

Ketamine promotes the neural differentiation of mouse embryonic stem cells by activating mTOR

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Abstract. Ketamine is a widely used general anesthetic and has been reported to demonstrate neurotoxicity and neuroprotection. Investigation into the regulatory mechanism of ketamine on influencing neural development is of importance for a better and safer way of relieving pain. Reverse transcription-quantitative polymerase chain reaction and western blotting were used to detect the critical neural associated gene expression, and flow cytometry to detect the neural differentiation effect. Hence, in the present study the underlying mechanism of ketamine (50 nM) on neural differentiation of the mouse embryonic stem cell (mESC) line 46C was investigated. The results demonstrated that a low dose of ketamine (50 nM) promoted the differentiation of mESCs to neural stem cells (NSCs) and activated mammalian target of rapamycin (mTOR) by upregulating the expression levels of phosphorylated (p)-mTOR. Furthermore, inhibition of the mTOR signaling pathway by rapamycin or knockdown of mTOR suppressed neural differentiation. A rescue experiment further confirmed that downregulation of mTOR inhibited the promotion of neural differentiation induced by ketamine. Taken together, the present study indicated that a low level of ketamine upregulated p-mTOR expression levels, promoting neural differentiation.

Introduction

Ketamine, an N-methyl-D-aspartate (NMDA) receptor antagonist, is widely used in pediatric anesthesia, periop-

erative sedation, analgesia and other diagnostic procedures in pediatrics for children 0-14 years old (1). It is often consumed as a drug of abuse by the public, including pregnant women (2); the fetuses of such pregnant patients, who received non-obstetric surgery, have an increasing incidence of exposure to ketamine through the placenta. Additionally, 0.75-2% of pregnant women require surgery associated with pregnancy or other medical issues (3,4). A series of experiments have revealed that ketamine can induce neuroapoptosis and damage in the developing brain (5-7). Repeated exposure to ketamine can be deleterious to neurodevelopment in infants (8). In contrast, increasing evidence also suggested that ketamine has neuroprotective function. Clinical studies have demonstrated that a single dose of ketamine mitigates postoperative cognitive dysfunction (8) and may offer specific protection towards post-operative cognitive dysfunction (9). Ketamine may additionally prevent stress-induced cognitive inflexibility in rats (10). Previous studies demonstrated that for traumatic brain injuries (TBIs), subarachnoid hemorrhage, malignant stroke and other neurological diseases, ketamine could inhibit the neuronal discharge across all injury modalities (11,12). The neuroprotective function of ketamine has also been demonstrated in hypoxia-ischemia and TBI, and as a fast-acting antidepressant (13-15). Dong *et al* (16) demonstrated that the phosphoinositide 3-kinase-protein kinase B/Akt signaling pathway was involved in ketamine-induced neurogenesis of cultured neural stem/progenitor cells (NSPCs). Furthermore, ketamine induces human neurotoxicity in neurons differentiated from human embryonic stem cells (hESCs) via the reactive oxygen species-mediated mitochondrial apoptosis pathway (17). These studies suggested that the effect of ketamine on neurodevelopment may be dose-dependent. Additionally, the underlying mechanism of ketamine on neurodevelopment may also depend on different developmental stages; however, the molecular mechanism of ketamine regulating the early development of neural cells remains unclear.

Mouse ESCs (mESCs) derived from embryos at the pre-implantation stage demonstrating an unlimited self-renewal ability and capacity to generate different cell types are valuable for clinical research (18). Therefore, mESCs are an important as an *in vitro* model to study ontogenetic development. Previous studies identified that there are specific

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critical genes regulating neural differentiation, for example, zing finger homeobox (*Zfhx1b*) has been reported to promote neural stem cell (NSC) colony formation by inducing Sex determining region Y-box (*Sox1*) expression (19). Sirtuin1 could mediate alterations in DNA methylation to modulate embryonic stem cell differentiation (20). The microRNA-134/methyl-CpG binding domain protein 3 axis could regulate the reprogramming and pluripotency of induced pluripotent stem cells, a type of ESC-like cells, from neural progenitor cells (NPCs) (21); however, the neuroprotective function of ketamine in mESCs on NSC differentiation and its downstream mechanism remains elusive.

Mammalian target of rapamycin (mTOR) is a critical regulator of growth and homeostasis (22–24). A growing number of studies have demonstrated that the mTOR-related signaling pathway is associated with the differentiation of NPCs and NSCs (25,26), and is important to regulate oligodendrocyte differentiation and remyelination (27). mTOR also serves an important role in regulating cortical interneuron number and autophagy during brain development (28). Rapamycin, the mechanistic target of mTOR, has been associated with improvements in neurological deficits and increased brain water content (29). However, whether mTOR could regulate the neural differentiation of ESCs has been rarely evaluated. Besides, whether mTOR participates in ketamine regulatory signaling pathway or not, is also unclear.

