

# FTY720 Attenuates 6-OHDA-Associated Dopaminergic Degeneration in Cellular and Mouse Parkinsonian Models

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**Abstract** FTY720 (fingolimod) is the first oral drug approved for treating relapsing-remitting forms of multiple sclerosis. It is also protective in other neurological models including ischemia, Alzheimer's disease, Huntington disease and Rett syndrome. However, whether it might protect in a 6-hydroxydopamine (6-OHDA) mouse model associated with the dopaminergic pathology of Parkinson's disease (PD), has not been explored. Therefore, in the present study, we investigated the effects of FTY720 on 6-OHDA-induced neurotoxicity in cell cultures and mice. Here we show that FTY720 protected against 6-OHDA cytotoxicity and apoptosis in SH-SY5Y cells. We also show that prior administration of FTY720 to 6-OHDA lesioned mice ameliorated both motor deficits and nigral dopaminergic neurotoxicity, while also reducing 6-OHDA-associated inflammation. The protective effects of FTY720 were associated with activation of AKT and ERK1/2 pro-survival pathways and an increase in brain derived neurotrophic factor (BDNF) expression *in vitro* and *in vivo*. These findings suggest that FTY720 holds promise as a PD therapeutic acting, at least in part, through AKT/ERK1/2/P-CREB-associated BDNF expression.

**Keywords** FTY720 · Parkinson's disease · 6-OHDA · BDNF · Apoptosis

## Introduction

Parkinson's disease (PD) is the most common neurodegenerative disease after Alzheimer's disease (AD), and is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) prior to motor symptom onset. Currently, most treatments for PD focus on the symptomatic treatment of motor symptoms related to SNc loss. Still, the development of new therapeutic approaches that can slow or halt PD progression remains a major goal of translational medicine.

FTY720 (fingolimod), when phosphorylated, is a structural analog of sphingosine-1-phosphate (S1P) and has been approved by the Food and Drug Administration (FDA) for treating relapsing-remitting multiple sclerosis (MS) [1, 2]. FTY720-P then acts as an immunomodulator; however, in the central nervous system (CNS) it appears that the drug has additional neuroprotective effects [3]. For instance, a recent study has shown that FTY720 increases BDNF levels and significantly prolongs survival in a mouse model of Rett syndrome [4]. FTY720 has also been shown to be neuroprotective in models of Huntington's disease [5], suggesting a potential therapeutic benefit for FTY720 in the treatment of brain disorders other than MS. It was also reported recently that S1P itself can enhance mitochondrial function in dopaminergic neurons in a mouse PD model, as well as in MPP<sup>+</sup>-treated MN9D cells [6]. We recently showed that FTY720 protects against neuroinflammatory cell death associated with tumor-necrosis-factor- $\alpha$  toxicity in dopaminergic cells and that it also reduces synucleinopathy and improves function in A53T parkinsonian mice

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[7, 8]. Together, these data strongly suggest that FTY720 is highly protective *in vivo*. Thus, in the present study, we investigated whether FTY720 was effective in protecting mice lesioned with 6-hydroxydopamine (6-OHDA) and if so, how that may occur. Our findings revealed that FTY720 attenuated 6-OHDA-induced neurotoxicity of SH-SY5Y cells as well as in 6-OHDA lesioned C57BL/6 mice. Moreover, the beneficial effect of FTY720 appears to be associated with an activation of both the AKT and ERK1/2 pro-survival signaling pathways, with a subsequent increase in BDNF expression.

## Materials and Methods

### Reagents and Chemicals

FTY720 was purchased from Cayman Chemical (Cayman Chemical Company, Ann Arbor, MI, USA). The toxin, 6-OHDA-HCl was purchased from Sigma (Sigma, St Louis, USA). All chemicals were dissolved according to the manufacturer's instructions. All cell culture reagents were purchased from Gibco if not otherwise indicated (Life Technologies, Madrid, Spain).

### Cell Death/viability Assessment and Flow Cytometry Analysis

Human neuroblastoma SH-SY5Y cells were grown in DMEM-F12 (Invitrogen) supplemented with 10% fetal calf serum. To assess the impact of FTY720 alone on cell viability, we first incubated SH-SY5Y cells with various concentrations of FTY720 (0.5, 1, 2 and 4  $\mu\text{M}$ ) and cell viability were measured 24 h later by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. We found that FTY720 alone (0.5–4  $\mu\text{M}$ ) did not affect cell viability and cell morphology. Then cells were pretreated with FTY720 at the indicated concentrations (0.5, 1, 2 and 4  $\mu\text{M}$ ) for 12 h before being exposed to 100  $\mu\text{M}$  6-OHDA for an additional 24 h. Cell viability was measured by MTT assay and 6-OHDA-induced cell death was quantified by measuring lactate dehydrogenase (LDH) release from damaged cells into the culture medium according to the manufacturer's instruction (Roche Diagnostic, Indianapolis, IN, USA).

6-OHDA-induced cell apoptosis was detected with an Annexin V-FITC/ Propidium iodide (PI) double staining Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instruction. Briefly, cells were treated with FTY720 (2 and 4  $\mu\text{M}$ ) for 12 h before being exposed to 100  $\mu\text{M}$  6-OHDA for an additional 24 h, and then  $1 \times 10^6$  cells were harvested, washed twice with ice-cold PBS, and evaluated for apoptosis by double staining with Annexin

V-FITC and PI in binding buffer using a Cytoflex flow cytometer (Beckman Coulter life sciences, USA).

### TUNEL Staining

Cells were treated with different concentrations of FTY720 for 12 h before being exposed to 100  $\mu\text{M}$  6-OHDA for an additional 24 h. Apoptotic cells were detected by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining using an *in situ* cell death detection kit (Cat. No. 11684817910, Roche Diagnostic) according to the manufacturer's protocol. Briefly, the slides were rinsed with PBS and fixed with fixation solution for 1 h at 4°C, washed with PBS for 30 min, and then incubated in blocking solution for 15 min. After incubation in permeabilization solution, the slides were further incubated in TUNEL reaction mixture for 1 h at 37°C; samples were then added Converter-POD for 30 min at 37°C before being rinsed with PBS. The samples were stained with diaminobezidine (DAB) and then observed by bright field microscopy. The percentage of apoptotic cells was calculated by counting approximately 500 cells.

### Western Blot Analysis

Protein extracts were prepared from SH-SY5Y cells and mouse striatum by homogenization in RIPA buffer with protease inhibitors and phosphatases inhibitors. Protein concentration was determined by bicinchoninic acid (BCA) method. The primary antibodies used in this study included: BDNF, P-ERK1/2, total ERK1/2, P-AKT, total AKT, P-CREB, total CREB, GAPDH and  $\beta$ -actin (Cell Signaling Technology, Beverly, MA). The blots were re-probed with  $\beta$ -actin or GAPDH as the loading control. Densitometry analyses were performed using Image J software [9].

