Cellular and Molecular Biology

Chen *et al. Cell. Mol. Biol.*2016, 62 (3): 105-114
ISSN: 1165-158X
doi: 10.14715/cmb/2016.62.3.18

Original Research

Hypoxia stimulates the proliferation of rat neural stem cells by regulating the expression of metabotropic glutamate receptors: an in vitro study

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Abstract: Neural stem cells (NSCs) reside in not only developing, but also adult brain with specialized microenvironments that regulate their function. In vitro and in vivo studies have revealed strong regulatory links between hypoxic/ischemic insults and activation of NSCs. However, the underlying mechanisms remain unclear. Here, we show that proliferating NSCs isolated from rat E15.5 cortex expressed functional metabotropic glutamate receptor (mGluR) subtype 3-7. Hypoxic exposure regulated their expression in NSCs in mRNA and protein levels. Activation of mGluRs by glutamate or Trans-ACPD (a non subtype-selective mGluRs agonist) sensitized NSCs to the growth effects of hypoxia. Pharmacological blockade of ionotropic glutamate receptor (iGluR) using MK-801 did not attenuate the action of glutamate in NSCs. Furthemore, we used the group specific mGluR agonists DHPG, LY 379268 and L-AP4 to explore which mGluR subtypes are responsible for stimulating NSCs proliferation after hypoxia. The results suggested that hypoxia increased expression of group I mGluR5 and significantly enhanced the NSCs proliferation. We conclude that hypoxia regulates the expression of mGluRs in proliferating NSCs and the dynamic expression of mGluRs induced by hypoxia may be one of the mechanisms of hypoxia stimulated NSCs activation. Regulation of mGluRs in NSCs might be a useful tool in the experimental cell therapy of hypoxic/ischemic injuries of CNS.

Key words: Hypoxia, neural stem cells, glutamate, metabotropic glutamate receptors, proliferation.

Introduction

Neural stem cells (NSCs) are primitive cells with self-renewal capacity and multi-potentiality to generate the neural lineages, which are present in not only developing, but also adult central nervous system (CNS) as well. In the adult brain, the two principal populations of NSCs are located in the subventricular zone (SVZ) lining the lateral ventricles and in the subgranular zone (SGZ) of the hippocampus dentate gyrus (1-2). NSCs reside in the specialized neurogenic niches that regulate their function, which might provide an access for brain repair after injuries (3-5).

Oxygen deficiency, resulting from ischemic/hypoxic insults, triggers a host of intrinsic adaptive processes designed to promote tissue and cell protection and regeneration (6-8). Previous investigations suggest that ischemia/hypoxia promote the division of NSCs located in the SVZ of the lateral ventricle and the SGZ of the hippocampus dentate gyrus in the adult brain. Newly proliferated cells can differentiate into neurons, integrate in neural network and participate in recovery of neurological deficit (9-12). Furthermore, studies also show that in vitro hypoxia could stimulate proliferating and neuronal differentiating of cultured NSCs (13-16). However, the intracellular and extracellular mechanisms that NSCs respond to ischemic/hypoxic insults remain unclear.

Glutamate is the predominant excitatory neurotransmitter in CNS. Following brain ischemia/hypoxia, release of glutamate generally induces neuronal death at high concentrations and contributes subsequently to the pathogenesis of brain injury (17). However, recent study suggests that glutamate is emerging as an important signal molecule in regulation of the survival, proliferation and differentiation of NSCs in developing CNS after brain ischemic/hypoxic injuries as well (18-21). Glutamate signals in CNS are mediated by ionotropic and metabotropic glutamate receptors (mGluRs). The mGluRs are a Family C subclass of G protein-coupled receptors. On the basis of sequence similarities, second messenger coupling and pharmacology, eight subtypes of mGluRs are divided into three groups. The group I (mGluR1 and mGluR5) is coupled to the activation of phospholipase C. The group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8) are coupled to the negative regulation of adenylyl cyclase activity (22-23).

Although mGluRs are generally considered to be expressed by mature neurons in adult CNS and involved in the regulation of synaptic plasticity (24), some of the mGluR subtypes are recently reported expressed in neurogenic regions of developing and adult brain. Cell lines expressing mGluR subtypes include NSCs, embryonic stem cells and some types of non-neuronal cells as well (18, 25-29). Furthermore, activation of mGluR subtypes by their ligands can regulate cell proliferation, differentiation and survival in vivo and in vitro (30-32). Specific enhanced mGluR4 reduces proliferation and promotes differentiation of cerebellar granule

Received January 27, 2016; **Accepted** March 27, 2016; **Published** March 31, 2016

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cell neuroprecursors (32). The mGluR5 knockout or blockage of the mGluR5 or mGluR3 with antagonists showed a significant reduction of dividing NPCs present in the SVZ and hippocampus dentate gyrus in mice (31). Group II mGluRs activation reduces basal levels of apoptosis and expands the number of NPCs present in culture from prenatal SVZs (21). Differentiation of embryonic stem cells into embryoid bodies is associated with the expression of mGluR4, the activation of which drives cell differentiation towards the mesoderm and endoderm lineages (26). These findings suggested that mGluR signaling might be a key regulating component in NSCs proliferation and differentiation in developing CNS cells. However, the link between the expression of mGluRs and NSCs activation after ischemia/hypoxia remains uncharacterized.

We previously reported that hypoxia could increase the proliferation of rat fetal cortical NSCs in vitro (14). Here, we observed in vitro expression profile of eight mGluR subtypes in the NSCs isolated from fetal rat cortex, and examined the effect of hypoxia on the expression of the mGluR subtypes in mRNA and protein levels. Further, the functions of mGluR subtypes in hypoxia regulating NSCs activation were investigated.

Materials and Methods

Animals

Adult male and female Sprague-Dawley rats (Weigh of 250-300 g) were obtained from Experimental Animal Center of Xi'an Jiaotong University College of Medicine (Certificate No. 22-9601018). Animals were housed in a temperature-controlled animal facility with a 12 h light/dark cycle, and free access to food and water. For mating and staging of embryos, mature female and male rats at oestrus were placed overnight into the same cage at 6:00 on the evening. Then the females were examined for mating by the presence of vaginal plug or spermatozoa in the vagina, which was considered as pregnant and counted as embryonic day 0.5 (E0.5) for fetus. Mothers at pregnant 15.5 d were used to isolate embryos. All experimental protocols were approved previously by the Animal Care and Use Regulation of Xi'an Jiaotong University College of Medicine, and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animals' suffering and to keep the numbers of animals used to a minimum.

Isolation and culture of rat embryonic NSCs

NSCs were prepared from rat embryonic cortex using the methods as described previously (33) with some modifications. Briefly, pregnant 15.5 d rats were anesthetized with 10% chloral hydrate (intraperitoneal, 50 mg/kg), the fetal brain were dissected, and washed three times in ice-cold sterile phosphate buffered saline containing 0.6% D-glucose(D-PBS, pH 7.4). After removal of the meanings carefully, the tissue was mechanically dissociated and incubated in dissecting buffer at 37°C for 10 min. The dissecting buffer consisted of D-PBS with 0.01% trypsin (Sigma, USA), 200 μM EDTA and 1 mM $MgCl_2$. Trypsin was neutralized using 0.2 mg/ml soybean trypsin inhibitor (Roche, Germany). The tissues were further dissociated into single-cell suspension with a fire-polished Pasteur pipette. Cells were seeded into substrate-free T75 flask at a concentration of 200000 cells/ml and grown in suspension in serum-free neurosphere initiation medium (NPIM), which consisted of Dulbecco's modified Eagle medium and Hams F12 (1:1), supplemented with 2% B27, 1% N2, 200 U/ml penicillin, 200 U/ml streptomycin, 10 ng/ml bFGF (all from Gibco, USA) and 2.5 μg/ml heparin (Sigma, USA). Cells were incubated in a humidified incubator (37°C, 5% CO_2/95% air) and half the medium was changed every 2-3 days.

