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ACTIVATION OF mGluR4 PROMOTES PROLIFERATION OF RAT NEURAL PROGENITOR CELLS WHILE MEDIATING ACTIVATION OF ERK1/2 SIGNALING PATHWAY

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Abstract

Metabotropic glutamate receptors (mGluRs) influence the proliferation and differentiation of neural progenitor cells (NPCs) in the brain. They may play a major role in neurogenesis during embryonic development and in the adult brain. In this study, we investigated the expression of mGluR4 in NPCs and its possible role in the proliferation of rat embryonic NPCs *in vitro*, the expression of cyclin D1 and the activation of signaling pathways of mitogen-activated protein kinases (MAPKs). The results showed that mGluR4 protein was expressed in NPCs *in vitro*. mGluR4 selective agonist VU0155041 promoted the proliferation of NPCs by increasing cell activity, diameter of neurospheres and cell division. In addition, mGluR4 siRNA decreased the proliferation of NPCs. The protein expression of cyclin D1 increased with VU0155041 treatment and decreased after siRNA treatment. We also demonstrated that activation of ERK1/2 signaling pathways was involved in the proliferation of NPCs. VU0155041 increased phosphorylation of p-ERK1/2 levels, and mGluR4 siRNA decreased p-ERK1/2 levels. Furthermore, p-p38 expression was decreased by VU0155041 but was increased by mGluR4 siRNA. ERK1/2 inhibitor U0126 attenuated the increase of proliferation and cyclin D1 induced by VU0155041. These findings indicate that mGluR4 promotes the proliferation of rat NPCs and cyclin D1 expression through activation of ERK1/2 signaling pathways *in vitro*, suggesting that mGluR4 may play an important role in brain development. This study will help to develop a new potential therapeutic agent for brain injury and for the prevention of neurodegenerative disorders.

Key words: Metabotropic glutamate receptor 4, neural progenitor cells, proliferation, mitogen-activated protein kinase, cyclin D1.

Article information

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INTRODUCTION

Neural progenitor cells (NPCs) have the capacity of self-renewal and differentiation in the mammalian central nervous system (CNS) during early development and throughout adulthood. They ultimately produce neurons, astrocytes, and oligodendrocytes in the CNS. The discovery of NPCs has practical implications for brain repair following injury. Recently, the use of NPCs as treatment has been considered to be very effective in the improvement of neurologic functions in ischemic brain, nerve injury, and neurodegenerative disorders (7,26,33). After isolation of NPCs, many efforts have been made to identify molecular mechanisms of proliferation, differentiation, and cell-fate determination of NPCs. However, the molecular mechanisms of controlling cell-fate of NSCs are still not fully understood.

Glutamate can regulate cell death and neurogenesis by activating metabotropic glutamate receptors (mGluRs) (34,36). mGluRs are a family, including eight G-protein-linked receptors, which regulate a variety of intracellular signaling pathways (32). Group III (mGluR4, 6, 7, 8) mGluRs are coupled to Gi/Go (11,15) . Previous studies have shown that mGluRs participate in the biology of nerve cells, such as cell proliferation and differentiation (28,30). It is reported that embryonic stem (ES) cells can differentiate into mesoblast and endoderm cells with a remarkably increased expression of mGluR4 in the process. Moreover, activation of mGluR4 accelerates differentiation of retinoic acid-induced ES cells into NPCs (4). Recent evidence indicates that mGluR7 promotes the proliferation and differentiation of NPCs (42). Since mGluR4 and mGluR7 belong to the same mGluR group, it is likely that mGluR4 may also regulate the proliferation of NPCs. However, research on the function of mGluR4 is quite limited because of the lack of effective tools. Recently, the selective mGluR4 agonist VU0155041 provides an effective means for exploring the functions of mGluR4 (45). It has been shown that mitogen-activated protein kinases (MAPKs), including extracellular signal-related protein kinases (ERKs), c-Jun NH2-termianl kinases (JNKs), and p38 MAP kinases, regulate proliferation of nerve cells (39,48). Group II/III mGluRs have been found to activate MAPK pathways through G subunits excluding the Gi subunit (44). It is reported that mGluR3 affects the proliferation and differentiation of glioma-initiating cells (GICs) through MAPK pathways (10). Recent evidence has shown that the activation of mGluR7 promotes the proliferation and differentiation of NSCs by activating MAPK signaling pathways (42). Based on these findings, the MAPK pathways seem critical in the proliferation and differentiation of NPCs mediated by mGluRs. However, little is known about how or whether MAPK pathways involve regulation of mGluR4-induced proliferation in NPCs.

In the development of NPCs, mGluR4 plays a critical role in neurogenesis (4). Thus, here we aimed to investigate the effect of mGluR4 on proliferation of NPCs in embryonic rat brain and explore its mechanisms related to cell signaling system.

