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Original Article

Glutamate influences neuronal differentiation of neural stem cells from the injured cortex of adult rats

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Abstract: Objective: To explore the influence of glutamate on the neuronal differentiation of neural stem cells (NSCs) from the injured cortex of adult rats. Methods: NSCs were isolated and cultured from the injured cortex after traumatic brain injury (TBI), cells were identified by immunofluorescence staining. NSCs were divided into four groups: control, Glutamate, Glutamate+MK-801 (0 h), Glutamate+MK-801 (24 h). Cells were measured by immunofluorescence, TUNEL assay, real-time PCR, Western blot on 1, 3, 7, 14 day *in vitro*. Results: NSCs from the injured cortex were BrdU and nestin double-labeled, and could differentiate into MAP-2$^+$ neurons, GFAP$^+$ astrocytes, and CNP$^+$ oligodendrocytes. Glutamate promoted NSCs to differentiate into DCX$^+$ neuronal progenitor cells (NPCs) at the initial stage; however it subsequently enhanced cellular apoptosis. N-methyl-D-aspartic acid (NMDA) receptor antagonist MK-801 could counteract the effect of glutamate when they were treated together. Interestingly, if MK-801 was added 24 h after glutamate treatment, the number of NPCs was increased and it was higher than that in the Glutamate group on day 3 and day 7 *in vitro*. On day 14, many MAP-2$^+$ mature neurons with large cell bodies and abundant processes were observed in the Glutamate+MK-801 (24 h) group. In addition, the expression of NR1 in the Glutamate+MK-801 (24 h) group were gradually increased, peaked on day 7 and then remained at a stable level. Conclusion: Glutamate promotes neuronal differentiation of NSCs at the initial stage, but it also induces neuronal apoptosis during differentiation, which may be related to the gradual increase of NMDA receptor expression during the development of neurons.

Keywords: Traumatic brain injury, neural stem cells, glutamate, differentiation, NMDA receptor

Introduction

It was previously thought that neurons in the central nervous system (CNS) fail to regenerate [1, 2], but Reynolds [3] and Richards [4] isolated neural stem cells (NSCs) from the striatum and hippocampus of adult rats in 1992. After that, there has been increasing evidence that the adult mammalian CNS has a neurogenesis process under physiological or pathological conditions [5-9], and this phenomenon exists in both the human and primate CNS [10-12]. Itoh [13] isolated and cultured neurospheres from the injured cortex after TBI. Richardson [14] and Arsenijevic [15] also reported that neurospheres were isolated and cultured from cerebral cortex which was surgically removed. The above studies suggest that there is neurogenesis in the adult cerebral cortex under pathological conditions. Our previous study has also observed that cortical endogenous neurogenesis happened in the peri-injured cortex of adult rats with TBI and cortical neural progenitor cells could differentiate into astrocytes and immature neurons [16].

Neurotransmitters are thought to be involved in the proliferation and differentiation of NSCs in the adult brain [17, 18]. In the CNS, glutamate is the main excitatory neurotransmitter, which also affects neurogenesis. Our previous results indicated that neural progenitor cells could emerge in the injured area and differentiate into immature neurons and astrocytes *in vivo*, but ultimately did not develop into mature neurons [16]. So, why do neural progenitor cells survive, but new neurons are on the road to death? What factors affect the fate of these cells? The level of extracellular glutamate in the brain tissue after the CNS injury is higher [19,

20]. Excessive glutamate has excitotoxic effects. Is it related to the new neurons death?

In the current research, we used a craniocerebral hydraulic injury device to prepare a TBI model in adult rats, isolated and cultured NSCs from the injured cortex *in vitro*, and then investigated the effect of glutamate on the differentiation of NSCs into neurons and its probable mechanism involved in this process.

Materials and methods

Animals

Sprague Dawley (SD) adult rats (220-250 g) were obtained from the Center of Experimental Animals, Nantong University, China. Rats were housed in standard cages with 65±5% humidity, 22±1°C, and light-controlled 12 hour light/dark cycle (lights on from 7:00 to 19:00) room, and the food and water were provided ad libitum. All animal experiments in this research were carried out following the National Institute of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Nantong University.

The preparation of the TBI model

The TBI model was prepared using AmScien Instruments FP302 craniocerebral hydraulic injury device as described in our previous study [16]. Rats were anesthetized and the head was fixed to a stereotactic device, and a circular bone window with a diameter of 4 mm was created with the center of 2.5 mm medio-lateral (M/L), 3.5 mm antero-posterior (A/P) with reference to the bregma. The meninges were kept intact. The impact pressure of 1.5 atm (1 atm = 101.3 kPa) was used to induce brain injury in rats. After the impact, the injured cortex immediately emerged with obvious hematoma, indicating that the model was successfully prepared.

Isolation and culture of cortical NSCs

Three days after injury the animals were humainely euthanized and the brains extracted. The cerebral cortex in the injured area was digested by Accutase enzyme (Sigma, USA) at 37°C for 20 min, and the digestion was terminated by adding fetal bovine serum (FBS, Sigma, USA). The tissue was centrifuged at 1000 × g for 10 min, and the deposit was gently dissolved by a pipette with HEBSS solution until the suspension became a milky white homogenate. After that, the suspension was filtered with a 200-mesh stainless steel sieve, then centrifuged, and added with 2 ml serum-free medium. After pipetting, 3 ml 8% bovine serum albumin (BSA) (Sigma, USA) was slowly added to the bottom of the suspension, centrifuged at 1000 × g for 5 min. Finally, the cells were cultured at a concentration of 5×10^5 in a 50 ml culture flask containing 10 ml the basal medium with 20 ng/ml EGF (Sigma, USA), 20 ng/ml bFGF (Sigma, USA) and BrdU (5 μmol/l, Sigma, USA) in a 37°C, 5% CO_2 incubator. When the size of the suspended cell spheres in the culture solution was almost the same, and no longer increased significantly, the primary clones were formed. They were passaged once a week. The second passage of cell spheres were digested into a single cell suspension and were diluted to 400 cells/ml with the basal medium, then were seeded into 96-well culture plates a 37°C, 5% CO_2 incubator, 50 μl per well. The cells were observed under an inverted microscope after 2 h, and the wells with only 1 cell were marked. The neurospheres originating from a single cell were prepared for nestin/BrdU immunofluorescence staining.

