Introduction

One of the more serious conditions affecting the central nervous system in the perinatal era is hypoxic-ischemic brain damage, which is brought on by suffocation or hypoxia, which can cause cerebral palsy, epilepsy and intellectual and hearing impairment or even death if left untreated (1). The brain, particularly the hippocampus, is sensitive to low concentrations of oxygen, which is important for learning and memory (2). In hypoxia-reoxygenation injury (HI), short- or long-term damage to the developing brain may impair these functions. Hippocampal pyramidal neurons are the most vulnerable neuronal cells in HI injury-induced apoptosis, and HI injury to hippocampal neuronal cells may lead to severe neurological deficits due to the important influence of hippocampal structure on cognitive function. This brings a heavy burden to the families of these patients and society. Recent studies have focused on neonatal HI encephalopathy, hoping to obtain more basic and clinical research on the pathogenesis and treatment of neonatal HI encephalopathy, so as to reduce the mortality rate and the chance of neurological sequelae in these children (3).

The pathogenesis of HI brain injury is complex, involving several mechanisms. The occurrence, progression, and regulatory mechanism of HI brain injury have been investigated in various studies. Studies have shown that when ischemia and hypoxia occur in brain tissue, they damage the oxidative defense system of brain tissue and break the balance between oxygen radical production and oxygen scavenging, allowing the body to build up a lot of reactive oxygen species will cause an oxidative stress reaction (4-6). Large amounts of oxygen free radicals, which attack mitochondria, increase mitochondrial permeability and release mitochondrial cytochrome c and apoptosis-inducing factors, leading to neurodegeneration and apoptosis, as well as memory impairment (1). At present, numerous therapies have been utilized in an attempt to resolve brain injury caused by HI. Researches on additional treatment options, such as erythropoietin (7), glutamate receptor antagonist (8), and stem cell transplantation (9) are currently ongoing.

MicroRNA (miRNA) regulates transcription factors by inducing the degradation of miRNA to regulate gene expression. In addition, miRNAs can act as independent transcripts to directly inhibit or increase the translation of target genes, and play a post-transcriptional regulatory role (10) mainly in cell differentiation, metabolism, apoptosis, neuronal development, and other biological processes (11). Ouyang and colleagues demonstrated that the upregulated expression of miR-181 in ischemic focal points promoted cell death in mice with cerebral ischemia (12). Other studies have found that distress hypoxic injury caused a clear decrease in the miR-23b expression...
of fetuses, thereby inhibiting the apoptotic peptidase activating factor 1 (APAF1) expression (13). MiR-27a in the course of traumatic brain injury, which played a neuroprotective role by inhibiting forkhead box O3 (FOXO3)-mediated autophagy (14). MiR-126 is an important potential target and regulatory site of ischemia-related angiogenesis and repair signals and also plays significant roles in cell growth, proliferation, and immune regulation (15).

Our study used a newborn Sprague-Dawley rat model of HI and oxygen and glucose deprivation (OGD) to examine the effects of miR-126-3p on cortical neurons and elucidate the associated pathways.

Materials and Methods

Animals

Newborn Sprague–Dawley rats of either sex (age: 7 days; weight: 13-19 g) (Beijing Vital River Laboratory Animal Technology Co., Ltd. SCXX (Jing) 20160006). Conditions included standard nutrition (23±2°C, 55±5% relative humidity) with a 12-h light-dark cycle. All animal care refers to the guidelines of the National Institutes of Health (NIH publication no. 85-23, revised 1996). These animal experiments were agreed upon by the Animal Care and Use Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University.

HI Model

As previous description (6), the left common carotid artery of neonatal rats was permanently ligated while they were under sevoflurane anesthesia. The operation time was dominated within 5 minutes, and neonatal rats went back to their mothers for 2 hours after waking. Then, the pups underwent perfusion with 8% O₂/92% N₂ for 120 min at 37°C. There was no occurrence of death during this process. Subsequently, the rats were fed as usual. Brain tissues were collected 24 hours or 34 days post-HI.

