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Research Article

Dexmedetomidine Suppresses Glutamate-Stimulated Neuronal Injury Via MicroRNA-433/Janus Kinase 2/Signal Transducer and Activator of Transcription 3 Pathway

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Background: Cerebral ischemia has been a hotpot in the prevention and treatment of cerebral ischemia. Dexmedetomidine (Dex) is a new type of highly selective α2 adrenergic receptor agonist with pharmacological properties.

Objective: Quantitative studies have shown that Dex has a protective effect on glutamate (Glu)-induced neuronal damage. however, its mechanism has not been fully elucidated. The purpose of this study was to explore the underlying molecular mechanism by which Dex ameliorates Glu-induced neuronal injury by regulating miR-433/JAK2/STAT3 axis.

Materials and Methods: A model of neuronal injury was constructed by Glu treatment and intervened with Dex. miRNA expression profiling assay was conducted to screen potential miRNAs affected by Dex. Cell viability, lactate dehydrogenase (LDH) release and apoptosis were detected by MTT assay, LDH kit, and TUNEL staining, respectively. Oxidative stress indicators were assessed by ELISA whereas mitochondrial membrane potential (MMP) was assessed by C11-BODIPY581/591 staining. The targeting relationship between the miR-433 and JAK2 was verified by dual-luciferase reporter assay and gene expression was analyzed by quantitative PCR and Western blot.

Results: Glu treatment decreased cell viability and MMP and promoted LDH release, apoptosis and oxidative damage. Glu-induced changes in neurons were reversed after Dex treatment through upregulating the miR-433 expression to block the activation of JAK2/STAT3 pathway.

Conclusions: Dex protects against Glu-induced neuronal injury by regulating miR-433/JAK2/STAT3 pathway, which provides new insights into the treatment of neuronal injury.

Keywords: Dexmedetomidine, HT22, Hypoxia, MicroRNA-433/Janus kinase 2/Signal transducer and activator of transcription 3

1. Background

Cerebral ischemia (CI), taking up over 80% of cerebrovascular diseases (CBD) in human, is characterized by elevated morbidity, disability, and mortality. It has been a hotpot in the prevention and treatment of CBD (1). The incidence of CBD in China is as high as 250/100,000, ranking second in the world. So far, there is still a lack of effective control methods for CI, being a new challenge at home and abroad.

When CI occurs, the neurons in the ischemic central

area are mainly necrotic and can die promptly within a few minutes. During the process, neurons are morphologically changed, which is characterized by increased cell membrane permeability, lysosome rupture with the release of lysosome enzymes, cell membrane rupture and cytoplasmic spillage, thereby inducing inflammatory response. The death form of neurons in the penumbra area around the ischemic central area is mainly apoptosis (2). In clinical practice, inhibition of ischemia-induced neuronal apoptosis is helpful for rational intervention in cerebral ischemia.

Dexmedetomidine (Dex) is a new type of highly selective α2 adrenergic receptor agonist with pharmacological properties such as analgesia and sympathetic inhibition. It is clinically used as a new clinical anesthetic for sedation of endotracheal intubation and mechanical ventilation in surgical patients under general anesthesia (3). Recently, studies have investigated the availability of Dex to protect neurons. For instance, Cheng *et al.* (4) suggest that Dex mitigates CI/reperfusion injury in neonatal rats via suppressing the toll-like receptor 4 signaling pathway. Wang *et al.* (5) have clarified that Dex post-treatment stimulates a neuroprotective effect in rats via activating the extracellular signal-regulated kinase signaling pathway in the animal model of subarachnoid hemorrhage. Ge *et al.* (6) elucidate that Dex is available to ameliorate the cognitive ability via suppressing brain inflammation in patients undergoing carotid endarterectomy. Nevertheless, the neuroprotective mechanism of Dex in ischemic brain injury needs to be further explored.

MicroRNAs (miRNAs) can interact with the 3'-untranslated region (UTR) of its target mRNA at the post-transcriptional level and accelerate the degradation of mRNA (7). Studies have manifested that miRNAs take a prominent position in modulating neuronal damage. For instance, Li *et al.* (8) clarify that miR-338-5p restrains neuronal apoptosis via targeting BCL-2-Like Protein 11 in APP/PS1 mice. Gao *et al.* (9) indicate that miR-29a-3p suppresses glucose-oxygen deprivation-stimulated neuronal injury in rats via targeting tumor-necrosis factor receptor and controlling the nuclear factor of κB signaling pathway. Gui *et al.* (10) report that miR-424 restrains neuronal apoptosis in rats under middle cerebral artery occlusion treatment via the transforming growth factor-β 1/Smad3 signaling pathway. However, whether Dex has neuroprotective effects on CI via the miRNA-mediated pathway remains to be further explored.

In this study, we explored the changes of miRNAs in neuronal injury models after Dex treatment by miRNA expression profiling array and examined the role of miR-433-mediated JAK2/STAT3 pathway in Dex-mediated neuroprotection through loss-of-function experiments.

2. Objective

Quantitative studies have shown that Dex has a protective effect on glutamate (Glu)-induced neuronal damage. however, its mechanism has not been fully elucidated. The purpose of this study was to explore the underlying molecular mechanism by which Dex ameliorates Glu-induced neuronal injury by regulating the miR-433/JAK2/STAT3 axis.

