Neurochemistry International 133 (2020) 104630

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

PDE9 inhibition promotes proliferation of neural stem cells *via* cGMP-PKG pathway following oxygen-glucose deprivation/reoxygenation injury *in vitro*

Xiao Huan[a,1], Cheng Oumei[b,1], Qiu Hongmei[a], Yang Junxia[a], Ma Xiaojiao[a], Jiang Qingsong[a,*]

[a] Department of Pharmacology,Chongqing Key Lab of Biochemistry and Molecular Pharmacology, Chongqing Medical University, Chongqing, 400016, China
[b] Department of Neurology, The First Affiliated Hospital of Chongqing Medical University, Chongqing, 400016, China

ARTICLE INFO

Keywords:
Phosphodiesterase 9 (PDE9)
Cerebral ischemia
Neural stem cells
Proliferation
Cyclic guanosine monophosphate (cGMP)
cGMP-dependent protein kinase (PKG)

ABSTRACT

Cerebral ischemia is one of leading causes of death and long-term disability worldwide. Stem cell-based therapy is promising some valuable strategies for the structural and functional recovery after ischemic insult. The inhibition of phosphodiesterases (PDEs) has wide spectrum neuroprotective properties by stimulating proliferation of neural stem cells (NSCs). However, the potential role of PDE9 on NSCs proliferation after cerebral ischemia is not well investigated. The present study aimed to assess the contribution of PDE9 inhibition on the proliferation of NSCs and to determine the details of its underlying mechanisms against cerebral ischemia. The survival and proliferation of NSCs were assessed by CCK-8 assay and BrdU immunofluorescence staining, respectively. PDE9 activity and cGMP level were measured by ELISA kits. The protein expression of PKG and BDNF was detected by Western blot. Exposing NSCs of cultured primary hippocampus to oxygen-glucose deprivation/reoxygenation (OGD/R) significantly decreased the survival rate, but increased the proliferation of NSCs. Meanwhile, PDE9 activity was decreased, cGMP level was increased, PKG and BDNF protein expression was increased. PF-04447953, a PDE9 inhibitor, increased the survival rate of NSCs, moreover, PDE9 activity reduced more, and NSCs proliferation, cGMP level, PKG and BDNF protein expression were increased further, compared with OGD/R model group. These effects of PF-04447953, except for PDE9 activity and cGMP level, were reversed by treatment with KT5823, a PKG inhibitor. Taken together, the inhibition of PDE9 can promote the proliferation of NSCs following OGD/R injury, which may be, at least partly, mediated by cGMP-PKG pathway.

1. Introduction

Stroke is one of leading causes of death and long-term disability worldwide. It has been found that more than 80% of stroke is induced by cerebral ischemia. At present, there is no neuroprotective agent that has been approved by FDA for treatment of ischemic stroke. Brain self-repair processes after ischemic stroke are attracting more attention. Accumulating studies have suggested that neural stem cells (NSCs), which occurs mainly in the subventricular zone and subgranular zone in hippocampus, are important to restore cerebral function after ischemia (Zhang et al., 2013). NSCs could be activated under pathological conditions. Then the activated NSCs can proliferate, differentiate, migrate, and integrate into the damaged area to promote the recovery of neurological function (Vandenbosch et al., 2009). However, these abilities of endogenous NSCs are remarkably poor. Meanwhile, the transplantation of exogenous NSCs is restricted because of limited sources, immunological rejection, and ethical considerations (De et al., 2012). Therefore, the activation of endogenous NSCs might be an effective therapeutic strategy after ischemic insult. Unfortunately, there is still a lack of knowledge about the involved signaling pathways to regulate and the specific targets to promote the proliferation of NSCs.

Altering intracellular signaling pathways involved in inflammation and immune regulation, neurotransmitters have shown therapeutic benefits in experimental models of neurological diseases (Whitney et al., 2009). One of the second messengers, cyclic guanosine monophosphate (cGMP), is intracellular signaling target, the elevation of which has produced beneficial cellular effects within a range of CNS pathologies, including promoting the proliferation of NSCs in cerebral ischemia (Chalimoniuk and Strosznajder., 1998). cGMP activates cGMP-dependent protein kinase (PKG), then induces cGMP response element-binding protein (CREB) phosphorylation and affect several other targets, such as brain-derived neurotrophic factor (BDNF), which possesses the well-established neurotrophic action and neuroprotective effect (Chen et al., 2017). The only known negative regulators of cyclic

* Corresponding author. Department of Pharmacology, Chongqing Medical University, Chongqing, 400016, China.
E-mail address: cqjiangqs@163.com (J. Qingsong).
[1] Contributed equally.

https://doi.org/10.1016/j.neuint.2019.104630
Received 24 September 2019; Received in revised form 2 December 2019; Accepted 6 December 2019
Available online 09 December 2019
0197-0186/

Abbreviations

AD	Alzheimer's disease
AMP	adenosine monophosphate
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BrdU	5′-bromo-2-deoxyuridine
CCK-8	cell counting kit-8
cGMP	cyclic guanosine monophosphate
CREB	cGMP response element-binding protein
DAPI	4′, 6-diamidino-2-phenylindole
DMEM	dulbecco's modified Eagle's medium
EGF	epidermal growth factor
FBS	fetal bovine serum
GC	guanylate cyclase
GMP	guanylate monophosphate
MS	multiple sclerosis
NSCs	neural stem cells
OD	optical density
OGD/R	oxygen-glucose deprivation/reoxygenation
PD	Parkinson's disease
PDEs	phosphodiesterases
PKG	cGMP-dependent protein kinase
PLL	poly-L-lysine

