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miRNA-384-5p Targets GABRB1 to Regulate Ketamine-Induced Neurotoxicity in Neurons

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ABSTRACT

AIM: To determine the function of miR-384-5p in ketamine-induced neurotoxicity.

MATERIAL and METHODS: Neonatal hippocampal neurons were isolated from rats and treated with varying doses of ketamine. RT–qPCR was utilized to measure the miR-384-5p level in ketamine-treated neurons. Neuronal viability was evaluated by MTT assay. TUNEL staining and flow cytometry were applied to measure neuronal apoptosis. H2-DCFDA staining was utilized to detect the intracellular ROS level. Protein levels were measured using Western blotting. A luciferase reporter experiment was used in HEK293T cells to verify the interaction of miR-384-5p with GABRB1.

RESULTS: Ketamine induced neurotoxicity and miR-384-5p upregulation in hippocampal neurons. miR-384-5p downregulation mitigated ketamine-induced neurotoxicity by restraining apoptosis and ROS activity in neurons. GABRB1 was demonstrated to be targeted by miR-384-5p. GABRB1 depletion worsened ketamine-induced neurotoxicity. Moreover, GABRB1 depletion lessened the protective effect of miR-384-5p inhibition against ketamine-mediated neurotoxicity.

CONCLUSION: miR-384-5p regulates ketamine-induced neurotoxicity in hippocampal neurons by targeting GABRB1.

KEYWORDS: miR-384-5p, Ketamine, Neurotoxicity, GABRB1

ABBREVIATIONS: miRNAs: MicroRNAs, **NMDA:** N-methyl-D-aspartate

INTRODUCTION

Ketamine, characterized as an N-methyl-D-aspartate (NMDA) receptor blocker, has been commonly applied in pediatric anesthesiology for decades (4,19). Ketamine has also gained widespread acceptance on account of its effective anesthetic properties, short duration of action, minimum respiratory inhibition, and hemodynamic stability (29). However, accumulating clinical studies have suggested that exposure to ketamine can contribute to long-term learning disabilities and behavioral disturbances during critical brain development stages (26). Moreover, prolonged or repeated inhalation of ketamine could cause severe hippocampal neurodegeneration, resulting in neuron pyroptosis, neurite degeneration, and cell death in the brain (6,36). Thus, it is crucial to elucidate the molecular mechanisms of ketamine-mediated neurotoxicity to seek therapeutic strategies for the prevention and treatment of ketamine-associated neurodegeneration in infant brains.

miRNAs are a group of conserved small RNAs (18-28 nucleotides)that control gene expression by binding to the 3'-UTR of downstream mRNAs posttranscriptionally (3,8). miRNAs are highly expressed in the brains of different species (10,12,28), and have significant roles in cell development, survival, synthesis of neuronal progenitors and neurogenesis (9,31). For instance, miR-9 and miR-124 promote the development of neurites by activating the MEF2C-GPM6A pathway and downregulating HDAC5 (13). miR-16-5p upregulation attenuates neuronal apoptosis and inflammatory

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reactions in spinal cord injury (39). miR-582-5p targets ROCK1 to inhibit propofol-induced neuronal apoptosis (38). Additionally, many miRs, such as miR-429, -34a, and -137, were revealed as epigenetic modulators of ketamine-induced hippocampal neurodegeneration (11,15,22). Recently, ethanol exposure was reported to alter miR-384-5p levels in the rat brain (17). Moreover, hippocampus-enriched miR-384-5p was considered a possible indicator of neurotoxicity (27); it was also dysregulated in dopamine 2 receptor-neurons after cocaine treatment (30). However, whether miR-384-5p is related to ketamine-mediated neurotoxicity in hippocampal neurons is unclear.

This study elucidated the role and mechanisms of miR-384-5p in ketamine-mediated neurotoxicity in hippocampal neurons. The present study may contribute to developing therapeutic strategies for the prevention of ketamine-induced neurodegeneration.

MATERIAL and METHODS

Isolation of Neonatal Rat Hippocampal Neurons

Cell isolation experiments were performed following the Guides for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Affiliated Hospital of Southwest Medical University (Sichuan, China). One- to two-day-old neonatal Sprague–Dawley (SD) rats were purchased from Vital River Co., Ltd. (Beijing, China). Neonatal rat hippocampal neurons were obtained as previously published (5). The rats were euthanized by intraperitoneal injection of 3% isoflurane (150 mg/kg), and then their hippocampal tissues were collected in pathogen-free conditions. Primary hippocampal neurons were obtained from the hippocampal tissues of the rats. Briefly, hippocampal tissues were cultured in Hanks' balanced salt solution (cat. no. C0218; Beyotime, China), cut into pieces and digested in 0.125% trypsin-EDTA (cat. no. 25200-072; Gibco, CA, USA) for 20 min at 37 °C. The suspension (1×10^9 cells/mL) was then seeded in culture dishes or 96-well plates in DMEM containing 10% FBS and 2% HEPES-buffered salt solution. Afterward, neurons were transferred to neurobasal medium containing 2% B27, 0.25% GlutaMAX-I, and 5% FBS (all from Gibco). After 2 h, primary neurons were cultured in FBS-free neurobasal medium in an incubator at 37 °C. The medium was changed every 2 days.