In the present study, it was determined whether ketamine was able to influence the neural differentiation from the mESCs and the marker expression of sex-determining region Y-box (*Sox1*) (30), N-cadherin (*N-cad*) (31) and Nestin (32). The present study suggested a safe dose of ketamine for clinical application and demonstrated that mTOR may be a potential target of better and safer therapeutics in the future.

Materials and methods

mESC culture. The mESC line 46C, containing the *Sox1* promoter and expressing green fluorescence protein (GFP), was employed to indicate the endogenous *Sox1* expression during the neural differentiation at NPCs stage and gifted by Dr Xiaoqing Zhang (Tongji University, Shanghai, China) (33). Cells were cultured on feeder cells that are the irradiated mouse embryonic fibroblasts in KnockOut™ Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA; cat. no. 10829018) with 15% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), leukemia inhibitory factor (Merck KGaA, Darmstadt, Germany; cat. no. LIF2050) and β -mercaptoethanol (β -Me; 1:10,000, Sigma-Aldrich; Merck KGaA) at 37°C, under a 5% CO₂ atmosphere. After 48 h, mESCs were digested into single cells using 0.05% trypsin (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 2520056) and seeded on new feeder cells for passaging. The feeder cells that were able to secrete leukemia inhibitory factor to support the growth of the ESCs were made in our lab. Feeder cells were made from x-irradiated day 13.5 embryonic fibroblasts. Day 13.5 embryonic fibroblasts were granted from Dr Liu lab in Tongji University.

Neural differentiation of mESCs to NSCs. The protocol was adapted from a previous study (34). The mESC line, 46C,

was dissociated into single cells by trypsin and counted. Subsequently, 2×10^4 cells/ml mESCs were washed with Glasgow's minimum essential medium (GMEM; Gibco; Thermo Fisher Scientific, Inc.) in a 6 cm dish and re-suspended in GMEM with 8% knockout serum replacement (Gibco; Thermo Fisher Scientific, Inc.), 1% sodium pyruvate, 1% L-glutamine (Thermo Fisher Scientific, Inc.), 0.1 mM β -Me. Cells were cultured in a 6 cm ultra-low attachment petri dish and passaged every 2 days at 37°C in a 5% CO₂ atmosphere. The culture medium was changed every day. Clones exhibiting GFP fluorescence at the stage of NSC derived from 46C mESCs were detected with an IX73 + DP80 inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan; magnification, $\times 20$). During the neural differentiation, ketamine (final concentration 50 nM) was added into the medium. The control group consisted of cells treated only with physiological saline (0.9% NaCl). For the treatment with MK-801, 10 μ g/ml MK-801 was used to treat cells during neural differentiation. Rapamycin (final concentration 50 μ M) was added to the medium during the neural differentiation. The control group was treated with dimethyl sulfoxide, which was additionally used as the solvent for rapamycin. All the treating or control culture media was changed every day during the 7 days of neural differentiation from mESCs.

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Total neural stem cell RNA was isolated by RNaiso plus (Takara Biotechnology Co., Ltd., Dalian, China), mRNA was reverse transcribed to cDNA at 37°C for 15 min using a RT reagent kit (Perfect Real Time; Takara Biotechnology Co., Ltd.). qPCR was performed using SYBR Green qPCR Mix (Takara Biotechnology Co., Ltd.). The primers are as follows: Nestin forward, 5'-CCCTGAAGT CGAGGAGCTG-3' and reverse, 5'-CTGCTGCACCTCTAA GCGA-3'; N-cadherin forward, 5'AGCGCAGTCTTACCGAA GG-3' and reverse, 5'-TCGCTGCTTTCATACTGAACCTT-3'; Sox1 forward, 5'-AAGGAACACCCGGATTACAAGT-3' and reverse, 5'-GTTAGCCCAGCCGTTGAC-3'; and GAPDH forward, 5'-AGGTCGGTGTGAACGGATTG-3' and reverse 5'-TGTAGACCATGTAGTTGAGGTCA-3'. The PCR thermocycling conditions were as follows: Initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 5 sec, primer annealing at 60°C for 20 sec, elongation at 70°C for 10 sec. In total, three independent experiments were performed. The relative gene expression was presented as $2^{-\Delta\Delta C_q}$ using the relative quantification method and normalized to the expression of GAPDH (35).

