RESEARCH ARTICLE

Neonatal ketamine exposure-induced hippocampal neuroapoptosis in the developing brain impairs adult spatial learning ability

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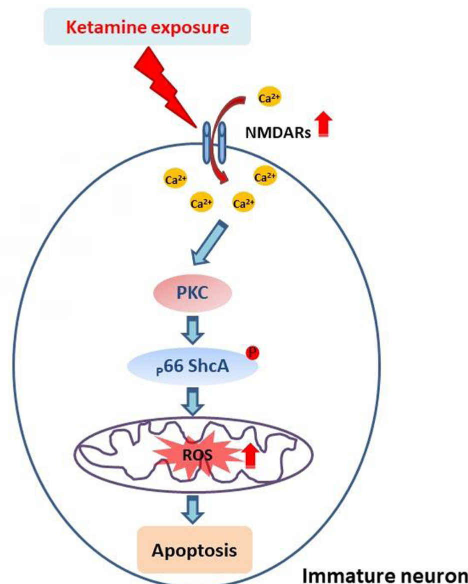
Graphical Abstract

Activation of p^{66}ShcA is involved in the neuroapoptotic cascade, which is induced by repeated ketamine exposure



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Abstract

Ketamine exposure can lead to selective neuroapoptosis in the developing brain. p^{66}ShcA, the cellular adapter protein expressed selectively in immature neurons, is a known pro-apoptotic molecule that triggers neuroapoptosis when activated. Sprague-Dawley rats at postnatal day 7 were subcutaneously injected in the neck with ketamine 20 mg/kg, six times at 2-hour intervals. At 0, 1, 3, and 6 hours after final injection, western blot assay was used to detect the expression of cleaved caspase-3, p^{66}ShcA, and phosphorylated p^{66}ShcA. We found that the expression of activated p^{66}ShcA and caspase-3 increased after ketamine exposure and peaked at 3 hours. The same procedure was performed on a different group of rats. At the age of 4 weeks, spatial learning and memory abilities were tested with the Morris water maze. Latency to find the hidden platform for these rats was longer than it was for control rats, although the residence time in the target quadrant was similar. These findings indicate that ketamine exposure resulted in p^{66}ShcA being activated in the course of an apoptotic cascade during the neonatal period. This may have contributed to the deficit in spatial learning and memory that persisted into adulthood. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas at Arlington, USA (approval No. A13.008) on January 22, 2013.

Key Words: *caspase-3; developmental neuroapoptosis; hippocampus; ketamine; Morris water maze; N-methyl-D-aspartate acid receptors; p^{66}ShcA; spatial learning*

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Introduction

Ketamine, a noncompetitive antagonist acting on glutamatergic N-methyl-D-aspartate acid receptors (NMDARs), is widely used in many pediatric procedures due to its potent analgesic and anesthetic properties, rapidly acting onset, short functional duration, and safe respiratory and hemodynamic profiles (Kohrs and Durieux, 1998; Hall and Shbarou, 2009; Durrmeyer et al., 2010; Collo and Merlo Pich, 2018). However, for more than a decade, numerous experimental studies confirm that ketamine selectively induces neuroapoptotic cell death in developing rodents and nonhuman primates, but not in the adult (Hayashi et al., 2002; Scallet et al., 2004; Slikker et al., 2007; Zou et al., 2009a; Soriano et al., 2010; Liu et al., 2011; Chen et al., 2012; Huang et al., 2012; Li et al., 2018a), and even a single dose—as might be used in clinics—induces extended neuronal apoptosis in certain encephalic regions of neonatal mice (Rudin et al., 2005). Studies by our group and others have demonstrated a higher sensitivity of NMDARs to ketamine block in immature neurons than in mature neurons, which could be the mechanism underlying the higher incidence of neuroapoptosis seen in neonatal brain neurons (Brady and Swann, 1986; Scallet et al., 2004; Slikker et al., 2007; Soriano et al., 2010; Huang et al., 2012; Jin et al., 2013; Felix et al., 2017a; Kokane et al., 2017; Obradovic et al., 2018). Because a robust increase in cell death occurs during ketamine withdrawal, scientists have proposed that the apoptosis is triggered by compensatory up-regulation of NMDAR subunit 1 (Wang et al., 2005, 2006; Suzuki et al., 2017; Nagels et al., 2018). Crucially, neonatal neuroapoptosis results in long-lasting cognitive impairment that can be seen in adulthood (Johnson et al., 1998; Paule et al., 2011; Buratovic et al., 2018; Dossat et al., 2018; Guarraci et al., 2018). Therefore, a revaluation of ketamine use in pediatric clinics, especially its neurotoxic effects that lead to long-lasting cognitive deficits, is of great significance. However, key molecules involved in the neuroapoptosis induced by repeated ketamine exposure in the developing brain are still unknown.