Animals Groups

On the basis of the random number table, 168 neonatal rats were taken into 6 groups (28 in each group, 14 males and 14 females), including sham group, HI group, miR-126-3p mimic (MiR-126-3p) group, mimic negative control (MiR-126-3p NC) group, inhibitor group, and inhibitor negative control (Inhibitor NC) group.

Neonatal rats in the sham group had the same procedure without ligation. In the miR-126-3p group, neonatal rats were treated with 10 μM miR-126-3p mimic before HI operation 48 hours, at 12-hour intervals, a total of 4 times (16). In the miR-126-3p NC group, neonatal rats were treated with 10 μM mimic negative control before HI operation 48 hours, at 12-hour intervals, a total of 4 times (16). In the inhibitor group, neonatal rats were administrated with 10 μM miR-126-3p inhibitor before HI operation 48 hours, at 12-hour intervals, a total of 4 times (16). In the inhibitor NC group, neonatal rats were administrated with 10 μM inhibitor negative control before HI operation 48 hours, at 12-hour intervals, a total of 4 times (16). The neonatal rats were anesthetized with 3% isoflurane (16).

For each dose, the neonatal rats were injected at 8:00 a.m. and 8:00 p.m. through intraventricular stereotactic injection (2 mm posterior, 1.5 mm lateral, 3 mm below the skull surface) before establishing the HI model (17). After the 4th dose, the HI model was established at 8:00 a.m. on the second day.

Samples collection

Twenty-four hours post-HI, neonatal rats (12/each group) were anesthetized by sevoflurane and sacrificed. The brain tissues were collected at 4°C. Some tissues (n=6, each group) were stored at −80°C for RT-PCR and western blot, while others were used for TTC staining.

2,3,5-TTC Staining

After 10 minutes of being frozen at -20°C, the entire brain tissue was cut into 5 slices. Slices were incubated with a 2% TTC solution for 30 min at 25°C. The images of slices were pictured and analyzed by ImagePro Plus software. The infarct volume (%) = cerebral infarction volume / total cerebral volume × 100%.

RT-PCR

After 24 hours post-HI, cortex tissues of the left brain were collected from the brains. Tissues were homogenized and centrifuged at 4°C (1000 × g, 20 min) for further detection. A Trizol Kit (R0016, Beyotime, Shanghai, China) was used to extract total RNA, and a BeyoFast™ probe one-step qRT-PCR kit (D7277M, Beyotime, Shanghai, China) was used to transcribe cDNA. According to the 2 − ΔΔCt method, the results of target mRNAs were calculated. The sequences of primers of this experiment are shown in Table 1.

Western Blotting

The cortex tissue of the left brain after 24 hours post-HI or cells treated in each group were centrifuged (1000 × g, 15 min) to obtain the supernatants. After analyzing the concentration of protein, the protein sample was electrophoresed on SDS-PAGE and then transferred to the membrane. After blocking, add PIK3R2 (1:500), orb76194, Biorbyt, Cambridge, UK), LC3B (1:1,000; 2775S, Cell Signaling Technology, Danvers, MA, USA), Beclin1 (1:1,000; 3738s, Cell Signaling Technology, Danvers, MA, USA), p62 (1:500, orb89844), Cleaved-capase-3 (1:500, orb106556), Bax (1:500, orb224426), Bcl-2 (1:500, orb228150), β-actin (1:2000, orb178392) for incubation. p62, Cleaved-capase-3, Bax, Bcl-2 and β-actin are all from Biorbyt. Finally, it was incubated with goat anti-rabbit IgG antibody (1:1,000; ABIN101988) for 60 minutes. For 3-5 minutes, the membranes were seen using an ECL luminous substrate. Using ImagePro Plus, proteins were scanned, measured, and normalized to β-actin.

