Cellular & Molecular Biology
Cell. Mol. Biol. 2014; 60 (2): 42-49
Published online September 6, 2014 (http://www.cellmolbiol.com)
Received on April 25, 2014, Accepted on August 19, 2014.
doi : 10.14715/cmb/2014.60.2.7

mGluR3 promotes proliferation of human embryonic cortical neural progenitor cells by activating ERK1/2 and JNK2 signaling pathway *in vitro*

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Abstract

Metabotropic glutamate receptors (mGluRs) regulate the proliferation and differentiation of neural progenitor cells (NPCs) in brain; however, the mechanisms remain unknown. In this study, we investigated the effect of mGluR3 on the proliferation of human embryonic neural progenitor cells (NPCs), the expression of cyclin D1 and the activation of signaling pathways of mitogen-activated protein kinases (MAPKs). The results showed that mGluR3 agonist N-Acetylaspartylglutamate (NAAG) increased the proliferation of NPCs by increasing cell activity, diameter of neurospheres and cell division. In addition, mGluR3 siRNA decreased the NPC proliferation. The protein expressions of cyclin D1 increased with NAAG treatment and decreased after siRNA treatment. It was also found that activation of extracellular signal-regulated protein kinase (ERK) and c-Jun N-terminal protein kinase (JNK) signaling pathways were involved in the proliferation of NPCs. NAAG increased phosphorylation of ERK1/2 and JNK2 levels, and meanwhile p-p38 level decreased; but p-ERK1/2 and p-JNK2 levels decreased after siRNA treatment, and p-p38 level increased. ERK1/2 inhibitor U0126 and JNK2 inhibitor SP600125 attenuated the increase of proliferation induced by NAAG. These findings demonstrated that mGluR3 promoted the proliferation of human embryonic cortical NPCs and increased cyclin D1 expression by activating ERK1/2 and JNK2 signaling pathways in vitro, suggesting that mGluR3 may be a target molecule for regulating NPC proliferation in brain development.

Key words: Metabotropic glutamate receptors, neural progenitor cells, mitogen-activated protein kinases, proliferation, cyclin D1.

Introduction

Neural precursor cells (NPCs), a kind of specific primitive nerve cell, exist in the mammalian central nervous system (CNS). NPCs can self-renew and give rise to the three major cell types-neurons, astrocytes, and oligodendrocytes. It has been reported that NPCs exist not only in the developing nervous system but also in the adult nervous system. These progenitor cells can be isolated from embryonic and adult brain, and expanded as neurospheres in the presence of mitogens such as epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) (1, 2). Control of the proliferation of NPCs may be important for the potential development of transplantation strategies and other therapeutic approaches for the treatment of neuronal injuries and neurodegenerative diseases (3, 4, 5). However, the molecular mechanisms of controlling proliferation of NPCs are still not fully understood.

A number of environmental signals support the proliferation of NPCs. Such as, glutamate is the major excitatory neurotransmitter in CNS, and play important roles in proliferation and maintenance of NPCs. Glutamate can regulate proliferation and cell death of NPCs by activating metabotropic glutamate receptors (mGluRs) (6, 7). mGluRs are potential attractive candidates as they are present in proliferating neural progenitor cells and their expression and function is differentially regulated during development (8, 9, 10, 11). mGluRs are a large family of eight receptor subtypes. Based on their diverse properties in terms of amino acid sequence, transduction coupling, pharmacology, and anatomical distribution, mGluRs have been classified into three groups: group I includes mGluR1 and mGluR5, which are coupled to polyphosphoinositide hydrolysis; group II (mGluR2 and mGluR3) and III (mGluR4, mGluR6, mGluR7, mGluR8) are negatively coupled to adenylyl cyclase (12, 13). Many studies have demonstrated that activation of mGluR3 can result in neuroprotection (14, 15). It is reported that cultured NPCs express mGluR3 (16, 17). ERK1/2 cascade transduces the activity of extracellular and intracellular signals into enduring changes in CNS structure and function by regulating cellular activities and gene transcription (18, 19). In addition, mGluR2 can couple to the mitogen activated protedin kinase (MAPK) cascade in chinese hamster ovary cells (20). Therefore, the MAPK signaling pathway may play an important role in the mGluRs mediated proliferation of NPCs.

In this experiment, we will explore mGluR3 would facilitate proliferation of human embryonic cortical NPCs and possible mechanisms of activation of mGluR3 on the relationship to cell signaling pathways.

Materials and methods

Isolation and culture of human neural progenitor cells

Human NPCs were isolated from 14-week fetuses cortex of electively terminating normal pregnant women at the 3nd Affiliated Hospital, College of Medi-

cine, Xi'an Jiaotong University. Informed consent was obtained from the pregnant woman before legal elective termination. The experimental protocols were approved by the Ethics Committee of College of Medicine, Xi'an Jiaotong University. The methods of specimen collection were conducted in accordance with the guidelines of National Institutes of Health and set out by Xi'an Jiaotong University. The cortex was removed in chilled sterile phosphate-buffered saline (PBS) containing 0.6% glucose under sterile condition. After removal of the meninges, the cortex was mechanically dissociated into single-cell suspensions in serum-free Dulbecco's Modified Eagles's Medium and Hams F12 (DMEM/F12) (GIBCO, USA). After centrifugation for 6 min at 800 rpm, the cells were cultured in culture flasks at a density of 100, 000 cells/ml of serum-free DMEM/F12 supplemented with 2% B27, 1% N2, 20ng/ml hEGF, 10ng/ml bFGF, 1% penicillin, 1% streptomycin and 2.5 μg/ml heparin. After 7 days of culture in vitro, the primary neurospheres were passaged by mechanical dissociation. The single cell was cultured at a density of 100, 000 cells/ml for 7 days until neurospheres were formed (passage 1 neurospheres). Passage 1 neurospheres were treated for below experiments.

siRNA synthesis and transfection

The pre-designed siRNA used for mGluR3 gene knockouting. Human mGluR3 siRNA (sense-5′GGG ACA AUG GAG AAU UAA ATT 3′, antisense-5′UUU AAU UCU CCA UUG UCC CAA 3′) and negative siRNA (NC-siRNA, sense-5′UUC UCC GAA CGU GUC ACG UTT 3′, antisense-5′ACG UGA CAC GUU CGG AGA ATT 3′) were chemically synthesiyed by Shanghai GenePharma Corporation (SGC, China). Lipofectamine™-2000 (Invitrogen, USA) were used to optimise siRNA transfection. All siRNA transfection were performed in serum-free DMEM/F12. Lipofectamine-siRNA complexes were initially formed with 300 nM siRNA and diluted to desired concentrations. Lipofectamine and siRNA were diluted in serum-free DMEM/F12 and incubated for 5 min at room temperature. After the two solutions were softly mixed, the mixed solutions were incubated for 15 min at room temperature. The complexes were added to the plated cells.

