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# Glutamate promotes neural stem cell proliferation by increasing the expression of vascular endothelial growth factor of astrocytes *in vitro*

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**Abstract**

The high levels of glutamate might involve in neurogenesis after brain injuries. However, the mechanisms are not fully understood. In this study, we investigated the effect of glutamate on the proliferation of rat embryonic neural stem/progenitor cells (NSCs) through regulating the vascular endothelial growth factor (VEGF) expression of astrocytes (ASTs) in vitro, and the cyclin D1 expression of NSCs. The results showed that glutamate promoted the expression and secretion of VEGF of rat astrocytes by activating group I mGluRs. Astrocyte conditioned medium-containing Glu [ACM (30%)] promoted the proliferation of embryonic NSCs compared with normal astrocyte conditioned medium+Glu [N-ACM (30%)+Glu (30 μM)] by increasing cell activity, diameter of neurospheres, bromodeoxyuridine (BrdU) incorporation and cell division; while ACM+VEGF neutralizing antibody [ACM (30%)+VEGF NAb (15 μg/ml)] significantly inhibited the proliferation of embryonic NSCs compared with ACM (30%). ACM (30%) increased the expressions of cyclin D1 and decreased cell death compared with N-ACM (30%)+Glu (30 μM). ACM (30%)+VEGF NAb (15 μg/ml) decreased the expressions of cyclin D1 and increased cell death compared with ACM (30%). These results demonstrated that glutamate could also indirectly promote the proliferation of rat embryonic NSCs through inducing the VEGF expression of ASTs in vitro, and VEGF may increase the expression of cyclin D1. These finding suggest that glutamate may be a major molecule for regulating embryonic NSC proliferation and facilitate neural repair in the process of NSC transplants after brain injuries.

***Key words:*** Neural stem cells, glutamate, vascular endothelial growth factor, proliferation, astrocytes, astrocyte conditioned medium.

## Introduction

Neural stem/progenitor cells (NSCs) are endowed with the capacity to self-renew and differentiate into neurons, astrocytes, and oligodendrocytes. They play a major role in the development of the embryonic central nervous system (CNS) and continue to function throughout adulthood (1,2). They are present in the newborn and adult in special brain areas. In the adult mammalian brain, NSCs are maintained in two neurogenic niches, the forebrain subventricular zone (SVZ) around the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus. At present, NSCs as treatment for CNS diseases have been considered to be very effective for the improvement of neurologic function in nerve injury, brain ischemia and neurodegenerative disorders (3,4). But the molecular mechanisms are still not fully understood. Therefore, it is very important to investigate the cellular biological processes that govern the proliferation of NSCs.

Glutamate, a major excitatory neurotransmitter in CNS, can be released from neurons following brain hypoxia/ischemia damage, which further causes consequent neuronal death through the activation of ionotropic glutamate receptors (iGluRs) in neurons (5). However, previous studies have shown that exogenous glutamate could promote survival and proliferation of NSCs derived from the SGZ through the activation of metabotropic glutamate receptors (mGluRs) (6). It is reported that the activation of mGluR5 and mGluR7, might promote the proliferation and differentiation of embryonic NSCs by influencing phosphorylation of RAS MAPK signaling pathways which include membrane-to-nucleus signaling modules that are involved in the regulation of multiple biological and physiological processes (7-11). In addition, astrocytes express a wide variety of cell surface ion channel and metabotropic receptors. Among the neurotransmitter receptors, metabotropic glutamate receptors have been proposed to be important in the development of CNS. Astrocytes are involved in the cell-to-cell communications with neurons in the CNS by generating cytokines and growth factors (12). For example, the production of vascular endothelial growth factor (VEGF) in astrocytes increased in response to brain hypoxia or ischemia (13). VEGF has been widely studied in the context of ischemic brain injury, and published studies using exogenous strategies of VEGF delivery have identified a variety of effects besides angiogenesis, such as the promotion of neuronal survival, anti-inflammatory, plasticity-promoting actions, and proliferation of NSCs. In injured brain, one significant way to promote differentiation and survival of transplanted NSCs is to modulate microenvironment and this might be accomplished by supplying additional neurotrophic growth factors such as brain derived neurotrophic factor (BDNF) and VEGF which

are known to play key roles in proliferation, differentiation and survival of NSCs (14,15). VEGF is one of such growth factors which could be used in combination with transplanted NSCs to improve therapeutic efficiency of cellular transplantation.

Embryonic cortex NSCs are usually used as therapeutic transplants into a mature brain. But the role of glutamate in regulating proliferation of transplanted embryonic NSCs after nerve injury and its mechanisms are not fully understood. In this experiment, we examined the effects of glutamate on proliferation of embryonic cortex NSCs by mediating astrocytes and investigated its mechanisms in vitro.

## Materials and methods

### *Animals and ethics statement*

Sprague-Dawley (SD) rats were housed and cared for by the Experimental Animal Center of Xi'an Jiaotong University College of Medicine. All animal experimentation was in accordance with the Society for Neuroscience's policy on the appropriate use of animals for neuroscience research and in accordance with the National Institutes of Health Guide for the care and use of laboratory animals (NIH Publication 80-23, rev. 1996). Experimental protocols were approved by the Ethics Committee of Xi'an Jiaotong University College of Medicine. All efforts were made to minimize the number of animals used and their suffering.

### *Isolation and culture of primary rat astrocytes*

Neonatal Sprague-Dawley (SD) rats (day 0–3) were obtained from the Experimental Animal Center of Xi'an Jiaotong University College of Medicine. To obtain astrocytes, cortical tissues were isolated as previously described with some modifications (16). Briefly, 10 postnatal day 1–3 SD rats were decapitated, and the cortices were removed and digested with 0.125% trypsin for 20 min at 37 °C. After trituration and centrifugation at 800 rpm for 6 min, the cells were resuspended and plated at an initial concentration of 100,000 cells/ml on culture flasks coated with 1 mg/mL poly-L-lysine. The cells were then cultured in DMEM and F12 (1:1) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin. After 1 day, the medium was changed to fresh DMEM/F12 and replaced every 3 days. These mixed glial cells were cultured for 9 days at 37 °C in a 5% $CO_2$ atmosphere incubator. Astrocytes were purified from the mixed culture by mild shaking (37 °C, 150 g/min, 15 h), as described previously (16,17). The isolated astrocytes were cultured in DMEM/F12 for experiments as described below. The supernatant from astrocytes cultured with fresh DMEM/F12 alone for 48 h was collected to be used as normal ACM (N-ACM). The supernatant from astrocytes cultured with fresh DMEM/F12 containing 100 μM Glu for 48 h was collected to be used as ACM. At least, three independent experiments were performed for each assay.