### Animal Surgery

All animal experiments were approved by Institutional Animal Care and Use committee of Shandong University and performed in accordance with the National institute of health guide for the care and use of Laboratory animals. Mice were maintained in a pathogen free facility and exposed to a 12-h light/dark cycle with food and water provided *ad libitum*. Ten-week-old male C57BL/6 mice were treated with 0.5 mg/kg FTY720 or vehicle by intraperitoneal injection for 7 days prior to lesioning. On the 7th day of treatment, 1 h after final dosing of FTY720, mice were placed in a stereotaxic device under anesthesia. We injected 6  $\mu\text{g}$  of 6-OHDA (in 2  $\mu\text{l}$  of normal saline with 0.02% ascorbic acid) or saline alone into two different sites of the right striatum using the following coordinates

from Bregma: AP+1.0 mm, ML +/- 2.1 mm, and DV -2.9 mm; as well as AP+0.3 mm, ML +/- 2.3 mm, and DV -2.9 mm as previously described [10]. Mice were sacrificed at different time points following 6-OHDA injection for biochemical or histological assessment.

### Apomorphine-induced Rotation Test

Apomorphine-induced rotations were monitored over 3 weeks' time, starting from 1 week post 6-OHDA lesioning according to previous published protocols [11]. Apomorphine was subcutaneously injected into mice at a dose of 0.1 mg/kg (Sigma), with mice placed individually in plastic beakers (diameter: 13 cm), and videotaped from above for 30 min. Quantitative analyses of completed (360°) left and right rotations were made off-line by an investigator blinded to the experimental conditions.

### Immunohistochemistry

Animals were anesthetized with sodium pentobarbital at 3 weeks after 6-OHDA administration, transcardially perfused with 0.9% normal saline, followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were dissected out, postfixed in 4% paraformaldehyde for 24 h, and cryopreserved in 30% sucrose for 48 h. Frozen brains were then coronally sectioned at 25 µM thickness on a cryomicrotome and sections were mounted on slides. Immunohistochemistry staining was performed on free floating sections using antibody raised against tyrosine hydroxylase (TH) (Chemicon) followed by biotinylated secondary antibody and streptavidin ABC solution (Vector Laboratories). Immunostaining was visualized after DAB staining (Vector Laboratories) using bright field microscopy (Olympus). For immunofluorescence staining, sections were first incubated with anti-GFAP or CD11b antibody at 4°C overnight, followed by incubation with Alexa Fluor-568-conjugated secondary antibody at room temperature for 2 h.

Assessment of the total number of TH<sup>+</sup> cells in the SNc was made according to the optical fractionator probe (MicroBrightfield) by an investigator blinded to the experimental design. Every fifth section covering the entire extent of these regions was included in the counting procedure. The data were expressed as a percentage of the corresponding area from the noninjected, intact side.

### Measurement of Striatal DA and its Metabolites

The striatal dopamine (DA) and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured using a highly sensitive High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method as previously described [12].

Briefly, the striatal tissues were removed at 3 weeks following 6-OHDA administration; tissue samples were individually weighed and homogenized in ice-cold 0.5 M formic acid with the concentration of 5 ml/g tissue. Lysates were centrifuged 15,000×g for 30 min at 4°C. The supernatant was separated and analyzed according to the established protocol.

### Statistical Analysis

Statistical significance between multiple groups was examined by one-way ANOVA followed by Tukey post hoc testing. All data are expressed as the mean ± SEM with  $p < 0.05$  considered statistically significant.

## Results

### FTY720 Protects SH-SY5Y Cells from 6-OHDA-Induced Cell Death

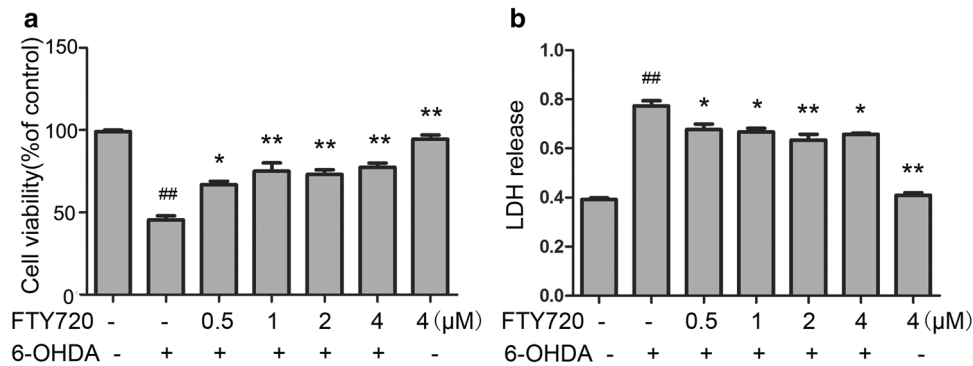
To test the ability of FTY720 to prevent 6-OHDA-induced cell death, we first performed MTT assay and LDH release assay after challenging cells with 100 µM 6-OHDA. Compared to vehicle-treated control cells, pretreatment of cells with FTY720 significantly reduced 6-OHDA-associated cell death as measured by MTT as well as by LDH release assays (Fig. 1a, b).

### FTY720 Attenuates 6-OHDA Induced Apoptosis in SH-SY5Y Cells

To further assess the neuroprotective effect of FTY720 against 6-OHDA neurotoxicity, we utilized flow cytometric analysis to measure the relative numbers of Annexin V and PI stained cells. As shown in Fig. 2a, b, 100 µM of 6-OHDA significantly increased apoptotic cell death in SH-SY5Y cells, with the total apoptotic rate reaching (26.96 ± 7.09)% by 24 h after 6-OHDA exposure. However, pretreatment of cells with FTY720 (2, 4 µM) for 12 h prior to 6-OHDA exposure significantly decreased the apoptotic cell number at 24 h. The anti-apoptotic effect of FTY720 was further confirmed using TUNEL staining (Fig. 2c).