Src homology 2/collagen domain (Shc) proteins belong to a family of intracellular signaling adapters in mammalian cells and include four members (ShcA–D). In the central nervous system, expression of ShcA is high in neural stem/progenitor cells. Further, it is continuously present during the early postnatal period, but absent in the mature brain (Conti et al., 2001; Cataudella et al., 2004). The isoform, p^{66}ShcA, is a known proapoptotic adaptor that promotes apoptosis when activated (Nemoto and Finkel, 2002; Giorgio et al., 2005; An et al., 2016). p^{66}ShcA has also been shown to activate caspase-3 and lead to apoptosis in a lung epithelial cell line (Lv et al., 2017).

We hypothesized that activation of p^{66}ShcA is involved in ketamine-induced neuroapoptosis in the neonatal brain, which leads to long-lasting cognitive deficits in adulthood. Using a validated rat model of neuroapoptosis induced by neonatal ketamine exposure (Zou et al., 2009b), here, we examined the time-course for changes in expression levels of activated caspase-3 and p^{66}ShcA in the hippocampus after ketamine exposure was withdrawn. Moreover, using the Morris water maze, we assessed behavioral changes in spatial learning and memory to determine whether neuronal cell death caused by neonatal ketamine exposure led to impairments in learning and memory that persisted into adulthood.

Materials and Methods

Animals

Male and female Sprague-Dawley rats at postnatal day 7 (PND 7, n = 31) were used in the study. All rats were from the breeding colony at the Animal Care Facility of the University of Texas, Arlington, USA. Animals were maintained in a temperature- and humidity-controlled environment on a 12-hour light/dark cycle with the food and water ad libitum. This study was approved by the Institutional Animal Care and Use Committee of the University of Texas at Arlington (IACUC) (approval No. A13.008) on January 22, 2013.

Drug administration

PND 7 rat pups, dosed subcutaneously with ketamine hydrochloride (Medical and Veterinary Supplies, Mettawa, IL, USA; 20 mg/kg; six injections at 2-hour intervals) using a 30-gauge needle (Zou et al., 2009b) in the neck, were randomly divided into four ketamine groups (4 rats in each group). To keep warmth and reduce potential stressors, pups were returned to their dam between injections. Rat groups were sacrificed at 0, 1, 3, or 6 hours after the last ketamine injection by performing a fast decapitation for protein extraction.

A second cohort of PND 7 rats were randomly divided into either ketamine (20 mg/kg; six subcutaneous injections at 2-hour intervals in the neck; n = 8) or control (0.02 mL saline instead of ketamine; n = 7) groups. To determine whether neonatal ketamine exposure induced long-lasting learning and memory deficits, spatial learning and memory ability was assessed via the Morris water maze at 4 weeks of age and compared between the two groups.