Cell Treatment and Transfection

To induce neurotoxicity in vitro, the medium was replaced with glucose-free neurobasal medium that contained 2.78 mmol/L glucose and 1% B27, as previously described (33). The isolated neurons were exposed to various doses of ketamine (0, 0.1, 1, 10, 20, 50, and 100 μM) for 24 h to induce neurotoxicity. Cells were then maintained for 24 h under normal conditions. Cells treated with PBS were set as the blank group.

A chemically synthetized rno-miR-384-5p inhibitor (ACAUUGCCUAGGAAUUGUUUACA), nonspecific control (NC inhibitor; UGUAAACAAUUCCUAGGCAAUGU), miR-384-5p mimic (UGUAAACAAUUCCUAGGCAAUGU), NC mimic (AUCAUCCAAUUGACAAAGUUGUG), shRNA targeting GABRB1 (sh-GABRB1; GCACCAATGAACCCAGCAACA), and sh-NC (AGCGCAACACCAAGCTCAACA) were obtained from RiboBio (Guangzhou, China). Neurons were seeded into 96-well plates at 1×10^7 cells/well and then transfected with 2 μg of vectors or 50 nM synthetic oligonucleotide after 24 h. Transfection was performed utilizing Lipofectamine 2000 (Invitrogen). Cells were treated with ketamine after 24 h of transfection.

Immunofluorescence

Hippocampal neurons were rinsed several times with PBS, incubated in 4% paraformaldehyde (cat. no. E672002; Sangon Biotech, Shanghai) for 20 min, and permeabilized with 0.5% Triton-100 X (cat. no. C0218; YM-MY808J; Shanghai YuanMu Biological Technology; China) for 30 min. Then, they were cultured in goat serum for 1 h to block nonspecific antigens. Primary anti-GFAP (ab254082) or βIII tubulin (ab18207) was applied to the cells overnight at 4 °C. The following day, the cells were washed and exposed to FITC-conjugated goat anti-rabbit antibody for 2 h in the dark. After washing, DAPI was applied for 30 min to stain the cell nuclei. Fluorescent images were captured using a Nikon ECLIPSE fluorescence microscope.

Detection of Reactive Oxidative Species (ROS) Level

Hippocampal neurons were cultured in 96-well plates at 1×10^4 cells/well at 37 °C. Then, the oxidation-selective fluorescent probe H2-DCFDA (10 μm; D6883, Sigma–Aldrich) was added to the culture for 40 min. Fluorescent images were acquired under a fluorescence microscope (Nikon ECLIPSE). A microplate reader (BioTek Instruments, USA) was employed to monitor the fluorescence intensity at 485 nm-535 nm.

RT–qPCR

A TRIzol kit (Invitrogen) was used to obtain total RNA from hippocampal neurons. Then, a Reverse Transcription Kit (Applied Biosystems, USA) or TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific) was applied to reverse transcribe total RNA into cDNA. RT–qPCR was conducted using a SYBR Premix MASTER Kit (Roche) with a CFX96 System (Bio–Rad, USA). RNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method. GAPDH/U6 was used as an endogenous control for GABRB1/miR-384-5p.

Western Blotting

Hippocampal neurons were lysed using RIPA buffer (Beyotime). Lysates were centrifuged for 15 min at 4°C. Proteins were quantified using the DC Protein Assay (Bio–Rad, USA), and 10 μg of samples were isolated using 10%-15% SDS–PAGE. After electrophoretic transfer onto PVDF membranes (Millipore, USA) at 300 mA for 100 min, the membranes were blocked in 5% defatted milk for 2 h, washed with TBST, and probed with primary antibodies at 4 °C overnight. The next day, the membranes were incubated with secondary antibodies for 2 h. Bands were visualized by an ECL chemiluminescent detection system (Beyotime) and analyzed by Image Software. The primary antibodies included Bax (ab182733, Abcam), Bcl-2 (ab194583), cleaved caspase-3 (PA5-23921; Thermo Fisher Scientific, USA), GABRB1 (PA5-116439), and β-actin (PA1-183-HRP).

MTT Assay

After treatment with ketamine, neurons in 200 µl of medium were seeded into 96-well plates at 3000 cells/well. Then, 0.5 mg/ml MTT solution (Sigma–Aldrich, USA) was added to the cells for 4 h. The absorbance value was recorded utilizing a microplate reader (Molecular Devices) at 490 nm. Each test was performed in 3 sets.

TUNEL

Neuronal apoptosis was detected by a TUNEL Apoptosis Assay Kit (Roche Applied BioSciences, USA). Nuclei were stained with DAPI (blue). Fluorescent images were acquired under a fluorescence microscope (Nikon ECLIPSE). TUNEL-positive cells were quantified by ImageJ software and calculated by GraphPad D Prism version 5.0.

