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Ketamine and propofol in combination induce neuroapoptosis and down-regulate the expression of *N*-methyl-D-aspartate glutamate receptor NR2B subunit in rat forebrain culture

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Background and objective: Ketamine has always been used in combination with propofol in paediatric patients. Ketamine interacts with *N*-methyl-D-aspartate glutamate receptor to exert its biologic actions. The NMDA receptor NR2B subunit is expressed at nearly adult level during forebrain development in the cortex and considered as the major type of functional NMDA receptor in the central nervous system. This study aimed to investigate whether ketamine or ketamine in combination with propofol induces apoptosis and regulates the expression level of NMDA receptor NR2B subunit in rat forebrain culture. *Methods*: Rat primary forebrain cultures were exposed to different concentrations of ketamine (1 μM, 10 μM, 20 μM) or 20 μM ketamine plus 5 μM propofol on the 6th day for 12 h. Cell viability was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Caspase-3 activity was measured and apoptotic morphology was examined by Hoechst dye staining. The expression of NMDA receptor NR2B subunit at mRNA and protein level was determined by qPCR and Western blot, respectively. *Results*: Ketamine (10 μM and 20 μM) or 20 μM ketamine plus 5 μM propofol resulted in a significant decrease in cell viability, and a significant increase in caspase-3 activity and apoptosis of primary rat forebrain culture. The expression of NMDA receptor NR2B subunit at both mRNA and protein levels were down-regulated by administration of ketamine (1 μM, 10 μM and 20 μM) and 20 μM ketamine plus 5 μM propofol. *Conclusion*: Ketamine (10 μM or 20 μM) alone or in combination with propofol (20 μM ketamine plus 5 μM propofol) induces neuroapoptosis and down-regulates of NMDA receptor NR2B subunit in rat forebrain culture.

1. Introduction

Glutamate (Glu) receptors are categorized into two major subclasses, ionotropic glutamate receptor (iGluR) and metabotropic glutamate receptor (mGluR) receptors (Slaughter and Miller 1983). *N*-Methyl-D-aspartate (NMDA) glutamate receptor is a subtype of iGluR widely distributed throughout the central nervous sysytem (CNS). The NMDA receptors are concentrated particularly in the hippocampus, in the outer layers of the cerebral cortex, and in the substantia gelatinosa of the spinal cord (Hudspith 1997). The NMDA receptor is implicated in various physiological and pathological processes, including memory and learning (Scheetz et al. 1994), synaptic plasticity (Meldrum et al. 1990), and abnormal CNS development (McDonald et al. 1990), NMDA receptor consists of an essential NR1 subunit, one or more regulatory NR2 subunits (NR2A–D), and NR3 subunit (Chatterton et al. 2002). NMDA receptor NR2A and NR2B, which are enriched in adult rat CNS, are considered as the main types of functional NMDA receptor channels in CNS neurons (Madden 2002). Especially, NR2B is expressed at nearly adult levels during forebrain development in cortex, and appears to play a more dominant role than NR2A in modulating NMDA receptors throughout the CNS during early postnatal development (Portera-Cailliau et al. 1996). Ketamine, a noncompetitive NMDA receptor antagonist, is widely used in paediatric anaesthesia including surgical or short diagnostic procedures and have also been used as a supplementary agent during regional anaesthesia. Clinically, ketamine is always used in combination with propofol that primarily interacts with gamma-aminobutyric acid-A ($GABA_A$) receptors. Ketamine inhibits the function of NR1/NR2A and NR1/NR2B receptors, which are activated by glutamate, consistent with its action on the NR1subunit (Liu et al. 2001). Furthermore, it has been demonstrated that ketamine up-regulates the expression level of NR1 subunits and induces apoptosis in rat forebrain culture (Wang et al. 2005, Zou et al. 2009). These previous findings led us to investigate whether the NR2B subunit is involved in apoptosis in rat forebrain culture induced by clinical concentrations of ketamine or ketamine in combination with propofol.

