Published in final edited form as:

Neuropharmacology. 2018 January ; 128: 63–75. doi:10.1016/j.neuropharm.2017.09.035.

Ketamine Induces Hippocampal Apoptosis through a Mechanism Associated with the Caspase-1 Dependent Pyroptosis

Zhi Ye^{1,2}, Qing Li^{1,3}, Qulian Guo², Yunchuan Xiong², Dong Guo¹, Hong Yang¹, and Yan Shu^{1,*}

¹Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland at Baltimore, Maryland, USA

²Department of Anesthesiology, Xiangya Hospital, Central South University, Changsha, 410078, China

³Institute of Clinical Pharmacology, Central South University, Hunan 410078, China

Abstract

Ketamine, a pediatric anesthetic, is widely used in clinical practice. There was growing evidence showing that ketamine can promote neuronal death in developing brains of both humans and animals. In this study, we used *in vivo* neonatal and juvenile mouse models to induce ketamine-related neurotoxicity in the hippocampus. Active caspase-3 and -9 proteins, which are responsible for the release of cytochrome C, and the mitochondrial translocation of p53, which is associated with mitochondrial apoptosis, were found to be significantly up-regulated in the ketamine-induced hippocampal neurotoxicity. Furthermore, we demonstrated that the levels of pyroptosis-related proteins, including caspase-1 and -11, NOD-like receptor family, pyrin domain containing 3 (NLRP3), and IL-1 β and IL-18, significantly increased after multiple doses of ketamine administration. We speculated that ketamine triggered the formation of NLRP3 and caspase-1 complex and its translocation to the mitochondria. In consistent with this, ketamine treatment was found to induce pyroptosis in mouse primary hippocampal neurons, which was characterized by increased pore formation and elevated lactate dehydrogenase release in mitochondria. Silencing caspase-1 with lentivirus-mediated short hairpin RNA (shRNA) significantly decreased the levels of not only pyroptosis-related proteins but also mitochondrial apoptosis-associated proteins in mouse primary hippocampal neurons. We conclude that caspase-1-dependent pyroptosis is an

*Correspondence to: Yan Shu, M.D., Ph.D., Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland at Baltimore, 20 N Pine Street, Pharmacy Hall N519, Baltimore, Maryland, MD 21201, USA; Phone: +01 410-706-7358, Fax: +01 410-706-0886, yshu@rx.umaryland.edu.

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AUTHOR CONTRIBUTION

Designed Research: Yan Shu, Zhi Ye, Qulian Guo

Performed Research: Zhi Ye, Qing Li, Yunchuan Xiong, Dong Guo, Hong Yang

Analyzed Data: Zhi Ye, Qing Li, Yunchuan Xiong, Dong Guo, Hong Yang

Wrote Manuscript: Zhi Ye, Yan Shu

CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no financial, personal, or any other conflicts of interest that influenced, or could be perceived to have influenced, this work.

important event which may be an essential pathway involved in the mitochondria-associated apoptosis in ketamine-induced hippocampal neurotoxicity.

Keywords

Ketamine; Neurotoxicity; Pyroptosis; Apoptosis; Caspase-1; Hippocampus

Introduction

There is a growing concern that the exposure of neonates to anesthetics, such as ketamine, might trigger brain damage resulting in behavioral changes later in life (Aligny et al., 2014; Ponten et al., 2012; Turner et al., 2012). Ketamine, a non-competitive N-methyl-D-aspartate (NMDA) antagonist, is widely used as an anesthetic in neonatal pediatrics. Even though it has been believed that NMDA antagonists could protect immature brains from excitotoxic injuries (Roux et al., 2015; Yuan et al., 2015), there is increasing evidence suggesting that ketamine have deleterious effects by inducing neuronal apoptotic death and subsequent cognitive dysfunction in adulthood (Cao et al., 2015; Jiang et al., 2014). Therefore, it is necessary to further explore the mechanism and formulate strategies to avoid or at least reduce the possible adverse effects from ketamine use.

Until now, the majority of studies on anesthetic neurotoxicity with neonates have been focused on neuronal apoptosis. The apoptotic or necrotic death of neuronal cells is critical pathogenesis of ketamine-induced neurotoxicity. Recently, a unique type of programmed cell death, pyroptosis, has been reported to be morphologically and mechanistically distinct from apoptosis and necrosis (Bergsbaken et al., 2009; Xu et al., 2014). As for pyroptosis, tissue injuries may lead to the formation and release of certain danger signals. Such danger signals may mediate the release of secondary intracellular danger signals, such as potassium efflux, that trigger the activation of NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome which activates caspase-1. Activation of caspase-1 results in the conversion of pro-IL-1 β and pro-IL-18 into active IL-1 β and IL-18 (Ruhl and Broz, 2015). Active IL-1 β and IL-18 would then bind to their receptors, leading to the activation of multiple cytokines that are involved in the inflammation cascade (Haldar et al., 2015). Caspase-1 dependence is the unique feature of pyroptosis (Karmakar et al., 2015; Tan and Chu, 2013). In addition, caspase-11, as an upstream regulator of caspase-1, has been reported to play a critical role in pyroptosis (Kayagaki et al., 2011; Rathinam et al., 2012). Caspase-11 could activate caspase-1 under the coordination of NLRP3 inflammasome. Whether pyroptosis is involved in anesthetic neurotoxicity has not been studied.

