REGULAR RESEARCH ARTICLE

# The Phosphodiesterase-4 Inhibitor Roflumilast, a Potential Treatment for the Comorbidity of Memory Loss and Depression in Alzheimer's Disease: A Preclinical Study in APP/PS1 Transgenic Mice

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## Abstract

**Background:** Depression is highly related to Alzheimer's disease (AD), yet no effective treatment is available. Phosphodiesterase-4 (PDE4) has been considered a promising target for treatment of AD and depression. Roflumilast, the first PDE4 inhibitor approved for clinical use, improves cognition at doses that do not cause side effects such as emesis.
**Methods:** Here we examined the effects of roflumilast on behavioral dysfunction and the related mechanisms in APPswe/PS1dE9 transgenic mice, a widely used model of AD. Mice at 10 months of age were examined for memory in the novel object recognition and Morris water-maze tests and depression-like behavior in the tail-suspension test and forced swimming test before killing for neurochemical assays.
**Results:** In the novel object recognition and Morris water-maze, APPswe/PS1dE9 mice showed significant cognitive declines, which were reversed by roflumilast at 5 and 10 mg/kg orally once per day. In the tail-suspension test and forced swimming test, the AD mice showed prolonged immobility time, which was also reversed by roflumilast. In addition, the staining of hematoxylin–eosin and Nissl showed that roflumilast relieved the neuronal cell injuries, while terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling analysis indicated that roflumilast ameliorated cell apoptosis in AD mice. Further, roflumilast reversed the decreased ratio of B-cell lymphoma-2/Bcl-2-associated X protein and the increased expression of PDE4B and PDE4D in the cerebral cortex and hippocampus of AD mice. Finally, roflumilast reversed the decreased levels of cyclic AMP (cAMP) and expression of phosphorylated cAMP response element-binding protein and brain derived neurotrophic factor in AD mice.
**Conclusions:** Together, these results suggest that roflumilast not only improves learning and memory but also attenuates depression-like behavior in AD mice, likely via PDE4B/PDE4D-mediated cAMP/cAMP response element-binding protein/brain derived neurotrophic factor signaling. Roflumilast can be a therapeutic agent for AD, in particular the comorbidity of memory loss and depression.

Key Words: Roflumilast, β-amyloid (Aβ), phosphodiesterase-4 (PDE4), Alzheimer's disease, depression

## Significance Statement

Depression is highly related to Alzheimer's disease (AD), yet no effective treatment is available. Phosphodiesterase-4 (PDE4) has been considered a promising target for treatment of AD and depression. The effect of PDE4 inhibitors on comorbidity of memory loss and depression remains largely unclear. The objective of the current study was to explore the effect of roflumilast, a selective PDE4 inhibitor that has been approved for clinic use, on the comorbidity. We found that roflumilast not only improved learning and memory, but also attenuated depression-like behavior in APP/PS1 mice, most likely via PDE4B/PDE4D-mediated cAMP/CREB/BDNF signaling. Roflumilast can be a therapeutic agent for AD, in particular the comorbidity of memory loss and depression.

## Introduction

Alzheimer's disease (AD), the most common neurodegenerative disorder, is characterized by a progressive loss of cognition. Depression is identified as the most frequent psychiatric comorbid condition in dementia and affects about 50% of AD patients (Chi et al., 2014; Romano et al., 2014). It has also been reported that depression, which can trigger, co-occur, or exacerbate dementia symptoms, is also responsible for the acceleration of cognition decline, a decreased quality of life, high levels of caregiver burden, and mortality in patients with AD (Bennett, 2014). A high comorbidity between AD and depression suggests similar mechanisms underlying the course of the 2 diseases, but there is no consensus on the etiology to date. Meanwhile, current antidepressants such as tricyclic agents, selective serotonin reuptake inhibitors, and monoamine oxidase inhibitors are limitedly effective for depression in AD (Byers and Yaffe, 2011). Therefore, revealing the biological mechanisms and identifying novel and effective treatment for the comorbidity of AD with depression are of particular importance and significance.

Phosphodiesterase-4 (PDE4), an important member of the PDE superfamily, is a key regulator of intracellular cyclic AMP (cAMP) levels (Zhang et al, 2009). As the second messenger, cAMP activates protein kinase A, which phosphorylates the subsequent downstream cAMP-response element binding (CREB) protein (Hansen and Zhang, 2013; Heckman et al., 2018). In the central nervous system, this signaling cascade exerts both pre- and post-synaptic effects and is essential for a variety of cellular functions, including neurotransmitter release and neuroprotection. Inhibition of PDE4 prevents cAMP breakdown, which is well recognized as the mechanism by which PDE4 inhibitors can treat impaired memory linked to several brain disorders (Hansen and Zhang, 2017; Ricciarelli and Fedele, 2018). In pre-clinical studies and early-stage clinical trials, PDE4 inhibitors such as rolipram have been shown to enhance memory (Li et al., 2011; Gurney and D'Amato, 2015; Heckman et al., 2015; Prickaerts et al., 2017). They also improve depressive-like behaviors induced by chronic unpredictable mild stress, lipopolysaccharide, or ethanol abstinence (Gong et al., 2017; Zhou et al., 2017; Yu et al., 2018). Consequently, it is reasonable to believe that PDE4 is a potential target for treatment of the comorbidity of depression and AD. However, the demonstration for this is lacking.

The clinical application of PDE4 inhibitors has been hampered due to nausea and emesis in patients, the major side effects of this class of drugs. Roflumilast is the first drug targeting PDE4 that was marketed for treatment of chronic obstructive pulmonary disease (COPD) with relatively weak potency inducing nausea and vomiting (Luo et al., 2018). Recently, preclinical studies have proven cognitive enhancement of roflumilast. Specifically, roflumilast improves memory in rodents at non-emetic doses (Vanmierlo et al., 2016), ameliorates hypertension-induced memory deficit in rats (Jabaris et al., 2015a, 2015b), and protects rat hippocampal neurons from sevoflurane-induced injury (Peng et al., 2018). More importantly, in clinical trials, roflumilast has been reported to not only improve memory in healthy adults (Van Duinen et al., 2018) and the elderly (Blokland et al., 2019) at lower doses than that used for COPD but also to block memory dysfunction of schizophrenia (Gilleen et.al., 2018). These appear to be attributed to the neuroprotective effect of roflumilast. However, whether roflumilast improves memory loss in AD and concurrently attenuates its comorbidity of depression is largely unknown. In the present study, we aimed to investigate the effects of roflumilast on memory deficits and the comorbid depression-like behavior using APPswe/PS1dE9 (APP/PS1) double transgenic mice, a widely used model of AD; the neurochemical mechanisms of roflumilast's action were also investigated.

## Materials and Methods

### Animals

APP/PS1 double transgenic mice were obtained from Beijing HFK Bioscience Co. Ltd (Beijing, China) and maintained on the C57BL/6J background. All mice used in this study were separately housed in individually ventilated cages on a 12-hour-light/-dark cycle (7:00 AM-7:00 PM) and were given food and water ad libitum in the SPF animal facilities at the Institute of Pharmacology, Shandong First Medical University (Tai'an, China). All experiments were performed in accordance to the protocols approved by the Laboratory Animals' Ethic Committee of Shandong First Medical University.

### Reagents and Antibodies

Roflumilast was purchased from Aladin Ltd. (Shanghai, China) and dissolved in dimethyl sulfoxide and kept at 4℃ before use. As previously reported (Vanmierlo et al., 2016), this stock solution was dissolved with 1% sodium cellulose solution, making the final dissolved in dimethyl sulfoxide concentration of 1.2%, which was used as vehicle.

Antibodies against PDE4A, PDE4B, PDE4D, B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), brain derived neurotrophic factor (BDNF), CREB, or phospho-CREB (pCREB) were purchased from Abcam (Cambridge, MA). cAMP enzyme-linked immunosorbent assay (ELISA) kits were purchased from Shanghai Blue Gene Biotech (Shanghai, China). BCA Protein Quantitation kits were purchased from Solarbio (Shanghai, China). Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL) In Situ Cell Death Detection kit was purchased from Roche Diagnostics (Mannheim, Germany).

