Journal of Integrative Neuroscience

J. Integr. Neurosci. **2026**; 25(1): 45490
https://doi.org/10.31083/JIN45490

Original Research

Roflupram Reduces 6-OHDA-Induced Cellular Damage in SH-SY5Y Cells by Inhibiting PDE4

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Academic Editor: Andrei Surguchov
Submitted: 2 August 2025 Revised: 27 November 2025 Accepted: 1 December 2025 Published: 23 January 2026

Abstract

Background: The main symptoms of Parkinson's disease (PD) include olfactory impairment and tremor. Current treatment methods for PD generally have limitations such as short duration and severe side effects. The novel phosphodiesterase 4 (PDE4) inhibitor Roflupram (Roflu) mitigates inflammatory responses and enhances cognitive functions in individuals with neurological conditions. However, it remains unknown whether Roflu provides neuroprotection in a PD model induced by 6-hydroxydopamine (6-OHDA). **Methods**: Cell viability was assessed using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry. The expression level of tyrosine hydroxylase (TH) was evaluated by immunoblotting or immunofluorescence. Lactate dehydrogenase (LDH) release was measured to assess cytotoxicity. Intracellular reactive oxygen species (ROS) levels and mitochondrial membrane potential (MMP) were determined using fluorescent probes. **Results**: Roflu significantly increased cell viability in 6-OHDA-treated cells, as demonstrated by both MTT assay (17.18%, $p < 0.001$) and flow cytometry (12.20%, $p < 0.001$). It also upregulated the expression level of TH by 28.53% ($p < 0.05$). Furthermore, Roflu reduced LDH release by 23.54% ($p < 0.001$), indicating decreased cellular damage. Roflu markedly suppressed 6-OHDA-induced ROS accumulation by 57.82% ($p < 0.001$) and enhanced mitochondrial membrane potential (MMP) by 21.07% ($p < 0.01$). In addition, Roflu downregulated *PDE4B* expression in 6-OHDA-treated cells by 88.40% ($p < 0.001$). Knockdown of *PDE4B* mimicked the protective effects of Roflu, increasing cell survival by 18.43% ($p < 0.001$) and reducing LDH release by 21.54% ($p < 0.001$). Conversely, overexpression of *PDE4B* completely abolished the protective effects of Roflu, reversing both the increase in cell survival and the reduction in LDH release induced by Roflu in 6-OHDA-treated cells. **Conclusion**: Roflu has demonstrated a clear protective effect against cell damage caused by 6-OHDA, which is closely related to the inhibition of *PDE4B*. These findings indicate that Roflu has substantial preclinical potential as a therapeutic candidate for PD and other neurodegenerative disorders involving oxidative damage.

Keywords: Parkinson's disease; cyclic nucleotide phosphodiesterases; neuroprotection; oxidopamine; oxidative damage

1. Introduction

The classic feature of Parkinson's disease (PD) is the massive production and aggregation of the pathological form of α-synuclein (α-Syn) [1]. Patients with PD present with various clinical symptoms, including both motor and non-motor symptoms [2]. The benefits of current medications for PD are frequently only temporary, and they cannot stop the pathological process of neuronal degeneration; furthermore, long-term use is prone to cause terminal phenomena, on-off phenomena, and cardiovascular adverse reactions [3,4]. Considering the multiplicity of pathological mechanisms and the uncontrollable irreversible loss of neurons, effective disease-modifying PD therapies are currently lacking [5]. Thus, elucidating the key molecular pathways of neuronal degeneration is vital for identifying the core pathogenesis of PD and developing targeted neuroprotective therapies and new interventional methods [6]. The fundamental mechanisms driving dopaminergic neuronal loss in the pathogenesis of PD remain unclear and constitute a major barrier to the development of disease-modifying interventions. Consequently, identifying novel molecular regulators that can attenuate neuronal deterioration is crucial and may accelerate the discovery of therapeutic targets.

Phosphodiesterase 4 (PDE4) regulates the cyclic adenosine monophosphate (cAMP) signaling pathway, and abnormal cAMP signaling activation can accelerate neuronal damage by promoting neuroinflammatory responses and oxidative stress; thus, PDE4 has been implicated in the pathogenic mechanisms of various neurodegenerative conditions [7,8]. PDE4 inhibitors can improve neuroinflammation and oxidative stress by enhancing the cAMP signaling pathway and exhibit protective effects in various neurodegenerative disease models [9,10]. PDE4 inhibitors are considered candidates for the treatment of PD because of their anti-inflammatory, antioxidant, and neuroplasticity-promoting properties [9]. Previous studies demonstrated that PDE4 inhibitors exert therapeutic benefits in PD mod-

els by safeguarding dopaminergic neurons and improving impaired spatial working memory; however, their exact molecular mechanisms are unclear [11,12]. Currently used PDE4 inhibitors generally cause dose-limiting adverse reactions (especially gastrointestinal toxicity, such as nausea and vomiting), e.g., roipram, which was discontinued in a clinical trial for depression because of intolerable gastrointestinal side effects [7].

Roflupram (Roflu; also termed FFPM) is a potent PDE4 inhibitor (Fig. 1A). Roflu demonstrated superior selectivity for the *PDE4A4*, *PDE4B2*, and *PDE4D4* subtypes compared with rolipram, along with notable anti-inflammatory and neuroprotective activities [13,14]. Further, Roflu shows a low incidence of adverse effects such as vomiting, and it can cross the blood-brain barrier [13]. Roflu enhances autophagy and inhibits the activation of the inflammatory body of microglia, thereby reducing inflammatory responses and neural damage. Its advantages have been confirmed in neurodegenerative disease models, i.e., it can significantly improve cognitive dysfunction, and exert neuroprotective effects through the cAMP/protein kinase A (PKA)/cAMP-response element binding protein (CREB) signal transduction axis. Roflu also alleviates neuronal damage in brain ischemic injury models [9,13,15], suggesting its potential as a safe therapeutic intervention for stroke. However, the therapeutic potential of Roflu in PD requires further research.

Cellular oxidative damage and stress play pivotal roles in the pathogenesis of PD. Oxidative stress is a state of stress caused by the overproduction of reactive oxygen species (ROS) [16,17]. The high metabolic rate and the propensity for dopamine auto-oxidation render dopaminergic neurons particularly vulnerable in PD. Oxidative stress leads to a vicious cycle of mitochondrial dysfunction. The deficiency in mitochondrial complex I results in excessive ROS production, which is further exacerbated by a significantly compromised antioxidant defense system in the brains of PD patients, preventing cells from clearing excessive ROS. Abnormal aggregation of α-Syn can occur because of protein oxidative damage, and aggregated α-Syn inhibits mitochondrial functioning, thus exacerbating oxidative stress [18,19]. 6-Hydroxydopamine (6-OHDA) exhibits highly selective toxicity toward catecholaminergic neurons, thus it has been used for generating animal PD models. 6-OHDA differs from dopamine by only one hydroxyl group and can be actively absorbed by cells via the dopamine transporter. Subsequently, dopaminergic neurons in the nigrostriatal pathway are selectively eliminated [20,21].

