Effect of NMDA on proliferation and apoptosis in hippocampal neural stem cells treated with MK-801

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Abstract. The purpose of the present study was to investigate effects of *N*-methyl-D-aspartate (NMDA) on proliferation and apoptosis of hippocampal neural stem cells (NSCs) treated with dizocilpine (MK-801). Cultures of hippocampal NSCs were randomly divided into four groups consisting of an untreated control, cells treated with MK-801, NMDA and a combination of MK801 and NMDA (M+N). Proliferative and apoptotic responses for each of the experimental groups were determined by MTS and flow cytometry. The results revealed that MK-801 and NMDA exerted significant effects on hippocampal NSCs proliferation. Cell survival rates decreased in MK-801, NMDA and M+N treated groups compared with the control group. Cells survival rates in NMDA and M+N treated groups increased compared with the MK-801 treated group. MK-801 and NMDA were demonstrated to significantly affect apoptosis in hippocampal NSCs. Total and early stages of apoptosis in MK-801 and NMDA groups significantly increased compared with the control group. Total and early apoptosis of NSCs in the M+N group significantly decreased compared with MK-801 and NMDA groups. Late apoptosis of NSCs in MK-801 and NMDA groups significantly decreased compared with the control group. Late apoptosis of NSCs in the M+N group significantly increased compared with MK-801 and NMDA groups. The present study revealed that MK-801 inhibited proliferation and increased apoptosis in hippocampal NSCs. NMDA may reduce the neurotoxicity induced by MK-801, which may be associated with its activity towards NMDA receptors and may describe a novel therapeutic target for the treatment of schizophrenia.

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Introduction

Schizophrenia is a severe mental disorder, which affects ~1% of the world's population (1). Approximately 70% of schizophrenia cases are inheritable and the condition has a major impact on the quality of life (2). Currently, the pathogenesis of schizophrenia remains unclear (2). Glutamate is an excitatory neurotransmitter, which interacts with the *N*-methyl-D-aspartate receptor (NMDAR), a subtype of glutamate receptors. NMDAR is associated with learning, memory, cognition and synaptic plasticity (3,4). NMDAR subunit 1 (NR1) is a major functional subunit of the NMDAR family (5). NMDAR is regarded as a major contributing factor in the development of schizophrenia (6) and is associated with specific symptoms induced by changes in the glutamatergic system (7).

Several studies have reported that non-competitive NMDAR antagonists, including dizocilpine (MK-801), phencyclidine and ketamine, impair spatial-delayed alternation performance and produce similar behavioral responses in psychosis (6,8,9). Animal models with psychosis have been established based on these agents (10). Furthermore, studies have demonstrated that NMDAR is dysfunctional in schizophrenia, particularly in the hippocampus (11,12). It is well-known that the hippocampus is the region of the brain associated with emotions, learning and memory and synaptic plasticity, which suggests that NMDARs, specifically a dysregulation or hypofunction of NMDARs, may serve a key role in the pathogenic process of schizophrenia (4). Previous studies have indicated that adult neurogenesis is presented within the hippocampus (13,14). However, studies are yet to determine associations between NMDARs and hippocampal neural stem cell (NSC) proliferation and apoptosis.

In a previous study, NMDAR antagonist MK-801 was evaluated to establish a schizophrenia-like mouse model, which produced behavioral responses that closely resembled those observed in hippocampal neurogenesis (14-16). Other studies

focused on MK-801 to establish schizophrenia-like symptoms and models of behavior (6,17).

In the present study, MK-801 was employed to establish a cell model of schizophrenia, which was used to investigate effects of NMDA on proliferation and apoptosis of hippocampal NSCs. To confirm an association between NMDARs and hippocampal NSCs proliferation and apoptosis induced by MK-801, the mechanism of NMDAR in the pathogenesis of schizophrenia and regulation of hippocampal NSCs was evaluated. Overall, the present study contributed to a better understanding of the mechanisms of schizophrenia and provided an experimental basis for further research in this area.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F-12 (F12), StemPro® Accutase® Cell Dissociation reagent, fetal bovine serum (FBS) and B27 were purchased from Life Technologies (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Anti-nestin antibody (cat. no. N5413), poly-L-lysine hydrobromide (PLL), MK-801 and NMDA were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). CellTiter 96® Aqueous One Solution Cell Proliferation assay (MTS) was purchased from Promega Corporation (Madison, WI, USA). Annexin V-fluorescein isothiocyanate (FITC) apoptosis assay kit was purchased from BestBio Company (Shanghai, China).