## Experimental Schedule

Thirty male APP/PS1 mice at 10 months of age and 10 age-matched male C57BL/6 mice as wild-type (WT) controls were used in the experiments. As shown in the experimental schedule (Figure 1), the mice were administered by gavage with roflumilast at the dose of 5 or 10 mg/kg or its vehicle for 30 days. The doses were selected based on the result of our pilot experiment using the dose conversed from the clinical dose (0.5 mg/kg/d, orally) for COPD (Nair and Jacob, 2016). From day 21 to 30 of administration, all mice were subjected to various behavioral tests in the following order: the novel object recognition (NOR), the Morris water-maze (MWM), the tail suspension test (TST), and the forced swimming test (FST); all behavioral experiments were conducted 2 hours after gavage administration. One hour after the last behavioral test (i.e., the FST), the mice were killed by decapitation and the brains were collected. The hemispheres of 3 mice in each group were collected for pathological staining. The cerebral cortex and hippocampus of the remaining mice were isolated from the hemispheres for western-blot and ELISA assays; tissues were stored at –80℃ before use.

## NOR Test

The NOR test was conducted in a blue rectangular open field apparatus (60 × 60 × 60 cm) for 3 consecutive days (Li et al., 2011; Antunes and Biala, 2012). On the first day, mice were individually habituated to the empty arena for 10 minutes. On the second day, mice were individually placed in the arena for 10 minutes to explore the 2 identical objects. The objects were counterbalanced between mice to avoid innate preference for a location. On the third day, mice were returned and allowed to freely explore the 2 objects, 1 of which was replaced by a novel object; the positions of the novel and familiar objects were still counterbalanced between mice. The time spent exploring each object was recorded, and recognition object index was calculated as a percentage of the time spent exploring the novel object over the total time spent exploring both objects. The tracking information was processed by the Topscan Package (Clever Sys Inc.).

## MWM Test

Spatial learning and memory were assessed using the MWM with the tracking information processed by the Topscan Package (Clever Sys Inc.). As previously described (Wang et al., 2014), mice were individually placed into an open swimming arena (diameter, 120 cm; height, 50 cm; water depth, 30 cm)
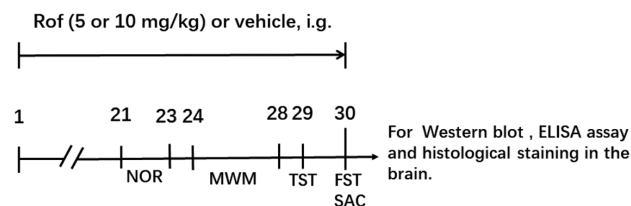
to locate an invisible underwater platform (1 cm under the water). During the acquisition trial, mice were successively placed from an equidistant location to the platform in each quadrant for 4 days. The maximum swimming time was 60 seconds, and mice were allowed to stay on the platform for 10 seconds. If the mouse did escape by swimming to the platform within 60 seconds, the escape latency, which was defined as the time spent in locating the hidden platform, was recorded as 60 seconds. During the probe trial on the fifth day, the platform was removed and mice were placed from the opposite side of the previous platform quadrant (i.e., the target quadrant) to swim for 60 seconds. The time required for the mouse to first reach the location of the target quadrant, the number of crossings into the target quadrant, and the swimming distance were recorded.

## TST

The TST was performed by suspending an individual mouse by the tail on a hook positioned horizontally 50 cm above the tabletop, using adhesive tape placed about 1 cm from the tip of the tail. The tracking information was analyzed by the Depressionscan Package (Clever Sys Inc.), and the immobility time was recorded during the last 4 minutes of a 6-minute session.

## FST

The FST was conducted in a cylinder (height, 45 cm; diameter, 20 cm) containing tap water (25 ± 2℃; depth, 25 cm). As previously described (Zhang et al., 2002), each mouse was introduced into the cylinder to swim freely and the total immobility time was recorded during the last 4 minutes of a 6-minute trial. Similar to the TST, the tracking information was processed by the Depressionscan Package.

## Western Blot

After completion of the behavioral tests, the mice were killed 1 hour after the last behavioral test (i.e., the FST; Figure 1) under anesthesia for collection of blood and tissues. The entire cerebral cortex and hippocampus from hemispheres were homogenized and protein extracts were prepared as previously described (Wang et al., 2014). Expression of PDE4A, PDE4B, PDE4D, CREB, pCREB, Bcl-2, Bax, and BDNF was determined by western blot. The protein signals were visualized and sensitometry analyses conducted using the Image-Pro Plus software version 6.0 (Media Cybernetics Corp, Bethesda, MD).

## ELISA Assay

Brain regions, including the entire cerebral cortex and hippocampus from hemispheres, were homogenized with ice-cold phosphate buffered solution containing 1% phenyl methane sulfonyl fluoride. Lysates were repeatedly thawed and refrozen 3 times, and the supernatant after centrifugation at 5000 × g for 10 minutes was collected for cAMP measurements using ELISA kits following the manufacturer's protocol (Shanghai Bluegene Biotech Co., Ltd., Shanghai, China). Colorimetric reaction was conducted and absorbance at 450 nm was recorded with a multifunctional microplate reader (TECAN). The bicinchoninic acid (BCA) method was used to measure the protein concentrations with the Enhanced BCA Protein Assay kit (Solarbio) according to the manufacturer's instructions.



**Figure 1.** The experimental process and schematic schedule. The mice were administered by gavage with vehicle or roflumilast at the dose of 5 or 10 mg/kg for 30 days. From day 21 to 30 of administration, all mice were subjected to various behavioral tests in the following order: the novel object recognition (NOR), Morris water maze (MWM), tail suspension test (TST), and forced swimming test (FST). The tests were carried out 2 hours after drug administration. One hour after the FST, the mice were killed by decapitation and brains were collected for western blotting, ELISA, and histological staining. i.g., intragastric administration; ELISA, enzyme-linked immunosorbent assay; Rof, roflumilast; SAC, sacrifice.

## Hematoxylin–eosin and Nissl Staining

The fresh brain tissues were washed with saline, followed by fixing overnight in 4% paraformaldehyde. After dehydration, the tissues were embedded in paraffin and then cut into 3-μm sections on a microtome (Leica, Nussloch, Germany) followed by staining with hematoxylin–eosin (HE) or Nissl (toluidine blue) staining. HE staining was carried out as follows: the slide-mounted brain sections were stained in hematoxylin for 3 to 5 minutes, rinsed with dH$_2$O, then decolorized in 1% hydrochloric acid, dehydrated in 85%, 95% alcohol and stained in eosin for 5 minutes. The sections were then dehydrated with gradient alcohol, made transparent in dimethyl benzene, and sealed with neutral resin. Nissl staining was carried out using the following protocol: the slides were stained in Toluidine Blue solution for 3 minutes, rinsed with dH$_2$O, then decolorized in 0.1% glacial acetic acid, cleared in xylene, and sealed with neutral resin. All sections were observed under the microscope (Nikon Eclipse E200).

## TUNEL Assay

To detect apoptotic cells, consecutive sections were performed by TUNEL assay using in situ cell death detection kits (Roche Diagnostics, Mannheim, Germany). Briefly, according to the manufacturer's protocol, the 3-μm paraffin-embedded tissue sections were dewaxed to water, permeabilized with proteinase K for 25 minutes at 37°C, and then washed 3 times in 0.01 M phosphate buffer solution. The sections were incubated in terminal deoxribosyl transferase buffer and fluorescein Isothiocyanate-labeled deoxyuridine triphosphates at 37°C for 2 hours and then stained with 4',6-diamidino-2-phenylindole after 3 additional washes. The fluorescence microscope was used to determine the number of apoptotic cells around the traumatic foci. Three random slides were selected from each group, and 5 randomly selected visual fields from each slide were observed. The apoptosis ratio was recorded for statistical analysis.

## Statistical Analysis

All quantitative data were expressed as the means ± SEM and analyzed using GraphPad Prism version 8 (GraphPad Software Inc., San Diego, CA). Comparisons among multiple groups were performed by 2-way (genotype × treatment) ANOVA followed by post hoc Dunnett's tests, and $P < .05$ was considered statistically significant.