### FTY720 Promotes Phosphorylation of Pro-survival Molecules, AKT and ERK1/2 and Increases BDNF Levels in SH-SY5Y Cells

Next we explored the ability of FTY720 to activate the pro-survival AKT and ERK1/2 pathways in SH-SY5Y cells. As shown in Fig. 3a, FTY720 stimulated a rapid increase in the phosphorylation of pro-survival molecules AKT and ERK1/2 in SH-SY5Y cells. After activation of ERK1/2,



**Fig. 1** FTY720 prevents 6-OHDA-induced cell death in SH-SY5Y cells. SH-SY5Y cells were pretreated with FTY720 for 12 h at the indicated concentrations and exposed to 100 μM 6-OHDA for 24 h, the protective effect of FTY720 was determined at 24 h after

6-OHDA exposure by **a** MTT assay and **b** LDH release. All data are presented as the mean ± SEM of triplicate independent experiments. <sup>##</sup> $p < 0.01$  vs. control; <sup>\*</sup> $p < 0.05$ ; <sup>\*\*</sup> $p < 0.01$  vs. 6-OHDA group

FTY720 also led to phosphorylation of CREB (P-CREB, Fig. 3b), which is downstream of ERK1/2, and is a transcription factor that when activated by phosphorylation is known to increase BDNF expression.

Previous studies have shown that FTY720 can increase BDNF expression in dopaminergic MN9D cells [7]. In order to assess whether it also increased BDNF expression in another dopaminergic cell line, we next treated SH-SY5Y cells with 2 μM of FTY720 at various time points (4, 8 and 24 h) then measured BDNF protein levels. Consistent with previous findings, we found a significant increase in BDNF protein levels in SH-SY5Y cells treated with FTY720 (Fig. 3c).

### FTY720 Attenuates Neurodegeneration in the Ventral Midbrain and Ameliorates Motor Deficits Associated with 6-OHDA

Following our in vitro observations described above, we then assessed if FTY720 could also be protective against 6-OHDA neurotoxicity in an in vivo model of PD. For this purpose, the analysis was carried out in mice that had received pretreatment with vehicle alone or FTY720 for 7 days prior to 6-OHDA lesioning of the striatum. Evaluation of tissues collected 21 days after 6-OHDA injections demonstrated that the 6-OHDA-induced neurodegeneration in the SNc and striatum were remarkably attenuated by FTY720 (Fig. 4a–c). These results were further supported by immunoblots of striatal extracts evaluated using an anti-TH antibody, which showed higher TH protein levels in FTY720-treated mice (Fig. 4d).

In order to correlate early biochemical changes in the striatum with long-term motor alterations induced by 6-OHDA, mice were monitored for apomorphine-induced rotation at 7, 14 and 21 days after 6-OHDA lesioning. Apomorphine-induced asymmetrical rotations contralateral

to the 6-OHDA injection site were significantly reduced by FTY720 treatment as compared to mice lesioned with 6-OHDA alone (Fig. 4e).

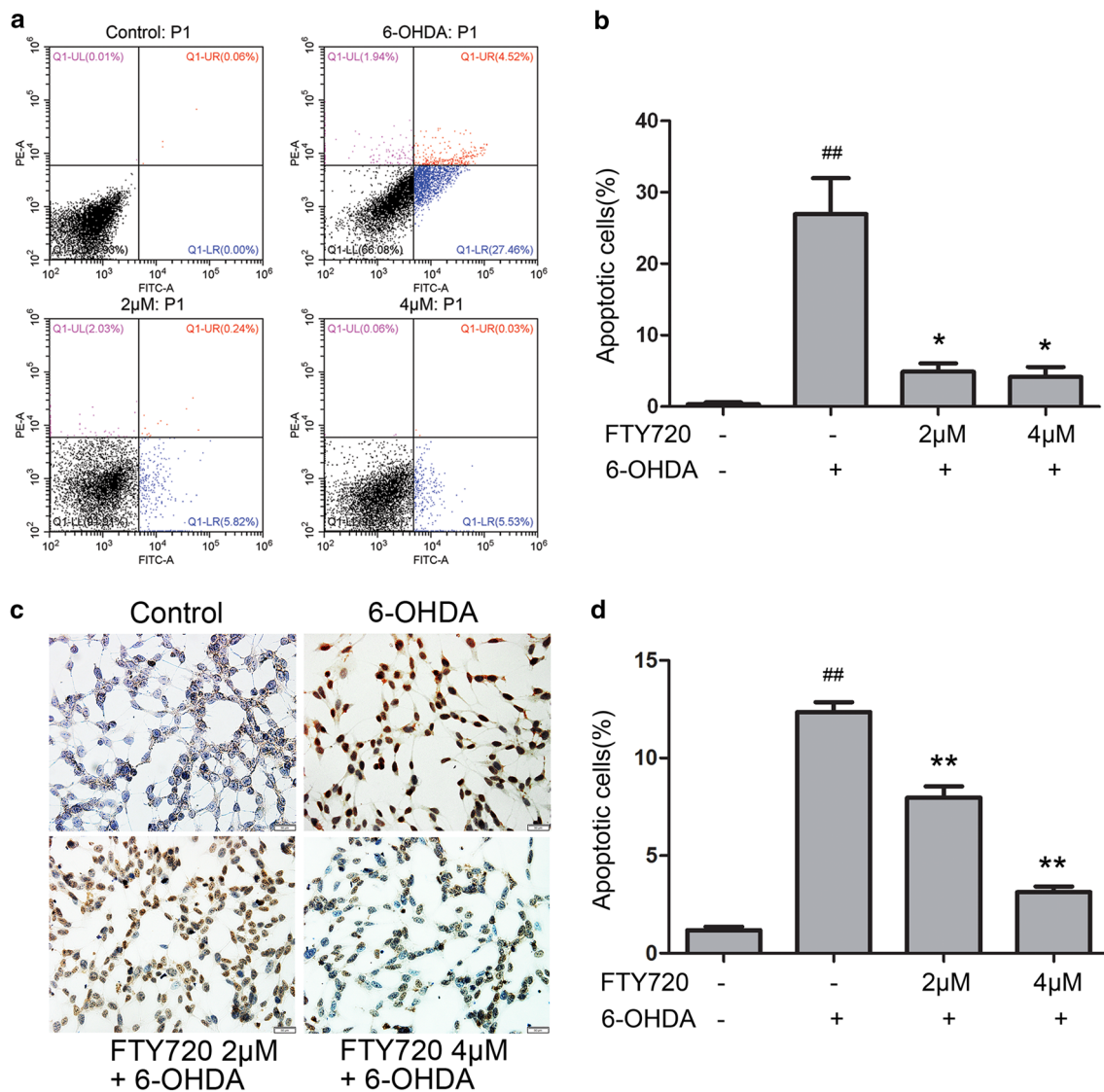
### FTY720 Prevents Striatal Dopamine Depletion Induced by 6-OHDA

The striatal levels of DA, DOPAC and HVA were measured 21 days after 6-OHDA administration using a highly sensitive HPLC-MS/MS system. Consistent with a loss of striatal dopaminergic terminals, we also noted a profound reduction in striatal DA and its metabolites after 6-OHDA lesioning that was significantly attenuated by FTY720 pretreatment, which also produced a significant elevation in striatal DA and the metabolites DOPAC and HVA at 21 days post 6-OHDA lesioning (Fig. 5a–c).