Interestingly, a recent study showed that stimulation of astrocytes with ATP, LPS or ethanol led to the recruitment of caspase-1 and NLRP3 to the mitochondria (Alfonso-Loeches et al., 2014). In this study, co-localization of NLRP3/caspase-1 within mitochondria stimulated the generation of mitochondria ROS (mROS) which induces cell apoptosis. Importantly, mitochondria can be rapidly dismantled in a caspase-1-dependent manner, as manifested by increased mROS production, loss of outer and inner membrane integrity, and fragmentation of the mitochondrial network. Ketamine can cause neuronal apoptosis as well as impaired

electrophysiologic and behavioral function in the hippocampus weeks or months after ketamine exposure (Brown et al., 2015; Gazal et al., 2015; Wu et al., 2014). The exact mechanism of such ketamine-induced hippocampal toxicity remains largely unknown. We speculated the involvement of pyroptosis with co-localization of NLRP3/caspase-1 in the mitochondria being a cross talk between pyroptosis and apoptosis. In the present study, neonatal and juvenile mice and prenatal mouse hippocampal neuron culture were used to test our hypothesis both *in vivo* and *in vitro*.

Materials and Methods

Induction of hippocampal neurotoxicity by ketamine in mice

The induction of hippocampal neurotoxicity by sub-chronic ketamine exposure *in vivo* was done according to previously reported protocols with slight modifications (Cao et al., 2015; Jiang et al., 2014). Ketamine (racemic ketamine HCl) was intraperitoneally administered to C57BL/6 mice (at postnatal 10 days or 30 days) at the dosage of 100 mg/kg/day for seven consecutive days. Saline was used in the control group. The experimental design is shown in Figure 1. All animal studies were approved by the Ethics Committee at Xiangya Hospital, Central South University, and the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland at Baltimore.

Immunohistochemistry and TUNEL staining

Mice were anesthetized with isoflurane. Then the mice were perfused with 200 ml 0.9% NaCl solution and subsequently with 4% paraformaldehyde in 0.1 mol/l phosphate buffer at pH 7.4. Brains were removed and postfixed for 24 h in the same fixative. The postfixed brains were cryoprotected in 25% sucrose in PBS. Then the brains were coronally sectioned on a cryostat (LEICA, CM1850, Germany) at 20 μ m thickness or paraffin embedded and sliced on a microtome at 5 μ m thickness. For the assay of the expression of caspase-1, the free-floating vibratome sections were processed immunohistochemically. Briefly, an ABC kit (Zhongshan Biotechnology Company, Beijing) was used to localize the primary antibody. The diaminobenzidine (DAB) kit (Zhongshan Biotechnology Company, Beijing) was used to visualize the catalyzed peroxidase-reaction product. The primary antibodies were the goat polyclonal antibody against caspase-1 (sc-1218, Santa Cruz Biotechnology) at 1:400 dilution. At the same time, the negative controls were performed, which were incubated without the primary antibodies. For TUNEL staining, sections (n = 8/group) were treated according to the manufacturer's instructions (Roche Molecular Biochemicals Inc., Mannheim, Germany). In brief, after pretreatment with Proteinase K and 0.3% H₂O₂, sections were incubated with terminal deoxynucleotidyl transferase enzyme for 1 h at 37 °C and then with peroxidase-conjugated antibody for 30 min, and 3'-diaminobenzidine (DAB) was used to visualize the apoptotic cells. The photographs were taken under high-power magnification (400 $\times$)

The numbers of caspase-1 and TUNEL positive stained cells were counted in five randomized area of 0.5 mm² in the hippocampal region. They were summed and the results were expressed as positive cells per 1 mm².

Primary mouse hippocampal neuron culture and ketamine treatment

Culture was prepared with hippocampus from wild type C57BL/6 mouse embryos (E17) as described previously (Repetto et al., 2014). Briefly, after dissection, mouse hippocampus tissues were digested with 0.25% trypsin and triturated mechanically. Neurons were seeded onto poly-L-lysine-coated coverslips at the low densities as describe below and placed upside down above a layer of astrocytes containing N2.1 medium. The cells were maintained in Neurobasal (Invitrogen) consisting of B27 supplement and antibiotics, and 2 mmol/L glutamine. One hundred cells per mm² were plated for transfection procedures and 50 cells per mm² for immunohistochemical staining. Previously, neurotoxicity could be induced by acute ketamine exposure *in vivo* with a single intraperitoneal (i.p.) injection or multiple (four or six) consecutive i.p. injections of ketamine at 1-h intervals (Huang et al., 2016; Huang et al., 2015). For our *in vitro* studies here, primary hippocampal cells were acutely exposed to ketamine treatment (0, 50, 100, 200 and 500 µg/ml in medium, respectively) to induce cytotoxicity within the timeframe of viable primary hippocampal cell culture. After 4 cycles of ketamine incubation (30 min ketamine exposure with 30 minute interval between cycles), the ketamine-containing culture medium was replaced with the regular medium. After 20 hours, the cell viability was measured with the MTT assays as described below. As indicated in our results, this treatment regimen could dose-dependently lead to cytotoxicity in primary hippocampal neurons.