MTT assay and Diameter measure of neurospheres

The cell proliferation of human NPCs under treatment conditions was estimated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide) assay. Passage 1 NPCs were seeded into 96-well plates (10,000 cells/well in 200 μl medium) and incubated with the test substances for 2 days at 37°C in 5% CO_2. Then, NPCs were treated with different concentrations of Lipofectamine-siRNA complexes (30 nM, 60 nM), mGluR3 agonist N-Acetylaspartylglutamate (NAAG, 3 μM, 30 μM, 300 μM) (Sigma, St Louis, MO, USA), mGluR5 antagonist 6-methyl-2-(phenylethynyl) pyridine hydrochloride (MPEP, 100μM) (Sigma, St Louis, MO, USA), ERK1/2 inhibitor U0126 (15 μM) and JNK2 inhibitor SP600125 (15 μM) for 1 day, 2 days and 3 days. NPCs cultured with complete medium were used as blank control, treated with negative siRNA (NC-siRNA) and used as negative control. At the end of culture, the diameters of neurospheres were measured using DP71 camera (ver 5.1, Olympus, Japan), following 20 μl of 5 mg/ml MTT (Sigma, St Louis, MO, USA) was added per well and allowed to incubate at 37 °C for another 2 hours. The supernatants were removed, and the resulting formazan crystals were dissolved in 150 μL of dimethylsulfoxide (Sigma, St Louis, MO, USA). Absorbance was determined at 492 nm using multi-microplate test system (POLARstar OPTIMA, BMG Labtechnologies, Germany). In each experiment, five parallel wells were made. Results were collected as the mean of more than three independent experiments.

Cell cycle analysis

For analyzing cell cycle after siRNA and NAAG treatment, DNA content per duplicate was analyzed using a flow cytometer. Passage 1 NPCs were seeded in 6-well plates and incubated for 2 days. Then, NPCs were treated by 60 nM siRNA, 300 μM NAAG, 15 μM U0126 and 15 μM SP600125 for 1 day, and dissociated into single-cell suspensions, followed by 75% ice cold ethanol fixation overnight at 4 °C. Fixed cells were stained with 50 μg/ml propidium iodide (PI) containing 50 μg/ml RNase A (DNase free) for 20 min in the dark, and subsequently analyzed by fluorescence activated cell sorting and Modfit LT software (FACSCalibur, BD Biosciences, San Jose, CA, USA). The cells were excited at 488 nm, the emission was collected through a 630 nm filter. 20, 000 cells were collected from each sample. The distribution of cells in the G0/G1, S and G2/M stages were analyzed. We evaluated the changes of cell cycle by calculating the proliferation index (PI) and S-phase cell fraction (SPF). The following formulas were used: PI=(S+G2/M)/(G0/G1+S+G2/M), SPF=S/(G0/G1+S+G2/M) (21).

Apoptosis analysis

Passage 1 NPCs were seeded in 6-well plates and treated with 60 nM siRNA and 300 μM NAAG for 1 day, and dissociated into single-cell suspensions, then harvested and washed twice with PBS. The cells were labeled by incubation with 5 μL FITC-Annexin V and 10 μL PI at 250 μg/ml for 15 min in the dark at room temperature. The cells were washed with PBS and examined using a flow cytometry. Quantification of apoptosis was analyzed by counting the number of cells stained by FITC-labeled Annexin V. The apoptosis of cells was detected using the Annexin V/PI Apoptosis Detection Kit by FACS.

Western blot analysis

To observe the expression of cyclin D1, nestin, mGluR3, mGluR5, phosphorylated extracellular signal regulated kinase1/2 (ERK1/2), c-Jun N-terminal protein kinase2 (JNK2) and p38, passage 1 NPCs were treated with 60 nM siRNA and 300 μM NAAG for 1 day. At the end of culture, the cells were lysed in RIPA lysis buffer supplied with protease inhibitor cocktail. Insoluble material was removed by centrifugation at 12, 000 rpm for 15 minutes at 4 °C. Protein was fractionated by 10% SDS-polyacrylamide gel electrophoresis for 2 h and transferred to a nitrocellulose membrane for 3 h. The membranes were blocked with 5% non-fat dry milk in TBST for 2 hours, then were washed 3 times with TBST shaking for 5 min each time. The following

primary antibodies were incubated overnight at 4 °C: mouse anti-cyclin D1 (1:1000, Neomarker, Fremont, CA, USA), rabbit anti-mGluR3 (1:2000, Santa Cruz, CA, USA), rabbit anti-mGluR5 (1:2000, Santa Cruz, CA, USA), mouse anti-nestin (1:1000, Santa Cruz, CA, USA), rabbit anti-ERK1/2 (1:2000, Cell Signaling, Danvers, MA, USA), mouse anti-P-ERK 1/2 (1:2000, Cell Signaling, Danvers, MA, USA), rabbit anti-JNK2 (1:1000, Santa Cruz, CA, USA), mouse anti-P-JNK2 (1:2000, Cell Signaling, Danvers, MA, USA), Rabbit anti-p38 (1:2000, Cell Signaling,Danvers, MA, USA), rabbit anti-P-p38 (1:1000, Cell Signaling, Danvers, MA, USA), mouse anti-β-Actin (1:5000, Santa Cruz, CA, USA). These membranes were incubated in the dark with ECL (Amersham). The luminescent signal was recorded and quantified with the Syngene G Box (Syngene, UK). The data were sent to the computer for analysis and documentation.

Statistical Analysis

Statistical analysis was performed using SPSS 13.0 software. The results were analyzed by one-way ANOVA. Tukey's post-hoc analyses were used to detect the difference between groups. The data were expressed as mean±SD. The $P < 0.05$ was considered statistically significant.

Results

mGluR3 promoted proliferation of human NPCs

For analyzing the effects of mGluR3 on the proliferation of human NPCs. The passage 1 NPCs were treated with mGluR3 siRNA and agonist NAAG for 1, 2 and 3 days. MTT assay and Diameter measure of neurospheres methods were fulfilled. MTT assay showed that 60 nM siRNA significantly inhibited the proliferation of human NPCs at 1, 2 and 3 days of treatment (Fig. 1A), but 300 μM NAAG significantly promoted the proliferation of human NPCs at 1, 2 and 3 days of treatment (Fig. 1B) ($P < 0.05$). NAAG plus MPEP significantly promoted the proliferation of human NPCs at 1, 2 and 3 days of treatment compared to MPEP group, NAAG plus siRNA group significantly inhibited the proliferation of human NPCs at 1, 2 and 3 days of treatment compared to NAAG group (Fig. 1C) ($P < 0.05$). After 1, 2 and 3 days of treatment in NPCs, the mean diameter of neurospheres significantly decreased with the 60 nM siRNA treatment compared with those of control groups (Fig. 1D) ($P < 0.05$). However, the mean diameter of neurospheres of 300 μM NAAG treatment group remarkably increased ($P < 0.05$). After 1, 2 and 3 days of 300 μM NAAG treatment, the diameter of neurospheres were 70.27±6.21 μm, 105.36±7.83 μm and 134.72±8.45 μm, respectively (Fig. 1E). The mean diameter of neurospheres significantly increased in NAAG plus MPEP group compared to MPEP group ($P < 0.05$), and significantly decreased in NAAG plus siRNA group compared to NAAG group (Fig. 1F) ($P < 0.05$). Since there were significant differences on the proliferation of human NPCs between the control group and 60 nM siRNA, 300 μM NAAG treatment groups ($P < 0.05$), they were chosen to be the concentration in all the other experiments performed in this experiment. These findings suggested that mGluR3 promoted the proliferation of human NPCs in vitro.