Morris Water Maze

29 days after HI, the positioning navigation experiment and spinal exploration experiment were carried out. The water temperature was 25 ± 1°C, and the light source and reference objects were consistent during the experiment. Positioning navigation experiment: A fixed water entry point was demarcated in the pool. The moment the rats realized they were on a platform was timed after they were thrown into the pool with their heads facing the wall. If the period was longer than 90 seconds, the rats were instructed to find the platform and to remain there for 20 seconds. Each rat was recorded for 5 consecutive days, 4 times/day. Spinal exploration experiment: Rats were dropped into the water from the opposite side of the original platform quadrant after the platform had been removed for the positio-
nal navigation experiment training, and the times that the rats traversed the platform within 90 seconds were noted.

Nissl and Hematoxylin-eosin (HE) Staining
Thirty-four days post HI (after Morris Water Maze), the rats were sacrificed. Brain tissue was fixed with formaldehyde and then sliced. Then treated with dimethylbenzene and a gradient of alcohol (absolute, 95%, 80%, and 70%) as usual. Next, the slices were stained with Nissl (C0117; Beyotime, Shanghai, China) or HE (G1120, Solarbio, Beijing, China) solution at 60°C for 50 min. The sections were washed with water first, then dehydrated with alcohol (70%, 80%, and 95%) for two minutes then dimethylbenzene for five minutes. Under a light microscope, the results were seen and captured in pictures (CX31; Olympus, Tokyo, Japan).

TUNEL Assay
Thirty-four days post-HI, the apoptosis of neurons in the cortex region of the left brain or neurons was quantitated using a cell apoptosis detection kit (C1098, Beyotime, Shanghai, China). Brownish-yellow or brownish-yellow particles with morphological characteristics of apoptotic cells are signs of cell apoptosis. The images were obtained by the light microscope. Apoptotic index = (apoptosis cells / total cells) × 100%.

Immunohistochemistry
Thirty-four days post HI, the silences of cortex tissues of the left brain were administrated with dewaxing, and rehydration as usual. Then, they were removed using boiled citric acid buffer (BCA) for 8 minutes after being incubated with a 3% H₂O₂ methanol solution for 20 minutes. The silences were blocked with 5% bovine serum albumin (BSA) for 18 minutes at room temperature before being incubated with PIK3R2 (1:300; orb76194; Biorbyt, China) antibody for 12 hours at 4°C. Afterward, the sections were rewarmed and incubated with a secondary antibody for 60 min. After visualization using DAB, the tissue specimens were counterstained, dehydrated, cleared, and fixed. Observe the slices under the microscope and count them.

Primary Cortical Neurons Culture
The cortical tissues of Sprague-Dawley rats aged 1 day were collected for separation and elution. The single cell suspension (2×10⁶/mL) was prepared using fresh complete culture medium and seeded on the poly-L-lysine and laminin-coated cover glasses. The cells were cultured at 37°C with 5% CO₂. The cells were then grown in a medium designed specifically for neurons (Gibco, Rockville, MD, USA), with medium changes occurring every three days.

Oxygen and Glucose Deprivation (OGD) Cell Model
Following the previously described (18), the hypoxic/reoxygenated injury model of cortical neurons was constructed. Cells were cultured in hypoxia for 18 h (5% CO₂, 95% N₂), and reoxygenation for 6 h (5% CO₂).

Cells Groups
Cell groups were included normal control (control), HI, HI+miR-126-3p overexpression (miR-126-3p), HI+PIK3R2 silencing (si-PIK3R2), HI+PIK3R2 silencing negative control (Si-PIK3R2 NC), HI+PIK3R2 overexpression (miR+PIK3R2), HI+PIK3R2 overexpression negative control (miR+ PIK3R2 NC), HI+miR-126-3p overexpression+PIK3R2 overexpression (miR+PIK3R2).

Cortical neuron cells were separately transfected with 100 pmol miR-126-3p mimic (19), 50 nM PIK3R2 siRNA (20), 50 nM PIK3R2 mimic or their equal negative control for 24 h, before HI.