Flow Cytometry

Hippocampal neurons were rinsed in PBS, digested by 0.025% trypsin, and treated with 70% methanol overnight. Subsequently, the cells were incubated with 5 µL annexin-V-fluorescein isothiocyanate (FITC) and 5 µL PI for 10 min at 24-26 °C away from light. Next, without washing, the cells were mixed with 400 µL binding buffer, and apoptosis was evaluated by flow cytometry.

Luciferase Reporter Assay

The 3'-UTR of the wild-type rat GABRB1 gene was inserted into a psiCHECK-2 luciferase vector (Promega, USA), and then the GABRB1-3'-UTR-Wt plasmid was generated. The rno-miR-384-5p binding site at the GABRB1-3'-UTR was mutated. The mutant rat GABRB1 gene was also inserted into a psiCHECK-2 luciferase vector to generate the GABRB1-3'-UTR-Mut plasmid. HEK293T cells (ATCC, USA) were plated on 24-well plates and transfected with 2.5 µg GABRB1-3'-UTR-Wt or GABRB1-3'-UTR-Mut plasmid together with 50 nM miR-384-5p mimics or controls. After 48 h, a luciferase reporter assay system (Promega) was used to examine the results.

Statistical Analysis

Data are shown as the mean ± SD and were analyzed with SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Student's t test and one-way ANOVA were applied to compare the differences between groups. All experiments were independently conducted a minimum of 3 times. A significant difference was confirmed if the p value was < 0.05.

RESULTS

Ketamine Induces Neurotoxicity and miR-384-5p Upregulation in Hippocampal Neurons

Figure 1A indicates that the isolated neurons expressed βIII tubulin rather than GFAP, demonstrating successful isolation of hippocampal neuronal cells. Previous studies suggested that high doses of ketamine could cause neuronal death in cultured hippocampal neurons within 24 h (2,7,18). To detect the toxicity of ketamine, hippocampal neurons were treated with different doses of ketamine for 24 h. After treatment, an MTT assay was performed and showed that the viability of neurons was markedly reduced with increasing ketamine concentration ($p<0.05$ at 10 µM, $p<0.01$ at 20 and 50 µM, and $p<0.001$ at 100 µM) (Figure 1B). Based on these results, 20 µM ketamine was selected for the subsequent assays. A TUNEL assay indicated significantly increased apoptosis of neurons after exposure to ketamine (Figure 1C). Next, the ROS level in neurons was assessed using H2-DCFDA. The results showed that ketamine potentiated the intensity of H2-DCFDA (Figure 1D), suggesting that ketamine promotes ROS activity in neurons. Furthermore, we utilized RT–qPCR to examine the miR-384-5p level in ketamine-treated hippocampal neurons. The data showed that ketamine upregulated miR-384-5p expression in neurons in a concentration-dependent manner (Figure 1E). These results showed that ketamine causes neurotoxicity and miR-384-5p upregulation in primary cultured hippocampal neurons.

miR-384-5p Downregulation Reduces Ketamine-Induced Neurotoxicity

To verify whether miR-384-5p plays a role in ketamine-mediated neurotoxicity, neurons were transfected with a synthetic miR-384-5p inhibitor before ketamine treatment. Neurons were transfected with miR-384-5p inhibitor or NC inhibitor, and untreated cells were used as the blank group. Then, RT–qPCR revealed that miR-384-5p expression in neurons was decreased in the miR-384-5p inhibitor group compared to the control (Figure 2A). Subsequent experiments were conducted in hippocampal neurons treated with 20 µM ketamine. A TUNEL assay revealed that the increase in apoptosis in ketamine-treated neurons was reversed by miR-384-5p inhibition (Figure 2B), and flow cytometry analysis yielded the same result (Figure 2C). This suggests that silencing miR-384-5p attenuates the harmful effects of ketamine in neurons. Moreover, the miR-384-5p inhibitor decreased cleaved caspase-3 and Bax expression while enhancing Bcl-2 expression in neurons exposed to ketamine (Figure 2D). Moreover, miR-384-5p inhibition eliminated the promotion of ROS expression by ketamine in neurons (Figure 2E). Correspondingly, in the context of ketamine, cells transfected with miR-384-5p inhibitor exhibited increased expression of antioxidant proteins (CAT and SOD1) compared with control cells (Figure 2F). Overall, these data indicate that miR-384-5p knockdown protects neurons from ketamine-induced neurotoxicity.

GABRB1 is Targeted by miR-384-5p

To investigate the mechanisms of miR-384-5p in neuroprotection, we searched the TargetScan database to identify its downstream targets. As shown in Figure 3A, 7 potential genes that can form complementary base pairing with miR-384-5p were found based on the cumulative weighted context++ score on the website. We selected GABRB1 because its expression was significantly upregulated in cells transfected with the miR-384-5p inhibitor (Figure 3B). Additionally, the GABRB1 protein level was elevated after miR-384-5p depletion (Figure 3C). The binding site of miR-384-5p at position 157-163 of the GABRB1 3' UTR is shown in Figure 3D. Next, miR-384-5p was overexpressed in neurons

after miR-384-5p mimic transfection (Figure 3E). We also discovered that luciferase activity was impeded in HEK293T cells cotransfected with miR-384-5p mimic and GABRB1-Wt, while the GABRB1-Mut group showed no alteration (Figure 3F). This demonstrates that miR-384-5p directly targets GABRB1. In addition, GABRB1 expression was downregulated in hippocampal neurons treated with ketamine, as indicated by RT–qPCR and Western blotting (Figure 3G, H).