nucleotides are phosphodiesterases (PDEs) family that hydrolyze cyclic nucleotides into adenosine monophosphate (AMP) or guanylate monophosphate (GMP). Mammalian PDE enzymes are divided into 11 families of proteins and three broad groups based on their catalytic selectivity to cyclic nucleotides: cAMP-specific PDEs (PDE4, PDE7 and PDE8), cGMP-specific PDEs (PDE5, PDE6, and PDE9), and PDEs that lack specificity, hydrolyzing both cyclic nucleotides (PDE1, PDE2, PDE3, PDE10, and PDE11) (Knott et al., 2017). Among them, PDE9, a specific hydrolase for cGMP, is highly abundant in hippocampus and cortex, which are related to learning and memory and emotion (Reyes-Irisarri et al., 2007). Studies have found that the inhibition of PDEs enhances the proliferation of NSCs. For example, PDE5 inhibition increases the proliferation in subventricular zone derived NSCs cultures (Santos et al., 2014); PDE7 inhibition promotes proliferation and neuronal differentiation in subventricular zone and subgranular zone of the adult brain (Morales-Garcia et al., 2017). Since PDE9 is also present in the central nervous system, some studies have reported that PDE9 inhibitors can significantly improve the learning and memory impairment induced by scopolamine and MK801 in rats (Van der Staay et al., 2008; Kroker et al., 2014), and can promote the elongation of nerve cells and synapse formation (Hutson et al., 2011). However, the effect of PDE9 inhibition on NSCs proliferation after cerebral ischemia is unknown.

Therefore, an oxygen glucose deprivation/reoxygenation (OGD/R) model was used in cultured hippocampal primary NSCs to mimic cerebral ischemia. PDE9 inhibitor, PF-04447943 and PKG inhibitor, KT5823 were applied to determine the action of PDE9 inhibition and the underlying regulation of cGMP-PKG signaling pathway.

2. Materials and methods

2.1. Chemical and regents

PF-04447943 ($C_{20}H_{25}N_7O_2$; MW: 395.46; purity ≥ 99.84%; LCMS-grade) was purchased from Medchem Express (Monmouth, NJ, USA). KT5823 was purchased from R&D Systems (Minnesota, MN, USA). Dulbecco's modified Eagle's medium (DMEM)/F12, B27, and StemPro™Accutase™Cell dissociation reagent were purchased from Gibco Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Sijiqing (Jiangsu, China). Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were purchased from Peprotech, Inc (Rocky Hill, NJ, USA). 5′-bromo-2-deoxyuridine (BrdU) and poly-L-lysine (PLL) were purchased from Sigma Aldrich (Santa Clara, CA, USA). Cell counting kit-8 (CCK-8) and 4′, 6-diamidino-2-phenylindole (DAPI) were purchased from Genview (Beijing, China). Dylight 594 and Alexa Fluor 488 were purchased from Immunoway Biotechnology (Plano, TX, USA). cGMP, PDE9 ELISA Kits were purchased from MEIBIAO Biological Co. Ltd (Jiangsu, China). RIPA lysate and BCA Protein Quantification Kits were purchased from Beijing Dingguo Changsheng Biotechnology Co. Ltd (Beijing, China). BDNF, BrdU and nestin antibodies were purchased from Abcam (Cambridge, UK). PKG antibody was purchased from Bioss Biotechnology Co. Ltd (Beijing, China). β-actin antibody was purchased from Proteintech Group, Inc (Hubei, China). The remaining reagents were analytical grade.

2.2. Isolation and cultivation of hippocampal NSCs

Animal experiments were carried out in strict accordance with the regulations in the Guide for the Care and Use of Laboratory Animals issued by the Ministry of Science and Technology of the People's Republic of China. All experiments involving rats were reviewed and approved by the Animal Laboratory Administration Center and the Ethics Committee of Chongqing Medical University [Permit Number: SCXK (Chongqing)-2018-0003]. Neonatal 1-day-old Sprague-Dawley rats, provided by the Experimental Animal Center of Chongqing Medical University, were used for primary hippocampal NSCs cultivation, as previously described with slight modifications (Reynolds et al., 1992; Andersen et al., 2007; Babu et al., 2011). Briefly, neonatal rats were decapitated and the bilateral hippocampus were isolated and minced in a 0.125% trypsin solution for 20 min at 37 °C. The tissue was washed in media containing 10% FBS, and then passed through a 200 mesh filter screen. The extracted cells were resuspended in complete medium containing DMEM/F12, 2% B27, 20 ng/mL recombinant rat bFGF, and 20 ng/mL recombinant rat EGF, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cell suspension was filtered through a 400 mesh filter screen, plated in 75 cm^2 glass culture flasks (1×10^6 cells/mL), and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Fresh complete medium was replaced every 2–3 days, and the cells were subcultured once every 7 days. Experiments were performed using the third passage cells. Cells were plated onto PLL–coated plastic culture plates or dishes containing complete medium.

2.3. Identification of NSCs

Briefly, the identification of NSCs has changed slightly as previously described (Cheng et al., 2015). Cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature; rinsed three times with PBS; and permeabilized with 0.3% Triton X-100 in PBS for 30 min. Cells were then blocked with 10% normal goat serum for 30 min at room temperature. Subsequently, the NSCs were incubated overnight at 4 °C with nestin antibody (1:100). The following day, cells were washed and incubated for 90 min at room temperature with Alexa Fluor 488 antibody (1:100). Cell nuclei were counterstained with DAPI for 3–5 min in the dark. Finally, the cells were examined by fluorescence microscopy.

