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Original Article

Fibroblast growth factor 1 attenuates 6-hydroxydopamine-induced neurotoxicity: an *in vitro* and *in vivo* investigation in experimental models of parkinson's disease

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Abstract: Parkinson's disease (PD) is a degenerative disorder of the central nervous system and is characterized by motor system disorders resulting in loss of dopamine producing brain cells. Acidic fibroblast growth factor, also called FGF1, promotes the survival of neurons. The aims of the present study were to confirm FGF1 could protect neurons cultures from 6-hydroxydopamine (6-OHDA) toxicity in vitro and in vivo. Our results demonstrated FGF1 administration improved the motor function recovery, increased the TH-positive neurons survival and up-regulated the levels of neurotransmitters in PD rats. Meanwhile, FGF1 prevents the death of DA neuron at least in part by reducing the levels of α-synuclein and ER stress. The administration of FGF1 activated downstream signals PI3K/Akt and ERK1/2. In conclusion, FGF1 diminished α-synuclein neurotoxicity by down regulating ER stress mediators and the level of apoptosis, and these effects may underlying the activation of the PI3K/Akt and ERK1/2 signal pathway.

Keywords: ER stress, α-synuclein, parkinson's disease, FGF1

Introduction

Parkinson's disease (PD) as the second most common neurodegenerative disorder, is characterized by selective neuronal death affecting the locus coeruleus, dorsal motor nucleus of vagus, nucleus basalis of Meynert and pathologically by marked dopaminergic (DA) neurons loss in the substantia nigra pars compacta (SNpc) and intracytoplasmic proteinaceous inclusion bodies formation [1]. Currently, the pathogenesis of PD is still unclear, accumulating evidence has suggested that both environmental and genetic factors collaborate and cause dopaminergic cell death. Aging, exposure to pesticides, and endogenous toxic agents, such as dopamine derivatives, mighty induce oxidative stress and lead to neurodegeneration.

Recently, studies showed that endoplasmic reticulum (ER) stress, in conjunction with abnormal protein degradation, plays a crucial role in the development of PD. Misfolded protein aggregation and disrupt calcium homeostasis in ER lead to ER stress and unfolded protein response (UPR) [2, 3]. ER stress signaling pathways have been unraveled: the first step in the UPR is the recognition of unfolded proteins by the HSP70-class chaperone glucose regulated protein 78 (GRP78). The titration of GRP78 by unfolded proteins leads to its dissociation from and activation of the three ER stress receptors: pancreatic ER kinase-like ER kinase (PERK), activating transcription factor (ATF6), and inositol-requiring enzyme 1 (IRE1), the PERK pathway is activated first, followed rapidly by ATF6, while IRE1 is activated last. Persistent and severe ER stress leads to activation of the

PERK-eukaryotic initiation factor 2 (eIF2)-activating transcription factor 4 (ATF4) pathway, and culminates in the induction of the CCAAT-enhancer-binding protein (C/EBP) homologous protein (CHOP), which up-regulates apoptosis-related genes including caspase-12, caspase-3 to promote cell death [3]. ER stress markers are known to be induced by 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenylpyridinium (MPP+), rotenone and paraquat, which cause degeneration of dopaminergic neurons in the SN and are used to model PD [2, 4]. The sustained increase of α-synuclein (α-syn) has also been shown to be involved in ER stress [5]. Elevated α-syn levels have been found to block ER to Golgi membrane trafficking and cause ER stress [6]. Thus modulation of ER Stress and α-syn constitutes an important therapeutic target for PD.

Neurotrophic factors have the ability to protect dopaminergic neurons or enhance their regeneration through activation of multiple intracellular pathways that oppose neurons apoptosis in PD [7]. For example, intrastriatal administration of glial cell-derived neurotrophic factor (GDNF), cerebral dopamine neurotrophic factor (CDNF) and neurturin (NTN) have all been shown to protect against 6-OHDA rat model of PD [8]. Acidic fibroblast growth factor (FGF1 or aFGF) and basic fibroblast growth factor (FGF2 or bFGF) are members of FGF-1 sub-family of FGFs that, in the adult, shows preferential expression in the brain, has proven to be highly efficient at the regeneration of neurons in multiple experimental animal models, including those of optic nerve injury and excitotoxic cell death [9]. Several groups have accordingly shown that FGF2 regulates dopaminergic neuron and nigrostriatal pathway development [10], enhances neuronal survivability and protects from 6-OHDA-induced cell death in the substantia nigra of PD mouse models [11]. However, the effect of FGF1 in PD received less attention. Only one study showed treatment of MPTP-depleted young mice with FGF1 results in partial recovery in the nigrostriatal DA system [12].

The aims of the present study were two-fold; firstly to confirm the single published study to date showing that FGF1 could protect neurons cultures from 6-OHDA toxicity *in vitro* and *in vivo* and, secondly, to examine for the first time whether FGF1 could inhibit ER stress and attenuate α-syn aggregation in PD rat model.

Materials and methods

Animals protocols

Young adult male SD rats (280-320 g) were purchased from the Animal Center of the Chinese Academy of Sciences, in accordance with the National Institutes for Health Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Animal Care and Use Committee of Wenzhou Medical University. The rats were maintained in a temperature/humidity-controlled environment under a 12 h light/dark cycle with *ad libitum* access to food and water. The animals were anesthetized with 5% chloral hydrate, shaven and the back of their heads were disinfected. Then, at the middle of the back of the head, longitudinal cuts were made, and the fascia were cut off by means of 30% hydrogen peroxide etching, thereby exposing the bregma on the skull and the word point. Then the rats were fixed to a stereotaxic apparatus (KOPF Company, Germany). The rat model of PD was reproduced by injecting 6-OHDA (10 μl, 1.5 μg/ul, dissolved in 0.2% ascorbic acid saline solution) unilaterally into the striatum on the right at a constant rate (coordinates: A: +0.7 mm from bregma, L: +2.8 mm from midline and H: +5.5 mm). 6-OHDA was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). The injection rate was 10 nl/sec and the needle was kept in place for 5 min after the injection. The sham operation model was injected with 0.9% saline and 0.2 mg/ml ascorbic acid at the same cerebral intervention as the physical damage.

Behavioral test

One week after right striatum stereotaxic injection of 6-OHDA, animals were subjected to rotational behavior testing. Rats were injection subcutaneously with apomorphine hydrochloride (0.5 mg/kg), placed in a round kettle (40 cm in diameter), and recorded contralateral turns in a 30 min period by a kind of equipment used to detect rotational behavior of rats, the rats with contralateral turns more than 7 cycles/min were used as valid pathology model [13]. The valid Parkinson's disease rats were randomly divided into two groups, FGF1 (80 μg/kg/day) group or vehicle (model group). The sham-lesion control was also included. FGF1 (Grostre Biotech Co., Wenzhou, China) in distilled water was injected by cauda vein for 2 weeks. The turns of rats were performed at 1, 2, and 3 weeks after the first FGF1 administration.

Cell culture and viability assay

PC12 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells grown in DMEM medium were supplemented with 10% fetal bovine serum and 1% antibiotics and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. PC12 cells were seeded on 96-well plates and treated with 6-OHDA (50, 100, 150, 200 and 400 μM) for 24 h with or without FGF1 (10, 20, 40, 80 ng/mL). Thapsigargin (TG, 1 nm), a specific ER stress activator, was added as a positive control to compare with 6-OHDA. Cell viability was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. During the last 4 h, MTT (600 mM) was added to the media. Cells were then washed with PBS (pH 7.4), and DMSO was added to solubilize the formazan crystals. The absorbance was measured at 570 nm. Optimal conditions of 150 μM 6-OHDA and 20 ng/ml FGF1 were used for the subsequent experiments.