GABRB1 Knockdown Reverses the Neuroprotective Function of miR-384-5p Inhibition

To further explore whether GABRB1 directly participates in the regulation of miR-384-5p in ketamine-induced neurotoxicity, neurons were transfected with a rat GABRB1-specific shRNA (sh-GABRB1) or a nonspecific control (sh-NC) for 24 h. RT–qPCR and Western blotting demonstrated that endogenous

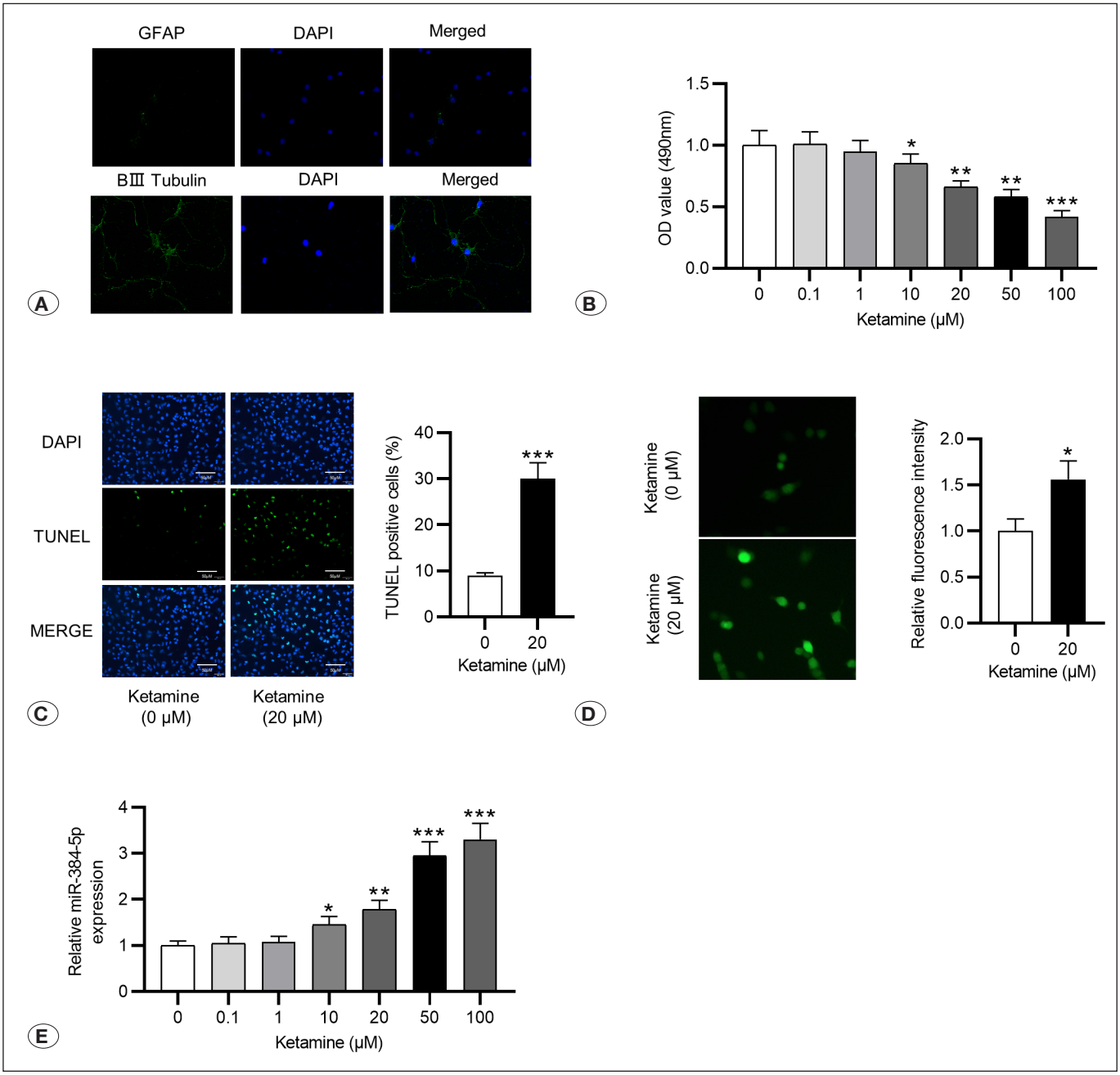

Figure 1: Ketamine induces neurotoxicity and upregulates miR-384-5p. **A)** Immunofluorescence staining for the expression of βIII tubulin and GFAP in hippocampal neurons. **B)** Hippocampal neurons were treated with ketamine at doses of 0, 0.1, 1, 10, 20, 50, and 100 μM for 24 h. Viability was assessed by MTT assay. **C)** Apoptosis in neurons treated with 0 mM or 20 mM ketamine was examined by TUNEL assay. **D)** The ROS level in neurons treated with 0 mM or 20 mM ketamine was measured using the fluorescent dye H2-DCFDA. **E)** The miR-384-5p level in neurons treated with different doses of ketamine was detected by RT–qPCR. *p<0.05, **p<0.01, ***p<0.001.