Cell viability by MTT assay

MTT assay was performed as previously reported (Tan et al., 2012). The cells from the steps described above were placed in 100 µl of culture medium containing 0.5 mg/ml thiazolyl blue tetrazolium bromide (MTT) and incubated at 37°C for 4 hours. Then the medium was aspirated off carefully without disturbing the crystal and 100 µl DMSO were added to each well. After 15 min incubation, optical density (OD) at wavelength of 550 nm was determined using an automatic microtiter plate reader (Epson LX-800, Molecular Devices).

Lentivirus-mediated short hairpin RNA (shRNA) knockdown of gene expression

The GV248-lentiviral expression vectors containing the caspase-1 shRNA sequence and a negative control sequence were purchased from Genchem Biotechnology Company (Shanghai, China), as previously reported (Zhang et al., 2015). The GV248 vector contains an eGFP reporter and expresses a puromycin-resistant gene. Lentiviral shRNA was produced by co-transfecting the TransLentiviral Packaging Mix with a recombinant lentivirus vector into HEK 293T cells. Supernatants containing the lentivirus expressing the caspase-1 shRNA or the negative control sequence were harvested 72 hours after transfection. The lentiviruses were purified using ultracentrifugation, and the titer of lentiviruses was determined. The primary hippocampal neurons were seeded in 6-well plates and then incubated with lentivirus for at least 3 days in the presence of Polybrene (10 µg/ml). Cells that were successfully transfected were selected by adding a fresh medium that contained 2 µg/ml puromycin (Sigma) and further incubated for 24 hours. Knockdown of caspase-1 protein expression was confirmed by Western blotting using rabbit anti-caspase-1 polyclonal antibody. The cells stably transfected with lentiviral shRNA were referred to as caspase-1 shRNA cells or control shRNA cells.

Isolation of mitochondria

The cytosolic and mitochondrial fractions of the harvested brain tissues (hippocampus) and primary hippocampal neurons were prepared by using Mitochondria Isolation Kit for Cultured Cells according to the manufacturer's instructions (ThermoFisher Scientific, USA). The efficient isolation of mitochondria was demonstrated by the lack of cytoplasmic calnexin in Western blotting analysis (data not shown).

Pore formation test

Pore formation was analyzed by ethidium bromide staining as previously reported (Kirby et al., 1998). Primary hippocampal neurons were seeded into 24-well plates at a density of 5×10^5 cells/well before they were stained with PBS containing 25 $\mu\text{g/ml}$ ethidium bromide and 5 $\mu\text{g/ml}$ acridine orange. All cells could be stained with acridine orange, whereas only cells with membrane pores would allow ethidium bromide to diffuse into the cell. Pore-forming activity was measured and expressed as the percentage of primary hippocampal neurons with positive ethidium bromide staining. Images were acquired using a Leica microscope (10 $\times$ 10) (DMI4000B, Leica Microsystems, Wetzlar, Germany) and were analyzed using ImageJ software.

Lactate dehydrogenase release assay

As previously reported (Kovarova et al., 2012), lactate dehydrogenase (LDH) release was measured by a colorimetric assay following the manufacturer's instructions (Takara Bio USA, Mountain View, CA). Results were compared with the total LDH released from cells after treated with 1% Triton X-100 (100% lysis control).

Western blot analysis

After protein concentrations were determined using bicinchoninic acid, equal amounts of protein lysates were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes followed by blockade with 3% BSA. Subsequently, membranes were incubated with anti-caspase-3 and -9 (1:500, #9662 and #9506; Cell Signaling Technology, Beverly, MA, USA), anti-Cytochrome C and anti-p53 (1:400, #4280 and #32532; Cell Signaling Technology, Beverly, MA, USA), anti-caspase-11 (1:1000, sc-28230; Santa Cruz Biotechnology, CA, USA), anti-caspase-1 (1:1,000, sc-1218, Santa Cruz Biotechnology, CA, USA), anti-NLRP3 (1:1000, ab214185, Abcam, Cambridge, MA, USA), or anti- β -actin (1:1000, #4970, Cell Signaling Technology, Beverly, MA, USA), anti-GAPDH (1:5000, sc-20358, Santa Cruz Biotechnology, CA, USA), and anti-VDAC (#4866, 1:5,000, Cell Signaling Technology, Beverly, MA, USA) at 4°C overnight. After membranes were washed, bound antibodies on membranes were immersed in horseradish peroxidase-conjugated secondary antibody (1:1,000) at room temperature for 1 h and visualized using enhanced chemiluminescence. Relative level of each protein to control protein was quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Immunoprecipitation

For immunoprecipitation, tissue and cell homogenates (each containing 400 μg of proteins) were diluted with 4-fold HEPES buffer (pH 7.4) containing 50 mM HEPES, 150 mM NaCl,