MATERIALS AND METHODS

Rat NPCs culture

Sprague Dawley (SD) rats were provided by the Experimental Animal Center of Xi'an Jiaotong University College of Medicine. The animal care was conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals. Experimental protocols were approved by the Ethics Committee of Xi'an Jiaotong University College of Medicine. Rat NSCs were prepared from the cortex of embryonic, day 15 (E15), fetal SD rats and cultured using the methods as described previously (2). The fresh cortex formation was microdissected in chilled, sterile phosphate-buffered saline (PBS) containing 0.6% glucose. The fetal cortex was incubated in a digestion solution containing 0.01% trypsin, 200 mM EDTA, 0.6% glucose, and 1 mM MgCl2 in PBS (all reagents from Sigma, St Louis, MO, USA) at 37 °C for 10 minutes (min). Next, the tissues were mechanically dissociated into a single cell suspension. The single cells were obtained by filtration using a three-hundreds-mesh stainless steel screen and cultured in serum-free-DMEM/F12 [Dulbecco's modified Eagle medium and Hams F12 (1:1) 10 ng/ml bFGF, 20 ng/ml EGF, 1% N2, 1% penicillin, 1% streptomycin, and 2% B27 supplement (all from Invitrogen, Carlsbad, CA, USA) and 2.5 μg/ml heparin (Sigma, St Louis, MO, USA)]. For culture, cells were plated at an initial concentration of 100,000 cells/ml in 50 ml cell culture flasks at 37 °C with 5% CO_2. After 5-7 days *in vitro* (DIV), the primary neurospheres were passaged. The neurospheres were dissociated with 0.05% trypsin and 200 μM EDTA for 10 min at 37 °C and mechanically triturated into a single cell suspension. The single cells were cultured at a density of 50,000 cells/ml for 4-5 days when neurospheres of 90 – 120 μm in diameter had been propagated (passage 1 neurospheres). Passage 1 neurospheres were used in this experiment. At least three independent experiments were performed for each assay.

siRNA synthesis and transfection

The pre-designed siRNA used for mGluR4 gene silencing. Rat mGluR4 siRNA (sense-5′CCA GGA AGG UAU GAA AUA ATT 3′, antisense-5′UUA UUU CAU ACC UUC CUG GAG 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, Carlsbad, CA, USA) were used to optimise siRNA transfection. All siRNA transfection were performed in serum-free DMEM/F12. Lipofectamine-siRNA complexes were initially formed with 250 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 diluted to the desired transfection concentration and added to the plated cells.

MTT assay and diameter measurement of neurospheres

We used the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based assay and measured the diameter of neurospheres to evaluate the effect of mGluR4 on proliferation of NPCs. The cells were incubated in 96-well plates (20,000 cells/well in 200 μl medium) and cultured for 2 days at 37 °C in 5% CO_2. Followed by incubation with different concentrations of Lipofectamine-siRNA complexes (30nM, 60nM), or 1 μM, 50 μM, 100 μM of selective mGluR4 agonist VU0155041 (Sigma, St Louis, MO, USA) or 10 μM, 20 μM, 40 μM of selective ERK1/2 inhibitor (U0126) (Cell Signaling, MA, USA) for 1 day, 2 days, and 3 days. Neurospheres cultured with complete medium were used as blank control. After culturing for 1, 2 and 3 days, 20 μl of 5 mg/ml MTT (Sigma, St Louis, MO, USA) was added per well and the cells were incubated for another 4 hours at 37 °C. Supernatants were removed and formazan crystals were then dissolved in 150 μL of dimethylsulfoxide (Sigma, St Louis, MO, USA). Finally, optical density was determined at 492 nm using multi-microplate test system (POLARstar OPTIMA, BMG Labtechnologies, Germany).

Diameter of neurospheres was measured using the methods as described previously and some modifications were made (42). Under monitored by inverted phase contrast microscope, passage 1 neurospheres (30-40 μm diameter) were individually transferred into the wells (one neurosphere/well) of nonadherent 96-well plate in 200 μl of serum-free medium using a sterile capillary tube. The neurospheres were cultured at 37 °C with 5% CO_2 for 12h, then the neurospheres were treated with siRNA, VU0155041 and U0126 for 1, 2 and 3d. The diameters of neurospheres were measured using DP71 camera and Image-Pro Express software (ver 5.1, Olympus, Japan). Results were collected as the average of more than three independent experiments.

Flow cytometric analysis of cell cycle

To determine the effects of mGluR4 on the cell cycle of rat NPCs, DNA content per duplicate was analyzed using a flow cytometer. The cells dissociated from the passage 1 neurospheres were seeded in 6-well plates and incubated for 2 days. Next, the cells were treated with 60 nM siRNA, 50 μM VU0155041, or 50 μM VU0155041 + 20 μM U0126 for 1 day and dissociated into single cell suspensions. The cells were fixed in 75% ice cold ethanol overnight at 4 °C. The fixed cells were stained with 50 μg/ml propidium iodide (PI) containing 50 μg/ml RNase A (DNase free) for 15 min at room temperature in the dark and analyzed by fluorescence activated cell sorting and Modfit LT software (FACSCalibur, BD Biosciences, San Jose, CA, USA). The cells were excited at 488 nm. At the same time, the emission was collected through a 630 nm filter. Twenty thousand cells were gathered from each sample. The distribution of the cell cycle included the G0/G1, S, and G2/M stages. We analyzed the distribution 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) (8).