This study evaluated the neuroprotective effects of Roflu, a PDE4 inhibitor, in a PD model. Previous study showed that PDE4 inhibitors exerted potential neuroprotective effects in PD, including 1-methyl-4-phenylpyridinium (MPP^+)/1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine (MPTP), rotenone, and α-Syn overexpression PD models [22–24]. The neuroprotection offered by PDE4 inhibitors is linked to improved regulation of autophagy and pathways involved in mitochondrial. However, it is unclear whether Roflu would exert a protective effect in the classic 6-OHDA model of PD, which is crucial for further validation of the role of Roflu in PD and its potential as a candidate drug. Here, we used a 6-OHDA-injured SH-SY5Y cell model to investigate the neuroprotective effects of Roflu and the involvement of a PDE4 target, showing that Roflu significantly increased cell viability and tyrosine hydroxylase (TH) expression levels induced by 6-OHDA while reducing lactate dehydrogenase (LDH) release. Furthermore, Roflu treatment promoted the recovery of mitochondrial membrane potential (MMP). We investigated *PDE4B* expression, which was lacking in previous studies, and found that Roflu downregulated 6-OHDA-induced *PDE4B* expression. *PDE4B* knockdown increased cell viability and reduced LDH release, whereas the protective effect of Roflu was antagonized by *PDE4B* overexpression. Our findings demonstrate that by inhibiting PDE4, Roflu alleviates 6-OHDA-induced oxidative damage, which confirms its considerable potential as a neuroprotective agent. This study identifies Roflu as a novel therapeutic candidate and provides a fresh mechanistic rationale for treating PD and other oxidative stress-related neurodegenerative disorders.

2. Materials and Methods

2.1 Materials

The manufacturers and item numbers of the reagents were as follows: MPP^+ (#D048, Sigma-Aldrich, St. Louis, MO, USA); LDH Cytotoxicity Assay Kit (#C0017, Beyotime Institute of Biotechnology, Shanghai, China); bicinchoninic acid (BCA) protein assay kit (#23225, Thermo Fisher Scientific, Waltham, MA, USA); si*PDE4B* was purchased from GenePharma (Shanghai, China); Roflu (#SML2106, Sigma-Aldrich); dimethyl sulfoxide (DMSO; #D2650, Sigma-Aldrich); 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; #M2128, Sigma-Aldrich); tetramethylrhodamine, ethyl ester, perchlorate (TMRE; #T669, Thermo Fisher Scientific); annexin V-FITC/PI double staining cell apoptosis detection kit (#KGA108, KeyGen Biotech, Nanjing, Jiangsu, China); phosphate buffered saline (PBS; #10010023, Gibco, Waltham, MA, USA); penicillin/streptomycin (#15140122, Gibco); anti-GAPDH (#2118, Cell Signaling Technology, Danvers, MA, USA); and anti-TH (#ab152, Merck-Calbiochem, La Jolla, CA, USA); anti-PDE4B (ab124772, Abcam, Cambridge, UK).

2.2 Methods

2.2.1 Cell Culture

The SH-SY5Y (American Type Culture Collection, Manassas, VA, USA, ATCC, #CRL-2266, RRID: CVCL-0019) was cultivated in a humidified incubator (#51036153,

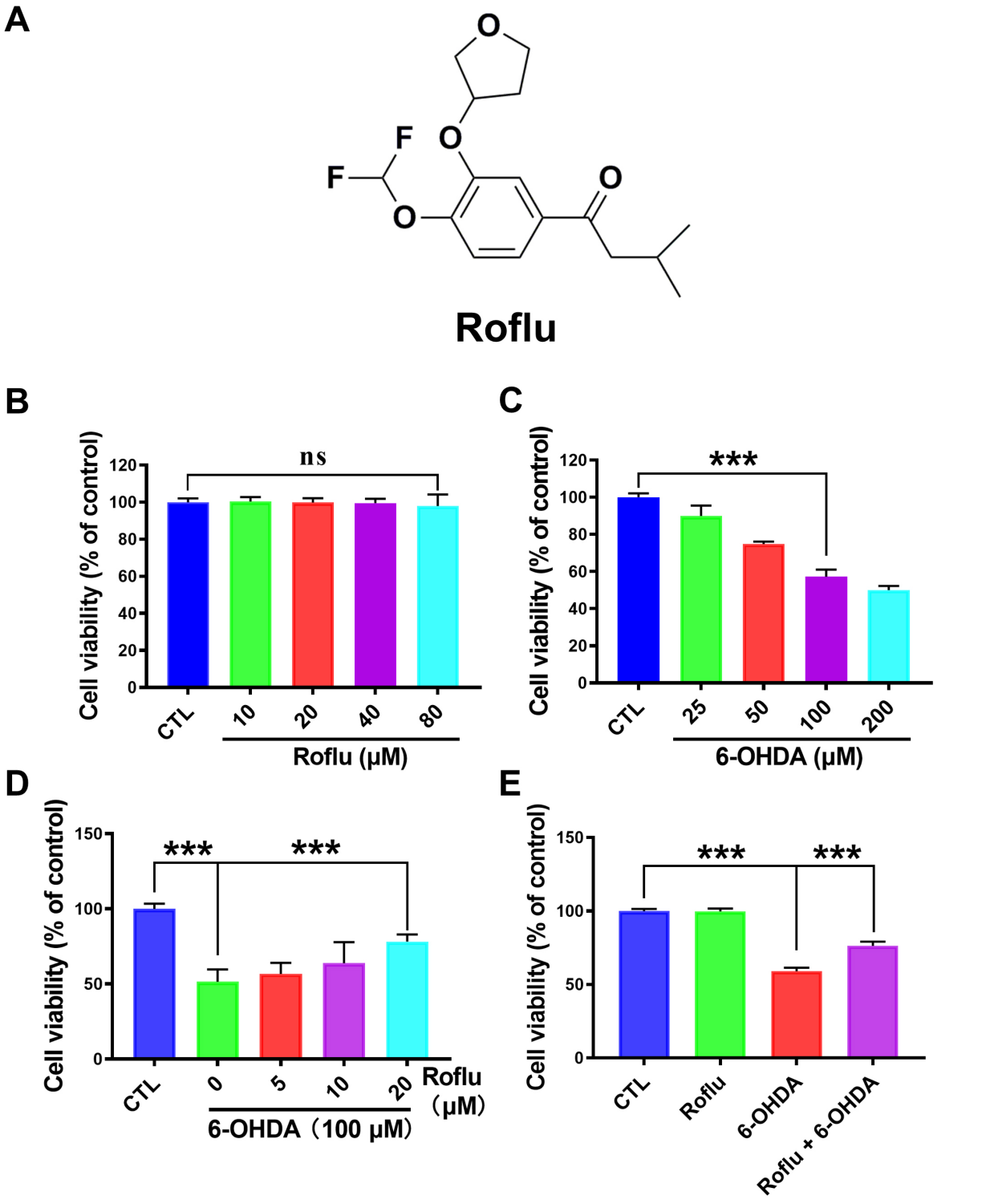

Fig. 1. Roflu increases the cell viability in the context of 6-OHDA-induced injury. (A) Structural diagram of Roflu. (B) The viability of cells treated with a gradient of Roflu concentrations (10–80 μM) for 48 h was determined by MTT assay. (C) The cytotoxicity of 6-OHDA (25–200 μM) following a 48 h exposure was assessed in SH-SY5Y cells using the MTT method. (D) A 1 h pre-incubation with Roflu (5–20 μM) was applied to cell cultures, and then exposed to 6-OHDA (100 μM) for 48 h. (E) SH-SY5Y cell viability was assessed by MTT after pretreatment with 20 μM Roflu for 1 h prior to exposure to 100 μM 6-OHDA for 48 h. Data are presented in the form of mean ± SD (n = 6). $^{***}p < 0.001$. ns: no difference. CTL, control; Roflu, Roflupram; 6-OHDA, 6-hydroxydopamine; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD, standard deviation.

