Circular RNA cerebellar degeneration-related protein 1 antisense RNA (Circ-CDR1as) downregulation induced by dexmedetomidine treatment protects hippocampal neurons against hypoxia/reoxygenation injury through the microRNA-28-3p (miR-28-3p)/tumor necrosis factor receptor-associated factor-3 (TRAF3) axis

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ABSTRACT
Cerebral ischemia/reperfusion (CI/R) injury results in serious brain tissue damage, thereby leading to long-term disability and mortality. It has been reported that dexmedetomidine (DEX) exerted neuroprotective effects in CI/R injury. Herein, we intended to investigate whether and how circular RNA (circRNA) cerebellar degeneration-related protein 1 antisense RNA (circ-CDR1as) was involved in the DEX-mediated protection on hippocampal neurons. In our work, the mouse hippocampal neuronal cells (HT-22) were used to construct a hypoxia/reperfusion (H/R) model for CI/R injury. Cell proliferation and apoptosis were evaluated by CCK-8 and flow cytometry. Gene expressions were detected by RT-qPCR. Levels of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) were measured by ELISA. The association between miR-28-3p and circ-CDR1as or TRAF3 was verified by dual-luciferase assay. The results indicated that DEX alleviated HT-22 cell dysfunction induced by H/R treatment. In addition, circ-CDR1as was downregulated after DEX treatment and reversed the effects of DEX on the proliferation, apoptosis, and inflammatory responses of H/R-treated HT-22 cells. Circ-CDR1as positively regulated TRAF3 expression via interaction with miR-28-3p in HT-22 cells. Circ-CDR1as aggravated H/R-treated HT-22 cell dysfunction through targeting miR-28-3p. Furthermore, TRAF3 inhibition partly abolished the effect of circ-CDR1as overexpression on cellular activities of H/R-treated HT-22 cells. To sum up, our findings, for the first time, demonstrated that DEX exerted neuroprotective effects on hippocampal neurons against H/R treatment via the circ-CDR1as/miR-28-3p/TRAF3 regulatory network, providing novel therapeutic targets for DEX administration in CI/R treatment.

Introduction
Stroke, an acute cerebrovascular disorder, is a leading cause of long-term disability and mortality in adults worldwide [1]. Nearly 85% of stroke cases might be attributed to cerebral ischemia [2,3]. At present, the most effective method for ischemia stroke treatment in clinical practice is thrombolytic therapy through which blood supply to brain tissues can be timely restored, thereby ameliorating ischemic stroke-induced brain injury [4]. However, sudden resumption of blood supply after cerebral ischemia may induce a succession of pathological reactions, such as aggravated apoptosis and inflammatory responses in neurons, and even cause secondary injury to local brain tissues, which is known as cerebral ischemia/reperfusion (I/R) injury [5]. Although numerous drugs are of neuroprotective capability, many of them fail to therapeutic effects in cerebral I/R (CI/R) treatment [6]. Hence, it is of great significance to find new therapeutic approaches for CI/R therapy.

Dexmedetomidine (DEX), an activator of Alpha2-adrenoceptor, is a widely applied anesthetic drug for sympathetic activity depression, analgesia, and sedation in clinical anesthesia, without causing respiratory depression [7]. Besides, DEX also exerts essential pharmacological effects on reducing apoptosis, diminishing inflammation, and relieving neuropathic pain [8]. For example,
Kang et al. revealed that DEX eliminated diabetes-induced neuropathic pain in mice by regulating P2X4 and NLRP3 expressions [9]. Li et al. found that DEX reduced renal I/R-induced apoptosis via the α2 Adrenoceptor/P13K/Akt signaling [10]. He et al. demonstrated that DEX alleviated doxorubicin-mediated apoptosis and inflammation of myocardial cells [11]. Recently, there is a heated topic on DEX-mediated neuroprotection in CI/R [12–14]. However, the pharmacological action of DEX in CI/R still needs further investigation. Hence, a deeper understanding of the underlying mechanisms of DEX in CI/R is imperative for the better improvement of DEX application in CI/R treatment.

Circular RNAs (circRNAs), a group of newly identified RNAs with covalent closed-loop structures, play vital roles in several diseases, including CI/R [15–18]. To cite an instance, Zhang et al. disclosed circRNA CAMK4 aggravated CI/R injury by accelerating neuron cell death [19]. Yang et al. revealed that circ_008018 exacerbated CI/R-induced neuronal cell apoptosis via regulating miR-99a [20]. Liu et al. circ_002664 promoted CI/R-induced neuron cell apoptosis via regulating Herpud1 through interaction with miR-182-5p [21]. As reported in a study by Quan et al., circRNA cerebellar degeneration-related protein 1 antisense (circ-CDR1as) was highly expressed in PD and cause cell damage in vitro [22], indicating its promoting role in neurological disorder. Nevertheless, the functions of circ-CDR1as in CI/R remains poorly understood.

