antagonir, miR-25-5p agomir, NC antagonir, and NC agomir. After the delivery of the antagonir or agomir for 48 h, the relative levels of miR-335, miR-674-3p, and miR-183-5p in the antagonir group were reduced to 10%, 20%, and 30%, respectively, relative to those in the SIH and NC antagonir groups, and the miR-25-5p expression level was significantly higher (1000-fold) in the agomir group than in the SIH and NC antagonir groups (Figure S2, ***p < 0.001). The HR, ABP, SBP, MAP, and MAP levels of the SIH rats were reduced by miR-335 and miR-674-3p silencing (Figure 4a, b, *p < 0.05, ***p < 0.001) but were not affected by miR-183-5p suppression and miR-25-5p over-expression (Figure 4c, d).

3.4 | Functional enrichment analysis revealed the close association of miR-335 and miR-674-3p with SIH

The target genes of miR-335 and miR-674-3p were predicted via combinatorial analysis by using TargetScan and miRanda programs. As shown in Table S3 and Table S4, the numbers of the predicted target genes of miR-335 and miR-674-3p were 897 and 281, respectively. GO and KEGG survey were performed on the target genes of miR-335. The results showed that 88 GO terms (Table S5, Figure 5a) and 19 KEGG pathways (Table S6, Figure 5b) were significantly enriched. The GO annotation consisted of 56 biological processes, 21 cellular components, and 11 molecular functions. For the biological process, the most enriched term was actin cytoskeleton mean ± standard error of the mean (SEM). The threshold for statistical significance was p < 0.05. The full statistical reports were shown in Table S2.
FIGURE 2. Dynamics of microRNA expression in the rostral ventrolateral medulla after 15 days of stress exposure in rats. (a) Examples of the original tracings of heart rate (HR), arterial blood pressure (ABP), systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and renal sympathetic nerve activity (RSNA) recording in stress-induced hypertension (SIH) and normotensive (control) rats. (b) Chronic stress increased the HR, ABP, SBP, DBP, MAP, and RSNA in rats. Data were expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by two-tailed unpaired Student’s t-test. N = 9 rats per group. **p < 0.01, ***p < 0.001 vs. control group. (c) Sequence length distribution of valid reads in the control and SIH groups. (d) Validation of miRNA expression by reverse transcription quantitative polymerase chain reaction (RT-qPCR). U6 was used as the internal control. Data were presented as mean ± standard error of the mean (SEM) and analyzed via two-tailed unpaired Student’s t-test. N = 3 rats per group. *p < 0.05, **p < 0.01 vs. control group. ns means nonsignificant vs. control group. (e, f) Principal component analysis (PCA) and cluster analysis of differentially expressed miRNAs in the control and SIH rats.
 organization (GO: 0030036). The cytoplasm (GO: 0005737) was found to be predominant in the cellular component category, while the dynein complex binding (GO: 0070840) was highly enriched in the molecular function. The top three KEGG pathways were ubiquitin-mediated proteolysis, cysteine and methionine metabolism, and synaptic vesicle cycle. Several SIH-associated terms and pathways were observed, such as the positive regulation of NF-kappa B transcription factor activity (GO: 0051092), mTOR signalling pathway, and apoptosis-multiple species. In summary, specific genes could be regulated by miR-335 involved in SIH. The target genes of miR-674-3p were also subjected to GO and KEGG analyses. The results indicated that 69 GO terms (Table S7, Figure 5c) and 13 KEGG pathways (Table S8, Figure 5d) were significantly enriched. The GO terms were classified into 3 categories including 38 biological processes, 18 cellular components, and 13 molecular functions. The intracellular signal transduction (GO: 0035556) was the most represented biological process. Of the cellular components, the most enriched term was membrane (GO: 0016020). For the molecular function, the most abundant term was protein binding (GO: 0030036). The cytoplasm (GO: 0005737) was highly enriched found to be predominant in the cellular component category, while the cytoplasm (GO: 0005737) was significantly increased. The expression level of miR-335 in the mimic group was increased by approximately 500-fold relative to that in the mimic NC group (Figure 6e, ***p < 0.001). RT-qPCR and western blot results showed that miR-335 over-expression significantly reduced Sphk1 expression in B104 cells (Figure 6f, g, *p < 0.05, #p < 0.01). MiR-335 antagonism was microinjected into RVLM of the SIH rats for 48 h. The knockdown efficiency of miR-335 antagonism was shown in Figure S2. Compared with the SIH group, miR-335 knockdown significantly increased Sphk1 expression at both the mRNA and protein levels in the RVLM of SIH rats (Figure 6h, i, *p < 0.05, #p < 0.01). These results showed that miR-335 was able to regulate the expression of Sphk1.