Identification of multiple differentiation potentials

The second passage of cell spheres were digested into a single cell suspension and cultured into a 24-well culture plate pre-coated with poly-L-lysine (Sigma, USA) at a density of 1×10^5 cells/ml with DMEM/F12 complete medium. The culture plate was put into an incubator, half of the culture medium was replaced every 3 days, and then immunofluorescence detection of MAP-2, GFAP and CNP was performed after 14 days.

Drug treatments

The second passage of NSCs were digested into a single cell suspension and divided into 4 groups: control, Glutamate, Glutamate+MK-801 (0 h), and Glutamate+MK-801 (24 h). The cells of the control group were cultured in DMEM/F12 serum-free medium. The cells in Glutamate group were cultured in DMEM/F12 serum-free medium with glutamic acid (50 μmol/l, Sigma, USA). The cells in Glutamate+MK-801 (0 h) group were cultured with DMEM/F12 serum-

free medium containing glutamic acid (50 μmol/l) and NMDA receptor antagonist MK-801 (10 μmol/l, Sigma, USA). The cells in Glutamate+MK-801 (24 h) group were cultured with DMEM/F12 serum-free medium containing glutamic acid (50 μmol/l) and then MK-801 (10 μmol/l) was added into the culture medium 24 h later. Subsequently, the cells were immunofluorescence stained on days 1, 3, 7, 14 *in vitro* (DIV), respectively.

Immunofluorescence staining

A solution of 4% paraformaldehyde was applied to fix the adherent cells for 15 min. To block non-specific blinding sites, the cells were exposed to 0.01 mol/l PBS containing 10% goat serum for 0.5 h at room temperature. The cells were incubated with 2N HCL for 30 min at 37°C, then incubated with primary antibodies overnight at 4°C: rabbit antibody specific BrdU (1:100, Abcam, UK), mouse antibody specific nestin (1:2000, Abcam, UK), mouse antibody specific GFAP (1:200, Abcam, UK), guinea pig antibody specific DCX (1:1000, Millipore, USA), mouse antibody specific MAP-2 (1:500, Abcam, UK), and mouse antibody specific CNP (1:800, Millipore, USA), respectively. The cells were washed 3 times with 0.01 mol/l PBS, then the cells were cultured with Alexa Fluor® 568 goat anti-rabbit secondary antibody (1:1000, Abcam, UK), Alexa Fluor® 488 goat anti-mouse secondary antibody (1:800, Abcam, UK) or Alexa Fluor® 488 goat anti-guinea pig secondary antibody (1:800, Invitrogen, USA) at room temperature for 2 h. After rinsing, Hoechst-33342 (1:3000, Sigma, USA) was added for 0.5 h at room temperature. A fluorescence microscope (Leica, Germany) was used to view and acquire images.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

The cells were fixed with the 4% paraformaldehyde at room temperature for 15 min. The cells were washed 3 times with 0.01 mol/l PBS, and then the cells were subjected to TUNEL staining. The steps were performed according to the instructions of the TUNEL kit (Roche, Switzerland).

Flow cytometry assay

The second passage of NSCs were digested into a single cell suspension and fixed with 4% paraformaldehyde for 15 min. Then the cells were exposed to 0.01 mol/l PBS containing 10% goat serum for 30 min at room temperature and incubated with rabbit antibody specific for NR1 (1:1000, Abcam, UK) overnight at 4°C. The cells were washed 3 times with 0.01 mol/l PBS, then the cells were cultured with Alexa Fluor® 568 goat anti-rabbit secondary antibody (1:1000, Abcam, UK) at room temperature for 2 h. The percentage of $NR1^+$ cells was analyzed by Flow cytometry.

Quantitative real-time polymerase chain reaction (PCR) analysis

The RNA isolation kit (Invitrogen, USA) was applied to extract the total RNA from the cells, and the total RNA was reversely transcribed using reverse transcription kit (Roche, Germany). The PCR reactions with SYBR green I comprised an initial step at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s. The murine primer pairs used in this research are as follows: NR1, 5'-AGCGGGTAAACAACAGCAACAAA-3' and 5'-GAATCGGCCAAAGGGACTGAA-3'; GAPDH, 5'-CATCACTGCCACTCAGAAGACT-3' and 5'-GGATACATTGGGGGTAGGAAC-3'. The instrument's software (Rotor-Gene software, version 6.0) was applied to analyze the relative gene expressions and the relative gene expressions were presented as $2^{-\Delta\Delta Ct}$ as described by the manufacturer (User Bulletin).