### FTY720 Reduces 6-OHDA-Induced Astrogliosis and Microgliosis in the SNc and Striatum

As 6-OHDA injection also induced a prominent inflammatory response in the SNc and striatum, we next examined astrocytic and microglial activation as an additional measure of tissue damage. The SNc and striatal sections were immunostained with anti-GFAP and anti-CD11b antibodies and counterstained with DAPI. As shown in Fig. 6a, b, 6-OHDA administration markedly increased markers of inflammation such as reactive astrocytes and microglia, whereas in vehicle-treated control animals, only a few faintly immunoreactive astrocytes and microglia were observed in the SNc and striatum. FTY720 administration resulted in significant decrease in 6-OHDA-induced astrogliosis and microgliosis. Taken together, these data suggest that the neuroprotective effects of FTY720 against 6-OHDA-neurotoxicity are associated with a marked reduction in inflammation.





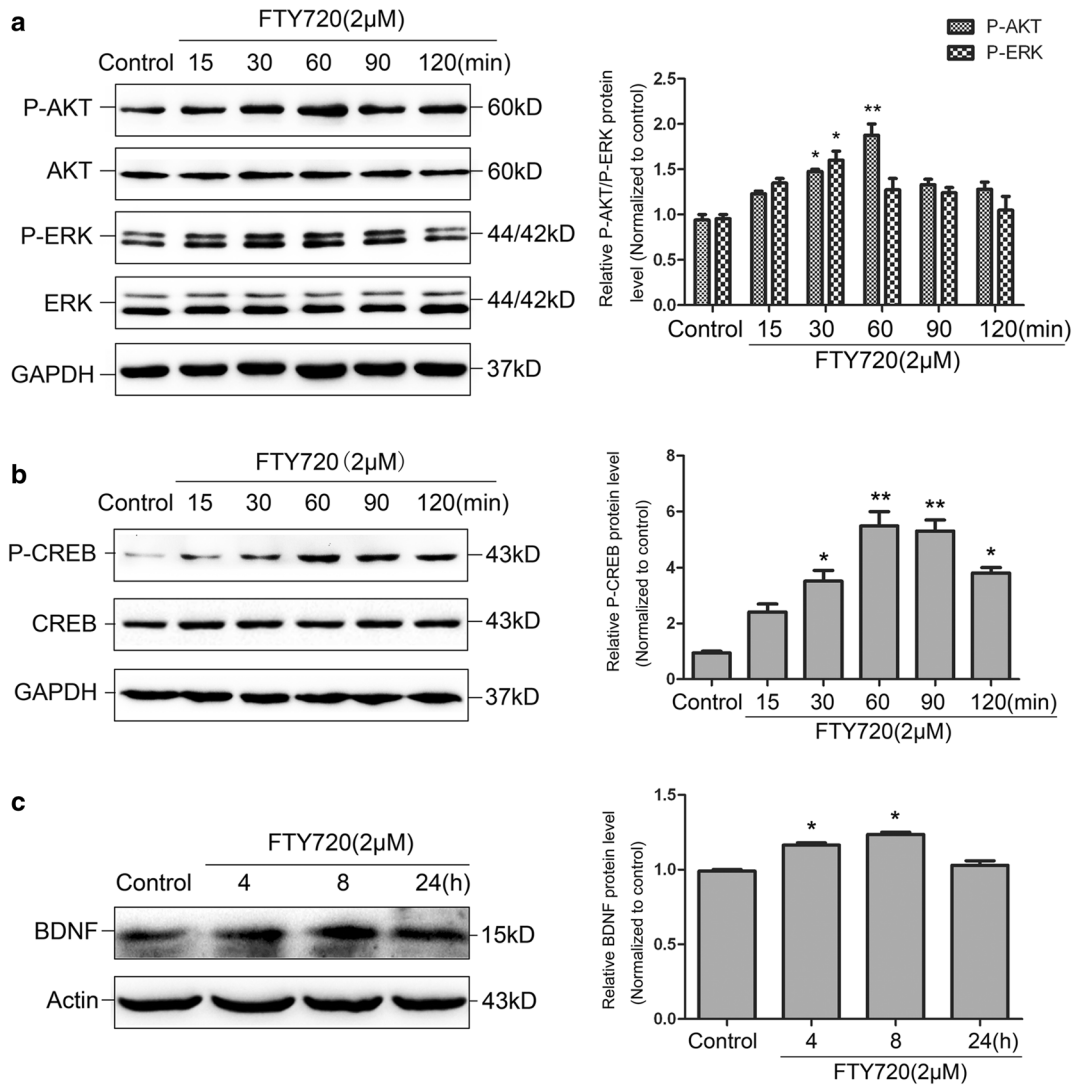
**Fig. 2** FTY720 protects SH-SY5Y cells from 6-OHDA-induced apoptosis. SH-SY5Y cells were pretreated with FTY720 2 and 4  $\mu$ M for 12 h and then subjected to 100  $\mu$ M 6-OHDA for 24 h. **a** Cell apoptosis was measured by Annexin-V/PI staining and summarized data showing apoptotic rate were shown in **(b)**; cell apoptosis was further determined by TUNEL staining and representative image of

TUNEL staining performed from control and 6-OHDA-treated cells were shown in **(c)** and summarized data showing apoptotic rate was shown in **(d)**. All data were represented as mean  $\pm$  SEM of triplicate independent experiments.  $##p < 0.01$  vs. control;  $*p < 0.05$ ,  $**p < 0.01$  vs. 6-OHDA group

### FTY720 Induces Phosphorylation of AKT, ERK1/2 and Increases BDNF Levels in vivo

Because we found that FTY720 has the ability to promote phosphorylation of AKT and ERK1/2 in vitro, we next explored the possibility that FTY720 induce the phosphorylation of AKT and ERK1/2 in vivo. For this purpose, ten-week-old C57BL/6 mice were given 0.5 mg/kg of FTY720 by intraperitoneal injection once daily for 7 consecutive days. Control animals received vehicle alone at the same frequency and volume as FTY720. Brains

were removed at the indicated time points after the final dosing and striatal tissues were collected and evaluated by western blot. As shown in Fig. 7a, b, administration of FTY720 significantly increased the phosphorylation of AKT, ERK1/2 and CREB in mouse striatal tissues. We also tested the level of BDNF in striatal tissues. In consistent with our in vitro data, striatal protein levels of BDNF were upregulated as early as 30 min after the last FTY720 injection and remained elevated at 24 h after the final dosing (Fig. 7c).