Animals. A total of 50 neonatal C57/BL mice pups <24 h old (weight, ~1.2 g) were provided by the Experimental Animal Centre of Ningxia Medical University (Yinchuan, China). The male:female ratio was 1:1. Animals were housed in an animal room (temperature, 22-26˚C; relative humidity, 40-60%) with a 12 h light/dark cycle. Maternal animals were individually housed in cages and had free access to standard laboratory chow and water. The newborn offspring were used for the present study within 24 h of birth. The mice were provided by the Experimental Animal Center of Ningxia Medical University (Yinchuan, China). All protocols were preapproved by the Ethics Committee of Ningxia Medical University (2014-014).

Primary culture of NSCs. The bodies of newborn C57/BL mice were disinfected with 75% ethanol. Mice were decapitated to obtain the brain tissue, which was kept on ice. Hippocampal tissues were dissected under an anatomic microscope and washed in cold Dulbecco's PBS (D-PBS). Accutase treatment was combined with mechanical separation to digest the hippocampal tissues and the isolated cells were suspended in DMEM/F12, then centrifuged at 200 x g at room temperature (RT) for 5 min and the supernatant was discarded. The cell suspension was collected following passing through a 400-mesh sieve and seeded in culture bottles (25 cm^3). The conditional culture medium was composed of DMEM/F12 (1:1), B27 (2%), bFGF (20 ng/ml), EGF (20 ng/ml) and penicillin-streptomycin (100 U/ml). The density of cells was 0.5-1.0x10^6/ml. The original volume of conditioned medium was 4 ml following culturing at 37˚C in an atmosphere of 5% CO_2 for 3-4 days an additional 1 ml conditioned medium was added to the culture bottle. At 5-7 days post-culture the diameter of the neurospheres were ~100 μm and subculturing was performed. NSCs were digested by accutase for ~5 min. The NSC suspension was collected and centrifuged at 200 x g at RT for 5 min. The supernatant was discarded and the cells were resuspended in conditional culture medium. The cellular density was 0.5-1.0x10^6/ml in a culture bottle. The cells were incubated in an incubator containing 5% CO_2 at 37˚C.

Identification of NSCs. Immunofluorescence staining of nestin (a maker of NSCs) was performed to confirm the successful culture of NSCs. In brief, 0.01% PLL was coated on the sterile cover slips in 24-well plates for 30 min. Then the 24-well plates were washed three times with PBS. On day 4, the 2nd generation of NSCs were seeded at a density of 1.0x10^5/ml in the 24-well plates coated with PLL for 24 h in an incubator containing 5% CO_2 at 37˚C. The NSCs were washed with 0.01 M PBS and fixed with 4% paraformaldehyde for 30 min at RT, rinsed with 0.01 M PBS and incubated with 10% BSA for 1 h at 37˚C. The cells were incubated overnight at 4˚C with the primary antibodies against nestin (1:200). In the control group, the primary antibodies were substituted with 0.01 M PBS. The glass plates were subsequently washed with 0.01 M PBS three times and incubated with the FITC-labeled goat anti-rabbit IgG (H+L) (1:200; cat. no. A0562; Beyotime Institute of Biotechnology, Haimen, China) secondary antibodies at RT for 2 h. Then slides were washed three times with 0.01 M PBS. DAPI was used to counterstain the nuclei at room temperature for 10 min and then washed three times with 0.01 M PBS. Images were acquired using an Olympus FV1000 confocal microscope. The positive cells were quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Preparation of MK-801 and NMDA. MK-801 was dissolved in DMEM/F12 at a concentration of 5 mM and stored at -20˚C. Aliquots were further diluted in DMEM/F12 to a final concentration of 50, 100, 200, 400 and 800 μM in wells. NMDA was dissolved in DMEM/F12 at a concentration of 50 mM and stored at -20˚C. Aliquots were further diluted in DMEM/F12 to a concentration of 5 mM and then to a final concentration of 50, 100, 200, 400 and 800 μM in wells.

MTS assay. To assess effects of MK-801 and NMDA on hippocampal NSCs proliferation, four experimental groups were defined: Control group; MK-801 (M) group, with cells treated with 200 μM MK-801 for 24 h in an incubator at 37˚C; NMDA (N) group, with cells treated with 100 μM NMDA for 2 h in an incubator at 37˚C; M+N group, with cells treated with 200 μM MK-801 for 24 h prior to adding 100 μM NMDA for 2 h in an incubator at 37˚C. The 96-well plates contained 1.0x10^4 cells per well and the NSCs were incubated in an incubator containing 5% CO_2 at 37˚C. Following the above treatment, 20 μl of MTS was pipetted into each well of the 96-well plate containing cells in 100 μl culture medium. Plates were incubated at 37˚C for 4 h in a humidified, 5% CO_2 atmosphere. Absorbance at 490 nm was recorded using a plate reader.