### *Culture of primary rat embryonic NSCs*

NSCs from cortex of 20 embryonic day 15 (E15) fetal SD rats were isolated and propagated using the neurosphere method as described previously (18). In brief, the cortex was microdissected, cut into small pieces, and incubated in a digestion solution (trypsin 0.01%, EDTA 200 mM, glucose 0.6%, MgCl2 1 mM in PBS [all from Sigma, St. Louis, MO]) at 37 °C for 10 min. Then, the tissues were mechanically dissociated into single-cell suspensions. The single-cell suspension was resuspended in serum-free DMEM/F12 (Dulbecco's modified Eagle medium and Ham's F12 [1:1] 10 ng/ml bFGF, 20 ng/ ml EGF, 1% penicillin, 1% streptomycin, 1% N2, and 2% B27 supplement [all from Invitrogen, Carlsbad, CA] and 2.5 lg/ml heparin [Sigma]). Cells were plated at an initial concentration of 100,000 cells/ml in 50-ml cell culture flasks at 37 °C with 5% $CO_2$ for culture. After 5-7 days in vitro (DIV), the primary neurospheres were passaged. The neurospheres were dissociated with 0.05% trypsin and 200 μM EDTA for 8 min at 37 °C and mechanically triturated into a single-cell suspension. The single cells were cultured at a density of 50,000 cells/ml for 5 days, when neurospheres of 90-120 μm in diameter had been propagated (passage 1 neurospheres). Passage 1 neurospheres were processed for experiments as described below, and at least three independent experiments were performed for each assay.

### *Immunocytochemistry*

For immunofluorescent staining, astrocytes and neurospheres were fixed with chilled methanol and acetone for 20 min and then blocked with 10% normal goat serum in PBS containing 0.3% Triton X-100 for 1 h at room temperature. The primary antibodies were diluted in PBS containing 3% bovine serum albumin. Polyclonal rabbit anti-glial fibrillary acidic protein (anti-GFAP) antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, USA) was used to identify astrocytes and monoclonal mouse anti-nestin antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, USA) was used to identify NSCs. respectively. Cells were incubated with primary antibodies at 4 °C overnight, and were then washed with PBS for 10 min three times. Subsequently, the anti-rabbit IgG-PE (1:1000; Santa Cruz Biotechnology, Santa Cruz, USA) or anti- mouse IgG-FITC (1:1000; Santa Cruz Biotechnology, Santa Cruz, USA) was added, followed by 2 h of incubation, and DAPI or PI was used to counterstain the nucleus for 5 min. Negative controls lacking the primary antibody were used to eliminate nonspecific staining. Immunostained cells were visualized by indirect fluorescence under a fluorescent microscope (Olympus BX51; Olympus, Tokyo, Japan) equipped with a DP70 digital camera and the DPManager (DPController) software (Olympus).

### *Real-time quantitative PCR analysis*

To analyze the expression of mRNA, astrocytes were treated by Glu (1, 10, 100, 1000 μM), or/and mGluRs antagonist MCPG (1 mM), group I mGluRs agonist DHPG (200 μM), group II GluRs agonist DCG-IV (50 μM), group III mGluRs agonist L-AP4 (50 μM), group I mGluRs antagonist AIDA (200 μM), group II mGluRs antagonist LY341495 (10 μM), group III mGluRs antagonist UBP1112 (100 μM) using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA was extracted from the cells, quantified by spectrophotometry (Nano-Drop™

Wilmington, USA). The RNA was reverse transcribed to cDNA using a reverse transcriptase kit (PrimeScript™ RT reagent Kit, TaKaRa Bio. Co. Ltd., USA). Relative abundance of each mRNA sample was quantified by Q-PCR using the corresponding primers and the SYBR® Premix Ex Taq™II (TaKaRa. Bio. Co. Ltd., USA). Primers for rat VEGF (forward 5′-AGGCTGCACC-CACGACAGAAG-3′; reverse 5′- TCACCGCCTTG-GCTTGTCAC-3′) and rat β-Actin (forward 5′- GGA-GATTACTGCCCTGGCTCCTA-3′; reverse 5′-GACT-CATCGTACTCCTGCTTGCTG-3′) were synthesized by TaKaRa. Real-time PCR reactions were carried out using iQ Multicolor Real-Time PCR Detection System (Bio-Rad, USA). Cycle threshold values were obtained from the Bio-Rad iQ5 2.0 Standard Edition optical System software (Bio-Rad, USA). Data were analyzed using the ΔΔCT method and β-actin served as an internal control. The results were presented as mean ± SEM of three separate experiments.

***Western blot analysis***

Astrocytes (100,000 cells/ml) seeded in 6-well plates were treated by Glu (1, 10, 100, 1000 μM), or/and MCPG (1 mM), MCPG (1 mM), DHPG (200 μM), DCG-IV (50 μM), L-AP4 (50 μM), AIDA (200 μM), LY341495 (10 μM), UBP1112 (100 μM) for detecting VEGF expression. Passage 1 rat NSCs were treated by N-ACM (30%), ACM (30%) and ACM (30%) + VEGF NAb (15 μg/ml) for analyzing Cyclin D1. Then, the cells were lysed in RIPA lysis buffer. Insoluble material was removed by centrifugation at 12,000 rpm for 12 min at 4 °C. Cell lysates were subjected to electrophoresis using 10% SDS polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with primary monoclonal antibodies overnight at 4 °C and then secondary antibody for 2 h at room temperature. The primary monoclonal antibodies included mouse monoclonal anti-VEGF (1:1000, Santa Cruz, CA, USA), mouse monoclonal anti-Cyclin D1 (1:1000, Neomarker, Fremont, CA, USA), and mouse monoclonal anti-β-Actin (1:5000, Santa Cruz, CA, USA). The membranes were incubated with ECL (Amersham) for chemiluminescence detection. The luminescent signal was detected by CCD camera, recorded and quantified with Syngene G Box (Syngene, UK).

***ELISA***

To determine the VEGF level secreted by astrocytes, 200 μl supernatant was collected from the media after 24 h culture in the presence of Glu (1, 10, 100, 1000 μM), MCPG (1 mM), MCPG (1 mM), DHPG (200 μM), DCG-IV (50 μM), L-AP4 (50 μM), AIDA (200 μM), LY341495 (10 μM), UBP1112 (100 μM). The concentration was measured using a VEGF ELISA kit (R&D, Minneapolis, MN) following the suggested protocol. The optical densities were determined within 30 min and recorded with a microplate reader (BioTek, EXL800, Winooski, VT) at 450 nm.