Western blotting. Cells were lysed by radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China; cat. no. P0013B) and quantified by a Bicinchoninic Protein Assay Kit (Beyotime Institute of Biotechnology; cat. no. P0009). A total of 40 μ g protein was loaded for electrophoresis on 10% SDS-PAGE gels. Proteins were transferred onto polyvinylidene fluoride membranes (Merck KGaA; cat. no. MH0323) and blocked with TBS and Tween 20 with 3% bovine serum albumin (Amresco, Inc., Framingham, MA, USA) for 1 h at room temperature and incubated with primary antibodies at 4°C overnight. The antibodies were as follows: mTOR (cat. no. 2972, Cell Signaling

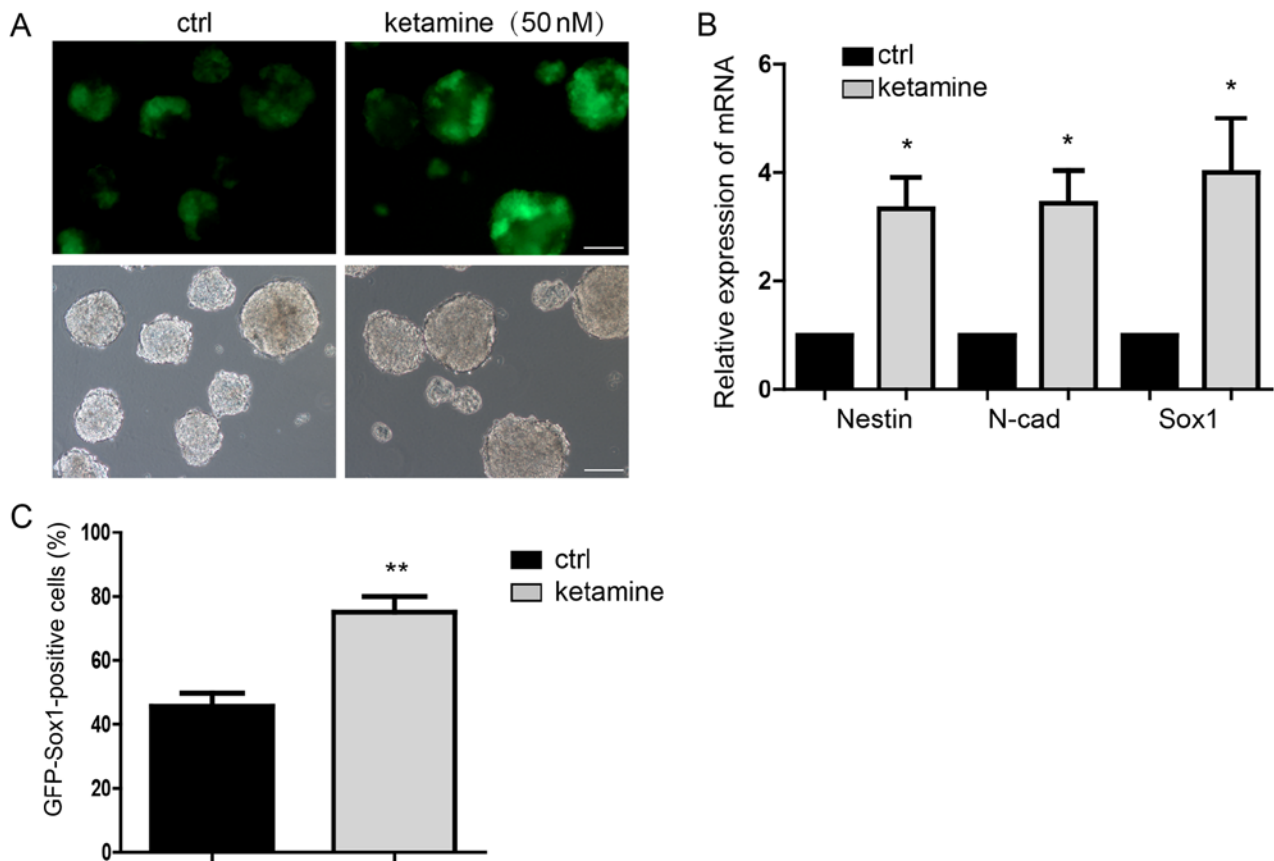


Figure 1. Ketamine promotes neural differentiation. (A) GFP indicated the Sox1 expression of NPCs, suggesting the differentiation potential. Scale bar, 100 μ m. (B) Detection of the NPCs markers expression by RT-qPCR. (C) Flow cytometry analysis indicated more GFP-Sox1-positive cells in the ketamine-treatment group. Data are presented as the mean \pm standard deviation ($n=3$). * $P<0.05$, ** $P<0.01$ vs. the ctrl. Ctrl, control; GFP, green fluorescence protein; NPC, neural progenitor cell; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Sox, sex-determining region Y-box.