2.4. OGD/R model

In brief, the OGD/R model was established with a slight change as described above (Chen et al., 2011; Wu et al., 2012). After washing three times with D-Hanks' solution, NSCs were maintained with a

glucose-free serum-free DMEM medium in a humidified anaerobic incubator containing 94% N_2, 1% O_2, and 5% CO_2 at 37 °C for 150 min. For reoxygenation, the a glucose-free serum-free DMEM medium was replaced with complete medium and the NSCs were incubated in a humidified normoxic atmosphere containing 5% CO_2 for 48 h. To investigate the action of PDE9 inhibition and the effect of cGMP-PKG signaling pathway, PF-04447943 (10^{-7} mol/L, PDE9 inhibitor) alone or with KT5823 (10^{-6} mol/L, PKG inhibitor) were used after OGD/R for 48 h.

2.5. CCK-8 assay

The CCK-8 assay was used to quantitatively assess cell survival. Briefly, the third passage NSCs (approximately 5×10^3 cells/well) were seeded in PLL-coated 96-well plates with six replicates in each group, and subjected to the various treatments described previously. 10% CCK-8 solution was added to each culture well, and NSCs were incubated for 4 h at 37 °C. Absorbance at 450 nm was measured with a microplate reader (Denley Dragon Wellscan MK3, Thermo). The results of three independent experiments were used for statistical analysis.

2.6. BrdU labeling of proliferative NSCs

To evaluate the proliferation of NSCs, cells in the different groups were incubated in 24-well plates (approximately 5×10^4 cells/well) with three replicates in each group with 10 μmol/L BrdU; for 6 h at 37 °C in a humidified atmosphere containing 5% CO_2. Briefly, cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature; rinsed three times in PBS; and permeabilized with 0.3% TritonX-100 in PBS for 30 min. Permeabilization was followed by 30 min incubation in 2 N HCl for DNA denaturation. Cells were then blocked with 10% normal goat serum for 30 min at room temperature. Subsequently, the NSCs were incubated overnight at 4 °C with BrdU antibody (1:200). The following day, cells were washed and incubated for 90 min at room temperature with Dylight 594 antibody (1:200). Cell nuclei were counterstained with DAPI for 3–5 min in the dark. Finally, the cells were examined by fluorescence microscopy. Positive cell rate (*i.e.*, total number of positive cells/total number of viable cells × 100%) was calculated by using image analysis (Image-pro plus 6.0., Media Cybernetics). The results of three independent experiments were used for statistical analysis.

2.7. ELISA assay

Briefly, the third passage NSCs (approximately 1×10^6 cells/well) were seeded in PLL-coated 6-well plates with three replicates in each group. According to the manufacturer's instructions, the cGMP level and PDE9 activity in the media of cultured cells were determined using ELISA Kits. The optical density (OD) values were measured at the wavelength of 450 nm using a microplate reader. Then the concentrations of cGMP and PDE9 were calculated by their specific standard curves, respectively. The results of three independent experiments were used for statistical analysis.

2.8. Western blot analysis

For extraction of total proteins, cells were rinsed twice with ice-cold PBS, lysed in RIPA lysis buffer containing 1% PMSF, and determined by BCA assay. Protein samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to the membrane by wet method. Antibodies [β-actin (1:2000), PKG (1:1000), and BDNF (1:1000)] were added and incubated overnight after closed with BSA. The membranes was incubated with secondary antibody (1:2000) for 2 h. The bands were visualized using the enhanced chemiluminescence method. Repeat three times for each group. The signal was quantified using Image Lab software (Bio-Rad).

2.9. Statistical analysis

All data are expressed as mean ± SD. Statistical analysis was performed using SPSS 17.0. The between group differences were evaluated by using one-way analysis of variance (ANOVA) and Tukey's range test. $P < 0.05$ was considered statistically significant. GraphPad Prism 5.01 software was used for drawing.

3. Results

3.1. Identification of NSCs

Nestin is a specific marker for NSCs. The nestin was in positive expression after 3 passages of purified culture. The percentage of marked DAPI of NSCs arrived at 100%, and the nuclei of NSCs expressed blue fluorescence. The results indicate that the cells are NSCs and can be used for subsequent experimental observations (Fig. 1).

3.2. Effect of PDE9 inhibition on the cell viability of NSCs

Exposing to OGD/R, the cell viability of NSCs was reduced by 37.8%, compared with normal NSCs ($P < 0.01$). Incubation with PF-04447943 at 10^{-7} mol/L significantly increased the OGD/R-decreased

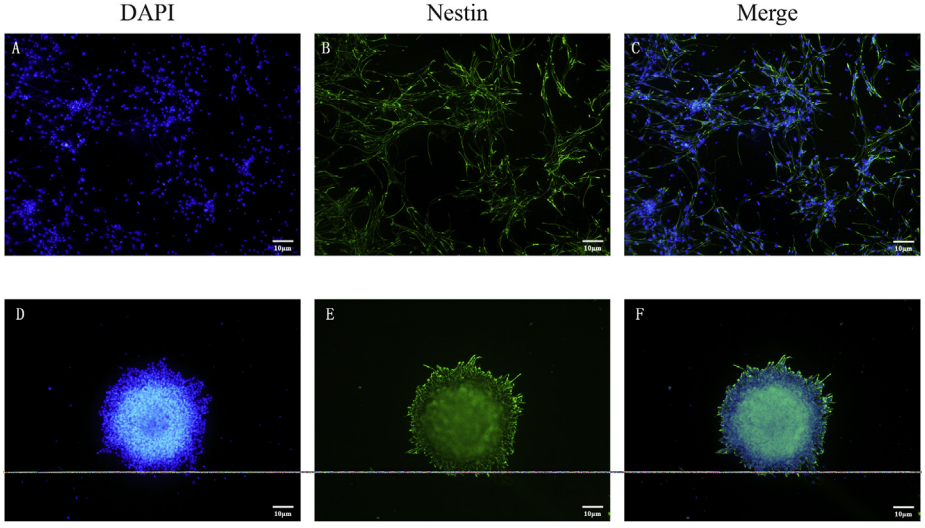

Fig. 1. Identification of NSCs by immunofluorescence (scale = 10 μm). A–C: NSCs were identified by the representative marker nestin as adherent monolayer culture. D–F: Neurosphere was identified by the representative marker nestin. Nuclei were labeled with DAPI (blue) (A, D). Primary hippocampal NSCs from neonatal rats were immunolabeled with an antibody against nestin (green) (B, E). Merged views of panels A/B and D/E (C, F).