Thermo Fisher Scientific) (37 °C; 5% CO_2). Cells were cultured in DMEM/F12 (#C11330500BT, Thermo Fisher Scientific) growth medium containing 10% fetal bovine serum (FBS) (#10099141, Thermo Fisher Scientific) and 1% penicillin–streptomycin. At 80%–90% confluence, as monitored daily via morphology, the cells were passaged every two cycles using a 0.25% trypsin-EDTA solution (#25200056, Thermo Fisher Scientific). All cell lines were authenticated via autosomal short tandem repeat (STR) profiling and confirmed negative for mycoplasma contamination.

2.2.2 MTT Assay

Following overnight culture in 96-well plates, cells were subjected to a 60 min pretreatment with serial dilutions of Roflu (10–80 μM) after the growth medium was aspirated. In the additional toxicity test, cells underwent a 48 h treatment with varying concentrations of 6-OHDA (25–200 μM). Moreover, cells were pretreated with Roflu (5–20 μM) for 1 h prior to a 48 h exposure to 100 μM 6-OHDA. The control (CTL) group was untreated. The Roflu group was treated with 20 μM Roflu, the 6-OHDA group was treated with 100 μM 6-OHDA, and the Roflu + 6-OHDA group was treated with 20 μM Roflu for 1 h and then 6-OHDA for 48 h. Cells in the above experiment were treated respectively, cell viability was assessed by the MTT assay. This involved a 4 h incubation using MTT reagent (0.5 mg/mL). The absorbance was measured at 570 nm after 10 min of orbital shaking.

2.2.3 LDH Determination

Before experimentation, the cells had reached approximately 70% confluence. The experiment comprised four treatment groups: an untreated CTL group, a group treated with 20 μM Roflu, a group treated with 6-OHDA (100 μM), and a group (Roflu + 6-OHDA) receiving a 1 h pretreatment with 100 μM Roflu prior to a subsequent 48 h challenge with 6-OHDA. Following 1 h pre-incubation with Roflu (20 μM), cultures were exposed to 100 μM 6-OHDA for 48 h. Plates were centrifuged (400 $\times g$, 5 min), after which 120 μL supernatant aliquots were transferred to fresh plates. Per the manufacturer's protocol, 60 μL LDH detection reagent was dispensed. Following a 30 min incubation in the dark, the absorbance was read at 490 nm, and the LDH release was calculated according to the instructions.

2.2.4 Flow Cytometry for Determining Cell Apoptosis

We divided the cells into four groups (CTL, Roflu, 6-OHDA, Roflu+6-OHDA), and the treatments for each group were carried out as described above. After discarding the culture medium, cells were treated with Roflu (20 μM) for 1 h and then challenged with 100 μM 6-OHDA for 24 h. Cells were then harvested by trypsinization. After centrifugation, following resuspension in 500 μL of binding buffer, the cells were stained with 5 μL Annexin V-FITC and 5 μL PI. Measurement of the apoptosis was performed using flow cytometry.

2.2.5 ROS Measurement

After being seeded in 24-well plates and cultured overnight. The experiment comprised four treatment groups: an untreated CTL group, a group treated with 20 μM Roflu, a group treated with 6-OHDA (100 μM), and a group (Roflu + 6-OHDA) receiving a 1 h pre-treatment with 100 μM Roflu prior to a subsequent 48 h challenge with 6-OHDA. After the cells are processed, they were washed three times. Subsequently, CellROX Deep Red Reagent (#C10422, Thermo Fisher Scientific) (10 μM) was applied to cells. Following three washes with PBS, ROS levels were assessed.

2.2.6 MMP Measurement

The CTL group was the untreated control group. The Roflu group was treated with a concentration of 20 μM Roflu, the 6-OHDA group was treated with a concentration of 100 μM 6-OHDA, and the Roflu + 6-OHDA group was treated with 100 μM Roflu for 1 h, with subsequent exposure to 6-OHDA for 24 h. Washing of the cells was gently carried out three times using PBS. We then stained the cells with 50 nM TMRE for 20 minutes. Following three additional PBS washes (5 min each) to remove unbound dye, the MMP was assessed by imaging TMRE fluorescence using an inverted confocal microscope (ECLIPSE Ti2-A, Nikon, Tokyo, Japan).

2.2.7 siRNA Transfection Experiment

For *PDE4B* knockdown, we utilized a siRNA duplex (designated si*PDE4B*) with the following strands: sense: 5′-CCUGCAAGAAGAAUCAUAUTT-3′; antisense: 5′-AUAUGAUUCUUCUUGCAGGTT-3′. The negative control (NC) consisted of a non-targeting scrambled siRNA. Transfection complexes were prepared by incubating Lipofectamine 3000 (#L3000001, Thermo Fisher Scientific) with Opti-MEM (#31985070, Thermo Fisher Scientific), and 50 nM siRNA for 20 min. Following a 6 h exposure to the complexes, the medium was replenished with fresh culture medium. Cell harvesting for analysis was subsequently performed 24 h post-transfection.

2.2.8 Transfection of PDE4B Plasmid

The transfection mixture was prepared by separately diluting the following in Opti-MEM: (1) the *PDE4B* expression plasmid complexed with P3000™ reagent, and (2) Lipofectamine 3000. To enable complex formation, the separately equilibrated solutions were pooled, mixed gently, and subjected to a 20 min incubation. To assess functional changes, the cells were analyzed 24 h after being treated with the transfection complexes.

2.2.9 Western Blotting

To extract cellular proteins, the samples were lysed using radioimmunoprecipitation assay buffer supplemented with the protease inhibitor PMSF (#P7626, Merck-Calbiochem) at a 1:100 volume ratio. Centrifugation was used to separate the soluble proteins into the supernatant fraction, which was retained. Loading consistency across samples was ensured by quantifying protein levels using a BCA assay. Size-based fractionation of proteins was achieved by electrophoresis on SDS-PAGE gels. Following separation, the proteins were blotted onto a polyvinylidene fluoride (PVDF) (#IPVH00010, Merck-Calbiochem) membrane. Following overnight incubation with primary antibodies at 4 °C, the membranes underwent extensive tris buffered saline with tween 20 (TBST) washes. Subsequently, they were probed with enzyme-conjugated secondary antibodies for 2 h to detect the target complexes. Finally, the target protein bands were visualized by chemiluminescence following the reaction with the necessary detection substrate, allowing visualization of their position and relative quantity.

2.2.10 Statistical Analysis

The convention of reporting the mean ± standard deviation (mean ± SD) was adopted for all data presentation. For analyses comparing two experimental groups, statistical significance was assessed using the Student's *t* test. Multi-group means were compared employing one-way analysis of variance (ANOVA), supplemented by the Bonferroni post hoc test for detailed two-group analysis. GraphPad Prism 8.0 (Insightful Science, San Diego, CA, USA) was used to generate the histogram plots. The threshold for statistical significance was set at $p < 0.05$.

3. Results

3.1 Roflu Increases the Cell Viability the Context of 6-OHDA-Induced Injury

As an initial evaluation of its neuroprotective properties, the cytotoxicity of Roflu was tested in SH-SY5Y cells via the MTT assay. The cell survival rate after treatment with Roflu (10–80 μM) did not show significant changes, and cell viability did not differ significantly ($p > 0.05$), indicating that Roflu exerted no cytotoxicity on SH-SY5Y cells (Fig. 1B). We examined the cytotoxicity of 6-OHDA in SH-SY5Y cells, and 6-OHDA significantly reduced cell viability in a dose-dependent manner. A 42.77% decrease in cell viability was recorded upon exposure to 100 μM 6-OHDA (Fig. 1C). Therefore, in the subsequent experiments, we used this concentration (100 μM of 6-OHDA. 6-OHDA-induced cell damage was concomitantly mitigated by Roflu (5–20 μM), whereas the protective effect was concentration-dependent (Fig. 1D). As illustrated in Fig. 1E, 6-OHDA exposure significantly reduced cell viability ($p < 0.001$, relative to the control). This effect was markedly reversed by pretreatment with 20 μM Roflu ($p < 0.001$). The PI-positive cells were significantly elevated by 6-OHDA ($p < 0.001$). In contrast, Roflu treatment markedly attenuated this increase ($p < 0.05$) (**Supplementary Fig. 1**). Collectively, these data suggest a protective role of Roflu against the cytotoxic effects of 6-OHDA in SH-SY5Y cells.