After 5-7 days in vitro (DIV), cells were passaged when generated neurospheres (primary neurospheres) of 80-120 μm in diameter were observed. Neurospheres were collected by centrifuge and dissociated using 0.05% trypsin and 200 μM EDTA in D-PBS, and mechanically triturated. Cells were reseeded into T25 flasks at a density of 50000 cells/ml in NPIM and cultured for 3-5 days when neurospheres (secondary neurospheres) of 80-100 μm in diameter had been propagated. Secondary neurospheres were used in the experiments described below, and at least three independent experiments were performed for each assay.

Hypoxia/reoxygenation procedure

The NSCs were made hypoxia by incubating cells in Bugbox-M microaerophilic incubation system (Ruskinn, UK) with humidified 0.3% O_2/94.7% N_2/5% CO_2, as described previously (14). In brief, the cells were placed into the incubator for up to 3, 6, 12 or 24 h. For reoxygenation, the neurospheres were returned into the standard incubator conditions of 95% air/5% CO_2 at indicated hypoxic time point, and cultured for different durations, depending on experimental design. The control cultures were incubated in standard conditions all the time for the same durations.

Immunofluorescent staining

The secondary neurospheres were collected by centrifugation and fixed for 20 min using 4% paraformaldehyde in PBS at room temperature (RT), then mounted to slides. After blocked for 1 h with 2% bovine serum albumin (BSA) in PBS containing 0.3% Triton X-100 (PBS-TX) at RT, the neurospheres were incubated with mouse monoclonal anti-nestin antibody (1:200, Chemicon, USA) overnight at 4°C. After washing, cells were reacted with FITC-conjugated anti-mouse IgG (1:200, Vector, USA) for 2 h at RT. Labeled cells were further counterstained with propidium iodide (PI, 50 μg/ml, Sigma, USA) and mounted using VECTASHIELD mounting medium. Nestin positive cells were observed and counted using a laser scanning confocal microscope (TCS SP2, Leica, Germany) and normalized to PI-stained cells.

For demonstrating the differentiating potential of the NSCs, the neurospheres were dissociated into single cells and plated onto poly-D-lysine (Sigma, USA) coated coverslips, and then incubated for 3 days in the presence of 1% fetal bovine serum (FBS) and absence of bFGF. The cells were fixed for 10 min using 4% paraformaldehyde in PBS at RT, washed by PBS, and blocked for 1 h with 2% BSA in PBS-TX at RT. The mouse anti-Tuj1 (1:200, Chemicon, USA), or anti-GFAP (1:2000, Neomarker, USA) was added and incubated

overnight at 4°C. After wishing in PBS, FITC-conjugated anti-mouse IgG (1:200, Vector, USA) was added and incubated for 2 h at RT. Labeled cells were further counter-stained using PI and mounted. The immunoreactive cells were observed using a fluorescence microscopy (BX51 and DP71, Olympus, Japan).

RNA extraction, cDNA synthesis and PCR

Neurospheres total RNA was extracted using the TRIzol Reagent (Invitrogen, USA) protocol according to the manufacturer's guidelines. The RNA pellets were briefly air-dried and dissolved in 30 µl RNase-free deionized water for 10 min at 55°C and stored at -80°C. The RNA concentration was measured using absorbance at 260 nm and the purity was determined using the ratio of absorbance at 260 and 280 nm. The integrity of total RNA was further checked by 1% agarose gel electrophoresis through viewing of the eithidium bromide stained 28S, 18S and 5S ribosomal RNA band. The total RNA was used as template in cDNA synthesis.

First-strand cDNA was synthesized using the ExScript™ RTase regent kit (TaKaRa, Japan) with Oligo (dT) primers. Reverse transcription was performed in a final reaction volume of 10 µl containing 500 ng of total RNA, 2 µl of 5×ExScript™ reverse transcriptase buffer, 0.5 mM of each deoxynucleotide triphosphate (dNTP), 2.5 µM of Oligo (dT) (12-18) primer, 10 units of RNase inhibitor, and 50 units of ExScript™ RNase H free reverse transcriptase. The mixture was incubated at 42°C for 10 min, and then heated at 95°C for 2 min to inactivate the reverse transcriptase. The resulted cDNA was subsequently stored at –20°C.

Amplification of first-strand cDNA was performed in a 50 µl reaction volume consisting of 10 mM Tris-HCl, pH 7.4, 500 mM KCl, 1.5 mM MgCl, 200 µM of each dNTP, 0.4 µM of each primer, 1.25 U of hot start *Taq* DNA polymerase (TaKaRa, Japan) and 1 ul cDNA sample. After denaturation at 94°C for 5 min, the 30 thermal cycles included denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 45 s, followed by final extension at 72°C for 5 min. The gene specific primers for eight mGluR subtypes and GAPDH were shown in Table 1. The PCR products were separated by electrophoresing on 2% agarose gel in 1 X TAE buffer and visualized using eithidium bromide and ultraviolet illumination. PCR controls without template, primers or *Taq* DNA polymerase, failed to yield products.

Real-time quantitative PCR

Real-time quantitative PCR analyses were performed using SYBR Green I detection method. First-strand cDNA synthesized from total RNA was used as template. PCR was run in a total volume of 25 µl containing 12.5 µl of 2× SYBR *Premix Ex Taq* (TaKaRa,Japan), 0.5 µl of 50× ROX Reference Dye, 0.2 µM of each primer and 2 µl cDNA template using an ABI Prism 7000 Sequence detection system (Applied Biosystems, USA). The thermocycling conditions were as follows: an initial denaturation at 95°C for 10 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. After the PCR cycles, the PCR products were checked by run a SYBR Green I melting analysis with ramping the temperature of the samples from 62°C to 95°C. Amplification was performed in triplicate for each sample.

The primers used in real-time quantitative PCR were designed with Oligo 5.0 software and synthesized by TakaRa Incorporation. To prevent possible extraneous amplification origin from trace contaminating genomic DNA, each pair of primers were designed to span two consecutive exons. The primer sequences and related information on each gene are shown in Table 1. To determine the similarity of the PCR efficiency of target and reference gene, a serial dilution of cDNA template was used for amplification of each target gene using gene primers and internal control (GAPDH). The slope of the linear regression (by plotting the Ct as a function of log concentration of template) shows a difference ranging from 3.1 to 3.6 cycles between each 10 × dilution, which is close to the theoretically expected value of 3.3 cycles.

Data were analyzed at the termination of each as-

Table 1. Gene targets and primer sequences used in real-time quantitative PCR.

Transcript	Primer sequence	Product (bp)	Accession
mGluR1	Forward, CTGTGTGCAGTGAGCCTTGCTTA Reverse, AGGTGAACTCGTCCTGCACAAAC	120	NM_017011
mGluR2	Forward, TGGGCGGTGTCTTCCTTT Reverse, ATGCGATTGGTCTTGGTGAG	142	XM_343470
mGluR3	Forward, AAATTCATCAGCCCCAGTTCTC Reverse, AAGCCACACGGACACCATC	81	XM_342626
mGluR4	Forward, CGTGTACTGTGTACGCCATCAAGAC Reverse, AAGCGCTCAGACTCACGGAGA	195	NM_022666
mGluR5	Forward, AGCAGATCCAGCAGCCTAGTCAA Reverse, GACAGACAGTCGCTGCCACAA	141	NM_017012
mGluR6	Forward, CGGTGCAAAGCACAGCTCA Reverse, TGACCTAGCACAGAAGCCTCAGAA	75	NM_022920
mGluR7	Forward, CCTTGCTGCTGGACCTGTGA Reverse, CACTCCAGTTTGATGATTGGGATG	140	NM_031040
mGluR8	Forward, TTTGGTACAGCCCAGTCAGCAG Reverse, TGGGCATATAGAGCATTCCCAGA	106	NM_022202
GAPDH	Forward, GACAACTTTGGCATCGTGGA Reverse, ATGCAGGGATGATGTTCTGG	133	NM_017008

The name and GenBank accession number for each gene target evaluated is listed. Sequences for the forward and reverse primers used for real-time quantitative PCR are given along with the expected product size in base pairs (bp).

say using the Sequence Detector 1.0 software (Applied Biosystems, USA). Default baseline and threshold setting were selected. The increase in SYBR Green I emission intensity (ΔRn) was plotted as a function of cycle number. The threshold cycle (C_T) was determined by software as the amplification cycle at which the ΔRn first intersects the established baseline. The transcript level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method (34-35), which one of the unknown samples is termed the calibrator sample for comparison of each unknown sample's mRNA level. GAPDH was chosen as an internal control gene for normalizing transcript level of eight mGluR subtypes. The formula used was as follow: Fold induction = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = [Ct_{GI\ (unknown\ sample)} - Ct_{GAPDH\ (unknown\ sample)}] - [Ct_{GI\ (calibrator\ sample)} - Ct_{GAPDH\ (calibrator\ sample)}]$, and GI is the gene of interest.