2. Investigations and results

2.1. *Ketamine or ketamine in combination with propofol inhibits the proliferation of rat forebrain cultures*

As shown in Fig. 1, MTT assay demonstrated that the proliferation of immature rat forebrain cultures was substantially decreased upon treatment with 10 μM ketamine, 20 μM ketamine or 20 μM ketamine plus 5 μM propofol compared to untreated control ($P < 0.05$), but not significantly decreased upon

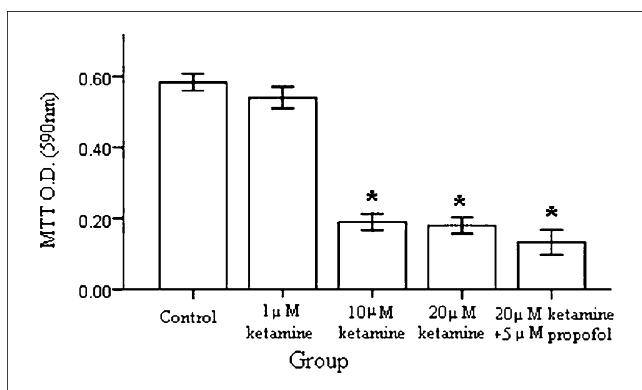


Fig. 1: Ketamine alone or in combination with propofol inhibits the proliferation of rat forebrain culture. Rat primary forebrain cultures were exposed to the indicated dose of ketamine or propofol and cell proliferation was determined by MTT assay. Experiments were repeated independently three times, each time in triplicates. Data were presented as mean ± SD.* indicated statistically significant differences at P< 0.05 vs control

treatment with 1 μM ketamine (P> 0.05, vs. untreated control). These data suggest that ketamine alone or in combination with propofol inhibited the proliferation of rat forebrain cultures.

2.2. *Ketamine or ketamine in combination with propofol promotes the apoptosis of rat forebrain cultures*

To further investigate the reduced proliferation of rat forebrain cultures treated with ketamine, we examined for apoptosis. Caspase-3 is one of the principal caspases that plays a central role in mediating apoptotic responses. As shown in Fig. 2, caspase-3 protease activity was significantly increased in a dose-dependent manner in the ketamine-treated groups in comparison with untreated control. Moreover, 20 μM ketamine plus 5 μM propofol induced further activation of caspae-3 which was about 4-fold that of control (P< 0.05) and 1.2–fold that of treatment by 20 μM ketamine alone (P< 0.05). The induction of apoptosis by ketamine or ketamine in combination with propofol was further confirmed by Hoechst dye H33258

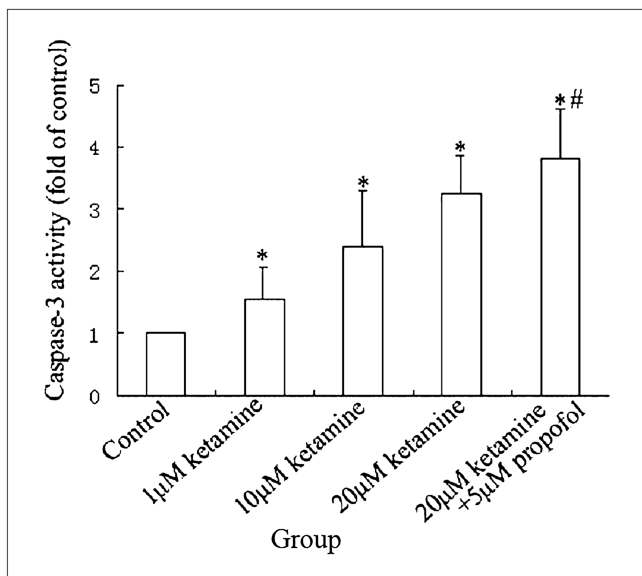
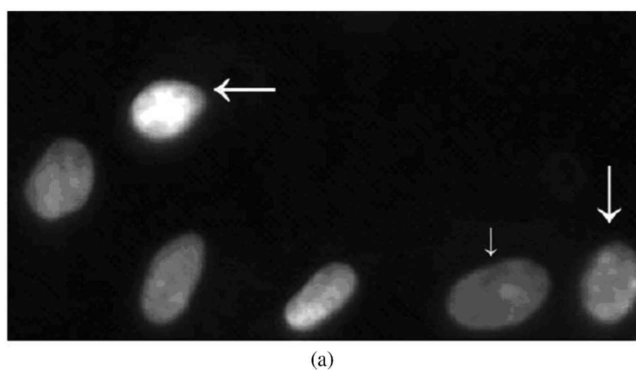
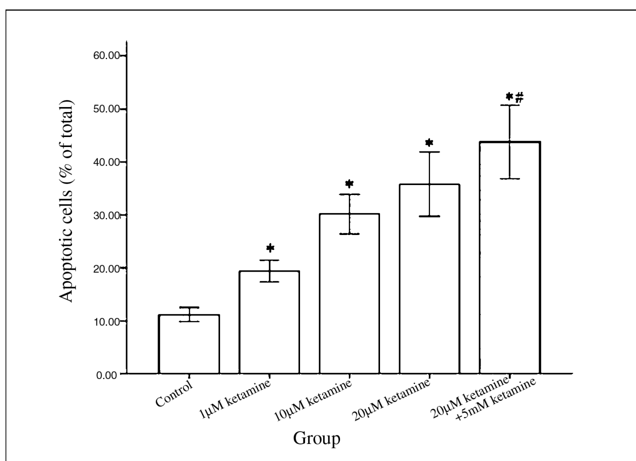