3. Materials and Methods

3.1. Cell Culture

HT22 cells were provided by Shanghai Institute of Biochemistry and Cell Biology. The cell culture conditions were as follows: Dulbecco's Modified Eagle Medium containing 10% (v/v) fetal bovine serum, 1% penicillin/streptomycin, $NaHCO_3$ (2 $mg.mL^{-1}$) and 15 mM HEPES. The cell injury model was established as previously described (11). HT22 cells were treated with 1, 5, 15, 30 and 60 mM glutamate (GLu) for 24 h and collected for further analysis.

3.2. (4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

Cells were seeded into 96-well plates (5×10^3 cells/well), supplemented with 20 μL of MTT solution (5 $mg.mL^{-1}$) for 4 h, and added with 150 μL of Dimethyl Sulfoxide to dissolve the purple formazan crystals. Optical $density_{570\ nm}$ was read using a microplate reader (12).

3.3. Lactate Dehydrogenase (LDH) Assay

The cell supernatant was transferred to a 96-well plate (60 $\mu L.well^{-1}$) and hatched with 60 μL of LDH detection solution (Beyotime, Shanghai, China, 060319190906). After 30 min, the absorbance at 490 nm was detected by a microplate reader (13).

3.4. Transferase-Mediated Deoxyuridine Triphosphate-Biotin Nick end Labeling (TUNEL) Staining

Cells were fixed and permeabilized with 4% paraformaldehyde and 0.1% citrate buffer (Sigma-Aldrich) containing 0.1% Triton X-100 (Sigma-Aldrich).
Thereafter, cells were incubated in TUNEL reaction solution and viewed under an inverted fluorescence microscope. Nuclei were blue after counter-staining with 4',6-diamidino-2-phenylindole whereas TUNEL positive cells were green.

3.5. MiRNA Expression Profiling Array

To examine the differentially expressed miRNAs in HT22 cells after Dex treatment, total RNA was extracted from HT22 cells through Trizol reagent and purified by RNeasy MinElute cleanup kit (Qiagen, Valencia, CA). miRNA gene profile analysis was performed by Affymetrix GeneChip miRNA array 3.0 and the details were described previously (11).

3.6. Cell Transfection

MiR-433 inhibitor/mimic and corresponding negative controls (GenePharma, Shanghai, China) were transfected into Dex-treated HT-22 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., CA, USA). Cells after 48-h transfection were harvested for subsequent experiments.

3.7. Enzyme-Linked Immunosorbent Assay (ELISA) Method

Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), CAT (Catalase), reactive oxygen species (ROS), and malondialdehyde (MDA) of HT22 cells was tested by ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

3.8. Western Blot

Cell lysates collected by radio-immunoprecipitation assay lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1% NP-40, 0.1% sodium lauryl sulfate) were quantified by BCA protein detection method (Pierce, IL, USA). After separation by sulfate polyacrylamide gel electrophoresis, 30μg of protein was transferred onto polyvinylidene fluoride membrane (Merck Millipore) and incubated with the primary and secondary antibodies. The primary antibodies included Bax (ab32503, Abcam, MA, USA), Bcl-2 (MA5-11950, Invitrogen), Cytochrome C (556433, BD Biosciences, NI, USA), active-caspase-3 (9661, Cell Signaling Technology, MA, USA), active-caspase-9 (ab2324, Abcam), JAK2 (3230, Cell Signaling Technology), p-STAT3 (9145, Cell Signaling Technology), STAT3 (9139, Cell Signaling Technology), GAPDH (60004-1-Ig, Proteintech, IL, USA). The protein membrane was developed by enhanced chemiluminescence (Merck Millipore, Darmstadt, Germany) and photographed by the LAS-4000 imager (GE Healthcare Biosciences, Pittsburgh, PA, USA).

3.9. Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from cells using Trizol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.), of which the concentration and quality were assessed using a NanoDrop™ 3000 Spectrophotometer (Thermo Fisher Scientific, Inc.). Reverse transcription of RNA into cDNA was performed using PrimeScript RT Master mix (Takara, Dalian, China), and PCR was implemented with SYBR Premix ExTaq Kit (Takara Bio, Inc.). JAK2 expression was calculated using the $2^{-\Delta\Delta Cq}$ method, with GAPDH as an internal reference gene.

3.10. Dual Luciferase Reporter Assay

The potential targets of miR-433 were searched Through an online bioinformatics tool. The JAK2-WT-3'-UTR and JAK2-MUT-3'-UTR regions were amplified using PCR and cloned into the psi-CHECK-2 Vector (Promega, WI, USA). HT22 cells (5×10^4 cells/well) were transfected with the above-mentioned reporter and mir-433 mimic via Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The changes of the fluorescence intensity were monitored in the luciferase reporter kit system (Promega).

3.11. Examination of Mitochondrial Membrane Potential (MMP)

Cells collected by trypsinization were stained by the C11-BODIPY581/591 staining kit (Abnova, Taiwan) and analyzed by flow cytometry.

3.12. Statistical Analysis

All results were reported as Mean ± standard deviation and compared by one-way analysis of variance (ANOVA) and Tukey's post hoc test. $P<0.05$ was indicative of significant difference.