cell viability by 45.9% ($P < 0.01$), which was close to the level of normal NSCs ($P > 0.05$). KT5823 at 10^{-6} mol/L could completely block the effect of PF-04447943 ($P < 0.01$) (Fig. 2).

3.3. Effect of PDE9 inhibition on the cell proliferation of NSCs

There were very few BrdU-positive cells in normal NSCs, but the number of BrdU-positive cells increased by 53.3% after OGD/R ($P < 0.05$). Notably, the BrdU-positive cells increased by 1.4 times by treatment with PF-04447943 at 10^{-7} mol/L, compared with OGD/R model group ($P < 0.01$). KT5823 at 10^{-6} mol/L could block the effect of PF-04447943 ($P < 0.01$) (Fig. 3).

3.4. Effect of PDE9 inhibition on PDE activity and cGMP level

PDE9 activity reduced by 20.9% but cGMP level augmented by 1.0 times after OGD/R ($P < 0.05$). PF-04447943 incubation decreased PDE9 activity by 37.3% and increased intracellular cGMP by 1.5 times, compared with OGD/R model group ($P < 0.01$). However, KT5823 had no significant effects on the actions of PF-04447943 ($P > 0.05$) (Fig. 4).

3.5. Effect of PDE9 inhibition on PKG and BDNF protein expression

PKG and BDNF protein expression increased by 72.7% and 30.9%, respectively, after OGD/R ($P < 0.05$). Compared with OGD/R model group, PF-04447943 (10^{-7} mol/L) significantly upregulated the expression of both PKG and BDNF, by 49.1% ($P < 0.05$) and 72.7%, respectively ($P < 0.01$), which significantly was inhibited by KT-5823 (10^{-6} mol/L) ($P < 0.05$)(Fig. 5).

4. Discussion

After cerebral ischemic injury, the endogenous NSCs proliferate and migrate to the damaged regions, where they may differentiate into neurons to help neural function recovery. But the number of proliferating cells is less and the survival is poor (Arvidsson et al., 2002; Parent et al., 2002a; Parent et al., 2002b). Meanwhile, the disease process itself may weaken and destroy the ability of NSCs to grow and repair (Liang et al., 2016). Enhancing the proliferation of endogenous NSCs is promising some valuable strategies for the structural and functional recovery after cerebral ischemic insult. Therefore, it is important to understand the involved mechanisms and to find the new targets on the proliferation of NSCs.

cGMP is a key intracellular mediator of signal transduction. It has now been found that increasing cGMP level promotes the proliferation of NSCs and protects against nerve damage. cGMP level is regulated by the balance between its production and removal, which is produced by the catalysis of guanylate cyclase (GC) and hydrolyzed by the action of PDE. In recent years, cGMP from GC has been enthusiastically investigated (Bian and Murad., 2014). The inhibition of PDE subtypes has also been demonstrated to enhance performance of animals in various cognition tasks and accordingly PDE inhibitors have been proposed as new approach for treatment of cognitive dysfunction (Reneerkens et al., 2009). PDE9 is cGMP-specific and is highly expressed in hippocampus. Studies have shown that PDE9 is involved in the development and progression of Alzheimer's disease (AD) (Zhang et al., 2018). However, the effects of PDE9 on the proliferation of NSCs are few studies after cerebral ischemia. OGD/R model is one way to better study ischemia/refusion injury *in vitro*. In this study, the cell viability decreased, suggesting that cerebral ischemic injury was appeared after OGD/R treatment in cultured hippocampal NSCs. Meanwhile, the increase of BrdU positive cells indicated that cerebral ischemia itself can promote the proliferation of NSCs. Concurrently, PDE9 activity decreased and cGMP level increased, suggesting that PDE9-cGMP-related pathway involved the proliferation of NSCs after cerebral ischemia.

PKG is the crucial downstream effector of cGMP. PDE inhibition can increase cGMP level, then PKG is activated. cGMP-PKG pathway is thought to play a crucial role in the development of several neurodiseases, such as AD, Parkinson's disease (PD), and multiple sclerosis (MS) (Fiscus, 2002; Calabresi et al., 2013; Mancini et al., 2018). The pathway can promote the transcription of BDNF (Alhaider et al., 2011), which can stimulate cell proliferation, differentiation, survival, etc. Thereby, cGMP-PKG plays an essential regulation by BDNF on the proliferation of endogenous NSCs (Yan et al., 2016) and has neuroprotective and repair effects (Liu et al., 2016). In this study, NSCs proliferation increased after OGD/R treatment, accompanied with the decrease of PDE9 activity, the increase of cGMP level, and the protein expression of PKG and BDNF, suggesting that PDE9-cGMP-PKG pathway involved the proliferation of NSCs mediated by BDNF after cerebral ischemia. Unfortunately, the spontaneous proceed was too poor to rescue the cell viability.