Flow cytometry analysis

To quantify the apoptotic cells of each group, PC12 cells and medium were harvested after treatment. The concentration was adjusted to 1×10^6 cells/ml. After triple centrifugation and washing in cold PBS, cells were re-suspended in 1× binding buffer, double-stained with PI/Annexin V-FITC kit (Invitrogen, Carlsbad, CA, USA) for 15 min at room temperature in the dark, and the apoptotic rate was analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

HPLC-ECD analysis

The standard curve was constructed with purified samples. DA, 3, 4-dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HA), 5-hydroxyindole acetic acid (5-HIAA), 5-hydroxytryptamine (5-HT), epinephrine (E), norepinephrine (NE), were purchased from Sigma-Aldrich Ltd. (St. Louis, MO, USA). Liquid chromatographic experiments were an Agilent 1100 liquid chromatography (Agilent Technologies, Santa Clara, CA, USA), and the column was Agilent Eclipse XDB-C18 (2.1 mm × 150 mm, 5 μm particle, Agilent Technologies, Santa Clara, CA, USA). The working electrode was a glassy carbon disc electrode (3 mm in diameter) or ABNs modified electrode. The rats' corpus striatum was accurately weighed and placed in a 2-mL centrifuge tube, added at a concentration of 0.1 M perchloric acid solution 10 ml/g and homogenized on ice. The soluble fraction was obtained by centrifugation at 4°C, 12,000 rpm for 10 min, 1-2 times. Before HPLC-ED analysis, the sample was unfrozen at 4°C prior to injection. The standard curve into software Clarity v.2.6.4.402 calculated for each sample in various concentrations of neurotransmitters, and then Graphpad Prism 5.0 was used for analysis.

Histology and immunohistochemistry

The rats were anesthetized with 4% choral hydrate (10 ml/kg IP) and then perfused with 4% paraformaldehyde in a 0.1 M phosphate buffer. Brains were dissected and post-fixed in the same fixative overnight at 4°C. The substantia nigra were embedded in paraffin and sliced. After the tissue hydration dewaxing and antigen retrieval at high temperature and pressure, sections were incubated with 3% hydrogen peroxide for 30 min and 1% BSA for 30 min at room temperature. Soon afterwards, we added the primary antibody against CHOP (1:150), GRP78 (1:200), caspase-12 (1:600) and TH (1:300) at 4°C overnight. Next, the sections were washed with PBS three times, and incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at 37°C. The treated sections were incubated with 3, 3-diaminobenzidine (DAB, 5% diluted with PBS) for 5 min. The saline injection group was considered to be the negative control. The results were analyzed by counting the number of positive cells at ×400 magnification using a Nikon ECLPSE 80i (Nikon, Tokyo, Japan). The optical density of CHOP, GRP78, caspase-12 and TH in the substantia nigra were counted at 5 randomly selected fields per sample.

Western blot analysis

The rats were perfusion with ice-cold 0.9% NaCl and decapitated. Total proteins were isolated from the striatum of the rats with mammalian tissue extraction reagent according to the manufacturer's protocol. The PC12 cells were lysed in protein extraction reagent with protease and phosphatase inhibitors. α-synu-

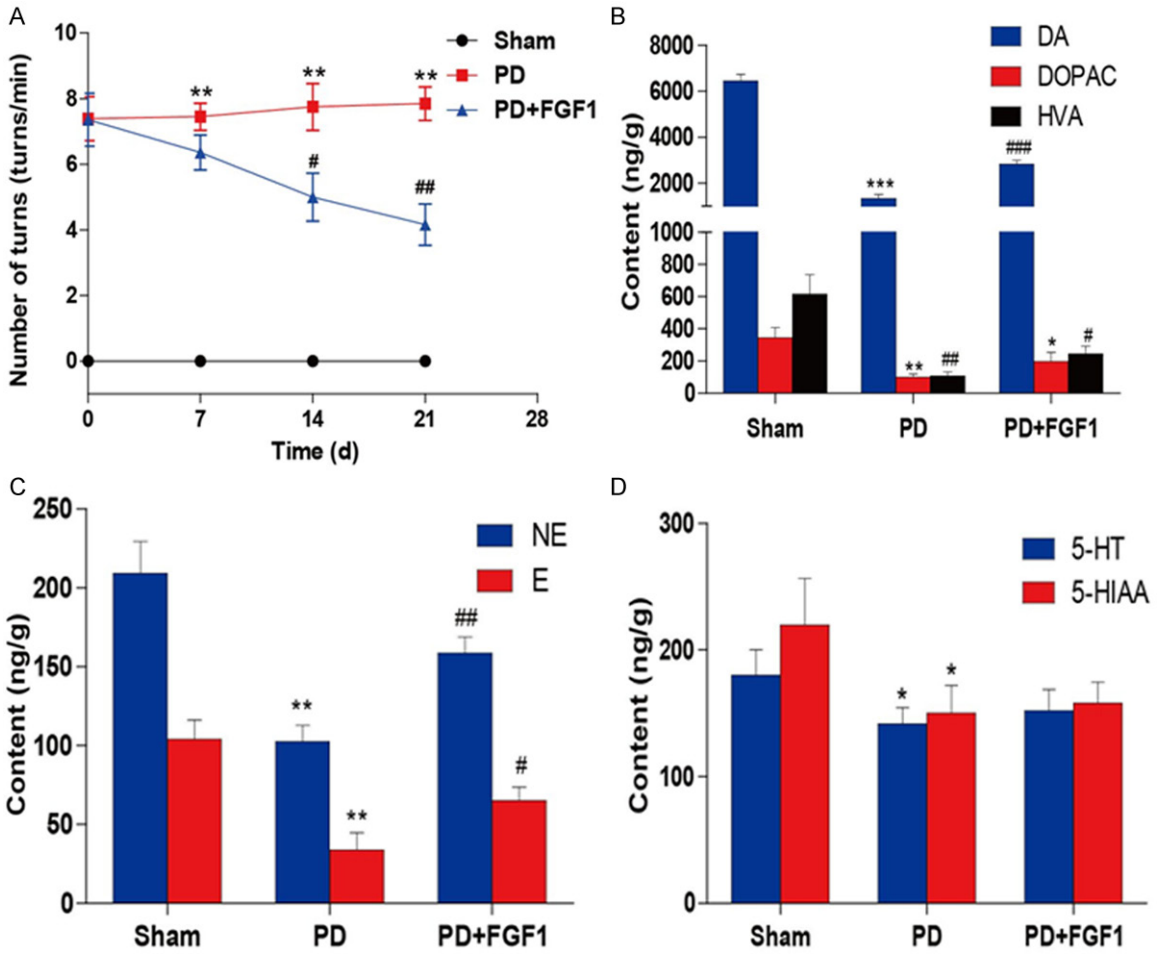


Figure 1. Effects of FGF1 infusions on amphetamine-induced rotation and neurotransmitters levels in the striatum of PD model rats. A. Effects of FGF1 administration on the apomorphine (APO)-induced ipsilateral rotations measured at 1, 2 and 3 weeks after lesion. B-D. The levels of monoamine neurotransmitters in the striatum detected by HPLC-ECD at 3 weeks post-lesion. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. PD group, n = 10.

clein was isolated like before describing [14]. All protein samples were separated on 10%-12% gradient SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA). The membranes were blocked in 5% skim milk for 1 h, and processed for immunolabeling for proteins of interest. Membranes were incubated with the following antibodies: Phospho-Akt (1:300), Akt (1:300), phospho-ERK (1:300), ERK (1:300), XBP-1 (1:300), ATF6 (1:300), CHOP (1:300), GRP78 (1:300), caspase-12 (1:1000), α-synuclein (1:300) and TH (1:800) (Santa Cruz Biotech, Santa Cruz, CA, USA) overnight at 4°C. Follow day, membranes were washed with Triton X-100 in TBS 3 times for 5 min, and incubated for 1 h with appropriate HRP-conjugated secondary antibodies at room temperature. Blots were washed and exposed by using a ChemiDocTM XRS+ Imaging System (Bio-Rad, Hercules, CA). Densitometry analysis band intensity was performed using Multi Gauge Software of Science Lab 2006 (FUJIFILM Corporation, Tokyo, Japan).

Statistical analysis

All values are expressed as the mean ± SEM. Data from at least 3 sets of independent experiments were analyzed by one-way analysis-of-variance (ANOVA), followed by a Dunnett's *post hoc* test. Values of $P < 0.05$ were considered significant.