In this study, a hypoxia/reoxygenation (H/R)-induced neuronal cell model serves as an effective tool for the research on the cellular dysfunction caused by CI/R injury [23]. It was hypothesized that DEX exerted protective effects on H/R-induced hippocampal neuron cells via regulating circ-CDR1as. Herein, we for the first time explored the specific role of circ-CDR1as/miR-28-3p/TRA3 competing endogenous RNA (ceRNA) network in the DEX-mediated protection against H/R-induced hippocampal neuronal dysfunction, thereby providing novel molecular targets for CI/R treatment with DEX.

Materials and methods

Cell culture and DEX treatment

Mouse hippocampal neuronal cells (HT-22) purchased from BeNa Culture Collection (Beijing, China) were cultured in DMEM supplemented with 10% FBS in an incubator (5% CO2; 37°C) as per standard protocols. For DEX treatment, HT-22 cells were exposed to DEX (100 μM) for 24 h.

Establishment of hypoxia/reoxygenation (H/R) cell model

To construct an H/R cell model, HT-22 cells were cultivated in a low-oxygen atmosphere (94% N2 + 5% CO2 + 1% O2; 37°C) for 6 h. Thereafter, the HT-22 cells were transferred to fresh DMEM supplemented with 10% FBS and cultured in a regular incubator (95% O2 + 5% CO2; 37°C) for 6 h. Untreated HT-22 cells were used as the blank control group (Control group) [24].

Cell transfection

Small interfering RNAs against circ-CDR1as (si-circ-CDR1as: 5’-UAAUGUGAGACGCUAUAGAC-3’, TRAF3 (si-TRA3F: 5’-AUAGAGAAUAUACCUGUCGA-3’), scramble control (si-NC: 5’-AUUGUAGAUAUACGCUUAUAU-3’), pcDNA3.1 overexpression vector for circ-CDR1as (oe-circ-CDR1as), empty vector (Vector), miR-28-3p overexpression and inhibition plasmids (miR-28-3p mimics: 5’-UAGAUUCACAGCUUUUGUUAU-3’ and miR-28-3p inhibitor: 5’-AUCUAGUGUCAGGAAACAAUAU-3’), and corresponding negative controls (NC mimics: 5’-AUCUAGUGUCAGCUUUUGUUAU-3’ and NC inhibitor: 5’-AUCUAGUGUCAGGAAACAAUAU-3’) were provided by GenePharma (Shanghai, China) and transfected into HT-22 cells via Lipofectamine 2000 (Invitrogen, USA).

CCK-8 assay

CCK-8 assay was utilized for cell viability assessment. HT-22 cells were seeded into six-well plates (3 × 10^5 cells/well) and cultured for 24 h. Afterward, 10 μl CCK-8 reagent was added into
each well. After incubation with CCK-8 reagent for 1 h, the absorbance (optical density) was measured with a microplate reader (Bio-Rad, USA) [25].

Flow cytometry
Flow cytometry was applied for the analysis of HT-22 cell apoptosis via the Annexin V-FITC Apoptosis Detection Kit (eBioscience, USA). HT-22 cells in each group were centrifuged at 400 × g for 5 min, rinsed 3 times with PBS, and then incubated with 5 μL Annexin V-FITC reagent and 10 μL PI reagent (Sigma-Aldrich, USA) for 15 min at room temperature in darkness. The apoptotic HT-22 cells were analyzed with a flow cytometer (Beckman Coulter, China) [26].

ELISA
In order to determine TNF-α, IL-6, and IL-1β levels in HT-22 cells, ELISA assays were performed with corresponding ELISA assay kits (Mlibio, China) according to the standard protocol [27].