To detect the possible therapeutic effects and the involved signal pathway of PDE9 inhibition on ischemia, a PDE9 inhibitor, PF-04447943, and a PKG blocker, KT5823, were used. PF-04447943 could improve cell viability, accompanied by the significant increase of NSCs proliferation, even by 1.4 times higher than the level of OGD/R model cells. Meanwhile, PDE9 activity was decreased, cGMP-PKG was increased, and BDNF was upregulated, which all were more than the levels of OGD/R-treated cells. Furthermore, the effects of PF-04447943 could be abolished by KT5823 except for PDE9 activity and cGMP level. These results suggested that PDE9-cGMP-PKG signaling pathway is involved in the proliferation of NSCs after ischemic insult, and the inhibition of PDE9 activity can enhance the proliferation of NSCs by reducing cGMP hydrolysis, then increasing intracellular cGMP concentration, activating PKG-related pathways (Fig. 6).

Taken together, our results indicated that the inhibition of PDE9 can activate cGMP-PKG-related signaling pathways, thereby promote BDNF and ultimately stimulate the proliferation of NSCs and protect cell viability against cerebral ischemia. PDE9-cGMP-PKG signaling pathway is involved in the proliferation of NSCs. PDE9 may be an important target for the treatment of cerebral ischemia-related diseases, especially for the recovery of learning and memory.

5. Conclusions

Our study provides evidences that PDE9 may be a promising target for the treatment of cerebral ischemia-related diseases. Inhibition of PDE9 promotes the increase of cGMP level and the up-regulation of

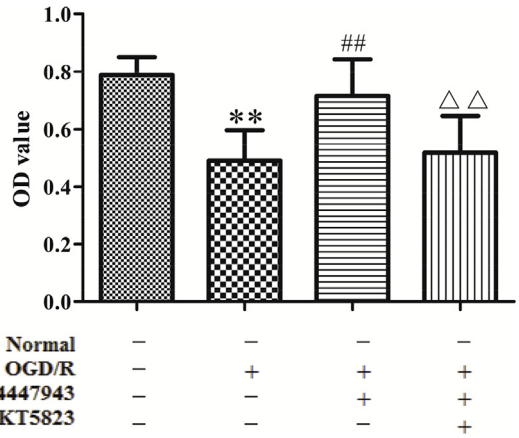

Fig. 2. Effect of PDE9 inhibition on cell viability of NSCs exposing to OGD/R was detected by CCK-8 assay. OGD/R: oxygen glucose deprivation/reoxygenation; PF-04447943: a PDE9 inhibitor (10^{-7} mol/L); KT5823: a PKG inhibitor (10^{-6} mol/L). Data are presented as the mean ± S.D. of three independent experiments. $^{**}P < 0.01$ *vs* normal; $^{\#\#}P < 0.01$ *vs* OGD/R; $^{\triangle\triangle}P < 0.01$ *vs* PF-04447943 (one-way ANOVA).