Results

FGF1 ameliorated the behavioral of 6-OHDA-induced PD rats

To evaluate the protective effects of FGF1 on 6-OHDA-induced PD animal model, PD rats were injected with exogenous FGF1 through the

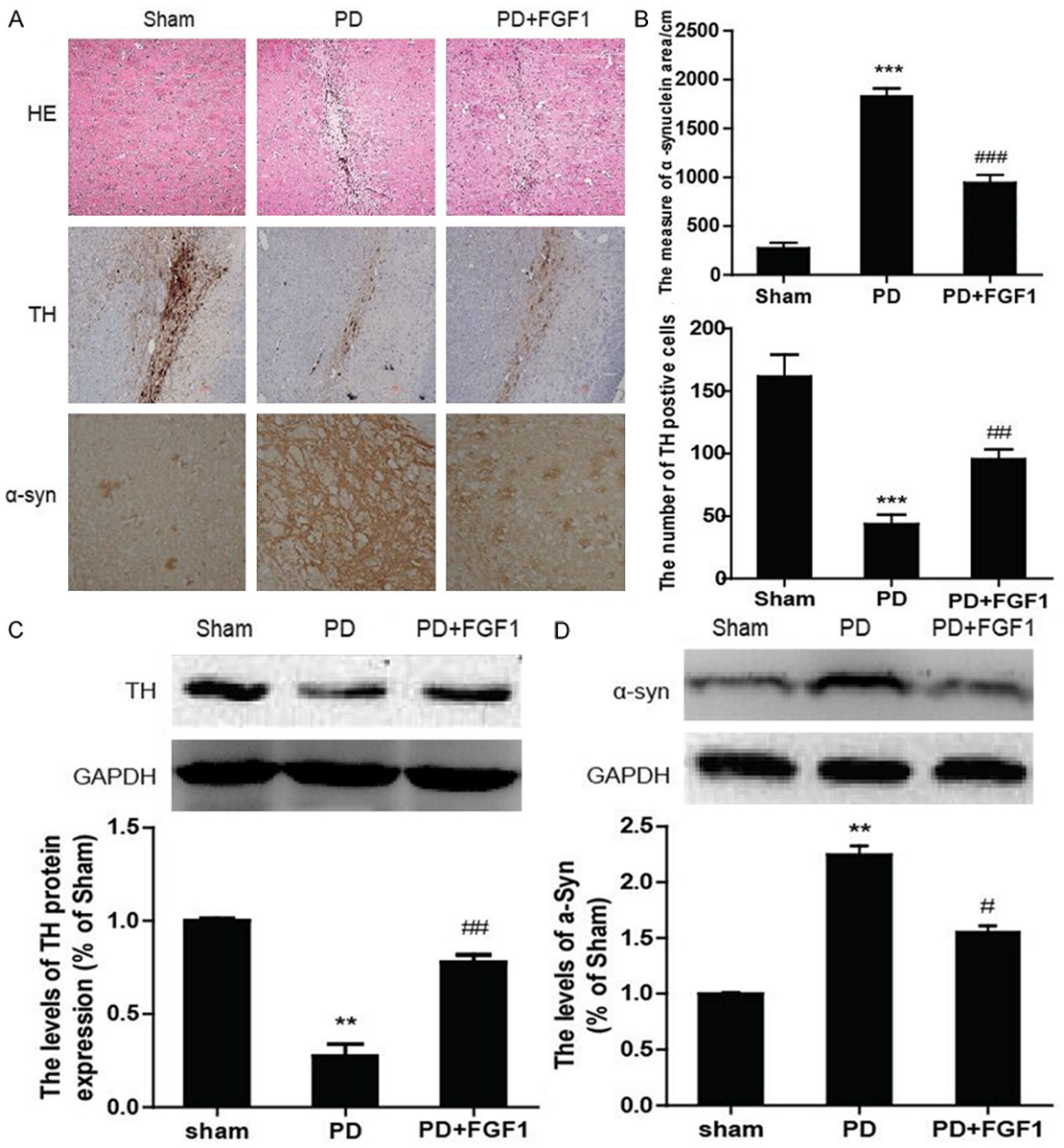


Figure 2. FGF1 inhibited α-synuclein protein and increased the level of TH in PD rats. A. The HE staining, immunohistochemistry of TH and α-synuclein in nigrostriatal. B. The optical density of α-synuclein-positive and TH-positive cells. C. The protein expressions of TH in nigrostriatal. D. The protein expressions of α-synuclein in nigrostriatal. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Sham group, $\#P < 0.05$ vs. PD group, n = 8.

tail vein for 14 days continuously. In regard to rats' ability to rotate in response to apomorphine was measured, as shown in **Figure 1A**, PD group and FGF1-treated rats rotated from the contralateral to the affected side, while the sham group showed no rotational behavior. As expected, the number of turns of FGF1 administrate remarkably decreased by 3.12 ± 0.54 versus of PD model rats ($P < 0.05$).

Effect of FGF1 on the striatal extracellular level of neurotransmitters

In addition, to rule out the possibility that FGF1 exert beneficial effect by improvement the monoamine neurotransmitters in the corpus striatum, HPLC-ECD was performed for detecting the neurotransmitters levels. Compared with sham group, the levels of neurotransmit-

ters in the PD group, including DA, DOPAC, HVA, NE, E and 5-HIAA, decreased remarkably. After the dose of 80 μg/kg/day FGF1 treatment, the levels of DA, DOPAC, HVA, NE and 5-HIAA markedly increased. It should be noted another two neurotransmitters, E and 5-HT, not be reversed by FGF1 significantly, implying FGF1 did not improve all of the neurotransmitters (**Figure 1B-D**). Taken together, these findings indicated that FGF1 alleviates 6-OHDA-induced motor dysfunction and increase some neurotransmitters secretion.

FGF1 attenuated the decrease of TH-positive neurons and suppressed the expression of α-synuclein

TH staining was performed to evaluate the survival of dopaminergic neurons. Morphological observations are shown in **Figure 2A**, in sham group, the cytoplasm and fibers of dopaminergic neurons were intensely stained and the cellular processes were evident. In contrast, rats in the model group showed a marked loss of DA-containing SN neurons in the right side, as few TH-positive cells were detected and the cellular processes were absent for most cells. FGF1 administration resulted in an increase by 80% fold in TH-positive cells with similar cell morphology to the model group (**Figure 2B**). Consistent with the immunohistochemical results, the protein levels of TH were significantly decreased in the right nigra region in PD rats, while treatment with FGF1 inhibited the activation of TH when compared with the sham group, indicating FGF1 could protect dopaminergic neurons from 6-OHDA neurotoxicity in rats (**Figure 2C**).

Alpha-synuclein is a protein with a natural tendency to aggregate into oligomers and is considered to be a key player in the pathophysiology of PD. As shown in **Figure 2A**, lots of positive neurons dense mesh and the fibrous protein exist in substantia nigra in PD group, and a small amount of expression of α-synuclein in sham group. After FGF1 treatment, the level of α-synuclein was decreased significantly (**Figure 2A**, **2B**). Western blot results also showed the same trend of the expression of α-synuclein (**Figure 2D**). Taken together, our data suggested that inhibiting α-synuclein aggregation and stimulating TH expression, might contribute to the neuroprotective activity of FGF1 in PD.

FGF1 inhibited ER stress-related protein and activated P13K/Akt and ERK1/2 in PD rats

To determine whether the neuroprotective effect of FGF1 is related to ER stress, we measured the expression of ER stress-related proteins. Our immunohistochemical results indicated that few ER stress-related proteins CHOP, GRP78 and cleaved caspase-12 were found in the cells of right nigra and corpus striatum region of the sham group. However, in the PD rat, cells positive for CHOP, GRP78 and cleaved caspase-12 were significantly increased in the same region when compared with the sham group. Moreover, FGF1 treatment suppressed by approximate 50% the activation of these ER stress-related proteins of PD rats (**Figure 3A**, **3B**). The expression of CHOP, GRP78 and cleaved caspase-12 were also determined by western blot analysis. Consistent with the immunohistochemical results, the protein levels of CHOP, GRP78 and cleaved caspase-12 were significantly upregulated in the right nigra region in PD rats, while treatment with FGF1 inhibited the activation of ER stress-related proteins when compared with the respective control group (**Figure 3C**, **3D**).