Hoechst 33258 staining

To analyze the effect of mGluR4 on apoptosis of NPCs, Hoechst 33258 labeling was used to identify the nuclei and to detect the chromosomal condensation and morphological changes. After being cultured for 2 days, the dissociated passage 1 neurospheres were treated with 60 nM siRNA, 50 μM VU0155041, or 50 μM VU0155041 + 20 μM U0126 for 1 day, and dissociated into a single cell sus-

pension. The single cells were plated onto the poly-L-lysine-coated coverslips in 24-well plates for another 6 hours (h). Next, the cells were fixed in 4% paraformaldehyde in PBS. Cells were stained with Hoechst 33258 (5 μg/ml) (Sigma, USA) for 5 min at room temperature. The stained cells were observed under UV illumination using an Olympus fluorescent microscope. The percentage of apoptotic cells was determined by counting the number of nuclear condensation cells versus total cells in each experimental condition. At least total 600 cells were used.

Measurement of apoptosis by Annexin-V/PI staining

After being cultured for 2 days, the dissociated passage 1 neurospheres were treated with 60 nM siRNA, 50 μM VU0155041, or 50 μM VU0155041 + 20 μM U0126 for 1 day, and dissociated into a single cell suspension. The cells were labeled by incubation with 5 μL FITC-Annexin V and 10 μL PI at 250 μg/ml for 10 min in the dark at room temperature. Cells then were washed with PBS and examined using flow cytometry. Quantification of apoptosis was determined 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. Early apoptotic cells were identified with PI negative and FITC Annexin V positive; cells that were in late apoptosis or already dead were both FITC Annexin V and PI positive.

Western blot analysis

To detect the expression of cyclin D1, mGluR4, phosphorylated extracellular signal regulated kinase (ERK), c-Jun N-terminal protein kinase (JNK), and p38, passage 1 rat NPCs were treated with 60 nM siRNA, 50 μM VU0155041, or 50 μM VU0155041 + 20 μM U0126 for 1 day. The cells were then collected and lysed in RIPA lysis buffer. Insoluble material was removed by centrifugation at 12, 000 rpm at 4 °C. Proteins were subjected to electrophoresis using 10% SDS polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were blocked for 2 h in 5% non-fat dry milk in PBST. The membrane was incubated with primary monoclonal antibodies overnight at 4 °C and with the secondary antibody for 4 h at room temperature. These antibodies included: anti-cyclin D1 (1:1000, Neomarker, Fremont, CA, USA), anti-mGluR4 (1:1000, Santa Cruz), anti-ERK1/2 (1:2000, Cell Signaling, Danvers, MA, USA), anti-P-ERK 1/2 (1:2000, Cell Signaling, Danvers, MA, USA), anti-JNK2 (1:1000, Santa Cruz), anti-P-JNK (1:2000, Cell Signaling, Danvers), anti-p38 (1:2000, Cell Signaling), anti-P-p38 (1:1000, Cell Signaling), anti-β-Actin (1:5000, Santa Cruz). These membranes were incubated in the dark with ECL (Amersham, USA).. The luminescent signal was recorded and quantified using the Syngene G Box (Syngene, UK). The data were then recorded in the computer for analysis and documentation.

Statistical analysis

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

RESULTS

mGluR4 was expressed in neurospheres of E15 rat cortex in vitro

To detect whether mGluR4 is expressed in rat E15 cortical NPCs, western blotting was performed using protein extracted from passage 1 neurospheres. The results showed that mGluR4 protein was expressed in rat NPCs. mGluR4 siRNA significantly decreased the expression of mGluR4 protein. Moreover, there was no change in the expression of mGluR4 protein after treatment with the selective mGluR4 agonist VU0155041 or VU0155041 + U0126 (Fig. 1).

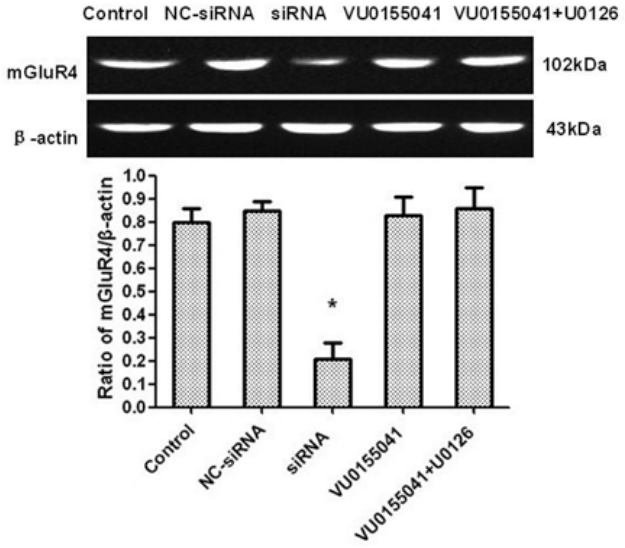

Figure 1. Protein expression of mGluR4 after NPCs spheres were treated with siRNA, VU0155041, or VU0155041+U0126. mGluR4 siRNA significantly decreased the expression of mGluR4 protein.