### 3.6 MiR-335 modulated apoptosis by targeting Sphk1 in SIH

Western blot was performed to evaluate the expression of BAX, cleaved Caspase 3, and BCL2 in the RVLM of SIH rats following miR-335 antagonist microinjection. As shown in Figure 7a, miR-335 down-regulation in SIH rats significantly reduced BAX and cleaved Caspase 3 expression and increased BCL2 level (*p < 0.05, **p < 0.01). Cleaved Caspase 3 protein was further determined by immunofluorescence. Cleaved Caspase 3-positive neural cells in the RVLM were more abundant in the SIH than in the normotensive (control) and SIH + miR-335 antagonist rats. (Figure 7b, **p < 0.01, ***p < 0.001). Furthermore, apoptosis level was detected by TUNEL staining in frozen section. Compared with the SIH group, the group treated with miR-335 antagonist had significantly decreased number of TUNEL-positive cells (Figure 7c, *p < 0.05, ***p < 0.001). In vitro study revealed that miR-335 over-expression significantly increased the expression of BAX and cleaved Caspase 3 and reduced the level of BCL2 (Figure 7d, *p < 0.05, **p < 0.01, ***p < 0.001) and decreased cell viability (Figure 7e, #p < 0.01, ##p < 0.001) in

**Table 2** Significantly differentially expressed miRNAs in the control and stress-induced hypertension (SIH) rats

| miRNA name | miRNA sequence | ControlRPM | SIHRPM | p value |
|------------|----------------|------------|--------|---------|
| miR-335    | TCAAGACGAAATACGAAAGAATG | 100.64     | 146.57 | 0.044   |
| miR-674-3p | CACGCTCCCCATCTCAGAAC | 328.54     | 427.05 | 0.031   |
| miR-183-5p | TATGGCAGTGGATATTCACT | 452.62     | 842.54 | 0.034   |
| miR-25-3p  | AGGCCGGACACGGGGCAATTGCT | 180.79     | 110.95 | 0.004   |

Abbreviation: RPM, reads per million mapped reads.