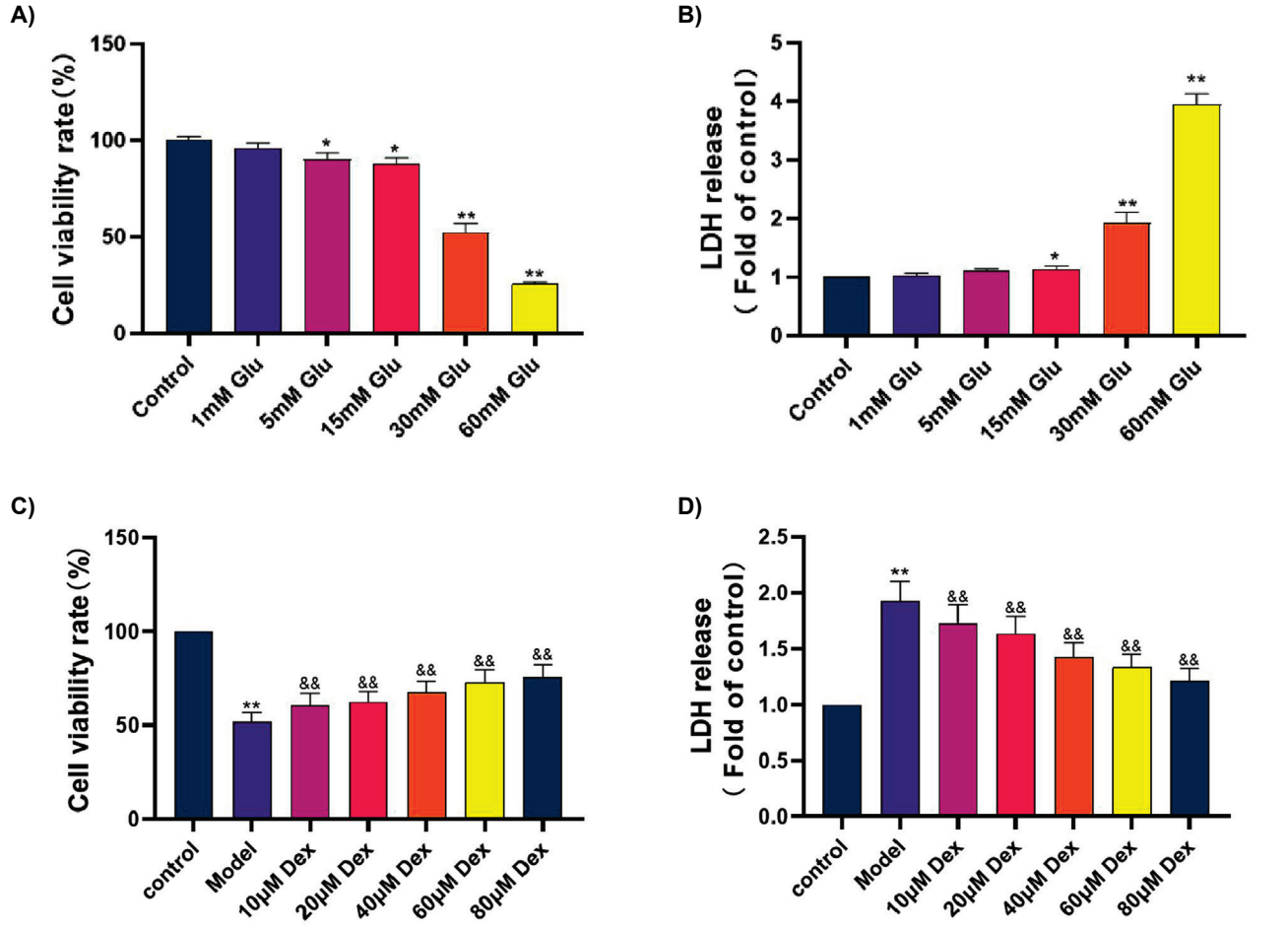

Figure 1. Dex treatment increases cell viability and reduces LDH release of GLu-treated HT22 cells. A) The impacts of Glu at different concentrations on cell viability rate analyzed by MTT test and **B)** the impacts of Glu at different concentrations on LDH release**. C**) The effects of different concentrations of Dex on GLu-stimulated HT22 cell viability rate analyzed by MTT, and **D**) the effects of different concentrations of Dex on GLu-stimulated HT22 cell LDH release. ** P<0.01 versus the control, && P<0.01.

4. Results

4.1. Dex Treatment Increases Cell Viability and Reduces LDH Release

After 24hours of treatment with different concentrations of GLu (1, 5, 15, 30, 60 mM), the viability rate of HT22 cells was suppressed and LDH release was induced **(Fig. 1A, 1B**). Effectively, Dex treatment inhibited LDH release and increased cell viability (**Fig. 1C, 1D**), indicating that Dex can improve Glu-induced neuronal injury.

4.2. Dex Suppresses GLu-Stimulated HT22 Cell Apoptosis

As shown in **Figure 2A, 2B**, Dex treatment reduced the TUNEL-positive cell rate and suppressed protein expression of Bax, Cytochrome C, active-caspase-9/3 whereas it promoted that of Bcl-2, implying that Dex could inhibit Glu-induced neuronal apoptosis.