Expression of nestin in human NPCs after siRNA knockdown mGluR3

Nestin was a marker of NPCs. Western blot results showed that nestin expressed in cultured human NPCs (Fig. 2). This confirmed that what we had cultured were surely NPCs. After knockdown of mGluR3 in human NPCs, the expression of nestin was no significant change in siRNA group and control group (Fig. 2). The results

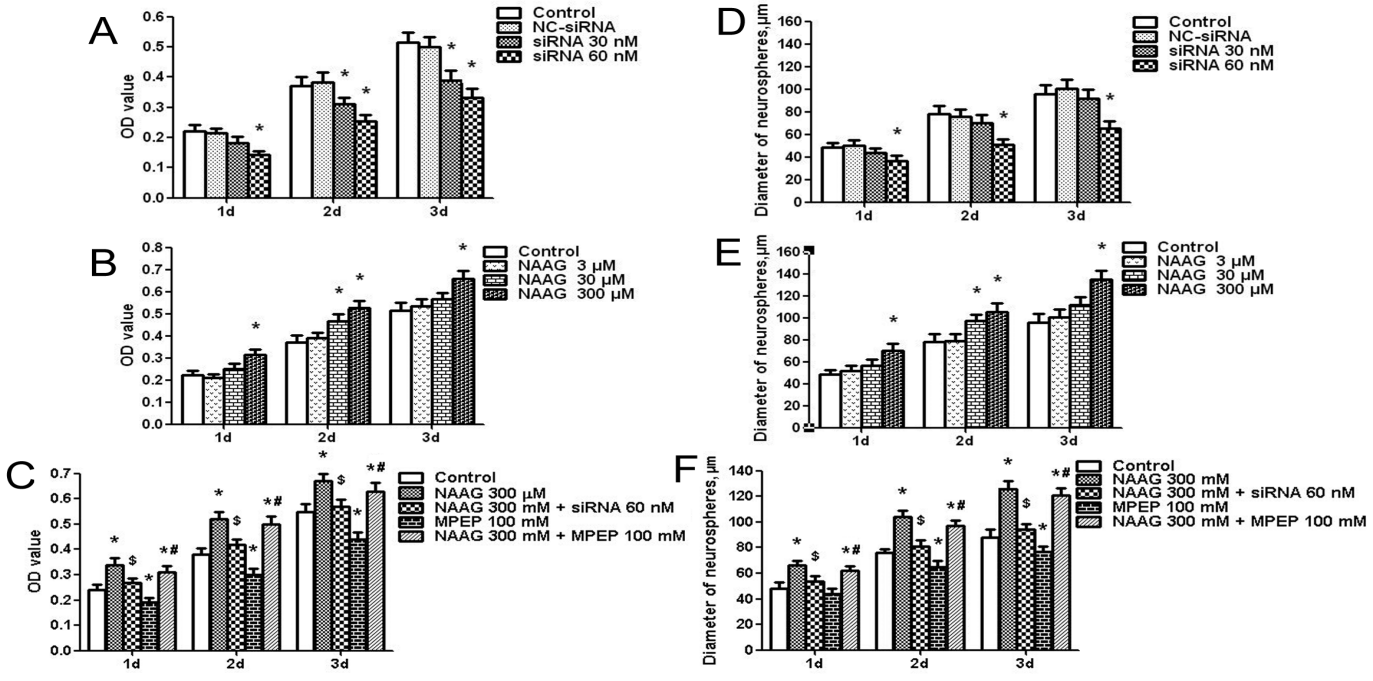

Figure 1. MTT assay for effects of mGluR3 on human NPCs proliferation (A, B, C) and neurosphere diameters (D, E, F). A & D: mGluR3 siRNA inhibited the proliferation of NPCs and decreased neurosphere diameters at 1, 2 and 3 d. B & E: mGluR3 agonist NAAG increased the proliferation of NPCs and increased neurosphere diameters at 1, 2 and 3 d. C & F: Effect of mGluR5 antagonist MPEP and mGluR3 siRNA on NAAG increasing NPC proliferation (* $P < 0.05$, compared to control group; $ $P < 0.05$, compared to NAAG group; # $P < 0.05$, compared to MPEP group, n=4).

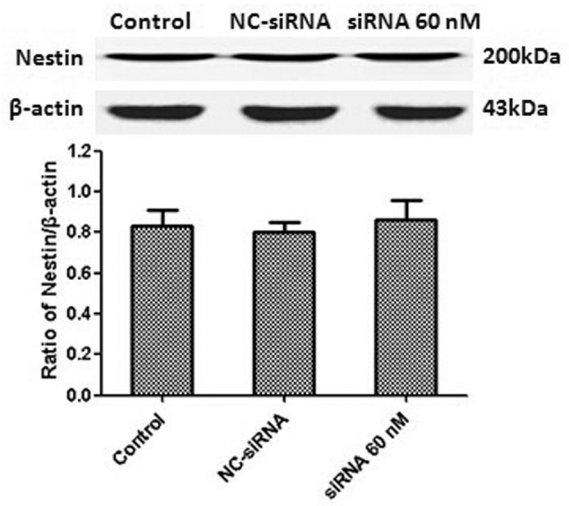

Figure 2. Effect of mGluR3 siRNA on expression of Nestin of human NPC. Western blotting results showed that nestin expression was no significant change after mGluR3 siRNA treatment (treatment versus control; * *P<0.05*, n=3).

confirmed that NPCs were still in undifferentiated state.

mGluR3 promoted human NPC proliferation by regulating the cell cycle and cyclin D1 expression

We next determined the cell cycle by fluorescence activated cell sorting (FACS) after human NPCs were treated by siRNA and NAAG. As illustrated in Fig 3A, only 20.31% of cells in mitotic phase were identified in the total NPCs at normoxic condition (PI=0.2031±0.0158). PI and SPF (PI=0.1142±0.0135, SPF=0.0733±0.0201) were significantly decreased in siRNA groups compared to control. But PI and SPF (PI=0.3168±0.0278, SPF =0.2287±0.021) were remarkbly increased in NAAG group ($P < 0.05$). The results indicated that mGluR3 significantly accelerated the cell division and DNA duplication of human NPCs.