Western blot analysis

The RIPA lysis buffer was applied to extract the total protein from the cells, and it was centrifuged at 4°C at 12,000 × g for 15 min to get the supernatant. Equal amount of protein was loaded onto a sodium dodecyl sulfate-polyacrylamide gel, then was subjected to electrophoresis. After that, the proteins were transferred to the polyvinylidene difluoride membrane (Pall, USA). Then, 5% skim milk was used to block nonspecific binding for the membrane, then the membrane was incubated with rabbit antibody specific for NR1 (1:800, Abcam, UK), or with mouse antibody specific for β-actin (1:2000, Sigma, USA) at 4°C for 12 h. Then, the membranes were incubated with the second antibody for 2 h at room temperature as follows: IRDye 700-conjugated affinity-purified goat anti-mouse IgG (1:5,000, Rockland Immunochemicals, USA) and with IRDye

800-conjugated affinity-purified goat anti-rabbit IgG (1:5,000, Rockland Immunochemicals, USA). The protein band was visualized by Odyssey laser scanning system (LI-COR Inc, USA). The protein band intensity was detected by the image analysis system (Odyssey 3.0 software).

Statistical analysis

The fluorescence images were imported into a computer and the number of positive cells was counted using JD-801 series morphological analysis software. Experimental data in this research were expressed as mean ± standard deviation (M ± SD). The Statistical Package for Social Science 21.0 (SPSS, 21.0) was used to analyze the data. The comparison between 2 groups was evaluated using Student's t test. Multiple comparisons among the groups were assessed by two-way analysis of variance (ANOVA). The difference was significant at $P < 0.05$.

Results

Identification of NSCs isolated from the injured cortex after TBI

The neurospheres originated from a single cell that was isolated from the injured cortex on day 3 after TBI in adult rats, and it was BrdU/nestin double-labeled positive (**Figure 1A**). The second-generation neurosphere could differentiate into MAP-2$^+$ neurons, GFAP$^+$ astrocytes, and CNP$^+$ oligodendrocytes (**Figure 1B**).

Effect of glutamate on the NPCs differentiation of NSCs

After culture for 1 DIV, the numbers of DCX$^+$ neuronal progenitor cells (NPCs) in the Glutamate and Glutamate+MK-801 (24 h) groups were more than those in the control and Glutamate+MK-801 (0 h) groups (**Figure 2A**, **2E**). After culture for 3 DIV, many DCX$^+$ NPCs were still observed in the Glutamate+MK-801 (24 h) group. The number of DCX$^+$ NPCs in the Glutamate group decreased but it was still more than those in the control and Glutamate+MK-801 (0 h) groups (**Figure 2B**, **2E**). After culture for 7 DIV, there were still many DCX$^+$ NPCs in the Glutamate+MK-801 (24 h) group but only a few DCX$^+$ NPCs were found in the other three groups (**Figure 2C**, **2E**). After culture for 14 DIV, DCX$^+$ NPCs were scarcely found in the all four groups (**Figure 2D**, **2E**). NMDA receptor antagonist MK-801 completely impaired the glutamate effect if MK-801 was added with glutamate together. Interestingly, if MK-801 was added 24 h after glutamate was treated, the number of NPCs was increased on 1 DIV, and it was even higher than that in the Glutamate group on 3 and 7 DIV. On 14 DIV, there were almost no DCX$^+$ NPCs in the four groups.

Effect of glutamate on mature neuron differentiation of NSCs

MAP-2 immunofluorescence assay was used for detecting mature neurons on 14 DIV. The result showed that there were many MAP-2$^+$ mature neurons in the Glutamate+MK-801 (24 h) group, with larger cell bodies and abundant processes. Only a small amount of MAP-2$^+$ neurons were seen in the other three groups (**Figure 3A**). The percentage of MAP-2$^+$ mature neurons in the Glutamate+MK-801 (24 h) group was statistically different from the other three groups (**Figure 3B**).

Effect of glutamate on cells apoptosis

The TUNEL kit was used to detect the cellular apoptosis. The result showed that a small number of TUNEL-positive apoptotic cells were observed in the four groups after culture for 1 DIV (**Figure 4A**). There were almost no changes in the numbers of apoptotic cells in the control, Glutamate+MK-801 (0 h) and Glutamate+MK-801 (24 h) groups on 3, 7, and 14 DIV, while the number of apoptotic cells in the Glutamate group was increased and reached a peak on 3 DIV. Although it decreased slightly on 7 DIV and 14 DIV with time, it was still higher than those in the other three groups at the corresponding time (**Figure 4B-D**), the differences were statistically significant (**Figure 4E**).

The expressions of NR1 in the NSCs and differentiated cells

NR1 is the constitutive subunit of the N-methyl-D-aspartic acid (NMDA) receptor. The result of Flow cytometry showed the positive rate of NR1 in NSCs was about 43.00% (**Figure 5A**). The mRNA or protein expression level of NR1 was detected by quantitative real-time PCR or Western blot analysis. The mRNA expression of