Western blot analysis

In order to show the expression of mGluRs protein in NSCs, secondary neurospheres were treated in hypoxic condition for 6, 12 and 24h. At the end of indicated time point, The neurospheres were collected by centrifuge and lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.5% sodium Deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 20 mM Na4P2O7, 10% glycerol) supplying with Protease Inhibitor Cocktail (Roche, Germany), and incubated for 30 minutes on ice. Cell lysate were cleared by centrifuging (12,000 g for 10 min at 4°C). Protein concentration of the samples was determined by BCA assay. Proteins (20-40 μg) were separated by 8% SDS-PAGE electrophoresis and electroblotted on nitrocellulose membrane (Bio-Rad, USA). The membranes were blocked for 1 h at RT with 5% non-fat dry milk in TBST (20 mM Tris, 150 mM NaCl and 0.05% Tween 20), and then incubated overnight at 4°C with primary antibodies. The following primary antibodies were used: Rabbit polyclonal anti-mGluR1 (1:500, Abcam); mouse monoclonal anti-mGluR2 (1:4000, Abcam); Rabbit polyclonal anti-mGluR3 (1:1000, Abcam); Rabbit polyclonal anti-mGluR4 (1:1000, Abcam); Rabbit polyclonal anti-mGluR5 (1:5000, Chemicon); Rabbit polyclonal anti-mGluR6 (1:1000, Abcam); Rabbit polyclonal anti-mGluR7 (1:1000, Upstate); Guinea Pig polyclonal anti-mGluR8 (1:4000, Chemicon, USA) and mouse monoclonal anti -β-actin (1:2000, Santa Cruz, USA). After washing in TBST, membranes were incubated for 1 h at RT using using horseradish peroxidase-labeled secondary antibodies (1:2000, KPL, USA). Immunoreactive bands were visualized by enhanced chemiluminescent substrate (Pierce, USA). Changes in mGluRs expression were normalized and expressed as ratio of optical density of the receptor and the corresponding β-actin bands.

MTT assay

To estimate relative levels of NSCs proliferation, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide]-based assay was performed as described previously (36). Briefly, NSCs were plated into 96-well plate at a concentration of 10 000 per well in 200 μl NPIM. The cells were treated using different concentration of glutamate, 1S, 3R-ACPD and/or MK-801 under normoxia or after hypoxia. Then 20 μl of 5 mg/ml MTT (Sigma, USA) was added in 200 μl medium per well and allowed to incubate at 37°C for 30 min. After removal of supernatant, the resulting formazan crystals were dissolved by adding 150 μl dimethyl sulphoxide (DMSO, Sigma, USA) per well and shaking. Absorbance was read at 492 nm using a microplate reader. Results are the average of three independent experiments.

BrdU Incorporation assay

The secondary neurospheres were dissociated into single cells and plated onto the poly-D-lysine-coated coverslips at a concentration of 50000 per well in 24-well plates. After incubated in hypoxia for 12 h and mGluR ligands treatment for 3 days, the proliferating cells were labeled with 10 μM bromodeoxyuridine (BrdU, Sigma, USA) for 2 h, then fixed in 4% paraformaldehyde and processed immunocytochemical staining. In brief, the cells were denatured in 2 M HCl for 30 min at 37°C, and HCl was neutralized with 0.1 M sodium borate buffer (pH8.5) for 10 min. Cells were washed with PBS and incubated overnight with mouse monoclonal anti-BrdU antibody (1:200, Chemicon, USA) at 4°C. After washing in PBS, the cells were reacted with FITC-conjugated anti-mouse antibody (1:200) for 2 h at room temperature. Labeled cells were further counter-stained using PI to demonstrate the total cells. BrdU-labelled cells were counted using fluorescence microscopy and normalized to the PI-stained cells.

Statistics

Statistical analysis was performed using one-way ANOVA. Tukey's post-hoc analyses were used to determine which treatments were different from others. All data are expressed as mean ± SEM. The p-value < 0.05 was considered statistically significant. All the calculations were performed using SPSS for Windows (version 10.0, SPSS, USA).

Results

Identification of the NSCs from fetal rat cortex

The NSCs from fetal rat cortex were grown in serum-free medium containing bFGF, N2 and B27. In the early of primary culture, many single cells attached to the flasks. With the growth, the dividing cells aggregated into small clusters, detached from the substrate and formed free-floating neurospheres during a period of 3-7 days. When the neurospheres with the diameter of 80-120 μm formed, the cells were passaged using trypsin and EDTA. The cells subsequently proliferated and formed secondary neurospheres (Fig. 1A) and were characterized by immunocytochemistry for the expression of nestin, a marker of NSCs. Over 95% of cells in the neurospheres were immunopositive for nestin (Fig. 1B). To identified the neural differentiating potential, the NSCs were induced differentiation by dissociated into single cells, plated onto poly-D-lysine-coated coverslips and cultured in the differentiating medium supplemented with 1% FBS and without bFGF. Immunocytochemical stain demonstrated that the differentiated cells can express Tuj1, a marker for neurons, or GFAP, a marker for astrocytes (Fig. 1C, D).

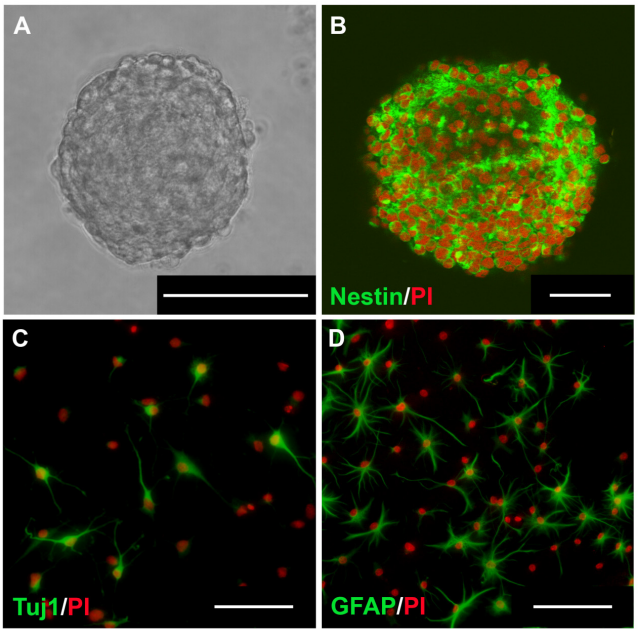

Figure 1. Culture and immunocytochemical characterization of the fetal rat cortex NSCs. NSCs were isolated from E15.5 fetal rat cortex and cultured in NPIM, neurospheres were formed after proliferating of 5-7 days in vitro (A). Nestin immunostaining NSCs were showed in neurospheres under proliferating conditions (B). NSCs were differentiated to Tuj1 and GFAP immunopositive cells by removal mitogens and adding 1% fetal bovine serum (C & D). The nuclei were counterstained with pripidium iodide (PI). Scale bars: A = 200 μm; B = 40 μm; C & D = 100 μm.

