Original Paper

Ketamine Inhibits Proliferation of Neural Stem Cell from Neonatal Rat Hippocampus *in Vitro*

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Key Words

Ketamine • Neural stem cell • Proliferation • Developing brain

Abstract

Background/Aims: Ketamine is a widely used anesthetic in obstetric and pediatric anesthesia. In the developing brain, the widespread neuron apoptosis triggered by ketamine has been demonstrated. However, little is known about its effect on neural stem cells (NSCs) function. This study aimed to investigate the effect of ketamine on proliferation of NSCs from neonatal rat hippocampus. **Methods:** Neural stem cells were isolated from the hippocampus of Sprague-Dawley rats on postnatal day 3. In dose-response experiments, cultured neural stem cells (NSCs) were exposed to different concentrations of ketamine (0-1000 μ M) for 24 hrs. The proliferative activity of NSCs was evaluated by 5-Bromo-2'-deoxyuridine (BrdU) incorporation assay. Apoptosis of neural stem cells were assessed using caspase-3 by western blot. The intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in NSCs was analyzed by flow cytometry. The activation of protein kinase C- α (PKC α) and the phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) were measured by western blot analysis. **Results:** Clinical relevant concentration of ketamine (10, 20 and 50 μ M) did not markedly alter the proliferation of NSCs from neonatal rat hippocampus *in vitro*. However, ketamine (200, 500, 800 and 1000 μ M) significantly inhibited the proliferation of NSCs and did not affect the expression of caspase-3. Meanwhile, ketamine (200, 500, 800 and 1000 μ M) also markedly decreased $[\text{Ca}^{2+}]_i$ as well as suppressed PKC α activation and ERK1/2 phosphorylation in NSCs. A combination of subthreshold concentrations of ketamine (100 μ M) and Ca^{2+} channel blocker verapamil (2.5 μ M), PKC α inhibitor chelerythrine (2.5 μ M) or ERK1/2 kinase inhibitor PD98059 (5 μ M) significantly produced suprathreshold effects on PKC α activation, ERK1/2 phosphorylation and NSC proliferation. **Conclusion:** Ketamine inhibited proliferation of NSCs from neonatal rat hippocampus *in vitro*. Suppressing Ca^{2+} -PKC α -ERK1/2 signaling pathway may be involved in this inhibitory effect of ketamine on NSCs proliferation.

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Introduction

Ketamine is an intravenous anesthetic that is being widely used in obstetric and pediatric anesthesia. The precise mechanisms for general anesthesia of ketamine are not completely elucidated but are considered to be dependent on blocking N-methyl-D-aspartate (NMDA) receptor ion channel. Initially, interest in the effect of NMDA-receptor antagonists on the developing brain was focused on preventing hypoxic/ischemic brain damage [1]. Subsequent studies, however, suggested that, in the developing brain, NMDA-receptor antagonists may also have direct neurotoxic effects. It was reported that blockade of NMDA glutamate receptors for only a few hours during late fetal or early neonatal life triggered widespread apoptotic neurodegeneration in the developing rodent and nonhuman primates brain [2-5].

Besides the acute toxicity characterized as apoptotic neurodegeneration, the long-term adverse effects produced by ketamine exposure in developing brain were also clearly demonstrated both in rodent and primates animals. It was showed that exposure to ketamine during a critical stage of brain development resulted in long-lasting behavioral and cognitive deficits in adulthood [6, 7]. In addition, two retrospective clinical studies [8, 9] suggest that anesthetic exposure early in life is closely associated with learning and behavioral abnormalities later in life.

Although more and more studies revealed that ketamine exposure in developing brain can cause neuronal apoptosis and lead to subsequent neurocognitive dysfunction, little is known about the effect of ketamine on hippocampal neurogenesis. Neurogenesis is critical to normal hippocampal function and even limited suppression of neurogenesis is associated with the development of significant cognitive deficits [10]. The process of hippocampal neurogenesis involves proliferation of neural stem cells (NSCs), differentiation into neurons and glia, migration and functional integration into the hippocampal neural circuit [11, 12]. In this complicated neurogenesis process the proliferation of NSCs was not only initial but also very crucial. This study aims to investigate the effect of ketamine on hippocampal NSCs proliferation and relevant mechanism.