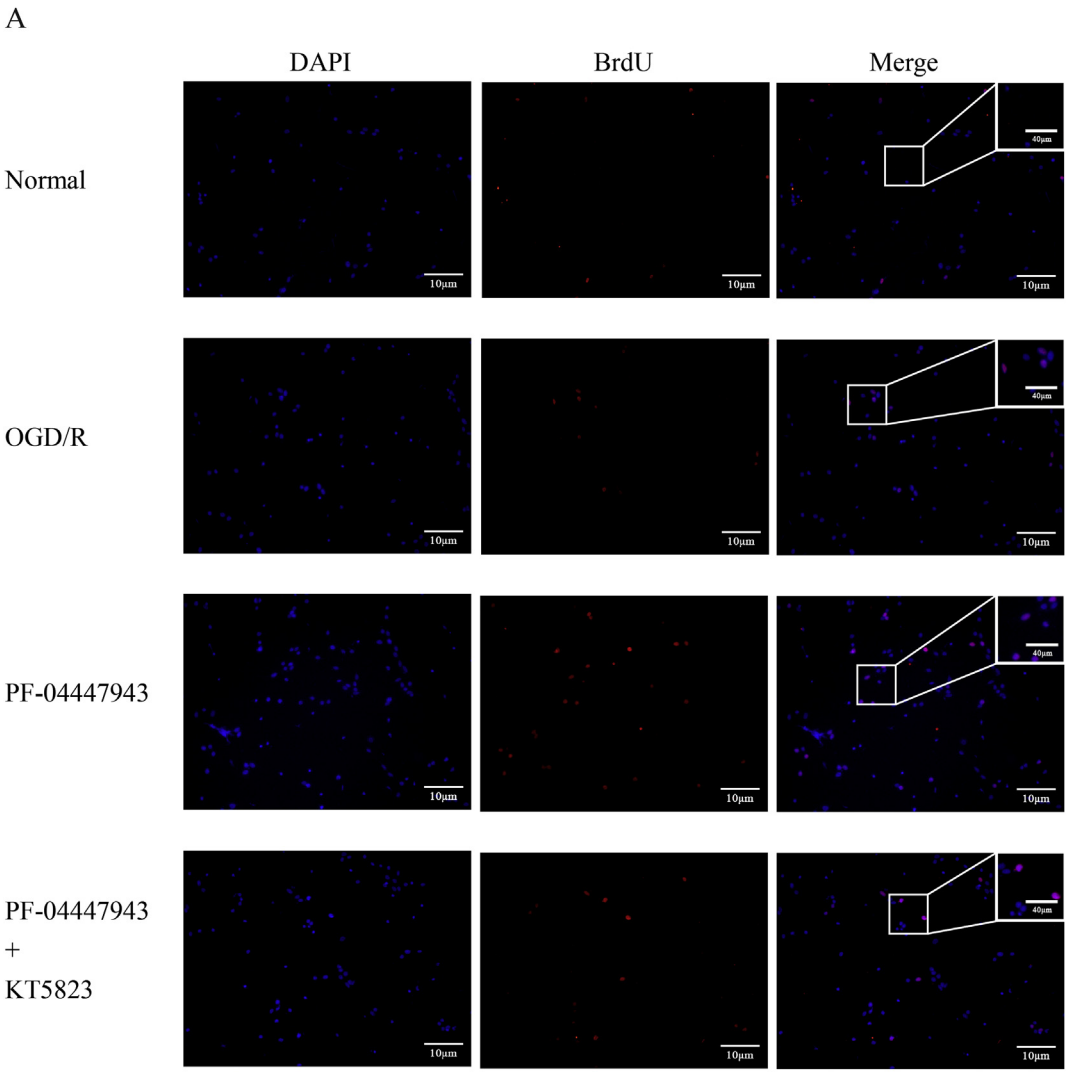

Fig. 3. Effect of PDE9 inhibition on cell proliferation of NSCs exposing to OGD/R was detected by immunofluorescence. A: Representative image showing NSCs proliferation (low magnification: scale = 10 μm and high magnification: scale = 40 μm). NSCs were immunostained with antibody to BrdU (red). Nuclei were labeled with DAPI (blue). B: Percentage of BrdU positive cells. OGD/R: oxygen glucose deprivation/reoxygenation; PF-04447943: a PDE9 inhibitor (10^{-7} mol/L); KT5823: a PKG inhibitor (10^{-6} mol/L). Data are presented as the mean ± S.D. of three independent experiments. $^{*}P < 0.05$ *vs* normal; $^{\#\#}P < 0.01$ *vs* OGD/R; $^{\triangle\triangle}P < 0.01$ *vs* PF-04447943 (one-way ANOVA).

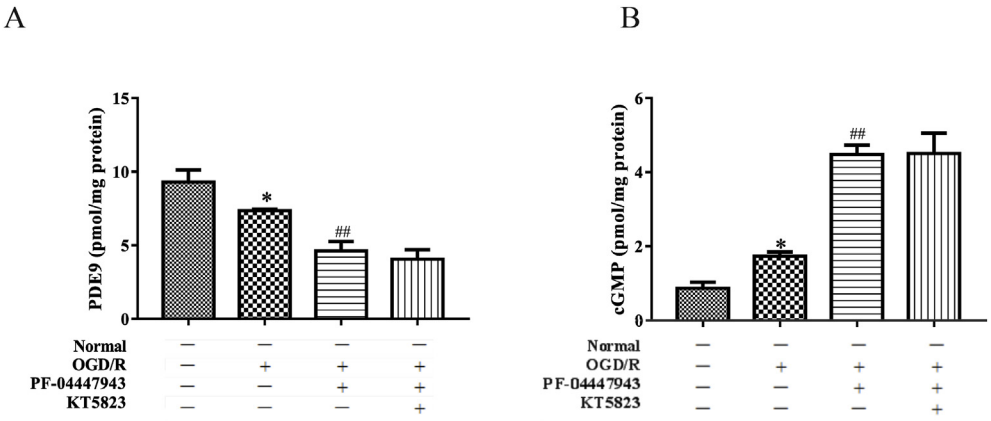

Fig. 4. Effect of PDE9 inhibition on PDE activity and cGMP level in NSCs exposing to OGD/R by ELISA. A: PDE activity; B: cGMP level. OGD/R: oxygen glucose deprivation/reoxygenation; PF-04447943: a PDE9 inhibitor (10^{-7} mol/L); KT5823: a PKG inhibitor (10^{-6} mol/L). Data are presented as the mean ± S.D. of three independent experiments. $^{*}P < 0.05$ *vs* normal; $^{\#\#}P < 0.01$ *vs* OGD/R (one-way ANOVA).

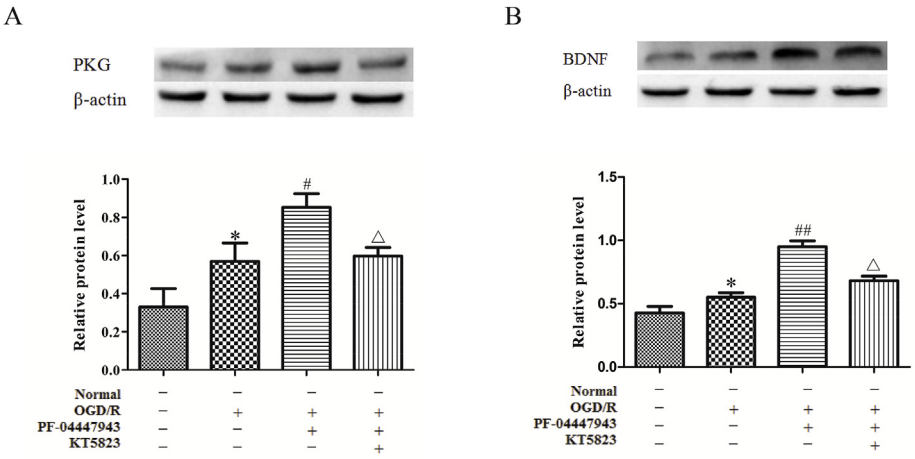

Fig. 5. Effect of PDE9 inhibition on PKG and BDNF protein expression in NSCs exposing to OGD/R by Western blot. Quantification of protein level was performed using densitometry. Data were normalized with β-actin protein. A: PKG protein expression; B: BDNF protein expression. OGD/R: oxygen glucose deprivation/reoxygenation; PF-04447943: a PDE9 inhibitor (10^{-7} mol/L); KT5823: a PKG inhibitor (10^{-6} mol/L). Data are presented as the mean ± S.D. of three independent experiments. $^{*}P < 0.05$ *vs* normal; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* OGD/R; $^{\triangle}P < 0.05$ *vs* PF-04447943 (one-way ANOVA).