## Results

### Roflumilast Reversed Cognition Deficits in APP/PS1 Mice

To determine learning and memory of mice in response to roflumilast treatment, the NOR and MWM were performed in APP/PS1 mice treated with or without roflumilast. In the NOR, APP/PS1 mice spent markedly less time exploring the novel object than WT mice (F [3, 27] = 6.35, $P < .05$; Figure 2A); this was reversed by roflumilast at 5 and 10 mg/kg orally, especially at the higher dose ($P < .05$; Figure 2A), as demonstrated by posthoc Dunnett's comparisons. Similarly, during the acquisition trial of the MWM, all mice gradually spent less time finding the hidden platform within 4 consecutive days, and the escape latency recorded in APP/PS1 mice was significantly prolonged compared with WT mice from day 2 (F [3, 27] = 6.61, $P < .05$; Figure 2B). Moreover, roflumilast at 10 mg/kg shortened the escape latency

in APP/PS1 mice on day 3 (F [3, 27] = 5.97, $P < .05$; Figure 2B), while at 5 or 10 mg/kg roflumilast significantly shortened the escape latency on day 4 (F [3, 27] = 6.94, $P < .05$) relative to APP/PS1 plus vehicle (Figure 2B, C). During the probe trial, the escape latency was evidently longer (F [3, 27] = 5.86, $P < .01$; Figure 2D) and the number of crossings into the target quadrant was less (F [3, 27] = 3.70, $P < .05$; Figure 2E) in APP/PS1 mice than in WT mice, both of which were reversed by roflumilast at 5 or 10 mg/kg ($P < .05$, Dunnett's tests; Figure 2D, E). However, the swimming distance of all mice was not changed significantly with or without roflumilast (F [3, 27] = 0.10, $P = .96$; Figure 2F), indicating that all mice displayed the same physical status.

### Roflumilast Alleviated Depression-Like Behavior in APP/PS1 Mice

The potential antidepressant-like effect of roflumilast was evaluated by TST and FST. The immobility time was significantly longer in APP/PS1 mice than in WT controls in either the TST (F [3, 27] = 6.70, $P < .05$; Figure 3A) or the FST (F [3, 27] = 5.04, $P < .05$; Figure 3B), which was reversed by roflumilast at the 10-mg/kg dose ($P < .05$, Dunnett's tests; Figure 3A, B).

### Roflumilast Attenuated Neuronal Cell Injuries and Apoptosis in APP/PS1 Mice

Neuronal damage in the cerebral cortex of APP/PS1 mice was shown by HE and Nissl staining (Figure 4). Several distorted neurons, which were shrunken or swollen, were observed in the neuropil of APP/PS1 mice (Figure 4B, F) compared with WT mice (Figure 4A, E). Most of the neurons were dark in appearance, lacking clear nuclei. The cytoplasm of neurons in many cases was condensed and showed vacuolization along with chromatolysis (Figure 4B, F). In contrast, in the brains of roflumilast-treated mice, little cytoplasmic swelling and vacuolation were detected; nuclear concentrations and abnormal nerve cells were also significantly reduced (Figure 4C, D and G, H). In the TUNEL assay of the cerebral cortex, APP/PS1 mice displayed significantly higher levels of apoptosis compared with the control (F [3, 6] = 10.49, $P < .01$; Figure 5B, C). Roflumilast at the same doses resulted in significant decreases in the percentage of TUNEL-positive cells ($P < .01$, Dunnett's tests; Figure 5B, C).

### Roflumilast Increased the Ratio of Bcl-2/Bax in APP/PS1 Mice

To further clarify the intrinsic molecular mechanism of pathological lesion, the Bcl family proteins were detected by western blotting in the brain tissues. For the apoptosis inhibitory protein Bcl-2, a significant downregulation was observed in the cerebral cortex of APP/PS1 mice compared with WT mice (F [3, 15] = 5.19, $P < .05$; Figure 6A, B); this was attenuated by roflumilast at 10 mg/kg, but it was statistically significant. A similar tendency to Bcl-2 changes was observed in the hippocampus, but it was not significantly different (Figure 6A, B). The apoptosis-promoting protein Bax was upregulated in the hippocampus and cerebral cortex of APP/PS1 mice compared with WT mice (F [3, 15] = 3.79, $P < .05$ and F [3, 15] = 5.74, $P < .05$, respectively; Fig. 6A, C); these were reversed by roflumilast at 5 and/or 10 mg/kg ($P < .05$, Dunnett's tests). The ratio of Bcl-2/Bax, the most important index of apoptosis, in the hippocampus and cerebral cortex of APP/PS1 mice was decreased significantly compared with WT mice (F [3, 15] = 15.30, $P < .05$ and F [3, 15] = 8.37, $P < .05$, respectively; Figure 6D); this was reversed by treatment of roflumilast at 5 or 10 mg/kg ($P < .05$, Dunnett's tests; Figure 6D).
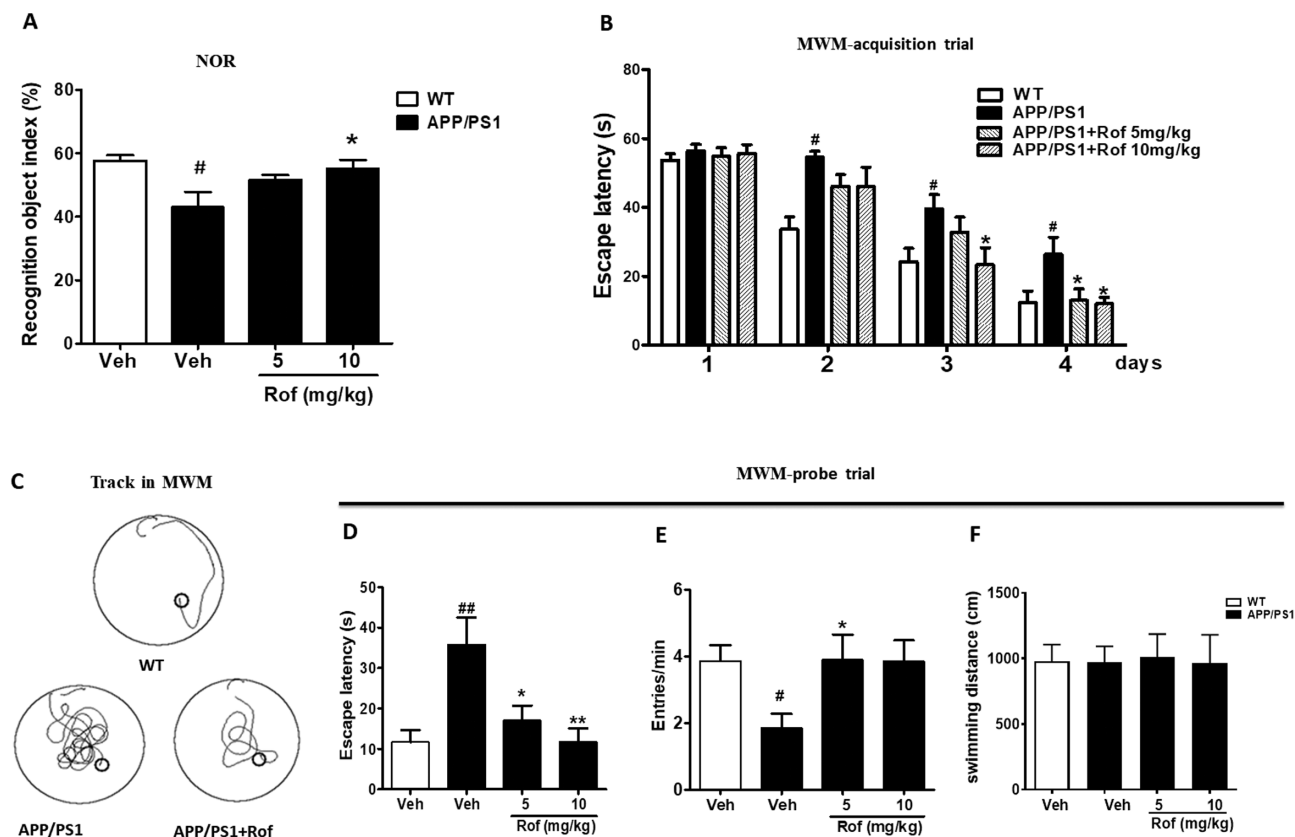
**Figure 2.** Roflumilast reversed the cognition deficits in APP/PS1 mice. Learning and memory of mice were assessed using the NOR and MWM tests. In the NOR, the time exploring each object was recorded and the recognition object index was calculated as the percentage of the time exploring the novel object over the total time exploring both objects (A). In the acquisition trial of MWM, all mice were tested for 4 consecutive days to locate the hidden platform; the escape latency (B) and swimming paths (C) were recorded. During the probe trial, the platform was removed, and the escape latency, which is the time entering the previous platform area (target quadrant) for the first time (D), entries into the target quadrant (E) and the swimming distance (F) in mice were recorded. Values shown represent the means ± SEM. [#]$P < .05$, [##]$P < .01$ vs WT plus vehicle; [*]$P < .05$, [**]$P < .01$ vs APP/PS1 plus vehicle; n = 10.
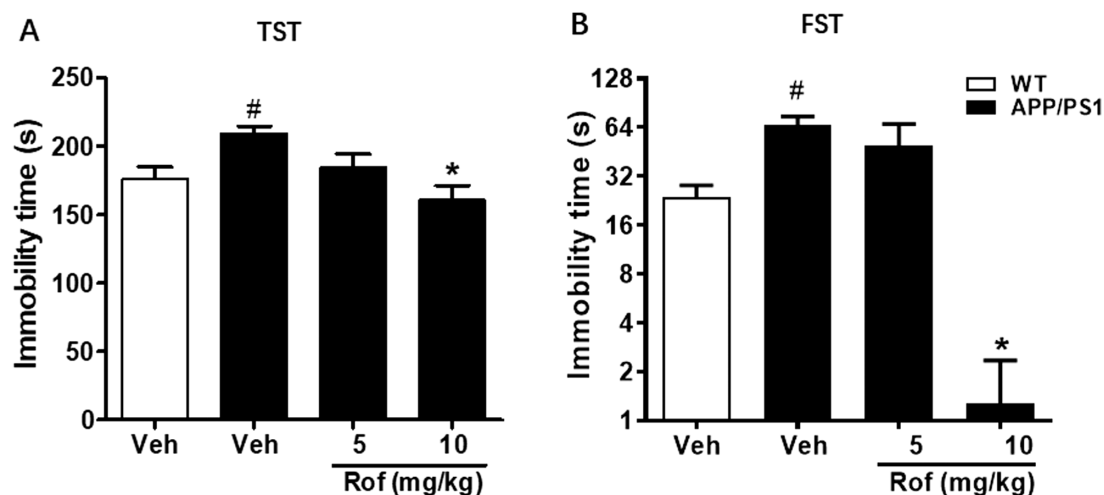


