Expression of NR1 and apoptosis levels in the hippocampal cells of mice treated with MK-801

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Abstract. The aim of the present study was to investigate the characteristics of N-methyl-D-aspartate receptor R1 (NR1) expression and apoptosis in the nerve cells of the hippocampus in schizophrenia-like mice. C57BL/6 mice were randomly allocated to the following groups: i) Blank group; ii) MK-801 group; iii) MK-801+NMDA group, according to body weight. The NMDAR antagonist, MK-801 (0.6 mg/kg/d) was intraperitoneally injected daily for 14 days to induce a schizophrenia-like phenotype mouse model, and the effect of the NMDA injection via the lateral ventricle was observed. The results demonstrated that the number of NR1 positive cells in the MK-801 group increased in the CA1 and DG regions, indicating that NMDA may reverse this change. The level of damage decreased in the MK-801 treated group when compared with the blank group in the CA3 region. The protein expression of NR1 increased however, at the mRNA expression level, NR1 was lower in the MK-801 treated group when compared to the blank group; NMDA also reversed this change. In addition, early and total apoptosis detected in the hippocampal nerve cells was significantly increased in the MK-801 group when compared with the blank group, which was reversible following treatment with NMDA. These results indicated that NMDA may regulate the expression of NR1 and suppress apoptosis in hippocampal nerve cells in schizophrenia-like mice. Thus, NR1 may be a promising therapeutic target for the treatment of schizophrenia.

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Introduction

Schizophrenia is a severe psychiatric disorder with a lifetime prevalence of around 1% (1). Cognitive impairments are the core symptoms of the disease which is characterized by disturbances in sensory information processing, attention, working memory and executive functions deficits (2) which are all hippocampus-dependent functions (3,4). Schizophrenia imposes a heavy financial burden and many potential safety hazards to families of sufferers and society as a whole.

Recent studies focusing on the pathogenesis and treatment of schizophrenia have made significant progress in understanding the biology underpinning the disease. The glutamatergic hypothesis of schizophrenia states that dysfunction of the glutamatergic system induces imbalance between the glutamatergic and dopaminergic systems in the central nervous system (CNS) and induces some symptoms of schizophrenia (5). N-methyl-D-aspartate receptors (NMDARs) may be a key factor in the pathogenesis of schizophrenia (6-8). NMDAR is one of the ionotropic glutamatergic receptors (5), is a tetrameric structure of seven subunits including at least one obligatory subunit, N-methyl-D-aspartate receptor R1 (NR1; it includes eight functional splice variants), and varying numbers of a family of NR2 (NR2A-D) or NR3 (NR3A-B) subunits (9,10). The properties of NMDARs are both complicated and diverse owing to their complex subunit compositions. The characteristics of NMDAR include high Ca^{2+} permeability and Mg^{2+} suppression closely associated with neurogenesis, neuronal survival, synaptic plasticity and the formation of learning and memory abilities (2,4,11-13). Studies have demonstrated that schizophrenia patients show abnormal synaptic plasticity, LTP and cognitive dysfunction (11,13-15). The hippocampus is involved with cognitive functions, particularly in learning and memory and so was chosen as the area of particular interest for the current study. NR1 subunits are essential components of NMDARs. NR1 is very important for understanding the distribution and functions of NMDARs (8), however, the expression and regulation of NR1 in the hippocampus of schizophrenia-like mice are not known.

To investigate these issues, our study examined the expression of the NR1 subunit in the granule cell layer CA1, CA3

and DG region, and discussed regulation of the NR1 subunit expression by NMDA. We also characterized the expression of the NR1 subunit mRNA and protein, and levels of apoptosis in the hippocampal nerve cells of schizophrenia-like mice. In conclusion, we demonstrated that NMDA can regulate the expression of NR1 and suppress apoptosis in the hippocampal nerve cells of schizophrenia-like mice.

Materials and methods

Materials. MK-801 (3 mg/ml in 0.9% saline) and NMDA (1.5 μg/ml in 0.9% saline) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The primary antibody of NR1 was purchased from Abcam (Cambridge, MA, USA), and β-actin antibody purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). The secondary antibody of goat anti-rabbit fluorescein isothiocyanate (FITC) was purchased from CWBIO (Beijing, China), and the HRP-conjugated goat anti-rabbit antibody purchased from ZSGB-BIO (Beijing, China). Annexin V-FITC/PI kit was purchased from Yeasen (USA). TRIzol reagent was purchased from Life Technologies (Thermo Fisher Scientific, Inc., Waltham, MA, USA). All PCR primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). RevertAid First Strand cDNA Synthesis kit and Maxima SYBR Green qPCR Master Mix were purchased from Thermo Fisher Scientific, Inc.