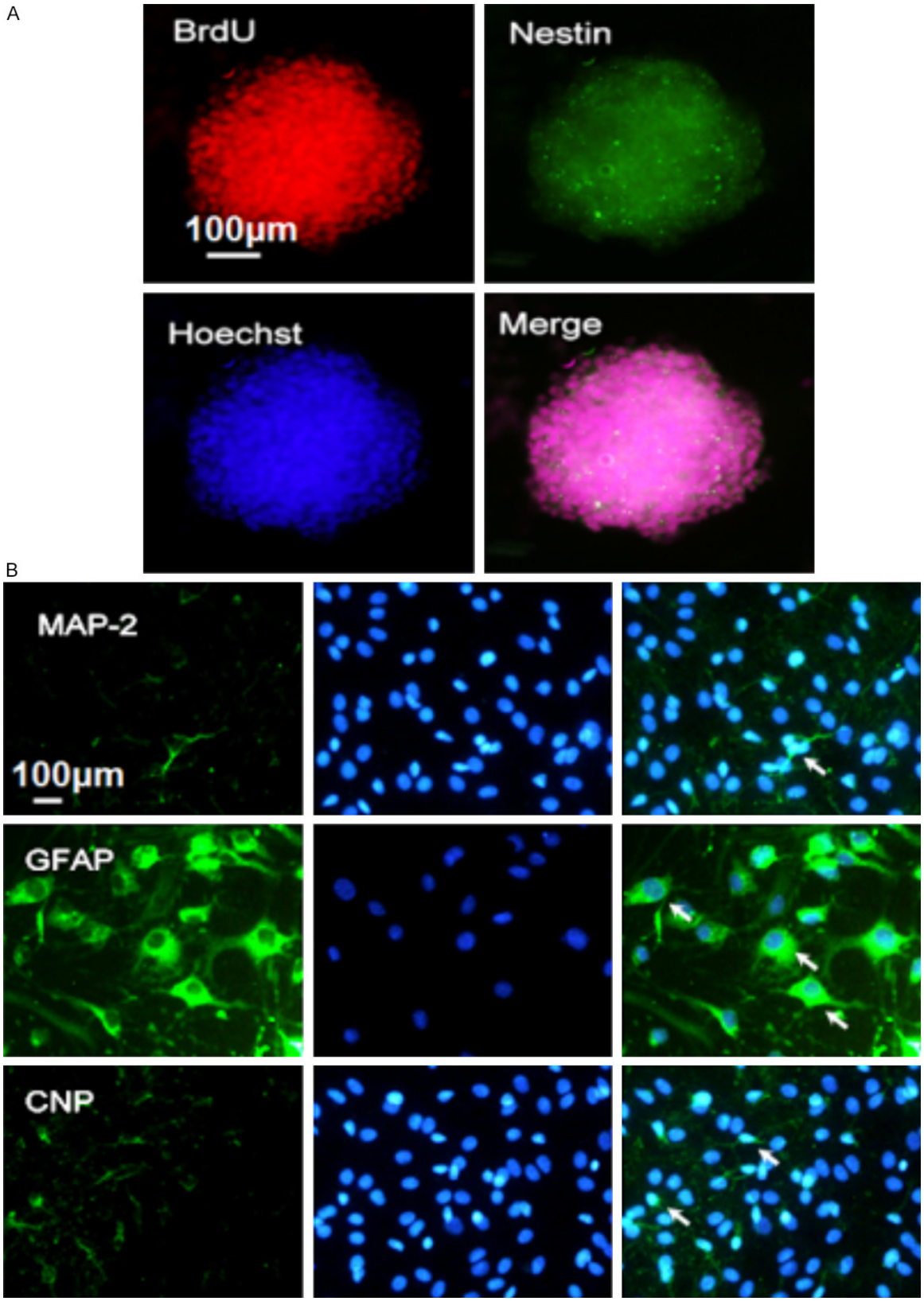

Figure 1. Identification of NSCs isolated from the injured cortex after TBI. A. The neurosphere was BrdU and nestin double-labeled positive. B. The neurosphere could differentiate into MAP-2$^+$ neurons, GFAP$^+$ astrocytes and CNP$^+$ oligodendrocytes. Arrows denote the double-positive cells.