mGluR4 promoted the proliferation of rat NPCs

To determine whether mGluR4 plays an important role in the proliferation of rat E15 cortical NPCs, secondary neurospheres were treated with mGluR4 siRNA (30, 60 nM), agonist VU0155041 (1, 50, 100 μM), and /orERK1/2 inhibitor (U0126) (10 μM, 20 μM, 40 μM) for 1, 2, and 3 days. Results of MTT assay showed that mGluR4 siRNA significantly inhibited cell activity of rat NPCs only at the concentration of 60 nM at different times examined, when compared with that in control Group (Fig. 2A) (P<0.05). By contrast, mGluR4 agonist VU0155041, at a concentration of 50 and 100 μM, significantly promoted the proliferation of NPCs (Fig. 2B) (P<0.05). Furthermore, 20 μM and 40 μM U0126 significantly suppressed the proliferation of NPCs increased by VU0155041, when compared with 50 μM VU0155041 group (Fig. 2C) (P<0.05).

To further determine the effects of mGluR4 on the proliferation rate of E15 cortical NPCs, we measured the diameter of neurospheres formed from NPCs; the mean diameter of neurospheres significantly decreased 1, 2, and 3 days after 60 nM siRNA treatment when compared with those of control groups (Fig. 2D, G) (P<0.05). In addition, the mean diameter of neurospheres remarkably increased after 50 and 100 μM VU0155041 treatment (Fig. 2E, G) (P<0.05). Meanwhile, 20 μM and 40 μM U0126 significantly reduced the increased size of neurospheres induced by 50 μM VU0155041 when compared with 50 μM VU0155041 group (Fig. 2F, G) (P<0.05). Since there

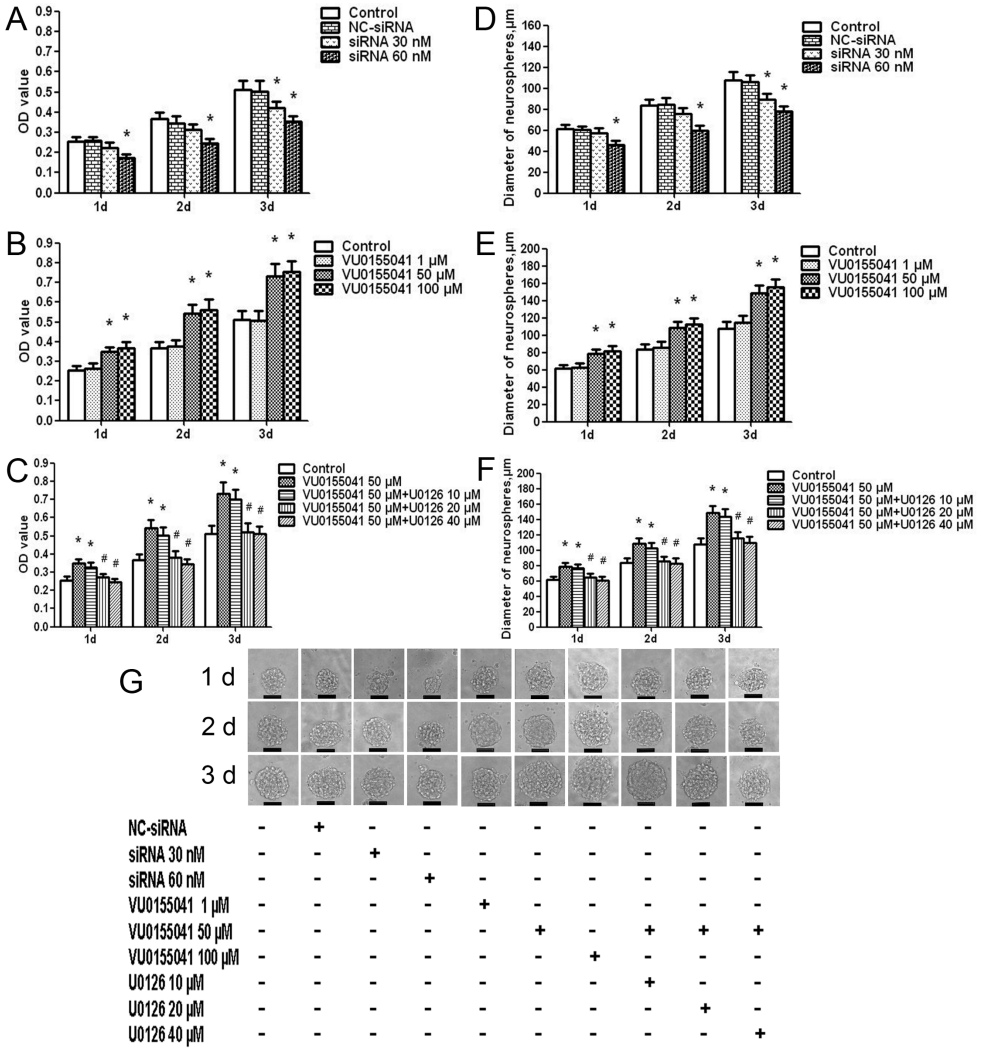

Figure 2. Effects of mGluR4 on proliferation of rat NPCs by MTT assay (A-C) and neurosphere diameters (D-F). (A and D) mGluR4 siRNA inhibited the proliferation of NPCs at 1, 2, and 3 d. (B and E) mGluR4 agonist VU0155041 increased the proliferation of NPCs at 1, 2, and 3 d. (C and F) ERK1/2 inhibitor U0126 decreased the proliferation of NSCs compared with VU0155041 group at 1, 2 and 3 d. (G) The change of phase contrast images of neurospheres (* $P<0.05$, compared to control group; # $P<0.05$, compared to VU0155041 group). Scale bar = 50 μm.