***MTT assay and diameter measuring of neurospheres***

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based assay was utilized to estimate the effect of ACM on rat NSC proliferation. Passage 1 rat NSCs were seeded into 96-well plates (20,000 cells/well in 200 μl medium) and incubated for 2 days at 37 °C in 5% $CO_2$. NSCs were treated with DMEM/F12+30% Normal ACM (N-ACM) (volume/volume), DMEM/F12+30% N-ACM+ Glu (30 μM), DMEM/F12+30% ACM-containing 100 μM Glu (ACM), and DMEM/F12+30% ACM-containing 100 μM Glu+VEGF Nab (15μg/ml) for 1, 2 and 3 days, respectively, ACM was obtained from medium which astrocytes were treated by Glu (100 μM) for 24 h. The concentration of Glu is around 30 μM in DMEM/F12+30% N-ACM+ Glu (30 μM), DMEM/F12+30% ACM-containing 100 μM Glu (ACM), and DMEM/F12+30% ACM-containing 100 μM Glu+VEGF Nab (15μg/ml) groups. Neurospheres cultured with complete medium at the same duration were used as blank control. At the end of culture, diameters of 10 neurospheres/well were measured using DP71 camera (ver 5.1, Olympus, Japan), total 50 neurospheres/group were measured; then 20 μl of 5 mg/ml MTT (Sigma, St Louis, MO, USA) solution was added per well and the cells were incubated for another 4 h at 37 °C. Supernatants were removed and formazan crystals were dissolved in 150 μl of dimethylsulfoxide (Sigma, St Louis, MO, USA). Finally, optical density was determined at 490 nm using multi-microplate test system (POLARstar OPTIMA, BMG Labtechnologies, Germany). In each assay, five parallel wells were made, and the assay was conducted in quadruplicate.

***Bromodeoxyuridine incorporation and immunocytochemistry***

The effect of ACM on rat NSC proliferation was investigated by bromodeoxyuridine (BrdU; Sigma) incorporation. Passage 1 NSCs were cultured for 2 days and treated with DMEM/F12+30% Normal ACM (N-ACM), Normal ACM (N-ACM)+ Glu (30 μM), DMEM/F12+30% ACM-containing 100 μM Glu (ACM), and DMEM/F12+30% ACM-containing 100 μM Glu+VEGF Nab (15μg/ml) for 2 days. The neurospheres were labeled with 10 μM BrdU for 4 h, then dissociated into single cells and plated onto poly-L-lysine-coated coverslips at a concentration of 50,000/ml per well in 24-well plates. After 6 h of attachment, the cells were fixed in 4% paraformaldehyde in PBS and processed for immunocytochemical staining. In brief, the cells were incubated in 2 N hydrochloric acid for 30 min at 37 °C, and 0.1 M sodium borate (pH 8.5) for 10 min. Cells were incubated overnight with mouse monoclonal anti-BrdU antibody (1:200; Chemicon, Billerica, MA) in PBS containing 0.1% Triton X-100 and 2% bovine serum albumin (BSA) at 4 °C. The cells were reacted with FITC-conjugated antimouse antibody (1:200) for 1 h at room temperature. Labeled cells were further counterstained using 50 μg/ml propidium iodide (PI; Sigma) and mounted. BrdU-labeled cells were counted using fluorescence microscopy and normalized to the total cell number (PI-stained cells).

***Cell cycle analysis***

DNA content and cell cycle distribution were determined using FACSort Cellquect software (BD Biosciences, San Jose, CA, USA). Passage 1 rat NSCs were cultured in 6-well plates (200,000 cells/well in 2 ml medium) and incubated for 2 days. NSCs were treated by N-ACM (30%), Normal ACM (N-ACM)+ Glu

(30 μM), ACM (30%) and ACM (30%) + VEGF NAb (15 μg/ml) for 2 days, dissociated into single-cell suspension, and then fixed in 75% ice cold ethanol overnight at 4 °C. The fixed cells were stained with 50 μg/ml Propidium Iodide (PI) containing 50 μg/ml RNase A (DNase free) for 25 min at room temperature in the dark, and then excited at 488 nm. The emission was collected through a 630 nm filter. The collected cells were analyzed by fluorescence activated cell sorting and Modfit LT software (BD Biosciences, San Jose, CA, USA). The cell cycle distribution was evaluated by calculating the proportion of cells in G0/G1, S, and G2/M stages. In each independent experiment, three parallel wells were made, and the procedures were carried out in triplicate.

### *Apoptosis analysis*

NSCs were cultured in 6-well plates (200,000 cells/well in 2 ml medium) for 2 days. Then, rat NSCs were treated with N-ACM (30%), Normal ACM (N-ACM)+ Glu (30 μM), ACM (30%) and ACM (30%) + VEGF NAb (15 μg/ml) for 2 days, and dissociated into a single cell suspension. The cells were labeled by incubation with 10 μL PI and 5 μL FITC-Annexin V at 250 μg/ml for 10 min in the dark at room temperature. Cells then were washed with PBS and examined using flow cytometry. The apoptosis of cells was detected using the Annexin V/PI Apoptosis Detection Kit by FACS. Early apoptotic cells were identified with PI negative and FITC Annexin V positive; cells that were in late apoptosis or already dead were both PI and FITC Annexin V positive.

### *Statistical analysis*

Statistical analysis was performed using one-way analysis of variance (ANOVA). Tukey's post-hoc analyses were used to assess the difference between groups. All the quantitative data were presented as mean ± SEM. Statistical significance was considered for $P$ values < 0.05.

## Results

### *Effect of glutamate on VEGF expression of rat astrocytes*

Using real-time Q-PCR, Western blotting and ELISA, we analyzed the change of VEGF expression and the level of VEGF secreted in rat astrocytes after treatment with Glu (1, 10, 100, 1000 μM) for 24 h. The culture astrocytes were identified by staining with GFAP (Fig 1A). The results indicated that the expression of VEGF and the level of VEGF secreted in supernatant increased remarkably after treatment with Glu (10, 100, 1000 μM), their values were max after treatment with 100 μM Glu (Fig. 1B-D; $P < 0.05$). The expression of VEGF and the level of VEGF secreted in supernatant increased significantly after treatment with 100 μM Glu for 16 h, 24 h and 48 h (Fig. 2A-C; $P < 0.05$).