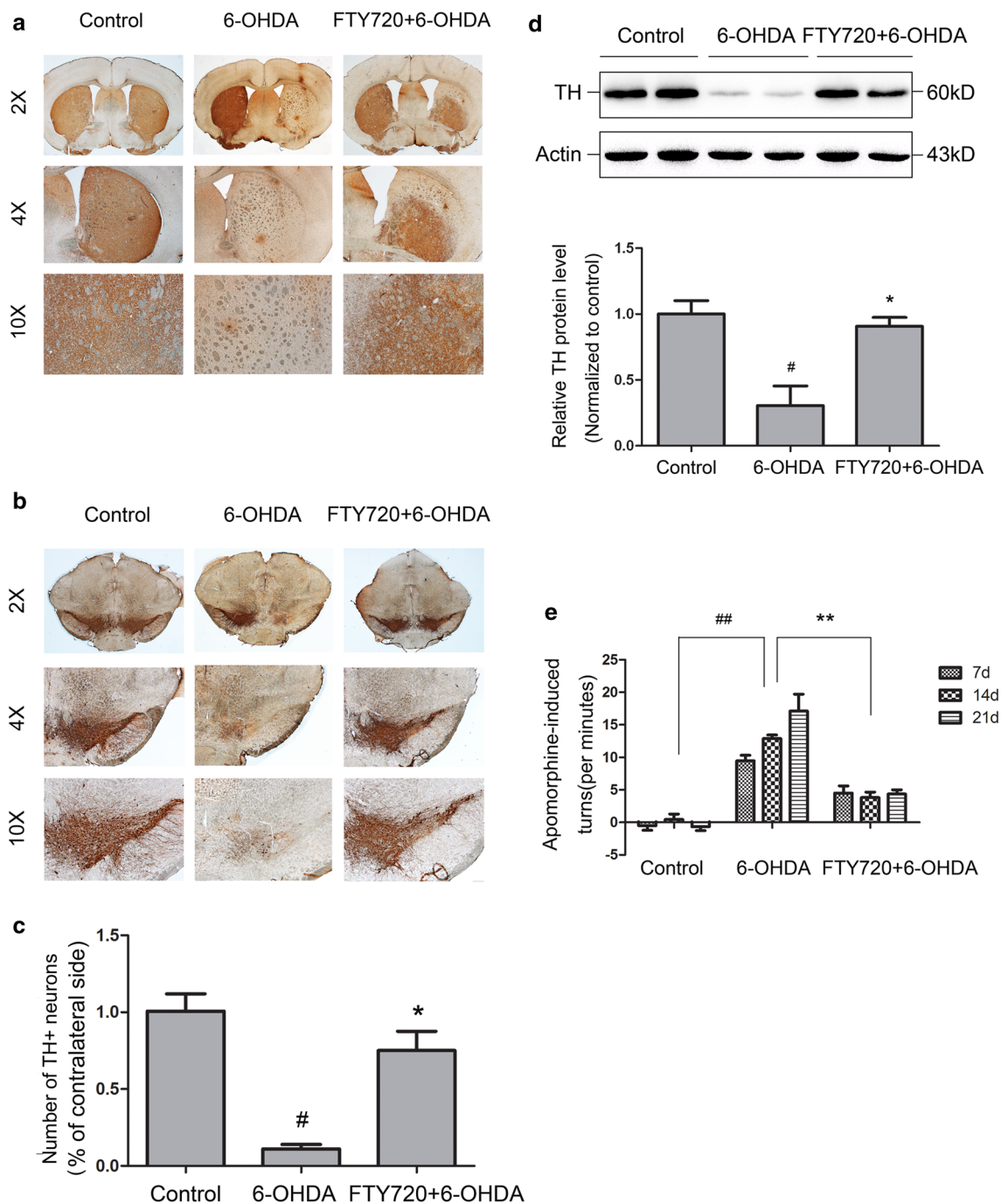
**Fig. 3** FTY720 promotes the phosphorylation of AKT, ERK1/2, CREB and increases BDNF level in SH-SY5Y cells. Cells were treated with 2 μM of FTY720 for different time points, total cell lysates were subjected to immunoblot analysis for **a** P-AKT, total

AKT, P-ERK1/2 and total ERK1/2, **b** P-CREB, total CREB and c BDNF. All data were represented as mean ± SEM of triplicate independent experiments. \**p* < 0.05, \*\**p* < 0.01 vs. control

**FTY720 also Increases Striatal BDNF Levels and Induces Phosphorylation of AKT and ERK1/2 in 6-OHDA Lesioned Mice**

We also tested the possibility that FTY720 may induce phosphorylation of AKT, ERK1/2 and increase striatal BDNF levels in 6-OHDA lesioned mice. To evaluate this, mice were treated with 0.5 mg/kg FTY720 or vehicle by intraperitoneal injection for 7 days prior to 6-OHDA lesioning, and mice were sacrificed 1 day after 6-OHDA injection. Consistent with the data obtained above, administration of FTY720 also significantly

stimulated the phosphorylation of AKT and ERK1/2 in 6-OHDA lesioned mice (Fig. 8a), highlighting the ability of the drug to modulate pro-survival molecules in neurons in vivo under PD context. We also observed robust increase in phosphorylation of transcription factor CREB in mouse striatal tissues (Fig. 8b), which is downstream of ERK1/2 activation and is known to stimulate BDNF expression. We next also tested the protein level of BDNF in striatal tissues. In line with the above data, administration of FTY720 also results in a significant increase in BDNF protein levels in 6-OHDA lesioned mice (Fig. 8c).



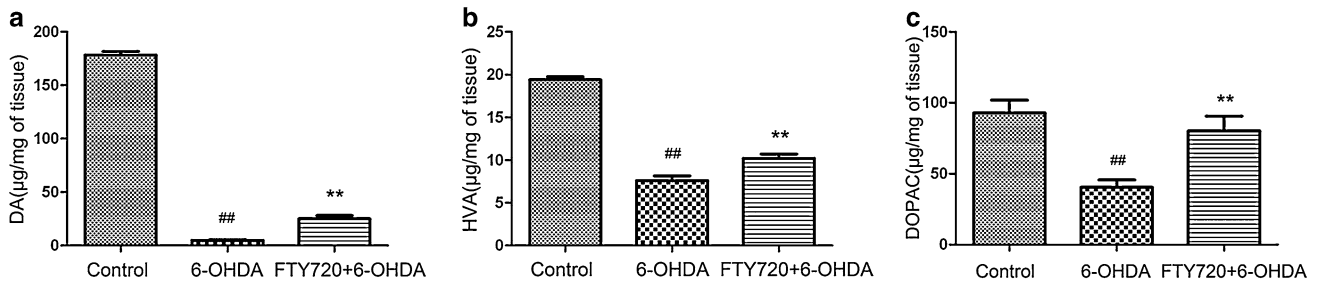
**Fig. 4** FTY720 attenuates 6-OHDA-induced loss of dopaminergic neurons in the SNc and striatum and improves motor deficits in 6-OHDA mice. Brain sections from the striatum and SNc were stained with TH (**a**, **b**) and number of TH<sup>+</sup> neurons in the SNc (**c**) were determined at 21 days after 6-OHDA injection by stereological counting. **d** Representative immunoblot documents and summarized