RT-qPCR
Isolation of total RNA from HT-22 cells was performed via TRIzol (Invitrogen). Then, cDNA was generated with a reverse transcriptase kit (Takara, Japan) or Thermo Fisher’s K1622 kit (Thermo Fisher Scientific, USA). Afterward, qPCR was accomplished with SYBR-Green PCR Master Mix kit (Takara, China). Relative gene expression was evaluated by 2^{-\Delta\Delta C_t} method, with GAPDH or U6 as the internal reference [28]. The primers used were as follows: circ-CDR1as forward (F): 5’-GTGTCTCCAGTGATCAGGC-3’ and reverse (R): 5’-TACTGGCAACACTGGAAACC-3’; TRAF3 F: 5’-CTTCCCCGGCTGATGATGG-3’ and R: 5’-GGCTGTATCTGACCGCTAGG-3’; GAPDH F: 5’-ACCCACTGCTCCACCTTGAC-3’ and R: 5’-TGTTGCTGAGCCCAATTGCCTT-3’; miR-28-3p F: 5’-ACGCCCACTGATTGAGGTGAGCT-3’ and R: 5’-AGTGAGGCTCCAGGATATT-3’; U6 F: 5’-CTCGTTCGGCAGCAGCATATACT-3’ and R: 5’-ACGCTTCACGAATATGGTCGTC-3’.

Dual-luciferase assay
The 3’ UTR regions of TRAF3 and circ-CDR1as with binding or mutant sequences for miR-28-3p were cloned into pmirGLO luciferase vectors (Promega, USA) to synthesize wild-type plasmids (TRAF3-WT and circ-CDR1as-WT) or mutant-type plasmids (TRAF3-MUT and circ-CDR1as-MUT). The above plasmids were respectively transfected into HT-22 cells, together with NC mimics or miR-28-3p with Lipofectamine 2000 (Invitrogen). 48 h later, the luciferase activity was determined via the dual-luciferase reporter assay kit (Promega, USA) and normalized to Renilla luciferase activity.

Statistical analysis
The data were expressed as mean ± standard deviation (SD). Each experiment was conducted three times. Student’s t test or one-way ANOVA was applied to accomplish difference comparison between two or multiple groups. Statistical analysis was performed using GraphPad Prism 6.0. A difference with P < 0.05 was deemed statistically significant.

Results
In this work, we intended to investigate the role and molecular mechanism of DEX in H/R-challenged hippocampal neuronal dysfunction. Our results demonstrated that DEX exerted neuroprotective effects on hippocampal neurons against H/R treatment via the circ-CDR1as/miR-28-3p/TRAF3 regulatory network, providing novel therapeutic targets for DEX administration in CI/R treatment.

DEX relieves HT-22 cell dysfunction induced by H/R treatment
To discover the possible effects of DEX treatment on H/R-induced HT-22 cells, we treated HT-22 cells with DEX after H/R treatment. As shown in Figure 1(a), HT-22 cell viability prominently dropped after H/R treatment, while DEX treatment exerted an ameliorative effect on cell viability. In addition, it was discovered that HT-22 cell
apoptosis rate was significantly increased after H/R treatment, while DEX led to an opposite result (Figure 1(b)), indicating that DEX reduced cell apoptosis. Moreover, the TNF-α, IL-6, and IL-1β levels were increased after H/R treatment, whereas DEX remarkably reversed such a phenomenon (Figure 1(c-e)). To sum up, DEX relieved H/R-induced apoptosis and inflammatory responses in HT-22 cells.

Circ-CDR1as is down-regulated after DEX treatment and reverses the effects of DEX on H/R-treated HT-22 cell proliferation, apoptosis, and inflammation

It was found that circ-CDR1as expression was markedly increased in HT-22 cells subject to H/R treatment; on the contrary, DEX led to down-regulated circ-CDR1as expression (Figure 2(b)). To further discover the potential functions of circ-CDR1as in DEX-mediated neuroprotection against H/R treatment, cell viability, apoptosis, and inflammatory responses of HT-22 cells were detected in each group. Firstly, circ-CDR1as was overexpressed in HT-22 cells (Figure 2(b)). As indicated by RT-qPCR assay, circ-CDR1as expression was evidently up-regulated by H/R, remarkably decreased by DEX, and increased again by circ-CDR1as overexpression (Figure 2(c)). Functional assays exhibited that circ-CDR1as upregulation reversed the effect of DEX on cellular processes of HT-22 cells, as indicated by decreased cell viability, elevated apoptotic rate, as well as increased inflammatory responses (Figure 2(d-h)).

MiR-28-3p directly binds to circ-CDR1as

StarBase predicted that circ-CDR1as contains a latent binding region for miR-28-3p (Figure 3(a)). The dual-luciferase reporter assay was performed to verify the binding condition between miR-28-3p and circ-CDR1as. Firstly, miR-28-3p was overexpressed in HT-22 cells (Figure 3(b)). The results showed that miR-28-3p upregulation
inhibited the luciferase activity of circ-CDR1as-WT but almost had no effect on the luciferase activity of circ-CDR1as-MUT (Figure 3(c)). RT-qPCR results revealed that miR-28-3p was lowly expressed in H/R-challenged HT-22 cells, while DEX upregulated miR-28-3p expression (Figure 3(d)). Next, circ-CDR1as was knockdown in HT-22 cells (Figure 3(e)). Furthermore, miR-28-3p expression was downregulated or overexpressed in HT-22 cells after circ-CDR1as overexpression or knockdown, respectively (figure 3(f)). These data showed that circ-CDR1as negatively regulated miR-28-3p expression.