Using Western blotting, we further analyzed the change of protein expression of mGluR3 and cyclin D1 (a key factor in the control of cell cycle). The mGluR3 expression only decreased in siRNA group (Fig. 3B) ($P < 0.05$). And the mGluR5 expression was no change in mGluR3 siRNA group compared to control group (Fig. 3C). The results confirmed that mGluR3-siRNA specifically silence mGluR3 expression. The protein expression of cyclin D1 of NPCs diminished remarkably in siRNA group compared to control group, but the expression of cyclin D1 of NPCs increased significantly in NAAG treatment (Fig. 3D) ($P < 0.05$).

mGluR3 inhibited apoptosis of human NPCs

To detect the possible effects of mGluR3 on cell death, we observed the apoptosis of human NPCs with Annexin V/PI staining. The proportion of early and late apoptosis (about 46.62±5.41%, 10.27±2.37%) increased significantly in siRNA group compared with control ($P < 0.05$). But the percent of early and late apoptosis (about 1.48±1.57%, 2.31±2.10%) decreased remarkably in NAAG group (Fig. 4) ($P < 0.05$). The results indicated that mGluR3 might interrupt apoptosis of human NPCs.

mGluR3 activated MAPK signaling pathway in human NPCs

To understand the possible molecular mechanism of mGluR3 promoting proliferation of human NPCs, we investigated the expression of the phosphorylated

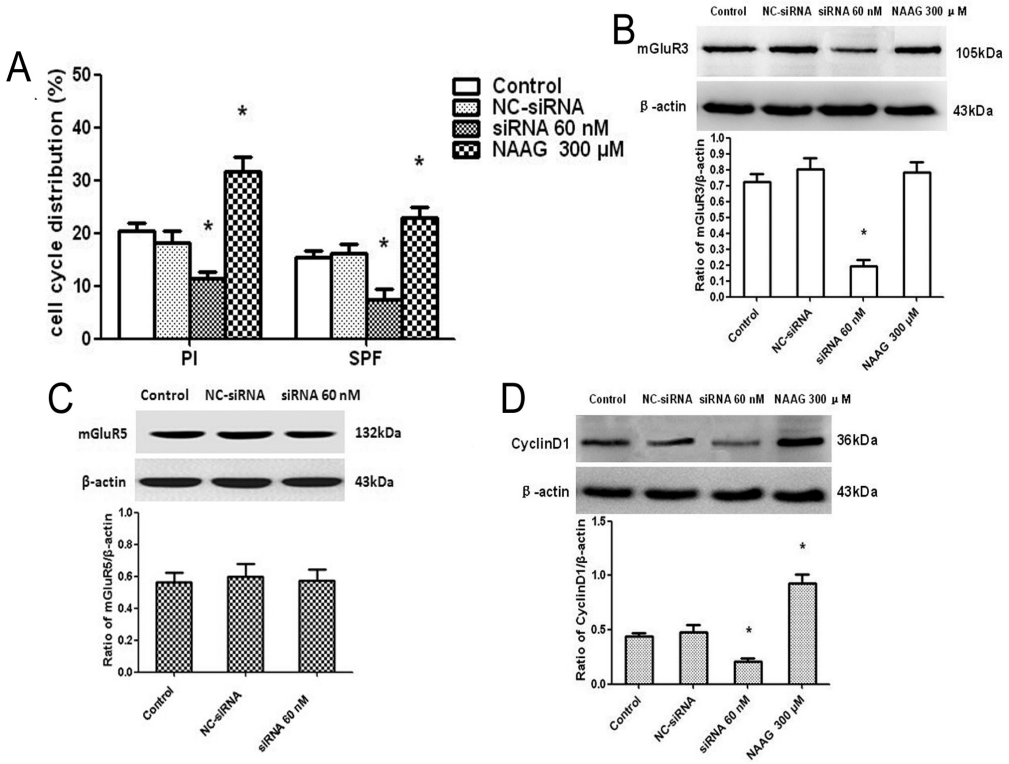

Figure 3. Effect of mGluR3 on cell cycle and expression of cyclin D1 of human NPC cultures. A: Cell cycle analysis showed that PI and SPF significantly changed by mGluR3. Proliferation index (PI)=(S+G2/M)/(G0/G1+S+G2/M), SPF=S/(G0/G1+S+G2/M). B: The protein expression of mGluR3 was only significantly decreased by siRNA. C: The protein expression of mGluR5 was no significant change after siRNA treatment. D: The protein expression of cyclin D1 significantly decreased after siRNA treatment, but increased after NAAG treatment (treatment versus control; * *P<0.05*, n=3).

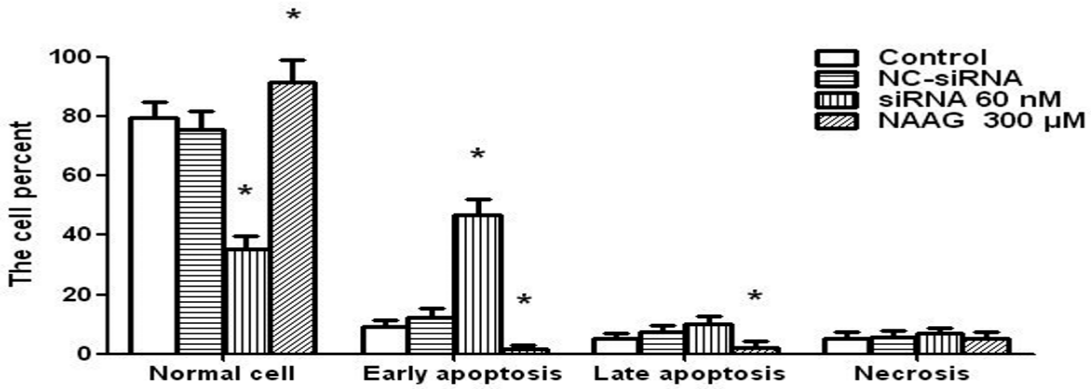

Figure 4. The effects of mGluR3 on apoptosis of human NPCs. The results of flow cytometry analysis of apoptosis in NPCs were visualized using Annexin-V/PI staining. The data show the percentage of normal cell, early apoptotic, late apoptotic, and necrotic NPCs. siRNA significantly increased apoptosis, but NAAG decreased apoptosis (treatment vs. control; * *P<0.05*, n=3).