To further understand the underlying mechanism behind the effect of FGF1 on PD model rats, the activation of PI3K/Akt and ERK1/2 downstream signals was also analyzed by western blot. As expected, FGF1 treatment increased the phosphorylation of Akt and ER1/2 when compared with controls (**Figure 3E**, **3F**). Taken together, these results demonstrate that the protective role of FGF1 in PD is related to the inhibition of ER stress through activation of the PI3K/Akt and ERK1/2 signaling pathways.

FGF1 inhibits 6-OHDA-induced apoptosis in PC12 cells

PC12 cells were treated with 6-OHDA to mimic the PD model *in vitro*. There was a dose-dependent decrease in cell viability following 6-OHDA exposures to PC12 cells (**Figure 4A**). Cell viability significant decreased by approximately 40% after PC12 cells were treated with 150 μM 6-OHDA for 24 h. Conversely, cells treated with various concentration of FGF1 for the addition of 6-OHDA (150 μM) for 24 h showed that cell

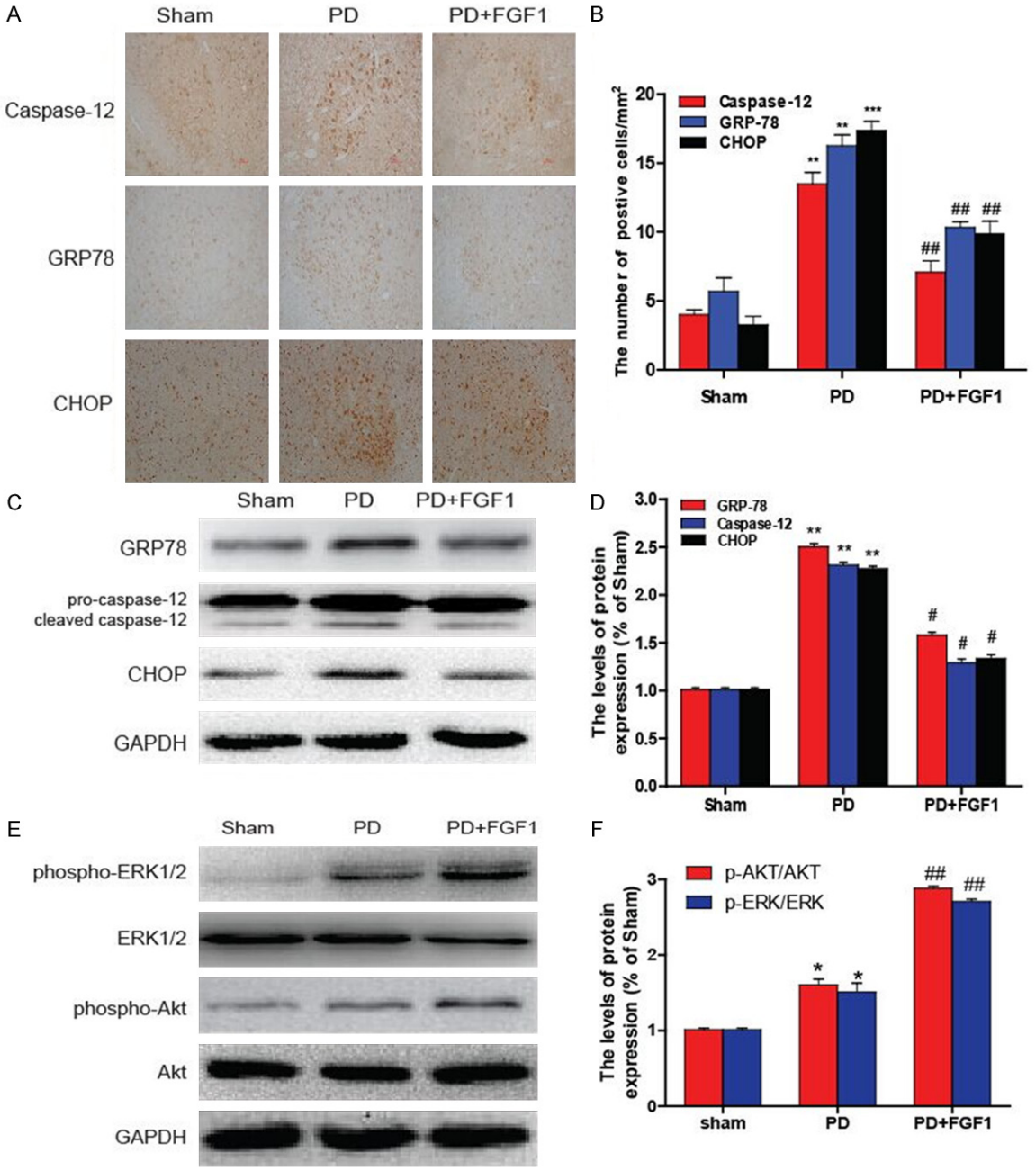


Figure 3. FGF1 inhibited the ER stress proteins and activates PI3K/Akt and ERK1/2 signals in PD rats. A. The results of immunohistochemistry for Caspase-12, GRP78 and CHOP. B. Analysis of the positive cells and optical density. C. The tissue lysates from striatum were analyzed by western blotting for the expression of GRP78, Caspase-12 and CHOP. D. The optical density analysis of GRP78, Cleaved-caspase 12 and CHOP protein. E. Western blotting for the expression of phospho-Akt, Akt, phospho-ERK and ERK. F. Bar diagram of phospho-Akt/Akt and phospho-ERK/ERK levels from three western blot analyses. $**P < 0.01$, $***P < 0.001$ vs. Sham group, $\#\#P < 0.01$ vs. PD group, n = 8.

viability increased significantly (**Figure 4B**). To confirm the effect of FGF1 on the 6-OHDA-induced apoptosis in PC12 cells, flow cytometry were performed. After 24 h of incubation with 150 μM 6-OHDA, the percentage of early apoptosis increased significantly, while 6-OHDA-induced apoptosis was inhibited by treatment of exogenous FGF1 (20 ng/ml) by approximately 50%, FGF1 alone did not display any obvious effect (**Figure 4C**, **4D**). Collectively, these data indicated FGF1 inhibits 6-OHDA-induced apoptosis in PC12 cells.