10% glycerol, 1% Triton X-100, and 1 mM each of EGTA, EDTA, PMSF and Na_3VO_4 . The mixture was pre-incubated for 1 h with 20 μL protein A/G before all proteins adhered nonspecifically to the protein A/G were removed through centrifugation. The supernatant was incubated further with 2–5 μg proper antibody or species-relevant non-specific IgG (n.s. IgG) for 4 h at 4 °C. After the addition of protein A/G-sepharose, the mixture was incubated at 4 °C for an additional 2 h, before the samples were washed three times with HEPES buffer and eluted by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer after 5-minute boiling. Western blot analysis was carried out on 4–20% SDS-PAGE and 50–100 μg proteins were electrotransferred onto nitrocellulose filter (pore size, 0.45 μm). After blocked for 2 h in phosphate-buffered saline (PBS) with 0.1% Tween 20 (PBST) and 3% BSA, the membranes were incubated overnight with primary antibody (1:500, anti-caspase-1, sc-1218, Santa Cruz Biotechnology, CA, USA; anti-NLRP3, ab214185, Abcam, Cambridge, MA, USA) in PBST containing 3% BSA. Detection was carried out using proper alkaline phosphatase conjugated IgG (1:20,000) and NBT/BCIP assay kit (Promega). After the immunoblotting, the band densities were measured using an image analyzer (LabWorks Software, UVP Upland, CA, USA)

ELISA

Concentrations of IL-1 β and IL-18 in hippocampal tissues and cell supernatants were measured by ELISA using a mouse IL-1 β (eBioscience) and IL-18 (MBL International) Mouse SimpleStep ELISA™ Kit according to the manufacturer's instructions.

Statistical analysis

Data were expressed as mean $\pm$ SD. Data were analyzed with analysis of variance (ANOVA) followed by Student-Newman-Keuls or Dunnet's test (SPSS, Inc., Cary, NC, USA). $P < 0.05$ was considered statistically significant.

Results

Mitochondrial apoptosis-related proteins were up-regulated in hippocampus with multiple doses of ketamine administration

Mitochondrial apoptosis has been characterized as important hippocampal neurotoxicity induced by constant ketamine exposure (Cao et al., 2015; Jiang et al., 2014). Therefore, firstly, we sought to confirm whether our regimen of ketamine treatment would induce hippocampal neuroapoptosis. Western blot analysis revealed that the levels of active caspase-3 and -9 in ketamine-treated mice were significantly higher than those in vehicle-treated mice after ketamine exposure for 7 consecutive days in both neonatal and juvenile mice ($P < 0.05$; Fig. 2A). Furthermore, to determine the release of cytochrome C and the mitochondrial translocation of p53, we measured the ratio of the amount of cytochrome C in the cytoplasmic fraction to the total cytochrome C and the ratio of p53 in the mitochondrial fraction to the total p53. In the ketamine-treated group, $41.4\% \pm 4.45\%$ and $38.9 \pm 3.61\%$ of cytochrome C were released in neonatal and juvenile mice, respectively, whereas only $8.82\% \pm 1.35\%$ and $7.65 \pm 1.24\%$ of cytochrome C were released in the vehicle groups ($P < 0.05$; Fig. 2B). Mitochondrial translocation of p53 was presented as the ratio of p53 in the mitochondrial fraction to that in both cytoplasmic and mitochondrial fractions together. In

the ketamine-treated group, mitochondrial translocation of p53 was $56.7\% \pm 6.57\%$ in neonatal mice and $54.0 \pm 4.87\%$ in juvenile mice, whereas in the vehicle groups, mitochondrial translocation of p53 was $7.92\% \pm 1.35\%$ and $8.50\% \pm 1.14\%$ respectively ($P < 0.05$; Fig. 2C).

Multiple doses of ketamine administration induced hippocampal pyroptosis and inflammation

Pyroptosis is a unique type of programmed cell death and is distinct from apoptosis and necrosis. In the present study, we revealed that the expressions of pro-caspase-1, caspase-1, procaspase-11, caspase-11, NLRP3, IL-1 β and IL-18 were significantly elevated in mouse hippocampus after multiple doses of ketamine administration (Fig. 3A and B). These changes are molecular characteristics of pyroptosis and inflammation.

To further confirm the induction of pyroptosis and apoptosis by the current treatment regimen of ketamine in mouse hippocampal, we also detected the numbers of caspase-1 and TUNEL positive cells, respectively. As indicated in Fig. 3C and D, remarkable immunoreactivity of caspase-1 and numerous TUNEL positive cells were observed in the hippocampal region of ketamine-treated group as compared with saline group both in neonatal and juvenile mice ($P < 0.05$).

Ketamine administration induced recruitment of caspase-1 and NLRP3 to mitochondria and the formation of NLRP3-caspase-1 complex in mitochondria

Previous studies have shown that NLRP3 is located at the cytoplasm (Bergsbaken et al., 2009; Ruhl and Broz, 2015). We noticed that ketamine treatment in the mice led to the recruitment of caspase-1 and NLRP3 to the mitochondria isolated from hippocampus, as demonstrated by the increased protein expression of both caspase-1 and NLRP3 ($P < 0.05$; Fig. 4A). Since caspase-1 is an NLRP3-binding protein that has been previously reported to facilitate pyroptosis, there might be a corresponding increase of NLRP3-caspase-1 complex formation in the hippocampus following ketamine administration. To test this, the proteins prepared from hippocampal mitochondria were immunoprecipitated with the antibody against NLRP3 then immunoblotted with antibodies against caspase-1. We found that ketamine administration induced sustained increases in the interactions between NLRP3 and caspase-1, as shown in Fig. 4B.