CCK-8 Assay
A 96-well plate with 2% B27-containing media was infected with a cell suspension and cultivated for 3 days at 37°C and 5% CO₂. Twenty-four hours after HI. For 4 hours, 10 L of the CCK-8 solution was added to each well. With the use of a microplate reader, the absorbance was determined at 450 nm.

Acridine Orange (AO) Staining
The cells were collected and cleaned with AO stain buffer (1×). Subsequently, they were resuspended with AO stain buffer (1×) at 1×10⁵ cells/well and incubated overnight. Thereafter, cells were stained with AO stain for 15 min without light and washed with PBS. The results were observed and pictured by a fluorescence microscope and analyzed using ImagePro Plus. In this study, we split the picture into red and green channels and analyzed the average gray mean of red fluorescence, which also represents the average fluorescence intensity.
High expression of miR-126-3p improved neuronal injury in the cortex

In Figure 1A, the expression of the miR-126-3p group was significantly higher than that of the miR-126-3p NC group, and the level of the inhibitor group was also significantly lower than that of the inhibitor NC group (P<0.05). As demonstrated in Figure 1B, the results of the positioning navigation experiment and spinal exploration experiment of rats in the miR-126-3p group are obviously better than those in other groups (P<0.01). In Figure 1C, the number of Nissl bodies was clearly decreased and the structure of neurons was destroyed in HR neonatal rats compared with normal rats. In addition, the structure and numbers of Nissl bodies were notably improved in the miR-126-3p group versus the HI group (Figure 1D, E). Silence of miR-126-3p aggravated neuron injury paralleled with the HI group.

High levels of miR-126-3p suppressed autophagy and apoptosis in the cortex

The apoptosis rate of the cerebral cortex was increased in the HI group (Figure 2A), decreased in the miR-126-3p group, and significantly increased in the inhibitor group (P<0.01). The levels of LC3II/I, Beclin-1, Bax and Cleaved-Caspase-3 increased in the HI group, while the levels of P62 and Bcl-2 decreased, while the protein expression level in the miR-126-3p group was completely opposite (Figure 2B), suggesting that miR-126-3p participated in HI-induced autophagy and apoptosis in the cerebral cortex of neonatal rats.

miR-126-3p overexpression inhibited the autophagy and death brought on by OGD

As shown in Figure 3A, the cell viability clearly declined after OGD, and miR-126-3p mimic transfection obviously increased the cell viability (P<0.01). The miR-126-3p expression in each group was confirmed (Figure 3B). The autophagy was increased after injury, but miR-126-3p overexpression inhibited the autophagy of cells through increasing P62 expression, decreasing red fluorescence, LC3II/I and Beclin-1 expression (Figure 3C). Meantime, the apoptosis of cortical neurons (Figure 3D), and Bax, Bcl-2, and cleaved-caspase-3 (Figure 3E) proteins were measured. Similar to this, miR-126-3p overexpression prevented cell death by upregulating Bcl-2 and downregulating Bax and cleaved-caspase-3 expression. However, miR-126-3p silencing increased the severity of autophagy and apoptosis in cells.

PIK3R2 was confirmed as the target gene of miR-126-3p

Treatment with the miR-126-3p mimic reduced the luciferase activity of PIK3R2 containing the wild-type 3’UTR sequence, whereas it did not reduce that of PIK3R2 containing the mutant 3’UTR sequence (Figure 4). This indicates that miR-126-3p can inhibit the transcription or translation of the target gene PIK3R2 by binding to the binding site on PIK3R2-3’UTR, and negatively regulate the expression of PIK3R2 protein. PIK3R2 may be the direct target gene downstream of miR-126-3p.
Effects of miR-126-3p on PIK3R2 expression in the damaged cortex caused by HI in neonatal rats and cortical neurons with OGD

The expression of PIK3R2 in the damaged cortex of each group was separately observed through immunohistochemistry (Figure 5A) and western blot (Figure 5B). The PIK3R2 levels in the cortical neurons of each group were analyzed by western blot (Figure 5C). The expression of PIK3R2 was clearly decreased by high miR-126-3p expression, whereas PIK3R2 was strengthened by low miR-126-3p expression.