Animals. Male C57BL/6 mice (23-25 grams, at 3-month old) were used in this study and provided by the Experimental Animal Center of the Ningxia Medical University. Mice were housed in groups of five in cages with free access to food and tap water for 7 days. The animal room temperature was maintained at 23±2˚C, with a 12/12 dark/light cycle (lights on 7:00-19:00) and 50% humidity. All animals were handled in accordance with the standards established by the institutional animal care and use committee of Ningxia Medical University.

Experimental groups and models. Male C57BL/6 mice were divided into 3 groups (n=12 in each group): A blank group, a MK-801 treated group and a MK-801+NMDA treated group. In the MK-801+NMDA treated group, mice were treated intra-peritoneally with MK-801 at dose of 0.6 mg/kg at the same time every day for 14 days (16). After 14 days, mice were anesthetized by intra-peritoneal injection of chloral hydrate and mounted on a stereotaxic frame for intra-cerebroventricular injection (mm from bregma: A -0.5, L -1.0, V-2.5) of NMDA (25 ng/μl, 3 ul) once each mouse (17). Brains were harvested 3 days after the operation (n=12). In the MK-801 group (schizophrenia-like group), saline was used instead of NMDA and all other operations were the same as intra-cerebroventricular injection groups (n=12). In the blank group, an equal volume of 0.9% saline was injected instead of MK-801 and NMDA (n=12).

Immunofluorescence (IF) staining. Mice (n=3 in each group) were transcardially perfused with ice-cold 0.01 M PBS (pH 7.4, 150 ml/mouse) and 4% paraformaldehyde solution (200 ml/mouse). Brains were harvested, and post-fixed for 24 h in 4% paraformaldehyde, followed by immersion in 20%, 30% sucrose solution overnight. Brains were washed once for 10 min with ice cold 0.01 M PBS, embedded in 1% gelose solution and 30 μm-thick coronal sections cut and used for immunofluorescent staining of NR1 in the hippocampal granule cell layer. Sections were permeabilized and blocked with blocking solution (7% BSA and 0.3% Triton X-100 in 0.01 M PBS) for 1 h at room temperature, and incubated overnight at 4˚C with rabbit anti-NR1 (1:800). Sections were washed three times for 30 min with 0.01 M PBS followed by second antibody incubation with goat anti-rabbit fluorescein isothiocyanate (FITC, 1:200) for 2 h at room temperature. Sections were washed three times for 30 min with 0.01 M PBS. Finally, the number of NR1 positive cells in granule cell layer of hippocampus was measured using confocal laser scanning microscope.

Western blotting. Mice (n=3 in each group) were decapitated and hippocampi were harvested. The hippocampi were put into glass homogenates with ice-cold buffer containing protease inhibitor for 45 min. The homogenates were centrifuged at 12,000 x g for 20 min and the supernatant was collected (stored at -80˚C). The protein levels were detected using the BCA method. Total protein in each group was separated (NR1, β-actin) by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes. PVDF membranes were immersed in blocking buffer (5% fat-free milk in PBST) for 1 h at room temperature and incubated overnight at 4˚C with the primary rabbit antibodies respectively (NR1, 1:500; β-actin, 1:3,000). The membranes were then washed three times for 30 min using PBST and incubated HRP-conjugated goat anti-rabbit antibody (1:5,000) for 2 h at room temperature and washed three times for 30 min in PBST. Immuno-reactive proteins were visualized using enhanced chemiluminescence (ECL) detection and the signals were quantified by densitometry using a western blotting detection system.