Expression of mGluR subtypes in fetal rat NSCs

To demonstrate the expression of eight mGluR subtypes in the fetal rat NSCs under proliferating condition, we examined the expression of mGluR subtypes at mRNA and protein levels in secondary neurospheres at 7 DIV. RT-PCR analysis showed that cultured NSCs expressed the transcripts of all mGluR subtypes (Fig. 2A). However, Western blot analysis showed that protein expression was detectable for mGluR5 of the group I subtype, mGluR3 of the group II subtype and mGluR4, mGluR6 and mGluR7 of the group III subtype (fig. 2B). Although the mRNA of mGluR1, mGluR2 and mGluR8 was detectable, the protein expression of these mGluR subtypes cannot observable in the normoxic condition (Fig. 2). Moreover, we further checked the protein of the three mGluRs after 12 h of hypoxic treatment, and did not found the protein expression of these receptors (data not shown).

Regulation of hypoxia on expression of mGluR subtypes in fetal rat NSCs

To determine the effects of hypoxia on the expression of mGluR subtypes in fetal rat NSCs in vitro, the secondary neurospheres were induced hypoxia for consecutive 3, 6, 12 or 24 h. Expression of mGluRs mRNA was examined using real time quantitative PCR analysis. The results showed hypoxia regulated the mGluR subtypes mRNA levels in the temporal dependent manner (Fig. 3). The mRNA level of mGluR5 in group I subtype was increased to 2.83 ± 0.25 folds at 24 h of hypoxia, compared to that of normoxia (Fig. 3A). In the group II, the level of mGluR3 mRNA was stably increased at 6, 12 and 24 h of hypoxia, which was about 2.5 folds increase (Fig. 3B). In the group III, mRNA level varied in the subtypes of mGluR4, 6 and 7 after hypoxic treatments. The mGluR6 mRNA was increased at 24 h of hypoxia. The mGluR7 transcript was increased as early as 3 h of hypoxic treatment, and was increased 3.83 ± 0.32 folds at 24 h of hypoxia. However, the mGluR4 mRNA was not observed to change at all hypoxic time points (Fig. 3C).

The level of mGluRs mRNA was further examined at 12, 24 h and 3 days reoxygenation after 12 h of hypoxia (RAH) (Fig. 3). The mGluR5 mRNA was kept at higher level up to 3 days RAH, while others were showed totally decreasing expression patterns after 12 h RAH. The mGluR3, mGluR6 and mGluR7 were rapidly decreased from 24 h to 3 days RAH. The mGluR4 was increased compared to hypoxic treatment at 12 and 24 h RAH, and then was slightly down-regulated at 3 days RAH.

Hypoxia-induced change of protein expression of mGluRs in NSCs was further determined at 6, 12, 24 h of hypoxia and 3 days RAH (Fig. 4). Consistent with mRNA expression pattern, the mGluR5 was showed an increase at 24 h of hypoxia and sustained higher level at 3 days RAH (Fig. 4A). The expression of mGluR3 was increased at 12 and 24 h of hypoxia, and then the protein level was dramatically decreased at 3 days RAH

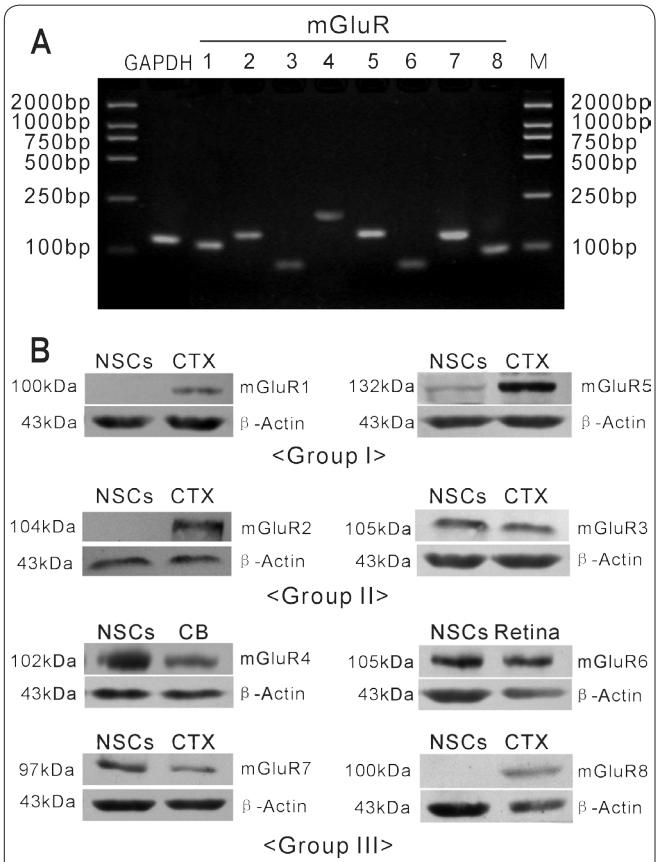

Figure 2. Expression of mGluR subtypes in the fetal rat cortex neurospheres. RT-PCR and immunoblot analysis of eight mGluR subtypes in three groups in secondary neurospheres at 7 DIV is showed in panel A and B, respectively. The transcripts of all the eight subtypes of mGluR were present in neurospheres (A). No proteins of mGluR1, 2 and 8 were detectable in the neurosphere cultures in the experiment (B). Each RT-PCR and immunoblot was performed in three independent neurosphere cultures. Protein extracts from adult rat CTX (cortex), CB (cerebellum) or retina were used as positive controls.

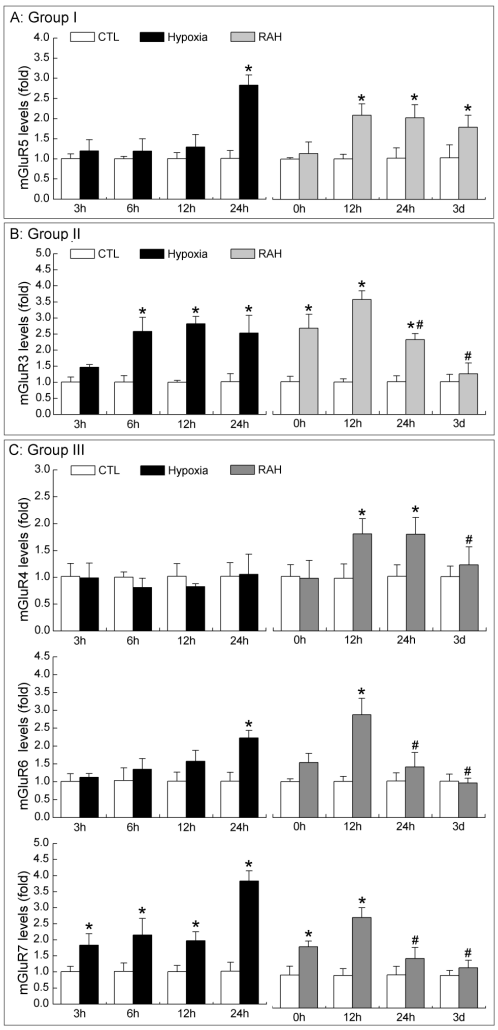

Figure 3. Effects of hypoxia and RAH on mRNA expression of mGluR subtypes in the neurosphere cultures. Neurospheres were made hypoxia for 3, 6, 12 and 24 hrs, or 12 hrs followed by reoxygenation of 12, 24 hrs and 3 days. The neurospheres in control were cultured in normal condition for the same durations. The transcripts of mGluR subtypes in three groups were analyzed using real time quantitative PCR. GAPDH was chosen as an internal control gene for normalizing transcript level of the mGluR subtypes. Each value represents the mean ± SEM in the three independent experiments. * P<0.05 versus controls, respectively; # P<0.05 versus 12 hrs of RAH (reoxygenation after hypoxia).