Technology, Inc., Danvers, MA, USA, 1:1,000), p-mTOR (cat. no. 5536, Cell Signaling Technology, Inc., 1:1,000), GAPDH (cat. no. 5174, Cell Signaling Technology, Inc. 1:1,500), p-p70 S6k antibody (cat. no. 9205, Cell Signaling Technology, Inc. 1:1,000) and p70 S6k antibody (cat. no. 2708, Cell Signaling Technology, Inc. 1:1,000). The horseradish peroxidase-conjugated secondary antibody used was anti-rabbit IgG (cat. no. 7074; Cell Signaling Technology, Inc.; 1:2,500) and was incubated with the membranes for 2 h at room temperature. The bands were detected by an enhanced chemiluminescence western blotting substrate (Thermo Fisher Scientific, Inc.). Amersham Imager 600 (GE Healthcare, Chicago, IL, USA) was used for detecting the signaling. ImageJ_v1.8.0 software (National Institutes of Health, Bethesda, MD, USA) was used for densitometry.

Knockdown of mTOR. The pLKO.1-puro vector (Addgene, Inc., Cambridge, MA, USA; cat. no. 8453) containing mTOR short hairpin (sh)RNA was constructed to downregulate mTOR expression. The sequence of shRNA-1 was: 5'-AGT ACTGTAGCACCTTGGG-3' and of shRNA-2 was: 5'-TCT TCTCTCTGTAGTCCCG-3'. The control vector used was the empty pLKO.1-puro vector. The vectors (1 μ g/6 cm dish) were transiently transfected into the cells during neural differentiation from mESCs at day 3 using the Lipofectamine[®] 2000 Transfection Reagent (Thermo Fisher Scientific, Inc.) and re-transfected at day 5 in order to maintain the knockdown

effect during the 7 days of neural transfection. Transfection efficiency was detected by RT-qPCR at day 7.

Flow cytometry. The mESC line, 46C is a cell line with GFP expression, indicating endogenous Sox1 expression during the differentiation from mESCs to NSCs. Flow cytometry was performed to detect the quantitative proportion of GFP-Sox1-positive cells to determine the differentiation efficiency. Clones of NSCs were digested to a single cell suspension by 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 2520056) at 37°C for 2 min. Cells were collected by centrifugation at 1,000 \times g for 2 min at room temperature and re-suspended with PBS to wash the cells. This step was repeated twice. The cell suspension in PBS was used for further analysis. A flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used to detect the GFP-Sox1-positive NSCs. The results were analyzed by using FlowJo software (version 7.6.1; FlowJo LLC, Ashland, OR, USA).

Statistical analysis. Each experiment was performed at least 3 times ($n\geq 3$). Statistical significance was detected by a Student's t-test between two groups. For multiple groups, one-way analysis of variance was used, followed by Tukey's honest significance test. Data are presented as the mean \pm standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