4.3. Dex Represses GLu-Stimulated Oxidative Stress in Neurons

The levels of cellular oxidative stress-related indicators were detected by ELISA. As shown in **Figure 3A**, Dex treatment was attributable to the increment of SOD levels and the reduction of MDA, ROS, GSH-px and CAT levels in GLu-treated neurons. In addition, detection of MMP by C11-BODIPY581/591 staining

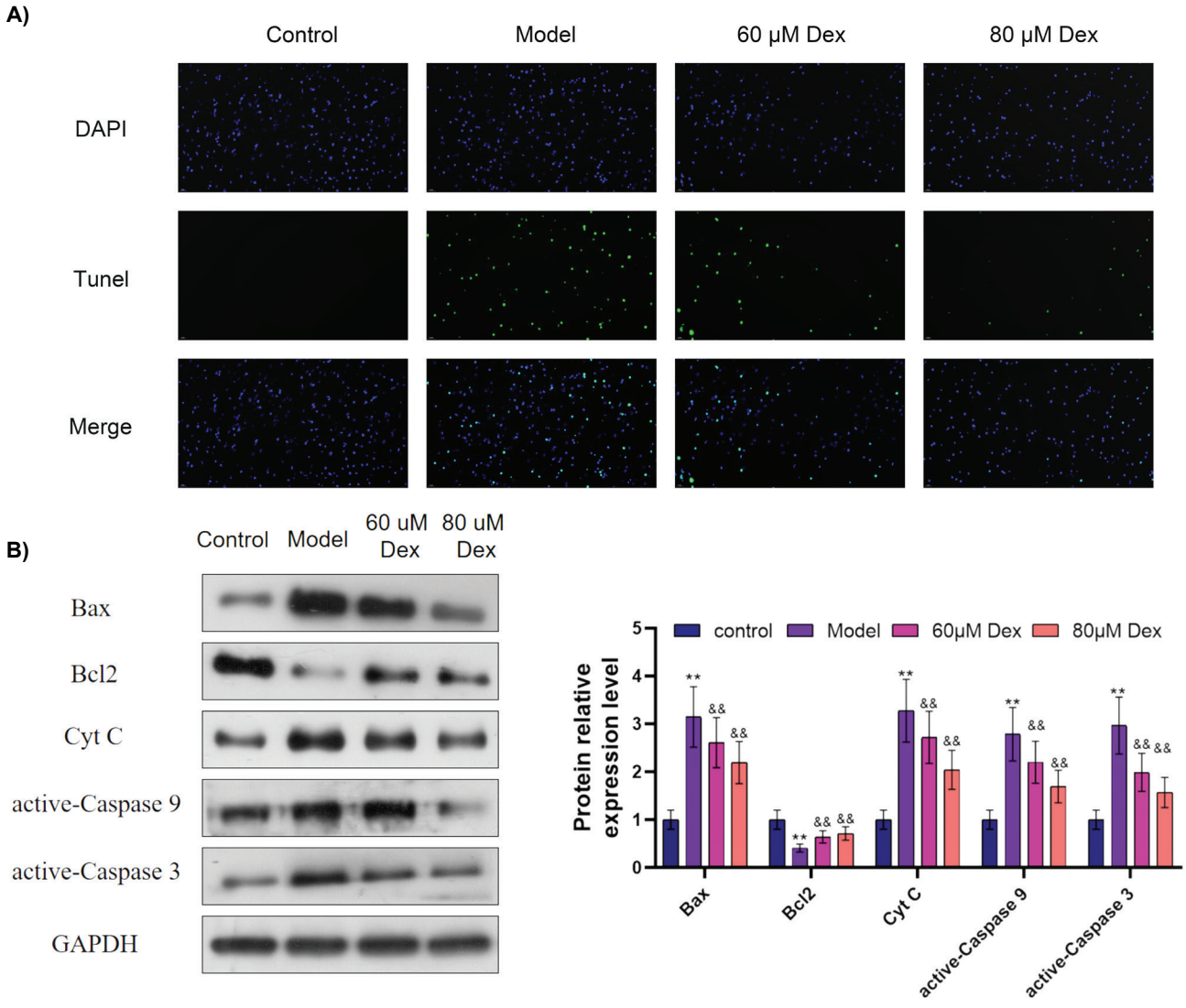

Figure 2. Dex represses GLu-stimulated neuronal apoptosis. A) The impact of Dex on GLu-stimulated HT22 cell apoptosis detected by TUNEL method and **B**) the influence of Dex on Bax, Bcl-2, Cytochrome C, Active-Caspase 9 and Active-Caspase 3 detected by western blot. ** P<0.01 versus the control, && P<0.01 versus the model.

revealed that after Dex treatment, MMP was elevated (**Fig. 3B**). Overall, Dex inhibits Glu-induced oxidative damage in neurons.

4.4. Dex in HT22 Cells Stimulates the Elevation of MiR-433 Expression

miRNAs exert a critical role in the process of Dex protecting against CI (14). Nevertheless, the mechanism by which Dex regulates miRNA was still largely unknown. In this study, miRNA gene expression profiles in neurons after Dex treatment were analyzed, showing that Dex induced the differential expression of miRNAs (72 miRNAs were upregulated and 71 miRNAs were downregulated) (**Fig. 4A**). To further verify the results, quantitative PCR was performed, finally discovering that miR-433 expression was obviously upregulated by Dex treatment in neurons (P < 0.05, **Fig. 4**B).