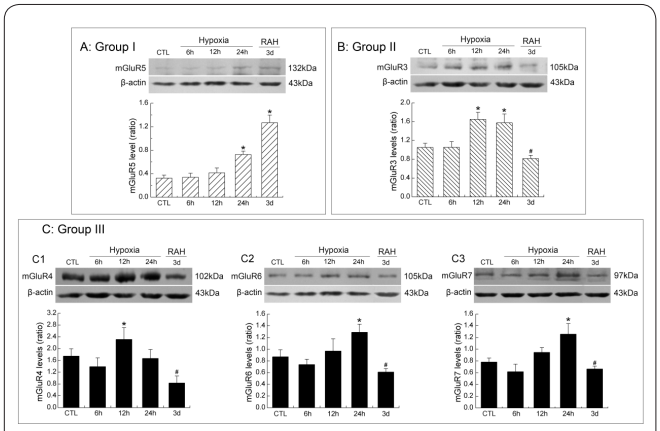

Figure 4. Effects of hypoxia and RAH on protein expression of mGluR subtypes in neurosphere cultures. Neurospheres were made hypoxia for 6, 12 and 24 hrs, or 3 days of RAH, the neurospheres in control were cultured in normal condition. β-actin was chosen as an internal control for normalizing the expression of mGluR subtypes. Protein expression of the mGluR subtypes was assayed using immunoblot analysis. * P<0.05 versus controls, respectively; # P<0.05 versus 12 (C1) or 24 (B, C2 & C3) hrs of RAH (reoxygenation after hypoxia).

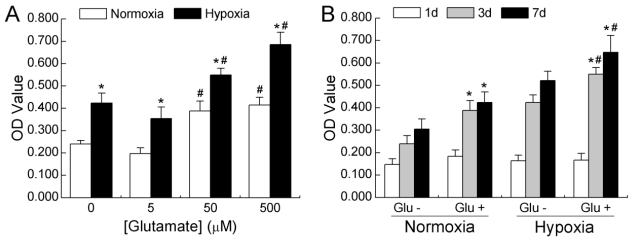

Figure 5. Dose- and time-dependent proliferation of NSCs exposure to glutamate under normoxic or hypoxic condition. A. The neurospheres were treated with 5, 50 and 500 μM of glutamate under 12 hrs of normoxic or hypoxic condition, then allowed to grow under normoxia up to 3 days. MTT assay was performed. * P<0.05 hypoxia versus normoxia; # P<0.05 with versus without glutamate. B. The neurospheres were made hypoxia for 12 hrs, and then incubated in nomoxia for 1, 3 and 7 days, with or without 50μM of glutamate. * P<0.05 with versus without glutamate; # P<0.05 hypoxia versus normoxia in the same glutamate treatment durations.

(Fig. 4B). In the group III, the expression of mGluR4 increased at 12 h of hypoxia, then declined to control level at 24 h of hypoxia, and went on decreasing to about half of control at 3 days RAH. The mGluR6 and mGluR7 expression increased at 24 h of hypoxia, and significantly declined to control level at 3 days RAH (Fig. 4C).

Effect of mGluRs activation on proliferation of the fetal rat NSCs under normoxic and hypoxic conditions

We further observed the effects of glutamate on the proliferation of NSCs in normoxic condition or after hypoxic treatment. Glutamate is the endogenous ligand of glutamate receptors involved in iGluRs and mGluRs. Previous report showed that high levels of glutamate could reduce basal levels of apoptosis and increase NSCs proliferation. In this study, we observed the effect of mGluRs activation by glutamate treatment on NSCs proliferation under hypoxic condition. The neurospheres were cultured in NPIM under normoxia or RAH, and supplemented with different concentration of glutamate (5, 50 or 500 μM), and MTT assay was employed to demonstrate the Dose- and time-dependent proliferating of NSCs (Fig. 5). Consistent with our previously report, hypoxia enhanced the NSCs proliferation(14). Further, the addition of 50 and 500μM glutamate significantly increased the proliferating of NSCs after hypoxic treatment compared to nomoxic control.

To determine that NSCs proliferation by glutamate stimulation is mediated by mGluRs or iGluRs, Trans-ACPD, an mGluRs agonist, and MK-801, an iGluRs antagonist were used. MK-801 (10 μM) did not attenuate the NSCs response to glutamate. Adding of Trans-ACPD (100 μM) also increased the proliferation of NSCs, most likely glutamate did under the same conditions (Fig. 6).

For confirming which groups are responsible for stimulating NSCs proliferation after hypoxia, Trans-ACPD (an mGluRs agonist), DHPG (selective group I mGluRs

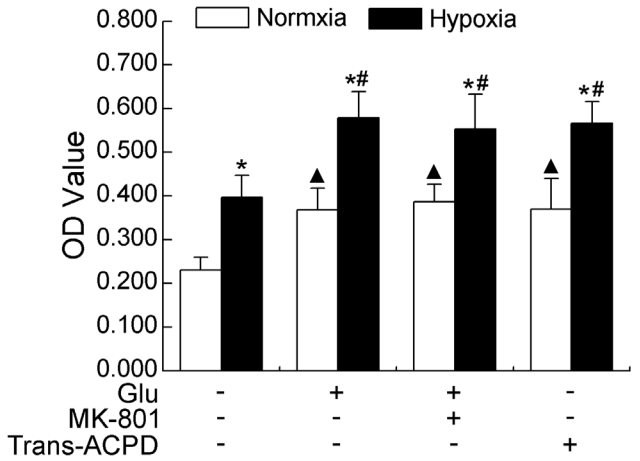

Figure 6. Proliferation of NSCs exposure to iGluRs antagonist and mGluRs agonist. The neurospheres were made hypoxia for 12 hrs or incubated in normoxia, treated with glutamate (50μM) and/or NMDA receptor antagonist MK-801 (10 μM), or mGluRs agonist trans-ACPD (100 μM) for 3 days. MTT assay was performed. * P<0.05 hypoxia versus normoxia, # P<0.05 treatment versus non-treatment of ligand after hypoxia, ▲ P<0.05 treatment versus non treatment in normoxic condtion.

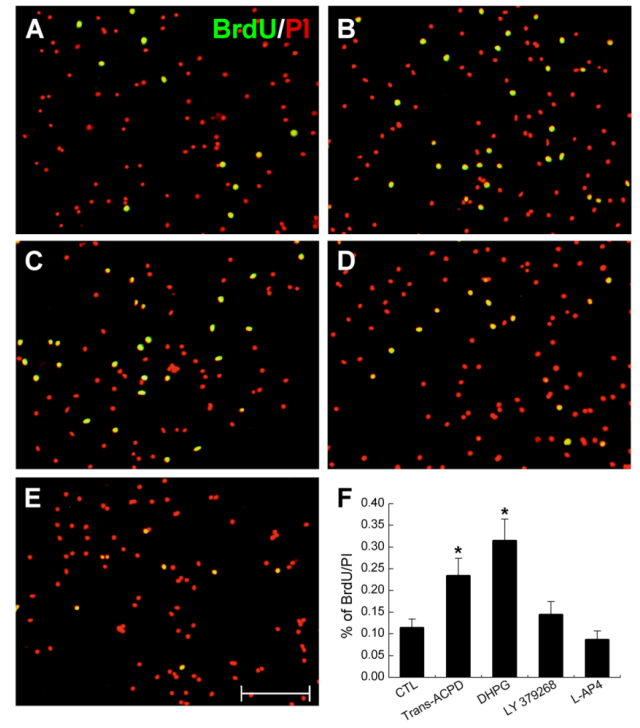

Figure 7. Proliferation of NSCs exposure to selective mGluRs agonists. The NSCs were cultured on poly-D-lysine-coated 24-well plate overnight. The cells were made hypoxia for 12 hrs, treated with mGluRs agonist trans-ACPD (100 μM), group I mGluRs agonist DHPG (100 μM), group II mGluRs agonist LY 379268 (1 μM), or group III mGluRs agonist L-AP4 (100 μM), for 3 days. Then the proliferating cells were labeled using BrdU (10 μM) for 2 hrs. * P<0.05 treatment versus non-treatment of ligands, respectively. Scale bars = 200 μm.

agonist), LY 379268 (selective group II mGluRs agonist) and L-AP4 (selective group III mGluRs agonist) were used. BrdU incorporation assay was employed to label the proliferating cells. Like the effect of Trans-ACPD on NSCs, DHPG (100 μM) significantly enhanced the NSCs proliferation, while LY 379268 (1 μM) did not show the same effect. However, L-AP4 (100 μM) decreased the NSCs proliferation (Fig. 7).