GABRB1 expression was knocked down in the sh-GABRB1 group compared with the blank and control groups (Figure 4A, B). Next, cells were transfected with miR-384-5p inhibitor, sh-GABRB1, and miR-384-5p inhibitor + sh-GABRB1 and then exposed to 20 μM ketamine. We found that GABRB1 protein expression was upregulated in cells transfected with miR-384-5p inhibitor and then decreased after transfection with sh-GABRB1 (Figure 4C). Apoptosis assays showed that GABRB1 knockdown restored the apoptosis of neurons inhibited by miR-384-5p inhibition (Figure 4D, E), which was also manifested by upregulation of cleaved caspase-3 and Bax protein expression and downregulation of Bcl-2 protein expression (Figure 4F). Furthermore, the inhibitory effects of the miR-384-5p inhibitor on ROS level were attenuated

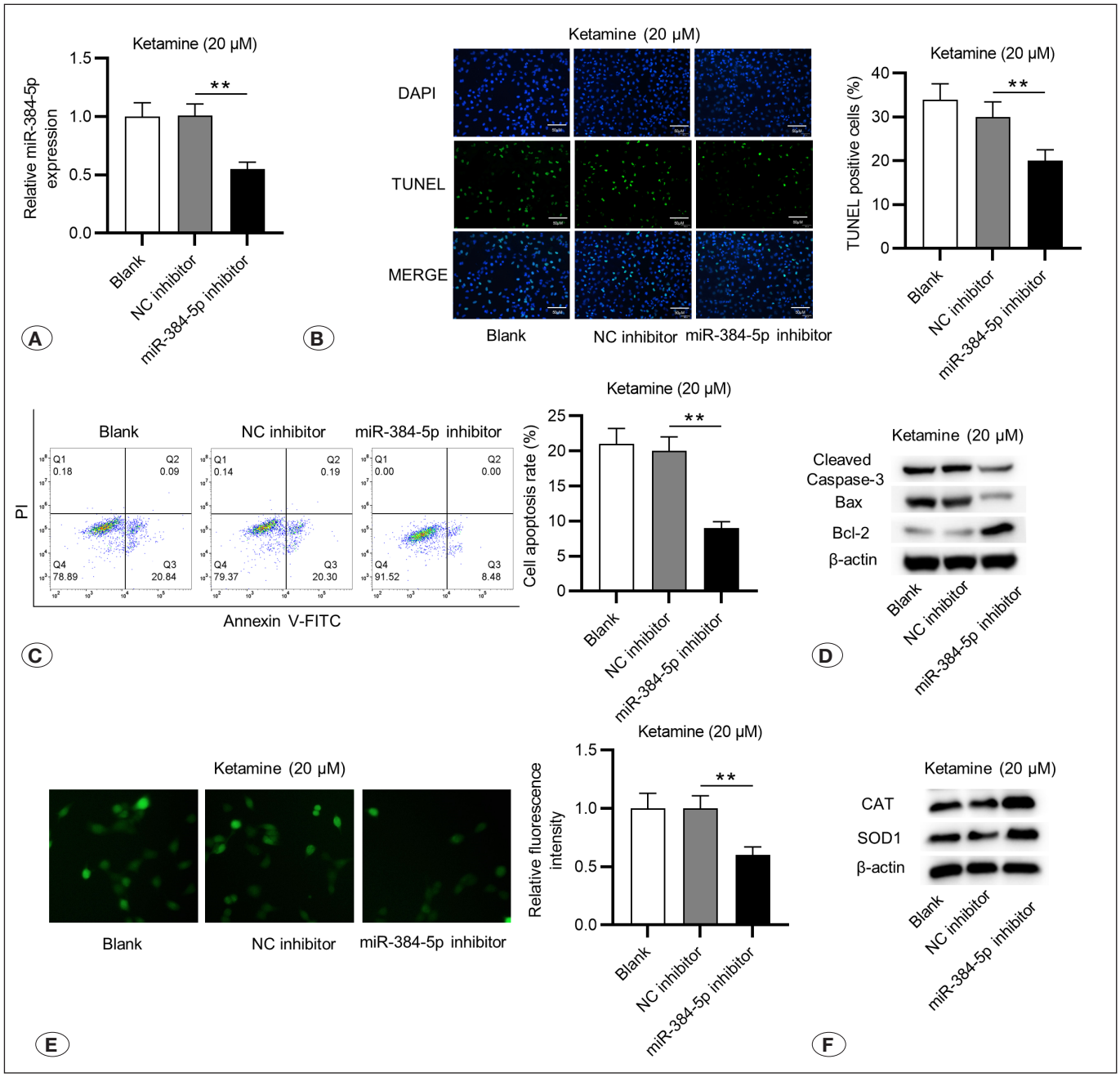

Figure 2: miR-384-5p downregulation reduces ketamine-induced neurotoxicity. Neurons transfected with miR-384-5p inhibitor or NC inhibitor were treated with 20 mM ketamine for 24 h. **A)** RT–qPCR for miR-384-5p expression in neurons. **B)** TUNEL staining for neuronal apoptosis upon miR-384-5p downregulation. **C)** Flow cytometry for neuronal apoptosis upon miR-384-5p downregulation. **D)** Western blotting for cleaved caspase-3, Bax, and Bcl-2 protein expression in neurons upon miR-384-5p downregulation. **E)** H2-DCFDA staining for the ROS level in neurons upon miR-384-5p downregulation. **F)** Western blotting for CAT and SOD1 protein expression in H2-DCFDA-stained neurons. ¨p< 0.01.

Human ortholog of target gene	Representative transcript
POU5F1B	ENST00000465342.2
C10orf11	ENST00000496424.2
MKRN3	ENST00000314520.3
CYP24A1	ENST00000216862.3
GABRB1	ENST00000295454.3
SRSF7	ENST00000313117.6
GPR63	ENST00000229955.3