**Figure 3.** Roflumilast alleviated depression-like behavior in APP/PS1 mice. The tail suspension test (TST) (A) and forced swimming test (FST) (B) were performed for assessing depression-like behavior of mice. In both experiments, the total immobility time was recorded during the last 4 minutes of the 6-minute session. Data shown represent the means ± SEM. [#]$P < .05$ vs WT plus vehicle; [*]$P < .05$ vs APP/PS1 plus vehicle; n = 10.

## Roflumilast Reduced Expression of PDE4 Subtypes in APP/PS1 Mice

To identify the mechanism mediating the effect of roflumilast on behavior, the PDE4 subtype proteins were measured by western blotting. The results showed that the expression of PDE4B and PDE4D was significantly increased in the cerebral cortex of APP/PS1 mice compared with WT mice (F [3, 15] = 13.65, $P < .05$ for PDE4B and F [3, 15] = 9.90, $P < .05$ for PDE4D; Figure 7A and C, D); these were reversed by roflumilast at 5 or 10 mg/kg (for both $P < .01$, Dunnett's
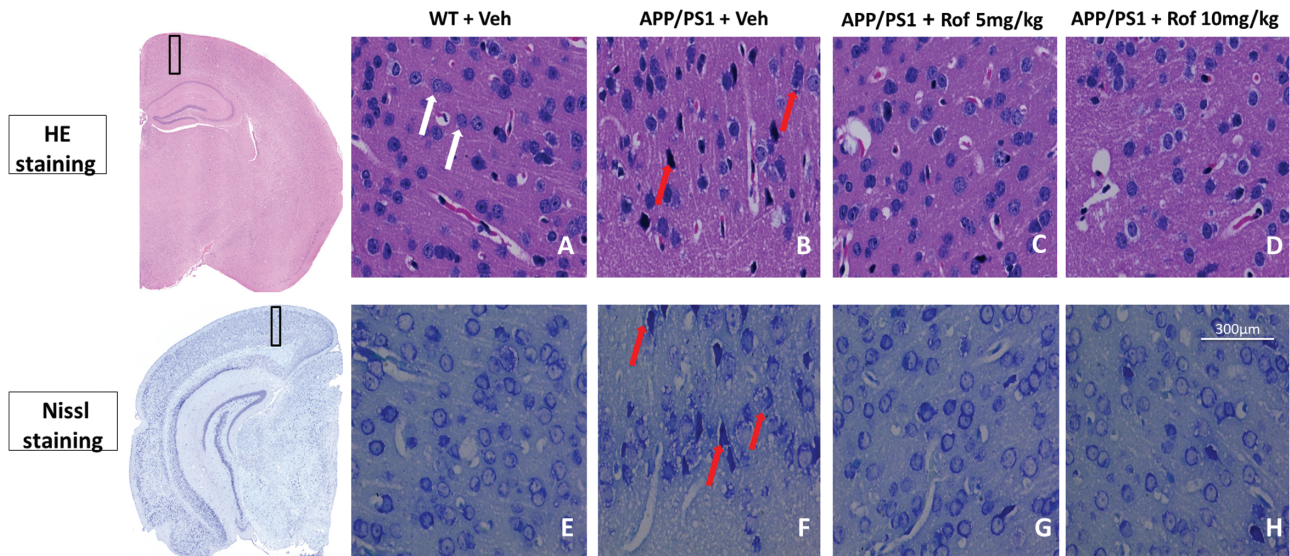
**Figure 4.** Roflumilast attenuated neuronal cell injuries in the brain of APP/PS1 mice. Neuronal injuries of the cerebral cortex were examined by HE and Nissl staining (upper and lower rows of panels, respectively) after exposure to Rof (5 or 10 mg/kg). The neurons in WT mice appeared regularly spaced within the layers with stained cytoplasm and nuclei located approximately at the center of the soma (white arrows). Damaged neurons in APP/PS1mice were darkly stained and exhibited shrunken and triangulated neuronal bodies, nucleus condensation, and cytoplasmic swelling (red arrows); n = 3.

tests). The expression of PDE4B and PDE4D was increased in the hippocampus of APP/PS1 mice, but it was not statistically significant compared with WT. Nevertheless, administration of roflumilast at 5 or 10 mg/kg still decreased both PDE4B (F [3, 15] = 8.96, $P < .05$; Figure A, C) and PDE4D (F [3, 15] = 10.03, $P < .05$; Figure 7A, D). In contrast, there was no significant change in PDE4A expression in any of the brain regions of APP/PS1 mice, regardless of the absence or presence of roflumilast (F [3, 15] = 2.20, $P = .13$ for the cortex and F [3, 15] = 3.03, $P = .06$ for the hippocampus; Figure 7A, B).