Discussion

NSCs offer the promise of novel repair strategy in CNS disorders. Hence, there is a growing interest in exploring the mechanisms that regulate their survival, proliferation, differentiation and migration of NSCs in development and after CNS injuries, especially in cerebral ischemia and brain hypoxia (12, 37). One of the important objects of investigation is aimed at identifying the mechanisms that allow their positive enrichment both in vitro and in vivo (38). Neurotransmitter-mediated signaling might play a role in activation of NSCs after CNS disorders, since some neurotransmitters have been demonstrated to regulate neurogenesis in the developing and adult CNS (18, 39-41). Recent evidences suggest that some mGluR subtypes are expressed in the proliferating NSCs, and the activation by endogenous or exogenous ligands can regulate cell survival and proliferation, although the mechanisms remain to be detailed (21, 27, 30-31, 42). The essential finding in the present study is that hypoxia regulated the expression of mGluR subtypes in the fetal rat NSCs in vitro, activation of them was involved in the NSCs proliferating action in response to hypoxia.

The first report that mGluR subtypes were expressed in the neural progenitors emerged in 1997, which demonstrated that eight mGluRs mRNA can express in P19 embryoarcinoma cells during neuronal differentiation induced by retinoic acid (43). Where after, the mGluR subtypes are found expressed at early developmental stages in the embryonic brain (42, 44). Functional mGluR subtypes were also found in cultured undifferentiated NSCs or neurospheres (18, 27-28, 31) and mouse embryonic stem cells (26, 45). For mGluR subtypes expression, proliferating NSCs isolated from the C57 mice forebrain at embryonic day 20 (E20) express mGluR3, 4, 5 and 7 mRNA (31); proliferating NSCs isolated from the Std-ddY mice neocortex at E15.5 express mGluR2, 3, 5 and 8 mRNA, but not mGluR1, 4, 6, and 7 mRNA (28); proliferating NSCs isolated from adult CD1 mice SVZ express the mRNA of all mGluR subtypes with the exception of mGluR2 and mGluR6, and only mGluR5 and a faint mGluR1 are detected at protein level (27). The present findings showed that the undifferentiated NSCs isolated from SD rat cortex at E15.5 expressed the mRNA of all eight mGluR subtypes, and only mGluR3, 4, 5, 6 and 7 proteins were detected. The difference of mRNA and protein among mGluR subtypes may refer to cellular post-transcriptional processes in the NCSs and relate to the functional of these receptors. Furthermore, the diversity of expression profiles of mGluR subtypes in NSCs among reports may due to different animal strains and/or developmental stage in different experiments.

Hypoxia affects the expression of numerous genes in NSCs, many of which are potential molecules in the regulation of NSCs response to ischemic/hypoxic insults (46-47). Our result demonstrated that hypoxia changed the mGluRs expression profile at mRNA and protein levels in NSCs in vitro. It was considered that mGluRs were involved in NSCs activation after hypoxia. In neu-

rons, activation of group I mGluRs induced cell death (48). Ischemic/hypoxic rats were showed a reduction in the expression of group I mGluRs in the hippocampus and cerebral cortex (49). Blockade of group I mGluRs is considered to protect neurons from hypoxia/ischemia injuries (50-52). Endogenous activation of mGluR5 also contributes to the development of hypoxia-induced liver cell injury. Mouse hepatocytes lacking mGluR5 are less sensitive to hypoxic damage (53). Furthermore, recent reports have been suggested that group I mGluRs expressing in NSCs can regulate their survival and proliferation (27, 31). Expression of mGluR5 was increased in a rat model of intracerebral hemorrhage and enhanced neurogenesis in the subventricular zone(54). It is possible that hypoxia and RAH up-regulating of mGluR5 in group I may be a mechanism in the proliferating response of NSCs to hypoxia in our experiment.

Activation of group II and III mGluRs in brain provides an essential protective mechanism against brain hypoxia/ischemia injuries. Expression of mGlu2/3 receptors was substantially higher in the brain of anoxia-tolerant species, and treatment the brain with mGlu2/3 receptor antagonist amplified anoxic damage and enabled the induction of cell death (55). In contrast, treatment with the mGlu2/3 receptor agonist was highly protective against anoxic brain damage (56) . Activation of Group III mGluRs with agonist (R, S)-4-phosphonophenylglycine protect neurons against hypoxic insult in rat hippocampal slices (57). In NSCs, group II mGluRs were showed increase the survival and reduce basal levels of apoptosis in NSCs (21, 31, 58). But for the effect of group II mGluRs on NSCs proliferation, there two opposite reports in previous study: enhancement (21, 31) or inhibition (30, 32). These reports also showed that activation of group II mGluRs promotes the differentiation of NSCs. To be similar, activation of group III mGluRs are showed to suppress the self-replication of NSCs isolated from neocortex (28). In our opinion, the dynamic expression of group II and III mGluRs after hypoxia and RAH may play different roles in regulation of the survival, proliferation and differentiation of NSCs in different developmental stage.

Glutamate is the endogenous ligand of glutamate receptors. The concentration of glutamate in the normal adult brain is 1.30-2.80 μM (59). Following cerebral ischemia/hypoxia, local extracellular glutamate concentration can reach >500 μM, while reach 100 μM in the cerebrospinal fluid, which triggers the death of neurons, causing mental or physical handicap (60-62). However, recent reports showed that the release of glutamate following brain hypoxia or ischemia may contribute to promote the activation of NSCs in the neurogenic region of brain. Previous report showed that high levels of glutamate (up to 1 mM) are not toxic to normal cultured NSCs, inversely reduce basal levels of apoptosis and increase NSCs proliferation (21). To be similarity, we found in the experiment that higher doses of glutamate (50 and 500 μM) promoted the proliferation of NSCs. Further, compared to normoxic condition, hypoxic exposure sensitized the proliferating response of NSCs to glutamate stimulation. It is possible that up-regulation of some mGluR subtypes contributes to the enhancement of NSCs proliferation after hypoxia.

Because iGluRs were also reported expressed in NSCs (63-64), we observed the effect of glutamate on NSCs proliferation at the present of MK-801, a NMDA iGluRs antagonist. The results showed MK-801 did not eliminate the action of glutamate on NSCs. We did not use the AMPA iGluRs antagonist to demonstrate the involvement of AMPA iGluRs in regulation of NSC proliferation in our experiments, for AMPA iGluRs expressed and functioned only when the cells have differentiated into young postmitotic neurons(65). Furthermore, Trans-ACPD, an mGluRs agonist, can mimic the action of glutamate in NSCs, suggesting that the growth effect of glutamate on the NSCs is mediated by mGluRs, other than iGluRs. It has been reported that different group of mRluRs may play different roles in regulation of NSCs survival, proliferation or differentiation (20-21, 27). We used group selective agonists to examine the effect of mGluR subtypes on NSCs proliferation after hypoxia. It was found that activation of group I mGluRs enhanced the NSCs proliferation, while group II or III did not promote the proliferation of NSCs. Compared to Trans-APCD treatment, activation of group I mGluRs had greater effect. The findings suggests group I mGluRs in NSCs plays an important role in hypoxia stimulating NSCs proliferation, while group II and III may have other functions, such as survival or differentiation, and the details need to be explored.