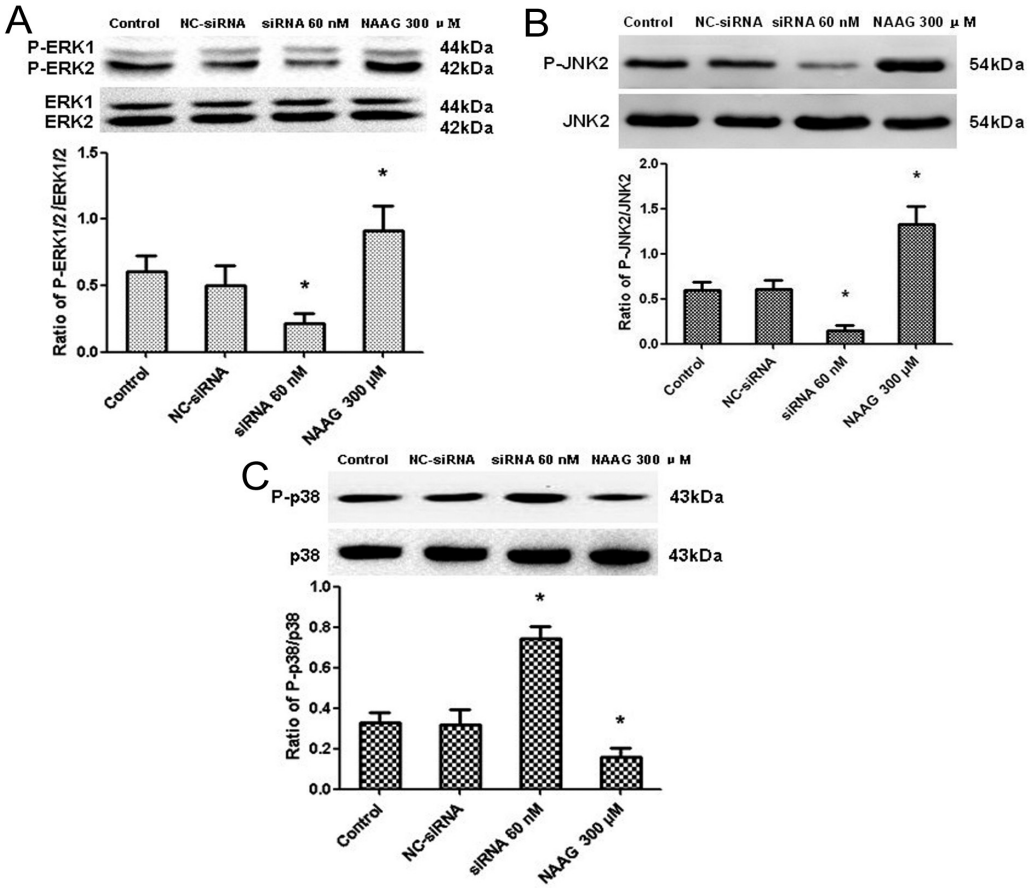

Figure 5. Expression of phosphorylated ERK1/2, JNK2 and p38 after siRNA and NAAG treatment in human NPCs. A: The expression of phosphorylated ERK1/2 significantly decreased in siRNA group, but increased in NAAG group. B: Phosphorylated JNK2 downregulated remarkably in siRNA group, upregulated in NAAG group. C: Phosphorylated p38 augmented in siRNA group, and diminished in NAAG group (treatment vs. control; * *P<0.05*, n=3).

ERK1/2, JNK2 and p38 in cell culture of human NPCs. No significant change was observed in the total protein expression of each signaling pathway (data not shown). The ratio of P-MAPK/total MAPK was used to indicate the phosphorylation and activation of the proteins. P-ERK1/2 and P-JNK2 level decreased remarkably in siRNA groups compared to control group, but increased significantly in NAAG groups (Fig. 5A, B) ($P < 0.05$). However, P-p38 upregulated significantly in siRNA groups, and it downregulated remarkably in NAAG group (Fig. 5C) ($P < 0.05$).

mGluR3 promoted human NPC proliferation by activating ERK1/2 and JNK2 signaling pathway

To determine whether the activation of MAPK participated in human NPCs proliferation induced by NAAG, we treated the NPCs with ERK1/2 inhibitor (U0126) and JNK2 inhibitor (SP600125), respectively, after NAAG administration 2 hours. MTT assay showed that NAAG plus U0126 significantly decreased the NPC activity compared to NAAG group at 1, 2 and 3 days of treatment, and NAAG plus SP600125 also diminished the cell activity compared to NAAG group (Fig. 6A) ($P < 0.05$). As illustrated in Figure 6B, when NPCs were treated with NAAG plus U0126, U0126 againsted human NPCs proliferation induced by NAAG, PI and SPF (PI=0.2372±0.0241, SPF=0.1411±0.019) were decreased significantly in NAAG+U0126 groups compared to NAAG group ($P < 0.05$). SP600125 produced similar effect. PI and SPF decreased remarkably com-

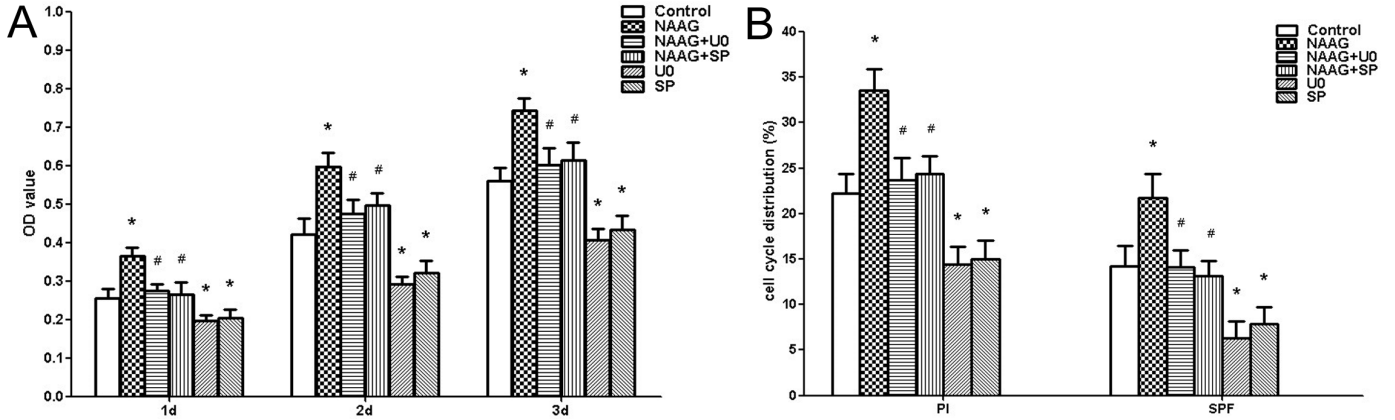

Figure 6. The effect of ERK1/2 and JNK2 inhibitors on human NPCs proliferation induced by NAAG. A: ERK1/2 inhibitor U0126 (15 μM) and JNK2 inhibitor SP600125 (15 μM) decreased the activity of NPCs at 1, 2, and 3 days. B: U0126 and SP600125 decreased significantly PI and SPF (* $P < 0.05$, compared to control group; # $P < 0.05$, compared to NAAG group, n=4).

pared to control groups when the cells were treated by SP600125 alone or U0126 alone without NAAG stimulation ($P < 0.05$). The results suggested that NAAG promotes human NPC proliferation by activating ERK1/2 and JNK2 signaling pathways.

