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Original Paper

Phosphodiesterase 4 Inhibitor Roflumilast Protects Rat Hippocampal Neurons from Sevoflurane Induced Injury via Modulation of MEK/ERK Signaling Pathway

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Key Words
Sevoflurane • Nerve damage • Roflumilast • CAMP • Phosphodiesterase 4 inhibitor (PDE-4i) • MEK/ERK pathway

Abstract
Background/Aims: Sevoflurane, a commonly used volatile anesthetic, recently has been found has neurotoxicity in the central nervous system of neonatal rodents. This study aimed to reveal whether phosphodiesterase 4 (PDE-4) inhibitor roflumilast has protective functions in sevoflurane-induced nerve damage. ***Methods:*** Hippocampal neurons were isolated from juvenile rats, and were exposed to sevoflurane with or without roflumilast treatment. Cell viability and apoptosis were respectively assessed by CCK-8 and flow cytometry. Western blot analysis was performed to detect the protein expressions of apoptosis-related factors, and core factors in MEK/ERK and mTOR signaling pathways. ***Results:*** Toxic effects of sevoflurane on hippocampal neurons were observed, as cell viability was reduced, apoptotic cell rate was increased, Bcl-2 was down-regulated, and Bax, cleaved caspase-3 and -9 were up-regulated after 1% sevoflurane exposure for 16 h. Sevoflurane exhibited a temporarily (less than 16 h) inhibitory effect on MEK/ERK pathway, but has no impact on mTOR pathway. Roflumilast promoted the release of cAMP and down-regulated the protein expression of PDE-4. Roflumilast (1 μM) alone has no impact on viability and apoptosis of hippocampal neurons. However, roflumilast increased cell viability and deceased apoptosis in sevoflurane-injured neurons. Besides, roflumilast could recover sevoflurane-induced deactivation of MEK/ERK pathway. ***Conclusion:*** To conclude, this study demonstrated a neuroprotective role of roflumilast in sevoflurane-induced nerve damage. Roflumilast promoted hippocampal neurons viability, and reduced apoptosis possibly via modulation of MEK/ERK signaling pathway.

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Introduction

Sevoflurane, a kind of volatile anesthetics, is commonly used as an inhalational agent preferred for its early awaking characteristic. Sevoflurane has low blood gas partition coefficient, aromatic odor, rapid onset, and low pungency into the airway [1], and because of this it has been widely applied during surgical operations and cesarean deliveries to induce and maintain pediatric anesthesia [2]. However, the occurrence of emergence agitation in children is the major disadvantage of this volatile anesthetic, with the reported incidence up to 80% [3]. Additionally, several lines of studies have shown that sevoflurane causes neurodegeneration in the central nervous system in neonatal rodents and long-lasting neurocognitive dysfunction, including learning disabilities [4-7]. Thus, it is necessary for us to investigate the effective strategies for protecting neurons from sevoflurane-induced cell damage.

Cyclic adenosine 3',5'-monophosphate (cAMP) is the first discovered second messenger for signal transduction [8]. cAMP signaling exists in all types of cells and contributes to multiple biological processes, including cell migration, differentiation, proliferation and apoptosis [9]. The cAMP signaling pathway also plays fundamental roles in the nervous system. cAMP can act as a local messenger upon physiological stimulation of a neuron, and it can be restricted at the micrometer level to induce high localized physiological responses [10]. The regulation of cAMP downstream of adenylyl cyclases is largely carried out by phosphodiesterases (PDEs), which are regulated by cAMP-dependent kinase [11]. In hippocampus, PDE-4 comprises the major cAMP-degrading PDE family [12].

Roflumilast is an oral PDE-4 selective inhibitor. By blocking PDE-4, roflumilast raises cAMP levels within airway smooth muscle cells and various inflammatory cells [13, 14]. Based on this fact, we speculated that roflumilast might have functional impacts on neurons by inhibition of PDE-4, and thus modulating the levels of cAMP. However, nowadays, most scholars focus their attentions on evaluating the clinical efficacy and safety of roflumilast in the treatment of chronic obstructive pulmonary disease (COPD) [15, 16]. Little was known about the neuroprotective functions of roflumilast on neuron.