Materials and Methods

Hippocampal NSCs culture

New-born Sprague-Dawley rats were purchased from Laboratory Animal Centre of Xuzhou Medical College. The experimental procedures were approved by XuZhou Medical College Committee on Animal Care. The NSCs from hippocampus of postnatal day 3 (P3) rats were isolated and propagated by a neurosphere method developed by Reynolds and Weiss [13]. The isolated cells were resuspended in free-serum medium of DMEM/F12 (1:1, Gibco, Grand Island, USA) which was supplemented with 2% B27 (Invitrogen, Grand Island, USA) without vitamin A, 20 ng/ml epidermal growth factor (EGF, PeproTech, Rocky Hill, USA), 20 ng/ml basic fibroblast growth factor (bFGF, PeproTech, Rocky Hill, USA), and 100 U/ml penicillin and phytomycin. A half of culture medium was replaced every 3 days. Cells were incubated for 7 days to form enough neurospheres. Then neurospheres were collected, dissociated with accutase (Sigma, Saint Louis, USA) for 10 min and then passaged at a cell density of 2×10^5 cells/mL.

5-Bromo-2'-deoxyuridine (BrdU) incorporation assay

Briefly, the single cell suspensions of NSCs from neurospheres on the sixth day were incubated with the proliferation marker BrdU (10 μ g/L) for 24 h at various concentrations of racemic ketamine (0, 10, 20, 50, 100, 200, 500, 800 and 1000 μ M). After incubation, the dissociated cells were seeded onto 100 μ g/mL poly-L-lysine-coated coverslips. Then they were stained for the NSC marker nestin and BrdU marker [14]. To detect nestin, the primary antibody was rabbit anti-nestin polyclonal antibody and the secondary antibody was Cy3 conjugated goat anti-rabbit IgG. To detect BrdU, the primary antibody was mouse anti-BrdU monoclonal antibody and the second antibody was fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG. Immunoreactive cells were visualized by fluorescence microscopy.

Ca²⁺ concentration measurement

The NSCs from neurospheres on the sixth day were incubated with various concentrations of ketamine for 24 h. Intracellular Ca²⁺ concentration ([Ca²⁺]_i) was determined with the Ca²⁺-sensitive fluorochrome Fluo-3/acetoxymethyl ester (Fluo-3/AM) by a Becton Dickinson FACS Calibur flow cytometer [15]. Cells were washed 2 times with D-Hanks' solution and resuspended to prepare 1x10⁶ cells per ml solution. Cells were incubated with 4 μM Fluo-3/AM (Dojindo, Kumamoto, Japan) at 37 °C for 30 min in the dark. Then the loaded cells were gently rinsed two times with D-Hanks' solution and the fluorescence intensity was analyzed by flow cytometry. The excitation wavelength and emission wavelength were 488nm and 525 nm.

Western blot analysis

The expressions of caspase-3, PKCα (membrane protein) and phosphorylated ERK1/2 were measured by western blot analysis after treatment with various concentrations of ketamine for 24 h. Cells were washed with ice-cold D-hanks' solution three times and then lysed with lysis buffer containing protease and phosphatase inhibitors (KeyGEN, China). The cell lysates were placed on ice for 15 min and centrifuged at 14000 rpm for 15 min at 4°C. Protein concentrations in the resulting lysates were determined with a BCA protein assay kit, bovine serum albumin (BSA) was used as a standard. Equal amounts of the resulting cell lysate was resolved by sodium dodecyl sulfate-polyacrylamide gel and the separated proteins were transferred to nitrocellulose membranes. Blots were incubated with blocking buffer for 1 h at room temperature and then incubated for 16 h at 4°C with primary antibodies raised to caspase-3 (1:1000, cell signaling technology), PKCα (1:200, Santa Cruz), phospho-ERK1/2 (1:1000, Millipore) and β-tubulin (internal standard, 1:10000). After incubation with primary antibody, blots were thoroughly washed and then incubated with alkaline phosphatase-conjugated secondary antibody (1:1000, Beyotime) for 2 h at room temperature. The immunoreactive bands were visualized with a NBT/BCIP color developing reagent.

Statistical analysis

Numerical data are presented as mean ± SD. Statistical analysis were conducted with the aid of SPSS 13.0. Differences were evaluated by one-way ANOVA followed by Dunnett's multiple comparison test. P<0.05 was considered statistically significant.