Dose-dependent effects of ketamine on the viability of primary hippocampal neurons

Primary hippocampal neurons were exposed to ketamine (50 to 500 $\mu\text{g/ml}$) for 4 cycles (each cycle with 30 min exposure and 30 min interval). Twenty hours after the last cycle, a significant decrease in cell viability of primary hippocampal neurons was observed in the groups treated with ketamine at 50 $\mu\text{g/ml}$ or higher ($P < 0.05$ as compared with the control). 500 $\mu\text{g/ml}$ ketamine decreased the cell viability up to $44 \pm 5.3\%$ as compared with the control ($P < 0.05$, Fig. 5). Thus, 500 $\mu\text{g/ml}$ ketamine was chosen for the following experiments.

Ketamine induced pyroptosis in mouse primary hippocampal neurons

As shown in Fig. 6A and B, pro-caspase-1, caspase-1, pro-caspase-11, caspase-11, NLRP3, IL-1 β , and IL-18 all had low expression in the control cells. However, their expression was significantly up-regulated after ketamine administration ($P < 0.05$). Since pyroptosis is characterized by the formation of pores in the plasma membrane of cells, pyroptosis can be measured by the pore formation assay or LDH release assay. Significant increases in pore formation and LDH level were observed in primary hippocampal neurons after ketamine exposure ($P < 0.05$, Fig. 6C).

Pyroptosis induced by ketamine exposure depended on caspase-1 activation

The relationship between caspase-1 and pyroptosis after ketamine exposure was investigated using primary mouse hippocampal neurons. A caspase-1 shRNA was used to knockdown the expression of pro-caspase-1 and the active caspase-1 as well (Fig. 7A and B). Silence of caspase-1 expression abolished the ketamine-induced up-regulation of pro-caspase-1, caspase-1, and IL-1 β and IL-18, but only partially decreased the expression of pro-caspase-11, caspase-11 and NLRP3 in primary hippocampal neurons (Fig. 7A, B and C). Furthermore, elimination of caspase-1 also reduced the pyroptotic response in primary hippocampal neurons, as indicated by the decreased pore formation and LDH release (Fig. 7D and 7E). These results revealed that ketamine might induce pyroptosis in primary hippocampal neurons through a caspase-1 dependent pathway.

Knockdown of caspase-1 by specific lentivirus shRNA mitigated ketamine-induced apoptosis in primary hippocampal neurons

To further validate the significance of caspase-1 in the ketamine-induced pyroptosis, we investigated the effects of caspase-1 silencing on mitochondria-mediated apoptosis in primary mouse hippocampal neurons. We found that knockdown of caspase-1 had a significant impact on the levels of active caspase-3 and -9, the release of cytochrome C, and the mitochondrial p53 translocation that were induced by ketamine treatment ($P < 0.05$; Fig. 8A and 8B). In contrast, caspase-1 silencing has little effects on the levels of these proteins in the primary hippocampal neurons received control treatment (Fig. 8). Western blotting and immunoprecipitation analysis indicated that the transduction with caspase-1-specific lentivirus shRNA not only reduced the translocation of caspase-1 and NLRP3 to mitochondria, but also inhibited the formation of NLRP3 and caspase-1 complex in the mitochondria in primary hippocampal neurons (Fig. 9A and 9B). These results demonstrated that ketamine-induced neuronal cell apoptosis was mediated by the caspase-1 dependent pyroptotic pathway.

Discussion

In mammals, the brain growth spurt covers the first 3–4 weeks of life, reaching its peak around 10 days after birth (Dobbing and Sands, 1979). The developing brains are highly susceptible to anesthetics when synaptogenesis is highly active during the brain growth spurt. Ketamine is widely used as an anesthetic, analgesic, and sedative in pediatric patients and acts primarily through the N-methyl-D-aspartate (NMDA) receptors. However, more and more studies have shown that repeated use of ketamine could trigger hydroxyl radical

generations, oxidative stresses and neuronal apoptotic deaths by directly blockading NMDA receptors in both neonates and adults (Aligny et al., 2014; Turner et al., 2012; Zou et al., 2009). Recently apoptosis and necrosis have been demonstrated as the major pathways responsible for ketamine-induced neurotoxicity and neuroapoptosis in developing brains (Cao et al., 2015; Jiang et al., 2014; Zou et al., 2009). Furthermore, *in vitro* cellular and *in vivo* animal models revealed that repetitive- or high-dose administration of ketamine suppressed neural excitability and induced apoptosis in hippocampus, the major component of brain that is associated with memory and learning (Brown et al., 2015; Cao et al., 2015; Huang et al., 2013; Jiang et al., 2014). However, the underlying mechanism through which ketamine mediates hippocampal neurotoxicity remains to be fully characterized. In the present study, neonatal (10 days post-birth) and juvenile (30 days post-birth) mouse models were chosen to elucidate the pathways involved in ketamine-mediated neurotoxicity in hippocampus.