**3.5 Sphk1 was a direct target of miR-335**

According to the TargetScan and miRanda algorithms, Sphk1 was one of the potential target genes of miR-335 (Table S3 and Figure 6a). Figure 2d showed that miR-335 was up-regulated in the RVLM of SIH rats. RT-qPCR and western blot experiments were performed to assess the Sphk1 expression in the RVLM of SIH and normotensive (control) rats. The results revealed that the mRNA and protein expression levels of Sphk1 in the SIH group were significantly lower than those in the control group (Figure 6b, c, *p < 0.05, **p < 0.01). An inverse correlation was observed between miR-335 and Sphk1 expression in the RVLM of SIH and control rats. GO and KEGG enrichment analysis indicated that Sphk1 was associated with sphingolipid metabolism. SPHK1 strongly affects apoptosis by catalyzing the conversion of sphingosine to sphingosine-1-phosphate (S1P, Maceyka et al., 2005; Maceyka et al., 2012; Alemany et al., 2007). Neuron cell apoptosis in RVLM increases the sympathetic nerve activity and contributes to the progression of hypertension (Kishi et al., 2010). Therefore, the focus was shifted to the Sphk1 gene. On the basis of the binding sequences, the wild-type (Sphk1-WT) and mutant (Sphk1-MUT) 3’ UTR vectors were constructed for dual-luciferase reporter assay. Figure 6d shows that miR-335 over-expression markedly inhibited the luciferase activity of the Sphk1-WT-3’ UTR reporter but not the Sphk1-MUT-3’ UTR reporter (**p < 0.01). MiR-335 mimics were transfected into B104 cells to further uncover the effects of miR-335 on Sphk1 expression. After 48 h of transfection, the expression level of miR-335 in the mimic group was increased by approximately 500-fold relative to that in the mimic NC group (Figure 6e, ***p < 0.001). RT-qPCR and western blot results showed that miR-335 over-expression significantly reduced Sphk1 expression in B104 cells (Figure 6f, g, *p < 0.05, #p < 0.01). MiR-335 antagonist was microinjected into RVLM of the SIH rats for 48 h. The knockdown efficiency of miR-335 antagonist was shown in Figure S2. Compared with the SIH group, miR-335 knockdown significantly increased Sphk1 expression at both the mRNA and protein levels in the RVLM of SIH rats (Figure 6h, i, *p < 0.05, #p < 0.01). These results showed that miR-335 was able to regulate the expression of Sphk1.
Up-regulated miR-335 and miR-674-3p expression in the rostral ventrolateral medulla contributed to the increase in the heart rate and blood pressure of normotensive (control) rats. (a, b) over-expression of miR-335 and miR-674-3p in RVLM increased the level of heart rate (HR), arterial blood pressure (ABP), systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) of control rats. (c, d) miR-183-5p and miR-25-5p did not participate in regulating the HR, ABP, SBP, DBP, and MAP of control rats. Data were shown as mean ± standard error of the mean (SEM). Statistical significance was determined using one-way ANOVA, followed by post hoc Bonferroni test. N = 4 rats per group. *p < 0.05, **p < 0.01 vs. control and NC agomir group. ns means nonsignificant vs. control and NC agomir/NC antagomir group. NC agomir, negative control agomir; NC antagomir, negative control antagomir.
MiR-335 and miR-674-3p knockdown in the rostral ventrolateral medulla lowered the heart rate and blood pressure of SIH rats. (a, b) inhibition of miR-335 and miR-674-3p expression in RVLM decreased the levels of heart rate (HR), arterial blood pressure (ABP), systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) of SIH rats. (c, d) miR-183-5p and miR-25-5p had no effect on the HR, ABP, SBP, DBP, and MAP of SIH rats. Data were shown as mean ± standard error of the mean (SEM). Statistical significance was determined using one-way ANOVA, followed by post hoc Bonferroni test. N = 4 rats per group. *p < 0.05, **p < 0.01, ***p < 0.001 vs. SIH and NC antagomir group. ns means nonsignificant vs. SIH and NC antagomir/ NC agomir group. NC agomir, negative control agomir. NC antagomir, negative control antagomir. SIH, stress-induced hypertension.
FIGURE 5 Histogram of the Gene Ontology (GO) classification and bubble chart of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for miR-335 (a, b) and miR-674-3p (c, d). GO categories, shown on the x-axis, are classified into three groups: biological process, cellular component, and molecular function. The y-axis indicates the number of genes in each category. The size of the bubbles corresponds to the number of target genes involved, and the color of the bubbles represents statistical significance. High enrichment scores indicate great enrichment degrees.
B104 cells. MiR-335 mimics and pEX-3-Sphk1 (a eukaryotic expression vector of Sphk1) were co-transfected into B104 cells to further confirm the role of Sphk1 in miR-335-induced apoptosis. As shown in Figure 7f, Sphk1 over-expression significantly reversed the effect of miR-335 on cell viability (\( p < 0.01 \)). All these results suggested that miR-335 induced apoptosis by inhibiting Sphk1 expression in the RVLM to increase sympathetic nerve activity, which participated in SIH pathogenesis.
4 | DISCUSSION

In 1993, Lee et al. and Wightman et al. identified the first miRNA (lin-4) from Caenorhabditis elegans (Lee et al., 1993; Wightman et al., 1993). Since then, knowledge about miRNAs has grown exponentially. Thousands of miRNAs have been identified in humans, animals, and plants. Given their ubiquity, miRNAs have been extensively studied and proven to contribute to pathological conditions, such as hypertension (Biancardi & Sharma, 2020; Li et al., 2018; Ozkan et al., 2019). However, research on the relationship between miRNAs and the neurogenic pathology of hypertension is limited. The RVLM, the vasomotor center, plays a key role in determining basal central sympathetic outflow, which is involved in the neural regulation of hypertension (Guyenet et al., 2018). The main purpose of the present study was to systematically investigate RVLM miRNAs involved in the pathology of SIH and further identify the potential regulatory mechanisms of these miRNAs at the molecular level. The identified valuable miRNAs might be potential candidates for future applications in the treatment of SIH.