were significant differences in the proliferation of NPCs between the control group and 60 nM siRNA, or 50μM VU0155041 treatment groups ($P<0.05$), 60 nM siRNA, 50 μM VU0155041 and 20 μM U0126 were the chosen concentrations used in the following experiments. The results suggested that mGluR4 promoted the proliferation of rat E15 cortical NPCs *in vitro.*

mGluR4 promoted the proliferation of rat NPCs by regulating cell cycle and expression of cyclin D1

As the cell cycle is involved in the regulation of cell growth, we determined the cell cycle using fluorescence activated cell sorting (FACS) after the cells dissociated from the neurospheres were treated with siRNA, VU0155041, or VU0155041+U0126. As illustrated in Fig 3, only 15.35% of cells in mitotic phase were identified out of the total NPCs at normoxic condition (PI = 0.1535 ± 0.0133). mGluR4 siRNA induced a significant decrease in PI and SPF by approximately 1.80 and 2.05 folds ($P<0.05$), respectively, when compared to the control. Moreover, PI and SPF were remarkably increased in VU0155041 group by approximately 1.85 and 1.86 folds ($P<0.05$). To further examine whether the cell cycle regulated by mGluR4 was mediated through ERK1/2, we assessed the effect of U0126 in the VU0155041-treated neurosphere cells. U0126 (20 μM) significantly decreased PI and SPF values by approximately 1.73 and 1.75 fold in the VU0155041 -treated neurosphere cells, a level similar to the control level, when compared to that in the VU0155041-treated neurosphere cells (Fig. 3A) ($P<0.05$). The results suggested antagonistic effect of U0126 on promotion of NPCs growth induced by VU0155041.

Based on the fact that cyclin D1 was a key factor in the control of cell cycle, next, we further analyzed the change of protein expression levels of cyclin D1 using western blotting. Protein expression of cyclin D1 of NPCs diminished remarkably in the siRNA group compared to the control group. Moreover, the expression of cyclin D1 of NSCs increased significantly in VU0155041 condition. Also, the expression of cyclin D1 decreased significantly in VU0155041+U0126 group compared to VU0155041 (Fig. 3B) ($P<0.05$). These results indicated that mGluR4 promoted the proliferation of NPCs via ERK1/2 regulating the expression of cyclin D1 *in vitro*.

Activation of mGluR4 inhibited apoptosis of rat NPCs

To examine the possible effects of mGluR4 on cell death, we observed the morphology of nuclei in the cells dissociated from primary neurospehres using Hoechst 33258 staining and Measurement of apoptosis by Annexin-V/PI staining. We found that the proportion of Hoechst 33258-stained nuclei was 14.73 ± 1.11% under normal

culture condition (Fig. 4A, F). The proportion increased remarkably after siRNA treatment (approximately 23.15 ± 2.12%, $P<0.05$) (Fig. 4C, F). By contrast, VU0155041 treatment induced a decrease in Hoechst 33258-stained nuclei by approximately 2.18-fold (Fig. 4D, F) ($P<0.05$). Furthermore, U0126 treatment significantly increased the number of Hoechst 33258-stained nuclei by 2.26-fold, when compared to that in VU0155041 group (Fig. 4E, F) ($P<0.05$). The proportion of early apoptotic and late apoptotic increased significantly after siRNA treatment, VU0155041 treatment induced a decrease, U0126 treatment increased remarkably the number of early apoptotic and late apoptotic, when compared to that in VU0155041 group (Fig. 4G) ($P<0.05$). These findings showed that mGluR4 may interrupt apoptosis of NPCs.

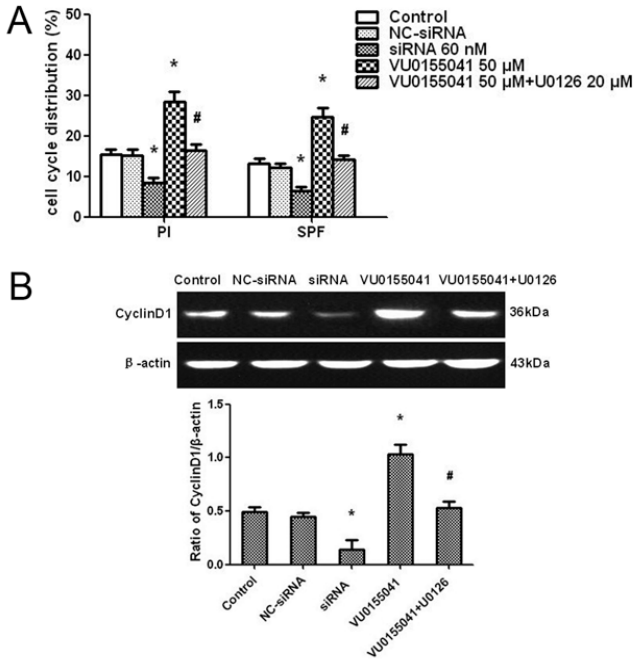

Figure 3. Effects of mGluR4 on cell cycle and cyclin D1 of rat NPCs. (A) The results of cell cycle analysis were performed by PI staining. The data showed that PI and SPF significantly changed by siRNA, VU0155041, and VU0155041+U0126. Proliferation index PI = (S + G2/M)/(G0/G1 + S + G2/M), SPF = S/(G0/G1 + S + G2/M). (B) The changes of protein expression of cyclin D1 (* $P<0.05$, compared with control group; # $P<0.05$, compared with VU0155041 group).