### *Glutamate promoted VEGF expression and secretion of rat astrocytes by acting at group I mGluRs*

To determine which mGluRs promotes VEGF expression and secretion of rat astrocytes activated by glutamate, astrocytes were treated with Glu (100 μM) or / and mGluRs antagonist MCPG (1 mM). Then, astrocytes were treated with DHPG (200 μM), DCG-IV (50 μM), L-AP4 ( 50 μM), AIDA (200 μM); LY341495 (10 μM), UBP1112 (100 μM) for 1 day, respectively. The results showed that 100 μM Glu increased significantly VEGF expression and secretion of rat astrocytes, but 1 mM MCPG decreased remarkably VEGF expres-

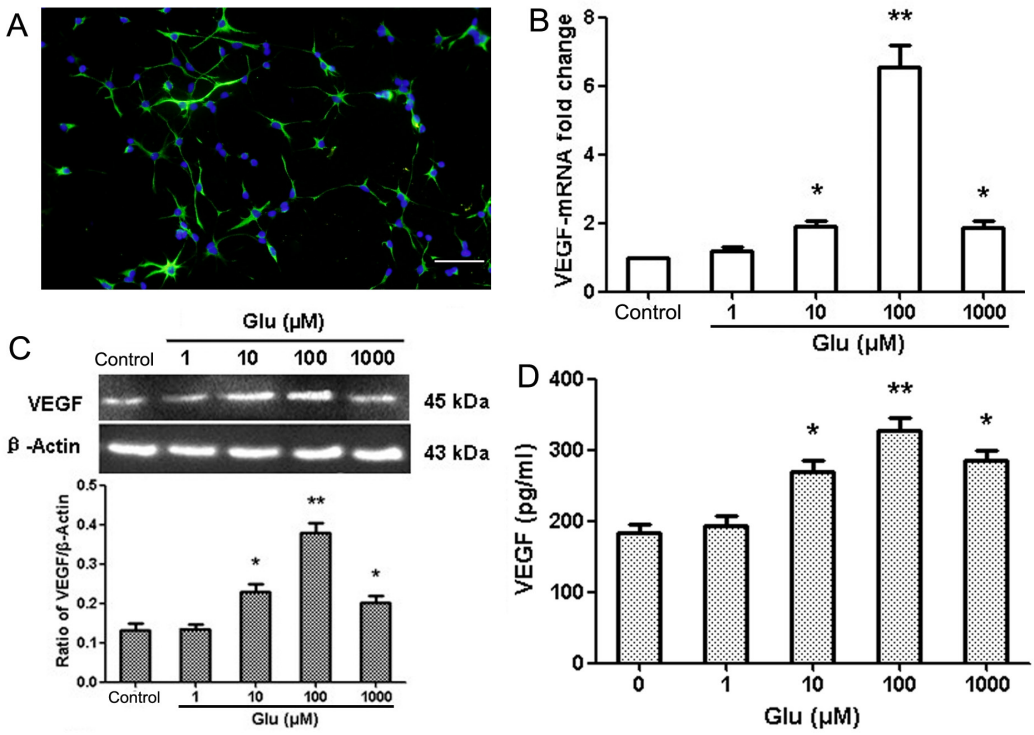


**Figure 1.** Effect of glutamate (Glu) on VEGF expression of rat astrocytes at different concentrations. (A) Astrocytes stained with GFAP (Green), nuclei were counterstained with DAPI (Blue), Scale bars=50 μm. (B) Real-time Q-PCR products. (C) Western blotting analysis, β-Actin was used as a loading control. (D) ELISA analysis (* $P < 0.05$, ** $P < 0.01$, compared with control group, n = 3).

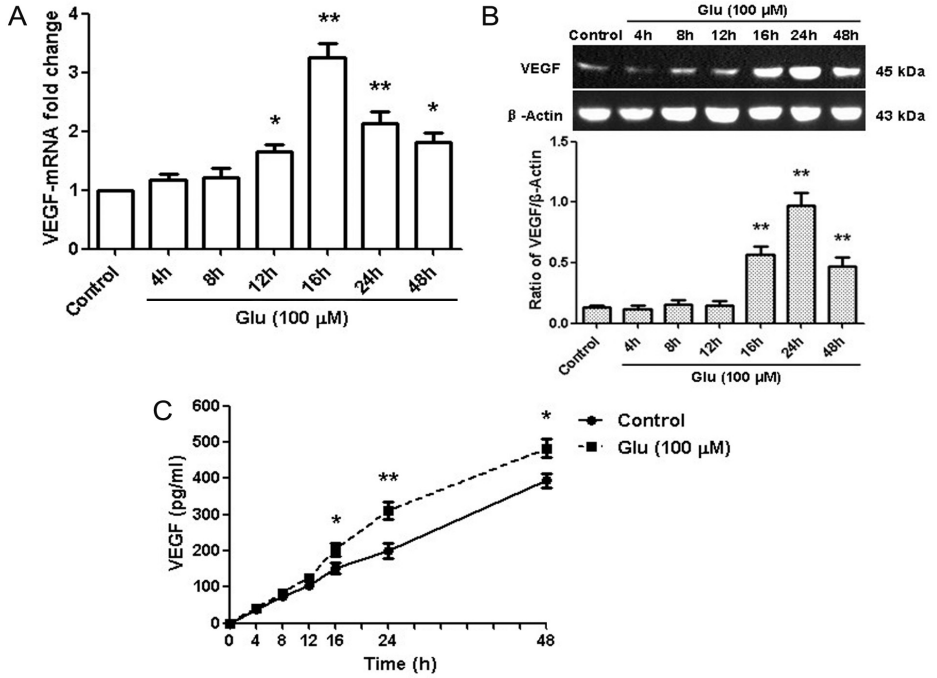


**Figure 2.** Effect of glutamate (100μM) on VEGF expression of rat astrocytes at different times. (A) Real-time Q-PCR results. (B) Western blotting results, β-Actin was used as a loading control. (C) ELISA results (* $P < 0.05$, ** $P < 0.01$, compared with control group, n = 3).