3.2 Roflu Protects SH-SY5Y Cells From Cytotoxic Effects Caused by 6-OHDA

The previous experimental results indicated that Roflu enhanced the viability following 6-OHDA exposure. To further confirm the neuroprotective effects of Roflu, we used flow cytometry to determine the cell survival rate. The experimental results were consistent with the aforementioned results. Roflu conferred a significant protective effect, enhancing the survival of 6-OHDA-challenged SH-SY5Y cells ($p < 0.001$), further verifying its protective effect on 6-OHDA-injured cells (Fig. 2A,B). The level of released LDH serves as a well-established indicator of membrane integrity and is widely utilized to assess cytotoxicity [25,26]. Relative to control, 6-OHDA treatment elevated LDH release in the culture medium ($p < 0.001$). In contrast, this effect was significantly suppressed by Roflu co-treatment ($p < 0.001$). Roflu thus could alleviate the cytotoxicity induced by 6-OHDA (Fig. 2C). TH is the first and most crucial rate-limiting step in the dopamine biosynthesis pathway, and it is a direct reflection of degeneration, apoptosis, and core pathological events [27]. In contrast to the significant reduction in TH protein caused by 6-OHDA ($p < 0.01$), Roflu treatment upregulated its expression ($p < 0.05$) (Fig. 2D,E; The original western blot images can be found in the **Supplementary Materials-original western blot images**). Collectively, these results indicate that Roflu plays a protective role against 6-OHDA-mediated neurotoxicity.

3.3 Roflu Protects SH-SY5Y Cells From the Oxidative Damage Caused by 6-OHDA

Intracellular oxidative damage and mitochondrial dysfunction play crucial roles in the neurotoxicity following 6-OHDA insult [28]. Previous findings demonstrate that Roflu attenuates the cytotoxic effects of 6-OHDA, thereby improving cell viability. However, its effect on intracellular oxidative damage is unknown. Exposure to 6-OHDA significantly elevated intracellular ROS levels ($p < 0.001$), whereas pretreatment with Roflu significantly reduced the intracellular ROS levels ($p < 0.001$) (Fig. 3A,B). In line with these findings, 6-OHDA treatment triggered a significant loss of MMP ($p < 0.001$), and Roflu significantly increased the MMP level of the cells ($p < 0.01$) (Fig. 3C,D). Thus, Roflu can resist mitochondrial damage induced by 6-OHDA and alleviate oxidative damage, thereby exerting neuroprotective effects.

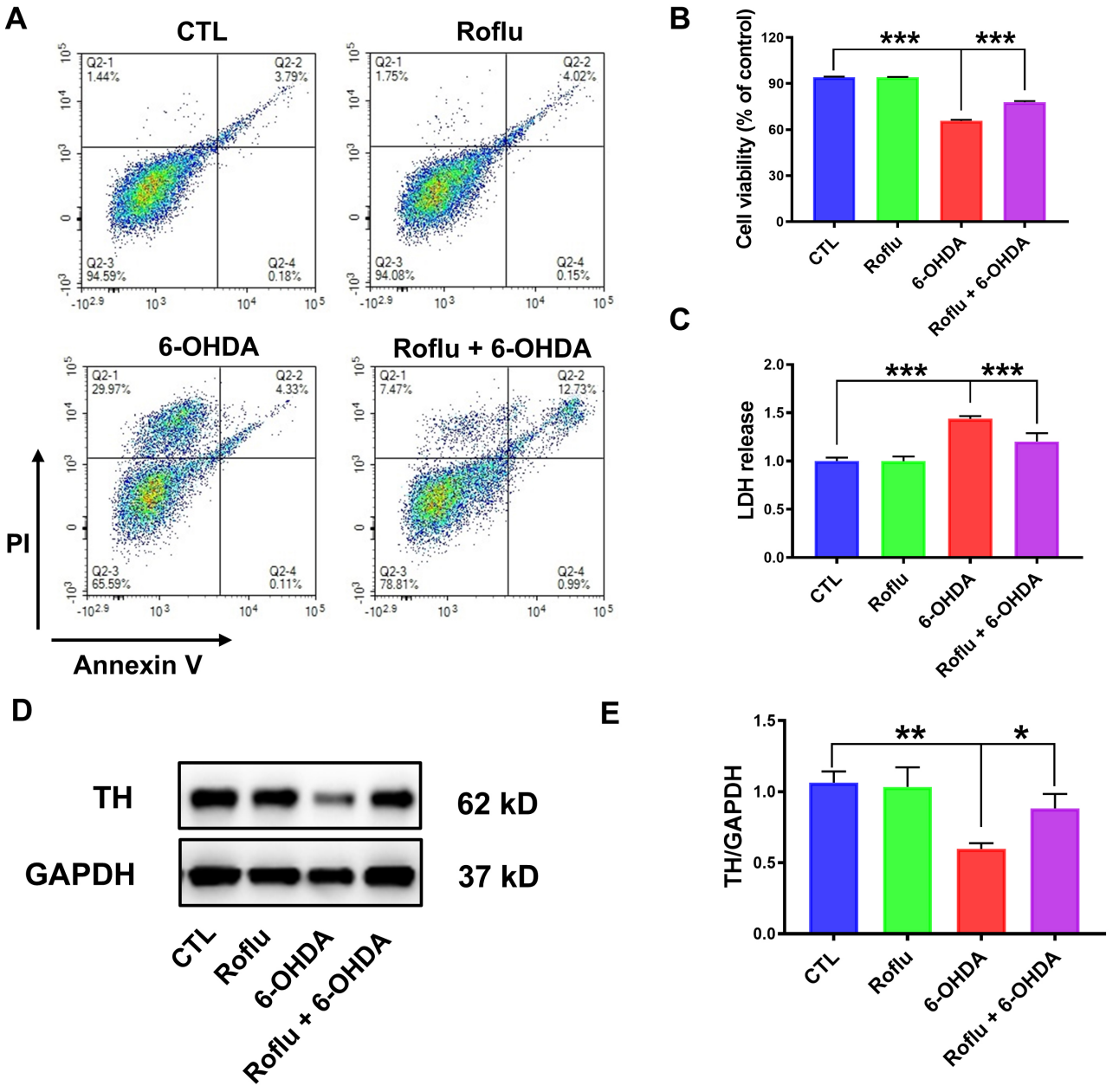

Fig. 2. Roflu protects SH-SY5Y cells from cytotoxic effects caused by 6-OHDA. (A,B) After a 1 h pretreatment with 20 µM Roflu, cells were subjected to 100 µM 6-OHDA for 24 h, with viability assessed using flow cytometry. Data are presented as mean ± SD (n = 3). $^{***}p < 0.001$. (C) Prior to exposure to 100 µM 6-OHDA for 48 h, cells were pretreated with 20 µM Roflu for 1 h. Cytotoxicity was then quantified by measuring LDH release with a kit. Data are presented as mean ± SD (n = 6). $^{***}p < 0.001$. (D,E) Assessment of TH expression by western blotting was conducted following a 1 h pretreatment with 20 µM Roflu and a subsequent 24 h incubation with 100 µM 6-OHDA. Data are presented as means ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$. LDH, lactate dehydrogenase; TH, tyrosine hydroxylase.