Herein, we aimed to reveal whether roflumilast could protect hippocampal neurons from sevoflurane-induced damage. In this study, hippocampal neurons were isolated from juvenile rats, and were exposed to sevoflurane treatment. Roflumilast was then added into cell culture medium, and the effects of roflumilast on cell viability and apoptosis were evaluated. The findings of this study will provide us with a novel perspective that roflumilast may have potentials for suppressing sevoflurane-induced nerve damage.

Materials and Methods

Cell culture

Hippocampal neurons were isolated from specific pathogen-free grade of Wistar rats (18 days) which were purchased from the Vital River Laboratories (Beijing, China). Animal experiments performed in this study were approved by the Animal Ethics Committee of Seventh People's Hospital of Shanghai University of TCM and were conducted in accordance with the instruction of our institute. The rats were anesthetized by intraperitoneal (IP) injection of chloral hydrate (350 mg/kg) and then euthanized by cervical dislocation for sampling. The hippocampus were then removed and cut into pieces and digested in 0.15% trypsin. After 30 min of incubation at 37°C, the cells were filtered and centrifuged, and single layer cells were obtained. The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), and were maintained in a humidified 5% CO_2 incubator at 37°C.

Sevoflurane treatment

Sevoflurane was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Cells were placed in an air-tight humidified modular incubator chamber (Billups-Rothenberg, Del Mar, CA) with inflow and outflow connectors. Sevoflurane treatment was performed as previously described [17]. Calibrated sevoflurane

vaporizer (Datex-Ohmeda) was used to deliver 1% sevoflurane mixed with 94% air and 5% CO_2 at 10 L/min. Cells were subjected with sevoflurane for 16 h. Control cells were exposed to normal condition with 95% air and 5% CO_2.

Roflumilast with purity higher than 98% (Sigma-Aldrich) was used for inhibition of PDE-4. The cells were treated with 0.1-5 μM of roflumilast for 16 h with or without sevoflurane exposure.

Cell viability assay

Hippocampal neurons were seeded in 96-well plate with a density of 5×10^3 cells/well, cell viability was assessed by a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). Briefly, after sevoflurane exposure with or without roflumilast treatment, 10 μL CCK-8 solution was added. The plates were then incubated at 37°C for 2 h, and the absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

Apoptosis assay

Hippocampal neurons were seeded in 6-well plate with a density of 5×10^5 cells/well, apoptosis was then tested by Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). In brief, after sevoflurane exposure with or without roflumilast treatment, cells were collected and resuspended in 200 μL binding buffer containing 10 μL Annexin V-FITC and 5 μL PI. The samples were then allowed to culture in the dark at room temperature for 30 min. After being filtered and mixed with 200 μL PBS, apoptotic cells in each sample were analyzed under a flow cytometer (Beckman Coulter, USA).

cAMP assay

The concentration of cAMP in cell culture supernatants was measured by an enzyme-linked immunosorbent assay (ELISA) kit (Amersham Bioscience, United Kingdom), according to the manufacturer's instructions.

Western blot

The protein used for western blotting was extracted using RIA lysis buffer (Beyotime Biotechnology, Shanghai, China). Protein concentrations were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Equal amounts of protein were resolved over 10-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into polyvinylidene fluoride (PVDF) membranes. The membranes were incubated in 5% non-fat milk for 1 h at room temperature, and then incubated with primary antibodies overnight at 4°C. Bcl-2 (ab196495, 1:1000 dilution), Bax (ab53154, 1:1000 dilution), caspase-3 (ab13586, 1:250 dilution), caspase-9 (ab32539, 1: 1000 dilution), p-mTOR (ab109268, 1:1000 dilution), mTOR (ab32028, 1:1000 dilution), p70S6K (ab47504, 1:500 dilution), p-p70S6K (ab59208, 1:500 dilution), p-MEK (ab30622, 1:1000 dilution), MEK (ab32091, 1:1000 dilution), ERK (ab196883, 1:500 dilution), p-ERK (ab214362, 1:1000 dilution), PDE-4 (ab14614, 1:500 dilution), and GAPDH (ab9485, 1:2500 dilution) were all purchased from Abcam (Cambridge, MA, USA). The membranes were then incubated with the secondary antibodies (Abcam) for 1 h at room temperature. Positive signals were visualized by the enhanced chemiluminescence (ECL) reagent (GE Healthcare, Little Chalfont, UK). Intensity of bands was quantified using Image Lab™ Software (Bio-Rad, CA, USA).

Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as the mean ± standard derivations (SD). Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). The P-values were calculated using a one-way analysis of variance (ANOVA). A P-value of <0.05 was considered to indicate a statistically significant result.

Results

Sevoflurane reduced cell viability and induced apoptosis of rat hippocampal neurons

To start with, we detected the effect of sevoflurane on the viability and apoptosis of hippocampal neurons. As results shown in Fig. 1A and 1B, neurons subjected with 1%

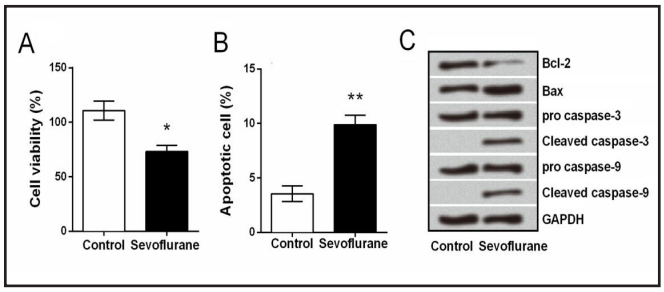

Fig. 1. Sevoflurane reduced cell viability and induced apoptosis of rat hippocampal neurons. (A) Cell viability, (B) apoptotic cell rate, and (C) protein expressions of apoptosis-related factors were measured after hippocampal neurons were exposed to 1% sevoflurane for 16 h. Results presented as means ± SD. n = 3. * P<0.05, and ** P<0.01 compared to control group (without sevoflurane exposure).

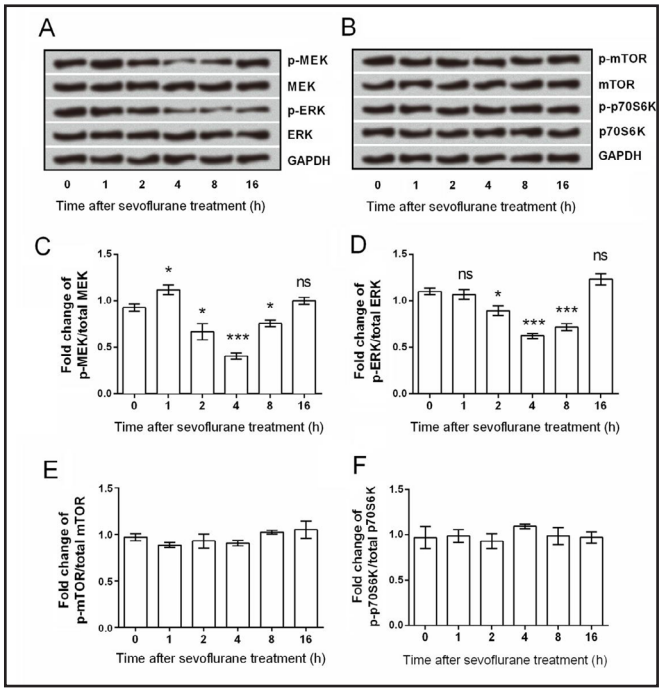

Fig. 2. Sevoflurane inhibited MEK/ERK signaling pathway. (A) Protein expression of core factors in MEK/ERK signaling, and (B) protein expression of core factors in mTOR signaling were detected in hippocampal neurons at 0-16 h post-treatment with sevoflurane. (C-F) Quantitative expression changes of core factors in MEK/ERK and mTOR signaling pathways. Results presented as means ± SD. n = 3. ns, not significant, * P<0.05, and *** P<0.001 compared to 0 h.

sevoflurane for 16 h resulted in a significant reduce in cell viability (P < 0.05), and a significance increase in apoptotic cell rate (P < 0.01). Western blotting results showed that anti-apoptotic protein Bcl-2 was down-regulated, the pro-apoptotic protein Bax was up-regulated, and caspase-3 and -9 were remarkably cleaved after sevoflurane stimulation (Fig. 1C). These data suggested the growth-inhibitory role of sevoflurane in hippocampal neurons.