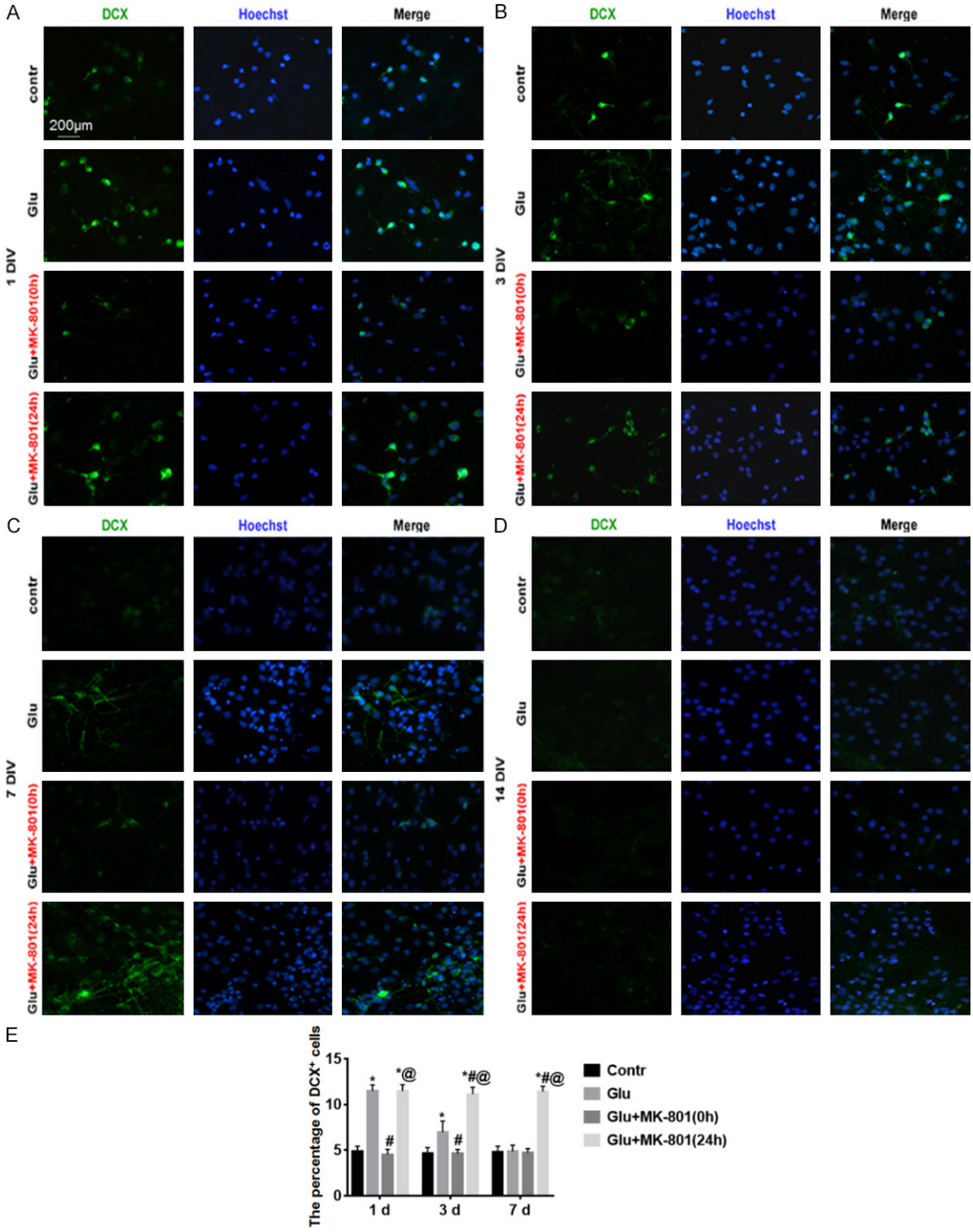

Figure 2. The effect of glutamate on the differentiation of NSCs into NPCs. A. The numbers of DCX^+ cells in the Glutamate and Glutamate+MK-801 (24 h) groups were more than those in the control and Glutamate+MK-801 (0 h) groups after culture 1 DIV. B. Many DCX^+ cells were found in the Glutamate+MK-801 (24 h) group after culture for 3 DIV. The number of DCX^+ cells in the Glutamate group was decreased but still higher than those in the control and Glutamate+MK-801 (0 h) groups. C. Many DCX^+ cells were still observed in the Glutamate+MK-801 (24 h) group after culture 7 DIV, and only a few DCX^+ cells were found in the other three groups. D. DCX^+ cells was hardly found in the four groups after culture 14 DIV. E. A statistic diagram for the percentage of DCX^+ cells in four groups after culture 1, 3, 7, 14 DIV. $^*P < 0.05$ VS. control; $^{\#}P < 0.05$ VS. Glutamate group; $^{@}P < 0.05$ VS. Glutamate+MK-801 (0 h) group. Data are represented as M±SD, n=6. Contr: Control; Glu: Glutamate.