data showing that FTY720 attenuates 6-OHDA-induced loss of TH in mice striatal tissues. **e** Apomorphine-induced turns were assessed at 7, 14 and 21 days after 6-OHDA injection (n=8 for each group of mice). For the *Western blot*, data were presented as the mean  $\pm$  SEM of triplicate independent experiments. ##*p*<0.01 vs. control, \**p*<0.05, \*\**p*<0.01 vs. 6-OHDA group

## Discussion

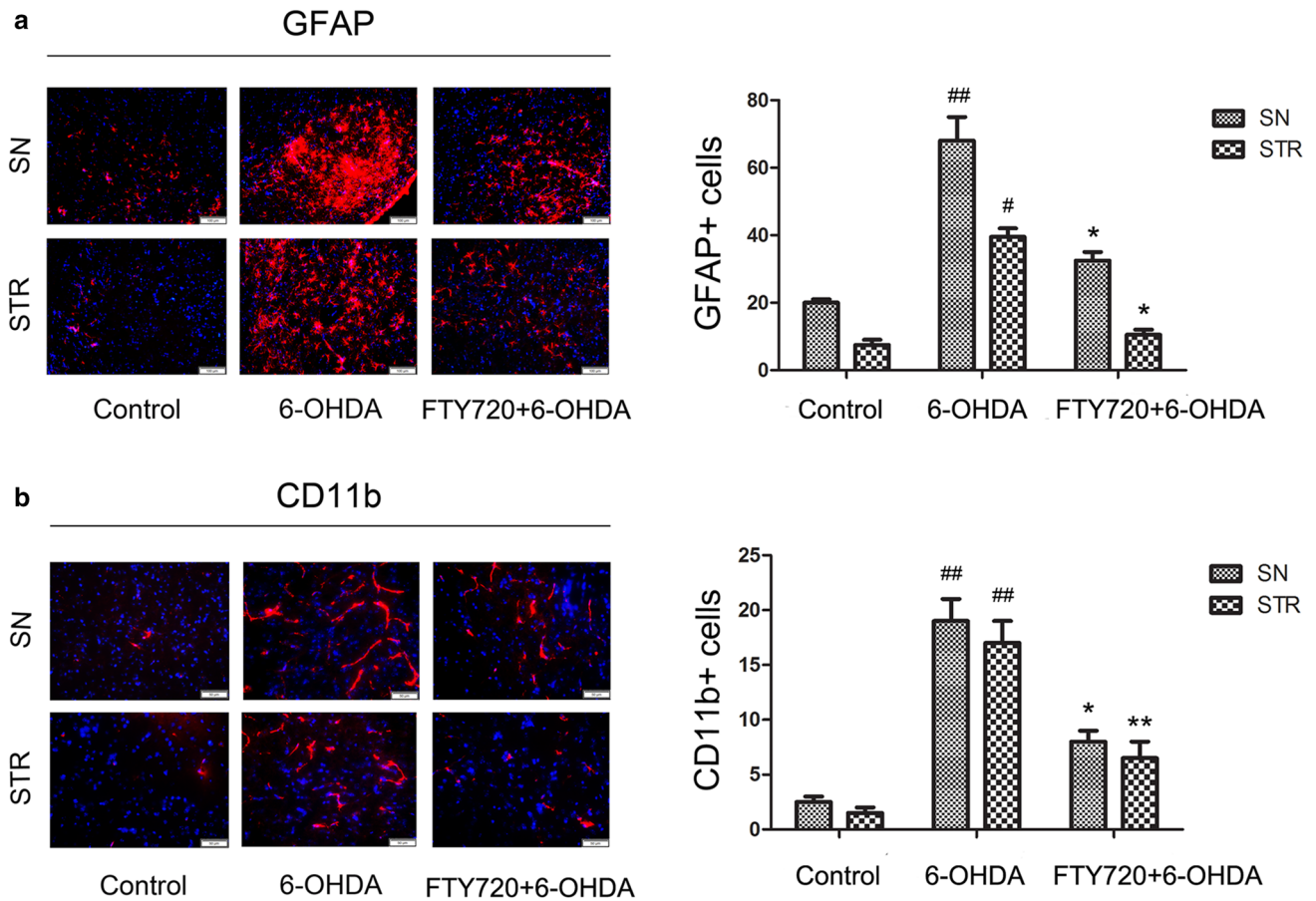
FTY720 was first characterized as an immunosuppressive drug for the treatment of MS. It was recently reported to

also have potent neuroprotective and neurorestorative properties, which make FTY720 a potential novel therapeutic for treating other neurodegenerative disorders [2, 3]. In the present study, we showed evidence that FTY720 may



**Fig. 5** FTY720 prevents the neurochemical imbalance induced by 6-OHDA at 21 days after the toxin was infused into the mice striatum. Striatal levels of DA (a) and its metabolites HVA (b) and DOPAC (c) were measured by HPLC analysis on the 21st day follow-

ing 6-OHDA injection. All data are presented as the mean ± SEM, n=6 for each group of mice. <sup>##</sup>*p*<0.01 vs. control; <sup>\*\*</sup>*p*<0.01 vs. 6-OHDA group