3.4 Roflu Reduces Cytotoxic Effects by Decreasing PDE4B in Cells Induced by 6-OHDA

PDE4 shows prominent expression in multiple brain areas, notably the striatum, hippocampus, and cortex, and cerebellum. Notably, among all PDEs, *PDE4B* mediates key aspects of striatal neuronal activity, suggesting a potential link to PD pathogenesis under pathological conditions [29]. 6-OHDA treatment induced a significant upregulation of *PDE4B* expression ($p < 0.001$). Conversely, Roflu treatment led to a notable downregulation of *PDE4B* ($p < 0.001$) (Fig. 4A,B). To confirm the role of *PDE4B* inhibition in Roflu-mediated protection against 6-OHDA cytotoxicity, we constructed cells with knocked-down *PDE4B*, and *PDE4B* levels showed a marked decrease in mRNA expres-

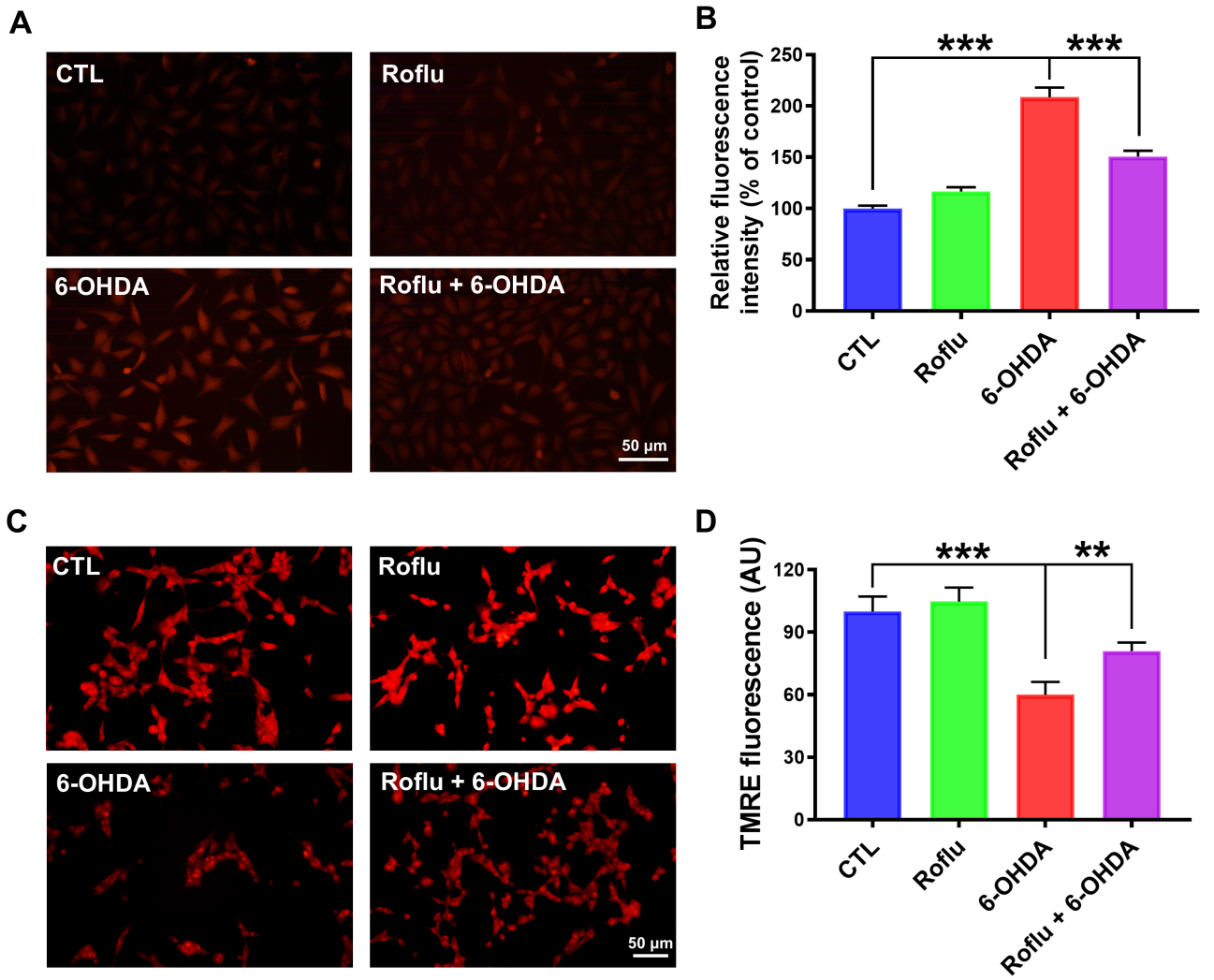

Fig. 3. Roflu protects SH-SY5Y cells from the oxidative damage caused by 6-OHDA. (A,B) SH-SY5Y cells were first treated with Roflu (20 μM) for 1 h, then exposed to 6-OHDA (100 μM) for 24 h. After the treatment, the ROS level was detected using CellROX Deep Red Reagent (10 μM). Scale bar = 50 μm. (C,D) SH-SY5Y cells were pre-incubated with Roflu (20 μM) for 1 h, followed by a 24 h treatment with 6-OHDA (100 μM), MMP was detected using TMRE (50 nM). Scale bar = 50 μm. Data are presented as means ± SD (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$. ROS, reactive oxygen species; MMP, mitochondrial membrane potential; TMRE, Tetramethylrhodamine, ethyl ester, perchlorate.

sion, with a concomitant reduction in protein abundance ($p < 0.01$) (Fig. 4C,D and **Supplementary Fig. 2**; The original western blot images can be found in the **Supplementary Materials-original western blot images**). Similarly, our results indicated that *PDE4B* knockdown protected against 6-OHDA-induced cytotoxicity, as evidenced by improved cell viability ($p < 0.001$) and reduced LDH release ($p < 0.001$) (Fig. 4E,F). In summary, Roflu confers neuroprotection against 6-OHDA-induced cytotoxicity via suppression of *PDE4B* expression.

3.5 PDE4B Overexpression Abolished Roflu's Protective Effect on SH-SY5Y cells From 6-OHDA

To establish a causal link between the *PDE4B* pathway and Roflu's neuroprotective effects against 6-OHDA in cells, *PDE4B* was overexpressed. We generated an SH-SY5Y cell line overexpressing *PDE4B*. A substantial increase in *PDE4B* expression was observed in these cells ($p < 0.01$) (Fig. 5A,B; The original western blot images can be found in the **Supplementary Materials-original western blot images**). We found that *PDE4B* overexpression abolished the protective effect of Roflu, as evidenced by a loss of the increase in cell viability following 6-OHDA treatment ($p < 0.001$) (Fig. 5C). At the same time, we also found that overexpressing *PDE4B* eliminated the effect of Roflu in reducing the LDH release in cells following 6-OHDA exposure ($p < 0.001$) (Fig. 5D). In conclusion, the protective effect of Roflu on SH-SY5Y cells against 6-OHDA-induced toxicity was negated by *PDE4B* overexpression.