3.5 mGluR4 promoted the proliferation of rat NPCs through activiating ERK1/2 signaling pathways in vitro

To examine whether MAPK signaling pathways involves proliferation and differentiation of rat E15 coritcal NPCs, we examined the expression of phosphorylated ERK, JNK, and p38 in cell cultures of rat NPCs. We found no significant changes in the total expression of each signaling pathway (ERK, JNK, and p38), Furthermore, the expression of phosphorylation of MAPK signaling pathways changed in all treatment groups. The ratio of p-MAPK/total MAPK was used to indicate the phosphorylation and activation of the proteins. We found the ratio of p-ERK1/2 to ERK1/2 was decreased 5.50-fold after treatment with siRNA, when compared to that in the control group (Fig. 5A) ($P<0.05$). The ratio was significantly increased by 2.88-fold in the VU0155041 group, and U0126 inhibited the increased ratio induced by VU0155041 to control level (Fig. 5A) ($P<0.05$). However, there was no significant difference in the phosphorylation of p-JNK2 after treatment with siRNA or VU0155041 and/or U0126 (Fig. 5B).

By contrast, the ratio of p-p38 to total p38 was significantly increased by nearly 3.23-fold in the group treated with siRNA compared to that in the control group (Fig. 5C) ($P<0.05$); In addition, the ratio was remarkably reduced by 1.94 fold in the VU0155041 groups; by contrast, U0126 inhibited the reduction induced by VU0155041 to control level when compared to the VU0155041 group (Fig. 5C) ($P<0.05$).

DISCUSSION

It is showed mGluRs play a critical role during cortical development and neurogenesis (12,13,36). In particular, some experiments demonstrate that mGluRs might support basic developmental processes, such as proliferation, survival, and differentiation of NPCs (6). For instance, mGluR Group I (mGluR1/5) have been shown to regulate survival, proliferation, and differentiation of neural progenitor cells, suggesting a role for these receptors in brain development and developmental disorders (6). The activation of mGluR5 may positively regulate proliferation, survival, and differentiation into neurons of NPCs (3,5,13,40). mGluR3 has been shown to facilitate proliferation of embryonic stem cells and NPCs (3,12,13). A positive role in NSC/NPC proliferation has also been suggested for group II receptors (2). Recent evidence suggests that mGluR Group III (mGluR4, 6, 7, 8) may be functionally expressed to regulate self-renewal capacity through a mechanism related to cAMP formation with promotion of subsequent differentiation of NPCs (29). It is reported that activation of mGluR7 promotes the proliferation and differentiation of neural progenitor cells and influences phosphorylation of MAPK signaling pathways (43). Previous studies have shown that activation of mGluR4 promotes retinoic acid-induced ES cell differentiation into NPCs (4). Since mGluR4 and mGluR7 belong to the same mGluR group, it is likely that mGluR4 may also possess the same functions in regulating the biological properties of NPCs. However, the mechanisms that mGluR4 regulates in NSC/NPC proliferation are uncertain. Recent studies indicate that mGluR4 regulats neurosphere proliferation, at least in part, through upregulation of mTOR pathway activity, and suggest that P-Ser or other mGluR4 effectors could be used to promote neurogenesis following stroke or other focal CNS injury (29,35). In the present study, we found that, GluR4 siRNA reduced DNA duplication and cell division of rat E15 cortical NPCs *in vitro*, as well as cell activity and size of neurospheres. In contrast, the selective mGluR4 agonist, VU0155041, had opposite effects. In addition, ERK1/2 inhibitor U0126 abolished the effects of VU0155041 on the increased DNA duplication, cell activity, and size of neurospheres. These findings indicate that mGluR4 positively regulates cell proliferation of rat E15 cortical NPCs via regulating ERK1/2.