Discussion

NPCs have the capacity for self-renewal and generation of new neurons, astrocytes and oligodendrocytes supporting cells. They are present not only in the fetal brain but also in the newborn and adult special brain areas (22). Recently, NPCs have be utilized to repair CNS diseases and replace lost neurons in neurological disorders. The proliferation of NPCs begins with the activation of membrane receptors by extracellular signals. These receptors activate intracellular signaling cascades that lead to changes in transcription of genes that are essential for proliferation NPCs (23). It has been reported that neurotransmitter receptors might regulate neurogenesis in the adult CNS (24). mGluRs are considered to be able to regulate proliferation of NPCs in the adult brain. For instance, Zhao et al. reported that mGluR5 promoted human embryonic cortical NPC proliferation in vitro (21, 25); Brazel et al. reported that mGluR2/3 agonists enhance survival and proliferation of cultured neuronal precursor cells derived from SVZ of newborn rats (16). However, the mechanisms of mGluR3 promoting NPC proliferation are uncertain.

NAAG is an endogenous dipeptide present in neuronal terminals of the hippocampal and cortical structures, areas which have long been implicated in the pathophysiology of schizophrenia (26). NAAG is synthesised enzymatically from N-acetylaspartate (NAA) and glutamate (27). NAAG has been demonstrated to activate mGluR2/3 (28, 29). In our experiments, only 300 μM NAAG can significantly promote the NPC proliferation. NAAG is considered as not a selective agonist for mGluR3, which can activate other mGluRs, and therefore some other mGluRs may also participate in regulating the NPC proliferation. To specify the effect of mGluR3 on NPC proliferation, siRNA, a popular reverse genetic tool which inhibits gene expression through sequence-specific degradation of a target mRNA, was adopted in our experiment to specifically silence mGluR3. mGluR3 shows a prominent expression in the developing human cortex at the early stages of cortical plate formation and cultured NPCs (16, 17). The activation of mGluR3 may positively regulate proliferation and survival of neural progenitor/stem cells derived from SVZ of newborn rats (16). We have found in this study that NAAG treatment may consistently enhance DNA duplication and cell division of human NPCs in vitro, and meanwhile increase cell activity and diameter of neurospheres. By contrast, siRNA treatments may reduce DNA duplication and cell division, as well as cell activity and diameter of neurospheres. It is also shown in our study that siRNA treatments increase cell death, while NAAG decreases cell death in vitro.

The first gap (G1) phase of cell cycle is a unique period when cells respond to environmental signals to determine cell fate such as proliferation, differentiation, cellular senescence and survival (30). D-type cyclins (including D1, D2, and D3) are important cell cycle regulators, which govern the cellular progression through the G1 phase of the cell cycle (31). The expression of three D-type cyclins fluctuates during the cell cycle and differs in a tissue-specific manner, indicating that the roles of different D-type cyclins are key in the control of cell cycle variations (32). Cyclin D1 and D2 are involved in the development of nerve system. Recent study suggests that cyclin D1 is important for the regulation of NPC proliferation during embryonic life, whereas cyclin D2 is more crucial in the adult brain (33, 34). After the extracellular mitogenic stimulation, D-cyclins can promote the release of E2F transcription factors and drive cell entry into S phase of the cell cycle (32). In this study, we demonstrate that NAAG may promote the expression of cyclin D1 and lead more cells into the S phase, while siRNA decrease the expression of cyclin D1 as well as the number of S phase cells. These results suggest that mGluR3 may increase the expression of cyclin D1 and drive more cells crossing G1/S node and entering into cell cycle, resulting in NPC proliferation.

The RAS MAPK signaling pathway includes membrane-to-nucleus signaling modules that are involved in the regulation of multiple biological and physiological processes such as survival, proliferation and differentiation. ERK, JNK and p38 MAPKs are main members of the MAPK family, which play an important role in the CNS development and differentiation (35). The ERK cascade can be activated by growth factors and transmit

signals to promote cell survival and proliferation (36). JNK can promote cell proliferation when it is activated by diverse cellular stresses, neurotransmitters, growth factors and cytokines (37). mGluR3 is coupled through G proteins to phospholipase C activation and acts on phosphatidylinositol bisphosphate. G-protein-coupled receptors can activate MAPK cascades through activation of PKC (38). It was reported that PKC/MAPK pathway can trigger cell proliferation (39). There have been several reports that PKC acts on the upstream of the ERK/JNK signaling pathway (40, 41). Zhao et al. demonstrated that mGluR5 may promote the proliferation and differentiation of neural progenitor cells by activating ERK and JNK signaling pathways (21, 25, 42). In this experiment, we find increased p-ERK and p-JNK in cultured human NPCs in response to mGluR3 agonist NAAG, but decreased p-ERK and p-JNK in response to siRNA, and NPC proliferation decreased when ERK1/2 and JNK2 signaling was inhibited by U0126 or SP600125. However, the signaling cascades underlying mGluRs seem to be far more complex. More work is needed to uncover the mechanisms of ERK and JNK pathways functioning on NPCs differentiation stimulated by mGluR3.

Activation of p38 MAPK could induce cell apoptosis, and inhibition of p38 with inhibitor SB203580 could diminish cell death (43, 44). In particular, it was reported that p38 MAPK signaling acts as a negative regulator of NSC proliferation (45). In this study, phosphorylation of p38 MAPK is found to have diminished in NPCs after the treatment with NAAG, while the expression of p-p38 has increased after siRNA treatment. These results suggest that mGluR3 may reduce apoptosis of NPCs by regulating the phosphorylation of p38 MAPK signaling.

In summary, our results demonstrate that mGluR3 promotes the proliferation of human embryonic cortical NPCs in vitro and reduce the apoptosis of the cells, and mGluR3 increases the expression of cyclin D1 through activating of ERK and JNK signaling pathways. This study suggests that mGluR3 may be a target molecule for regulating NPC proliferation and play a role in neural repair after brain injury and neurodegenerative disorders.

Acknowledgement

The authors would like to thank Professor Wang Jian Hua in Xi'an Jiaotong University, contributing to language revision of the manuscript.

References

1. Ciccolini, F. and Svendsen, C.N., Fibroblast growth factor 2 (FGF-2) promotes acquisition of epidermal growth factor (EGF) responsiveness in mouse striatal precursor cells: identification of neural precursors responding to both EGF and FGF-2. *J Neurosci.* 1998, **18**: 7869–7880.