Real-time PCR. Total RNA from the hippocampal tissue (n=3 in each group) was extracted using TRIzol reagent. One microgram of the purified total RNA was reverse transcribed using a RevertAid First Strand cDNA Synthesis kit. The mRNA level of NR1 was determined using the Maxima SYBR Green qPCR Master Mix. All PCR primers were synthesized by Sangon Biotech (Shanghai, China). The forward and reverse primer sequences for NR1 were 5'-CTTCCTCCAGCCACT ACCC-3' and 5'-AGAAAGCACCCCTGAAGCAC-3', respectively; for β-actin, forward and reverse primer sequence were 5'-CCTAAGGCCAACCGTGAAAAG-3'and 5'-ACCAGA GGCATACAGGGACAAC-3', respectively. β-actin was used as an internal reference to standardize each gene. Each sample was investigated in triplicate. The relative amount of mRNA was measured using the comparative threshold (Ct) method by normalizing target cDNA Ct values to that of β-actin, and the fold expression changes were calculated according to the $2^{-\Delta\Delta Ct}$ method (18,19).

Flow cytometry analysis. Analysis of apoptosis was performed by flow cytometry of the hippocampi (n=3 in each group) isolated from each mouse. Samples were digested with 0.25% trypsin at 37˚C for 20 min and cell suspension were then filtered through a cell strainer (20) and the number of

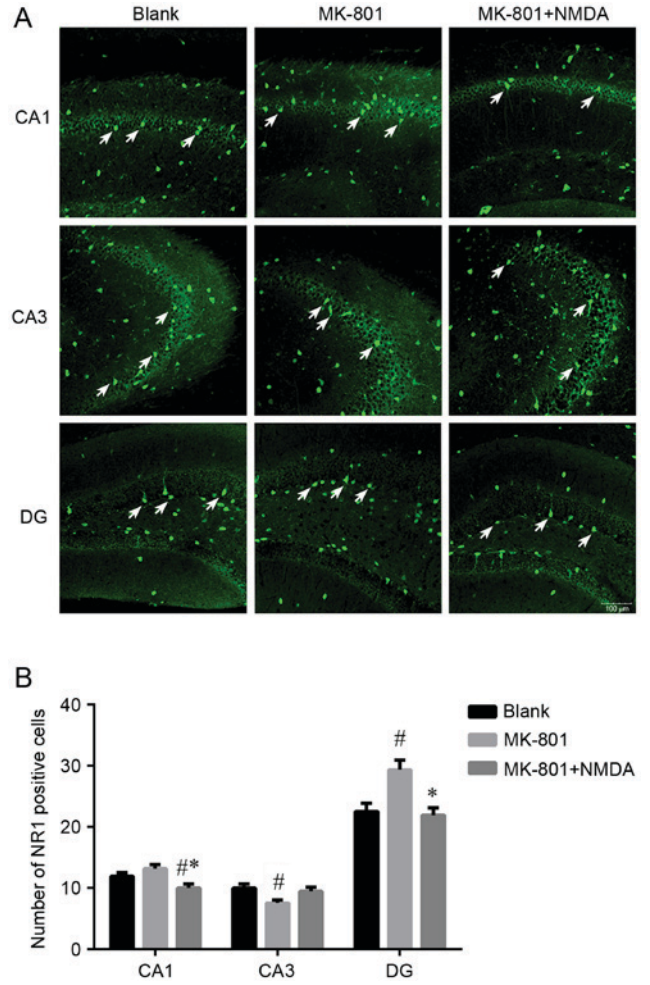

Figure 1. The NR1 positive cells distribution in different regions in hippocampus. (A) The distribution pattern of NR1 positive cells. Bars, 20 μm. (B) The number of NR1 positive cells. Three brain region of hippocampus: CA1, CA3, DG. Three groups: Blank, MK-801 and MK-801+NMDA. MK-801, NMDA receptor antagonist, MK-801 group (schizophrenia-like group); NMDA, N-methyl-D-aspartate; NR1, N-methyl-D-aspartate receptor R1. Data are presented as the mean ± standard deviation. $^{\#}$P<0.05 vs. blank group, *P<0.05 vs. MK-801 group. The number of NR1 positive cells in the MK-801 group increased in the CA1 (P=0.053) and DG (P<0.001) regions and this change was reversible by NMDA (P<0.001, P<0.001). The MK-801 group showed a decrease in NR1 compared with the blank group in the CA3 region (P<0.005) and again these changes were not reversible with NMDA (P=0.054).