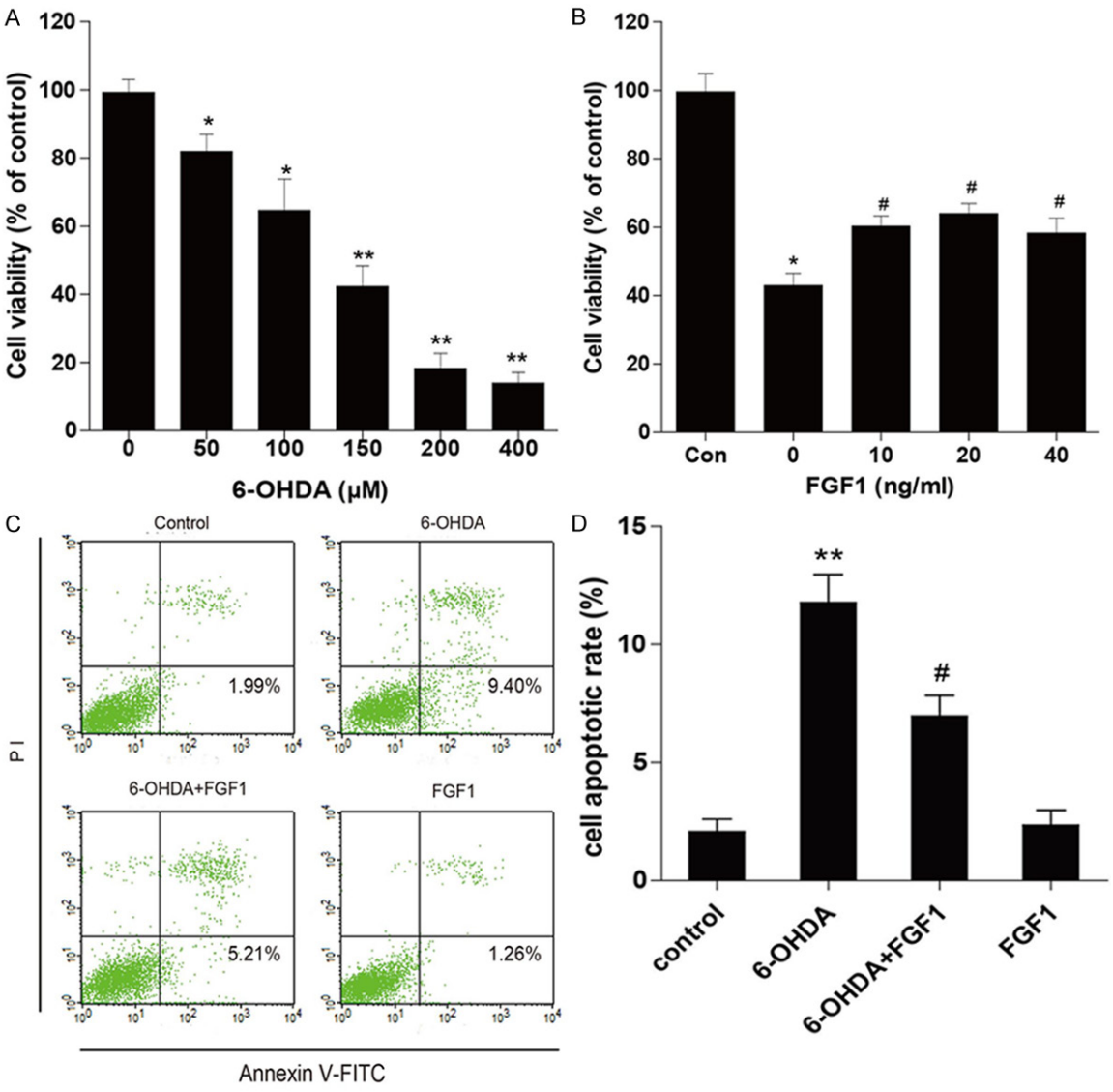


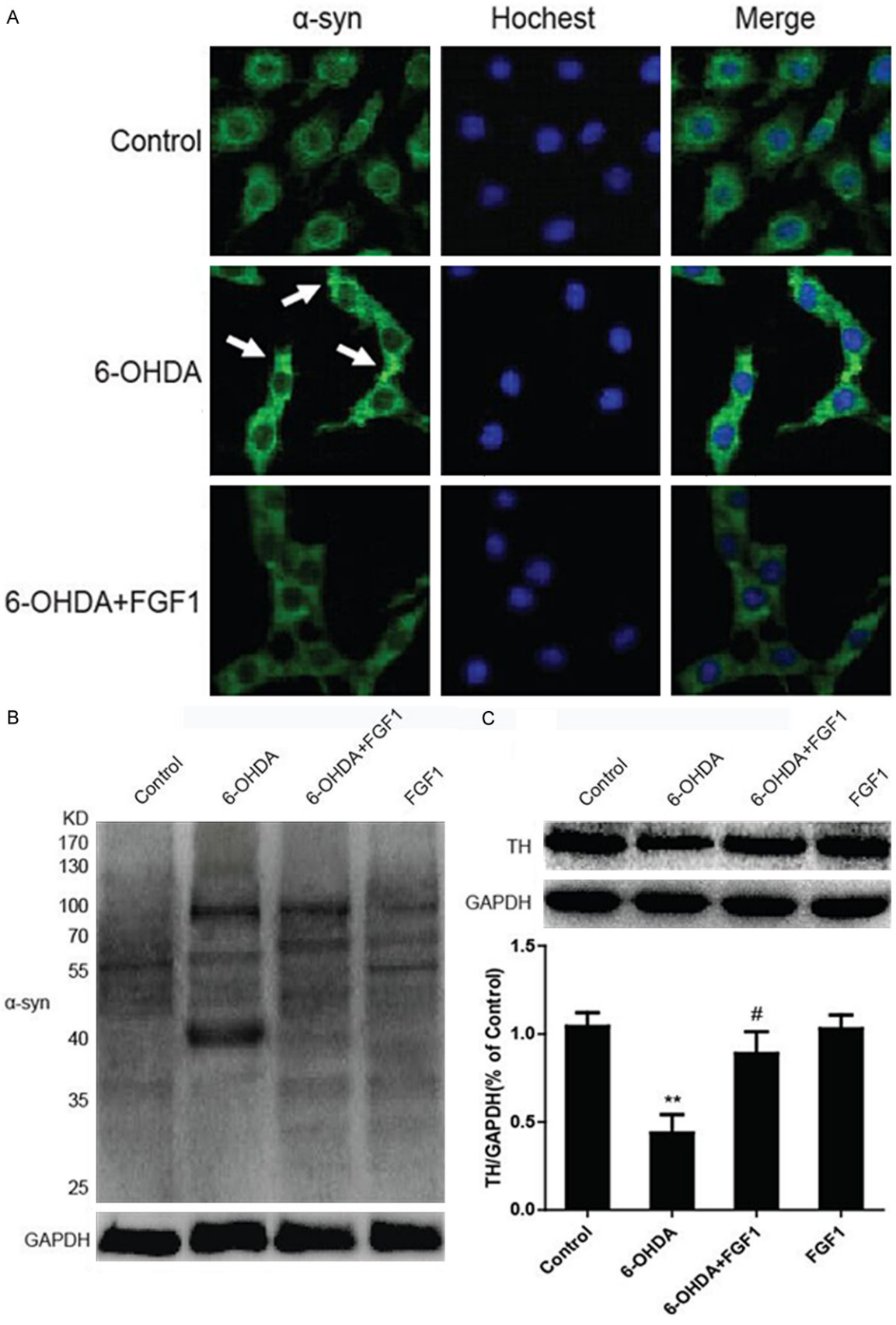
Figure 4. FGF1 inhibited 6-OHDA-induced apoptosis in PC12 cells. A. PC12 cells were treated with different concentrations of 6-OHDA for 24 h, cell viability was assessed by MTT assay. B. PC12 cells were treated with FGF1 and 6-OHDA for 24 h, cell viability was assessed by MTT assay. C. PC12 cells were treated with FGF1 and 6-OHDA for 24 h, cells were then stained with annexin V-FITC/propidium iodide and detected by flow cytometry; the lower right panel indicates the apoptotic cells. D. Bar diagram of the apoptotic cell rates. *$P < 0.05$ compared with control group, #$P < 0.05$ compared with 6-OHDA group, n = 3.

FGF1 attenuated the accumulation of α-synuclein and stimulated TH expression in PC12 cells

To determine if FGF1 attenuates α-synuclein aggregate and subsequent fibrillogenesis, immunocytochemistry and western blot with α-synuclein antibody were performed. After 6-OHDA treatment, PC12 cells showed marked α-synuclein accumulation compare with control group. However, FGF1 incubation remarkably reduced the expression of α-synuclein (**Figure 5A**). Western blot results also confirmed FGF1 significantly decreased the accumulation of α-synuclein and increased TH levels in 6-OHDA induced PC12 cells (**Figure 5B**, **5C**).

FGF1 inhibited 6-OHDA-induced ER stress and activated PI3K/Akt and ERK1/2 pathways in PC12 cells

To illustrate whether the molecular mechanism of FGF1 is related to the regular of ER stress in PC12 cells, the protein expression of ER stress-


A
α-syn
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Merge
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6-OHDA
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170
130
100
70
55
α-syn
40
35
25
GAPDH
C
Control
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TH/GAPDH(% of Control)
1.5
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Control
6-OHDA
6-OHDA+FGF1
FGF1

Figure 5. FGF1 increased the level of TH and inhibited α-synuclein protein accumulation induced by 6-OHDA in PC12 cell. A. The immunofluorescence staining of α-synuclein in control, 6-OHDA and FGF1 group. B. The protein expressions of α-synuclein in control, FGF1, 6-OHDA and FGF1 group. C. The protein expressions and optical density analysis of TH in control, FGF1, 6-OHDA and FGF1 group. $^{**}P < 0.001$ vs. control group; $\#P < 0.05$ vs. PD group, n = 3.