Western blot assay

Western blot assays were performed as described in (Tang et al., 2016), but with a little modification. The hippocampus was isolated immediately from neonatal rats after fast decapitation. Each animal's hippocampus was kept separately and temporarily stored in wet ice. The tissue was homogenized and detergent-extracted on ice using precooled RIPA cell-lysis buffer with repeated pipetting. The lysates were gathered and centrifuged at a 12,000 × g for 10 minutes at 4°C. The total amount of protein was quantified using a bicinchoninic acid protein-assay kit (Thermo Scientific Fisher, Waltham, MA, USA). Forty μg of total protein was used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which employed 10% or 15% gradient Tris/glycine gels under reducing conditions. Next, proteins were electrotransferred to a nitrocellulose membrane in a Trans-Blot SD Semi-dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). Nonspecific bands were blocked in phosphate-buffered saline with Tween 20 (PBST) (8 g NaCl; 0.2 g KCl; 1.42 g

Na_2HPO_4; 0.27 g KH_2PO_4; and 0.2% Tween 20 in 1000 mL distilled water) containing 5% skimmed milk (phosphorylated antibody using 2% bovine serum albumin) for 1 hour at room temperature. Membranes were subsequently incubated with antibodies of cleaved caspase-3 (1:500, Catalog No. C8487; Sigma, St. Louis, MO, USA), p^{66}ShcA (1:2000, Catalog No. 610879; BD Biosciences, San Jose, CA, USA), phosphorylated p^{66}ShcA (p-p^{66}ShcA, Catalog No. ab54518, 1:1000; Abcam, Cambridge, MA, USA), β-actin (1:1000, Catalog No. sc-47778; Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C overnight, separately. After membranes were washed three times in PBST for 10 min/time, bound antibodies were stained with anti-mouse or anti-rabbit horseradish peroxidase conjugated IgG (1:5000, Sigma) at room temperature for 1 hour. Finally, after washing the membrane four times in PBST for 10 min/time, specific bands were detected using an enhanced chemiluminescence kit (Thermo Scientific, Waltham, MA, USA). The band intensities were measured using FluorChem FC2 software (Bio-Rad, Santa Clara, CA, USA).

Morris water maze

To examine the effect of neonatal ketamine exposure on spatial learning and memory ability, the Morris water maze test was performed by the 4-week old rats that had been neonatally treated with ketamine. The maze was composed of a 160-cm plastic pool filled with opaque water and a 12-cm invisible plastic platform supported by a base resting on the pool bottom and placed around 1.5 cm below the water surface. An aquarium heater kept the water at a constant temperature between 25.5–27.0°C. Visual signs were on the walls surrounding the Morris water maze to assist the animals. The platform was placed in one specific target quadrant (the southwest quadrant), and was immobile throughout the trial until the probe was administered. The room light was indirect so as not to cause reflection on the water. The animals were trained for five days with two trials per day; the interval between the trials was at least 10 minutes. On each day, the rats were placed in one of two quadrants of the maze (North and East) facing the wall. Both quadrants were equidistant from the platform. Each rat was given 120 seconds to successfully locate and climb onto the platform. The rats were allowed to remain on the platform for 10 seconds after finding it. If the animals failed to locate the platform within 120 seconds, they were guided by the researcher and put on the platform for 10 seconds. The researchers left the room at the beginning of each trial to eliminate any cues that the rats might recognize. The probe trials also lasted 2 minutes. During the probe trial, the platform was removed from the pool and the rats were allowed to "free swim" in order to test for memory recall. After 2 minutes, each rat was removed, dried off and put back in its respective cage. A Noldus Ehto-Vision system (Noldus Information Technology, Leesburg, VA, USA) was used for all tracking and data collection. Latency to reach the platform, swimming distance, velocity, and time spent in each quadrant were calculated and saved on disk.

Statistical analysis

All results are expressed by mean ± SEM. Data were analyzed with SPSS 25 (IBM Corp., Armonk, NY, USA). The data obtained from the western blot assay were analyzed by one-way analysis of variance (Fisher's protected least significant difference test), while those from the Morris water maze test were assessed by unpaired *t* test or one-way analysis of variance. A value of $P < 0.05$ was considered statistically significant.