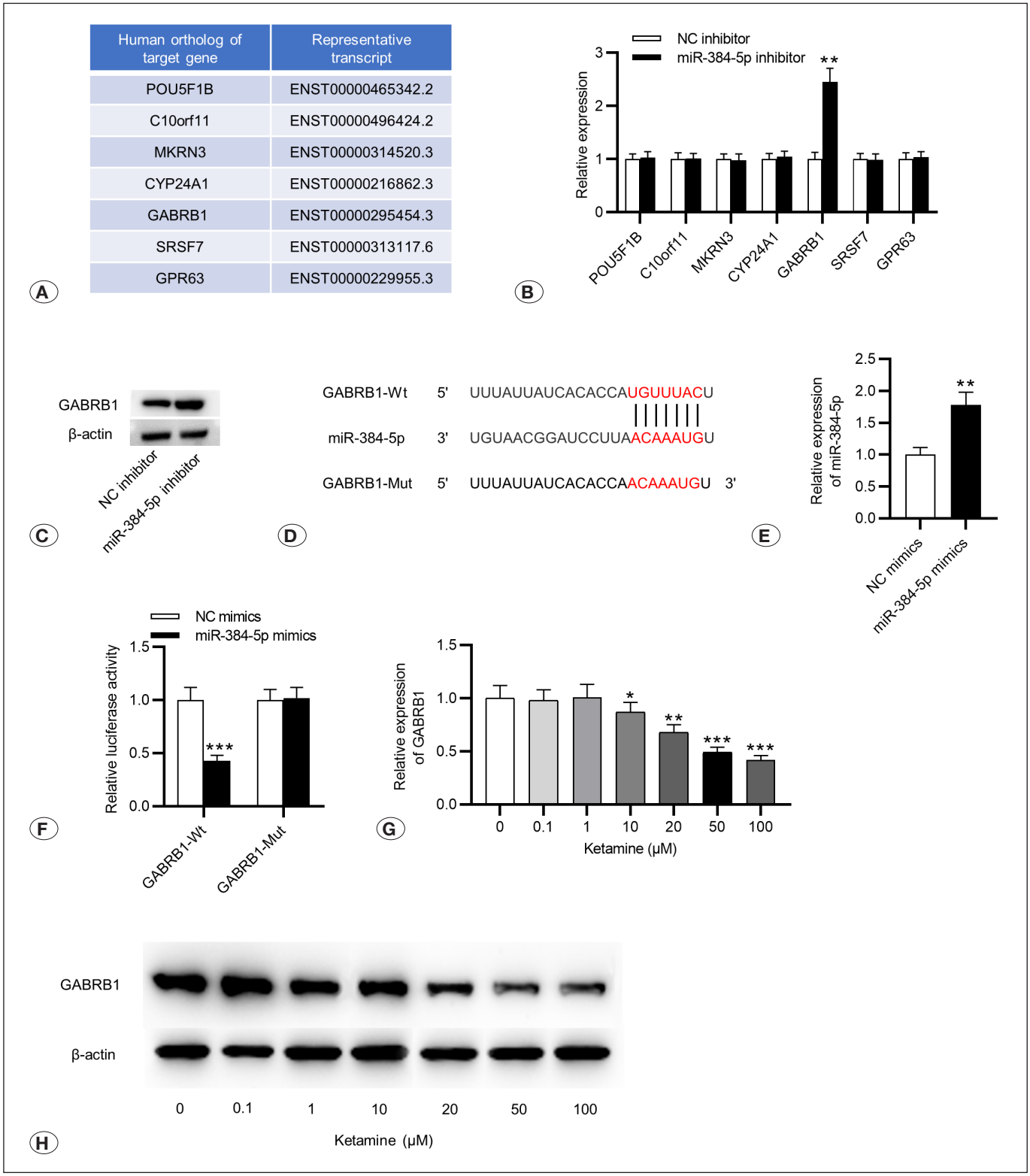

Figure 3: GABRB1 is targeted by miR-384-5p. **A)** The downstream targets of miR-384-5p found in TargetScan. **B)** RT–qPCR for mRNA expression in neurons transfected with miR-384-5p inhibitor. **C)** Western blotting for GABRB1 protein expression in neurons upon miR-384-5p downregulation. **D)** Binding site of miR-384-5p at the GABRB1 3' UTR. **E)** RT–qPCR for overexpression efficiency of miR-384-5p in neurons. **F)** HEK293T cells were double transfected with miR-384-5p mimic and GABRB1-Wt/Mut, and luciferase activity was analyzed 48 h later. **G, H)** RT–qPCR and Western blotting for GABRB1 expression in neurons treated with different concentrations of ketamine. *p<0.05, **p<0.01, ***p<0.001.