### The Role of Roflumilast in cAMP/CREB/BDNF Signaling in APP/PS1 Mice

PDE4 inhibitors produce pharmacological effects primarily via cAMP/CREB/BDNF signaling (Hansen and Zhang, 2013, 2017). Therefore, we investigated these signal molecules in the mouse brain. Consistent with increased PDE4 expression, cAMP levels determined by ELISA were significantly reduced in both the hippocampus and the cerebral cortex in APP/PS1 mice (F [3, 15] = 3.591, $P < .01$ and F [3, 15] = 4.526, $P < .05$, respectively; Figure 8A). In parallel with decreased PDE4 expression in APP/PS1 mice treated with roflumilast, the decreased levels of cAMP in APP/PS1 mice were reversed following i.g. administration of roflumilast (10 mg/kg) ($P < .01$ for the hippocampus or $P < .05$ for the cortex; Dunnett's tests; Figure 8A).

A similar pattern of changes in pCREB and BDNF was also observed. The decreased expression of pCREB and BDNF was revealed in the hippocampus (F [3, 15] = 11.37, $P < .05$ for pCREB, Figure 8B, C; and F [3, 15] = 7.77, $P < .05$ for BDNF, Figure 8D, E) and the cerebral cortex (F [3, 15] = 10.48, $P < .01$ for pCREB, Figure 8B, C; and F [3, 15] = 12.67, $P < .01$ for BDNF, Figure 8D, E) in APP/PS1 mice; these were also reversed by roflumilast at 5 or 10 mg/kg ($P < .05$ or $P < .01$; Dunnett's tests; Figure 8B–E), suggesting that roflumilast reduced PDE4B and PDE4D, leading to increases in cAMP, pCREB, and BDNF and subsequently improving behavioral and neuropathological impairments in AD.

## Discussion

Depression, the most common comorbidity of AD, is partly responsible for the predicament of treatment and caregiving in AD patients (Chi et al., 2014; Romano et al., 2014). The development of an effective treatment for AD with the comorbidity of depression is in high demand. Our study demonstrated that roflumilast enhanced cognition and reversed depression-like behavior in APP/PS1 mice by inhibiting PDE4B and PDE4D and subsequently regulating cAMP downstream molecules. This suggests the clinical potential of roflumilast for the comorbidity of AD and depression.

Aβ is the major component of senile plaques (SP). Regardless of the suspicion for the Aβ hypothesis owing to the clinical failures of anti-AD candidates (such as β- and γ-secretase inhibitors) targeting Aβ production, the neurotoxicity of Aβ is undoubtedly involved in AD (Sun et al., 2018). Therefore, it is reasonable to use APP/PS1 mice to evaluate potential anti-AD agents. As reported elsewhere (Jin et al., 2014; Fidelis et al., 2019), the current study also revealed that APP/PS1 mice at 10 months of age exhibit obvious impairment of cognition and simultaneously display depression-like behavior, suggesting that APP/PS1 transgenic mice can be an ideal model for the comorbidity of AD with depression.

The relationship between depression and AD is still not well understood, but it is true that a high prevalence of depression occurs in patients with AD. Consequently, there is a great search for drugs that improve both cognition and depressive behavior, and PDE4 inhibitors can be considered the most promising drugs. The classic PDE4 inhibitor rolipram ameliorates cognitive dysfunction and depression-like behavior in various animal models, and earlier clinical studies demonstrated moderate antidepressant effects of rolipram treatment (O'Donnell and Zhang, 2004; Zhang, 2009). However, the emetic effect of rolipram and other related PDE4 inhibitors has significantly hampered their clinical application (Robichaud et al., 2001; Wu et al., 2018). Roflumilast, which is the second generation of PDE4 inhibitors, has reduced emetic effects compared with rolipram, attracting a particular interest regarding its neuroprotective
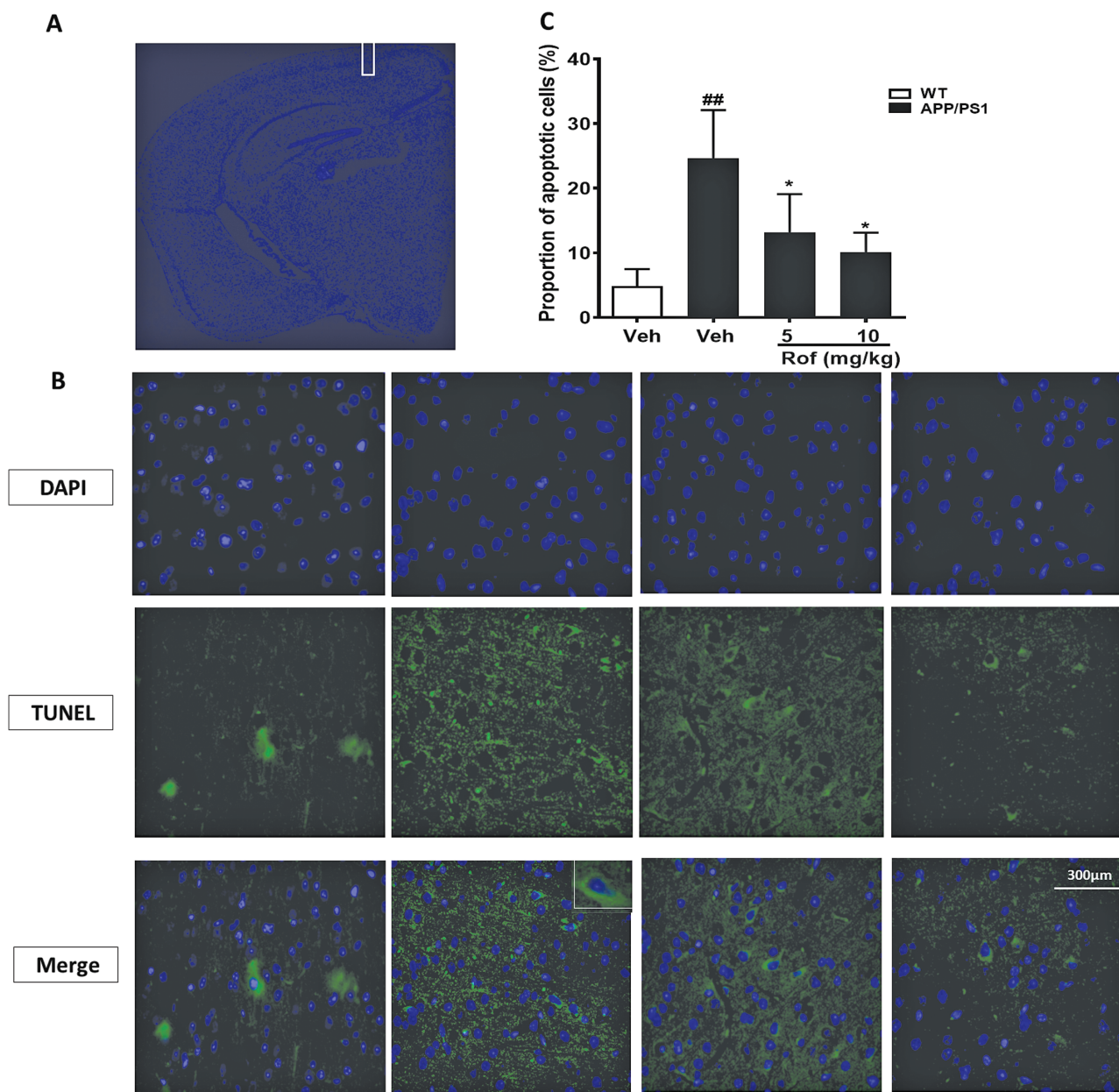
**Figure 5.** Roflumilast decreased cell apoptosis in the brain of APP/PS1 mice. (A, B) Cell apoptosis of the cerebral cortex by TUNEL staining of paraffin sections. Fluorescence colors: DAPI: blue; TUNEL: green. (C) Quantification of apoptotic cells affected by roflumilast (Rof, 5 and 10 mg/kg). The values shown are means ± SEM; $^{\#\#}P < .01$ vs WT plus vehicle; $^{*}P < .05$ vs APP/PS1 plus vehicle; n = 3.

effect. Recent demonstrations indicate that roflumilast, alone or in combination with PDE5 inhibitors such as vardenafil, reverses cognitive impairment induced by various methods (Gulisano et al., 2018). Evidence has also pointed out that roflumilast offers a more favorable window for treatment of cognitive deficits compared with rolipram (Jabaris et al., 2015a, 2015b; Vanmierlo et al., 2016). Moreover, a recent study has shown that roflumilast significantly ameliorates cognitive impairment in APP/PS1 mice (Feng et al., 2019), which is consistent with our study. Our study further demonstrated that roflumilast ameliorated the depression-like status in APP/PS1 mice, which has been rarely reported elsewhere, suggesting more favorable potential for AD treatment.