Although our and other studies show the roles of mGluR4 in regulating proliferation of NPCs, the underlying mechanisms are unclear. One possibility is that mGLR4 promotes proliferation via controlling cell cycle. Here, we found that VU0155041 treatment stimulated the expression of cyclin D1 but siRNA decreased the expression, moreover, ERK1/2 inhibitor U0126 abolished the effects of VU0155041. These findings suggest that mGluR4 increases the expression of cyclin D1 and drives more cells

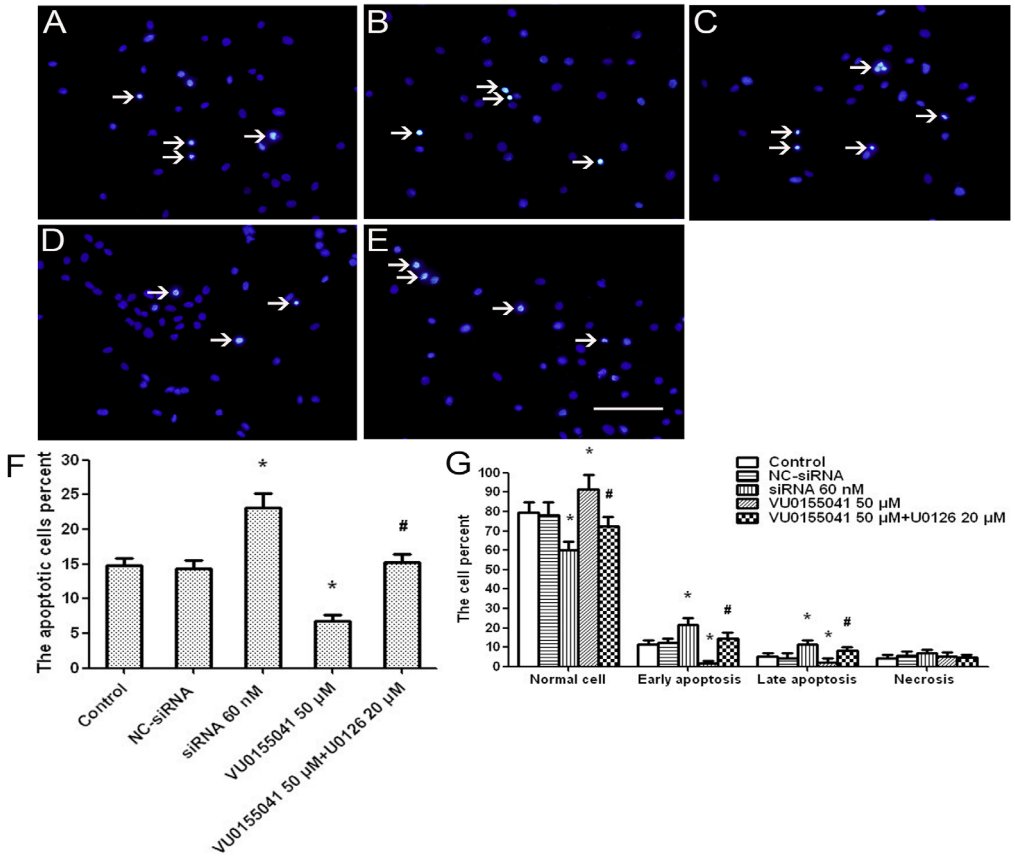

Figure 4. The effects of mGluR4 on the cell death of NPCs by nuclei stained with Hoechst 33258 and flow cytometry analysis of apoptosis. Panel (A–E) showed Hoechst 33258-stained apoptotic cells in Control, NC-siRNA, siRNA, VU0155041 and VU0155041+U0126 groups respectively. Panel (F) showed the percentage of apoptotic cells in all groups. siRNA increased the apoptotic cell. VU0155041 decreased the apoptotic cell, but U0126 increased apoptosis. Panel (G) The results of flow cytometry analysis of apoptosis were visualized using Annexin-V/PI staining. The data show the percentage of normal cell, early apoptotic, late apoptotic, and necrotic. (* P<0.05, compared with control group; # P<0.05, compared with VU0155041 group). White arrowheads indicate apoptotic cells. Scale bar = 100 μm.