Expression alteration is crucial in understanding the biological differences between diseased and healthy states and is valuable for seeking diagnostic biomarkers and therapeutic targets. MiRNA sequencing and RT-qPCR identified four miRNAs, namely, miR-335, miR-674-3p, miR-183-5p, and miR-25-5p, which were dysregulated between the control and SIH groups. In vivo refers to a type of experiment that is performed within a whole, living organism and is important for function validation and novel therapy development. Gain-of-function studies on miR-335 and miR-674-3p revealed that the agomirs administered into the RVLMs of normotensive rats increased the levels of HR, ABP, SBP, DBP, and MAP. Meanwhile, selective antagonirs targeting miR-335 and miR-674-3p reduced the HR, ABP, SBP, DBP, and MAP in the SIH rats. MiR-183-5p and miR-25-5p had no such effects and instead could produce a response or compensation. Therefore, the stress-induced changes in miR-335 and miR-674-3p expression in RVLM participated in the progression of hypertension.

MiR-335 is conserved across species, such as humans (hsa-miR-335-5p), rats (rno-miR-335), and mice (mmu-miR-335-3p). Among numerous miRNAs, miR-335 has attracted considerable attention. MiR-335 plays critical roles in tumorigenesis, tumor progression, invasion, and metastasis, and is widely dysregulated in a number of cancers, including bladder cancer (Liu et al., 2019), osteosarcoma (Xie et al., 2019), non-small cell lung cancer (Du et al., 2019), gallbladder carcinoma (Wang et al., 2019), and prostate cancer (Zhang et al., 2019). Li & Zhang summarized the function of miR-335 as an apoptosis promoter in type 2 diabetes (Li & Zhang, 2021). Tang & Qin reported that miR-335 induces insulin resistance in the gestational diabetes mellitus mice (Tang & Qin, 2019). MiR-335 activates autophagy and thus relieves chondrocyte inflammation, which is associated with the progression of osteoarthritis (Zhong et al., 2019). Therefore, miR-335 is implicated in various grave illnesses. The present study reported the elevated expression of miR-335 in the RVLM of SIH rats for the first time. Up-regulated miR-335 caused sympathetic activation and had a deleterious effect on the maintenance of blood pressure. Strong scientific evidence has indicated that miR-674-3p plays an important role in the deceleration of ciliary motility and age-dependent ventricular enlargement (Eom et al., 2020) and participates in inflammatory response during ischemic stroke (Xiao et al., 2015). Balakathiresan et al. proposed miR-674-3p as a potential biomarker of post-traumatic stress disorder (Balakathiresan et al., 2014). The expression of miR-674-3p is regulated by docetaxel and Fuzheng Yiliu decoction in the treatment of castration-resistant prostate cancer (Fu et al., 2019). Thus, the aberrant expression of miR-674-3p has been linked to various physiological or pathological processes. Current results revealed a higher level of miR-674-3p expression in the RVLM of SIH rats than in healthy controls. The miR-674-3p over-expression led to an increase in blood pressure in response to hypertension progression.