2. Tropepe, V., Sibilia, M., Ciruna, B.G., Rossant, J., Wagner, E.F. and van der Kooy, D., Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Dev. Biol.* 1999, **208**: 166–188. doi: 10.1006/dbio.1998.9192

3. Okano, H., Stem cell biology of the central nervous system. *J. Neurosci. Res.* 2002, **69**: 698–707. doi: 10.1002/jnr.10343

4. Marshall, C.T., Lu, C., Winstead, W., Zhang, X., Xiao, M., Harding, G., Klueber, K.M. and Roisen, F.J. The therapeutic potential of human olfactory-derived stem cells. *Histol Histopathol.* 2006, **21**: 633–643.

5. Chaturvedi, R.K., Shukla, S., Seth, K. and Agrawal, A.K., Zuckerkandl's organ improves long-term survival and function of neural stem cell derived dopaminergic neurons in Parkinsonian rats. *Exp Neurol.* 2008, **210**: 608–623. doi: 10.1016/j.expneurol.2007.12.016

6. Schlett, K., Glutamate as a modulator of embryonic and adult neurogenesis. *Curr Top Med Chem*. 2006, **6**: 949–960. doi: 10.2174/156802606777323665

7. Recasens, M., Guiramand, J., Aimar, R., Abdulkarim, A. and Barbanel, G., Metabotropic glutamate receptors as drug targets. *Curr Drug Targets.* 2007, **8**: 651–681. doi: 10.2174/138945007780618544

8. Meguro, R., Ohishi, H., Hoshino, K., Hicks, T.P. and Noria, M., Metabotropic glutamate receptor 2/3 immunoreactivity in the developing rat cerebellar cortex. *J. Comp. Neurol.* 1999, **410**: 243–255. doi: 10.1002/(SICI)1096-9861(19990726)410:2%3C243::AID-CNE6%3E3.0.CO;2-I

9. Jokel, E.S., Garduno, E.R., Ariano, M.A. and Levine, M.S., Metabotropic glutamate receptors mGluR1alpha and mGluR2/3 display dynamic expression patterns in developing rat striatum. *Dev. Neurosci.* 2001, **23**: 1–6.

10. Defagot, M.C., Villar, M.J. and Antonelli, M.C., Differential localization of metabotropic glutamate receptors during postnatal development. *Dev. Neurosci.* 2002, **24**: 272–282. doi: 10.1159/000066741

11. Di Giorgi Gerevini, V.D., Caruso, A., Cappuccio, I., Ricci Vitigni, L., Romeo, S., Della Rocca, C., Gradini, R., Melchiorri, D. and Nicoletti, F., 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**: 17–22. doi: 10.1016/j.devbrainres.2004.02.003

12. Nakajima, Y., Iwakabe, H., Akazawa, C., Nawa, H., Shigemoto, R., Mizuno, N. and Nakanishi, S., Molecular characterization of a novel retinal metabotropic glutamate receptor mGluR6 with a high agonist selectivity for L-2-amino-4-phosphonobutyrate. *J. Biol. Chem.* 1993, **268**: 11868–11873.

13. Saugstad, J.A., Kinzie, J.M., Shinohara, M.M., Segerson, T.P. and Westbrook, G.L., Cloning and expression of rat metabotropic glutamate receptor 8 reveals a distinct pharmacological profile. *Mol. Pharmacol.* 1997, **51**: 119–125.

14. Flor, P.J., Lindauer, K., Puttner, I., Ruegg, D., Lukic, S., Knopfel, T. and Kuhn, R., Molecular cloning, functional expression and pharmacological characterization of the human metabotropic glutamate receptor type 2. *Eur J Neurosci.* 1995, **7**: 622–629. doi: 10.1111/j.1460-9568.1995.tb00666.x

15. Bruno, V., Battaglia, G., Copani, A., D'Onofrio, M., Di Iorio, P., De Blasi, A., Melchiorri, D., Flor, P.J. and Nicoletti, F., Metabotropic glutamate receptor subtypes as targets for neuroprotective drugs. *J Cereb Blood Flow Metab.* 2001, **21**: 1013–1033. doi: 10.1097/00004647-200109000-00001

16. Brazel, C.Y., Nunez, J.L., Yang, Z. and Levison, S.W., Glutamate enhances survival and proliferation of neural progenitors derived from the subventricular zone. *Neuroscience.* 2005, **131**: 55–65. doi: 10.1016/j.neuroscience.2004.10.038

17. Di Giorgi-Gerevini, V., Melchiorri, D., Battaglia, G., Ricci-Vitiani, L., Ciceroni, C., Buscati, C.L., Biagioni, F., Iacovelli, L., Canudas, A.M., Parati, E., De Maria, R. and Nicoletti, F., Endogenous activation of metabotropic glutamate receptors supports the proliferation and survival of neural progenitor cells. *Cell Death Differ.* 2005, **12**: 1124–1133. doi: 10.1038/sj.cdd.4401639

18. Valjent, E., Corvol, J.C., Trzaskos, J.M., Girault, J.A. and Herve, D., Role of the ERK pathway in psychostimulant-induced locomotor sensitization. *BMC Neurosci.* 2006, **7**: 20–32.