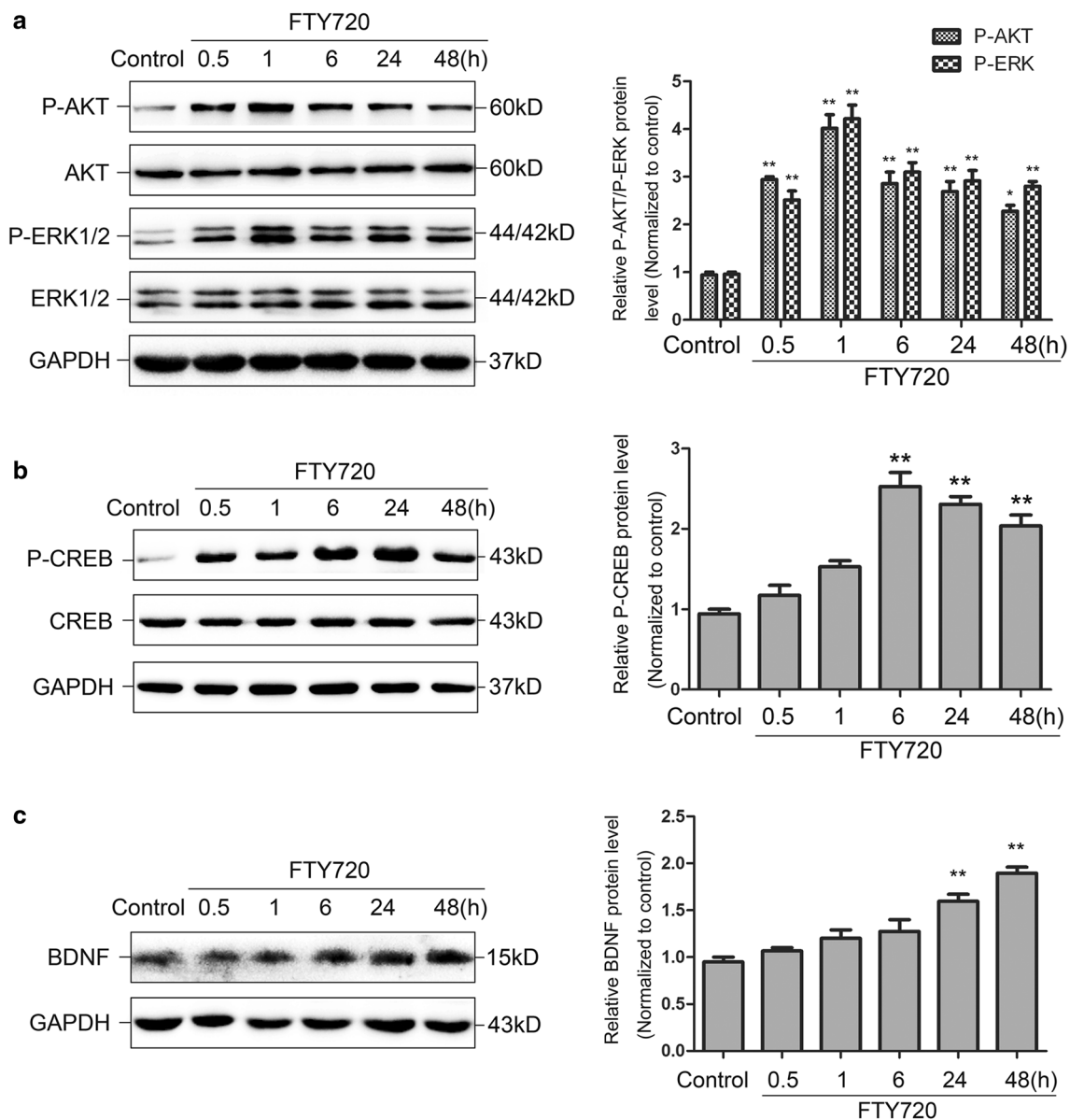
**Fig. 6** FTY720 attenuates 6-OHDA-induced astrocyte and microglia activation 21 days after 6-OHDA exposure in mice SNc and striatum. **a** Immunofluorescence for GFAP-positive astrocytes (red) and DAPI (blue) and summarized data showing that FTY720 produced a significant decrease in 6-OHDA-induced astrogliosis. **b** Immunofluores-

cence for CD11b immunoreactive microglia (red) and DAPI (blue) in SNc and striatum with summarized data showing that FTY720 administration produced a significant decrease in 6-OHDA-induced microglia. <sup>#</sup>*p*<0.05, <sup>##</sup>*p*<0.01 vs. control, <sup>\*</sup>*p*<0.05, <sup>\*\*</sup>*p*<0.01 vs. 6-OHDA group

represent a possible pharmacological intervention for treating patients in the early stages of PD. The results of this study revealed that FTY720 increased BDNF levels both in vitro and in vivo and significantly reduced apoptosis

in SH-SY5Y cells in association with activation of the pro-survival signals AKT and ERK1/2. Our in vivo studies in 6-OHDA lesioned mice demonstrated that FTY720 could ameliorate dopaminergic neurodegeneration and





**Fig. 7** FTY720 enhances phosphorylation of AKT, ERK1/2 and CREB and increases BDNF levels in mice striatum. Mice were given 0.5 mg/kg of FTY720 by intraperitoneal injection once daily for 7 consecutive days, brains were removed at the indicated time points after the final dosing and striatal tissues were collected and evaluated by *western blot* analysis. Increase in protein levels of P-AKT, ERK1/2 (**a**) and P-CREB (**b**) analyzed by *western blotting* and den-