Fig. 2: Ketamine alone or in combination with propofol enhances caspase-3 activity in rat forebrain culture. Rat primary forebrain cultures were exposed to the indicated dose of ketamine or propofol, and caspase-3 activity was determined by caspase-3 activity assay. Experiments were repeated three times independently and each experiment was performed in at least triplicates. Data were presented as mean ± SD.* indicated statistically significant differences at P< 0.05 vs control. # indicated statistically significant differences at P< 0.05 vs 20 μM ketamine alone



(a)



(b)

Fig. 3: Ketamine alone or in combination with propofol induces apoptosis in rat forebrain culture. Rat primary forebrain cultures were exposed to the indicated dose of ketamine or propofol, and apoptosis was determined by Hoechst dye staining. a. The small arrow indicated normal neuronal nucleus and the big arrow indicates neurons with apoptotic morphology. b. Experiments were repeated three times independently, each time in at least triplicates. Data were presented as mean ± SD.* indicated statistically significant differences at P< 0.05 vs control. # indicated statistically significant differences at P< 0.05 vs 20 μM ketamine alone

staining, which was used to assess the morphological changes of apoptosis in neurons. As shown in Fig. 3, ketamine (1 μM, 10 μM, or 20 μM), or 20 μM ketamine plus 5 μM propofol increased apoptotic cells death compared with untreated control (P< 0.05). Notably, 20 μM ketamine plus 5 μM propofol treatment induced more extensive apoptosis than 20 μM ketamine treatment alone (P< 0.05). Up to 40% of neurons showed apoptotic cell death after treatment with 20 μM ketamine plus 5 μM propofol for 12 h. Taken together, these results demonstrate that ketamine alone or in combination with propofol promoted the apoptosis of rat forebrain Fig. 4 cultures.

2.3. *Ketamine or ketamine in combination with propofol downregulates the expression of NMDA NR2B subunit in rat forebrain cultures*

By qRT-PCR, we observed that the expression of NMDA receptor NR2B subunit at mRNA level was decreased in rat forebrain cultures treated with ketamine in a dose-dependent manner. The lowest expression of NR2B mRNA was detected in the cultures treated with 20 μM ketamine plus 5 μM propofol (Fig. 3a). To provide further support that ketamine or ketamine in combination with propofol downregulates the expression of NMDA NR2B subunit in rat forebrain cultures, we performed Western blot assay and showed that NR2B subunit expression at the protein level was notably decreased in forebrain cultures exposed to 1 μM, 10 μM, 20 μM ketamine, or 20 μM ketamine plus 5 μM propofol (Band intensity of the control group: 273.81 ± 10.22,