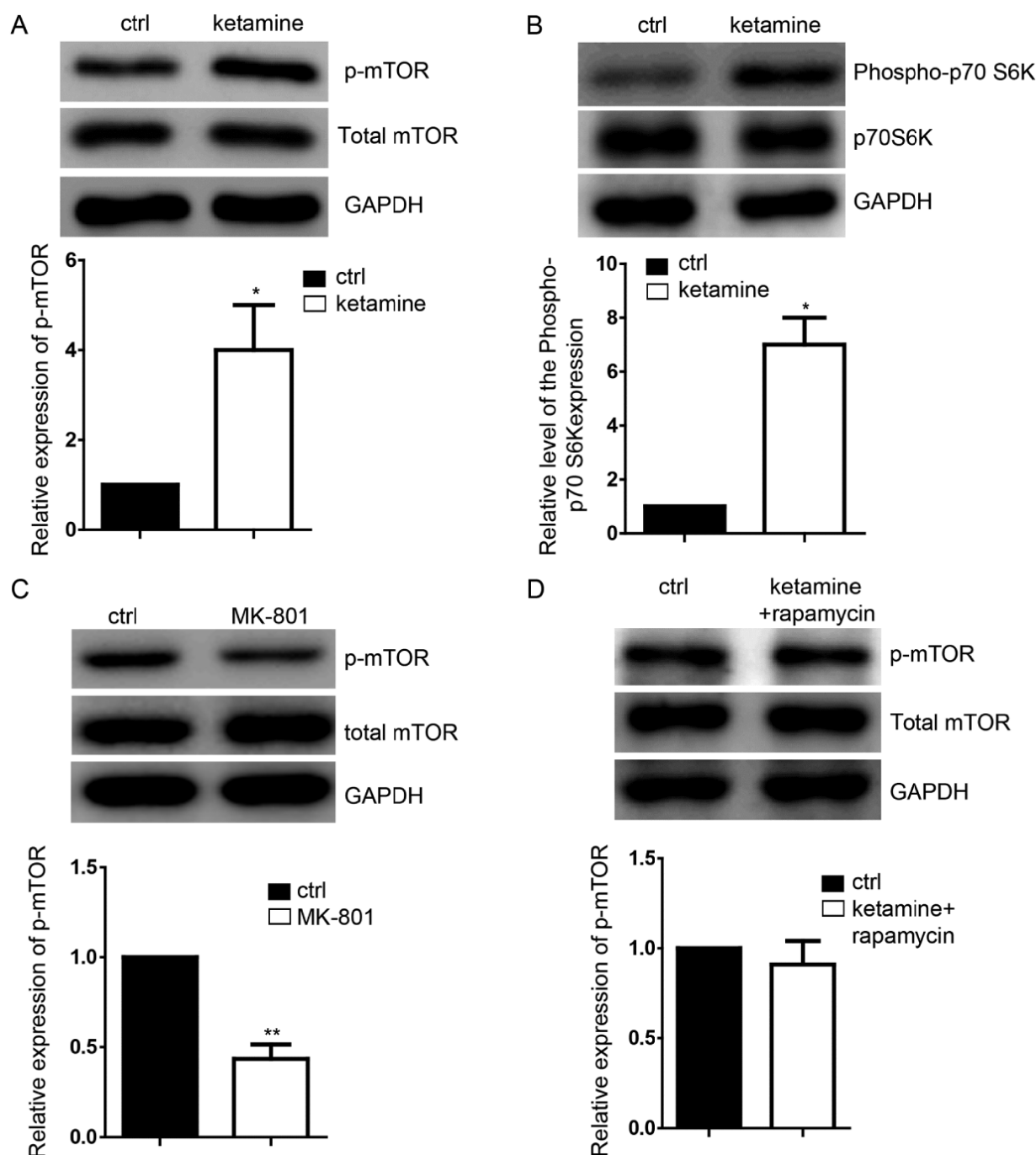


Figure 2. Ketamine activates the mTOR signaling pathway. (A) Representative images of the expression of p-mTOR in neural progenitor cells derived from ESCs as detected by western blotting; the ratio of p-mTOR normalized to GAPDH/total mTOR is presented. (B) Ketamine-treatment group demonstrated the upregulation of p-70S6K. (C) Inhibition of the NMDA signaling pathway by the NMDA receptor antagonist MK-801 decreased the expression levels of p-mTOR; the ratio of p-mTOR normalized to GAPDH/total mTOR is presented. (D) 50 μ M rapamycin markedly reduced the activation of the mTOR signaling, which attenuated the function of ketamine on regulating the level of p-mTOR. Data are presented as the mean \pm standard deviation (n=3). *P<0.05, **P<0.01 vs. the ctrl. Ctrl, control; ESC, embryonic stem cell; NMDA, N-methyl-D-aspartate; mTOR, mammalian target of rapamycin; p, phosphorylated.

Results

Ketamine promotes neural differentiation. Neural differentiation of mESCs to NSCs demonstrated that 50 nM ketamine added into the medium significantly promoted the neural differentiation detected on day 7 (Fig. 1A). Subsequently, the expression of NSCs markers was investigated demonstrating that Nestin, N-cad and Sox1 were significantly upregulated in

the ketamine-treatment group compared with the control group (Fig. 1B). Flow cytometry further confirmed that the proportion of GFP-Sox1-positive cells was significantly higher in the ketamine-treatment group compared with the control group (Fig. 1C). These results indicated that ketamine may not only be an anesthetic; however, additionally regulates neural differentiation. This suggested the potential influence of ketamine on individual neural differentiation at the early development stage.