In conclusion, our study indicates that NSCs can express mGluR subtypes and hypoxia regulates the expression of mGluRs. The dynamic expression profile of mGluRs induced by hypoxia may be one of the mechanisms that hypoxia stimulates NSCs activation, at least increased expression of group I mGluR5 significantly enhanced the NSCs proliferation. Further studies attempting to characterize the functions of different mGluR subtypes in NSCs response to hypoxic/ischemic insults in vitro and in vivo are now possible. The results also suggest that regulation of mGluRs in NSCs biology is useful in the experimental cell replacement therapy of hypoxic/ischemic injuries of CNS.

Acknowledgements

This study was supported by a grant from National Natural Science Foundation of China (No. 81070998, No. 31070943, No. 31271151).

References

1. Zhao CM, Deng W, Gage FH. Mechanisms and functional implications of adult neurogenesis. Cell 2008; 132(4): 645-60.
2. Taupin P. Adult neurogenesis in the mammalian central nervous system: functionality and potential clinical interest. Med Sci Monitor 2005; 11(7): Ra247-Ra52.
3. Watt FM, Hogan BL. Out of Eden: stem cells and their niches. Science 2000; 287(5457): 1427-30.
4. Miller FD, Gauthier-Fisher A. Home at last: neural stem cell niches defined. Cell Stem Cell 2009; 4(6): 507-10.
5. Taupin P. Adult neural stem cells, neurogenic niches, and cellular therapy. Stem Cell Rev 2006; 2(3): 213-9.
6. Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med 2002; 8(9): 963-70.
7. Parent JM, Vexler ZS, Gong C, Derugin N, Ferriero DM. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. Ann Neurol 2002; 52(6): 802-13.

8. Yang Z, Levison SW. Hypoxia/ischemia expands the regenerative capacity of progenitors in the perinatal subventricular zone. Neuroscience 2006; 139(2): 555-64.
9. Kawai T, Takagi N, Nakahara M, Takeo S. Changes in the expression of Hes5 and Mash1 mRNA in the adult rat dentate gyrus after transient forebrain ischemia. Neurosci Lett 2005; 380(1-2): 17-20.
10. Zhang P, Liu Y, Li J, Kang Q, Tian Y, Chen X, et al. Cell proliferation in ependymal/subventricular zone and nNOS expression following focal cerebral ischemia in adult rats. Neurol Res 2006; 28(1): 91-6.
11. Felling RJ, Snyder MJ, Romanko MJ, Rothstein RP, Ziegler AN, Yang Z, et al. Neural stem/progenitor cells participate in the regenerative response to perinatal hypoxia/ischemia. J Neurosci 2006; 26(16): 4359-69.
12. Lichtenwalner RJ, Parent JM. Adult neurogenesis and the ischemic forebrain. J Cereb Blood Flow Metab 2006; 26(1): 1-20.
13. Zhu LL, Wu LY, Yew DT, Fan M. Effects of hypoxia on the proliferation and differentiation of NSCs. Mol Neurobiol 2005; 31(1-3): 231-42.
14. Chen X, Tian Y, Yao L, Zhang J, Liu Y. Hypoxia stimulates proliferation of rat neural stem cells with influence on the expression of cyclin D1 and c-Jun N-terminal protein kinase signaling pathway in vitro. Neuroscience 2010; 165: 705-14.
15. Burgers HF, Schelshorn DW, Wagner W, Kuschinsky W, Maurer MH. Acute anoxia stimulates proliferation in adult neural stem cells from the rat brain. Exp Brain Res 2008; 188(1): 33-43.
16. Studer L, Csete M, Lee SH, Kabbani N, Walikonis J, Wold B, et al. Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. J Neurosci 2000; 20(19): 7377-83.
17. Grow J, Barks JD. Pathogenesis of hypoxic-ischemic cerebral injury in the term infant: current concepts. Clin Perinatol 2002; 29(4): 585-602, v.
18. Schlett K. Glutamate as a modulator of embryonic and adult neurogenesis. Curr Top Med Chem 2006; 6(10): 949-60.
19. Luk KC, Kennedy TE, Sadikot AF. Glutamate promotes proliferation of striatal neuronal progenitors by an NMDA receptor-mediated mechanism. J Neurosci 2003; 23(6): 2239-50.
20. Melchiorri D, Cappuccio I, Ciceroni C, Spinsanti P, Mosillo P, Sarichelou I, et al. Metabotropic glutamate receptors in stem/progenitor cells. Neuropharmacology 2007; 53(4): 473-80.
21. Brazel CY, Nunez JL, Yang Z, Levison SW. Glutamate enhances survival and proliferation of neural progenitors derived from the subventricular zone. Neuroscience 2005; 131(1): 55-65.
22. Moldrich RX, Chapman AG, De Sarro G, Meldrum BS. Glutamate metabotropic receptors as targets for drug therapy in epilepsy. Eur J Pharmacol 2003; 476(1-2): 3-16.
23. Kenny PJ, Markou A. The ups and downs of addiction: role of metabotropic glutamate receptors. Trends Pharmacol Sci 2004; 25(5): 265-72.
24. Gerber U, Gee CE, Benquet P. Metabotropic glutamate receptors: intracellular signaling pathways. Curr Opin Pharmacol 2007; 7(1): 56-61.
25. Guo J, Zhou X, Chen Y, Bai M, Yang X, Zhao K, et al. mGluR3 promotes proliferation of human embryonic cortical neural progenitor cells by activating ERK1/2 and JNK2 signaling pathway in vitro. Cell Mol Biol 2014; 60(2): 42-49.
26. Cappuccio I, Verani R, Spinsanti P, Niccolini C, Gradini R, Costantino S, et al. Context-dependent regulation of embryonic stem cell differentiation by mGlu4 metabotropic glutamate receptors. Neuropharmacology 2006; 51(3): 606-11.
27. Castiglione M, Calafiore M, Costa L, Sortino MA, Nicoletti F, Copani A. Group I metabotropic glutamate receptors control proliferation, survival and differentiation of cultured neural progenitor cells isolated from the subventricular zone of adult mice. Neuropharmacology 2008; 55(4): 560-7.
28. Nakamichi N, Yoshida K, Ishioka Y, Makanga JO, Fukui M, Yoneyama M, et al. Group III metabotropic glutamate receptor activation suppresses self-replication of undifferentiated neocortical progenitor cells. J Neurochem 2008; 105(5): 1996-2012.
29. Duan Z, Zhang X, Zhu GX, Gao Y, Xue X. ACTIVATION OF mGluR4 PROMOTES PROLIFERATION OF RAT NEURAL PROGENITOR CELLS WHILE MEDIATING ACTIVATION OF ERK1/2 SIGNALING PATHWAY. Cell Mol Biol 2013; 59: 1809-17.
30. Yoshimizu T, Chaki S. Increased cell proliferation in the adult mouse hippocampus following chronic administration of group II metabotropic glutamate receptor antagonist, MGS0039. Biochem Biophys Res Commun 2004; 315(2): 493-6.
31. Di Giorgi-Gerevini V, Melchiorri D, Battaglia G, Ricci-Vitiani L, Ciceroni C, Busceti CL, et al. Endogenous activation of metabotropic glutamate receptors supports the proliferation and survival of neural progenitor cells. Cell Death Differ 2005; 12(8): 1124-33.
32. Canudas AM, Di Giorgi-Gerevini V, Iacovelli L, Nano G, D'Onofrio M, Arcella A, et al. PHCCC, a specific enhancer of type 4 metabotropic glutamate receptors, reduces proliferation and promotes differentiation of cerebellar granule cell neuroprecursors. J Neurosci 2004; 24(46): 10343-52.
33. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 1992; 255(5052): 1707-10.
34. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25(4): 402-8.
35. Tian YF, Zhang PB, Xiao XL, Zhang JS, Zhao JJ, Kang QY, et al. The quantification of ADAMTS expression in an animal model of cerebral ischemia using real-time PCR. Acta Anaesthesiol Scand 2007; 51(2): 158-64.
36. Brannvall K, Korhonen L, Lindholm D. Estrogen-receptor-dependent regulation of neural stem cell proliferation and differentiation. Mol Cell Neurosci 2002; 21(3): 512-20.
37. Kokaia Z, Lindvall O. Neurogenesis after ischaemic brain insults. Curr Opin Neurobiol 2003; 13(1): 127-32.
38. Brazel CY, Romanko MJ, Rothstein RP, Levison SW. Roles of the mammalian subventricular zone in brain development. Prog Neurobiol 2003; 69(1): 49-69.
39. Nguyen L, Rigo JM, Rocher V, Belachew S, Malgrange B, Rogister B, et al. Neurotransmitters as early signals for central nervous system development. Cell Tissue Res 2001; 305(2): 187-202.
40. Hagg T. Molecular regulation of adult CNS neurogenesis: an integrated view. Trends Neurosci 2005; 28(11): 589-95.
41. Liu CX, Xu X, Chen XL, Yang PB, Zhang JS, Liu Y. Glutamate promotes neural stem cell proliferation by increasing the expression of vascular endothelial growth factor of astrocytes in vitro. Cellular and molecular biology 2015; 61(4): 75-84.
42. Di Giorgi Gerevini VD, Caruso A, Cappuccio I, Ricci Vitiani L, Romeo S, Della Rocca C, et al. The mGlu5 metabotropic glutamate receptor is expressed in zones of active neurogenesis of the embryonic and postnatal brain. Brain Res Dev Brain Res 2004; 150(1): 17-22.
43. Heck S, Enz R, Richter-Landsberg C, Blohm DH. Expression of eight metabotropic glutamate receptor subtypes during neuronal differentiation of P19 embryocarcinoma cells: a study by RT-PCR and in situ hybridization. Brain Res Dev Brain Res 1997; 101(1-2): 85-91.
44. Lujan R, Shigemoto R, Lopez-Bendito G. Glutamate and GABA receptor signalling in the developing brain. Neuroscience 2005;