Sevoflurane inhibited MEK/ERK signaling pathway

To reveal the underling molecular mechanisms of which sevoflurane inhibited hippocampal neurons growth, we focused on MEK, ERK, and mTOR signaling pathways. Western blot analysis was performed to detect the expression changes of main proteins in the three signaling pathways. As results shown in Fig. 2A, 2C-2D, down-regulations of p-MEK and p-ERK were observed at 2 h post-treatment with sevoflurane, and the protein levels of p-MEK and p-ERK were continued to decrease with the prolongation of time. Minimum protein levels of p-MEK and p-ERK were observed at 4 h post-treatment. At 8 h, these two protein expressions began to pick up, and returned to the original (0 h) levels at 16 h post-treatment. No significant change of total levels of MEK and ERK, as well as total and phosphorylated forms of mTOR and p70S6K (Fig. 2B, 2E-2F) was observed in sevoflurane-treated cells. These data showed a temporarily inhibitory activity of sevoflurane on MEK/ERK signaling pathway, which might be via which sevoflurane inhibited hippocampal neurons growth.

Roflumilast promoted the release of cAMP via down-regulation of PDE-4

A previous study has pointed out that cAMP level was decreased in rat hippocampus in response to sevoflurane-nitrous oxide anesthesia [18]; herein, we found that the release of cAMP was reduced by sevoflurane treatment. As data shown in Fig. 3A, cAMP concentration in the culture supernatants began to reduce at 2 h post-treatment with sevoflurane. cAMP level reached to a minimum at 4 h post-treatment, and a slight pickup was observed at 8 h post-treatment. Then, the functional impacts of roflumilast on hippocampal neurons were evaluated by measuring cAMP release. We found that high concentrations (0.5, 1, 2, and 5 μM) of roflumilast significantly increased the content of cAMP in cell culture supernatants (P < 0.01 or P < 0.001, Fig. 3B). Besides, we detected the protein expression of PDE-4, which was responsible for selectively modulating the concentration of cAMP in individual subcellular compartments [19]. It was shown in Fig. 3C that high concentrations (0.5, 1, 2, and 5 μM) of roflumilast remarkably down-regulated PDE-4 protein expressions. Since, 1 μM roflumilast possessed the highest level of cAMP, 1 μM was selected as a roflumilast treating condition for use in the following experiments.

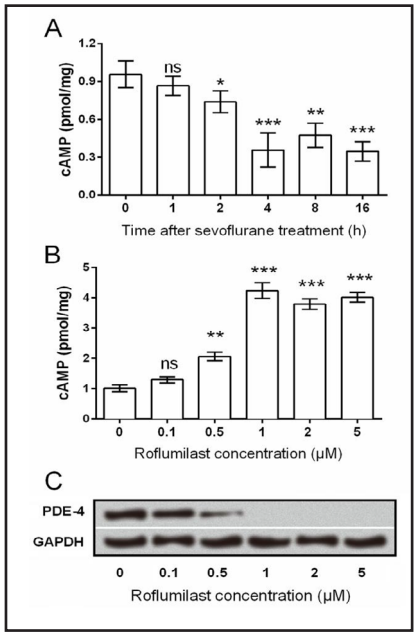

Fig. 3. Roflumilast promoted the release of cAMP via down-regulation of PDE-4. (A) The concentration of cAMP in culture supernatants was measured after hippocampal neurons were treated with sevoflurane for 0-16 h. (B) The concentration of cAMP in culture supernatants, and (B) the protein expressions of PDE-4 in cell were respectively assessed after hippocampal neurons were pre-treated with 0-5 μM of roflumilast for 16 h. Results presented as means ± SD. n = 3. ns, not significant, * P<0.05, ** P<0.01, and *** P<0.001 compared to 0 h or 0 μM groups.