Flow cytometry. Apoptosis in NSCs was measured by flow cytometry following staining with Annexin V-FITC and

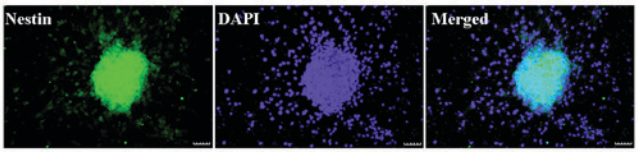

Figure 1. Identification of NSCs. Microscopy images presenting the morphology of neurospheres, with nestin, an NSC or neural progenitor marker in green and DAPI, a cell nuclear counterstaining in blue. Scale bar, 50 μm. NSC, neural stem cell.

propidium iodide (PI). Cells were treated as described previously and resuspended in binding buffer at $1.0x10^5$ cells/ml. Cell sample solution (100 μl) was incubated with FITC-conjugated annexin V (5 μl) and PI (5 μl) for 15 min at room temperature in the dark. Cells were analyzed by flow cytometry with FlowJo software 7.6.5 (FlowJo LLC, Ashland, OR, USA). Annexin V is a sensitive indicator for detecting early apoptosis in cells. The nucleus of cells in middle and late stages of apoptosis is stained by PI.

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical significance was calculated by one-way analysis of variance followed by the LSD post hoc test. All experiments were performed in triplicate. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of NSCs. Neurosphere with nestin expression (green) and DAPI staining (blue) are presented in Fig. 1. Nestin is an identification marker of NSCs or neural progenitor marker. DAPI is an identification marker for cell nuclei. The merged image demonstrated that nestin (green) and DAPI (blue) were co-localized in the cultured cells. The result indicated that NSCs had a high purity and may be applied in the proposed study.

Concentration dependence of MK-801 and NMDA treatments on NSCs viability. The concentrations of MK-801 and NMDA were confirmed through the MTS test. MK-801 and NMDA inhibited growth of NSCs in a dose-dependent manner. The research dosage of drugs is always confirmed using the IC_{50}, which is the dosage at which 50% of the cells die. Typically the dosage of a drug selected for treatment use will cause 50-80% cell viability. MK-801 at 200 μM is ~60% cell viability (200 μM for 24 h; Fig. 2). Therefore, MK-801 (200 μM for 24 h) was used in the present experiments. NMDA at 100 μM is ~60% cell viability (100 μM for 2 h; Fig. 3). Therefore NMDA (100 μM for 2 h) was selected for use in the present experiments.

MK-801 and NMDA affect hippocampal NSCs proliferation. Compared with the control group, cell survival rates of M, N and M+N groups were significantly decreased (P<0.01). Compared with the M group, cell survival rates of N and M+N groups increased significantly (P<0.01; Fig. 4).

Influence of NMDA and MK-801 treatment on NSCs apoptosis. Total apoptosis rates of NSCs in M and N groups increased

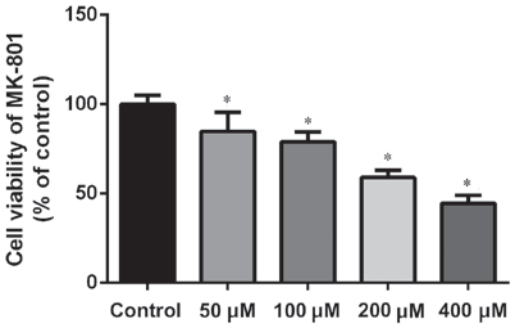

Figure 2. Concentration dependence of MK-801 on cell viability of hippocampal neural stem cells. Cell viability was measured by MTS assay. The final concentration of MK-801 was 200 μM for 24 h. Experiments were performed in triplicate. *P<0.01 vs. control. MK-801, dizocilpine.