Results

The effects of ketamine on NSCs proliferation by BrdU incorporation assay

It was found that the percentage of Nestin⁺/BrdU⁺ cells in cultured NSCs was not markedly changed by exposure to 10, 20 and 50 μM ketamine compared to control group (p>0.05, Fig. 1A). However, the percentage of nestin and BrdU double-positive cells was markedly decreased when cultures were exposed to 200, 500, 800 and 1000 μM ketamine (Fig. 1B and C).

The effect of ketamine on caspase-3 expression by western blot analysis

We detected the caspase-3 expression in NSCs exposed to different concentrations of ketamine (100, 200, 500, 800 and 1000 μM) for 24 h. The result showed that ketamine did not significantly affect the expression of caspase-3 (Fig. 2A and B).

Effect of ketamine on [Ca²⁺]_i of NSCs

As shown in Fig. 3A and B, the [Ca²⁺]_i of NSCs was markedly decreased after the cells were exposed to 100, 200, 500, 800 and 1000 μM ketamine for 24 h in DMEM/F12 medium containing calcium ions.

Effects of ketamine on PKCα activation and ERK1/2 phosphorylation

Western blot analysis of the protein lysates from NSCs exposed to ketamine at different concentrations for 24 h revealed that the activity of PKCα was markedly inhibited by 200, 500, 800 and 1000 μM ketamine. Meanwhile, the phosphorylation level of ERK1/2 was also significantly decreased by 200, 500, 800 and 1000 μM ketamine (Fig. 4 and Fig. 5).

Fig. 1. Effect of ketamine on proliferative activity of NSCs by BrdU incorporation assay. NSCs were cultured and treated with various concentrations of ketamine. The DNA synthesis of NSCs were measured after ketamine treatment for 24 hours. The NSCs were labeled by primary antibodies against NSCs marker nestin and proliferation marker BrdU. Immunoreactive cells were visualized by fluorescence microscopy. The percentage of nestin/BrdU double positive cells was calculated. Data are presented as mean $\pm$ SD. ** $P < 0.01$, vs control (0 μ M ketamine) group.

Fig. 2. Effect of ketamine on the expression of caspase-3 in NSCs. NSCs were cultured and treated with various concentrations of ketamine for 24 hours. The caspase-3 expression was detected by western blot analysis. Data are presented as mean $\pm$ SD.

Ca²⁺ is involved in the inhibition of PKCα activation by ketamine

To determine whether Ca²⁺ plays a role in the PKCα activation inhibited by ketamine, we used a Ca²⁺ blocker verapamil to treat NSCs. As shown in Fig. 6A and B, either ketamine (100

Fig. 6. The effect of ketamine and verapamil on PKC- α activation in NSCs. The expression of cell membrane PKC- α was measured by western blot analysis. Subthreshold concentrations of either ketamine (100 μ M) or verapamil (2.5 μ M) did not significantly inhibit PKC α activation. However, supra-threshold effect was produced by the combined administration of 100 μ M ketamine and 2.5 μ M verapamil. The data are presented as mean $\pm$ SD. **P < 0.01, vs control group. ##P < 0.01, vs ketamine group. \$\$P < 0.01, vs verapamil group.

Fig. 7. The effect of ketamine and chelerythrine on ERK1/2 phosphorylation in NSCs. The expression of phosphorylated ERK1/2 was measured by western blot analysis. Subthreshold concentrations of either ketamine (100 μ M) or chelerythrine (2.5 μ M) did not significantly inhibit ERK1/2 phosphorylation. However, supra-threshold effect were produced by the combined administration of 100 μ M ketamine and 2.5 μ M chelerythrine. The data are presented as mean $\pm$ SD. **P < 0.01, vs control group. ##P < 0.01, vs ketamine group. \$\$P < 0.01, vs chelerythrine group.

Fig. 8. The effect of ketamine and PD98059 on NSC proliferation by BrdU incorporation assay. Subthreshold concentrations of either ketamine (100 μ M) or PD98059 (5 μ M) did not significantly inhibit NSC proliferation. However, the supra-threshold effect was produced by the combined administration of 100 μ M ketamine and 5 μ M PD98059. The data are presented as mean $\pm$ SD. **P < 0.01, vs control group. ##P < 0.01, vs ketamine group. \$\$P < 0.01, vs PD98059 group.

μ M) or verapamil (2.5 μ M) did not significantly inhibit PKC α activation. However, when the cells were treated with 100 μ M ketamine combined with 2.5 μ M verapamil, the effect was markedly suppressed.