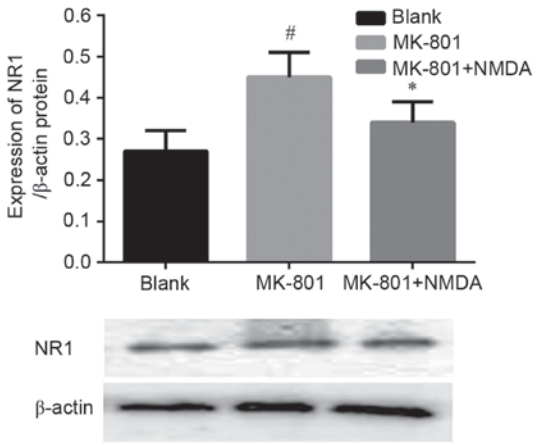

Figure 2. Expression of NR1 protein in the hippocampus of mice. Western blotting detects NR1 (105KD). NR1 levels normalized to β-actin. Three brain region of hippocampus: CA1, CA3, DG. Three groups: Blank, MK-801 and MK-801+NMDA. MK-801, NMDA receptor antagonist, MK-801 group (schizophrenia-like group); NMDA, N-methyl-D-aspartate; NR1, N-methyl-D-aspartate receptor R1. Data are presented as the mean ± standard deviation. $^{\#}$P<0.05 vs. blank group, *P<0.05 vs. MK-801 group. The expression of NR1 subunit increased in the MK-801 treated group (P=0.006) compared with to the blank group and again this trend was reversible by NMDA (P=0.035).

apoptotics cells was determined by flow cytometry assay using Annexin V-FITC/PI kit (21) and analyzed using the FACS express v2.0 software.

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analysis was carried out in SPSS11.5 (SPSS, Inc., Chicago, IL, USA) using one-way ANOVA. P<0.05 was considered as statistically significant.

Results

Expression of NR1 subunits in the CA1, CA3 and DG of the hippocampus. The distribution patterns of NR1 positive cells (white arrows) in the CA1, CA3 and dentate gyrus (DG) of the hippocampus are shown in Fig. 1A. The number of NR1 positive cells is shown in Fig. 1B. The results showed that the number of NR1 positive cells in the MK-801 group increased in the CA1 (P>0.05) and DG (P<0.05) regions and this change was reversible by NMDA. The MK-801 group showed a decrease in NR1 compared with the Blank group in the CA3 region (P<0.05).

Expression of NR1 subunit protein levels in the hippocampus. Fig. 2 shows the expression of NR1 subunit protein levels in the hippocampus of experimental mice. The data showed the expression of NR1 subunit increased in the MK-801 treated group (P<0.05) compared with to the Blank group and again this trend was reversible by NMDA.

Expression of NR1 subunit mRNA levels in hippocampus. The mRNA levels of the NR1 subunit normalized to β-actin in hippocampus of mice are shown in Fig. 3. The data show that the mRNA expression of NR1 subunit decreased in the MK-801 treated group (P<0.05) compared to the Blank group. This change was reversed by NMDA although no statistical significance was detected (P>0.05).

Apoptosis of hippocampal nerve cells. From Fig. 4 it can be seen that the early and total levels of apoptosis in the hippocampal nerve cells significantly increased (P<0.05) in the MK-801 group compared to the Blank group. In contrast, these changes were reversible by NMDA (P<0.05). The normal live cells (NLC) are representative of the survival rate of hippocampus nerve cells. The number of NLC decreased in the MK-801 group compared to the blank group (P<0.05) and again these changes were reversible with NMDA.

Discussion

The prevalence of schizophrenia increased to 1.0% between 1990 and 2010 all over the world (22). The underlying

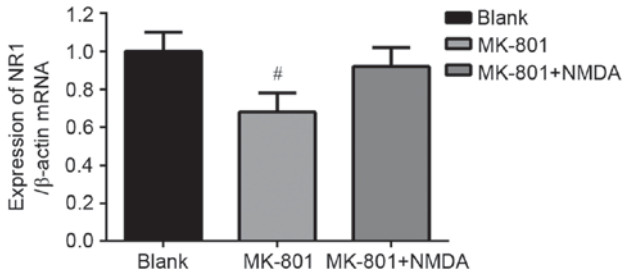

Figure 3. Expression of NR1 mRNA in the hippocampus of mice. Expression of NR1 relatives to β-actin. Three brain region of hippocampus: CA1, CA3, DG. Three groups: Blank, MK-801 and MK-801+NMDA. MK-801, NMDA receptor antagonist, MK-801 group (schizophrenia-like group); NMDA, N-methyl-D-aspartate; NR1, N-methyl-D-aspartate receptor R1. Data are presented as the mean ± standard deviation. #P<0.05 vs. blank group. The mRNA expression of NR1 subunit decreased in the MK-801 treated group (P<0.005) compared to the Blank group. This change was reversed by NMDA although no statistical significance was detected (P=0.057).