Effects of miR-126-3p on autophagy by targeting PIK3R2 in cortical neurons with OGD

The roles of silence of PIK3R2 were similar with miR-126-3p overexpression (Figure 6A and B) that autophagy of cells was suppressed through increasing P62 expression, decreasing red fluorescence, LC3II/I and Beclin-1 expression (Figure 6C and D). High expression of PIK3R2 weakened the effects of miR-126-3p overexpression.

Effects of miR-126-3p on apoptosis-related proteins by targeting PIK3R2 in cortical neurons with OGD

When compared to the HI group, miR-126-3p or si-PIK3R2 transfection resulted in higher levels of Bcl-2 and lower levels of Bax and cleaved-caspase-3 (P<0.01) (Figure 7). Furthermore, in contrast to the miR-126-3p group, the Bax and cleaved-caspase-3 protein in the miR+PIK3R2 group was markedly increased, whereas that of the Bcl-2 protein was markedly reduced (P<0.01). It revealed that miR-126-3p suppressed apoptosis of cortical neuron cells with OGD by targeting PIK3R2.

Discussion

The target gene regulated by miRNA accounts for at least 20% of the total genome, which involves the biological process of growth and development of various cells, and then regulates the pathophysiological process of many nervous system diseases. In the process of nervous system...
be reduced by extracellular vesicles from VSC4.1 neurons repaired by hypoxia that are miR-126-3p-enriched. (17). These reports demonstrated that high levels of miR-126-3p play a protective role in neuronal injury.

Interestingly, Shi et al reported that PIK3R2 is involved in the P13K-AKT pathway, which involves the signal cascade of cell growth, survival, proliferation, movement and morphology, and participates in the cortical stratification of the embryonic brain (27). Reducing or blocking the P13K-AKT pathway will reduce the physiological effect of neuronal apoptosis, thus promoting brain development. Here, we investigated the role of miR-126-3p in the regulation of autophagy and apoptosis in cortical neurons and HI rats. The results showed that miR-126-3p mimic therapy can inhibit PIK3R2 to decrease autophagy and apoptosis in the brain and cortical neurons. Additionally, miR-126-3p overexpression and PIK3R2 silencing reduced the expression of proteins involved in apoptosis. The data obtained from this research were consistent with previous studies (19, 28-30). Additionally, we found that miR-126-3p participated in neuronal autophagy by regulating PIK3R2, and miR-126-3p overexpression inhibited autophagy caused HI in neonatal rats and cortical neurons through down-regulating LC3II/I and Beclin-1, upregulating P62 expression (28, 31, 32). In clinical practice, drug administration is followed after injury. In our study, we were given the drug administration before the injury, which indicated that miR-126-3p pre-treatment for HI injury was effective. However, the effectiveness of miR-126-3p treatment after HI injury for neonatal rats is not unclear, which needs more experiments to explore its clinical effectiveness.

Autophagy is the adaptive response of cells when nutrition is deficient. Under the stimulation of various factors such as ischemia, cells start an autophagy mechanism to remove damaged mitochondria, prevent apoptosis factors from being released into the cytoplasm, and improve the tolerance of cells to hypoxia, which plays a certain protective role for cells (33-35). Autophagy gene Beclin-1 is involved in the nucleation process of autophagy, and excessive autophagy can significantly up-regulate Beclin-1 expression during the reperfusion period, which will aggravate I/R damage (36, 37). In autophagy fluid, LC3 is present as LC3 I and LC3 II. LC3 II joins the creation of the autophagy membrane by adhering to the membrane of the autophagosome. A selective autophagy receptor is created when LC3 II attaches to the p62 protein; p62 protein specifically binds to ubiquitinated proteins, moves them to autophagosomes, and subsequently destroys them in the autophagy pathway along with the ubiquitinated proteins. The activity of selective autophagy is inversely linked with protein levels (38-40). p62 protein specifically binds to ubiquitinated proteins, moves them to autophagosomes, and subsequently destroys them in the autophagy pathway along with the ubiquitinated proteins. The activity of selective autophagy is inversely linked with protein levels (41-45).