19. Zhao, L.Y., Jiao, Q., Xu, X., Yang, P.B., Song, T.S., Huang, C., Zhang, J.F. and Liu, Y., The decreased self-renewal potential of NPCs during human embryonic brain development with reduced activity of MAPKs. *Cell Mol Biol.* 2012, **58**: OL1730–1736.
20. Phillps, T., Barnes, A., Scott, S., Emson, P. and Rees, S., Human metabotropic glutamate receptor 2 couples to the MAP kinase cascade in chinese hamster ovary cells. *NeuroReport.* 1998, **9**: 2335–2339. doi: 10.1097/00001756-199807130-00034
21. Zhao, L., Jiao, Q., Chen, X., Yang, P., Zhao, B., Zheng, P. and Liu, Y., mGluR5 is involved in proliferation of rat neural progenitor cells exposed to hypoxia with activation of MAPK signaling pathway. *J Neurosci Res.* 2012, **90**: 447–460.
22. Mckay, R., Stem cells in the central nervous system. *Science.* 1997, **276**: 66–71. doi: 10.1126/science.276.5309.66
23. Chao, M.V., Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat. Rev. Neurosci.* 2003, **4**: 299–309. doi: 10.1038/nrn1078
24. Hagg, T., Molecular regulation of adult CNS neurogenesis: anintegrated view. *Trends Neurosci.* 2005, **28**: 589–595.
25. Zhao, L., Jiao, Q., Yang, P., Chen, X., Zhang, J., Zhao, B., Zheng, P. and Liu, Y., Metabotropic glutamate receptor 5 promotes proliferation of human neural stem/progenitor cells with activation ofmitogen-activated protein kinases signaling pathway in vitro. *Neuroscience*. 2011, **192**: 185–94.
26. Shave, E., Pliss, L., Lawrance, M.L., FitzGibbon, T., Stastny, F. and Balcar, V.J., Regional distribution and pharmacological characteristics of [3H]N-acetylaspartyl-glutamate (NAAG) binding sites in rat brain. *Neurochem. Int.* 2001, **38**: 53–62.
27. Gehl, L.M., Saab, O.H., Bzdega, T., Wroblewska, B. and Neale, J.H., Biosynthesis of NAAG by an enzyme-mediated process in rat central nervous system neurons and glia. *J. Neurochem.* 2004, **90**: 989–997. doi: 10.1111/j.1471-4159.2004.02578.x
28. Schweitzer, C., Kratzeisen, C., Adam, G., Lundstrom, K., Malherbe, P., Ohresser, S., Stadler, H., Wichmann, J., Woltering, T. and Mutel, V., Characterization of [(3)H]-LY354740 binding to rat mGlu2 and mGlu3 receptors expressed in CHO cells using semliki forest virus vectors. *Neuropharmacology.* 2000, **39**: 1700–1706.
29. Wroblewska, B., Wegorzewska, I.N., Bzdega, T., Olszewski, R.T. and Neale, J.H., Differential negative coupling of type 3 metabotropic glutamate receptor to cyclic GMP levels in neurons and astrocytes. *J. Neurochem.* 2006, **96**: 1071**–**1077. doi: 10.1111/j.1471-4159.2005.03569.x
30. Sherr, C.J., G1 phase progression: cycling on cue. *Cell*. 1994, **79**: 551–555. doi: 10.1016/0092-8674(94)90540-1
31. Sherr, C.J., D-type cyclins. *Trends Biochem Sci.* 1995, **20**: 187–190. doi: 10.1016/S0968-0004(00)89005-2
32. Kozar, K., Ciemerych, M.A., Rebel, V.I., Shigematsu, H., Zagozdzon, A., Sicinska, E., Geng, Y., Yu, Q., Bhattacharya, S., Bronson, R.T., Akashi, K. and Sicinski, P., Mouse development and cell proliferation in the absence of D-cyclins. *Cell.* 2004, **118**: 477–491. Doi: 10.1016/j.cell.2004.07.025
33. Kowalczyk, A., Filipkowski, R.K., Rylski, M., Wilczynski, G.M., Konopacki, F.A., Jaworski, J., Ciemerych, M.A., Sicinski, P. and Kaczmarek, L., The critical role of cyclin D2 in adult neurogenesis. *J Cell Biol.* 2004, **167**: 209–213. doi: 10.1083/jcb.200404181
34. Sundberg, M., Savola, S., Hienola, A., Korhonen, L. and Lindholm, D., Glucocorticoid hormones decrease proliferation of embryonic neural stem cells through ubiquitin-mediated degradation of cyclin D1. *J Neurosci.* 2006, **26**: 5402–5410. doi: 10.1523/JNEUROSCI.4906-05.2006
35. Yang, S.R., Cho, S.D., Ahn, N.S., Jung, J.W., Park, J.S., Jo, E.H., Hwang, J.W., Kim, S.H., Lee, B.H., Kang, K.S. and Lee, Y.S., The role of p38 MAP kinase and c-Jun N-terminal protein kinase signaling in the differentiation and apoptosis of immortalized neural stem cells. *Mutat Res.* 2005, **579**: 47–57. Doi: 10.1016/j.mrfmmm.2004.11.020
36. Matsuzawa, A. and Ichijo, H., Molecular mechanisms of the decision between life and death: regulation of apoptosis by apoptosis signal-regulating kinase 1. *J Biochem.* 2001, **130**: 1–8. doi: 10.1093/oxfordjournals.jbchem.a002947
37. Malemud, C.J., Inhibitors of stress-activated protein/mitogen-activated protein kinase pathways. *Curr Opin Pharmacol.* 2007, **7**: 339–343. doi: 10.1016/j.coph.2006.11.012
38. Liebmann, C., Regulation of MAP kinase activity by peptide receptor signalling pathway: paradigms of multiplicity. *Cell Signal.* 2001, **13**: 777–785. doi: 10.1016/S0898-6568(01)00192-9
39. Iijima, Y., Laser, M., Shiraishi, H., Willey, C.D., Sundaravadivel, B., Xu, L., McDermott, P.J. and Kuppuswamy, D., c-Raf/MEK/ERK pathway controls protein kinase C-mediated p70S6K activation in adult cardiac muscle cells. *J Biol Chem.* 2002, **277**: 23065–23075. doi: 10.1074/jbc.M200328200
40. Clark, J.A., Black, A.R., Leontieva, O.V., Frey, M.R., Pysz, M.A., Kunneva, L., Woloszynska-Read, A., Roy, D. and Black, J.D., Involvement of the ERK signaling cascade in protein kinase C-mediated cell cycle arrest in intestinal epithelial cells. *J Biol Chem.* 2004, **279**: 9233–9247. doi: 10.1074/jbc.M312268200
41. Huang, X., Yang, N., Ou, X., Li, D., Wang, Z., Xie, Q., Chen, Y., Lin, H., Yin, G. and Wen, F., Sequential activation of protein kinase C delta and JNK is required for interferon-alpha-induced expression of IFIT4. *Cell Signal.* 2008, **20**: 112–119. Doi: 10.1016/j.cellsig.2007.08.020
42. Zhao, L., Jiao, Q., Huang, C., Hou, N., Chen, X., Zhang, J., Yang, P., Xu, X., Song, T. and Liu, Y., mGluR5 promotes the differentiation of rat neural progenitor cells into cholinergic neurons and activation of extracellular signal-related protein kinases. *Neuroreport.* 2014, **25**: 427–434. doi: 10.1097/WNR.0000000000000134
43. Cheng, A., Chan, S.L., Milhavet, O., Wang, S. and Mattson, M.P., p38 MAP kinase mediates nitric oxide-induced apoptosis of neural progenitor cells. *J Biol Chem.* 2001, **276**: 43320–43327. doi: 10.1074/jbc.M107698200
44. Kim, H.J., Oh, J.E., Kim, S.W., Chun, Y.J. and Kim, M.Y., Ceramide induces p38 MAPK-dependent apoptosis and Bax translocation via inhibition of Akt in HL-60 cells. *Cancer Lett*. 2008, **260**: 88–95. doi: 10.1016/j.canlet.2007.10.030
45. Kim, J. and Wong, P.K., Loss of ATM impairs proliferation of neural stem cells through oxidative stress-mediated p38 MAPK signaling. *Stem Cells.* 2009, **27**: 1987–1998. doi: 10.1002/stem.125