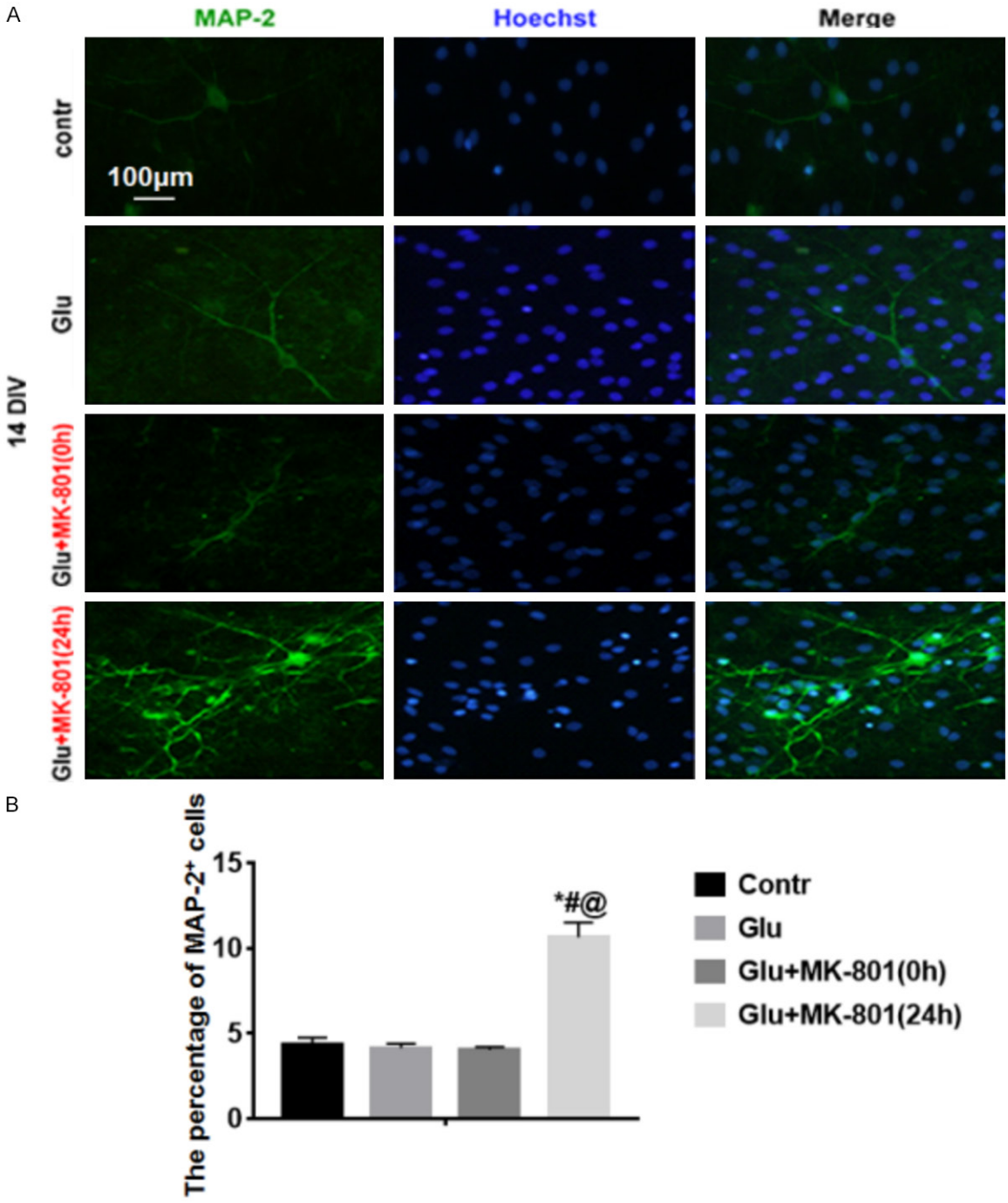

Figure 3. The effect of glutamate on the differentiation of NSCs into mature neurons. A. Many MAP-2$^+$ cells were observed in the Glutamate+MK-801 (24 h) group after culture for 14 DIV. Only a few MAP-2$^+$ cells were found in the other three groups. B. A statistic diagram for the percentage of MAP-2$^+$ cells in four groups. *P < 0.05 VS. control; $^{\#}P$ < 0.05 VS. Glutamate group; $^{@}P$ < 0.05 VS. Glutamate+MK-801 (0 h) group. Data are represented as M±SD, n=6. Contr: Control; Glu: Glutamate.

NR1 in the Glutamate+MK-801 (24 h) group was gradually increased, peaked on 7 DIV and then remained in a stable high level. The mRNA expression of NR1 in the control group was in a low level at all times. The level of NR1 mRNA in the Glutamate+MK-801 (24 h) group was higher than that in the control group at the corresponding time (**Figure 5B**).