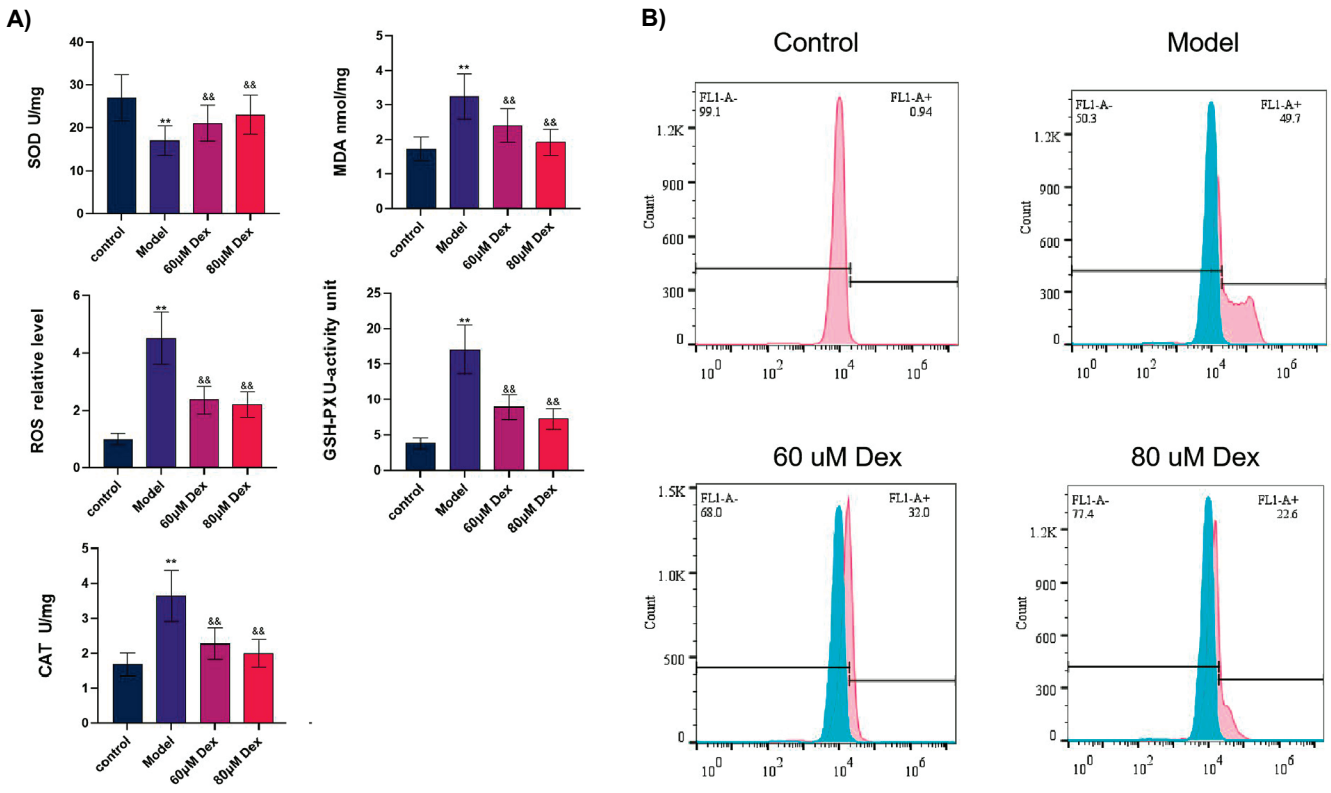

Figure 3. Dex suppresses GLu-induced oxidative stress in neurons. A) The influence of DEX on SOD, GSH-P, CAT, MDA and ROS content detected by ELISA, and **B**) the impact of Dex on MMP detected by C11-BODIPY581/591 staining. ** P<0.01 versus the control, && P <0.01 versus the model.

4.5. JAK2 Performs as a Target of MiR-433

It is currently recognized that miRNA interacts with the 3′-UTR of its target mRNA at the post-transcriptional level, thereby promoting the degradation of mRNA (7). In order to explore the possible mechanism of the miR-433, the bioinformatics tool (https://starbase.sysu.edu.cn) was applied to predict the existence of potential binding sites between miR-433 and JAK2 (**Fig. 5A**). Furthermore, it was checked that the miR-433 mimic inhibited the expression of JAK3 whereas miR-433 inhibitor had the opposite action (**Fig. 5B**), which indicated that JAK3 was regulated by miR-433. The dual luciferase reporter assay showed that the luciferase activity was decreased after co-transfection of JAK2-WT-3'-UTR with miR-433 mimic (**Fig. 5C**), confirming the targeting relation between miR-433 and JAK3.

4.6. Dex Suppresses the Activation of JAK2/STAT3 Signaling Pathway Via Upregulating MiR-433 Expression

Subsequently,the miR-433 inhibitor was transfected into Dex-treated HT-22 cells. As shown in **Figure 6**, Dex treatment inhibited the expression of JAK2 and p-STAT3, but this effect could be restored by knockdown of miR-433, hinting that Dex inhibits the activation of JAK2/STAT3 pathway by regulating miR-433 expression.