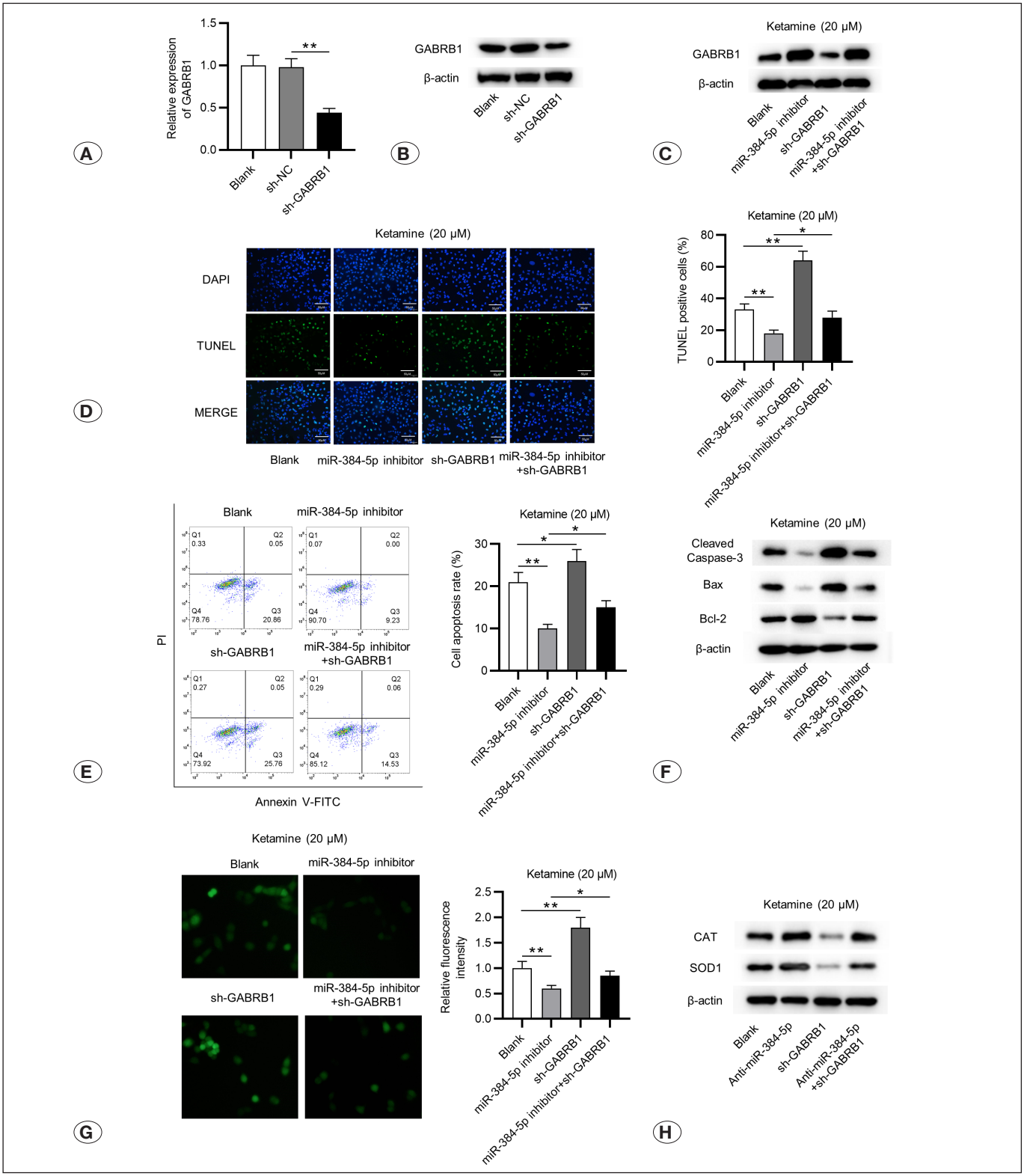

Figure 4: GABRB1 knockdown reverses the protective function of miR-384-5p inhibition in ketamine-induced neurotoxicity. **A, B)** RT–qPCR and Western blotting to determine the interference efficiency of GABRB1 in neurons. Cells were treated with miR-384-5p inhibitor, sh-GABRB1, and miR-384-5p inhibitor + sh-GABRB1 and then exposed to 20 μM ketamine. **C)** Western blotting for GABRB1 protein expression in neurons. **D, E)** TUNEL and flow cytometry analyses of neuronal apoptosis. **F)** Western blotting for cleaved caspase-3, Bax, and Bcl-2 protein expression in neurons. **G)** The ROS level in neurons was measured using H2-DCFDA staining. **H)** Western blotting for CAT and SOD1 protein expression in neurons. *p<0.05, **p<0.01.