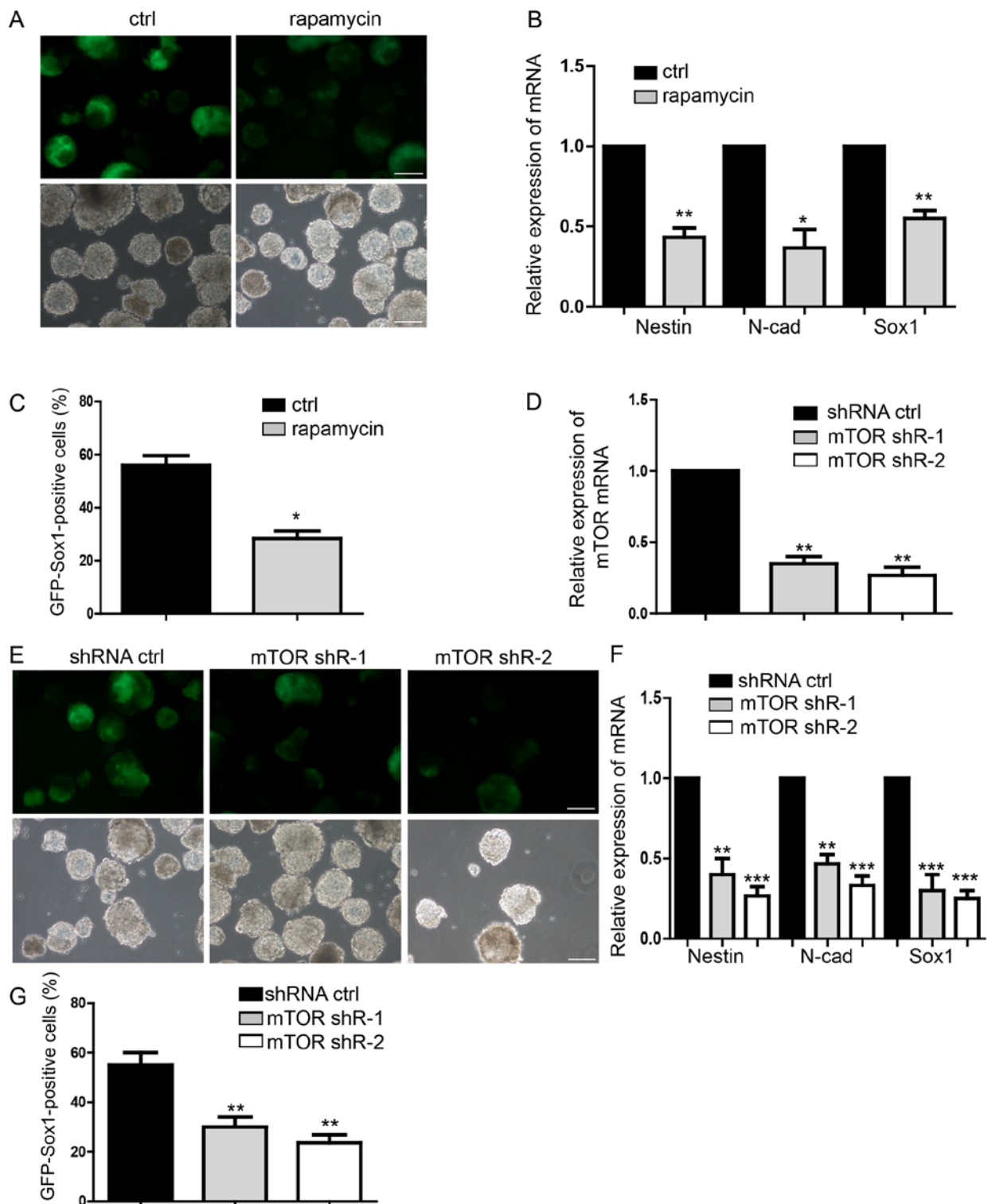


Figure 3. Inhibition of mTOR suppresses neural differentiation. (A) Representative images of neural differentiation in the rapamycin-treatment and control groups. (B) Expression of NPCs markers of Nestin, N-cad and Sox1 by RT-qPCR. (C) Flow cytometry analysis of rapamycin-treatment and control group. (D and E) Detection of the mTOR knockdown effect by shRNA, which suppressed neural differentiation. (F) Expression levels of NPCs markers, as measured by RT-qPCR. (G) Flow cytometry analysis indicating less NPCs following transfection with shRNA. Scale bar, 100 μ m. Data are presented as the mean \pm standard deviation (n=4). *P<0.05, **P<0.01 and ***P<0.001 vs. the ctrl. Ctrl, control; N-cad, N-cadherin; NPC, neural progenitor cell; m-TOR, mammalian target of rapamycin; p, phosphorylated; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; shR, short hairpin RNA; Sox, sex-determining region Y-box.