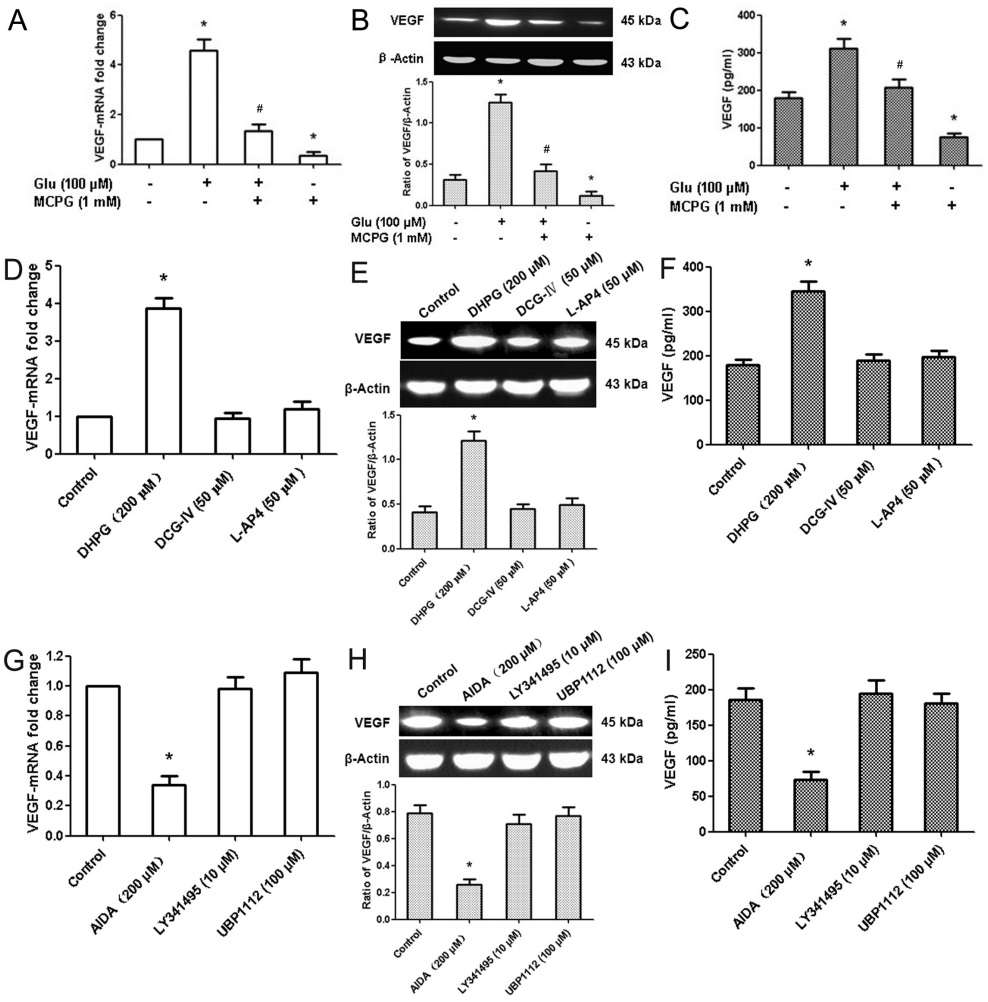


**Figure 3.** Glutamate promoted VEGF expression and secretion of rat astrocytes by acting at group I mGluRs. (A) Effect of Glu and MCPG on VEGF mRNA of rat astrocytes. (B) Effect of glu and MCPG on VEGF protein of rat astrocytes. (C) Effect of glu and MCPG on VEGF secretion of rat astrocytes. (D) Group I mGluRs agonist DHPG increased significantly VEGF mRNA expression of rat astrocytes. (E) Group I mGluRs agonist DHPG increased significantly VEGF protein expression of rat astrocytes. (F) Group I mGluRs agonist DHPG increased significantly VEGF secretion of rat astrocytes. (G) Group I mGluRs antagonist AIDA decreased significantly VEGF mRNA expression of rat astrocytes. (H) Group I mGluRs antagonist AIDA decreased significantly VEGF protein expression of rat astrocytes. (I) Group I mGluRs antagonist AIDA decreased VEGF secretion of rat astrocytes. (* $P < 0.05$, compared with control group; # $P < 0.05$, compared with 100 μM Glu group, n = 3).

sion and secretion of rat astrocytes, 100 μM Glu+1 mM MCPG decreased VEGF expression and secretion of rat astrocytes compared with the 100 μM Glu group (Fig. 3A-C; $P < 0.05$). In addition, 200 μM DHPG increased significantly VEGF expression and secretion of rat astrocytes, but the VEGF expression and secretion of rat astrocytes had no change in 50 μM DCG-IV and 50 μM L-AP4 groups compared with control (Fig. 3D-F; $P < 0.05$). 200 μM AIDA decreased VEGF expression and secretion of rat astrocytes, while the VEGF expression and secretion of rat astrocytes had no change in 10 μM LY341495 and 100 μM UBP1112 groups compared with control (Fig. 3G-I; $P < 0.05$).

### *Effect of ACM on the proliferation of rat embryonic NSCs*

To detect whether ACM-containing Glu plays an important role in the proliferation of rat NSCs, passage 1 neurospheres were treated with N-ACM (30%), N-ACM (30%)+Glu (30 μM), ACM (30%) and ACM (30%)+VEGF NAb (15 μg/ml) for 1, 2 and 3 days. MTT assay was employed to analyze the cell activity, and diameter measuring of neurospheres was applied to represent the cell growth. The culture rat NSCs were showed in Fig 4A. Neurospheres were stained with nestin, and plenty of cells were nestin-positive cells (Fig 4B). The results showed that N-ACM (30%), N-ACM (30%)+Glu (30 μM), ACM (30%) and ACM (30%)+VEGF NAb (15 μg/ml) increased significantly the activity of rat NSCs compared with control ($P < 0.05$); ACM (30%) increased remarkably the activity of rat NSCs compared with N-ACM (30%)+Glu (30 μM) ($P < 0.05$); meanwhile, there were no differences in ACM (30%)+VEGF NAb (15 μg/ml) groups compared with N-ACM (30%)+Glu (30 μM); and the cell activity were remarkably decreased in ACM (30%)+VEGF NAb (15 μg/ml) group compared with the ACM (30%) group (Fig. 4C; $P < 0.05$). N-ACM (30%), N-ACM (30%)+Glu (30 μM), ACM (30%) and ACM (30%)+VEGF NAb (15 μg/ml) increased the mean diameter of neurospheres compared with control ($P < 0.05$); there were no differences in and ACM (30%)+VEGF NAb (15 μg/ml) groups compared with N-ACM (30%)+Glu (30 μM); ACM (30%) increased the mean diameter of neurospheres compared with N-ACM (30%)+Glu (30 μM) ($P < 0.05$); however, ACM (30%)+VEGF NAb (15 μg/ml) significantly