**Circ-CDR1as expedited apoptosis and inflammatory responses of H/R-treated HT-22 cells via regulating miR-28-3p**

To explore the regulating role of circ-CDR1as and miR-28-3p in H/R-induced HT-22 cell dysfunction, H/R-treated HT-22 cells were transfected with
Vector, oe-circ-CDR1as, oe-circ-CDR1as+NC mimics, or oe-circ-CDR1as+miR-28-3p mimics, respectively. RT-qPCR assay revealed that miR-28-3p expression was decreased after circ-CDR1as overexpression and then increased after miR-28-3p addition (Figure 4(a)). Next, we measured cell viability, apoptotic rate, and levels of pro-inflammatory cytokines in each group. It was revealed that circ-CDR1as upregulation significantly inhibited proliferation and increased apoptosis of H/R-treated HT-22 cells; while miR-28-3p upregulation partly reversed such phenomena (Figure 4(b,c)). In addition, the TNF-α, IL-6, and IL-1β levels in H/R-treated HT-22 cells were remarkably lifted after circ-CDR1as amplification and declined after miR-28-3p overexpression (Figure 4(d-f)). Taken together, circ-CDR1as exacerbated H/R-induced apoptosis and inflammatory responses via miR-28-3p in HT-22 cells.

**MiR-28-3p targets TRAF3**

Via 5 miRNA databases (microT, miRmap, PITA, PicTar, and TargetScan), 10 candidate downstream genes (MARCH6, RNF216, FAF2, ARF6, ZFP91, C15orf48, FUBP3, FAM168B, NR3C2, and TRAF3) were predicted for miR-28-3p (Figure 5(a)). As Yao et al. disclosed that TRAF3 (tumor necrosis factor
receptor-associated factor-3) expression was upregulated in neurons from hippocampus after cerebral ischemia [29], TRAF3 was selected for subsequent experiments. The putative binding site between miR-28-3p and 3’UTR of TRAF3 was presented in Figure 5(b). Dual-luciferase activity assay manifested that miR-28-3p upregulation remarkably reduced the luciferase activity of TRAF3-WT, while the luciferase activity of TRAF3-MUT was nearly unchanged (Figure 5(c)). RT-qPCR revealed that TRAF3 expression in HT-22 cells were significantly lifted after H/R and substantially declined after DEX treatment (Figure 5(d)). Then, the efficiency of miR-28-3p inhibition was detected via RT-qPCR (Figure 5(e)). TRAF3 expression were apparently decreased after miR-28-3p addition and distinctly increased after miR-28-3p inhibition; however, such phenomena were partly abrogated by circ-
CDR1as amplification or circ-CDR1as silencing, respectively (figure 5(f,g)). These results indicated that circ-CDR1as positively regulated TRAF3 expression in HT-22 cells via interaction with miR-28-3p, suggesting that circ-CDR1as exerting its effects via the miR-28-3p/TRAF3 pathway. Functional assays exhibited that TRAF3 knockdown partly neutralized the effects of circ-CDR1as overexpression on viability, apoptosis, and inflammatory responses of HT-22 cells subject to H/R treatment (Figure 6(c-g)).

**Discussion**

DEX has not only effects on the hippocampus but also sensory processing, which involves the sensory cortex [30]. Moreover, DEX significantly regulates the thalamus, the pulvinar nucleus in particular [31], which plays a vital role in visual and auditory processing [32,33]. Moreover, accumulating evidence shows that DEX exerts significant neuroprotective effects in neurological diseases [34–36]. In this work, the protective effect and regulatory mechanism of DEX on H/R-induced HT-22 cell dysfunction were further investigated. Our findings substantiated that DEX considerably attenuated H/R-induced apoptosis.
and inflammation in hippocampal neurons via the circ-CDR1as/miR-28-3p/TRAF3 cascade.