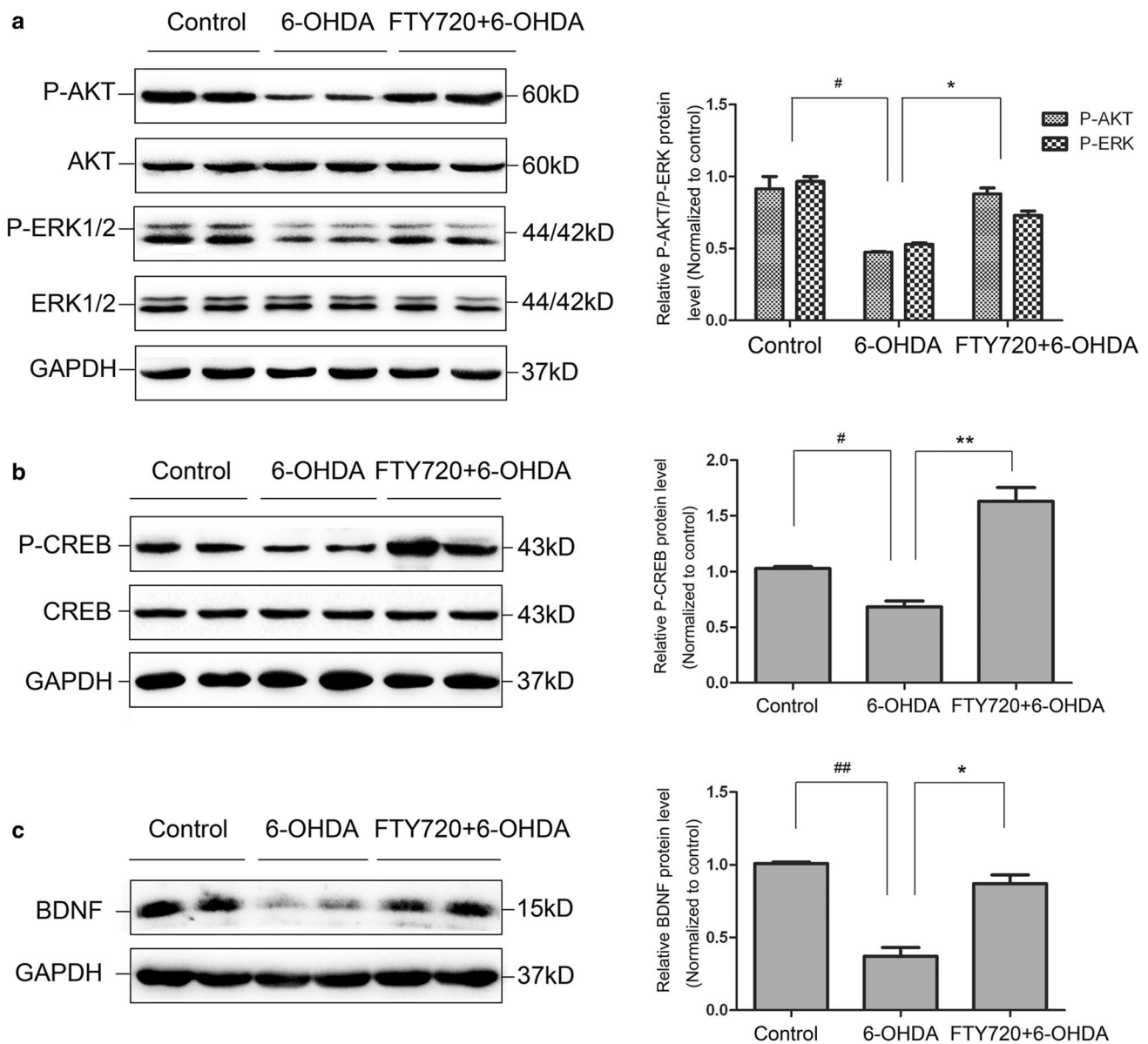
sitometry at different time points after FTY720 treatment in mice striatum, the untreated control mice striatum were used to show the *baseline* levels of these proteins. **c** Representative *blots* and densitometry analysis showing relative changes of BDNF at different time points after FTY720 treatment in mice striatum. Data are presented as mean  $\pm$  SEM,  $n=3$  for each group of mice. \* $p<0.05$ ; \*\* $p<0.01$  vs. control

neuroinflammation in mouse brain. Taken together, these results support the hypothesis that FTY720 can protect against 6-OHDA neurotoxicity *in vitro* and *in vivo*.

Neuroprotective effects of FTY720 have been demonstrated in several neurological models including Huntington's disease, Rett disease, stroke, and Alzheimer's disease [4, 5, 13–15]. We noted similar protective effects of FTY720 against neurodegeneration caused by 6-OHDA *in vitro* and *in vivo*. The neuroprotective effects of FTY720

observed in our studies appears to be related with increased levels of BDNF. Since recent findings suggest a fundamental and more general role of BDNF release as a factor in FTY720-mediated neuroprotection [4, 5, 13]. In Vidal-Martínez et al. we recently reported that long term treatment with FTY720 increases BDNF expression in A53T transgenic mice; while blocking BDNF signaling stimulates parkinsonian pathology [8]. In PD patients, both brain and peripheral levels of BDNF are significantly reduced





**Fig. 8** FTY720 induces phosphorylation of AKT, ERK1/2 and CREB and enhances BDNF levels in 6-OHDA lesioned mice striatum. Mice were treated with 0.5 mg/kg FTY720 or vehicle by intraperitoneal injection for 7 days prior to 6-OHDA lesioning, and mice were sacrificed 1 day after 6-OHDA injection. **a**Western blot analysis of P-AKT, P-ERK1/2 levels and its densitometric analysis in striatal tissues 1 day after 6-OHDA lesioning. **b** Representative western blot-

ting and densitometric analysis of CREB phosphorylation 1 day after 6-OHDA lesioning in striatal tissues. **c**Western blot analysis of BDNF levels and its densitometric analysis in striatal tissues 1 day after 6-OHDA lesioning. Data are presented as mean  $\pm$  SEM, n=3 for each group of mice. #*p*<0.05, ##*p*<0.01 vs. control; \**p*<0.05; \*\**p*<0.01 vs. 6-OHDA group

as compared to levels found in healthy subjects [16]. Data from PD animal models also show that BDNF plays a protective role for DA neurons [17]. Because reduced levels of BDNF typically correlate with impaired neuronal function, increasing endogenous BDNF levels with a well-tolerated drug may ameliorate many of the functional deficits associated with PD and related disorders [18]. In the current study, we found a robust increase in the phosphorylation of AKT, ERK1/2 and CREB after FTY720 treatment,

which in turn increased BDNF expression in mouse striatum, while also protecting dopaminergic neurons from degeneration.

The neuroprotective effects of FTY720 observed here may relate to its potent anti-inflammatory effects. Accumulation of microglia at the site of injury typically occurs in response to 6-OHDA-induced neurodegeneration and may contribute to neuronal damage by increasing local levels of pro-inflammatory cytokines, free radicals, and enzymes

that further worsen the outcome of the injury. Accordingly, we saw a large increase in the number of microglial cells with an activated morphology in both SNc and striatum of mice 21 days after 6-OHDA injection. This was significantly reduced in mice with FTY720 treatment. These findings are consistent with studies by others showing that FTY720 also promotes neuroprotective effects of microglia and attenuates kainic acid-induced neurodegeneration and associated microgliosis in rats [19]. In addition, astrogliosis also contributes to 6-OHDA-induced neurodegeneration, and FTY720 can attenuate 6-OHDA-induced astrogliosis. The mechanism(s) by which FTY720 specifically reduces microgliosis and astrogliosis to contribute to neuroprotection against 6-OHDA-induced neurotoxicity awaits further study.

Although the neuroprotective mechanisms of FTY720 are not fully understood, in this study, the increase of BDNF and the associated anti-inflammatory effects of FTY720 both appear to have contributed to neuroprotection in our models. Further investigations will help to elucidate the precise mechanisms exerted by this compound as it relates to PD.

In summary, we found that FTY720 (1) attenuated the neuropathology associated with 6-OHDA in mice, (2) reduced neuroinflammation, and (3) stimulated a neuroprotective increase in endogenous BDNF expression. These pre-clinical data support the efficacy of FTY720 *in vivo*, and further highlight the drug's therapeutic potential during the early PD before extensive loss of SNc dopaminergic neurons. Repurposing a drug that is already FDA-approved could provide a much needed neuroprotective PD therapeutic that may benefit affected individuals worldwide.

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#### Compliance with Ethical Standards

**Conflict of interest** These authors declare that there are no conflicts of interest.

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