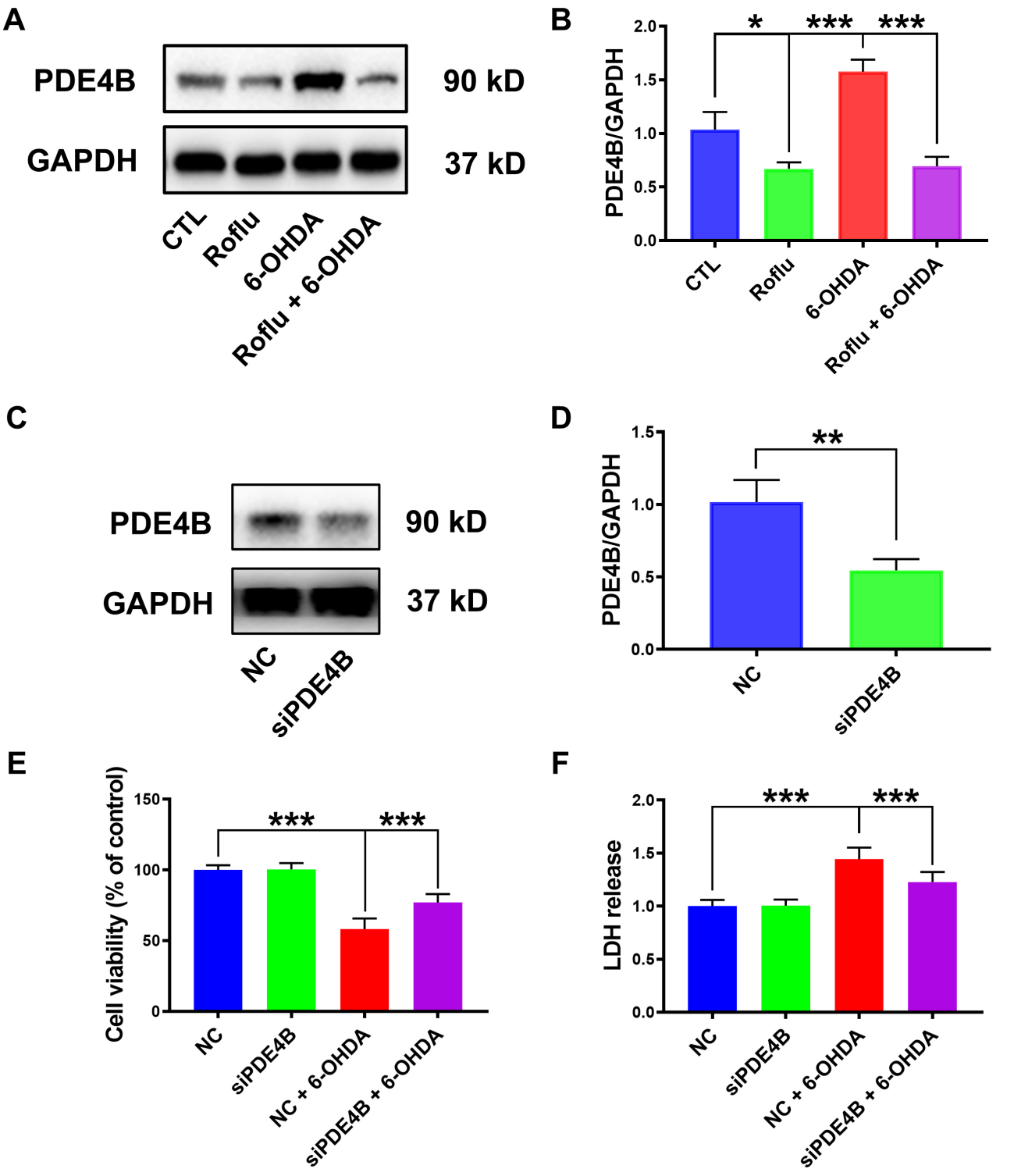

Fig. 4. Roflu reduces cytotoxic effects by decreasing *PDE4B* in cells induced by 6-OHDA. (A,B) Western blot analysis was performed to assess *PDE4B* expression in cells following pretreatment with 20 µM Roflu for 1 h and subsequent co-culture with 100 µM 6-OHDA for 24 h. The data are presented in the form of mean ± SD (n = 3). $^{*}p < 0.05$, $^{***}p < 0.001$. (C,D) To explore the knockdown efficiency of the *PDE4B* gene, SH-SY5Y cells were transfected with si*PDE4B* according to the instruction manual process, and the knockdown effect of *PDE4B* was verified by western blotting. The data are presented in the form of mean ± SD (n = 3). $^{**}p < 0.01$. (E,F) After transfection of SH-SY5Y cells with si*PDE4B*, the cells were treated with 100 µM 6-OHDA. After cell treatment was completed, cell viability and LDH levels were measured. Data are presented as means ± SD (n = 6). $^{***}p < 0.001$. PDE4, phosphodiesterase 4; NC, negative control.