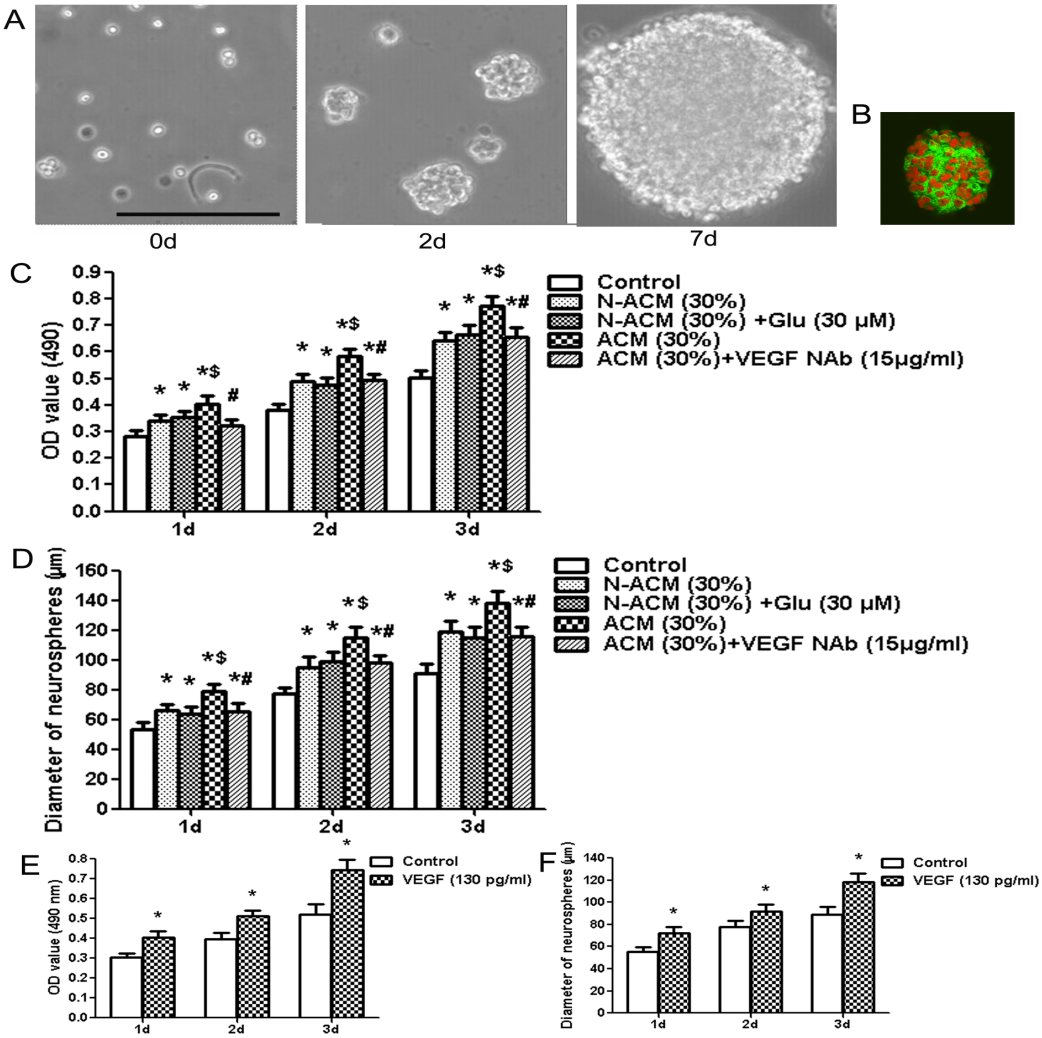


**Figure 4.** ACM affected the proliferation of rat NSCs. NSCs were cultured in DMEM/F12 alone (Control), DMEM/F12+30% Normal ACM (N-ACM), DMEM/F12+30% Normal ACM (N-ACM)+30μM Glu, DMEM/F12+30% ACM-containing 100 μM Glu (ACM), and DMEM/F12+30% ACM-containing 100 μM Glu+VEGF Nab (15μg/ml) for 1, 2, 3 days, respectively. (A) Culture of rat NSCs, Scale bars=100 μm. (B) Neurospheres stained with nestin (Green), nuclei were counterstained with PI (Red). (C) MTT assay for effects of ACM on rat NSCs activity in culture. (D) ACM affected rat neurosphere diameters in culture. (E) MTT assay for effects of VEGF on rat NSCs activity in culture. (F) VEGF affected rat neurosphere diameters in culture (* $P < 0.05$, compared with control group; $ $P < 0.05$, compared with N-ACM+30μM Glu group; # $P < 0.05$, compared with ACM (30%) group, n = 5).

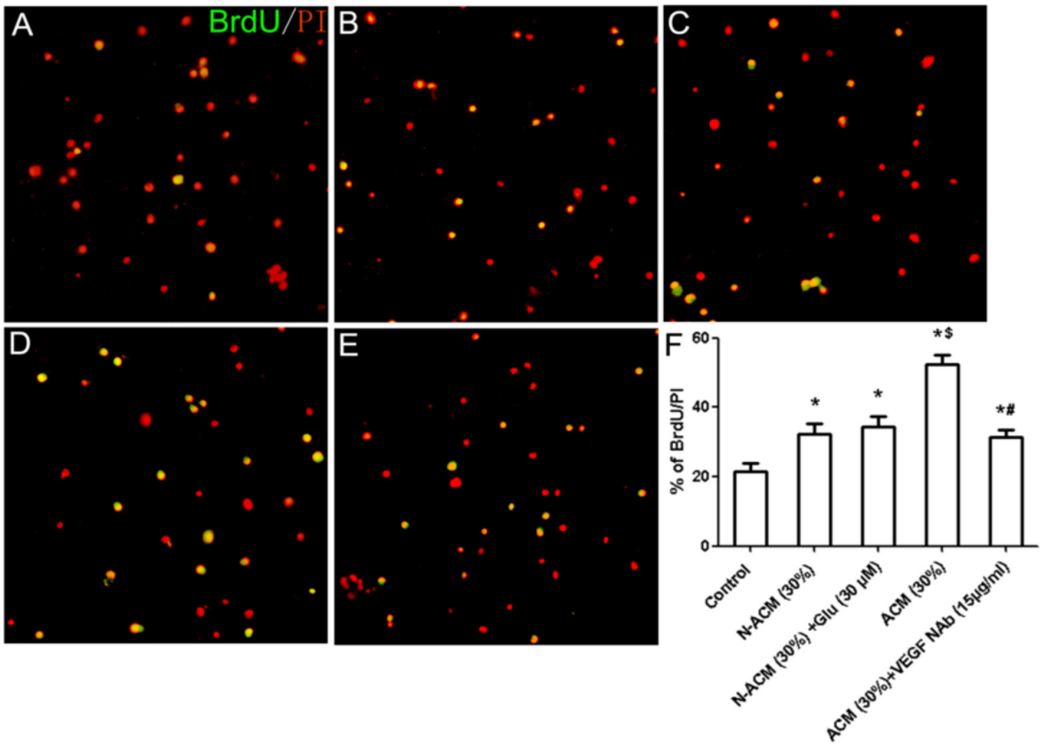


**Figure 5.** Effects of ACM on BrdU incorporation of rat NSCs. A-E show BrdU-labeled cells in DMEM/F12 alone (Control), DMEM/F12+30% Normal ACM (N-ACM), DMEM/F12+30% Normal ACM (N-ACM)+30μM Glu, DMEM/F12+30% ACM-containing 100 μM Glu (ACM), and DMEM/F12+30% ACM-containing 100 μM Glu+VEGF Nab (15μg/ml) groups, respectively. F shows the statistical percentage of BrdU-labeled cells in all groups (* $P < 0.05$, compared with control group; $ $P < 0.05$, compared with N-ACM+30μM Glu group; # $P < 0.05$, compared with ACM (30%) group, n = 3). Scale bars=100 μm.