It has been widely recognized that circRNAs play crucial roles in the development and progression of neurological diseases, such as cerebral I/R [37], Alzheimer’s disease (AD) [38], Parkinson’s disease (PD) [39], and Hirschsprung’s disease (HSCR) [40]. Circ-CDR1as, also known as ciRS-7, is deeply involved in the biological processes of cells, including cell viability and apoptosis. For example, Mao et al. found that circ-CDR1as inhibited the proliferation of bone microvascular endothelial cells by regulating FIH-1 via interaction with miR-135b [41]. Geng et al. revealed that circ-CDR1as...
contributed to apoptosis of hypoxia-treated mouse cardiac myocytes [42]. Besides, Zhang et al. uncovered that circ-CDR1as also exacerbated inflammatory responses [43]. In this study, we found that DEX exerted protective effects on hippocampal neuronal cells against H/R treatment, which was closely related to the downregulation of circ-CDR1as expression. However, circ-CDR1as overexpression could markedly weaken the protective effect of DEX on H/R-induced apoptosis and inflammation in HT-22 cells. Hence, DEX could attenuate H/R-induced dysfunctions in hippocampal neurons via down-regulating circ-CDR1as.

Emerging evidence has demonstrated that circRNAs can post-transcriptionally regulate the transcription and translation of messenger RNAs (mRNAs) as endogenous competitive RNAs for specific microRNAs (miRNAs) [44]. Moreover, such a circRNA-miRNA-mRNA regulating network also exerts key effects in diverse neurological disorders, including cerebral I/R [39,45,46]. Interestingly, our data demonstrated that circ-CDR1as sponged miR-28-3p as a ceRNA and miR-28-3p targeted TRAF3. Previous studies demonstrated that miRNAs also play important roles in CI/R-induced nerve damage. For instance, a report from Ma et al. showed that miRNA-589 protected against CI/R-induced inflammatory responses in primary cortical neurons via mediating TRAF6 [47]. Xing et al. disclosed that miR-374 targeted WNT5A to alleviate CI/R injury [48]. Besides, Liu et al. revealed that miR-211 exerted protective effects on OGD/R-challenged PC12 cells via reducing cell apoptotic rate [49]. MiR-28-3p is differently regulated in human diseases and deeply involved in the regulation of cellular functions [50]. In addition, Fan et al. disclosed that miR-28-3p expression was negatively related to IL-1β level in colorectal cancer [51]. In the present study, we found that miR-28-3p was downregulated in H/R-induced HT-22 cells, while DEX increased miR-28-3p expression. Further experiments revealed that miR-28-3p could partially abolish the effects of circ-CDR1as overexpression on H/R-induced HT-22 cells by enhancing proliferation, reducing apoptosis, and impairing the secretion of pro-inflammatory cytokines. Therefore, circ-CDR1as aggravated cellular dysfunction of H/R-induced HT-22 cells via interaction with miR-28-3p.

As a member of the TRAF adaptor protein family, TRAF3 exerts critical functions in regulating cellular activities in multiple diseases [52]. Sun et al. disclosed that TRAF3 upregulation substantially enhanced the inflammatory responses of caerulein-induced AR42J cells [53]. Liu et al. revealed that TRAF3 promoted apoptosis and inflammatory responses of oxygen-glucose deprivation/reperfusion (OGD/R)-induced PC12 cells [54]. Zhang et al. found that TRAF3 impaired the proliferation of MDA-MB-231 cells via inhibiting miR-29b-3p [55]. Also, Liu et al. demonstrated that TRAF3 aggravated cardiac I/R-induced apoptosis and inflammation [56]. Consistent with the above findings, TRAF3 expression was significantly increased in HT-22 cells after H/R treatment and remarkably after DEX administration. Rescue experiments showed that the promoting effects of circ-CDR1as upregulation on cellular dysfunction of H/R-treated HT-22 cells were partly abrogated by TRAF3 silencing. Taken together, circ-CDR1as upregulated TRAF3 expression, thereby promoting apoptosis and inflammatory responses in H/R-treated HT-22 cells.

Conclusion

In summary, this work demonstrated that DEX exerted neuroprotective effects against H/R-induced HT-22 cell dysfunction through regulating the circ-CDR1as/miR-28-3p/TRAF3 cascade. This study explored the neuroprotective effects and potential mechanisms of DEX in hippocampal neuron damage induced by cerebral I/R, providing a theoretical basis and certain targets for DEX application in cerebral I/R. In the future, in vivo experiments should be performed to further confirm the role of circ-CDR1as/miR-28-3p/TRAF3 axis in CI/R injury.

Research highlights

1. DEX attenuated H/R-induced dysfunctions of HT-22 cells by regulating circ-CDR1as
2. circ-CDR1as upregulated TRAF3 expression by targeting miR-28-3p
3. circ-CDR1as promoted dysfunction of H/R-induced HT-22 cells via miR-28-3p/TRAF3 axis
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