PKC α is involved in the inhibition of ERK1/2 phosphorylation by ketamine

It was found that either ketamine (100 μ M) or chelerythrine (2.5 μ M) did not significantly inhibit ERK1/2 phosphorylation. However, when the cells were treated with

100 μ M ketamine combined with 2.5 μ M chelerythrine, the phosphorylation of ERK1/2 was markedly decreased (Fig. 7A and B).

ERK1/2 is involved in the inhibition of NSC proliferation by ketamine

Ketamine (100 μ M) and PD98059 (5 μ M) could not significantly inhibit NSC proliferation respectively. However, when the cells were treated with 100 μ M ketamine combined with 5 μ M PD98059, NSC proliferation was markedly inhibited (Fig. 8).

Discussion

Ketamine, a noncompetitive NMDA receptor antagonist, has been reported to induce apoptotic neurodegeneration in the developing brain [16-19]. However, the effect of ketamine on neurogenesis during a critical stage of brain development has not been clarified. Given the importance of neural stem cells (NSCs) proliferation in neurogenesis, we firstly investigated the effect of ketamine on proliferation of hippocampal NSCs from neonatal rats *in vitro* and relevant mechanisms.

NSCs are self-renewal and multipotential cells that can generate differentiated progeny such as neurons and glia. NSCs play an important role in neurogenesis and gliogenesis in the development of the central nervous system. In humans, the brain growth spurt (BGS) proceeds from the third trimester until approximately 2 years after birth. In rats, this period corresponds to the first 21 postnatal days (PNDs) [20]. In this study, to observe NSC proliferation during the period equivalent to human BGS, we investigated the effects of ketamine on hippocampal NSC proliferation *in vitro* using PND3 rats. The process of hippocampal neurogenesis involves proliferation of NSCs, differentiation into neurons and glia, migration and functional integration into the hippocampal circuitry [11, 12]. In this complicated neurogenesis process, the proliferation of hippocampal NSCs was not only initial but also very crucial. In the present study, NSCs formed enough neurospheres. Then immunofluorescence for nestin (a specific protein in NSCs) was performed to identify that neurospheres were constitutive of NSCs. In addition, we detected differentiated neurons and astrocytes from cultured NSCs by immunofluorescence technology using specific antibodies. All the results demonstrated that the cells obtained for experiments were NSCs.

Our findings documented that ketamine (10, 20, 50 and 100 μ M) could not significantly affect the proliferation of NSCs. Our results were different from previous finding by Bai et al which reported that exposure to 100 μ M ketamine for 6 hours could promote the proliferation of NSCs from human embryonic stem cells (hESCs) *in vitro* [21]. This discrepancy may be attributed to the different NSCs source and ketamine exposure duration. Another study by Dong et al reported that ketamine (20, 50 and 100 μ M) could inhibit the proliferation of NSCs derived from the cortex of Sprague-Dawley rat on embryonic day 17 *in vitro* [22]. We consider that the NSCs derived from the cortex of embryonic day 17 rat may have different biological characteristics compared to NSCs isolated from the hippocampus of postnatal day 3 rat, which lead to different reaction to anesthetics. In addition, our results showed the higher concentrations of ketamine (200, 500, 800 and 1000 μ M) could significantly inhibit proliferation of hippocampal NSCs from neonatal rats by BrdU incorporation assay. Meanwhile, these higher concentrations of ketamine did not markedly promote NSCs apoptosis (Fig. 2). It suggested that the decreased percentage of Nestin/BrdU double-positive cells was mainly caused by inhibiting the proliferation of NSCs by ketamine.

The NMDA-R is a tetramer or pentamer consisted of NMDAR-1 subunit (NR1) and several NMDAR-2 subunit (NR2). Previous research had reported that NR1, NR2A and NR2B were expressed in the NSCs [22]. However, the role of NMDA-R in the proliferation of hippocampal NSCs remains controversial [23, 24]. NMDAR is a specific type of ionotropic glutamate receptor, which can allow the passage of Ca^{2+} and Na^{+} into the cell and K^{+} out of the cell, mainly the flux of Ca^{2+} [25]. The Ca^{2+} can in turn function as a second messenger in various signaling pathways. Intracellular calcium signal plays an important role in the

control of cellular proliferation [26, 27]. To clarify the mechanism by which ketamine exerts its inhibitory effect on NSCs proliferation, we observed the effect of ketamine on intracellular Ca^{2+} concentration in the presence of B27 without vitamin A, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). The results showed that ketamine markedly decreased intracellular Ca^{2+} concentration of NSCs from neonatal rat hippocampus (Fig. 3). It indicated that ketamine may exert its suppressive effect on NSCs proliferation through inhibiting intracellular calcium signal.