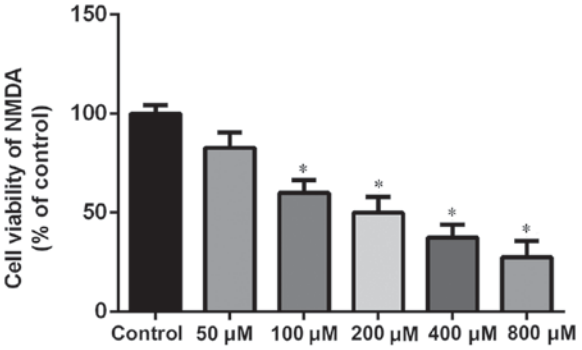

Figure 3. Concentration dependence of NMDA on cell viability of hippocampal neural stem cells. Cell viability was measured by MTS assay. The final concentration of NMDA was 100 μM for 2 h. Experiments were performed in triplicate. *P<0.01 vs. control. NMDA, *N*-methyl-D-aspartate.

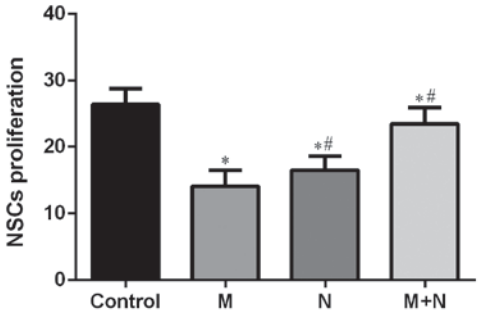

Figure 4. Effects of MK-801 and NMDA treatment on proliferation of hippocampal NSCs. *P<0.01 vs. control; #P<0.01 vs. M. MK-801, dizocilpine; NMDA, *N*-methyl-D-aspartate; NSC, neural stem cell; M, MK-801 (200 μM) treatment for 24 h; N, NMDA (100 μM) treatment for 2 h; M+N, MK-801 (200 μM) treatment for 24 h followed by NMDA (100 μM) treatment for 2 h.

compared with the control group (P<0.01). Compared with M or N group, the total apoptosis rate of the M+N group decreased (P<0.05). No significant difference was observed between the M and N groups (Figs. 5 and 6). Early stage apoptotic rates of NSCs in M and N groups were revealed to increase compared with the control group (P<0.001). Compared with M and N groups, the early stage apoptotic rate of the M+N group decreased (P<0.001). No significant difference was observed between M and N groups (Fig. 7). Late stage apoptotic rates of NSCs in M and N groups were significantly reduced

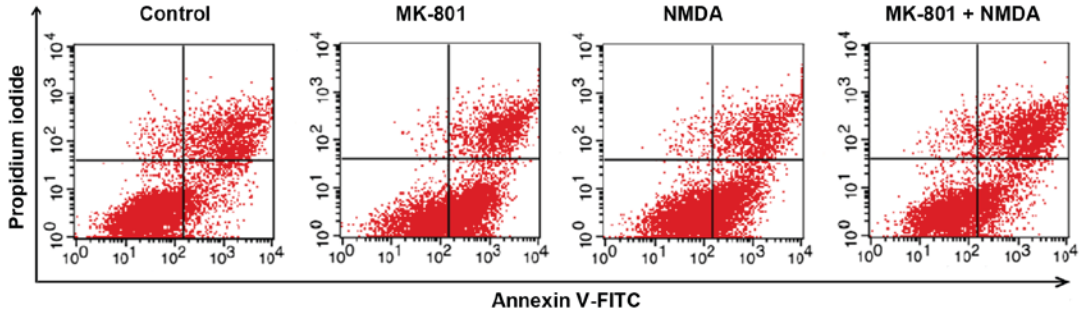

Figure 5. Effects of MK-801 and NMDA treatment on apoptosis of hippocampal stem cells. Flow cytometry analysis of hippocampal neurospheres for cell survival, necrosis and apoptosis following treatment with MK-801 (200 μM) for 24 h, NMDA (100 μM) for 2 h or MK-801 (200 μM) for 24 h followed by NMDA (100 μM) for 2 h. MK-801, dizocilpine; NMDA, *N*-methyl-D-aspartate.

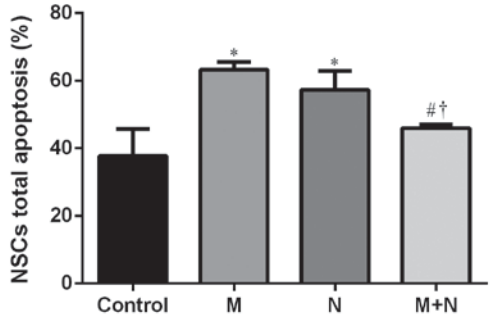

Figure 6. Analysis of MK-801 and NMDA treatment on apoptosis of hippocampal NSCs. *P<0.01 vs. control; #P<0.01 vs. M; †P<0.05 vs. N. MK-801, dizocilpine; NMDA, N-methyl-D-aspartate; NSC, neural stem cell; M, MK-801 (200 μM) treatment for 24 h; N, NMDA (100 μM) treatment for 2 h; M+N, MK-801 (200 μM) treatment for 24 h followed by NMDA (100 μM) treatment for 2 h.