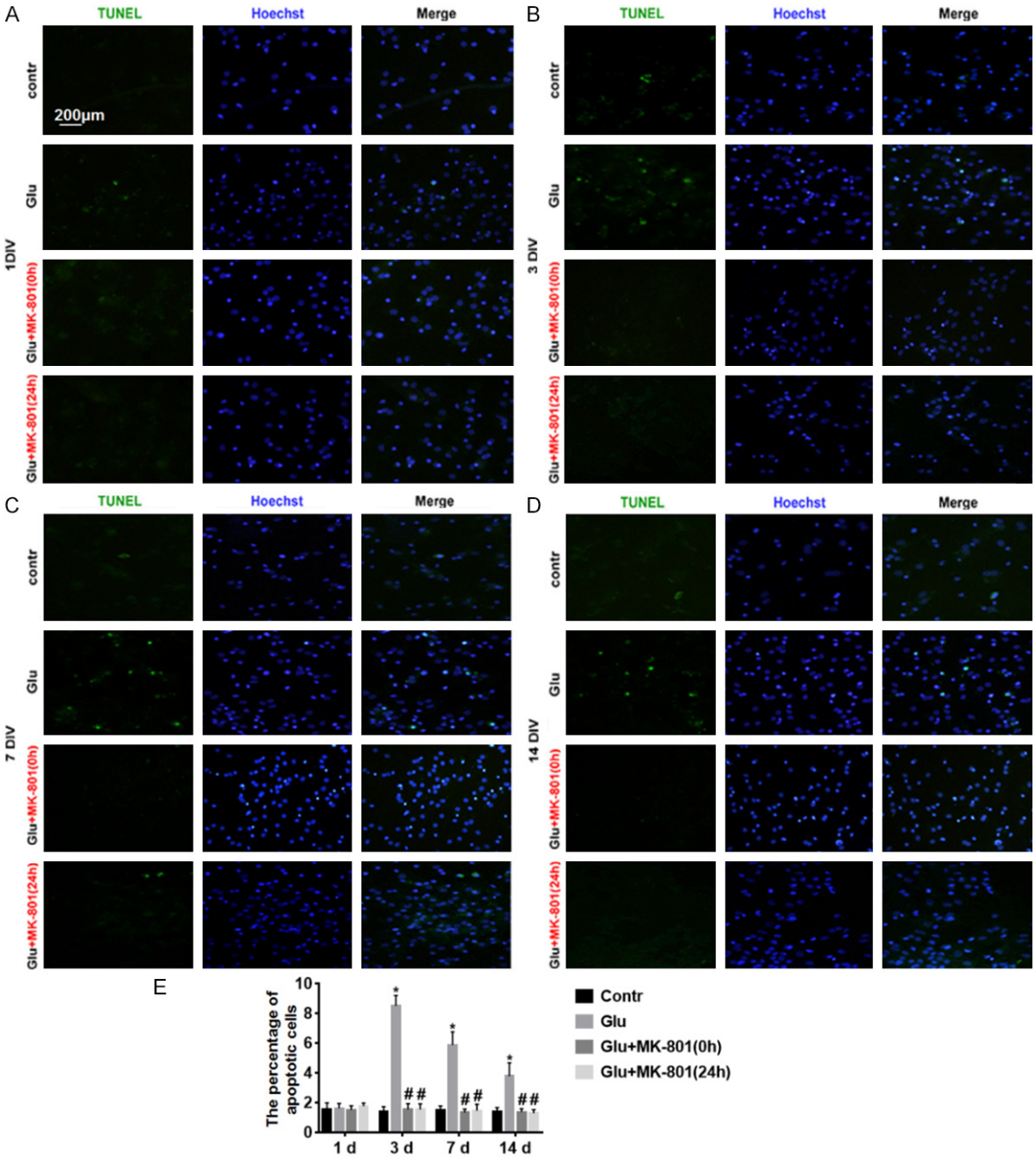

Figure 4. Effect of glutamate on cells apoptosis. A. A small number of apoptotic cells were found in the control, Glutamate, Glutamate+MK-801 (0 h) and Glutamate+MK-801 (24 h) groups on 1 DIV. B-D. After culture 3, 7, 14 DIV, the percentages of apoptotic cells in the control, Glutamate+MK-801 (0 h) and Glutamate+MK-801 (24 h) groups remained in a stable state but the percentage of apoptotic cells in the Glutamate group was increased and peaked on 3 DIV then decreased, but was still higher than those in the other three groups at the corresponding time. E. A statistic diagram for the percentages of TUNEL$^+$ cells in four groups. *P < 0.05 VS. control; $^{\#}P$ < 0.05 VS. Glutamate group; $^{@}P$ < 0.05 VS. Glutamate+MK-801 (0 h) group. Data are represented as M±SD, n=6. Contr: Control; Glu: Glutamate.

The differences were statistically significant. The change of NR1 protein expression was consistent with that of NR1 mRNA expression (**Figure 5C**).

Discussion

Our previous research showed that nestin$^+$/sox-2$^+$ NPCs appeared in the cortex around the

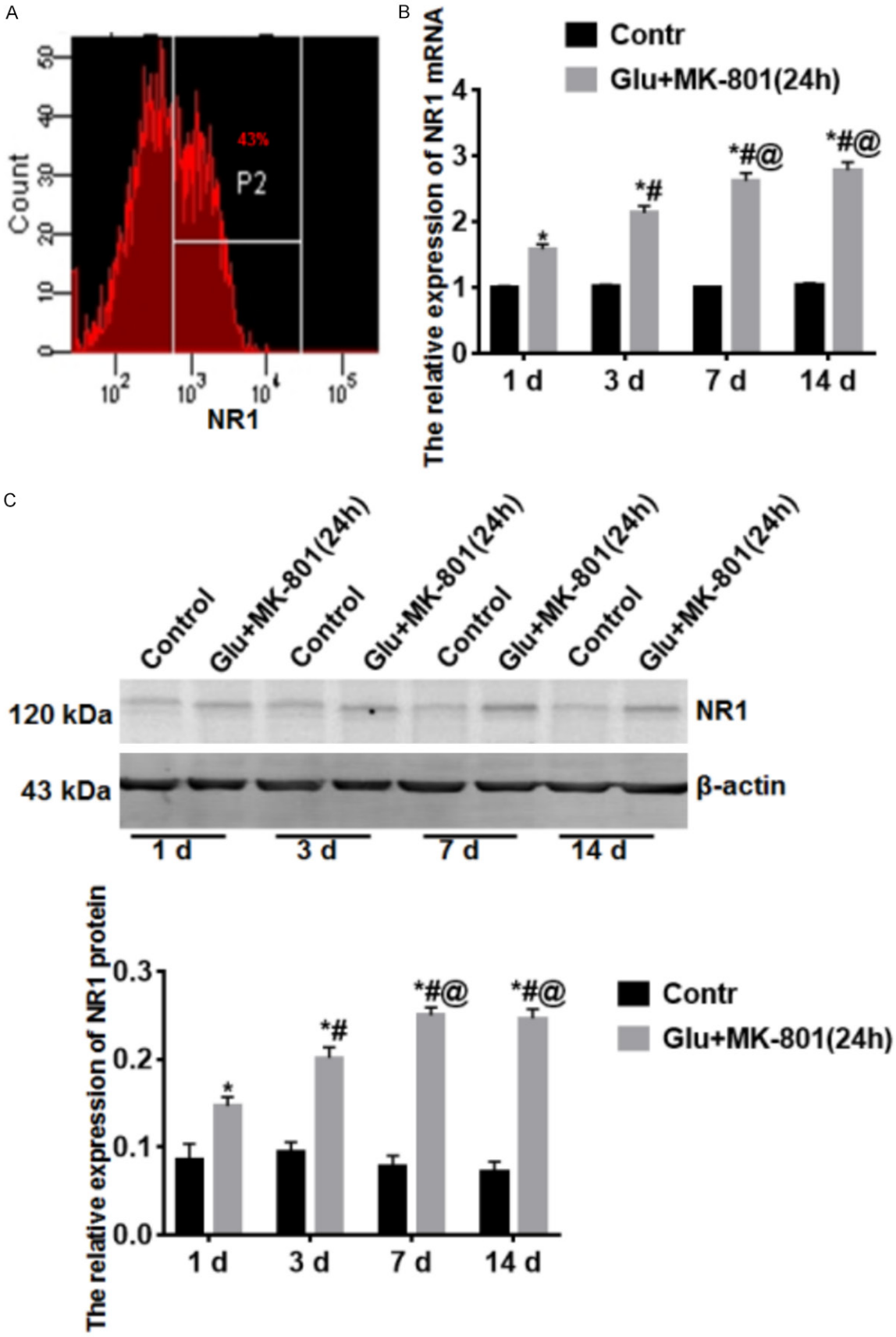
A
Count
43%
P2
NR1
B
Contr
Glu+MK-801(24h)
The relative expression of NR1 mRNA
1 d
3 d
7 d
14 d
C
Control
Glu+MK-801(24h)
120 kDa
NR1
43 kDa
β-actin
The relative expression of NR1 protein