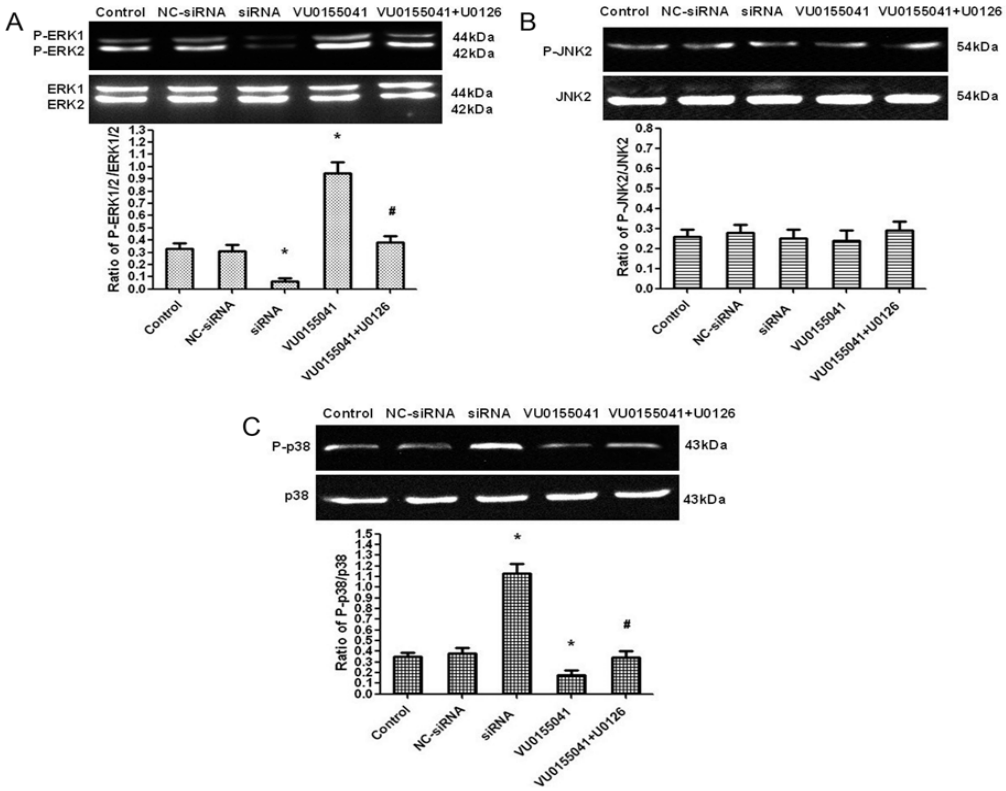

Figure 5. Western blotting analysis of phosphorylated JNK, ERK, p38 after treatments. (A) Phosphorylated and total ERK1/2. (B) Phosphorylated and total JNK2. (C) Phosphorylated and total p38 (* P<0.05, compared with control group; # P<0.05, compared with VU0155041 group). Data were expressed as a ratio of the normalized percentage of p-MAPKs and MAPKs.

crossing G1/S node and enters into cell cycle by activating ERK1/2, consequently, resulting in proliferation of NPCs. It is known that cyclinD1 is an important cell cycle protein responsible for the promoted transition into the proliferative stage. Over-expression of cyclinD1 leads to shortening of G1 phase, subsequently, leading to cross the G1/S point to rapidly enter into S phase (19,38). The expression of cyclinD1 is mainly regulated at the transcriptional level with little involvement of post-transcriptional processes (46). Mice lacking cyclin D1 show developmental neurological abnormalities and hypoplastic retinas, while cyclin D2-deficient animals have cerebellar defects (16,21). Recent studies show that cyclin D1 is a major regulator to control proliferation of NPCs during embryonic life, whereas cyclin D2 is more crucial in the adult brain (24,41). After the extracellular mitogenic stimulation, D-cyclins result in the release of the E2F transcription factors and drive cell entry into the S phase of the cell cycle (14,25).

Besides regulating neurosphere proliferation via controlling cell cycle, we also demonstrated that MAPK signaling pathways could be involved in proliferation. Here, we found that increased phosphorylation of ERK in response to activation of mGluR4 induced by VU0155041, decreased p-ERK in the VU0155041+U0126 group. Furthermore, in siRNA treatment group, our results reveal decreased p-ERK. These findings indicate that mGluR4 might promote the proliferation of NPCs by activating phosphorylation of p-ERK. Ferraguti et al previously showed that the stimulation of mGluR4 could activate MAPK in CHO cell model (17). Previous studies showed mGluR5 and mGluR7 also regulate the proliferation of neural progenitor cells through the activating of MAPK pathway (43,47), so mGluR4, mGluR5 and mGluR7 may promote the proliferation of NSCs/NPCs through the activating of MAPK pathway together. Shaw et al. have reported that MAPKs are downstream effectors of regulating NSC/NPC proliferation (37). It is known that MAPK signaling pathways are activated by stimuli at the extracellular membrane and culminate in the phosphorylation and activation of MAPKs including JNKs, ERKs, and p38, which promote translation and activate transcription factors, which increase protein synthesis and regulate multiple biological and physiological processes, such as survival, proliferation, differentiation, and transformation (18). In particular, JNK, ERK, and p38 MAPK play an important role in CNS development and differentiation. The ERK cascade could be stimulated by growth factors and transmit signals to facilitate cell survival, proliferation (20,27). Interestingly, it has been recently shown that group I mGluRs activates ERK and phosphoinositide 3-kinase (PI3K)–mTOR signaling pathways (1,31,40) following stroke or other focal CNS injury (29,35). Thus, it would be interesting to further investigate the roles of mGluR4 in neurogenesis after brain injury.

In addition, we further demonstrated that mGluR4 promoted the proliferation by inhibiting the apoptosis of NPCs and by reducing the phosphorylation of p38, as demonstrated by the fact that phosphorylation of p38 is diminished after VU0155041 treatment. Moreover, siRNA treatment increased the expression of p-p38. It has been shown that phosphorylation of p38 pathway mediates cell apoptosis, and inhibition of p38 with selective inhibitor SB203580 can reduce the cell death (9,22). Recent studies suggest that p38 MAPK signaling acts as a negative regulator of NSC/NPC proliferation (23).

In conclusion, we found that mGluR4 was expressed in rat NPCs, and mGluR4 promoted the proliferation of rat NSCs/NPC *in vitro* and reduce the apoptosis of the cells, and activation of mGluR4 may increase the expression of cyclin D1 with phosphorylation of ERK1/2. The results will provide novel insights to aid in the development of new strategies facilitating the expansion of NPCs and neural repair for nerve injury and neurodegenerative disorders. However, the signaling cascades underlying mGluR4 are very complicated and more work is needed to explore the mechanisms of MAPK signaling pathways on the proliferation of NPCs induced by mGluRs.

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