Ketamine-induced developmental neurotoxicity has been previously characterized by neuronal apoptosis (Brown et al., 2015; Cao et al., 2015; Huang et al., 2013; Jiang et al., 2014). In the present study, we adopted a similar approach and successfully induced hippocampal apoptosis in developing mouse brains. Activated (cleaved) caspase-3 and -9 are highly specific and sensitive biomarkers for apoptosis detection. The release of mitochondrial cytochrome C to the cytosol is considered as a critical step for apoptosis. Cytoplasmic leakage of cytochrome C from the mitochondrial intermembrane space quickly activates caspase-9 which cleaves caspase-3, thus facilitating the activation of caspase-dependent cell death pathway (Ray et al., 2012). Moreover, p53 may contribute to apoptosis by direct signaling at mitochondria and mitochondrial p53 has been suggested to sufficiently trigger apoptosis through a transcription-independent pathway (Endo et al., 2006a; Endo et al., 2006b). Therefore, in the present study, several molecular events, including caspase-3 and -9 activation (cleave), cytochrome C(Cyt C) release, and p53 mitochondrial translocation were used to determine the effects of ketamine on apoptotic death both *in vitro* and *in vivo*. Our *in vivo* animal studies indicated that the levels of caspase-3 and -9, the release of cytochrome C and the mitochondrial p53 translocation in the hippocampus significantly increased in the mice treated with ketamine (7 consecutive days of ketamine exposure in both neonatal and juvenile mice) as compared with control mice. Consistent with our *in vivo* results, ketamine exposure also increased the expression of mitochondria-associated pro-apoptotic proteins in primary hippocampal neurons.

It has been reported that constant exposure to high concentrations of ketamine could trigger cell death and inflammation (Lin et al., 2015; Sun and Pei, 2012). Pyroptosis is a novel and unique type of pro-inflammatory form of cell death, distinct from apoptosis and necrosis. Pyroptosis has unique features such as caspase-1 dependence, rapid plasma membrane rupture and release of pro-inflammatory intracellular contents. Several studies have indicated that pyroptosis may not only lead to cell death but also play an important role in the cascade of reactions resulting in tissue damages (Bergsbaken et al., 2009; Ruhl and Broz, 2015; Xu et al., 2014). Nevertheless, whether pyroptosis is involved in ketamine-induced hippocampal neurotoxicity and how pyroptosis, inflammatory cytokines, and apoptosis are related remains unknown.

Caspase-1 is an important regulatory molecule in the induction of pyroptosis (Cerqueira et al., 2015). NLRP3 contains a pyrin (PYD) domain that interacts with the adapter protein ASC (apoptosis-associated speck-like protein containing a CARD). ASC has a caspase activation and recruitment domain (CARD) that binds to and activates caspase-1 (Bergsbaken et al., 2009; Ruhl and Broz, 2015). Once activated, caspase-1 can lead to the cell death referred to as pyroptosis. Recent studies have shown that inflammatory factors, such as IL-1 β and IL-18, could be activated by caspase-1 cleavage in the presence of NLRP3 inflammasomes to induce caspase-1-dependent pyroptosis (Bergsbaken et al., 2011; Miao et al., 2010). In addition to caspase-1, caspase-11 is another key molecule that can induce pyroptosis once stimulated with various factors. CHOP-caspase-11 has been reported to mediate caspase-1-dependent pyroptosis and the related inflammatory response to renal ischemic reperfusion injuries (Yang et al., 2014). Our *in vivo* study has shown that constant exposure to ketamine could trigger pyroptosis that is characterized by increased expression of both pro-caspases-1 and pro-caspases-11, their processed forms active caspase-1 and caspase-11, NLRP3 and the generation of mature IL-1 β and IL-18. Consistent results were also found in our *in vitro* study in primary hippocampal neurons with increased pore formation and LDH release. NLRP3 inflammasome contains NLRP3, ASC, and the associated pro-caspase-1. It is likely that ketamine treatment could induce the activation and assembly of NLRP3 inflammasome and subsequently result in activation of caspase-1 and later caspase-11 *via* autocatalytic processes. This is consistent with our observation that the magnitude of increase in protein levels by ketamine treatment is similar between pro and active forms for both caspase-1 and caspase-11. However, silence of CHOP, which is a key transcription factor in ER stress, has been reported to markedly suppress both mRNA and protein expression of caspase-11 as well as pyroptosis in renal tubule epithelial cells upon ischemic injury (Yang et al., 2014). Whereas the expression increases in the pro-forms of caspase-1 and caspase-11 certainly contributes to those increases in their active forms in this study, it remains unclear that how important the catalytic cleavage is in the process of these active forms. Overall, our findings have demonstrated that pyroptosis and inflammatory responses are closely associated with the development and progression of ketamine-induced hippocampal neurotoxicity.