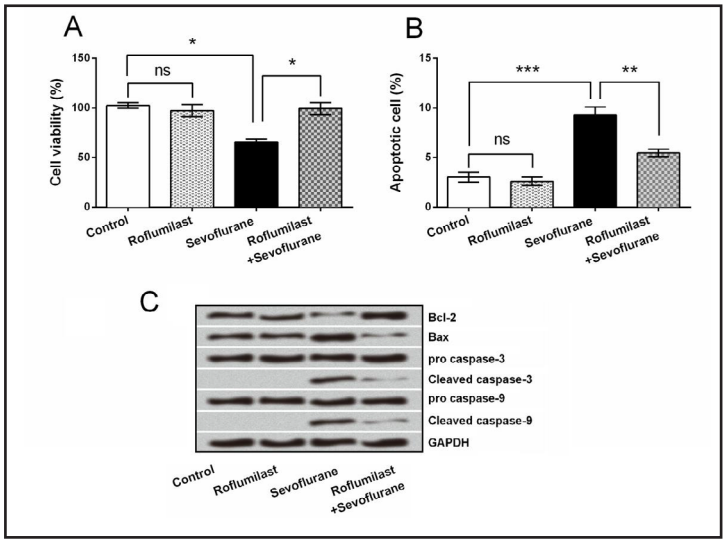

Fig. 4. Roflumilast rescued sevoflurane-induced hippocampal nerve damage. (A) Cell viability, (B) apoptotic cell rate, and (C) protein expressions of apoptosis-related factors were respectively detected, after hippocampal neurons were exposed to sevoflurane (16 h) and were treated with 1 μM roflumilast. Results presented as means ± SD. n = 3. ns, not significant, * P<0.05, ** P<0.01, and *** P<0.001 compared to the indicated group.

Roflumilast rescued sevoflurane-induced hippocampal nerve damage

To reveal whether roflumilast exhibited protective functions on sevoflurane-induced nerve damage, hippocampal neurons exposed to sevoflurane (16 h) were treated with 1 μM roflumilast. We found that roflumilast alone has no impact on cell viability, apoptosis and the

expressions of apoptosis-related proteins (Fig. 4A-4C). However, roflumilast could protect neurons from sevoflurane-induced cell damage, as roflumilast increased cell viability ($P < 0.05$), deceased apoptotic cell rate ($P < 0.01$), up-regulated Bcl-2 expression, and down-regulated Bax, cleaved caspase-3 and -9 expressions in sevoflurane-injured neurons.

Roflumilast recovered the inhibitory effects of sevoflurane on MEK/ERK signaling pathway

We then detected whether roflumilast protected sevoflurane-injured hippocampal neurons via modulation of MEK/ERK signaling pathway. Western blot analytical results showed that roflumilast treated alone remarkably up-regulated the protein expression levels of p-MEK and p-ERK ($P < 0.05$, $P < 0.01$ or $P < 0.001$, Fig. 5A-5F). Moreover, roflumilast could recover sevoflurane-induced down-regulations of p-MEK and p-ERK ($P < 0.05$, $P < 0.01$ or $P < 0.001$) during 16 h of sevoflurane treatment. The strongest recovery impacts of roflumilast against sevoflurane-induced blockage of MEK/ERK signaling pathway were observed at 4 h post-treatment.

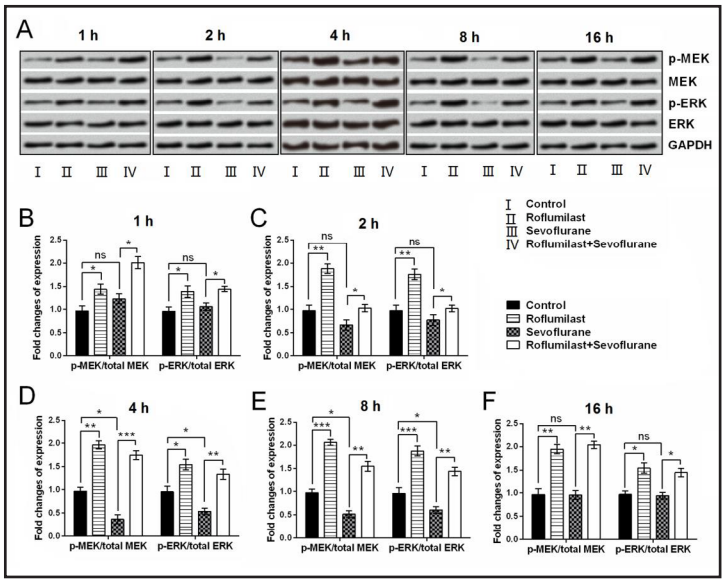

Fig. 5. Roflumilast protected sevoflurane-injured hippocampal neuron via modulation of MEK/ERK signaling pathway. (A) Protein expressions of core factors in MEK/ERK signaling were detected, after hippocampal neurons were exposed to sevoflurane (1, 2, 4, 8, and 16 h) and were treated with 1 μM roflumilast. (B-F) Quantitative expression changes of core factors in MEK/ERK and mTOR signaling pathways. Results presented as means ± SD. n = 3. ns, not significant, * P<0.05, ** P<0.01 and *** P<0.001 compared to the indicated group.