Given that PDE4 inhibitors do not affect the levels of Aβ or the SP, we simply focused on HE and Nissl staining for nerve damage in brain slices here, although SPs are reported to appear in the brain of APP/PS1 mice at 10 months of age. Consistent with behavioral improvement, roflumilast also relieved the neuronal damage and decreased cell apoptosis in the cerebral cortex of APP/PS1 mice. Bcl-2 family members form heterodimers or homodimers and act as regulators of survival or neuronal death following Aβ insult. Bcl-2 can form a dimer with apoptotic Bax. If the relative amount of Bax is higher than Bcl-2, cell death is facilitated. Studies have confirmed that PDE4 inhibitors such as rolipram regulate the expression of Bcl-2 and Bax to play an anti-apoptotic role (Wang et al., 2012). In this study, we found that roflumilast increased the ratio of Bcl-2/Bax in the brain of APP/PS1 mice, which is supported by a recent study (Peng et al., 2018). These results suggest that the cognition-enhancing effect of roflumilast in APP/PS1 mice may
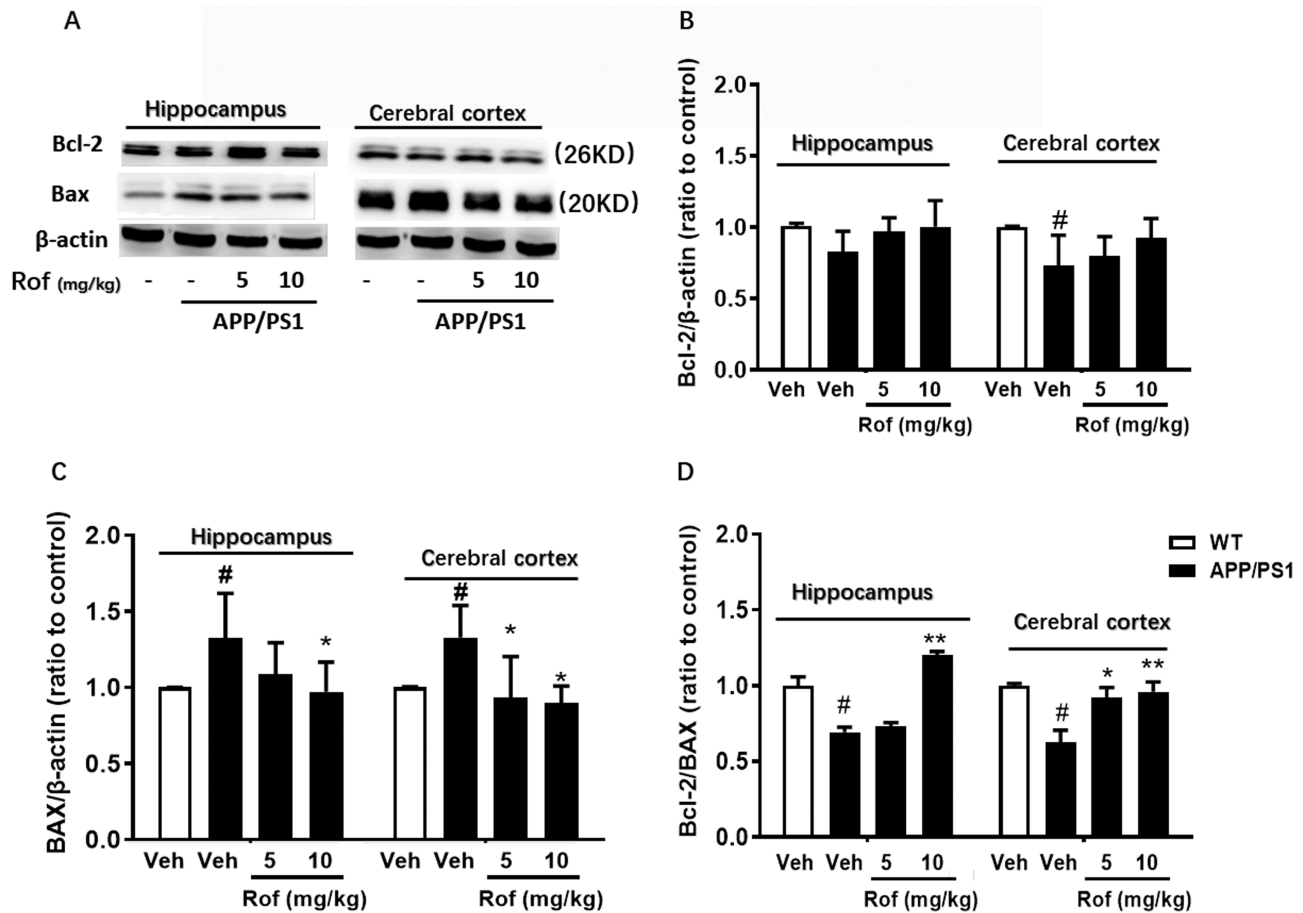
**Figure 6.** Roflumilast increased the ratio of Bcl-2/Bax in the brain of APP/PS1 mice. (A) Representative images by western blotting showing the expression of Bcl-2 and Bax in the hippocampus and cerebral cortex in WT and APP/PS1 mice treated with vehicle or Rof (5 or 10 mg/kg). (B–D) Quantitative analysis of Bcl-2, Bax, and the ratio of Bcl2/Bax. The values shown are means ± SEM, normalized by β-actin to WT mice treated with vehicle. $^{#}P<.05$ vs WT plus vehicle; $^{*}P<.05$, $^{**}P<.01$ vs APP/PS1 plus vehicle; n=6.

be related to its anti-apoptotic activity via modulation of the Bcl-2 family.

The PDE4 family consists of 4 subtypes (PDE4A-D) encoded by 4 distinct genes, which, except for PDE4C, are all highly but region-specifically expressed in the brain (Perez-Torres et al., 2000). PDE4 in the brain is involved in various functions, including depression (Zhang et al., 2002; Wang et al., 2015b), anxiety (Zhang et al., 2008; Li et al., 2009; Hansen et al., 2014), memory (Zhang et al., 2000; Hansen and Zhang, 2013; Vanmierlo et al., 2016; Blokland et al., 2019), and schizophrenia (Gilleen et al., 2018). These may be mediated by different PDE4 subtypes. For instance, inhibition of PDE4B has better antidepressant-like activity, while inhibition, knockdown, or knockout of PDE4D produces memory-enhancing and antidepressant-like effects (Li et al., 2011; Wang et al., 2015a; Ricciarelli et al., 2017; Zhang et al., 2002, 2008; Zhang et al., 2017). Roflumilast is a selective PDE4 inhibitor, which inhibits all the PDE4 subtypes to a similar extent. However, although roflumilast has neuroprotective effects, it is not clear if 1 or more PDE4 subtypes in the brain are involved. Here we examined all 3 PDE4 subtypes (PDE4A, PDE4B, and PDE4D) that are highly expressed in the brain to further clarify the innermost mechanism by which roflumilast improved the comorbidity. We found that the expression of PDE4B and PDE4D in the cerebral cortex and hippocampus of APP/PS1 mice was increased, with more significant changes in the cerebral cortex. In either brain region, the changes in PDE4 subtype (PDE4B and

PDE4D) expression were reversed by roflumilast. The specific responses of PDE4B and PDE4D to APP/PS1 transgene and treatment of roflumilast are consistent with our recent studies, in which PDE4D inhibitors enhanced memory while PDE4B inhibitors produced antidepressant-like effects (Zhang et al., 2017, 2018; Gurney et al., 2019). No changes in PDE4A were found in either the cerebral cortex or the hippocampus regardless of the presence of roflumilast. This indicates that PDE4A may not be the major PDE4 involved in the comorbidity of AD and depression or the beneficial effects of roflumilast.