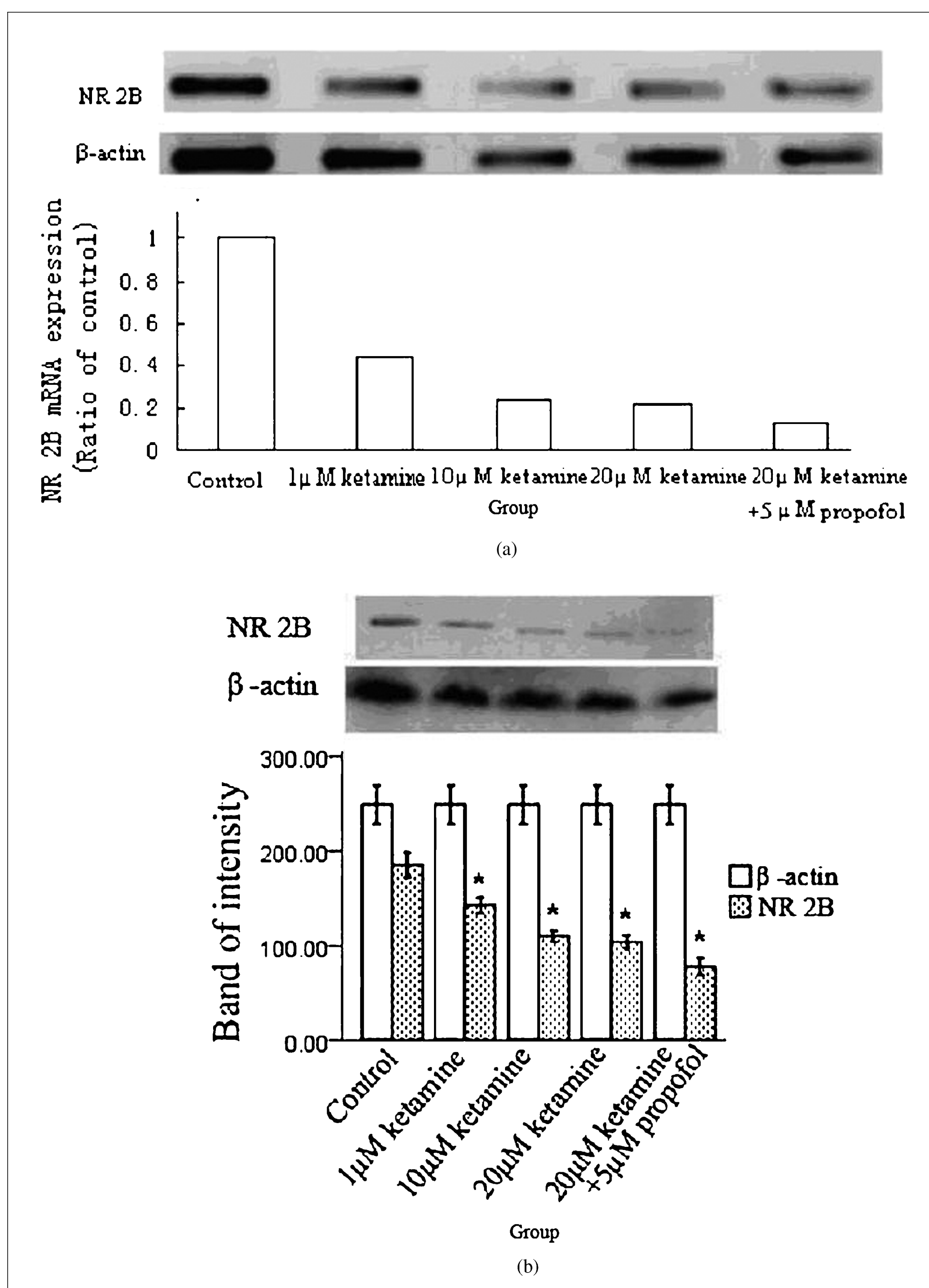


Fig. 4: Ketamine alone or in combination with propofol downregulates NR2B subunit expression in rat forebrain culture. Rat primary forebrain cultures were exposed to the indicated dose of ketamine or propofol and NR2B subunit expression was determined by qRT-PCR (a) and Western blot (b). Experiments were independently repeated three times, each time in at least triplicates. Data were presented as mean ± SD.* indicated statistically significant differences at P<0.05 vs control

1 μM ketamine-treated group: 171.67 ± 9.07, 10 μM ketamine-treated group: 155.33 ± 12.34, 20 μM ketamine-treated group: 121.67 ± 10.41, 20 μM ketamine plus 5 μM propofol: 83.33.39 ± 12.80, $P<0.05$, vs. control group. Fig. 3b).