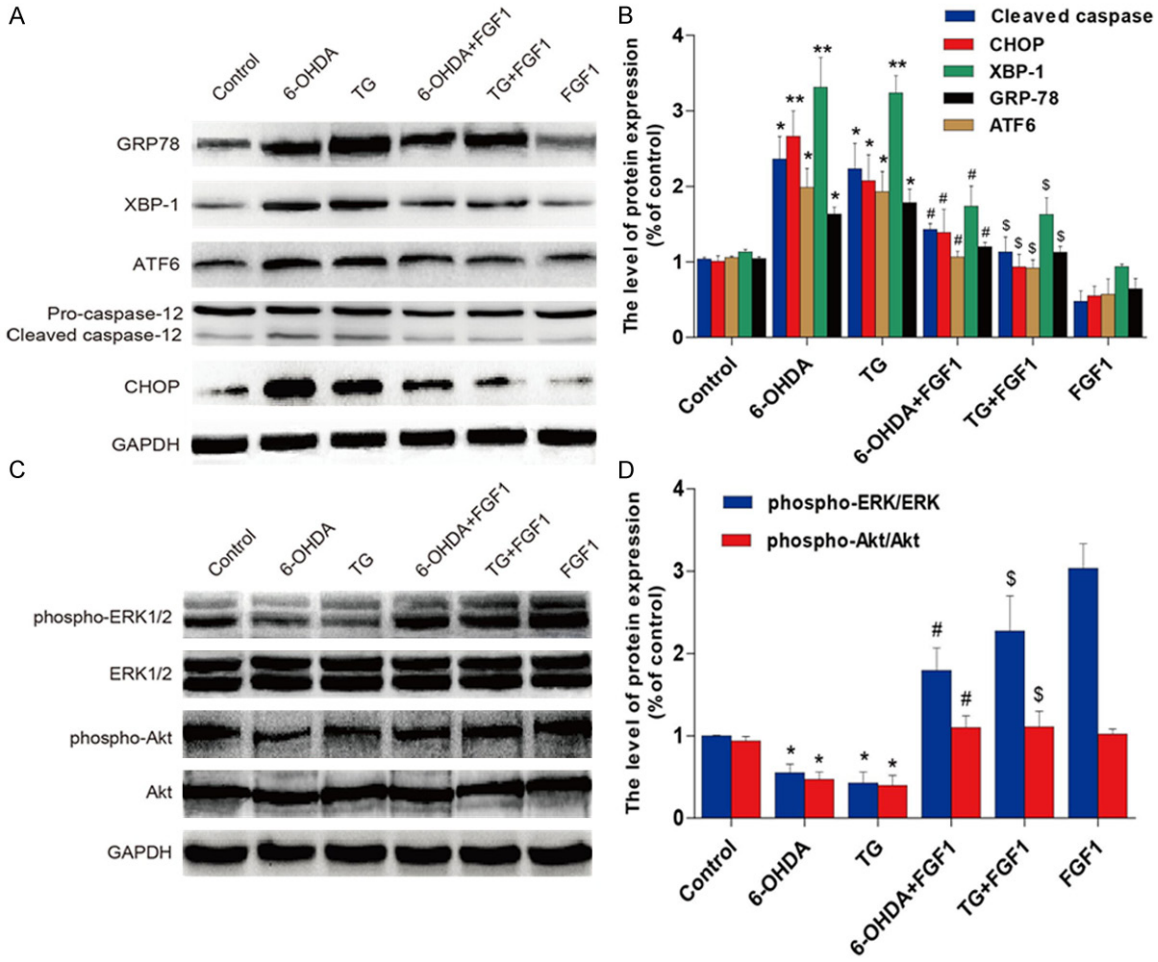

Figure 6. FGF1 activated PI3K/Akt and ERK1/2 signals and inhibited ER stress proteins induced by 6-OHDA and TG in PC12 cells. A. The results of western blot for XBP-1, ATF-6, Caspase-12, GRP78 and CHOP. B. The optical density analysis of XBP-1, ATF-6, Caspase-12, GRP78 and CHOP. C. Western blotting for the expression of phospho-Akt, Akt, phospho-ERK and ERK in PC12 cells. D. Bar diagram of phospho-Akt/Akt and phospho-ERK/ERK levels from three western blot analyses. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group, $\#P < 0.05$, $\#\#P < 0.01$ vs. 6-OHDA group, $\$P < 0.05$ vs. TG group, n = 3.

induced apoptosis were detected by western blot. As shown in **Figure 6A**, the expression of GRP78, XBP-1, ATF-6, cleaved caspase-12 and CHOP was significantly increased in the 6-OHDA incubated cells when compared with the control group, which was consistent with ER stress activator TG. FGF1 treatment alone had no effect on the level of the ER stress response proteins. However, GRP78, CHOP, cleaved caspase-12, XBP-1 and ATF-6 was down regulated in the group exposed to 6-OHDA/TG plus FGF1 (**Figure 6A-C**), indicating that the protective role of FGF1 may involve the inhibition of ER stress-induced proteins.

PI3K/Akt and ERK1/2 pathways are the main downstream signals of FGF1, we hypothesized that the PI3K/Akt and ERK1/2 pathways may be involved in the downstream of FGF1 downstream effects of FGF1-induced inhibition of ER stress in 6-OHDA-induced PD model. As shown in **Figure 6D**, an increase in p-Akt and p-ERK1/2

was observed in the PC12 cells exposed to 6-OHDA when compared with control cells. The treatment of FGF1 significantly increased the activation of the PI3K/Akt and ERK1/2 pathways in the PC12 cells exposed to 6-OHDA (**Figure 6D**). These data suggest that both the PI3K/Akt and ERK1/2 pathways may involve in the protective effect of FGF1.

Discussion

FGF1 was first found as a mitogen in the mammalian brain in 1990s [15]. The bioactivities of FGF1 include stimulating neuron survival, differentiation, growth and response to injury of DA neurons [16-18]. FGF1 have been shown to support survival of embryonic dopaminergic neurons with varying degrees of potency and specificity [19]. FGF1 improve akinesia and striatal uptake of F-DOPA measured by positron emission tomography (PET) [20]. In this study, we observed treatment with FGF1 produced a protective effect by increasing TH-positive neuron cells and improving locomotor activity of the PD rats. That is, consistent with the single published study about FGF1 and PD [12], our data proved FGF1 exist neuroprotection in 6-OHDA-indcued PD model rats.

HPLC with a new acetylene black nanoparticles modified electrode (HPLC-ED) were performed on the monoamine neurotransmitters and their metabolites change in this study. Acetylene black nanoparticles (ABNs) modified electrode had excellent catalytic activity for the oxidation of monoamine neurotransmitters and their metabolites with relatively high sensitivity, stability and long life [21]. Our results showed 6-OHDA reduced the neurotransmitters in PD rats. However, bFGF reversed the decreasing neurotransmitters by 6-OHDA, such as DA, DOPAC, HVA, NE and 5-HIAA, suggested bFGF exerts neuroprotective activity in PD animal model. This study is valuable for neurochemical measurements, because of the level of monoamine neurotransmitters and their metabolites in rat striatum of conscious and freely moving PD rats could be easily assessed when combined with *in vivo* microdialysis.

Growing evidence from studies in human PD, genetic or toxicological models, indicates that ER Stress is a common feature of the disease and contributes to neurodegeneration [22, 23]. The brains of PD patients were detected by higher levels of phosphorylated PERK and downstream eIF2α [24]. 6-OHDA and MPP+ were found to increase the phosphorylation of UPR proteins, XBP-1, PERK and eIF2α in a MN9D cell line [25], Grp78 and CHOP are well-known indicators of ER stress, and an increase in levels signals ER stress induction; moreover, CHOP pathway is a major regulator of ER stress-induced apoptosis, as $CHOP^{-/-}$ cells exhibit less programmed cell death [26]. CHOP is the initial signal to trigger the apoptosis pathway, and it represses the promoter of bcl-2 gene and renders cells sensitive to the proapoptotic effects [27, 28]. In this study, we investigated the ER stress induced by 6-OHDA *in vivo* and *in vitro.* As has been reported in previous research [29, 30], we found that exposure to 6-OHDA decreased TH express, cell viability with increased expression of GRP78, CHOP and caspase-12, suggesting that ER stress plays a potential role in 6-OHDA-induced neuron death in PD models.