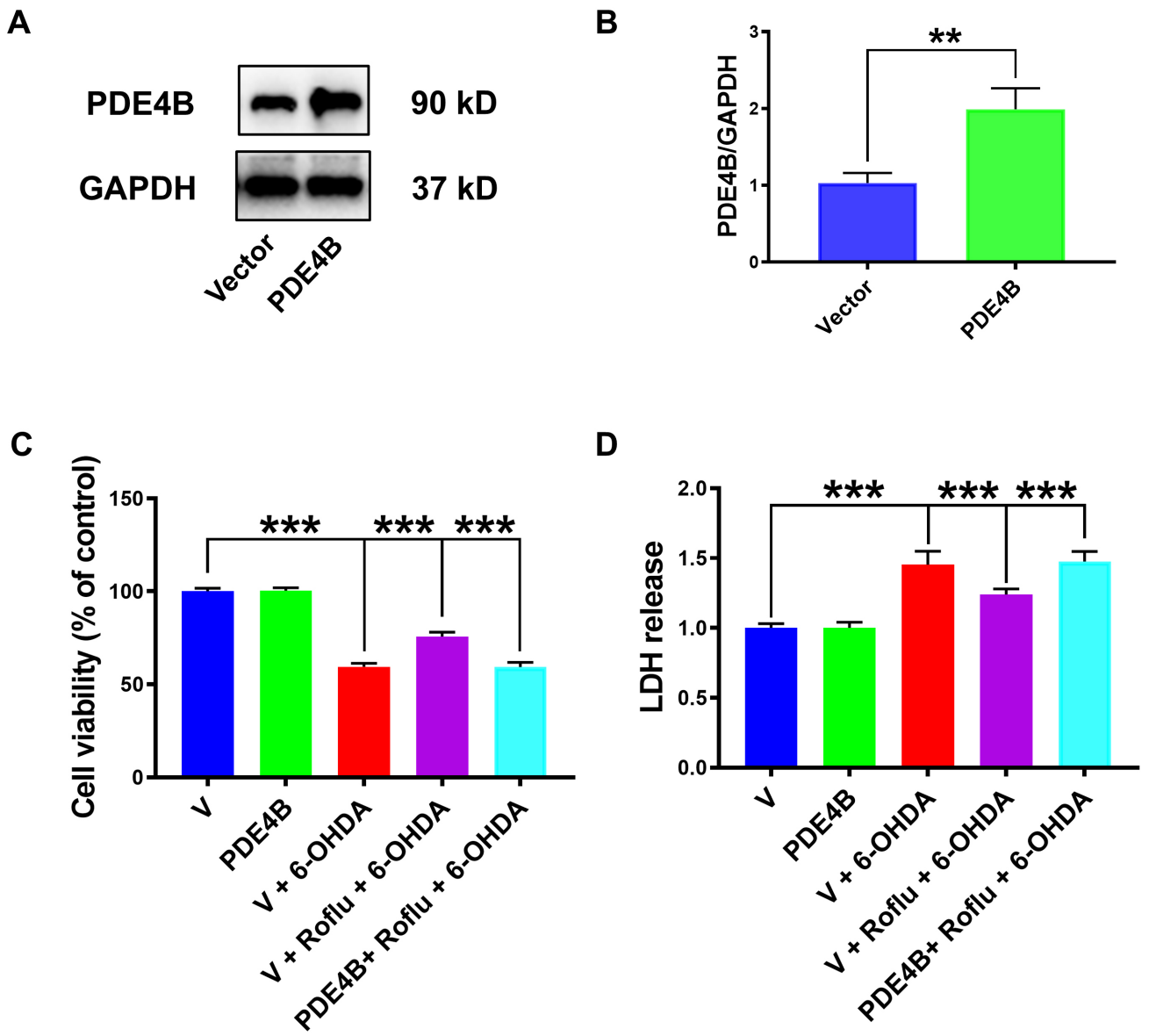

Fig. 5. *PDE4B* overexpression abolished Roflu's protective effect on SH-SY5Y cells from 6-OHDA. (A,B) Following the protocol provided by the transfection reagent manufacturer, transfection of SH-SY5Y cells with a *PDE4B* plasmid led to robust protein expression, as detected by western blot. Data are presented as mean ± SD (n = 3). $^{**}p < 0.01$. (C,D) After transfection with the *PDE4B* plasmid, cells were treated with 20 μM Roflu for 1 h, followed by exposure to 100 μM 6-OHDA for 48 h. After the cell treatment was completed, cell viability and LDH levels were measured. Data are presented as means ± SD (n = 6). $^{***}p < 0.001$. V, vector.

3.6 Overexpression of PDE4B Blocked the Ability of Roflu to Attenuate Oxidative Damage in 6-OHDA-Treated SH-SY5Y Cells

Our findings confirmed that the antioxidative protection offered by Roflu against 6-OHDA in SH-SY5Y cells, which was previously observed but mechanistically unexplained, was specifically mediated through *PDE4B* inhibition, as *PDE4B* overexpression completely prevented the suppression of ROS generation with Roflu ($p < 0.001$) (Fig. 6A,B). Simultaneously, the Roflu-induced increase in MMP following 6-OHDA insult was abolished by *PDE4B* overexpression ($p < 0.001$) (Fig. 6C,D). The protective effects of Roflu against 6-OHDA-induced oxidative stress in SH-SY5Y cells are critically dependent on *PDE4B*.

4. Discussion

We found that specific inhibition of *PDE4B* by Roflu underlies its neuroprotective effects in a PD model established by 6-OHDA. Specifically, Roflu mitigated oxidative stress during the injury of SH-SY5Y cells, thereby reversing the degeneration of dopaminergic neurons. These findings highlight *PDE4B* inhibition as a potential therapeutic strategy for PD and deepen the understanding of the regulatory mechanism of the PDE4/cAMP signaling

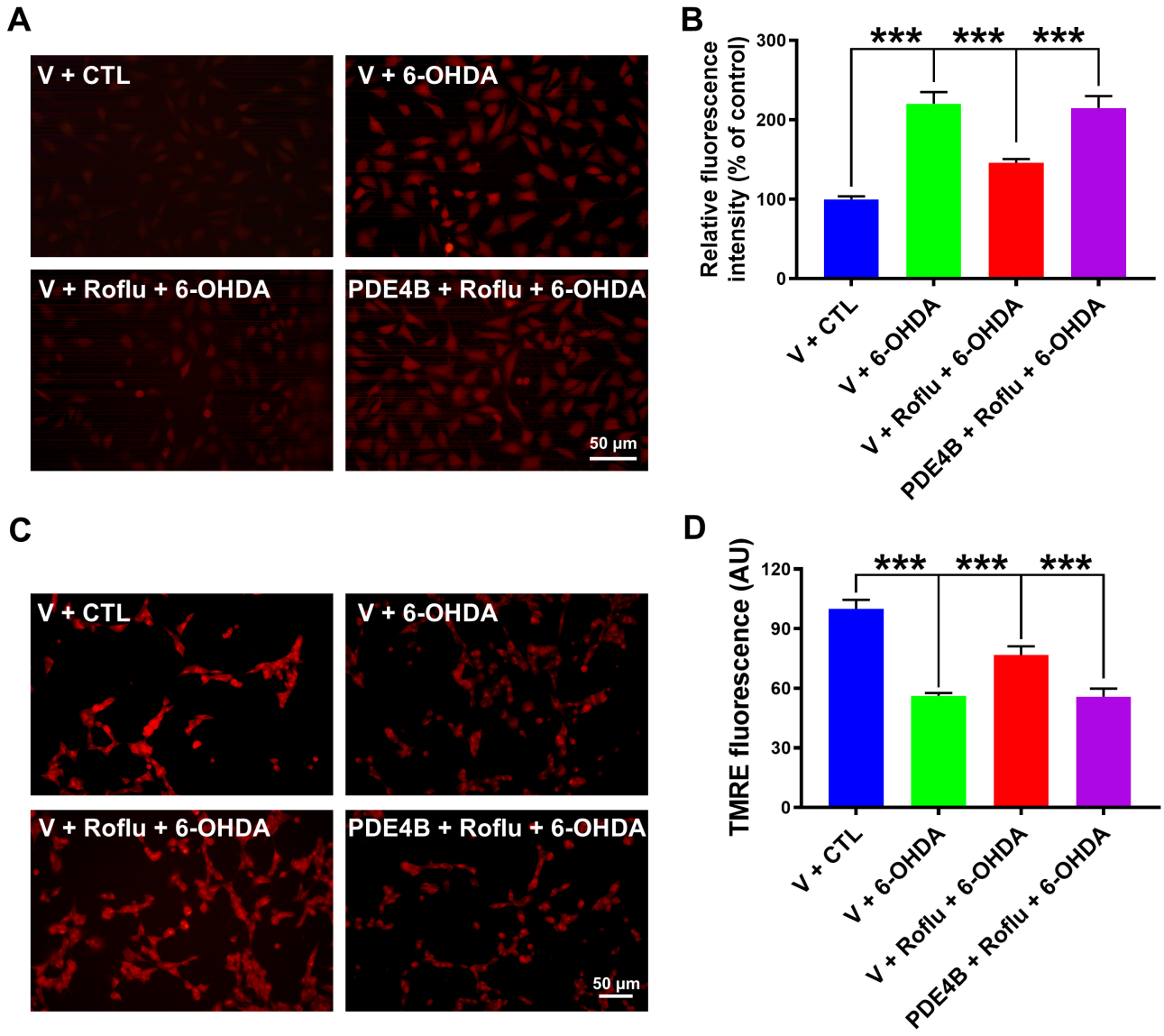

Fig. 6. Overexpression of *PDE4B* blocked the ability of Roflu to attenuate oxidative damage in 6-OHDA-treated SH-SY5Y cells. (A–D) Following transfection with the *PDE4B* plasmid, prior to a 24 h treatment with 100 μM 6-OHDA, SH-SY5Y cells were pretreated with 20 μM Roflu for 1 h. Subsequently, intracellular levels of ROS and MMP were measured. Scale bar = 50 μm. Data are presented as means ± SD (n = 3). $^{***}p < 0.001$.