3. Discussion

Our results demonstrate that ketamine (1 μM, 10 μM and 20 μM) induced apoptosis in immature rat forebrain cultures in a dose-dependent manner. Furthermore, ketamine in combination with propofol (20 μM ketamine plus 5 μM propofol) induced more extensive apoptosis compared with ketamine treatment alone. NMDA receptor NR2B subunit is likely to be involved in the apoptosis-inducing effect of ketamine or ketamine in combination with propofol.

We examined the expression level of the NMDA receptor NR2B subunit in rat forebrain primary cultures after 5 days, since a previous study had demonstrated that functional synapses form after 5 days in culture and that NR2B subunits initially predominate the NMDA receptors present at these sites (Tovar et al. 1999). NR2A expression in the rodent CNS begins only from 6–10 days postnatal (Zhong et al. 1994). During development, NR2B-rich receptors are supplemented with NR2A subunits (Monyer et al. 1994) and the mRNA level of NR2B increases between days 1 to 7 with little further changes after 7 days in cultured cortical neurons (Zhong et al. 1994). Thus the experi-

ments in the present study were performed after a culture period of 7–8 days at a developmental stage where NR2A expression is minimal.

Based on the ketamine concentration (about 1 μM) required for blocking NMDA receptors (Yamakura et al. 1993), the free clinical plasma concentration of ketamine (White et al. 1980), and the concentration of IC_{50} values (half-maximal inhibition) (Yamakura et al. 2000). The cultures were exposed to 1 μM, 10 μM or 20 μM ketamine for 12 h before we examined the effects of ketamine. The 5 μM concentration of propofol used in our experiments is based on some animal research and human brain concentration of propofol during maintenance of surgical anaesthesia (Kahraman et al. 2008).

Growing evidence indicates that ketamine can cause apoptosis (Zou et al. 2009; Brewer et al. 1993; Wang et al. 2000; Braun et al. 2010). Although the detailed mechanism underlying ketamine-induced apoptosis remains unknown, several events have been implicated, such as increased Ca^{2+} ion influx (Scheetz et al. 1994), Ca^{2+} overload-induced mitochondrial dysfunction (Stanika et al. 2009), the mitochondrial pathway (Braun et al. 2010), and changes in NMDA receptor subunit expression (Zou et al. 2009). In the present study, the cell viability approximately decreased 60% in the 10 μM and 20 μM ketamine administration groups compared to control, consistent with previous report that ketamine administration resulted in an approximately 50–70% loss in cell viability after exposure for 12–24 h (Wang et al. 2005). Caspase-3 is one of the key proteases in early stage of apoptosis. We observed that Caspase-3 activity was significantly enhanced by ketamine or ketamine in combination with propofol treatment even though no obvious effect on cell viability was detected upon treatment with 1 μM ketamine. Moreover, more extensive apoptosis was induced by ketamine in combination with propofol than the same concentration of ketamine alone. These results suggest that propofol treatment alone may induce apoptosis and the observed effect is additive, or a synergistic effect on inducing apoptosis occurs between ketamine and propofol. Propofol interacts primarily with $GABA_A$ receptors, but could also interact with NMDA receptors (Irifune et al. 2003). In addition, propofol induces apopotosis of neurons by activating extracellular signal-regulate kinase (ERK) (Straiko et al. 2009). On the other hand, ketamine may induce apoptosis of immature GABAergic neurons (Desfeux et al. 2010).