As a second messenger, Ca^{2+} can activate a series of Ca^{2+} -dependent protein kinase C (PKC). PKC is a family of serine/threonine kinases that plays an important role in modulating a variety of biological responses including the regulation of cell growth. There are three well-characterized MAPK subfamilies in mammalian cell: extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs) and p38 MAPK. The activation of ERK1/2 has been shown to lead to cellular proliferation, whereas JNK and p38 MAPK activation is associated with inflammatory cytokine action, cellular stress and apoptosis [28, 29]. MAPK/ERK1/2 can be activated through PKC-dependent mechanism [30]. To clarify the mechanism by which ketamine exerts its inhibitory effect on NSC proliferation, we observed the effect of ketamine on PKC α and ERK1/2 activation. The results showed that ketamine (200, 500, 800 and 1000 μM) markedly suppressed PKC α activation (Fig. 4) and ERK1/2 phosphorylation (Fig. 5) in NSCs from neonatal rat hippocampus.

To determine whether the inhibitory effect on PKC α activation by ketamine was mediated through interfering Ca^{2+} signaling, the effects of subthreshold concentrations of ketamine (100 μM) and verapamil (2.5 μM) on PKC α activation were studied. The result showed neither 100 μM ketamine nor 2.5 μM verapamil significantly inhibited PKC α activation in NSCs. However, the PKC α activation was markedly suppressed by combined treatment with 100 μM ketamine and 2.5 μM verapamil (Fig. 6), suggesting that ketamine and verapamil should mutually enhance the effect of the other side by the same mechanism and then reach to a suprathreshold effect. Based on the results above, we considered that ketamine may suppress PKC α activation by inhibiting calcium signaling in NSCs.

To determine whether PKC α is involved in the inhibitory effect of ketamine on ERK1/2 phosphorylation, the effects of subthreshold concentrations of ketamine (100 μM) and PKC α inhibitor chelerythrine (2.5 μM) on ERK1/2 phosphorylation were explored. The result showed neither 100 μM ketamine nor 2.5 μM chelerythrine significantly inhibited ERK1/2 phosphorylation in NSCs. However, combined treatment with 100 μM ketamine and 2.5 μM chelerythrine markedly suppressed ERK1/2 phosphorylation (Fig. 7). It suggested that ketamine and chelerythrine may mutually enhance the effect of the other side by the same mechanism and then reach to a suprathreshold effect. Based on the results above, we considered that ketamine may suppress ERK1/2 phosphorylation by inhibiting PKC α activation in NSCs.

It was also showed that neither 100 μM ketamine nor 5 μM PD98059 markedly inhibited NSC proliferation. However, the combined treatment with 100 μM ketamine and 5 μM PD98059 significantly inhibited NSC proliferation (Fig. 8). This result indicated that ketamine and PD98059 at their respective subthreshold concentrations may mutually enhance each other's effects by the same mechanism, resulting in a suprathreshold effect. Based on these results, we considered the possibility that ketamine can suppress NSC proliferation by inhibiting ERK1/2 phosphorylation.

A previous study demonstrated that plasma levels of ketamine varied from 37.8-108.4 μM in patients 1 minute after intravenous ketamine injection at a dose of 2.0-2.2 mg/kg [31]. Thus, a ketamine concentration of within 100 μM is observed *in vivo*. The present study indicated that ketamine (10, 20, 50 and 100 μM) failed to alter the proliferation of NSCs from neonatal rat hippocampus *in vitro*. While ketamine at the concentrations of 200, 500, 800 and 1000 μM significantly inhibited the proliferation of NSCs through attenuating Ca^{2+} -PKC α -ERK1/2 signal *in vitro*. Our results suggested that the direct inhibitory effect of ketamine on NSCs proliferation *in vitro* was evident only at the higher concentrations. While clinical

relevant concentrations of ketamine were not enough to directly alter the proliferation of NSCs *in vitro*. However, the circumstances for NSCs development *in vivo* were very different from those *in vitro* and it is possible that ketamine interferes the proliferation of NSCs by both direct and indirect pathways *in vivo*. Therefore, it is necessary to further investigate the effects of clinical relevant doses of anesthetics on NSC function *in vivo*.

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