Figure 5. The expression of NR1 in NSCs and differentiated cells. A. The expression of NR1 in NSCs was detected by Flow cytometry. The positive rate of NR1 in NSCs was about 43.00%. B. The relative expression of NR1 mRNA in the Glutamate+MK-801 (24 h) group increased gradually and peaked on 7 DIV then remained in a sable level. The mRNA expression of NR1 in the control group almost remained at a stable low level. The level of NR1 mRNA in the Glutamate+MK-801 (24 h) group was more than that in the control group at the corresponding time. C. The protein expression changes of NR1 in the control and Glutamate+MK-801 (24 h) groups were consistent with the changes of NR1 mRNA expression. $^{*}P < 0.05$ VS. control; $^{\#}P < 0.05$ VS. 1 d; $^{@}P < 0.05$ VS. 3 d. Data are represented as M±SD, n=6. Contr: Control; Glu: Glutamate.

injured area at 1, 3, 7, and 14 days after TBI in rats, and reached a peak at 3 days [16]. We chose to isolate and culture NSCs on 3 days after TBI in this research and BrdU/nestin immunofluorescence double labeling *in vitro* was applied to detect the exist of NSCs in the injured cortex. The results showed that BrdU/nestin double-labeled positive neurosphere originated from an isolated single cell from the injured cortex was obtained and it could differentiate into MAP-2^{+} neurons, GFAP^{+} astrocytes and CNP^{+} oligodendrocytes. These results suggest that NSCs appear in the injured cortex after TBI in adult rats. Therefore, it is basically consistent with the result of other scholars [13].

In the current research, the NSCs were treated with glutamate and NMDA receptor antagonist MK-801 (added with glutamate or 24 h later) *in vitro*, the neuronal differentiation of NSCs was detected by DCX and MAP-2 immunofluorescence, and the effect of glutamate on NSCs apoptosis was measured by TUNEL assay at the same time. Results showed that glutamate could promote the NSCs to differentiate to the NPCs at the initial stage of differentiation, but then the number of NPCs decreased. If MK-801 was treated at the same time and the above effect of glutamate was suppressed. Interestingly, if MK-801 was given 24 h after the treatment of glutamate, the percentage of neuronal differentiation could be significantly increased, and it was higher than that in the Glutamate group on 3 DIV and 7 DIV. On 14 DIV, there were almost no DCX^{+} NPCs in the four groups. The cells might have been dead or have matured and expressed DCX^{-}. These results indicate that glutamate initially promotes the differentiation of NSCs into NPCs, but it makes NPCs decrease with time. Only a small amount of MAP-2^{+} mature neurons were observed in the control, glutamate and Glutamate+MK-801 (0 h) groups on 14 DIV, while many MAP-2^{+} mature neurons were found in the Glutamate+MK-801 (24 h) group, with larger cell bodies and abundant processes. Moreover, we observed that glutamate promoted the cellular apoptosis during the cell development. There were almost no changes in the numbers of apoptotic cells in the control, Glutamate+MK-801 (0 h) and Glutamate+MK-801 (24 h) groups on 3, 7, and 14 DIV, while the number of apoptotic cells in the Glutamate group was increased and reached a peak on 3 DIV. Although it decreased slightly on 7 DIV and 14 DIV, it was still higher than those in the other three groups at the corresponding time. All these results indicate that glutamate may initially promote the neuronal differentiation of NSCs and subsequently induce the apoptosis of developing neurons by acting on NMDA receptors.

Why did glutamate act on the same NMDA receptors to promote the neuronal differentiation of NSCs at the beginning but then induce cellular apoptosis? What are the mechanisms of the different effects of glutamate? A functional NMDA receptor must contain the NR1 subunit, and multiple NR2 subunits and NR1 together form a tetramer (or pentamer). In the present research, flow cytometry was used to analyze the expression of NR1 in NSCs. The results showed that the positive rate of NR1 was about 43.00%, which was consistent with the results of other scholars who have observed lower expression of NMDA receptors in NSCs [21], while almost all neurons *in vivo* expressed NMDA receptors. Do gradually expressed NMDA receptors on the neuron during the development induce the two different effects of glutamate? In order to verify this hypothesis, real-time PCR and Western blot analysis were used to detect the expression of NR1 mRNA and protein in cells. Our results showed that the relative expressions of NR1 mRNA and protein in the Glutamate+MK-801 (24 h) group increased gradually and reached the peak on the 7th day, which preliminarily confirmed our hypothesis. Glutamate could promote the differentiation of NSCs into neurons at the begin-

ning stage; however, it subsequently induced neuronal apoptosis during differentiation, which may be related to the gradual increase of NMDA receptor expression in neurons during development. Some reports have shown that glutamate promotes hippocampal neurogenesis in rats through NMDA receptors [22], but other scholars have found that glutamate inhibits the neurogenesis [23]. So, glutamate seems to have a dual effect on neurogenesis and the results of our research may explain this phenomenon. But the concrete mechanisms underlying the process needs the further research.

Conclusions

NSCs appear in the cortex of the injured area after TBI in adult SD rats. Glutamate promotes the differentiation of NSCs into neurons at the initial stage. However, glutamate also induces neuronal apoptosis during differentiation, which may be related to the gradual increase of NMDA receptor expression during the development of neurons. NMDA receptor antagonist MK-801 can counteract the effect of glutamate.

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Disclosure of conflict of interest

None.

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