4.7. Dex Protects Against Glu-Induced Neuronal Injury Via MiR-433

Finally, whether miR-433 is involved in the process by which Dex protects against neuronal injury was explored. It was examined that the protective effect of Dex on neurons was reversed by knockdown of miR-433, resulting in reduced viability and MMP and promoted apoptosis (**Fig. 7A-7C**). Taken together, Dex protects against Glu-induced neuronal injury by regulating miR-433.

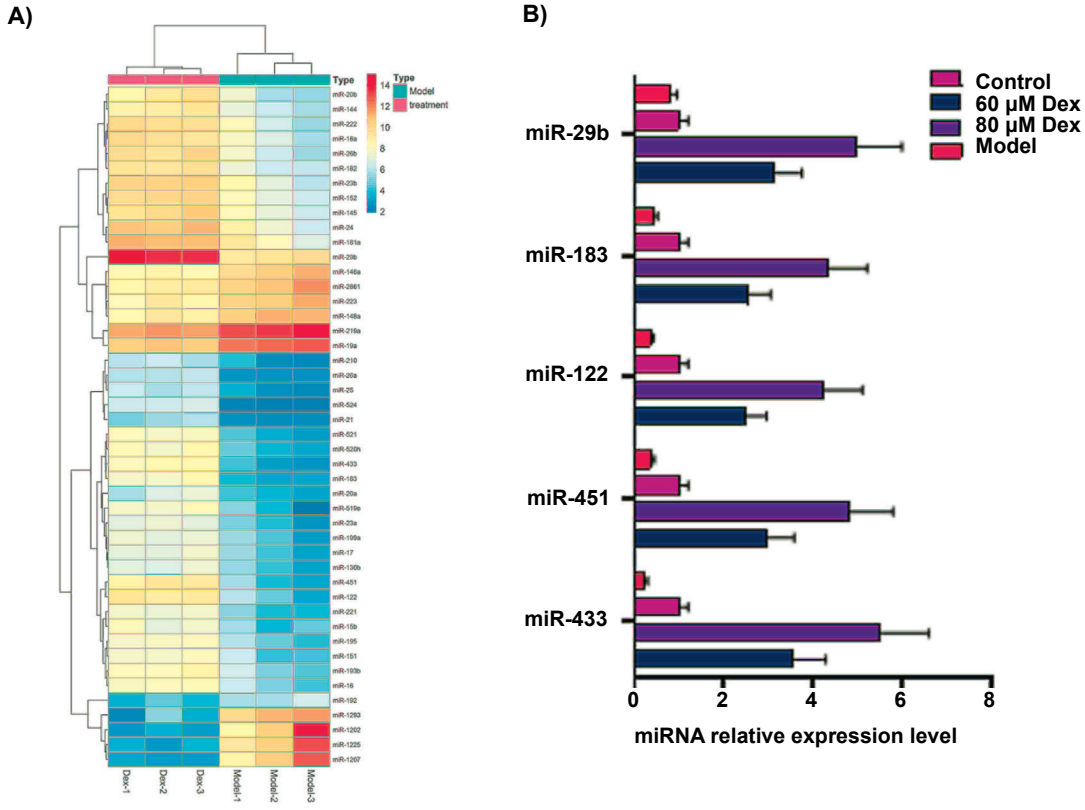

Figure 4. Dex in HT22 cells stimulates the elevation of miR-433 expression. A) Cluster heatmap of differential miRNA after Dex treatment and **B**) miRNA relative expression mediated by Dex. ** P<0.01 versus the control, && P<0.01 versus the model.