Results

Activation of caspase-3 and p^{66}ShcA after ketamine exposure

To assess the neuroapoptosis induced by ketamine exposure, we examined expression of activated caspase-3 at different times (0, 1, 3, and 6 hours) after the last ketamine injection. As shown in **Figure 1A** and **B**, the expression of cleaved caspase-3 in the 3-hour and 6-hour groups was significantly higher than that in the 0-hour and 1-hour groups ($F = 46.28$, $P = 0.002$, 3 hours *vs.* 0 hours; $F = 18.32$, $P = 0.014$, 3 hours *vs.* 1 hour; $F = 46.67$, $P = 0.002$, 6 hours *vs.* 0 hours; and $F = 15.94$, $P = 0.016$, 6 hours *vs.* 1 hour). The results indicated that significant hippocampal neuroapoptosis occurred at 3 hours and persisted until at least 6 hours after the last ketamine injection. To determine whether p^{66}ShcA was activated and involved in ketamine-induced hippocampal neuroapoptosis, the expression of activated p^{66}ShcA was detected by measuring the phosphorylated form of p^{66}ShcA at different time points (0, 1, 3, and 6 hours) after the last ketamine injection. Data in **Figure 1C** shows that p-p^{66}ShcA levels 3 hours after the last ketamine injection were significantly higher than those at 0 hours ($n = 4$, $P = 0.04$), although the expression levels of total p^{66}ShcA were not significantly different. Specifically, phosphorylation levels of p^{66}ShcA in the 3-hour group were 72.6 ± 1.2% higher than that in the 0-hour group. Statistical analyses indicated no significant difference in expression levels of p-p^{66}ShcA among the 0-, 1- and 6-hour groups (**Figure 1D**).

Neonatal ketamine exposure leads to deficits in spatial reference learning ability in adulthood

Results from the Morris water maze test showed that for both groups of 4-week old rats (control and ketamine), latency to find the platform gradually declined during the five-day training. However, the neonatal ketamine-treated group took significantly longer than the control group to find the hidden platform on the first 3 days of training (Day 1: $n = 7–8$, $P = 0.009$; Day 2: $n = 7–8$, $P = 0.017$; Day 3: $n = 7–8$, $P = 0.038$; **Figure 2A**). Analysis of total time spent swimming (**Figure 2B**) also showed that rats in the neonatal ketamine group took longer to find the platform than did the control group (control: 311.47 ± 18.29 seconds, $n = 7$; ketamine: 393.6 ± 29.6 seconds, $n = 8$; $P = 0.045$). **Figure 2C** shows that swimming speed did not differ significantly between groups (control: 22.1 ± 1.1 cm/s, $n = 7$; ketamine: 23.3 ± 1.1 cm/s, $n = 8$; $P = 0.479$). The probe trial (**Figure 2D**) showed that duration spent in the target quadrant did not differ between groups (control: 113.5 ± 3.2 seconds, $n = 7$; ketamine: 114.9