pathophysiological mechanisms of the disease are complex and remain to be fully elucidated. NMDAR is a major glutamate receptor subtype that is known to play a key role in learning and memory. NMDARs include NR1, NR2 (2A-2D), NR3 (3A-3B). NR1 subunits are the essential and obligatory components of NMDARs. NR1 subunits play an important role in determining the properties of NMDARs. Recently, NMDAR has received great attention on schizophrenia research. Dysfunction of NMDAR in the hippocampus is very important for the formation of schizophrenia (8).

We adopted an MK-801 administrated mouse model to investigate NR1 expression pattern and hippocampal neuron survival deficit in schizophrenia. MK-801 is a NMDAR antagonist per se. We worried changes released in this model may not be schizophrenia-related, but just a classical receptor-ligand feedback response of physiological process. Actually, no animal model can accurately reproduce all aspects of schizophrenia but mouse models can mimic several important aspects of the disease. MK-801 has been used for inducing a schizophrenia-like phenotype in rodents (23,24) which leads to different degrees of cognitive impairment (25-27). Some studies have shown hypofunction of the NMDA receptor by chronic treatment with MK-801 (28) or cell death in the hippocampus following NMDAR hypofunction (29). NMDAR subunits are known to play an important role in determining neurotoxic (30), functional and psychiatric effects in the hippocampus (31,32).

In this study, the results showed that the number of NR1 positive cells significantly increased in the hippocampal granule cell layer of the DG and CA1 regions, but decreased in