Interestingly, pyroptosis shares certain features with apoptosis, such as DNA fragmentation and nuclear condensation (Lamkanfi and Dixit, 2010). Recent studies have suggested that mitochondrial damage could be triggered by NLRP3 inflammasomes (Yu et al., 2014). However, how pyroptotic inflammasomes mediate mitochondrial cell apoptosis is an important yet unanswered question. It is likely that activated caspase-1 (p10 subunit) could migrate to mitochondria, which would allow caspase-1 to directly cleave certain mitochondrial proteins and trigger mitochondrial apoptosis (Puri et al., 2012; Yu et al., 2014). Moreover, a recent study has showed that ethanol, ATP or LPS promotes NLRP3/caspase-1 complex recruitment within the mitochondria to accelerate cell death (Alfonso-Loeches et al., 2014). Therefore, we speculated that NLRP3/caspase-1 complex recruitment in mitochondria might regulate mitochondrion-mediated apoptosis in ketamine-induced hippocampal neurotoxicity. In this study, we demonstrated that ketamine exposure could induce the translocation of NLRP3 and caspase-1 to mitochondria and form the NLRP3-caspase-1 complex in the hippocampus of mouse brain and in primary hippocampal cells.

Furthermore, we showed that knockdown of caspase-1 significantly alleviated the increased expression of those critical proteins related to both pyroptosis and apoptosis in our *in vitro* cell model. Here, for the first time, our data have indicated that pyroptosis plays an important role in the development and progression of ketamine-induced neurotoxicity. Specifically, the formation of NLRP3-caspase-1 complex and its mitochondrial translocation may be a molecular mechanism for the ketamine-induced hippocampal apoptosis. However, how the inflammation pathway is initiated by ketamine treatment remains to be an unanswered fundamental question. Ketamine is a NMDA receptor blocker. Interestingly, it has been recently reported that activation of NMDA receptor downregulates inflammasome activity and liver inflammation *via* a β -arrestin-2 pathway (Farooq et al., 2014). Further studies may be directed to investigate whether the block of NMDA receptors by ketamine would upregulate inflammasome activity and trigger pyroptosis in hippocampal neurons *via* the β -arrestin-2 pathway.

In conclusion, our findings, for the first time, showed that caspase-1 dependent pyroptosis was an important biological event in ketamine-induced hippocampal neurotoxicity both *in vivo* and *in vitro*. NLRP3/caspase-1 complex recruitment to mitochondria, serving as a pivotal cross talk between pyroptosis and apoptosis, might be a novel molecular mechanism for ketamine-induced apoptosis. Further uncovering the cross talk of these complex pathways will expand our understanding of the underlying mechanism of anesthetic-induced neurotoxicity.

Acknowledgments

The present study was partly supported by the National Institute of General Medical Sciences of the National Institutes of Health (NIH) under Award R01GM099742 (Y.S.). Dr. Zhi Ye received funding support from Xiangya Hospital, Central South University to conduct research in the US.

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- Ketamine, a widely used anesthetic, can cause neuronal death in developing brains
- Apoptosis has been a focus in studying ketamine-induced neurotoxicity
- We demonstrated a role of pyroptosis in ketamine-induced hippocampal neurotoxicity
- Hippocampal apoptosis by ketamine was *via* pyroptotic NLRP3-caspase-1 complex
- Cross talk between pyroptosis and apoptosis may lead to anesthetic neurotoxicity

Fig. 1. Protocol of ketamine administration

C57BL/6 mice, 10 days and 30 days after birth respectively, were intraperitoneally administered with ketamine (100 mg/kg/day) for 7 consecutive days. Normal saline was used for the control group. Mice were euthanized on the 8th day.

B

C

Fig. 2. Mitochondrial pro-apoptosis proteins in hippocampal tissues were induced by ketamine administration in mice

A. Western blots (top panel) and relative optical densities (bottom panel) for active caspase-3 and -9 in hippocampal tissues from mice after ketamine (KTM) or saline (S) administration were determined by Western blot assays. β -actin was the loading control. **B.** Western blots (top panel) and relative optical densities (bottom panel) for Cytochrome C (Cyt C) in cytosol and mitochondria of hippocampal tissues, showing the translocation or release of Cytochrome C induced by ketamine administrations. **C.** Western blots (top panel) and relative optical densities (bottom panel) for p53 in cytosol and mitochondria of hippocampal tissues, showing the translocation of p53 to mitochondria that was induced by

ketamine administrations. B & C, Voltage-dependent anion channel (VDAC) was the loading control. A–C: KTM, ketamine; S, saline. Data were expressed as mean $\pm$ S.D. $n=8$ mice for each group. $\times P < 0.05$ compared with saline group.

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A

B

Fig. 3. Multiple ketamine administrations in mice induced hippocampal pyroptosis and inflammation

A. Western blots (left panel) and relative optical densities (right two panels) for pro-caspase-1, caspase-1, pro-caspase-11, caspase-11 and NLRP3 in hippocampal tissues, showing pyroptosis induced by ketamine (KTM) administrations as compared to saline (S) treatment. β -actin was the loading control. **B.** Levels of IL-1 β and IL-18, which were determined by ELISA, in hippocampal tissues. **C and D.** Repeated administration of ketamine induced neuronal pyroptosis (C) and apoptosis (D) in hippocampal region. *Arrow* showing the caspase-1 (C) and TUNEL staining (D) positive cells in hippocampus. Original magnification, $\times 400$. Scale bar = 100 μ m. A-D: KTM, ketamine; S, saline. Data were expressed as mean $\pm$ S.D. $n = 8$ mice in each group. * $P < 0.05$ compared with the saline group.