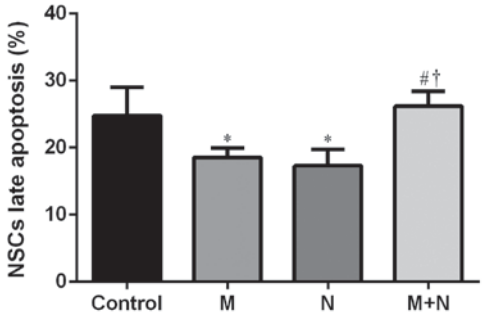

Figure 8. Effects of MK-801 and NMDA treatment on late stage apoptosis of hippocampal NSCs. *P<0.01 vs. control; #P<0.01 vs. M; †P<0.05 vs. N. MK-801, dizocilpine; NMDA, N-methyl-D-aspartate; NSC, neural stem cells; M, MK-801 (200 μM) treatment for 24 h; N, NMDA (100 μM) treatment for 2 h; M+N, MK-801 (200 μM) treatment for 24 h followed by NMDA (100 μM) treatment for 2 h.

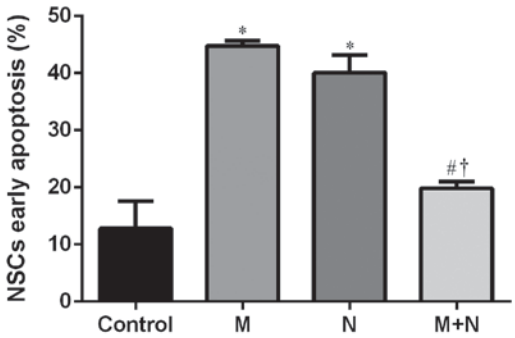

Figure 7. Effects of MK-801 and NMDA treatment on early stage apoptosis of hippocampal NSCs. *P<0.001 vs. control; #P<0.001 vs. M; †P<0.001 vs. N. MK-801, dizocilpine; NMDA, N-methyl-D-aspartate; NSC, neural stem cells; M, MK-801 (200 μM) treatment for 24 h; N, NMDA (100 μM) treatment for 2 h; M+N, MK-801 (200 μM) treatment for 24 h followed by NMDA (100 μM) treatment for 2 h.

compared with the control group (P<0.01). Compared with the M and N groups, the late stage apoptotic rate of the M+N group increased (P<0.01). There was no significant difference between the M and N groups (Fig. 8).

Discussion

Relapse rates of schizophrenia have become a focus of attention as its etiology and pathogenesis remain unclear (2). Glutamatergic dysfunction is known to be associated with the pathology of schizophrenia (17). The glutamate receptor hypothesis postulates that schizophrenia occurs in the hippocampus through NMDAR glutamate neurotransmitter system exceptions (5). Glutamic acid caused by glutamate ester is unusual and may result in symptoms of schizophrenia (5). Research has revealed that NMDAR agonists improve symptoms of schizophrenia, indicating that glutamate NMDAR serves an important role in the pathogenesis of schizophrenia (10).

NMDAR is an ionic, excitatory glutamate receptor in the CNS, which is expressed in the hippocampus and cerebral cortex area (18). NMDAR stimulates nerve growth and synaptogenesis and the excited mature receptor promotes learning, memory and other higher order functions (19). Excessive activation of NMDAR causes Ca^{2+} accumulation in neurons, inducing neurotoxicity and leading to cell death (5). Glycine is an NMDAR agonist, which is used as adjuvant for antipsychotic drugs, combined with traditional antipsychotic medicines, it improves negative symptoms of schizophrenia (20). NMDA and its receptor serve a role in the CNS and affect schizophrenia at least partially mediated by ion channel activity (2). NR1 is a functional subunit of the NMDAR family, which is representative of NMDAR expression levels and is referred to as the core subunit of NMDAR (21). Further studies have revealed that low NR1 expression is associated with low spatial learning and memory function in schizophrenia,