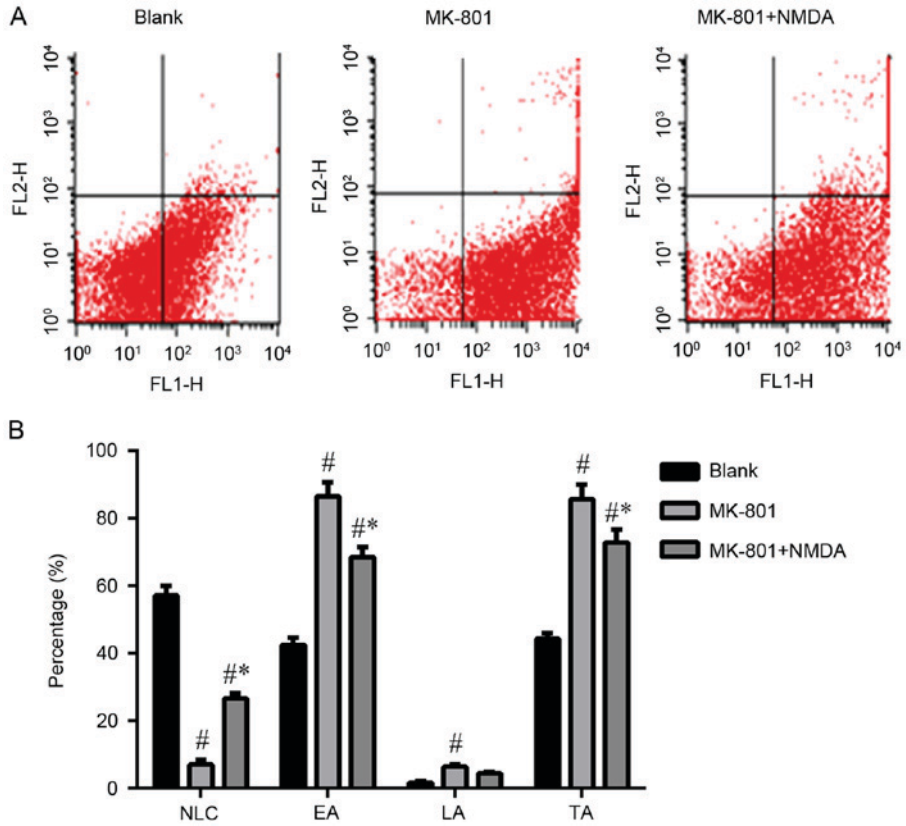

Figure 4. Apoptosis of hippocampal nerve cells. (A) Flow cytometry with apoptosis markers (FITC-Annexin V and PI). Three brain region of hippocampus: CA1, CA3, DG. Three groups: Blank, MK-801 and MK-801+NMDA. MK-801, NMDA receptor antagonist, MK-801 group (schizophrenia-like group); NMDA, N-methyl-D-aspartate; NR1, N-methyl-D-aspartate receptor R1. NLC, normal live cells; EA, early apoptosis; LA, late apoptosis; TA, total apoptosis. (B) Data are presented as the mean ± standard deviation. #P<0.05 vs. blank group, *P<0.05 vs. MK-801 group. The early and total levels of apoptosis in the hippocampal nerve cells significantly increased (P<0.001, P<0.001) in the MK-801 group compared to the Blank group. In contrast, these changes were reversible by NMDA (P<0.001, P<0.005). The number of NLC decreased in the MK-801 group compared to the blank group (P<0.001) and again these changes were reversible with NMDA (P<0.005). The number of LA increased in the MK-801 group compared to the blank group (P=0.046) and again these changes were not reversible with NMDA (P=0.308).

the CA3 region of schizophrenia-like mice induced by MK-801. NMDA was shown to reverse these changes. The NR1 total protein levels significantly increased, whilste the mRNA levels were reduced in the hippocampus of schizophrenia-like mice induced by MK-801. NMDA could also reverse these changes. Other studies of NMDAR expression in schizophrenia have shown variable changes at the transcript and protein expression levels in different areas of the brain. For example, study have shown that the protein expression of NR1 in the anterior cingulate cortex increased (33) or was found to be unchanged in hippocampus (34). However, another study found that NR1 protein expression was reduced in the prefrontal cortex, hippocampus and hippocampal DG region (35). Furthermore, at the mRNA level, expression has been shown to be decreased in the hippocampus (36,37), and the expression of the NR1 protein and mRNA levels were contradictory in the cortex of schizophrenia mice (33).

Another study examined NR1 expression in postmortem samples from patients with schizophrenia and comparison subjects. The data showed that at the transcriptional level, NR1 levels were lower in the thalamus of schizophrenia patients compared to control subjects (38). These studies support our experimental results to a certain extent. The current study has demonstrated that transcriptional changes of the NMDAR subunit expression in cortical areas appear to be associated with specific regions (33). These data may be explained by three supporting hypotheses. Firstly, the regional specificity expression of NR1 protein levels comes from the mRNA and the differential expressions in the CA1, CA3 and DG region contribute to the function of different areas (4). Secondly, it is possible that the peak of expression is not the same point at the protein and mRNA levels. Finally, NR1 may be involved in regulation of other complex components of glutamatergic pathways that are associated with schizophrenia (33).

The levels of apoptosis in the hippocampal nerve cells increased in schizophrenia-like mice induced by MK-801 which could be reversed by NMDA. Studies have shown that hyperfunction of glutamate receptors such as NMDA receptors increases intracellular Ca^{2+} levels. The continual influx of Ca^{2+} through the open NMDA receptors results in mitochondrial stress which attempts to sequester and buffer Ca^{2+}. As mitochondrial membrane potential decreases due to the overflow of Ca^{2+} and mitochondria reverse their ATP synthase in an attempt to restore Ca^{2+} homeostasis. Eventually, the excess Ca^{2+} uptake causes loss of mitochondrial membrane potential, mitochondrial swelling, opening of the mitochondrial permeability transition pore (39,40), outer membrane rupture and loss of Ca^{2+}, and apoptogenic factors into the cytoplasm. This process ultimately results in neuronal cell death (38). Our experimental data show that the apoptosis of hippocampal nerve cells significantly increased in schizophrenia-like mice induced by MK-801 and these changes could be reversed by NMDA.

We demonstrated that NR1 significantly contributes to the acquisition and apoptosis of hippocampal nerve cells in schizophrenia-like mice. NMDA can effectively regulate the expression of NR1 and levels of apoptosis in hippocampal nerve cells in schizophrenia-like mice, whereas the exactly underprinning molecular mechanisms need to be further clarified.

In summary, we have demonstrated that the expression of NR1 markedly increased in the hippocampus of schizophrenia-like mice, but showed differential trends in the CA1, CA3 and DG regions. NMDAR hyperfunction induced apoptosis in hippocampal nerve cells. NMDA contributes to positive function in schizophrenia, and may be offer an important molecular pathway for therapeutic intervention. NR1 might hold much potential as target in the therapy of schizophrenia.

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