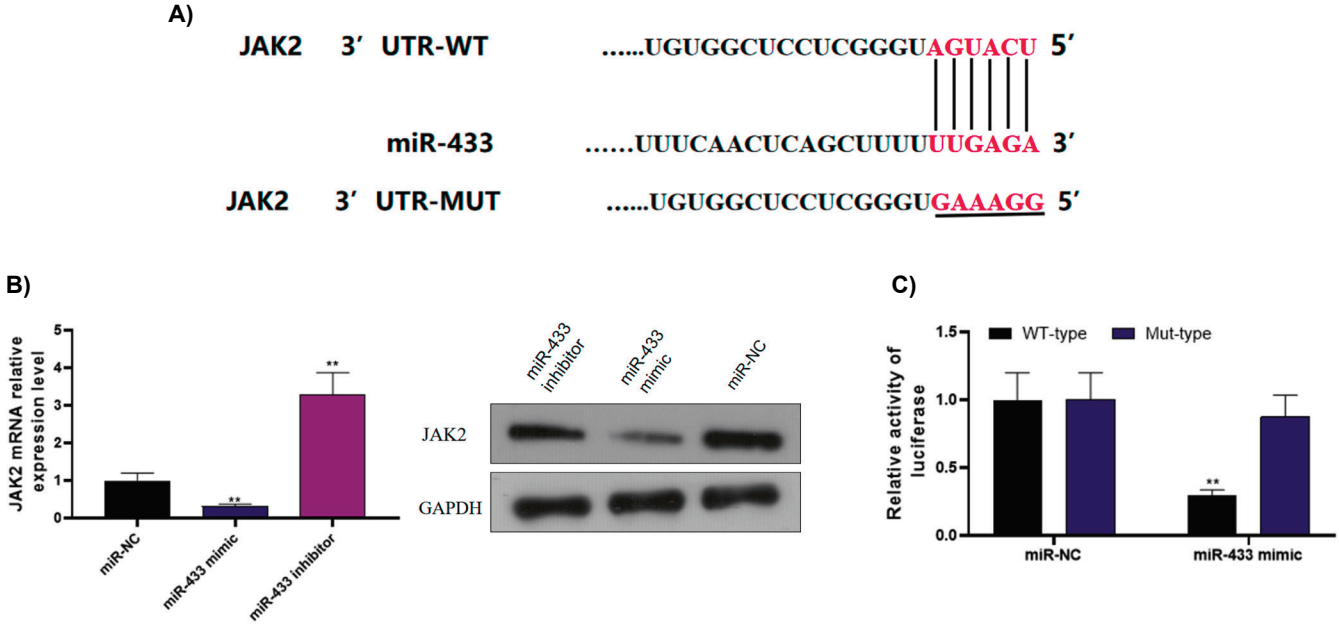

Figure 5. JAK2 performs as a target of miR-433. A) The complementary sequences of miR-433 and the 3'UTR of wild-type and mutant JAK2, **B**) the influence of miR-433 on JAK2 expression, and **C**) verification of the targeting relation between miR-433 and JAK2 by dual luciferase reporter test.

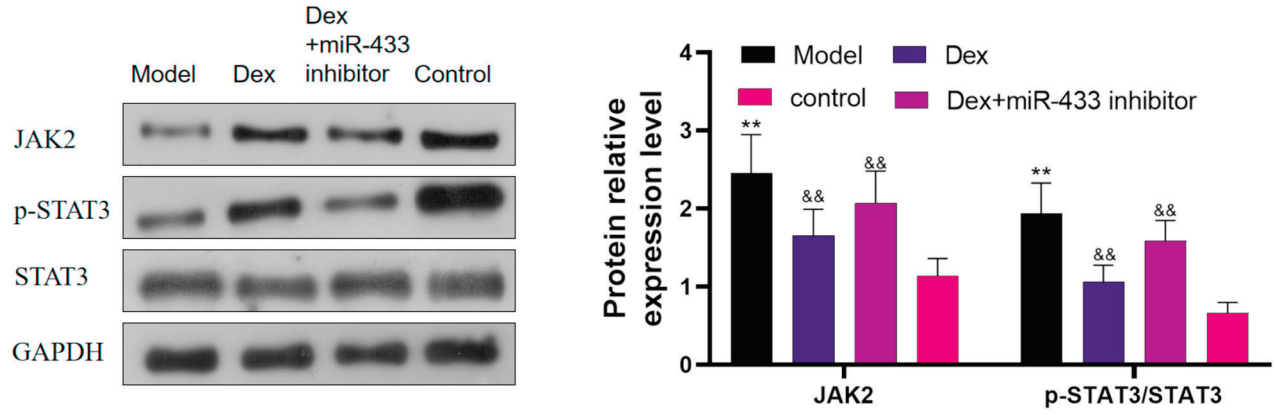

Figure 6. Dex suppresses the activation of JAK2/STAT3 signaling pathway via upregulating miR-433 expression. JAK2/STAT3 pathway-related proteins analyzed by western blot. ** P<0.01 versus the control, && P<0.01 versus the model.