Ketamine activates the mTOR signaling pathway. In order to detect the downstream targets of ketamine, western blotting was performed, which demonstrated the significant upregulation of p-mTOR (Fig. 2A) and of its downstream target,

p-70S6K compared with the control group (Fig. 2B), without influencing their total expression levels. Inhibition of the NMDA signaling pathway by the NMDA receptor antagonist MK-801 significantly decreased p-mTOR expression levels

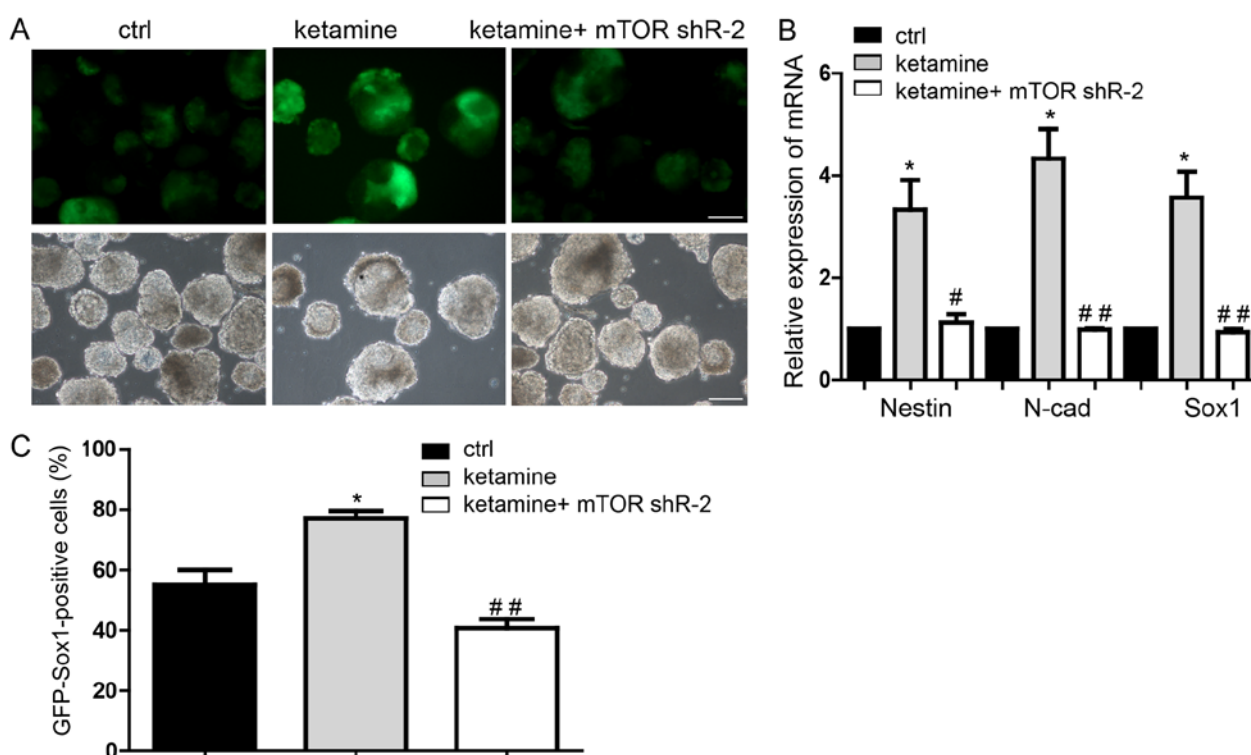


Figure 4. mTOR mediates the function of ketamine-regulated neural differentiation. (A) Representative images of neural differentiation in rescue experiments. (B) Downregulation of mTOR restored neural progenitor cell marker expression levels, in the ketamine-treatment group. (C) Flow cytometry assay also indicated that mTOR knockdown inhibited the neural differentiation promoted by ketamine. Scale bar, 100 μ m. Data are presented as the mean \pm standard deviation (n=3). * P <0.05 vs. respective ctrl; # P <0.05, ## P <0.01 vs. respective ketamine. Ctrl, control, cells transfected with the empty vector pLKO.1 and treated with 0.9% saline; mTOR, mammalian target of rapamycin; shR, short hairpin RNA; Sox, sex-determining region Y-box.

(Fig. 2C), which suggested that inhibition of NDMA signaling is not able to increase the mTOR expression level. Rapamycin (50 μ M) was used to notably reduce the activation of mTOR signaling caused by ketamine. Subsequently, the cells were treated with ketamine and rapamycin together to perform the rescue experiments and it was identified that rapamycin was able to block the p-mTOR expression level increased by ketamine (Fig. 2D).

Inhibition of the mTOR suppresses neural differentiation.