A

B

Fig. 4. Multiple ketamine administrations induced caspase-1 and NLRP3 recruitment to mitochondria and NLRP3-caspase-1 complex formation in mitochondria in mouse hippocampal tissues

A. Western blots (left panel) and relative optical densities (right panels) for active caspase-1 and NLRP3 in the isolated mitochondria. **B.** NLRP3-caspase-1 complex formation in the isolated mitochondria. The protein lysate was co-immunoprecipitated (IP) with the NLRP3 antibody (Ab), and the immune complexes were detected by Western blotting with caspase-1 Ab (WB) (top panel). The relative levels of caspase-1 determined in WB were quantitated by optical density analysis (bottom panel). A & B: KTM, ketamine; S, saline. Data were expressed as mean $\pm$ S.D. $n = 8$ rats for each group. * $P < 0.05$ compared with saline group.

Fig. 5. Dose-dependent effects of ketamine exposure on cell viability in mouse primary hippocampal neurons

Mouse primary hippocampal neurons were exposed to ketamine (0 to 500 µg/ml) for 4 cycles (each cycle with 30 min exposure and 30 min interval). The cell viability was determined 20 hours after the last ketamine treatment cycle. Data were expressed as mean $\pm$ S.D. (n = 3) for each group of cells with three separate repeats. * P < 0.05 compared with control (no ketamine treatment, 0 µg/ml) cells.

A

B

Fig. 6. Ketamine treatment induced pyroptosis in mouse primary hippocampal neurons

A. Western blots (left panel) and quantitative analysis (right panel) of relative levels for the proteins associated with pyroptosis. **B.** Effect of ketamine (500 µg/ml) treatment on IL-1 β and IL-18 levels, which were determined by ELISA, in the supernatants of cellular lysates. **C.** Effect of ketamine (500 µg/ml) on pore formation and lactate dehydrogenase (LDH) release. A–C: KTM, ketamine; control, no ketamine treatment. Data were expressed as mean $\pm$ S.D. (n = 3) for each group of cells with three separate repeats. * P < 0.05 compared with control cells.

C

A

B

D

Fig. 7. Knockdown of caspase-1 ameliorated ketamine-induced pyroptosis in mouse primary hippocampal neurons

A, B. Western blots (A) and quantitative analysis of the levels (B) for the proteins associated with pyroptosis including: pro-caspase-1, caspase-1, pro-caspase-11, caspase-11 and NLRP3. β -actin was the loading control. **C.** Levels of IL-1 β and IL-18, which were determined by ELISA, in the supernatants of cellular lysates. **D.** Effect of caspase-1 knockdown on ketamine-induced pore formation. Quantitative analysis (left panel) and representative photographs (right panel) for pore formation. The green fluorescence indicates the normal neurons, while orange fluorescence indicates the pore-forming cells (e.g., red arrows). **E.** Effect of caspase-1 knockdown on ketamine-induced lactate dehydrogenase (LDH) release. A-E: KTM, ketamine (500 μ g/ml); control, no ketamine treatment. Data were expressed as mean $\pm$ S.D. (n = 3) for each group of cells with three separate repeats. * $P < 0.05$ compared with control treatment, # $P < 0.05$ between caspase-1 shRNA and control shRNA with ketamine treatment.

A

B

Fig. 8. Knockdown of caspase-1 ameliorated ketamine-induced apoptosis in mouse primary hippocampal neurons

A. Western blots of activated caspase-3 and 9, the release of Cytochrome C, and the mitochondrial translocation of p53 in ketamine-treated and control cells. **B.** Relative optical

densities of the proteins determined in A for groups of mice. A & B: KTM, ketamine (500 $\mu\text{g/ml}$); control, no ketamine treatment. Data were expressed as mean $\pm$ S.D. ($n = 3$) for each group of cells with three separate repeats. $\ast P < 0.05$ compared with control treatment; $\# P < 0.05$ between caspase-1 shRNA and control shRNA with ketamine treatment.

A

B

Fig. 9. Knockdown of caspase-1 not only reduced mitochondrial translocations of caspase-1 and NLRP3, but also inhibited the formation of NLRP3 and caspase-1 complex in mitochondria in mouse primary hippocampal cells

A. Western blots (top panel) and relative optical densities (bottom panel) for active caspase-1 and NLRP3 in the isolated mitochondria. **B.** NLRP3-caspase-1 complex formation in the isolated mitochondria. The protein lysate was co-immunoprecipitated (IP) with the NLRP3 antibody (Ab), and the immune complexes were detected by Western blotting with caspase-1 Ab (WB) (top panel). The relative levels of caspase-1 determined in WB were quantitated by optical density analysis (bottom panel). A & B: KTM, ketamine (500 µg/ml); control, no ketamine treatment. Data were expressed as mean $\pm$ S.D. ($n = 3$) for each group of cells with three separate repeats. * $P < 0.05$ compared with control treatment, # $P < 0.05$ between caspase-1 shRNA and control shRNA with ketamine treatment.