130(3): 567-80.
45. Spinsanti P, De Vita T, Di Castro S, Storto M, Formisano P, Nicoletti F, et al. Endogenously activated mGlu5 metabotropic glutamate receptors sustain the increase in c-Myc expression induced by leukaemia inhibitory factor in cultured mouse embryonic stem cells. J Neurochem 2006; 99(1): 299-307.
46. Panchision DM. The role of oxygen in regulating neural stem cells in development and disease. J Cell Physiol 2009; 220(3): 562-8.
47. Zhu LL, Zhao T, huang X, Liu ZH, Wu LY, Wu KW, et al. Gene expression profiles and metabolic changes in embryonic neural progenitor cells under low oxygen. Cellular reprogramming 2011; 13(2): 113-20.
48. Hilton GD, Nunez JL, Bambrick L, Thompson SM, McCarthy MM. Glutamate-mediated excitotoxicity in neonatal hippocampal neurons is mediated by mGluR-induced release of Ca++ from intracellular stores and is prevented by estradiol. Eur J Neurosci 2006; 24(11): 3008-16.
49. Casolini P, Zuena AR, Cinque C, Matteucci P, Alema GS, Adriani W, et al. Sub-neurotoxic neonatal anoxia induces subtle behavioural changes and specific abnormalities in brain group-I metabotropic glutamate receptors in rats. J Neurochem 2005; 95(1): 137-45.
50. Moroni F, Attucci S, Cozzi A, Meli E, Picca R, Scheideler MA, et al. The novel and systemically active metabotropic glutamate 1 (mGlu1) receptor antagonist 3-MATIDA reduces post-ischemic neuronal death. Neuropharmacology 2002; 42(6): 741-51.
51. Nadlewska A, Car H, Wisniewska RJ, Wisniewski K. Behavioral effects of the selective blockade of metabotropic glutamate receptor subtype 5 in experimental hypoxia. Pol J Pharmacol 2002; 54(2): 95-102.
52. Makarewicz D, Duszczyk M, Gadamski R, Danysz W, Lazarewicz JW. Neuroprotective potential of group I metabotropic glutamate receptor antagonists in two ischemic models. Neurochem Int 2006; 48(6-7): 485-90.
53. Storto M, Battaglia G, Gradini R, Bruno V, Nicoletti F, Vairetti M. Mouse hepatocytes lacking mGlu5 metabotropic glutamate receptors are less sensitive to hypoxic damage. Eur J Pharmacol 2004; 497(1): 25-7.
54. Xu X, Zhang J, Chen X, Liu J, Lu H, Yang P, et al. The increased expression of metabotropic glutamate receptor 5 in subventricular zone neural progenitor cells and enhanced neurogenesis in a rat model of intracerebral hemorrhage. Neuroscience 2012; 202: 474-83.
55. Poli A, Beraudi A, Villani L, Storto M, Battaglia G, Di Giorgi Gerevini V, et al. Group II metabotropic glutamate receptors regulate the vulnerability to hypoxic brain damage. J Neurosci 2003; 23(14): 6023-9.
56. Cai Z, Lin S, Rhodes PG. Neuroprotective effects of N-acetylaspartylglutamate in a neonatal rat model of hypoxia-ischemia. Eur J Pharmacol 2002; 437(3): 139-45.
57. Sabelhaus CF, Schroder UH, Breder J, Henrich-Noack P, Reymann KG. Neuroprotection against hypoxic/hypoglycaemic injury after the insult by the group III metabotropic glutamate receptor agonist (R, S)-4-phosphonophenylglycine. Br J Pharmacol 2000; 131(4): 655-8.
58. Zhang Z, Ma W, Wang L, Gong H, Tian Y, Zhang J, et al. Activation of Type 4 Metabotropic Glutamate Receptor Attenuates Oxidative Stress-Induced Death of Neural Stem Cells with Inhibition of JNK and p38 MAPK Signaling. Stem Cells Dev 2015; 24(22): 2709-22.
59. Osuga H, Hakim AM. Relationship between extracellular glutamate concentration and voltage-sensitive calcium channel function in focal cerebral ischemia in the rat. J Cereb Blood Flow Metab 1996; 16(4): 629-36.
60. Takagi K, Ginsberg MD, Globus MY, Dietrich WD, Martinez E, Kraydieh S, et al. Changes in amino acid neurotransmitters and cerebral blood flow in the ischemic penumbral region following middle cerebral artery occlusion in the rat: correlation with histopathology. J Cereb Blood Flow Metab 1993; 13(4): 575-85.
61. Vannucci RC, Brucklacher RM, Vannucci SJ. CSF glutamate during hypoxia-ischemia in the immature rat. Brain Res Dev Brain Res 1999; 118(1-2): 147-51.
62. Choi DW, Rothman SM. The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. Annu Rev Neurosci 1990; 13: 171-82.
63. Nacher J, Rosell DR, Alonso-Llosa G, McEwen BS. NMDA receptor antagonist treatment induces a long-lasting increase in the number of proliferating cells, PSA-NCAM-immunoreactive granule neurons and radial glia in the adult rat dentate gyrus. Eur J Neurosci 2001; 13(3): 512-20.
64. Hirasawa T, Wada H, Kohsaka S, Uchino S. Inhibition of NMDA receptors induces delayed neuronal maturation and sustained proliferation of progenitor cells during neocortical development. J Neurosci Res 2003; 74(5): 676-87.
65. Jansson LC, Akerman KE. The role of glutamate and its receptors in the proliferation, migration, differentiation and survival of neural progenitor cells. Journal of neural transmission 2014; 121(8): 819-36.