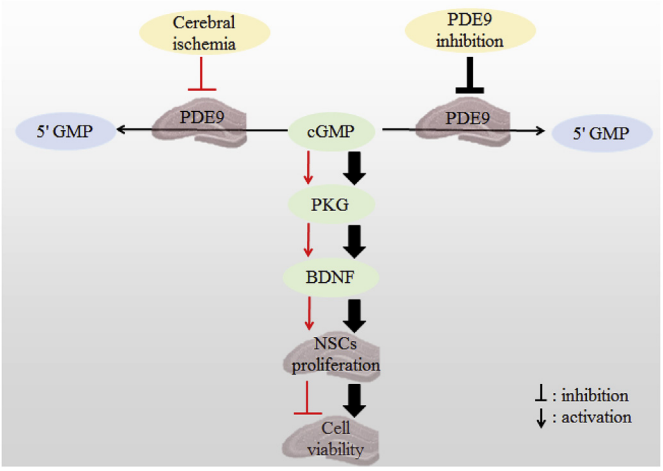

Fig. 6. The underlying effect of PDE9 inhibition on the proliferation of neural stem cells (NSCs) in hippocampus after cerebral ischemia. PDE9-cGMP-PKG signaling pathway is involved in the proliferation of NSCs after ischemic insult, however, the spontaneous proceed is too poor to rescue the cell viability. The inhibition of PDE9 activity can improve cell viability by stimulating more NSCs proliferation, which may be, at least partly, mediated by cGMP-PKG pathway.

PKG and BDNF protein expression to stimulate the proliferation of NSCs and to improve cell viability in hippocampus after ischemic insult.

Declaration of competing interest

None of the authors have any conflicts of interest.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (81871002, 81471334, 81100981) and the National Key Clinical Specialties Construction Program of China.

References

Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z., Lindvall, O., 2002. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat. Med. 8, 963–970.
Andersen, R.K., Johansen, M., Blaabjerg, M., Zimmer, J., Meyer, M., 2007. Neural tissue-spheres: a microexplant culture method for propagation of precursor cells from the rat forebrain subventricular zone. J. Neurosci. Methods 165, 55–63.
Alhaider, I.A., Aleisa, A.M., Tran, T.T., Alkadhi, K.A., 2011. Sleep deprivation prevents stimulation-induced increases of levels of P-CREB and BDNF: protection by caffeine. Mol. Cell. Neurosci. 46, 742–751.
Babu, H., Claasen, J.H., Kannan, S., Rünker, A.E., Palmer, T., Kempermann, G., 2011. A protocol for isolation and enriched monolayer cultivation of neural precursor cells from mouse dentate gyrus. Front. Neurosci. 5 5:89.
Bian, K., Murad, F., 2014. sGC-cGMP signaling: target for anticancer therapy. Adv. Exp. Med. Biol. 814, 5–13.
Chalimoniuk, M., Strosznajder, J.B., 1998. Aging modulates nitric oxide synthesis and cGMP levels in hippocampus and cerebellum. Effects of amyloid beta peptide. Mol. Chem. Neuropathol. 35, 77–95.
Chen, T., Liu, W., Chao, X., Qu, Y., Zhang, L., Luo, P., Xie, K., Huo, J., Fei, Z., 2011. Neuroprotective effect of osthole against oxygen and glucose deprivation in rat cortical neurons: involvement of mitogen-activated protein kinase pathway. Neuroscience 183, 203–211.
Calabresi, P., Di, Filippo M., Gallina, A., Wang, Y., Stankowski, J.N., Picconi, B., Dawson, V.L., Dawson, T.M., 2013. New synaptic and molecular targets for neuroprotection in Parkinson's disease. Mov. Disord. 28, 51–60.
Cheng, W., Yu, P., Wang, L., Shen, C., Song, X., Chen, J., Tang, F., Yang, Q., 2015. Sonic hedgehog signaling mediates resveratrol to increase proliferation of neural stem cells after oxygen-glucose deprivation/reoxygenation injury in vitro. Cell. Physiol. Biochem. 35, 2019–2032.
Chen, S.D., Wu, C.L., Hwang, W.C., Yang, D.I., 2017. More insight into BDNF against neurodegeneration: anti-apoptosis, anti-oxidation, and suppression of autophagy. Int.