axis in neurodegenerative diseases, offering new candidate compounds and mechanism-based evidence for the targeted treatment of PD and related oxidative damage-induced neurodegenerative diseases. Although current mainstream drugs for PD, such as levodopa, can temporarily improve motor function, they cannot stop the pathological process of neuronal degeneration and are prone to cause adverse reactions, such as the wearing-off and on-off phenomena, when used for a long time [3]. Therefore, new intervention targets and drugs that can delay neuronal damage are needed. Conventional PDE4 inhibitors, such as rolipram, were discontinued in Phase II trials owing to adverse gastrointestinal reactions such as vomiting [7]. Compared with rolipram, Roflu shows higher selectivity for PDE4A4, PDE4B2, and PDE4D4 subtypes (IC_{50} values for PDE4A4/B2/D4 subtypes are 3 to 10 fold lower than those of rolipram), and demonstrates minimal emetic potential along with favorable blood-brain barrier penetration in animal studies [13,14], suggesting that its therapeutic window may be significantly better than that of rolipram. Roflu demonstrates significant advantages in safety, with no cytotoxicity observed within the concentration range of 10 to 80 μM in experiments. Additionally, Roflu has been proven to enhance the autophagic clearance of β-amyloid in Alzheimer's disease models [15], suggesting that it cooperatively regulates abnormal aggregation of α-Syn in PD [18]. This multi-target intervention is particularly important in complex neurodegenerative diseases.

Previous research showed that PDE4 inhibitors have potential neuroprotective effects in PD, including in MPP^+/MPTP, rotenone, and α-Syn overexpression PD models [22–24]. However, it was unclear whether Roflu would exert protective effects in the classic 6-OHDA-induced PD model. The 6-OHDA model is one of the most classic and widely used pharmacological induction models in PD research, particularly for simulating the pathological hallmarks of PD, the distinctive pathological changes in nigrostriatal dopaminergic neuronal loss [30,31]. In the present study, an injury model of SH-SY5Y cells induced by 6-OHDA was employed to systematically assess the neuroprotective efficacy of Roflu. Our findings indicated that 6-OHDA treatment markedly decreased SH-SY5Y cell viability, promoted LDH release, and reduced the expression of TH. A key enzyme in dopamine synthesis, TH's expression level directly reflects the functional state of dopaminergic neurons [27]. Roflu pretreatment significantly reversed these changes, indicating that Roflu can exert protective effects at multiple levels, such as cell survival, functional maintenance, and oxidative stress regulation. Thus, our results demonstrate that Roflu exerts antioxidant effect, thereby corroborating the established role of PDE4 inhibition in mitigating neural damage across diverse experimental models [22–24] and further supporting the potential of PDE4 inhibitors as neuroprotective agents. Roflu needs to be further examined in 6-OHDA and α-Syn transgenic rodent models with regard to its protective effects on motor symptoms, dopamine content, and substantia nigra-striatum pathology, and to systematically evaluate the safety of long-term administration.

The core manifestations of oxidative damage in PD include an overproduction of ROS and a compromised antioxidant system (such as glutathione) [32]. Dopamine metabolism is a major source of ROS, while mitochondria, the cellular powerhouses, are also the primary site for its generation. Further amplify this oxidative burden, their dysfunction (especially the reduced activity of complex I) directly leads to an ATP synthesis disorder, causing neurons to be in an energy crisis which initiates a vicious cycle of sustained ROS overproduction [33]. Excessive ROS can directly attack and oxidatively damage lipids, proteins (e.g., α-Syn), and DNA, thus damaging cell structure and function. Abnormal α-Syn protein aggregates into Lewy bodies, potentially damaging mitochondria themselves and interfering with the phosphatase and tensin homolog PTEN-induced (PINK1)/Parkin pathway-mediated mitochondrial autophagy. Consequently, this results in the accumulation of dysfunctional mitochondria and creates a vicious cycle exacerbating mitochondrial dysfunction and oxidative damage [16,34]. The selective degeneration of dopaminergic neurons is driven by a self-perpetuating cycle of mitochondrial impairment and persistent oxidative stress. In this study, 6-OHDA exposure resulted in significant ROS accumulation and MMP reduction, establishing oxidative injury as a key contributing factor in 6-OHDA-mediated neurotoxicity. These findings are consistent with those of a previous study demonstrating that PDE4 inhibitors attenuate MPP^+-induced MMP loss in cells [22–24]. Roflu significantly decreased ROS levels induced by 6-OHDA and restored MMP, indicating that Roflu exerts protective effects at multiple levels, including cell survival and oxidative stress regulation. This observation aligns with reported antioxidant and mitochondrial protective effects of PDE4 inhibitors in other neurological injury models [9,35,36], underscoring their promise as neuroprotective agents.

Next, we investigated the connection between the neuroprotective effects of Roflu and PDE4. The *PDE4B* expression within the substantia nigra is the highest among all phosphodiesterases and is related to the function of the striatum and the pathology of PD [29]. We detected a marked upregulation of *PDE4B* expression in the 6-OHDA-treated SH-SY5Y cell model, and Roflu could markedly inhibit this trend. Moreover, knockdown of *PDE4B* simulated the effect of Roflu, whereas overexpression of *PDE4B* counteracted the improvement effect of Roflu on cell viability and LDH release and even reversed its regulatory effects on ROS and MMP. These findings collectively demonstrate that the neuroprotective mechanism of Roflu involved specifically through the inhibition of the *PDE4B* subtype and that *PDE4B* is the key target mediating its neuroprotective effect. This study addresses the lack of attention to *PDE4B* in previous studies, clarifying the role of *PDE4B* with regard to the protective effects of Roflu. This study confirmed that Roflu can exert neuroprotection in the PD model by attenuating oxidative stress. However, the specific mechanism of action still requires further research. The nuclear factor-erythroid 2-related factor 2 (Nrf2)/ heme oxygenase-1 (HO-1) pathway is the core regulatory hub for the cellular capacity to combat oxidative stress. Previous study have shown that inhibiting PDE4 can regulate it [10], which provides a direction for further exploration of more downstream molecular pathways.

5. Conclusion

Our study thus provides experimental evidence that Roflu exerts neuroprotective effects in a well-established 6-OHDA-induced PD model, complementing the previous data from different PD models such as MPP^+/MPTP. These findings strengthen the universality of PDE4 inhibitors in various models of PD. Our results clearly identify *PDE4B* as the core target for Roflu in regulating oxidative damage, resolving the key controversy of "functional heterogeneity of PDE4 subtypes" in previous studies [22–24]. Given its high selectivity and favorable safety profile, Roflu shows significant promise as a drug candidate targeting of PD and other neurodegenerative diseases that share common pathological features.

Availability of Data and Materials

All data generated or analysed during this study are included in the research article.

Author Contributions

JHZ: Conceptualization, Methodology, Investigation, Funding acquisition, Writing—original draft. XHY: Conceptualization, Methodology, Investigation, Writing—original draft. JLX: Conceptualization, Methodology, Investigation. QMW: Data curation, Formal analysis, Software. ZML: Conceptualization, Funding acquisition, Project administration, Resources, Writing—review & editing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This work was supported by National Nature Science Foundation of China (Grant No. 82304434), Guang-Dong Basic and Applied Basic Research Foundation (Grant No. 2023A1515111199), China Postdoctoral Science Foundation (Grant No. 2022M713263), Scientific Research Starting Foundation for High-level Talents of Meizhou People's Hospital (Grant No. KYQD202501, KYQD202502), PSM Guangdong Pharmaceutical Popular Science Research Foundation (Simcere Foundation) (Grant No. 2025KP178, 2025KP78), Shining Across China-Medicinal Research Fund (Grant No. Z04J2023E095), Social Development Science and Technology Plan Project of Meizhou (Grant No. 2024C0301079).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/JIN45490.

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