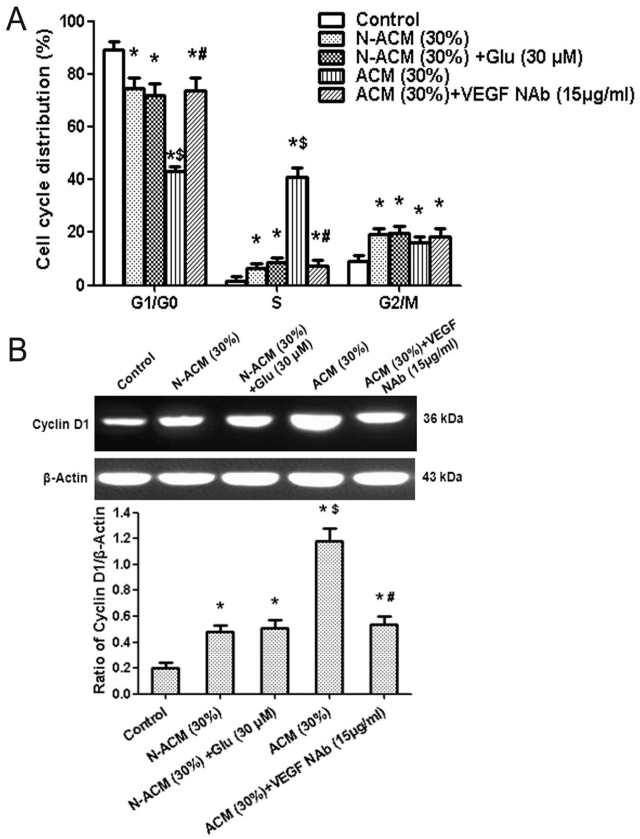


**Figure 6.** ACM regulated cell cycle and cyclin D1 expression of rat NSCs via VEGF. (A) Effect of ACM on cell cycle in rat NSCs, the percentages of cells at G1/G0, S and G2 phases were shown. (B) Western blotting results showed that the expression of cyclin D1 (* $P < 0.05$, compared with control group; $ $P < 0.05$, compared with N-ACM+30μM Glu group; # $P < 0.05$, compared with ACM (30%) group, n = 3).

diminished the mean diameter of neurospheres compared with the ACM (30%) group ($P < 0.05$) (Fig. 4D; $P < 0.05$). VEGF (130 mg/ml) increased significantly the activity of rat NSCs compared with control (Fig. 4E; $P < 0.05$), and increased the mean diameter of neurospheres compared with control (Fig. 4F; $P < 0.05$). BrdU incorporation assay demonstrated that N-ACM (30%), N-ACM (30%)+Glu (30 μM), ACM (30%) and ACM (30%)+VEGF NAb (15 μg/ml) enhanced BrdU incorporating into NSCs ($P < 0.05$); ACM (30%) increased BrdU incorporation into NSCs compared with N-ACM (30%)+Glu (30 μM) ($P < 0.05$); ACM (30%)+VEGF NAb (15 μg/ml) reduced BrdU incorporation into NSCs compared with the ACM (30%) group (Fig. 5; $P < 0.05$).

### *Effect of ACM on cell cycle and cyclin D1 expression of rat NSCs*

Cell cycle, which is involved in the regulation of cell growth, was examined using a flow cytometer 2 day after treatment. As illustrated in Figure 6A, the number of S phase cells increased significantly in N-ACM (30%), N-ACM (30%)+Glu (30 μM), ACM (30%) and ACM (30%)+VEGF NAb (15 μg/ml) groups compared to control ($P < 0.05$); it increased significantly in ACM (30%) group compared to the N-ACM (30%)+Glu (30 μM) group; there were no differences in ACM (30%)+VEGF NAb (15 μg/ml) groups compared with N-ACM (30%)+Glu (30 μM) group; but the number of S phase cells decreased remarkably in ACM (30%)+VEGF NAb (15 μg/ml) group compared with ACM (30%) group ($P < 0.05$). Cyclin D1 was a key factor in the control of cell cycle. The protein expressions of cyclin D1 were analyzed using Western blotting. The protein expression of cyclin D1 of rat NSCs increased

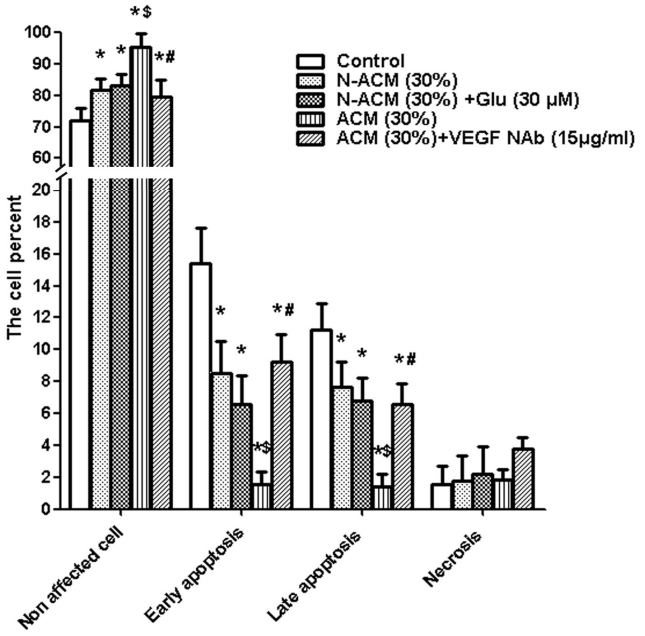


**Figure 7.** The effects of ACM on the cell death of NSCs by flow cytometry analysis of apoptosis. The results of flow cytometry analysis of apoptosis were visualized using Annexin-V/PI staining. The data show the percentage of normal cell, early apoptosis, late apoptosis, and necrosis (* $P < 0.05$, compared with control group; $ $P < 0.05$, compared with N-ACM+30μM Glu group; # $P < 0.05$, compared with ACM (30%) group, n = 3).

significantly in N-ACM (30%), N-ACM (30%)+Glu (30 μM), ACM (30%) and ACM (30%)+VEGF NAb (15 μg/ml) groups compared to control ($P < 0.05$); it increased significantly in ACM (30%) group compared with N-ACM (30%)+Glu (30 μM) group ($P < 0.05$); however, the protein expression of cyclin D1 of NSCs diminished remarkably in ACM (30%)+VEGF NAb (15 μg/ml) groups compared with ACM (30%)+Glu (30 μM) group (Fig. 6B; $P < 0.05$). These results indicate that ACM promoted the proliferation of rat NSCs by VEGF regulating the expression of cyclin D1 in vitro.