J. Mol. Sci. 18, 545.
De, F.D., Merlini, A., Laterza, C., Martino, G., 2012. Neural stem cell transplantation in central nervous system disorders: from cell replacement to neuroprotection. Curr. Opin. Neurol. 25, 322–333.
Fiscus, R.R., 2002. Involvement of cyclic GMP and protein kinase G in the regulation of apoptosis and survival in neural cells. Neurosignals 11, 175–190.
Hutson, P.H., Finger, E.N., Magliaro, B.C., Smith, S.M., Converso, A., Sanderson, P.E., Mullins, D., Hyde, L.A., Eschle, B.K., Turnbull, Z., Sloan, H., Guzzi, M., Zhang, X., Wang, A., Rindgen, D., Mazzola, R., Vivian, J.A., Eddins, D., Uslaner, J.M., Bednar, R., Gambone, C., Le-Mair, W., Marino, M.J., Sachs, N., Xu, G., Parmentier-Batteur, S., 2011. The selective phosphodiesterase 9 (PDE9) inhibitor PF-04447943 (6-[(3S,4S)-4-methyl-1-(pyrimidin-2-ylmethyl)pyrrolidin-3-yl]-1-(tetrahydro-2H-pyran-4-yl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one) enhances synaptic plasticity and cognitive function in rodents. Neuropharmacology 61, 665–676.
Kroker, K.S., Mathis, C., Marti, A., Cassel, J.C., Rosenbrock, H., Dorner-Ciossek, C., 2014. PDE9A inhibition rescues amyloid beta-induced deficits in synaptic plasticity and cognition. Neurobiol. Aging 35, 2072–2078.
Knott, E.P., Assi, M., Rao, S.N., Ghosh, M., Pearse, D.D., 2017. Phosphodiesterase inhibitors as a therapeutic approach to neuroprotection and repair. Int. J. Mol. Sci. 18, 696.
Liang, A.C., Mandeville, E.T., Maki, T., Shindo, A., Som, A.T., Egawa, N., Itoh, K., Chuang, T.T., McNeish, J.D., Holder, J.C., Lok, J., Lo, E.H., Arai, K., 2016. Effects of aging on neural stem/progenitor cells and oligodendrocyte precursor cells after focal cerebral ischemia in spontaneously hypertensive rats. Cell Transplant. 25, 705–714.
Liu, S., Wu, M., Shi, B.Q., 2016. Human umbilical cord mesenchymal stem cells protects against Aβ-induced impairment partly through up-regulation of expression of neurotrophins. Chin. Pharmacol. Bull. 32, 980–985.
Morales-Garcia, J.A., Echeverry-Alzate, V., Alonso-Gil, S., Sanz-SanCristobal, M., Lopez-Moreno, J.A., Gil, C., Martinez, A., Santos, A., Perez-Castillo, A., 2017. Phosphodiesterase7 inhibition activates adult neurogenesis in hippocampus and subventricular zone in vitro and in vivo. Stem Cells 35, 458–472.
Mancini, A., Tantucci, M., Mazzocchetti, P., de, Iure A., Durante, V., Macchioni, L., Giampà, C., Alvino, A., Gaetani, L., Costa, C., Tozzi, A., Calabresi, P., Di, Filippo M., 2018. Microglial activation and the nitric oxide/cGMP/PKG pathway underlie enhanced neuronal vulnerability to mitochondrial dysfunction in experimental multiple sclerosis. Neurobiol. Dis. 113, 97–108.
Parent, J.M., Vexler, Z.S., Gong, C., Derugin, N., Ferriero, D.M., 2002a. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. Ann. Neurol. 52, 802–813.
Parent, J.M., Valentin, V.V., Lowenstein, D.H., 2002b. Prolonged seizures increase proliferating neuroblasts in the adult rat subventricular zone-olfactory bulb pathway. J. Neurosci. 22, 3174–3188.
Reynolds, B.A., Tetzlaff, W., Weiss, S., 1992. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J. Neurosci. 12, 4565–4574.
Reyes-Irisarri, E., Markerink-Van Ittersum, M., Mengod, G., de Vente, J., 2007. Expression of the cGMP-specific phosphodiesterases 2 and 9 in normal and Alzheimer's disease human brains. Eur. J. Neurosci. 25, 3332–3338.
Reneerkens, O.A., Rutten, K., Steinbusch, H.W., Blokland, A., Prickaerts, J., 2009. Selective phosphodiesterase inhibitors: a promising target for cognition enhancement. Psychopharmacology (Berlin) 202, 419–443.
Santos, A.I., Carreira, B.P., Nobre, R.J., Carvalho, C.M., Araújo, I.M., 2014. Stimulation of neural stem cell proliferation by inhibition of phosphodiesterase 5. Stem Cell. Int. 2014, 878397.
Van der Staay, F.J., Rutten, K., Bärfacker, L., Devry, J., Erb, C., Heckroth, H., Karthaus, D., Tersteegen, A., van, Kampen M., Blokland, A., Prickaerts, J., Reymann, K.G., Schröder, U.H., Hendrix, M., 2008. The novel selective PDE9 inhibitor BAY 73-6691 improves learning and memory in rodents. Neuropharmacology 55, 908–918.
Vandenbosch, R., Borgs, L., Beukelaers, P., Belachew, S., Moonen, G., Nguyen, L., Malgrange, B., 2009. Adult neurogenesis and the diseased brain. Curr. Med. Chem. 16, 652–666.
Whitney, N.P., Eidem, T.M., Peng, H., Huang, Y., Zheng, J.C., 2009. Inflammation mediates varying effects in neurogenesis: relevance to the pathogenesis of brain injury and neurodegenerative disorders. J. Neurochem. 108, 1343–1359.
Wu, X., Zhao, J., Yu, S., Chen, Y., Wu, J., Zhao, Y., 2012. Sulforaphane protects primary cultures of cortical neurons against injury induced by oxygen-glucose deprivation/reoxygenation via antiapoptosis. Neurosci. Bull. 28, 509–516.
Yan, C., Liu, Y.W., Wu, L.L., 2016. Regulatory of Jiaweisinisan on expression of hippocampal BDNF, NR1 and dental gyrus neurogenesis in rats with chronic stressed-depression. Chin. Pharmacol. Bull. 32, 569–574.
Zhang, L., Chopp, M., Meier, D.H., Winter, S., Wang, L., Szalad, A., Lu, M., Wei, M., Cui, Y., Zhang, Z.G., 2013. Sonic hedgehog signaling pathway mediates cerebrolysin-improved neurological function after stroke. Stroke 44, 1965–1972.
Zhang, C., Zhou, Q., Wu, X.N., Huang, Y.D., Zhou, J., Lai, Z., Wu, Y., Luo, H.B., 2018. Discovery of novel PDE9A inhibitors with antioxidant activities for treatment of Alzheimer's disease. J. Enzym. Inhib. Med. Chem. 33, 260–270.