Given the verified relationship between ER Stress and PD, researchers are constantly searching for new targets for the treatment of PD based on ER Stress-related mechanisms. In organotypic hippocampal slice cultures, mithramycin were found to confer resistance to ER Stress-induced neurotoxicity [31]. Our pervious study showed neurotrophic factors, such as FGF2, protect oxidative injury or ischemia and reperfusion injury in stroke and spinal cord injury is related to the inhibition of ER stress-induced neurocyte apoptosis [32, 33]. In this study, the levels of these ER stress-induced apoptosis proteins after dopaminergic neuronal injury were detected to investigate the molecular mechanism of FGF1 in PD. We found FGF1 decrease in the ER stress response protein GRP78, CHOP, caspase-12 in the cytoplasm and fibers of dopaminergic neurons in PD rats. Moreover, a suitable concentration of FGF1 could also suppress the upregulation of ER stress response proteins and significantly decrease 6-OHDA-induced apoptotic cell rate. These results indicate that ER stress was involved in PD and could be inhibited by exogenous FGF1 administration.

PD is characterized by the accumulation of Lewy bodies, which are mainly composed by aggregated α-synuclein. The pathological aggregation of α-synuclein within dopaminergic cells induced cell death and α-synuclein over-

expression triggers apoptotic mechanism. Recent evidence showed α-synuclein may act as a neuronal sensor, whose misfolding, aggregation and accumulation, induced as a consequence of cell stress, then in turn activate ER stress-related responses [34]. Treating the α-synuclein-transgenesis mouse model the rat AAV2/6 model of α-synucleinopathy with Salubrinal, a pharmacological inhibitor of ER-stress toxicity, dramatically delays the onset of motoric symptoms and decreases accumulation of α-synuclein oligomers [6, 35]. Thus, we hypothesis that FGF1 modulates the UPR, blocks apoptosis, and promotes the survival of nigral dopamine neurons in a rat model of PD induced by elevated level of α-synucleinopathy. In 6-OHDA-induced PD model rats, we found FGF1 diminished α-synuclein neurotoxicity by down regulating ER stress mediators and the level of apoptosis, prompted survival of nigral tyrosin TH-positive cells and resulted in higher levels of striatal DA. *In vitro* PD model, aggregation of α-synuclein was also observed both in 6-OHDA and TG, a specifically ER stress inducer, induced PC12 cells. At the same time, FGF1 attenuated the accumulation of α-synuclein, inhibited ER stress signal pathway, decreased apoptosis rates in 6-OHDA and TG induced PC12 cells. Collectively, FGF1 prevents the death of DA neuron at least in part by reducing the levels of α-synuclein and ER stress. It should be note, our study did not figure out the relationship of ER stress and α-synuclein by FGF1 treatment, which need to further research.

The PI3K/Akt and ERK1/2 pathways are two main downstream signals activated by bFGF. The PI3K/Akt pathway is necessary for mediating neuronal survival under a variety of circumstances and for the trophic factor-induced cell survival of several neuronal cell types [36]. Activated ERK kinase was also found to induce the neuronal transdifferentiation of dopamine neuron cells [37]. For instance, Akt and Erk phosphorylate the pro-apoptotic Bcl-2 family member Bax and thereby inhibit Bax's pro-apoptotic functions [38]. Although the PI3K/Akt and ERK1/2 signals activation is generally considered a prosurvival signal, the role of the PI3K/Akt and MAPK/ERK cascade in the protective effects of bFGF and ER stress in 6-OHDA-induced PD models needs to be characterized. In our study, FGF1 also shows a neuronal protective effect in a PD rat model and in 6-OHDA-induced PC12 cell model via the activation of both the PI3K/Akt and ERK1/2 signals. Further studies are needed to demonstrate whether the protective effect of FGF1 and activation of the PI3K/Akt pathway are mediated by the reduction the aggregation of α-synuclein to the ER.

Over the past 20 years, FGF1 have generated considerable excitement for the potential as therapy for neurodegenerative disease including PD [11]. FGF1 appeared to be efficacious in phase II and phase I clinical trials in spinal cord injury [39, 40], to explore the appropriate therapeutic time window and special drug delivery system combined with factors still need long-term study. It also need to be addressed that FGF1 is not stable enough which is easy to be degraded by various enzymes in vitro, resulting in the loss of biological activity. So the combination with other drugs or delivery systems to increase its stability and smoothly through the blood-brain-barrier may contribute to the functions of FGF1. Moreover, the mechanism of PD also undefined clearly, this study lays the groundwork for future translational confidence of FGF1 in neurodegenerative disease, especially the relations to ER stress. Collectively, the translational application study of FGF1 may not only focus on the effect in the neurogenesis, the combination with special biomaterials which can improves the efficiency and prolongs the effective time should also be addressed.

Conclusion

In summary, the present study demonstrated that FGF1 significant ameliorates 6-OHDA-induced PD, as evidenced by improved rotational test, diminished α-synuclein neurotoxicity by down regulating ER stress mediators and the level of apoptosis, prompted survival of nigral tyrosin TH-positive cells. FGF1 inhibits ER stress-induced apoptosis may via activation of the PI3K/Akt and ERK1/2 pathways. Our study demonstrates that FGF1 therapy may be benefit for recoveries in neurodegenerative diseases include PD, while the underlying mechanism is necessary in the further studies.

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Disclosure of conflict of interest

None.

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References

[1] Bandopadhyay R and de Belleroche J. Pathogenesis of Parkinson's disease: emerging role of molecular chaperones. Trends Mol Med 2010; 16: 27-36.

[2] Lee JS, Mendez R, Heng HH, Yang ZQ and Zhang K. Pharmacological ER stress promotes hepatic lipogenesis and lipid droplet formation. Am J Transl Res 2012; 4: 102-113.

[3] Sano R and Reed JC. ER stress-induced cell death mechanisms. Biochim Biophys Acta 2013; 1833: 3460-3470.

[4] Ryu EJ, Harding HP, Angelastro JM, Vitolo OV, Ron D and Greene LA. Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. J Neurosci 2002; 22: 10690-10698.

[5] Colla E, Jensen PH, Pletnikova O, Troncoso JC, Glabe C and Lee MK. Accumulation of toxic alpha-synuclein oligomer within endoplasmic reticulum occurs in alpha-synucleinopathy in vivo. J Neurosci 2012; 32: 3301-3305.

[6] Colla E, Coune P, Liu Y, Pletnikova O, Troncoso JC, Iwatsubo T, Schneider BL and Lee MK. Endoplasmic reticulum stress is important for the manifestations of alpha-synucleinopathy in vivo. J Neurosci 2012; 32: 3306-3320.

[7] Sullivan AM and Toulouse A. Neurotrophic factors for the treatment of Parkinson's disease. Cytokine Growth Factor Rev 2011; 22: 157-165.

[8] Chiocco MJ, Harvey BK, Wang Y and Hoffer BJ. Neurotrophic factors for the treatment of Parkinson's disease. Parkinsonism Relat Disord 2007; 13 Suppl 3: S321-328.

[9] Beenken A and Mohammadi M. The FGF family: biology, pathophysiology and therapy. Nat Rev Drug Discov 2009; 8: 235-253.

[10] Baron O, Ratzka A and Grothe C. Fibroblast growth factor 2 regulates adequate nigrostriatal pathway formation in mice. J Comp Neurol 2012; 520: 3949-3961.

[11] Timmer M, Cesnulevicius K, Winkler C, Kolb J, Lipokatic-Takacs E, Jungnickel J and Grothe C. Fibroblast growth factor (FGF)-2 and FGF receptor 3 are required for the development of the substantia nigra, and FGF-2 plays a crucial role for the rescue of dopaminergic neurons after 6-hydroxydopamine lesion. J Neurosci 2007; 27: 459-471.