Discussion

The major findings of the current study are that sevoflurane treatment impairs cell viability and induces apoptosis of hippocampal neurons via modulation of MEK/ERK signaling pathway. Besides, roflumilast, an inhibitor of PDE-4, protects hippocampal neurons from sevoflurane-induced nerve damage via MEK/ERK signaling pathway.

In this study, we revealed a toxic effect of sevoflurane on hippocampal neurons, which were isolated from 18 days of rat. Sevoflurane with a concentration of 1% decreased neurons viability, increased apoptotic cell rate, up-regulated Bcl-2 protein expression, and down-regulated Bax, cleaved caspase-3 and -9 protein expressions. These findings are consistence with previous studies [4-7], suggesting that neonatal sevoflurane exposure is neurotoxic.

Recently biological studies have reported that aberrant activation of MEK/ERK and mTOR signaling pathways was pivotal for cell growth, survival and motility [20-22]. Neuronal ERK activation is a central mean of transducing neurotransmitter and neurotrophic signals, and thereby participates in a wide range of activities including neural survival, protection, and stress-related behavioral responses [23]. Suppression of MEK/ERK and mTOR phosphorylation is critically involved in the mechanism underlying anesthetic-induced toxicity in the developing brain [24-26]. In the current study, by performing western blot analysis, we found that sevoflurane inhibited the activation of MEK/ERK signaling, but has

no impact on mTOR signaling. These data indicated that sevoflurane injured hippocampal neurons via modulation of MEK/ERK signaling but not mTOR signaling.

Roflumilast, a PDE-4 selective inhibitor, has the ability of raising cAMP levels [13, 14]; this was also confirmed in this study. That roflumilast with the concentration of 0.5-5 μM remarkably suppressed PDE-4 protein expressions, and significantly increased cAMP levels. Further investigations revealed that 1 μM roflumilast exerted protective functions on sevoflurane-injured hippocampal neurons, as cell viability was increased, apoptotic cell rate was decreased, Bcl-2 was up-regulated, and Bax, cleaved caspase-3 and -9 were down-regulated by addition of roflumilast. Actually, several previous studies have revealed the neuroprotective functions of roflumilast. For instance, an *in vivo* study has reported that subcutaneous administration of roflumilast (3 mg/kg) significantly improved neurological deficits in the rat subarachnoid hemorrhage model [27]. Another *in vitro* study reported that roflumilast at concentrations of 1 nM or greater could attenuate blast-induced traumatic brain injury [28]. However, the findings in this study provided the first evidence that roflumilast could attenuate sevoflurane-induced nerve damage. Furthermore, we found that roflumilast activated MEK/ERK signaling pathway, even in the presence of sevoflurane. Together, we inferred that roflumilast protected hippocampal neurons from sevoflurane-induced nerve damage possibly via increasing cAMP levels and activation of MEK/ERK signaling.

PDE-4 inhibitors (PDE-4i) play a role in inhibiting cAMP hydrolysis by PDE-4. By doing so, PDE-4i have currently gained much attention for their therapeutic potency of respiratory diseases such as COPD [15, 16]. In terms of nervous system, immunocytological data have shown that rolipram, a kind of PDE-4i, has potential to improve spiral ganglion neurons survival [29]. Another kind of PDE-4i, HT-0712 was reported as an effective drug to treat age-associated memory impairment in human [30]. Additionally, rolipram, roflumilast, and PF-06266047 mitigated behavioral deficits and cell-specific pathology in the $Cln3^{\Delta ex7/8}$ mouse model of juvenile neuronal ceroid lipofuscinosis [31]. The findings of this study will add to the growing literature that PDE-4i may represent a therapeutic strategy for the treatment of a variety of nerve diseases. However, there existed several limitations in this study. First, this study revealed the functions of roflumilast in rat primary hippocampal neurons. Further studies are needed to investigate whether the functional effects can be reproduced in human primary cells. Second, the detailed regulations between roflumilast, cAMP, MEK/ERK signaling pathway, and neuron growth should be revealed. Third, *in vivo* investigations are needed, which may largely improve the findings.

Conclusion

This study demonstrated a neuroprotective role of roflumilast in sevoflurane-induced nerve damage. Roflumilast promoted hippocampal neurons viability, and reduced apoptosis possibly via modulation of MEK/ERK signaling pathway.

Acknowledgements

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Disclosure Statement

No conflict of interests exists.

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