by sh-GABRB1 (Figure 4G). miR-384-5p inhibition led to upregulation of CAT and SOD1 in ketamine-exposed neurons; however, GABRB1 depletion negated these effects (Figure 4H). Taken together, miR-384-5p regulates ketamine-induced neurotoxicity in primary cultured hippocampal neurons by targeting GABRB1.

DISCUSSION

Ketamine has been broadly used in pediatric anesthesiology. However, accumulating studies have suggested that exposure to a high dose of ketamine can cause neurotoxicity in brain development stages (32). It is well established that miRNAs are involved in the regulation of ketamine-mediated neurotoxicity. As previously published, lentiviral suppression of miR-34a expression reduces ketamine-induced memory impairment and hippocampal neuron apoptosis (22). Depletion of miR-375 attenuates neural cell apoptosis and neural toxicity induced by ketamine (40). miR-107 downregulation protects embryonic stem cell-derived neurons against ketamine-induced injury (20). In this study, rat hippocampal neurons were treated with ketamine to induce neurotoxicity, and the miR-384-5p level was assessed to explore its biological role in ketamine-induced neurotoxicity. miR-384-5p expression is increased with the progression of neuronal apoptosis in rats treated with trimethyltin and, thus, is considered a potential indicator of neurotoxicity (27). It has been shown that miR-384-5p can accelerate anesthesia injury to neurons in the abutment nucleus of rats through various signaling pathways and biological processes (35). Additionally, miR-384-5p is upregulated after rotenone treatment, and its depletion alleviates neurotoxicity induced by rotenone in SH-SY5Y cells by suppressing endoplasmic reticulum stress (21). Consistent with these studies, we discovered that miR-384-5p was elevated in primary cultured neurons treated with ketamine, and inhibition of miR-384-5p prevented ketamine-induced neuronal apoptosis.

Oxidative stress disrupts the oxidative and antioxidant balance. The balance between ROS generation and elimination is crucial in oxidative stress, which plays a protective or harmful role in disease (1). Alterations in ROS levels have been found to be related to various pathological and physiological processes (16). Increasing evidence shows that an excess of ROS can cause oxidative injury to cells (23,34). In the present investigation, inhibition of miR-384-5p was found to reduce ROS levels and increase antioxidant protein (CAT and SOD1) expression levels in neurons. This suggests that miR-384-5p downregulation can negatively regulate ketamine-mediated oxidative stress.

We further investigated the targets of miR-384-5p. As a subunit of gamma-aminobutyric acid type A (GABAA) receptors, GABAA receptor beta1 (GABRB1) plays a critical role in rapid inhibitory synaptic transmission (25). GABAergic neurons account for approximately 30% of all neurons in the thalamus (24). A study showed that the GABRB1 gene participates in processes associated with immune and neuronal systems, such as Toll-like receptor signaling, neurotrophin signaling, and the adherens junction pathway (37). As reported previously, downregulation of GABRB1 expression is found during early neuronal differentiation, which may lead to abnormal brain development (14). Here, miR-384-p was found to negatively regulate GABRB1 expression. Additionally, we found that GABRB1 expression was depleted in hippocampal neurons after ketamine treatment. More importantly, GABRB1 depletion aggravated ketamine-induced neurotoxicity. In neurons damaged by ketamine, GABRB1 downregulation attenuated the neuroprotective effect of silencing miR-384-5p. Therefore, GABRB1 may be a protective factor against ketamine-induced neurotoxicity.

CONCLUSION

We verified that miR-384-5p downregulation exerts neuroprotective effects against ketamine-induced neurotoxicity in neonatal rat hippocampal neurons, and these effects may be related to the regulation of GABRB1. The results of our study may contribute to the identification of more effective biomarkers associated with ketamine-induced neurotoxicity and may provide clues for the development of novel clinical interventions to treat or prevent ketamine-induced neural damage in the brain. However, our study was limited to in vitro experiments; thus, conducting *in vivo* experiments in the future could provide stronger evidence to support our findings.

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AUTHORSHIP CONTRIBUTION

Study conception and design: QY, FL
Data collection: QY
Analysis and interpretation of results: QY, FL
Draft manuscript preparation: QY
Critical revision of the article: QY, FL
Other (study supervision, fundings, materials, etc...): QY
All authors (QY, FL) reviewed the results and approved the final version of the manuscript.

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