[12] Date I, Notter MF, Felten SY and Felten DL. MPTP-treated young mice but not aging mice show partial recovery of the nigrostriatal dopaminergic system by stereotaxic injection of acidic fibroblast growth factor (aFGF). Brain research 1990; 526: 156-160.

[13] Truong L, Allbutt H, Kassiou M and Henderson JM. Developing a preclinical model of Parkinson's disease: a study of behaviour in rats with graded 6-OHDA lesions. Behav Brain Res 2006; 169: 1-9.

[14] Lee HJ, Shin SY, Choi C, Lee YH and Lee SJ. Formation and removal of alpha-synuclein aggregates in cells exposed to mitochondrial inhibitors. J Biol Chem 2002; 277: 5411-5417.

[15] Olson L. Grafts and growth factors in CNS. Basic science with clinical promise. Stereotact Funct Neurosurg 1990; 54-55: 250-267.

[16] Garcia de Yebenes J, Yebenes J and Mena MA. Neurotrophic factors in neurodegenerative disorders: model of Parkinson's disease. Neurotox Res 2000; 2: 115-137.

[17] Baird A. Fibroblast growth factors: activities and significance of non-neurotrophin neurotrophic growth factors. Curr Opin Neurobiol 1994; 4: 78-86.

[18] Giacobini MM, Stromberg I, Almstrom S, Cao Y and Olson L. Fibroblast growth factors enhance dopamine fiber formation from nigral grafts. Brain Res Dev Brain Res 1993; 75: 65-73.

[19] Recchia A, Debetto P, Negro A, Guidolin D, Skaper SD and Giusti P. Alpha-synuclein and Parkinson's disease. FASEB J 2004; 18: 617-626.

[20] de Yebenes JG, Pernaute RS, Garrido JM, Rabano A, Albisua J, Rojo A, Mena MA, Ruiz PG, Jorge P, Correa C, Leenders K, Antonini A, Gunther I, Psylla M and Vontobel P. Long-term intracerebral infusion of fibroblast growth factors restores motility and enhances F-DOPA uptake in parkinsonian monkeys. Parkinsonism Relat Disord 1998; 4: 147-158.

[21] Lin L, Yang J, Lin R, Yu L, Gao H, Yang S and Li X. In vivo study on the monoamine neurotransmitters and their metabolites change in the striatum of Parkinsonian rats by liquid chromatography with an acetylene black nanoparti-

cles modified electrode. J Pharm Biomed Anal 2013; 72: 74-79.
[22] Mercado G, Valdes P and Hetz C. An ERcentric view of Parkinson's disease. Trends Mol Med 2013; 19: 165-175.
[23] Zhou JX, Zhang HB, Huang Y, He Y, Zheng Y, Anderson JP, Gai WP, Liang ZG, Wang Y, Ren XM, Wang Q, Gong XL, Yang J, Wang X, Halliday G and Wang XM. Tenuigenin attenuates alpha-synuclein-induced cytotoxicity by down-regulating polo-like kinase 3. CNS Neurosci Ther 2013; 19: 688-694.
[24] Hoozemans JJ and Scheper W. Endoplasmic reticulum: the unfolded protein response is tangled in neurodegeneration. Int J Biochem Cell Biol 2012; 44: 1295-1298.
[25] Holtz WA and O'Malley KL. Parkinsonian mimetics induce aspects of unfolded protein response in death of dopaminergic neurons. J Biol Chem 2003; 278: 19367-19377.
[26] Silva RM, Ries V, Oo TF, Yarygina O, Jackson-Lewis V, Ryu EJ, Lu PD, Marciniak SJ, Ron D, Przedborski S, Kholodilov N, Greene LA and Burke RE. CHOP/GADD153 is a mediator of apoptotic death in substantia nigra dopamine neurons in an in vivo neurotoxin model of parkinsonism. J Neurochem 2005; 95: 974-986.
[27] Zhang HY, Wang ZG, Lu XH, Kong XX, Wu FZ, Lin L, Tan X, Ye LB and Xiao J. Endoplasmic Reticulum Stress: Relevance and Therapeutics in Central Nervous System Diseases. Mol Neurobiol 2014; [Epub ahead of print].
[28] Wan XS, Lu XH, Xiao YC, Lin Y, Zhu H, Ding T, Yang Y, Huang Y, Zhang Y, Liu YL, Xu ZM, Xiao J and Li XK. ATF4- and CHOP-dependent induction of FGF21 through endoplasmic reticulum stress. Mol Neurobiol 2014; 2014: 807874.
[29] Tanaka K, Fukuoka S, Kawahara S, Kimoto N and Ogawa N. Effect of cabergoline on increase of several ER stress-related molecules in 6-OHDA-lesioned mice. Neurol Sci 2013; 34: 259-261.
[30] Kosuge Y, Taniguchi Y, Imai T, Ishige K and Ito Y. Neuroprotective effect of mithramycin against endoplasmic reticulum stress-induced neurotoxicity in organotypic hippocampal slice cultures. Neuropharmacology 2011; 61: 252-261.
[31] Hung CC, Ichimura T, Stevens JL and Bonventre JV. Protection of renal epithelial cells against oxidative injury by endoplasmic reticulum stress preconditioning is mediated by ERK1/2 activation. J Biol Chem 2003; 278: 29317-29326.
[32] Zhang HY, Zhang X, Wang ZG, Shi HX, Wu FZ, Lin BB, Xu XL, Wang XJ, Fu XB, Li ZY, Shen CJ, Li XK and Xiao J. Exogenous basic fibroblast growth factor inhibits ER stress-induced apoptosis and improves recovery from spinal cord injury. CNS Neurosci Ther 2013; 19: 20-29.
[33] Wang Z, Zhang H, Xu X, Shi H, Yu X, Wang X, Yan Y, Fu X, Hu H, Li X and Xiao J. bFGF inhibits ER stress induced by ischemic oxidative injury via activation of the PI3K/Akt and ERK1/2 pathways. Toxicol Lett 2012; 212: 137-146.
[34] Bellucci A, Navarria L, Zaltieri M, Falarti E, Bodei S, Sigala S, Battistin L, Spillantini M, Missale C and Spano P. Induction of the unfolded protein response by alpha-synuclein in experimental models of Parkinson's disease. J Neurochem 2011; 116: 588-605.
[35] Gorbatyuk MS, Shabashvili A, Chen W, Meyers C, Sullivan LF, Salganik M, Lin JH, Lewin AS, Muzyczka N and Gorbatyuk OS. Glucose regulated protein 78 diminishes alpha-synuclein neurotoxicity in a rat model of Parkinson disease. Mol Ther 2012; 20: 1327-1337.
[36] Liao Y and Hung MC. Physiological regulation of Akt activity and stability. Am J Transl Res 2010; 2: 19-42.
[37] Zhang Q, Zhang J, Jiang C, Qin J, Ke K and Ding F. Involvement of ERK1/2 pathway in neuroprotective effects of pyrroloquinoline quinine against rotenone-induced SH-SY5Y cell injury. Neuroscience 2014; 270: 183-191.
[38] Sanchez A, Tripathy D, Yin X, Luo J, Martinez J and Grammas P. Pigment epithelium-derived factor (PEDF) protects cortical neurons in vitro from oxidant injury by activation of extracellular signal-regulated kinase (ERK) 1/2 and induction of Bcl-2. Neurosci Res 2012; 72: 1-8.
[39] Wu JC, Huang WC, Chen YC, Tu TH, Tsai YA, Huang SF, Huang HC and Cheng H. Acidic fibroblast growth factor for repair of human spinal cord injury: a clinical trial. J Neurosurg Spine 2011; 15: 216-227.
[40] Wu JC, Huang WC, Tsai YA, Chen YC and Cheng H. Nerve repair using acidic fibroblast growth factor in human cervical spinal cord injury: a preliminary Phase I clinical study. J Neurosurg Spine 2008; 8: 208-214.