The neural differentiation of mESCs was analyzed and rapamycin (50 μ M) was added to the medium to investigate whether the number of NSCs was decreased compared with the control group on day 7 (Fig. 3A). Expression levels of Nestin, Sox1, N-cad were significantly downregulated by rapamycin compared with the control (Fig. 3B). Flow cytometry assay also indicated significantly fewer GFP-Sox1-positive cells following rapamycin treatment compared with the control (Fig. 3C). Transfection with mTOR-shRNA during the differentiation of mESCs to NSCs (Fig. 3D) notably suppressed neural differentiation (Fig. 3E). The expression levels of the NSCs markers were significantly downregulated in response to mTOR silencing compared with in the control (Fig. 3F). Finally, the proportion of GFP-SOX1-positive cells in the mTOR knockdown groups were significantly decreased compared with the control, as measured by flow cytometry (Fig. 3G). These results suggested that inhibition of mTOR signaling was able to significantly repress neural differentiation, which

is contrary to the function of ketamine and suggested the possible regulatory mechanism of ketamine/mTOR signaling during neural differentiation.

mTOR mediates the function of ketamine-regulated neural differentiation. Transfection with mTOR-shRNA (Fig. 3D) demonstrated that shRNA-2 induced more of a decrease of average mTOR expression and a more marked inhibitory effect on neural differentiation compared with shRNA-1. Therefore, shRNA-2 was selected for further study. Downregulation of mTOR significantly inhibited the promotion of neural differentiation induced by ketamine, on day 7 (Fig. 4A). Expression levels of NSCs markers were significantly restored by mTOR knockdown in the ketamine-treatment group (Fig. 4B). Flow cytometry also confirmed the rescue effect of mTOR downregulation following ketamine treatment (Fig. 4C). These results suggested that repression of mTOR blocked neural differentiation promoted by ketamine, which suggested the novel involvement of the ketamine/mTOR signaling pathway during neural differentiation.

Discussion

In the present study, it was revealed that ketamine activated mTOR to promote the neural differentiation of mESCs, providing the theoretical basis for the rational use of ketamine.

Ketamine, a widely used anesthetic, has potential neuro-degenerative and long-term cognitive deficits, affecting brain development (7,36-39). Methods of safe ketamine

application is an important research goal in clinical practice. The effects of ketamine are not only dependent on its dose, but also on the frequency of exposure (40-43). Ketamine has a relative neuroprotective function by relieving pain and inhibiting inflammation (44). Ketamine serves an important role in regulating nerve development (16). In the present study, ketamine at 50 nM promoted neural differentiation and upregulated NSC marker expression levels. The process of neural differentiation occurs during early development (45,46). The present results additionally demonstrated the positive effects of ketamine at a low dose, suggesting the safe clinical use in surgery for pregnant patients and children in the future.

ESCs have been extensively used for studying development, particularly neural development (47-51). Numerous genes serve an important role in the differentiation into neural stem cells (52,53). A recent study demonstrated that fibronectin type III domain-containing 5 facilitated neural differentiation by increasing the expression of brain derived neurotrophic factor (54). *Zfhl1b* gene expression has been confirmed to be notably upregulated via the fibroblast growth factor signaling pathway in mESCs cultured in a permissive neural-inducing environment (19). Ketamine was proposed to regulate mTOR activity by upregulating the expression levels of p-mTOR in the present study. This was reversed by adding the mTOR inhibitor rapamycin or by downregulating mTOR. This suggested a potential molecular mechanism of ketamine regulation; however, further investigation is required.

In neural progenitors, insulin has been demonstrated to induce neurogenesis of NPCs by activating mTOR (26). mTOR is also needed for the of dendritic arbors development and stabilization in the newly born olfactory bulb neurons (55). The mTOR signaling pathway was reported to mediate valproic acid-induced neural differentiation of NSCs (56). Inhibition of the mTOR signaling pathway by rapamycin was observed to suppress neural differentiation in the present study. The promotion of neural differentiation caused by ketamine was also inhibited by silencing mTOR. The expression of Nestin, Sox1 and N-cad was also restored by downregulating mTOR. The NMDA signaling pathway, was inhibited during the neural differentiation and the levels of p-mTOR were also suppressed. This result indicated the regulatory function of ketamine via a non-NMDA signaling pathway during neural differentiation. However, a limitation of the present study is that whether the NMDA receptor may influence neural differentiation remains unknown. mTOR complex 1 (mTORC1) was closely associated with the neuron-associated biological process downstream target (57). The activity of p70S6K, the downstream target of mTORC1, was increased by ketamine, indicating that it may participate in the regulation of ketamine. These results determined that the ketamine/mTOR signaling pathway regulated the neural differentiation process of NSCs derived from mESCs; however, further investigation is required.

In summary, the present study revealed the ketamine/mTOR signaling pathway on regulating the neural differentiation and suggested a potential dose of ketamine. The ketamine/mTOR signaling pathway needs to be further investigated for its potential use in clinic.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XZ performed the experiments. XL performed the reverse transcription-quantitative polymerase chain reaction assays and wrote parts of the manuscript. LZ performed the western blotting. JY conducted the statistical analysis. RH performed microscopy. YS cultured and prepared the cells. SX analyzed the expression level of western blot. HJ provided guidance and analyzed some of the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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