Activation of NMDA receptors requires the simultaneous binding of two co-agonists: glutamate and glycine. The ABD (agonist binding domain) binds glycine in NR1 and NR3, whereas NR2 ABDs bind glutamate (Furukawa et al. 2005; Yao et al. 2006). This is demonstrated by the NMDA composition including binary assemblies such as NR1/NR2A or NR1/NR2B in many brain regions, triheteromeric assemblies such as NR1/NR2A/NR2B in the forebrain or NR1/NR2A/NR2C in the cerebellum (Hatton et al. 2005). Although ketamine discriminates poorly among NMDA receptor subunits (Yamakura et al. 1993), it displays over a 50-fold 'preference' for NR1/NR2A or NR1/NR2B receptors compared with NR1/NR2C or NR1/NR2D receptors (Raditsch et al. 1993; Chao et al. 1997). Thus NR1/NR2A or NR1/NR2B receptors are likely to play an important role in mediating the effects of ketamine. It was demonstrated that ketamine administration (10 or 20 μM for 48 h) to DIV 4 rat forebrain cultures produced a significant upregulation of NR1 expression at the protein level (Wang et al. 2005). In contrast, here we found that treatment of DIV 6 rat forebrain cultures with ketamine (1 μM, 10 μM and 20 μM for 12 h) led to a down-regulation of NR2B subunit at both mRNA and protein levels. Our data is consistent with the result of a recent microarray analysis in which it was demonstrated that slightly decreased expression of NR2B were observed in postnatal day 7 rats following subcutaneous injection of 20 mg kg^{-1} ketamine in six successive doses at 2 h intervals (Shi et al. 2010). Furthermore, ketamine is considered to be an effective treatment for different chronic pain conditions (Backonja et al. 1994; Eide et al. 1995; Felsby et al. 1995), and it has been proposed that NR2B subunits in the spinal cord are implicated in such pain conditions (Gu et al. 2010). An additional study demonstrated that NR2B subunits were involved in the discriminative stimulus effects of ketamine (De Vry et al. 2003). All these studies suggest that NR2B subunits may mediate the effects of ketamine, at least partly. On the other hand, ketamine treatment could change the NMDA receptor function associated with other subunits. These data indicate that the effects of ketamine most likely depend on the dose given, the route of administration, the duration of exposure, the tissue sites, the receptor type or subtype activated, and the stage of maturity at the time of exposure.

In summary, our data demonstrate that ketamine (1 μM, 10 μM and 20 μM) or ketamine in combination with propofol (20 μM ketamine plus 5 μM propofol) induced apoptosis and downregulation of the NMDA receptor NR2B subunit expression in rat forebrain culture. Although the results presented remain to be verified in the clinical situation, significant attention should be paid if ketamine is to be used in combination with propofol over a long period of time in pediatric patients.

4. Experimental

4.1. *Primary forebrain cultures*

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Chinese Medical University, Shenyang, China, and all experiments were performed in accordance with the NIH (National Institutes of Health) Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and reduce the number of animals used. Primary forebrain cultures were prepared from Sprague-Dawley newborn rat pups (less than 24 h). Cells were diluted to a concentration of 1×10^6 cells ml^{-1} with complete medium containing DMEM (dulbecco's modified eagle's medium)/F12 (Gibco BRL, Gaithersburg, MD, USA), 10% fetal bovine serm, 10% horse serum, 100 Uml^{-1} penicillin, 100 U ml^{-1} streptomyc in sulfates and seeded on poly-lysine-coated culture bottles or plates and cultured with 5% CO2 at 37 °C. After one day, the medium was changed to serum-free DMEM/F12 + 2% B27 (Gibco BRL, Gaithersburg, MD, USA) supplement, subsequently half of cell culture medium was replaced every 2 days. On the 6th day *in vitro* (DIV), cells were treated by 1 μM, 10 μM, 20 μM ketamine (Sigma-Aldrich Inc. St, Louis, MO, USA), or 20 μM ketamine combination with 5 μM propofol (AstraZeneca S. p. A., Caponago, Italy) for 12 h. The media containing ketamine or propofol was removed and the cultures were kept in medium containing DMEM/F12 + 2% B27 for 12 h. All subsequent experiments were carried out on 7 DIV. Glial cells were reduced to less than 0.5% of the nearly pure neuronal population using serum-free B27 neurobasal medium as assessed by immunocytochemistry for glial fibrillary acidic protein and neuron-specific enolase (Brewer et al. 1993).

4.2. *MTT assay*

Forebrain cells were cultured in 96-well plates. 3-(4,5 -Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) assay was performed on DIV 7 by replacing media with 250 μl of serum-free media containing 0.5 mg ml^{-1} MTT (Sigma, USA) and incubated at 37 °C for 3 h. Then 150 μl of dimethyl sulphoxide (DMSO) (Sigma, USA) was added to each well after MTT was washed out. The optical density (OD) was read on a microplate reader (Sunrise RC TECAN, Switzerland) at 590 nm. All experiments were performed in triplicates.