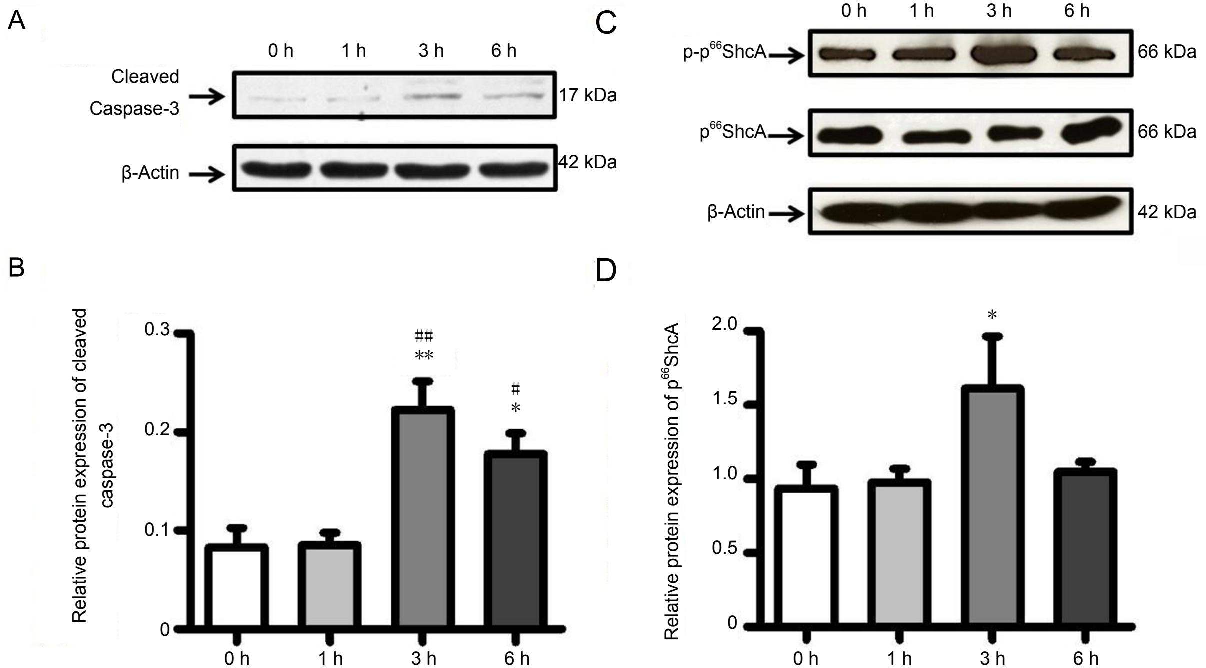

Figure 1 Expression of cleaved caspase-3 and p66ShcA over time in the neonatal hippocampus after the last ketamine injection.
(A) Representative immunoblots of activated caspase-3 protein. (B) Quantification of cleaved caspase-3/β-actin ratio. (C) Representative immunoblots of p-p66ShcA. (D) Quantification of p-p66ShcA/p66ShcA ratio. All results are represented as the mean ± SEM (n = 4; one-way analysis of variance followed by Fisher's protected least significant difference test). *P < 0.05, **P < 0.01, *vs.* 0-h group; #P < 0.05, ##P < 0.01, *vs.* 1-h group. Experiments were conducted in triplicate at least.