Figure 6. miR-126-3p targeted PIK3R2 to regulate autophagy in cortical neurons with OGD. The expression of miR-126-3p (A) and PIK3R2 miRNA (B) was measured by RT-PCR. (C) The autophagy of cells was observed through AO staining, scale = 50 µm; (D) The autophagy-related (LC3II/I, Beclin-1 and P62) proteins were detected by western blot, and their relative expression was quantified by Image-Pro Plus software. *P<0.05, **P<0.01, compared with sham group; P<0.05, ***P<0.01, compared with HI group; ^P<0.05, ^^P<0.01, compared with MIR-126-3p group; %P<0.05, %%P<0.01, compared with si-PIK3R2 NC group; @P<0.05, @@P<0.01, compared with Mir+PIK3R2 NC group.

Figure 7. miR-126-3p targeted PIK3R2 to regulate apoptosis-related proteins in cortical neurons with OGD. The apoptosis-related (Bax, Bcl-2 and Cleaved caspase-3) proteins in the damaged cortex were detected by western blot, and their relative expression was quantified by Image-Pro Plus software. *P<0.01, compared with sham group; #P<0.05, ##P<0.01, compared with HI group; %P<0.05, %%P<0.01, compared with MiR+PIK3R2 NC group; @P<0.05, @@P<0.01, compared with si-PIK3R2 NC group; ^P<0.05, ^^P<0.01, compared with sham group; ! ! P<0.01, compared with si-PIK3R2 group; ! ! ! P<0.01, compared with MiR+PIK3R2 group; ! ! ! ! P<0.01, compared with Hi group; ##P<0.01, compared with MiR+PIK3R2 group; $P<0.05, $ $ P<0.01, compared with sham group; ! ! ! ! ! P<0.01, compared with Hi group; $P<0.05, $ $ P<0.01, compared with MiR+PIK3R2 group; ! ! ! ! ! ! P<0.01, compared with si-PIK3R2 group; ! ! ! ! ! ! ! P<0.01, compared with Hi group.

damage, there is abnormal inactivation or over-expression of miRNA (21-23). MiR-126-3p is specifically expressed in many systems, such as respiratory, digestive, hematopoietic, reproductive and embryonic tissues. The cardiovascular system is where MiR-126-3p exhibits the highest expression specificity (24-26). A study reported that inhibiting P13K/Akt signaling in the cerebral vascular endothelium, lowering PIK3R2 levels, and reducing blood-brain barrier (BBB) damage after intracerebral hemorrhage are all possible effects of high levels of miRNA-126-3p (19). Wang et al. found that by controlling PIK3R2-mediated inosine 3-kinase (P13K)/AKT and nuclear factor kappa B (NF-B), I/R-induced pain hypersensitivity can...
to be further investigated to achieve precision medical treatment based on the regulatory mechanism of miR-126-3p and its target genes.

In conclusion, by controlling PIK3R2, miR-126-3p reduced cortical autophagy and apoptosis in HI neonatal rats and cortical neurons, offering a new therapeutic target for brain damage in neonates.

Data Availability Statement
The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Conflict of Interests
The authors declare that no conflicts of interest.

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Author Contributions
JG: Conceptualization, Methodology, and Software data analysis. JG and XJ: Data curation and Writing of the original draft of the manuscript. XJ and HL: Visualization, Investigation, and Supervision. JG and HL: Writing, Reviewing, and Editing of the manuscript. All authors read and approved the final version of the manuscript.

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