and the glutamic acid system and other specific regions of the brain expressing NMDAR are closely associated with schizophrenia (11,22). A previous study using a rat model demonstrated that depression following cerebral hemorrhage was caused by enhanced expression of the NMDARs NR1 and NR2B in the hippocampus, accompanied by decreased bromodeoxyuridine expression (23). The results presented in the present study demonstrated that NMDAR antagonist MK-801 and NMDAR agonist NMDA decreased cell viability. NMDARs were significantly inhibited by MK-801 or activated by NMDA and demonstrated neurotoxicity through decreased cell viability. MK-801 inhibited the NMDAR inducing neurotoxicity. Subsequent addition of NMDA activated NMDAR and neutralized the inhibitory effect of MK-801. NMDA may inhibit neurotoxicity induced by MK-801.

Neurogenesis involves proliferation and differentiation of neural precursor cells in the developing and adult brain, which is associated with NMDAR (19). The dysfunction of adult neurogenesis is associated with the etiology of schizophrenia (23). At a cellular level, cell proliferation is a major biological characteristic. The data presented in the present study were obtained using MTS assays and flow cytometry to determine effects of MK-801 and NMDA on proliferation and apoptosis in hippocampal NSCs. MK-801 and NMDA inhibited cell proliferation when compared with the control cells, as using MK-801 or NMDA alone significantly inhibited or activated NMDARs, respectively, leading to neurotoxicity and decreased cell proliferation. Treatment with MK-801, to inhibit NMDARs, followed by treatment with NMDA, to reactivate NMDARs, was demonstrated to neutralize the initial effect of MK-801. NMDA reversed the inhibitory effect of MK-801 on cell proliferation. MK-801 and NMDA demonstrated to induce apoptosis when compared with the control cells. Whereas MK-801 treatment inhibited NMDARs, subsequent treatment with NMDA suppressed stimulatory effects of MK-801 on cell apoptosis. Apoptotic rates of hippocampal NSCs significantly decreased, suggesting that NMDARs may be associated with the process of hippocampal NSC apoptosis and proliferation.

MK-801 inhibits the function of glutamate in hippocampal neurons (24). By blocking Ca^{2+} channels, it induces neurotoxic effects, which prevent hippocampal neurogenesis and promote apoptosis of hippocampal NSCs (25,26). The NMDAR antagonist ketamine alters neurogenesis by inhibiting proliferation of NSCs (27). In the present study, treatment with MK-801 for 24 h followed by treatment with NMDA for 2 h revealed that cell vitality partially recovered and total and early apoptotic levels significantly decreased. The underlying mechanisms may be associated with the activation of NMDAR. NMDA activate NMDAR on hippocampal neurons, change synaptic plasticity and release excitatory neurotransmitters that condition and inhibit MK-801 in a non-competitive role to improve cell vitality (28,29). A further study has revealed that MK-801 treatment [0.2 mg/kg, intraperitoneal (IP)] increases NSC proliferation in the hippocampus of a rat model with Parkinson's disease (30). MK-801 treatment results in enhanced hippocampal neurogenesis in the model. Differences between this study and the current study were the dose of MK-801 treatment (0.2 mg/kg, IP, rat) compared with 200 μM added to the cell supernatant, respectively, and alterations in the model type. Observed effects of MK-801 were therefore different. Effects of low and high dosage of MK-801 varied on proliferation and neurogenesis. Excitatory amino acid toxicity exists in Parkinson's or Alzheimer's disease and MK-801 may be useful in the treatment of these (31). However, effects of MK-801 may be harmful for normal cells. A cell model using MK-801 was established.

In conclusion, on a cellular level, NMDA may inhibit neurotoxic effects of MK-801 on hippocampal NSCs and significantly improve hippocampal NSCs activity and rate of apoptosis, which serves an essential role in proliferation and apoptosis. Limitations of the current study include missing NMDAR knockout or gene silencing experiments. However, MK-801 and NMDA were used to evaluate the association between NMDAR and proliferation and apoptosis of hippocampal NSCs. Exploring regulatory effects of NMDARs on the functioning of the hippocampus and schizophrenia is of importance for an increased understanding of the underlying biology of mental health and it may facilitate identifying novel treatment targets.

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Availability of data and materials

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

Authors' contributions

JD performed the data analyses and drafted the manuscript. YS performed the experiments. HHZ assisted with the experiments. QRM, YWZ, YXD and YQH collected and interpreted the data. JL conceived the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental protocol was approved by the Ethics Committee of Ningxia Medical University (approval no. 2014-014).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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