While roflumilast simply inhibits PDE4 activity, we analyzed the brain expression of PDE4 subtypes in the present study. This was based on 3 considerations. First, chronic PDE4 inhibitors such as rolipram differentially alter the expression of PDE4 subtypes in the brain of normal mice (Dlaboga et al., 2006). Second, APP/PS1 transgenic mice displayed increases in expression of PDE4 subtypes, especially PDE4B and PDE4D in the cerebral cortex (Figure 7). Third, antidepressant activity and memory enhancement are primarily contributed by inhibition of PDE4B and PDE4D, respectively (Zhang et al., 2017), although PDE4D is involved in both behavioral effects (Zhang et al., 2002; Li et al., 2011; Wang et al., 2015a; Ricciarelli et al., 2017). Consistent with these findings, our results suggest that roflumilast attenuates depression-like behavior and memory deficit in AD mice via downregulation of PDE4B and PDE4D, respectively. Therefore, measuring the expression of PDE4 subtypes helped
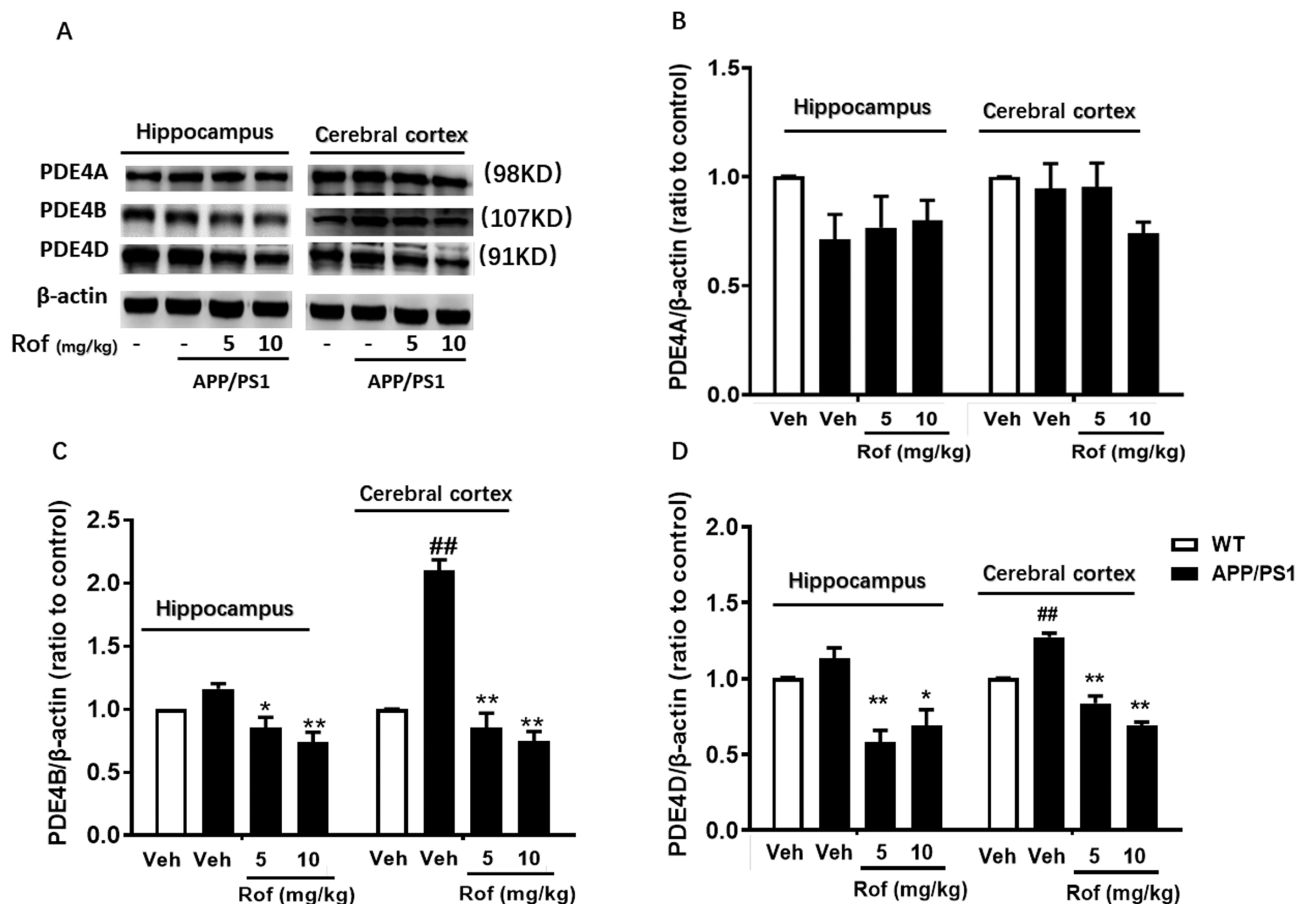
**Figure 7.** Roflumilast reduced the expression of PDE4 in the brain of APP/PS1 mice. (A) Representative images by western blotting for the expression of PDE4A, PDE4B, and PDE4D in the hippocampus and cerebral cortex of WT or APP/PS1 mice treated with vehicle or Rof (5 or 10 mg/kg). (B–D) Quantitative analysis of PDE4A, PDE4B, and PDE4D. The values shown are means ± SEM, normalized by β-actin to WT mice treated with vehicle. $^{\#\#}P<.01$ vs WT plus vehicle; $^{*}P<.05$, $^{**}P<.01$ vs APP/PS1 plus vehicle; n = 6.

us understand which PDE4 subtypes are involved in the unique effects of roflumilast on the comorbidity of AD and depression.

It was noted that the dose (10 mg/kg) of roflumilast for producing the antidepressant-like effect was much higher than that (5 mg/kg) for enhancing learning and memory. Since the oral emetic doses of roflumilast in mice and rats could be extrapolated at 9 to 30 mg/kg (Kobayashi et al., 2011; Vanmierlo et al., 2016), the dose of roflumilast for an antidepressant action appears to reach a therapeutic limit. Given that inhibition of PDE4B and PDE4D predominantly results in antidepressant activity and memory enhancement, respectively, as described above, and that roflumilast improves memory at much lower doses (Vanmierlo et al., 2016), it is reasonable to believe that roflumilast may produce antidepressant activity primarily via inhibition of PDE4B. While roflumilast is assumed to inhibit all PDE4 subtypes similarly, it is possible that PDE4B needs a higher dose of roflumilast to influence affective behavior compared with the dose for improving memory, which is more likely via PDE4D. The near-therapeutic-limit dose of roflumilast for antidepressant activity could limit the clinical usefulness of roflumilast to target both memory and affect at the same time in AD.

It is not clear what caused the difference in the effective doses between the present and other studies (Vanmierlo et al., 2016; Feng et al., 2019). A similar high dose (10 mg/kg) of roflumilast appears to be required as well for reducing alcohol consumption

in mice in our recent study (Liu et al., 2017). The discrepancies in response to roflumilast treatment may be contributed by animal models and the age, genetic background, or even cohorts of AD mice. Further studies are needed to clarify this.

Of note, we did not include WT mice treated with the 2 doses of roflumilast given that there are many reports on pharmacological profiles of roflumilast in normal mice (Suzuki et al., 2013; Vanmierlo et al., 2016; Liu et al., 2017); instead, we focused on the potential of anti-AD effects of roflumilast using AD mice in order to keep the study more innovative.