4.3. *Caspase-3 activity assay*

Caspase-3 activity was measured using a colorimetric kit according to the manufacturer's instructions (Beyotime, Haimen, China). Cell lysate was prepared after treatment and 10 μl cell lysate was added to 96-well microtitre plates and incubated in 80 μl reaction buffer at 37 °C for 4 h. The OD was measured on a microplate reader (Sunrise RC TECAN, Switzerland) at 405 nm. Caspase-3 activity was expressed as the percentage of enzyme activity compared to control. All experiments were performed in triplicates.

4.4. *Quantitation of apoptosis*

The cells were fixed with a 4% formalin neutral phosphate buffer solution (pH 7.4) for 30 min at room temperature. Subsequently, the cells were stained with 5 μg ml^{-1} Hoechst dye 33258 (Molecular Probes, Oregon, USA) for 15 min. The nuclear morphology was observed under a fluorescence microscope (Leica DMI4000, Germany). The percentage of cells with apoptotic nuclear morphology was calculated from 5 randomly selected fields. At least 200 cells were counted per condition from three independent experiments performed in triplicates.

4.5. *Total RNA extraction and cDNA synthesis*

Cultures were homogenized in trizol reagent (Invitrogen) and total RNA was extracted following the manufacture's protocol. The total RNA from each sample was tested by 1% denaturing agarose gel (28S/18S ratio: 1–2) and the RNA purity and quality were determined by OD values at 260/280 using NanoPhotometer™ (Germany). Equal amount of RNA from each sample was reverse transcribed to cDNA using with PrimeScript® RT kit (Takara Biotechnology Dalian Co., Ltd) according to the manufacturer's instructions.

4.6. *RT-qPCR*

Premix Ex Taq™ kit (Takara Biotechnology Dalian Co., Ltd) was used for RT-qPCR. The 25 μl reaction system contained 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM), 1 μl probe (10 μM), Premix Ex Taq™ 12.5 μl, ultra pure water 8.5 μl and cDNA 2 μl. The mixtures were predenatured at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s using the Rotor-Gene 3000 (Corbett Research) instrument. The forward (f), reverse (r) primers and probe (p) for NMDA receptor NR2B subunit were: f, 5′-TGGAGCTGGTAGCCATGAAC-3′; r, 5′-GAGAGTCTGAGCAGAAATGAAGTC-3′, and p, 5′-(FAM) TGACCCAAAGAGCATCATCACCCGT (Eclipse)-3′; For β-actin: f, 5' -TATGAGGGTTACGCGCTCCC - 3'; r, 5'-TCTTTAATGTCACG CACGATTTCC - 3'; and p, 5'-(FAM) CCTGCGTCTGGACCTGGCTGGC (Eclipse)-3'. All reactions were performed in triplicates.

4.7. *Western Blot*

Western blot analysis was performed as described previously (Chen et al. 2008). After total protein concentration was determined according to Lowry's method (Lowry et al. 1951) using bovine serum albumin as the standard, 50 μg of protein was separated by SDS-PAGE (polyacrylamide gel electrophoresis) and electrotransferred onto PDVF membranes (Invitrogen), which were probed with anti-NR2B (1:1,000, Abcam Hongkong Ltd.) or anti-β-actin (1:3000, Sigma). The membranes were incubated with horseradish peroxidase conjugated secondary antibodies (anti-rabbit IgG for the primary antibodies), and developed using ECL (enhanced chemiluminescent) system (Amersham. Piscataway, New Jersey, USA). The protein bands were analyzed by densitometric analysis.

4.8. *Statistical analysis*

Results were expressed as mean ± SD. Normality was assessed using the one-sample Kolmogorov–Smirnov test. Differences among the groups were determined by one-way analysis of variance (ANOVA), followed by the Dunnett post-hoc test. A value of $P < 0.05$ was considered significantly. RT-qPCRs data were analyzed using the software provided by Corbett Research.

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