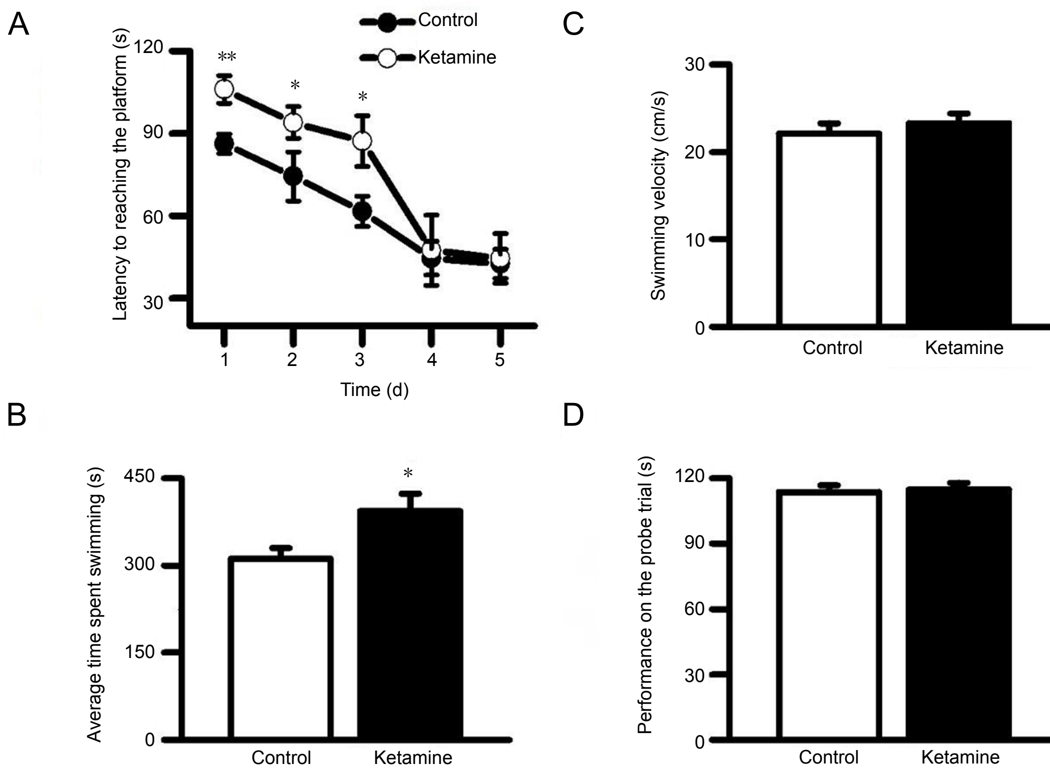

Figure 2 Learning and memory ability in 4-week old control rats and rats after neonatal treatment with ketamine.
(A) Mean latencies to reaching the platform during the 5-day learning period in control (saline-treated) and ketamine groups. Latency in the ketamine group was prolonged during the first 3 days. (B) Average time spent swimming during the 5-day trials. Total time spent in the water maze over the session was prolonged in the ketamine group, indicating a significant impairment in learning ability. (C) Swimming velocity did not differ between groups. (D) Performance on the probe trial (duration spent in the target quadrant) did not differ between groups. Results are represented as the mean ± SEM (n = 7 in control group; n = 8 in ketamine group; unpaired t test or one-way analysis of variance). *P < 0.05, **P < 0.01, *vs.* control group.

± 3.0 seconds, n = 8; P = 0.758). These results suggest that ketamine exposure at neonatal age leads to deficits in spatial reference learning ability at adulthood, but does not seem to adversely influence memory retention once it has been learned.

Discussion

Several studies report that exposing developing brains to ketamine leads to neuronal apoptotic death through the compensatory up-regulation of NMDAR subunit 1, and that the resulting apoptosis is widespread and dose-dependent (Wang et al., 2005, 2006, 2017; Kokane et al., 2017; Dossat et al., 2018; Gao et al., 2018; Liu et al., 2019). Up-regulation of NMDAR subunit 1 initiates a cascade process starting with Ca^{2+} influx, which results in the expression of proteins relating to reactive oxygen species (ROS) (Li et al., 2018b) and mitochondrial membrane depolarization. The apoptotic cascade is triggered in the course of ketamine withdrawal (Shan et al., 2019). Furthermore, *in vitro* studies provide evidence that ketamine-induced apoptosis is mediated via the mitochondrial pathway in which cells reenter an aberrant cycle, as evidenced by increases in the expression of Cyclin D1, cyclin-dependent kinase 4, E2F1, Bim, and activated caspase-3 (Braun et al., 2010; Ye et al., 2018). Consistent with these results, our data showed a significant up-regulation of activated caspase-3 in the hippocampus of PND 7 rat pups at 3 and 6 hours after ketamine withdrawal.

Behavioral studies demonstrate that prolonged ketamine exposure in the developing brain results in subsequent long-lasting cognitive impairment (Walker et al., 2010; Paule et al., 2011; Huang et al., 2012; Felix et al., 2017b; Onaolapo et al., 2017; Reus et al., 2017; Buratovic et al., 2018). The present study examined whether neonatal exposure to ketamine has long-lasting effects on spatial learning in later life. Four-week-old rats that had been pretreated with ketamine at PND 7 exhibited impaired ability to learn the location of the hidden platform during Morris water maze training. Swimming velocity did not differ between treated and untreated rats, indicating that the spatial learning deficit in the neonatally ketamine-treated rats was not due to changes in motor coordination or physical ability. In the probe trial, the time spent in the target quadrant did not differ between groups, suggesting that neonatal ketamine exposure caused a deficit of spatial reference learning ability, but not in memory recall. Therefore, these results provide further evidence that neonatal ketamine exposure impairs the performance of spatial learning acquisition. It is well accepted that long-term potentiation in the hippocampus and glutamatergic transmission may play an important role in spatial learning (Morris, 1989; Bannerman et al., 1995; Guo et al., 2018; Li et al., 2018b). An *in vitro* study by our group has shown the impairment of long-term potentiation in the anterior cingulate cortex caused by neonatal ketamine exposure (Wang et al., 2014). Thus, we propose that the impairment of long-term synaptic plasticity in the hippocampus due to neuroapoptosis caused by neonatal ketamine exposure contributes to the deficit of spatial learning. Our results were also supported by data from other groups showing that ketamine induced a loss in ability to navigate toward a hidden platform but not toward a visible one (Wesierska et al., 1990; Coronel-Oliveros and Pacheco-Calderon, 2018). However, the downstream signaling pathway and the pathophysiological changes resulting from neonatal ketamine exposure still remain to be investigated.