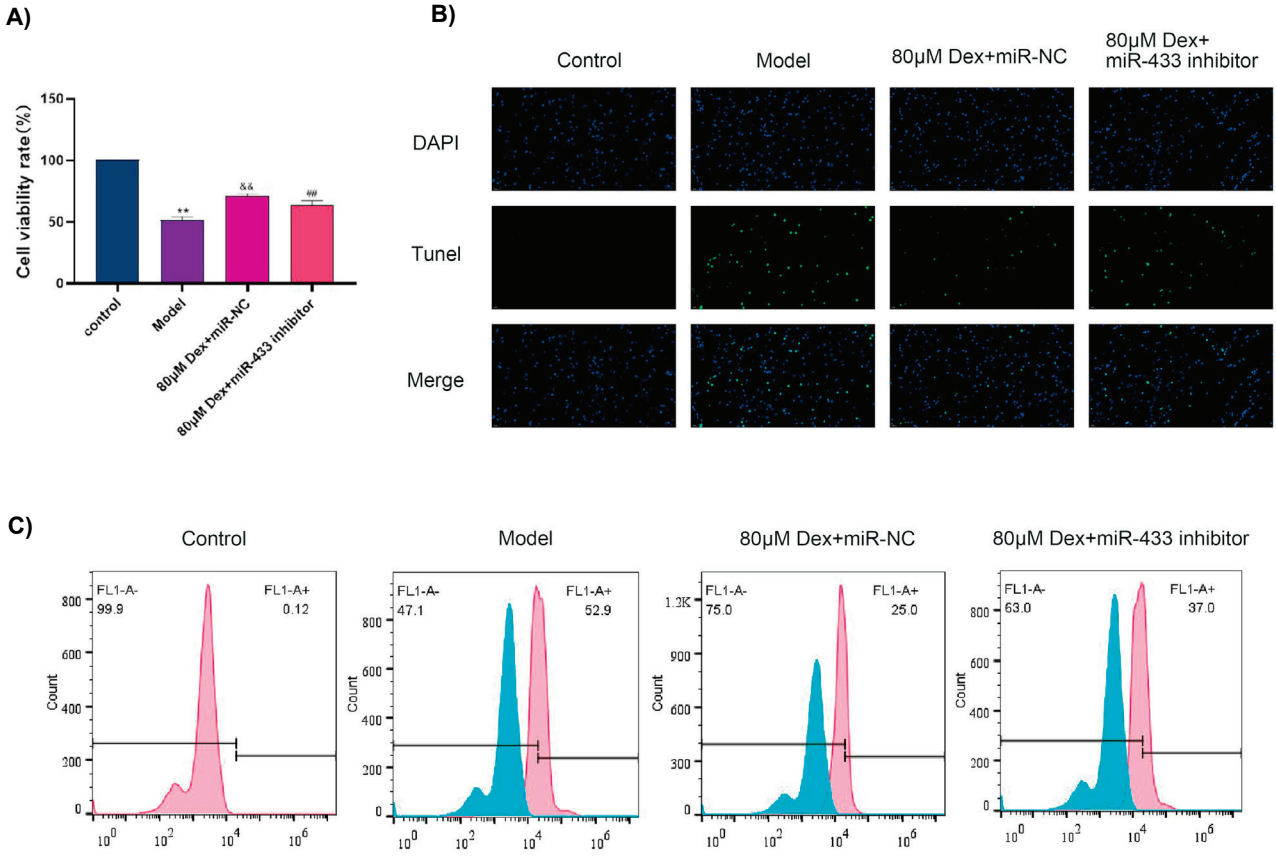

Figure 7. Dex protects against Glu-induced neuronal injury via miR-433. A) Cell viability detected by MTT method, **B**) cell apoptosis detected by Tunel method and **C**) MMP detected by C11-BODIPY581/591 staining. ** P< 0.01 versus the control, && P< 0.01 versus the model.

5. Discussion

Dex, a brand-new type of extremely selective α2 adrenergic receptor agonist, has pharmacological properties like analgesia and sympathetic inhibition. Dex could combine with transmembrane G protein-binding receptors in brain and spinal cord tissues, therefore acquiring neuroprotective effects in multiple brain injury models. Tu *et al.* (15) state that Dex is available to mitigate propofol-induced cytotoxicity in hippocampal neurons. Additionally, Wang *et al.* (16) clarify that Dex could suppress H_2O_2-stimulated hippocampal neuron damage via the miR-223-3p/TIAL1 signaling axis. Few studies have explored the mechanism of Dex in GLu-induced neuronal injury. In the present study, we found that Dex effectively protected against Glu-induced neuronal injury, which mainly exerted its effect by regulating the miR-433/JAK2/STAT3 pathway.

The experimental data found that Dex effectively decreased the apoptosis rate and protein expression of apoptotic indicators, inhibited oxidative stress, increased MMP and viability to protect Glu-injured neurons. GLu induces neuronal apoptosis through oxidative stress, calcium overload, and endoplasmic reticulum stress (17). The release of Cytochrome C from mitochondria is a key step in apoptosis. Cytochrome C released into the cytoplasm can combine with apoptosis-related factor 1 in the presence of dATP to form multimers and to promote its binding with Caspase-9 to form apoptotic bodies. The activated caspase-9 can activate other caspases such as caspase-3, thereby inducing apoptosis. Bax can promote the opening of mitochondrial mPTP, thereby promoting the release of Cytochrome C. Bcl-2 is mainly located on the mitochondrial membrane, preventing Bax activation and mitochondrial orientation, or combining with Bax and Bak to form a complex on the mitochondrial membrane to prevent the formation of Bax or Bak homologous polymers, thereby preventing cell apoptosis. The study elucidated that Dex repressed GLu-stimulated HT22 cell apoptosis through elevating Bax/Bcl2 expression and suppressing Cytochrome C, Active-caspase 9 and Active-caspase 3 expression.

JAK2/STAT3 is an intracellular signaling pathway activated by cytokine receptors. IL6/IL6R complex could activate JAK2 and phosphorylate and activate STAT3. STAT3, as a vital transcription factor, has been reported to modulate gene transcription in CI injury (18). It has been mentioned that the JAK2/STAT3 signaling pathway is aberrantly activated in ischemic brain tissue (19). Our study revealed that Dex repressed the activation of the JAK2/STAT3 signaling pathway. Similarly, Feng *et al.* (20) indicate that Dex boosts the survival of astrocytes via suppressing the JAK2/STAT3 pathway in the OGD-stimulated astrocyte damage model. Xiong *et al.* (18) state that Dex exerts a protective function in the brain via suppressing the JAK2/STAT3 pathway during cardiopulmonary bypass. In addition, our study pointed out that Dex treatment upregulated miR-433 expression. MiR-433 expression is reduced in the hypoxia-stimulated rat neuronal injury cell model (19). Also, the collected data confirmed that JAK2 performed as a target of miR-433. Wang *et al.* (21) state that miR-433 could target JAK2 to suppress Aβ-stimulated neuronal damage.

Taken together, the results of this study suggest that Dex protects against Glu-induced neuronal injury by upregulating miR-433 and inhibiting JAK3/STAT3 pathway activation. This study may expand the current understanding of Glu toxicity and provide further insights into the identification of new treatments for neurological deficits.

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Not applicable

Competing interests

The authors declare that they have no competing interests.

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