It is well demonstrated that the inhibition of PDE4 reduces the degradation of cAMP, leading to activation of the cAMP/protein kinase A/CREB signaling pathway. We then investigated the downstream molecules of this cAMP pathway and demonstrated that roflumilast reversed the decreases in cAMP and pCREB in the cerebral cortex and hippocampus of APP/PS1 mice. This is supported by the recent studies using APP/PS1 mice (Feng et al., 2019) and deoxycorticosterone acetate salt hypertensive rats (Jabaris et al., 2015b). However, it has been noted that the PDE4D inhibitor GEBR-7b improves cognition of APP/PS1 mice without restoring CREB phosphorylation (Sierksma et al., 2014). While it is not clear what causes the different responses of PDE4 inhibitors to pCREB, the dose and duration of the use of the PDE4 inhibitors and simultaneous inhibition of both PDE4B and PDE4D by roflumilast may contribute to the discrepancy. This needs to be further studied for clarification.
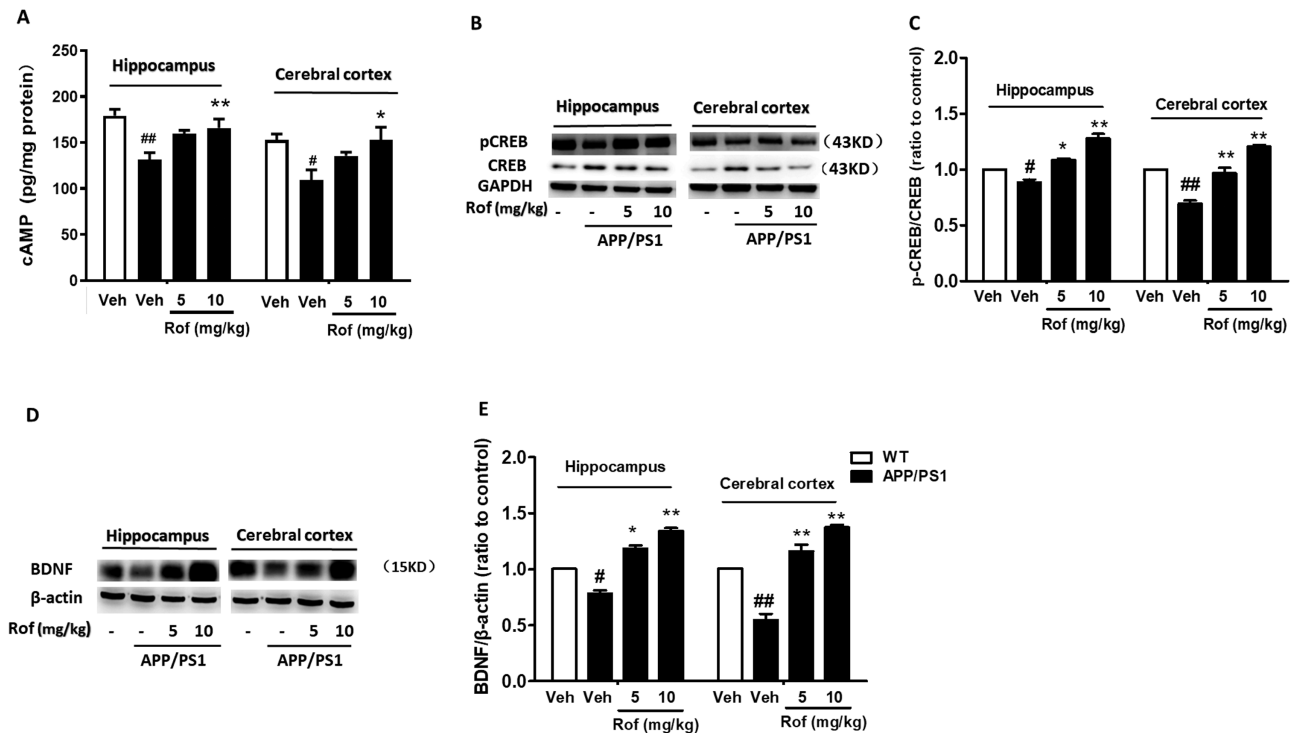
**Figure 8.** The effects of roflumilast on cAMP, pCREB/CREB, and BDNF in the brain of APP/PS1 mice. (A) Effect of Rof (5 or 10 mg/kg) on cAMP levels measured by ELISA in the hippocampus and cerebral cortex of APP/PS1 mice. (B, C) Representative images by Western blotting (B) and quantitative analysis (C) of pCREB and CREB in the hippocampus and cerebral cortex of WT and APP/PS1 mice treated with vehicle or Rof. (D, E) Representative images by western blotting (D) and quantitative analysis (E) of BDNF in the hippocampus and cerebral cortex of WT and APP/PS1 mice treated with vehicle or Rof. The values shown are means ± SEM, normalized by β-actin and expressed as the ratio of pCREB/CREB (for C) to WT mice treated with vehicle (for C and E). $^{\#}P<.05$, $^{\#\#}P<.01$ vs WT plus vehicle; $^{*}P<.05$, $^{**}P<.01$ vs APP/PS1 plus vehicle; n=6.
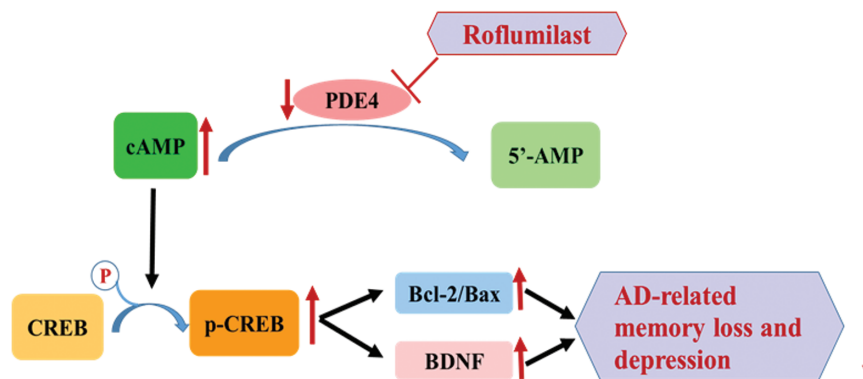


**Figure 9.** The possible mechanisms whereby roflumilast reverses AD-related memory loss and depression. Roflumilast not only improves learning and memory but also attenuates depression-like behavior in AD mice, most likely via the PDE4-mediated cAMP/CREB/BDNF signaling pathway, in which Bcl-2/Bax may also be involved. More specifically, roflumilast inhibits PDE4, in particular PDE4B and PDE4D, leading to accumulation of cAMP and subsequent increases in pCREB, BDNF, and Bcl2/Bax in the brain. Therefore, roflumilast can be a therapeutic agent for AD, in particular the comorbidity of memory deficits and depression.

CREB regulates the transcription of downstream signaling molecules, such as BDNF and the Bcl-2 gene family. BDNF is recognized as a key mediator of brain function, including neurodevelopment, synaptic structure, neurotransmitter release, and learning and memory (Jabaris et al., 2015b; Sartor et al., 2018). Roflumilast reversed the decreased expression of BDNF after restoring pCREB in APP/PS1 mice. The Bcl-2 gene family is an apoptosis regulator, and the effect of roflumilast on Bcl-2 and Bax may also be a downstream event of pCREB. In addition, the downstream targets of pCREB also include inflammatory cytokines, such as interleukin-1, interleukin-6, and nuclear factor kappa-B (Wang et al., 2012; Hansen and Zhang, 2013). Recent studies have demonstrated that roflumilast reduces inflammatory cytokines and exhibits a neuroprotective role via cAMP/CREB signaling (Feng et al., 2019). This is consistent with our previous finding (Wang et al., 2012). These results suggest that roflumilast may improve cognition and produce antidepressant-like behavior through a variety of signaling mechanisms after inhibition of PDE4. Nevertheless, other intracellular mechanisms that may be involved in behavioral consequences remain to be further investigated.

## Conclusion

Taken together, we provided solid demonstration that roflumilast not only improves learning and memory but also attenuates depression-like behavior in APP/PS1 mice, most likely via

PDE4D/4B-mediated cAMP/CREB/BDNF signaling. This signaling pathway mediates the neuroprotective, anti-apoptotic, and anti-inflammatory effects of roflumilast, which also contribute to its behavioral benefits. Therefore, roflumilast can be a therapeutic agent for AD, in particular the comorbidity of memory deficits and depression (Figure 9), although the potential side effect of emesis can still be a concern as a high dose appears to be required. Further studies are needed to clarify this issue.

## Acknowledgments

## Statement of Interest

Han-Ting Zhang is currently an adjunct professor of the Institute of Pharmacology, Shandong First Medical University. The other authors do not have conflicts of interest to disclose.

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