ShcA is selectively expressed in neural stem/progenitor cells, is continuously present in the developing brain, but is almost absent in mature neurons (O'Bryan et al., 1996; Conti et al., 1997, 2001; Nakamura et al., 1998). Further, ShcA is only present in restricted areas of adult neurogenesis, such as the olfactory epithelium (Conti et al., 1997) and the sub-ventricular zone (Ponti et al., 2005). The ShcA gene encodes three isoform proteins: p^{46}ShcA, p^{52}ShcA, and p^{66}ShcA, each containing an SH2-phosphotyrosine binding domain and a second phosphotyrosine binding domain (Pelicci et al., 1992; Pawson and Scott, 1997). p^{66}ShcA is the longest of these three adapter proteins and contains a unique collagen homologous region 2 (CH2) domain. It is involved in a signaling network that contributes to lifespan regulation, stress responses, and aging (Gertz and Steegborn, 2010). Studies have demonstrated that p66ShcA can upregulate endogenous ROS production (Nemoto and Finkel, 2002; Gertz and Steegborn, 2010), which is known as a trigger for initiating apoptosis (Giorgio et al., 2005). Protein kinase Cβ has been reported to be activated under oxidative conditions, and may phosphorylate p^{66}ShcA and trigger mitochondrial protein accumulation. In turn, phosphorylated p^{66}ShcA (p-p^{66}ShcA) causes alterations in mitochondrial Ca^{2+} responses and three-dimensional structure, contributing to the incidence of apoptosis (Pinton et al., 2007). Thus, p^{66}ShcA plays a critical role in ROS signaling and apoptosis initiation. All these findings lead us to propose that p66ShcA is involved in ketamine-induced hippocampal neuroapoptosis in the neonatal brain. In the present study, we detected the expression of p-p^{66}ShcA and total p^{66}ShcA using western blot analysis. Quantification of the p-p^{66}ShcA/p^{66}ShcA ratio showed that p-p^{66}ShcA had significantly increased by 3 hours after the last ketamine injection. Critically, activated caspase-3 also had a peak increase at 3 hours. Thus, these findings indicate that p^{66}ShcA is activated after repeated ketamine administration, which may trigger an apoptotic cascade in the hippocampus. Based on previous findings that compensatory up-regulation of NMDARs after ketamine exposure is the pathophysiological basis of neuroapoptosis in the developing brain (Wang et al., 2005; Zou et al., 2009b; Kokane et al., 2017), we propose that ketamine exposure-induced upregulation of NMDARs leads to Ca^{2+} overloading and subsequently activates the protein kinase Cβ that phosphorylates p^{66}ShcA. In turn, phosphorylation of p^{66}ShcA increases the production of endogenous ROS (Nemoto and Finkel, 2002; Gertz and Steegborn, 2010), causing high expression of active caspase-3. As a result, apoptosis is eventually triggered in the developing brain. Future studies are needed to determine the contribution of other signaling molecules to this pathway in order to deepen our understanding of the underlying mechanism.

In summary, the behavioral data from the present study provides further evidence that neonatal ketamine exposure is linked to spatial learning deficits seen later in life. Neurochemical studies indicate that such learning deficits are strongly associated with neuroapoptotic cell death in the hippocampus. Importantly, activation of p^{66}ShcA is involved in the neuroapoptotic cascade. Given the potential neurotoxic effects of repeated ketamine exposure on human neurodevelopment, studying the clinical pharmacology and neurotoxicology of ketamine is necessary in the future. Although the current *in vivo* study begins to explore the molecules critical for developing ketamine-induced apoptosis in immature neurons, *in vitro* experiments are still needed in future studies.

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