### *Effect of ACM on apoptosis of rat NSCs*

To examine the possible effects of ACM on cell death, we observed the measurement of apoptosis by Annexin-V/PI staining. We found that the proportion of early apoptosis and late apoptosis decreased significantly after N-ACM (30%), N-ACM (30%)+Glu (30 μM), ACM (30%) and ACM (30%)+VEGF NAb (15 μg/ml) treatment, ACM (30%) treatment decreased significantly the number of early apoptosis and late apoptosis compared with N-ACM (30%)+Glu (30 μM) group; ACM (30%)+VEGF NAb (15 μg/ml) treatment increased remarkably the number of early apoptosis and late apoptosis compared with ACM (30%) group (Fig. 7; $P < 0.05$). These findings showed that ACM may interrupt apoptosis of NSCs via VEGF.

## Discussion

Glutamate receptors are expressed widely throughout the CNS. It has been found that the activation of mGluRs can also have a neuroprotective effect on neurons and enhance survival and proliferation of NSCs (6). The mGluRs have been divided into three subgroups comprising group I (mGluR1, 5), group II (mGluR2, 3) and group III (mGluR4, 6, 7, 8). Zhao et al. reported that the activation of mGluR5 could promote the proliferation and differentiation of embryonic NSCs in the development of CNS (8,11). A recent line of evidence proves that brain injuries enhance the proliferation of postnatal NSCs (19-21). In recent years, embryonic NSCs have been used for treating nerve injuries. However, the mechanisms which embryonic NSCs were activated for increased proliferation after brain injuries are not fully understood. Interestingly, it is reported that neurons and astrocytes are all subjected to high levels of glutamate following brain injuries. Meanwhile, VEGF expression is greatly enhanced in astrocytes after brain injuries, which can also induce neuronal growth and provide neuroprotection beyond vascular growth (22). Therefore, it is supposed that glutamate might promote postnatal NSC proliferation through inducing expression of VEGF of astrocytes during brain injuries. In our experiment, the results demonstrated that glutamate could promote VEGF expression and secreting of rat astrocytes through activating group I mGluRs in vitro.

Previous studies documented that in addition to the intrinsic properties of NSCs, local microenvironment or "niche", such as cell-cell contact, cytokines and growth factors, plays important roles in the cell fate determination of NSCs. It is demonstrated that astrocytes construct a major component of neural microenvironment in the CNS. Astrocytes, as one of the key players mediating inflammatory response, might produce anti-inflammatory cytokines and diverse trophic factors (23,24) in CNS diseases or injuries, which participate in neurogenesis (25,26). In this study, our results showed that ACM significantly promoted proliferation of embryonic NSCs, suggesting that astrocytes exerted effects in cell fate determination of embryonic NSCs. Up to now, accumulating evidence has also demonstrated that astrocytes play crucial roles in regulating the proliferation and differentiation of postnatal NSCs (26,27). In addition, some studies reported that astrocytes are all subjected to high levels of glutamate after brain injuries. This indicates that glutamate might affect proliferation of postnatal NSCs by stimulating astrocytes in the injured CNS. Therefore, the mechanism of effect of glutamate on NSC proliferation is two aspects including direct and indirect effect. The direct effect: glutamate may promote NSC proliferation by activation group I mGluRs of NSCs. The indirect effect: glutamate could promote the proliferation of NSCs through inducing the VEGF expression of ASTs.

It is reported that astrocytes may produce a plethora of molecules, including cytokines, growth factors and trophic factors (28,29). Horner reported that astrocytes stimulated by ischemia or injuries significantly accelerate the proliferation and differentiation of NSCs (30,31). It is reported that the bioactive molecules produced by astrocytes participate in regulating the behavior of NSCs (26,27). In the present study, the results showed that ACM (30%) may promote embryonic NSC proliferation compared with N-ACM+Glu (30 μM); Q-PCR, Western Blot and ELISA results showed that VEGF expression was significantly up-regulated from glutamate stimulating astrocytes and the amounts of VEGF secreted in ACM (30%); ACM+VEGF NAb remarkably inhibited embryonic NSC proliferation

compared with ACM (30%). It is indicates that glutamate might promote embryonic NSC proliferation by inducing the VEGF expression of ASTs. In addition, our previous findings showed that glutamate promotes embryonic NSC proliferation through activating mGluRs. Therefore, glutamate might promote embryonic NSC proliferation by increasing VEGF expression of ASTs and activating mGluRs of NSCs in the process of NSC transplants after nerve injuries. Identification of the molecular cues that modulate NSCs fate choice is a prerequisite for their therapeutic applications.

VEGF was originally characterized as a potent stimulator of angiogenesis. Subsequently, multifaceted effects of VEGF have been uncovered in nervous tissue (22). Recent studies indicate that VEGF stimulates neurogenesis in vitro and in vivo (32,33). The versatile actions of VEGF has been expanded to stimulating survival and proliferation of endogenous neural stem/progenitor cells, and VEGF was shown to increase endogenous neurogenesis after stroke (32,34). Cerebral ischemia stimulated neurogenesis both in the rostral SVZ of the lateral ventricles and in the SGZ of the dentate gyrus (DG) (35,36). VEGF enhanced neurogenesis in both regions (33,37,38). In VEGF-transgenic mice, brains examined after ischemia showed significantly increased SVZ neurogenesis, with chains of neuroblasts extending from the SVZ to the peri-infarct cortex, along with an increase in the number of newly generated cortical neurons after ischemia (33). Other studies suggested that astroglia cells, which express VEGF, stimulate self-renewal and expand neurogenesis of postnatal NSCs and may serve as a niche for NSCs (26). In addition, it is reported that VEGF from endothelial cells might regulate the proliferation of embryonic NSCs in vivo. Our results showed that ACM + VEGF NAb remarkably inhibited proliferation of embryonic NSCs and increased cell death compared with ACM, this indicates that ACM might promote embryonic NSC proliferation via VEGF.

Important cell cycle regulators include D-type cyclins protein kinase complexes, which govern the cellular progression through the G1 phase of the cell cycle (8). Cyclin D1 are involved in the development of nerve system. After the extracellular mitogenic stimulation, D-cyclins result in release of the E2F transcription factors and drive cell entry into the S phase of cell cycle. In this experiment, we demonstrate that ACM-containing Glu may stimulate the expression of cyclin D1 and lead more cells into the S phase, while VEGF Nab decrease the expression of cyclin D1 as well as the number of S phase cells. These results suggest that glutamate may increase the expression of cyclin D1 and drive more cells crossing G1/S node and entering into cell cycle by inducing the VEGF expression of ASTs, resulting in proliferation of embryonic NSCs.

In conclusion, our results demonstrate that glutamate could also indirectly promote the proliferation of rat embryonic NSCs through inducing the VEGF expression of ASTs in vitro, and VEGF may increase the expression of cyclin D1. This study suggests that glutamate may be a major molecule for regulating embryonic NSC proliferation and facilitate neural repair in the process of NSC transplants after ischemic brain injury and neurodegenerative disorders. However, more work is needed to uncover the role and mechanism of glutamate promoting the proliferation of NSCs in vivo.

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