In animals, mature miRNA is incorporated into the RNA-induced silencing complex that pairs with the 3′-untranslated region (3′ UTR) of target mRNA through the seed sequence (nucleotides 2–8 from the 5′ end of miRNA) to cleave mRNA or inhibit its translation (Bartel, 2009). On the basis of this theory, 897 predicted target genes of miR-335 and 912 of miR-674-3p were identified by utilizing TargetScan and miRanda tools. The specific GO and KEGG enrichments for the associations of miR-335-mRNA and miR-674-3p-mRNA were then investigated. The pathology of SIH might be regulated by miR-335 and miR-674-3p in different ways, such as the neuron apoptotic process (GO: 0051402) and apoptosis-multiple species pathway. Kishi et al. reported that the neural apoptosis in RVLM regulates sympathetic vasomotor tone, which in turn is involved in the pathogenesis of hypertension (Kishi et al., 2010). The present results revealed that the in vivo miR-335 down-regulation significantly...
**FIGURE 7** Sphk1 is involved in miR-335-mediated apoptosis in SIH. (a–c) After 48 h microinjection of miR-335 antagonomir, BAX, cleaved Caspase 3, and BCL2 protein levels were detected by western blot analysis, and cleaved Caspase 3 protein was determined by immunofluorescence, and apoptosis level was tested by TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining in SIH rats. (d) After transfection of miR-335 mimics for 48 h in B104 cells, BAX, cleaved Caspase 3, and BCL2 protein levels were assessed by western blot analysis. (e, f) Cell viability was tested by cell counting kit-8 (CCK-8) in B104 cells after transfection of miR-335 mimics for 48, 72, and 96 h or co-transfection with miR-335 mimics and pEX-3-Sphk1 for 72 h. GAPDH was used as the internal control (a, d). Data were shown as mean ± standard error of the mean (SEM). Statistical significance was determined using one-way ANOVA followed by post hoc Bonferroni test (a–c, f) and two-tailed unpaired Student’s t-test (d, e). N = 3 rats per group (a–c). N = 3 of independent cell culture preparations (d). N = 5 of independent cell culture preparations (e, f). *p < 0.05, **p < 0.01, ***p < 0.001 vs. SIH group. #p < 0.05, ##p < 0.01, ###p < 0.001 vs. miR-335 mimics group. Mimics NC, mimics negative control; SIH, stress-induced hypertension.
suppressed cell apoptosis and showed potent antihypertensive effect in SIH rats. By contrast, the transfection of miR-335 mimics in vitro significantly promoted the apoptosis of B104 cells. Sphk1 was then characterized as a direct target of miR-335. Its expression was decreased and negatively correlated with miR-335 expression in the RVLM of SIH rats. MiR-335 inhibition dramatically enhanced Sphk1 expression in SIH rats. Meanwhile, the 3' UTR activity and mRNA and protein expression of Sphk1 decreased following miR-335 over-expression in vitro. Sphingosine kinase-1 (SphK1), which is encoded by Sphk1, is a lipid kinase that catalyzes the phosphorylation of sphingosine to sphingosine-1-phosphate (S1P) and exerts an anti-apoptotic effect in various diseases (Alemany et al., 2007; Maceyka et al., 2005; Maceyka et al., 2012). MiR-335 over-expression reduced Sphk1 expression and stimulated apoptosis. These effects were reversed by Sphk1 over-expression. Therefore, targeting Sphk1 by miR-335 increases apoptosis, which is involved in SIH. Notably, our current study has some limitations. First, all experiments were carried out on a small number of samples (n = 3 or 4), limiting the statistical power. Next, only male rats were used. Nevertheless, Ramirez & Sullivan summarized the sex differences in hypertension (Ramirez & Sullivan, 2018). Last, further studies are still required to explore other target genes and signals participating in miR-335- and miR-674-3p-regulated SIH progression.

This study indicated that miR-335 and miR-674-3p were up-regulated in the RVLM of SIH rats. Their suppressed expression in RVLM preferentially reduced the stress-induced increase in HR, ABP, SBP, DBP, and MAP levels. Mechanistically, a miR-335/Sphk1/apoptosis axis was found to play a key role in the pathology of SIH. The findings first suggested that the changes in miRNA expression participated in the sympathetic excitability driven in RVLM that contributed to SIH pathogenesis. MiR-335 and miR-674-3p could be beneficially exploited as potential therapeutic targets in SIH.

ACKNOWLEDGMENTS
We thank Mr. Min Peng at LC-BIO and Dr. Mao Wang at DUKENUS Medical Center for their assistance. This work was supported by grants from the Chinese National Natural Science Foundation 32071111, 31871151, 31571171, and 31100838 (to D. S. Du), the Shanghai Natural Science Foundation 15ZR1414900 (to D. S. Du), and the Zhejiang Chinese Medical University Research Funding 111100E017/005/007/002 (to S. Zhang).

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to declare.

AUTHOR CONTRIBUTIONS
S.Z. and D.D.S. conceived and designed research. S.Z., M.Y.X, G.J.C., L.T., and H.L.Z. performed the experiments. S.Z., M.Y.X, and G.J.C. analyzed and interpreted the data. S.Z. wrote the manuscript, which was read, edited, and approved by all the authors. S.Z. and D.D.S. contributed reagents, materials, and analysis tools.

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DATA AVAILABILITY STATEMENT
All materials are available in the Methods section of the article as well as https://zenodo.org/record/5910043#.YfJsh_tBzD5. The miRNA sequencing raw data reported in this manuscript has been deposited in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA797958 (https://www.ncbi.nlm.nih.gov/sra/PRJNA797958). The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**How to cite this article:** Zhang S., Xing M., Chen G., Tong L., Zhang H., Du, D. (2022). Up-regulation of miR-335 and miR-674-3p in the rostral ventrolateral medulla contributes to stress-induced hypertension. Journal of Neurochemistry. 161:387–404. [https://doi.org/10.1111/jnc.15589](https://doi.org/10.1111/jnc.15589)