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Activation of Wnt/β-catenin Pathway by Exogenous Wnt1 Protects SH-SY5Y Cells Against 6-Hydroxydopamine Toxicity

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Abstract Wnt1, initially described as a modulator of embryonic development, has recently been discovered to exert cytoprotective effects in cellular models of several diseases, including Parkinson's disease (PD). We, therefore, examined the neuroprotective effects of exogenous Wnt1 on dopaminergic SH-SY5Y cells treated with 6-hydroxydopamine (6-OHDA). Here, we show that 10–500 μM 6-OHDA treatment decreased cell viability and increased lactate dehydrogenase (LDH) leakage. SH-SY5Y cells treated with 100 μM 6-OHDA for 24 h showed reduced Wnt/β-catenin activity, decreased mitochondrial transmembrane potential, elevated levels of reactive oxidative species (ROS) and phosphatidylserine (PS) extraversion, increased levels of Chop and Bip/GRP78 and reduced level of p-Akt (Ser473). In contrast, exogenous Wnt1 attenuated 6-OHDA-induced changes. These results suggest that activation of the Wnt/

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β-catenin pathway by exogenous Wnt1 protects against 6-OHDA-induced changes by restoring mitochondria and endoplasmic reticulum (ER) function.

Keywords Wnt1 · Oxidative stress · Endoplasmic reticulum (ER) stress · Parkinson's disease

Introduction

Parkinson's disease (PD), characterized by loss of dopaminergic neurons in substantia nigra, is the second most common neurodegenerative diseases in elderly people. Studies have shown that several mechanisms are involved in pathogenesis of PD including oxidative stress, mitochondrial dysfunction, and elevated brain iron levels (Schapira et al. 1989; Dexter et al. 1991; Andersen 2004; Schapira and Gegg 2011). Recent evidence implies that endoplasmic reticulum (ER) stress may also play an important role in PD (Imai et al. 2001; Chen et al. 2004) and multiple signal pathways regulating apoptosis or survival are also implicated in the disease process (Weinreb et al. 2006).

Wnt signaling pathway is an autocrine–paracrine signal transduction pathway which has been demonstrated to participate in embryonic development, cell differentiation, and oncogenesis (Yamaguchi 2001; Li et al. 2006; Mazemondet et al. 2011; Vidya Priyadarsini et al. 2012). A main Wnt signaling pathway branch is the wnt/β-catenin pathway, which initiates with Wnt proteins binding to Frizzled receptors and activates Dishevelled. Activation of Dishevelled results in inhibition of glycogen synthase kinase-3β (GSK3β), which, in turn, causes stabilization of β-catenin. Stabilized β-catenin accumulates and is taken into the nuclear where it regulates expression of numerous genes (Nusse 1999).

Extensive research has confirmed the vital role of Wnt/β-catenin signaling in midbrain dopaminergic neuronal development (Castelo-Branco et al. 2003, 2004). The protective effects of Wnt/β-catenin pathway have also been demonstrated in animal and cellular models of Alzheimer's disease, retinal degeneration, and cerebral ischemia (De Ferrari et al. 2003; Lin et al. 2009; Chong et al. 2010). Recent studies found that Wnt1 acts as a candidate component of neuroprotective pathways in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced nigrostriatal dopaminergic plasticity (L'Episcopo et al. 2011b), and Wnt1/β-catenin pathway exerts its protective effects in dopamine (DA) toxicity models through Frizzled-1 receptors (L'Episcopo et al. 2011a). Consistent with these findings, we report that in 6-hydroxydopamine (6-OHDA)-treated dopaminergic SH-SY5Y cells, a cellular model of PD, exogenous Wnt1 protected cells from 6-OHDA neurotoxicity by a mechanism that involved maintenance of normal mitochondrial and ER function.

Materials and Methods

Cell Culture

Human neuroblastoma SH-SY5Y cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), maintained in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (Invitrogen, USA) supplemented with 10 % fetal bovine serum (FBS, Invitrogen), and cultured in a humidified incubator with 5 % CO_2 at 37 °C. Cells with 20–30 passages were used. For experiments, cells were seeded at a density of 1×10^5 cells/cm^2 in the plastic flasks or plates. Different concentrations of 6-OHDA and different incubation times were carried out according to corresponding experiments and with vehicle as control. To study the protective effects of Wnt1, human recombinant Wnt1 protein (Sigma-Aldrich, USA) or vehicle were added to the cultures 20 min prior to 6-OHDA.

Cell Viability Assay

SH-SY5Y cells were seeded in a 96-well plate at a density of 1×10^3 cells per well. After attachment, cells were treated with 6-OHDA (0–300 μM) or Wnt1 (1–100 ng/ml) for the indicated times. After treatment, cells were incubated with 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) for 4 h at 37 °C (Luo et al. 2012). Following aspiration of the MTT solution, the same volume of Dimethyl sulfoxide (DMSO) was added into each well to dissolve the purple formazan crystals. Absorbance was read in a microtiter plate reader at 490 nm. Cell viability was expressed as a percentage of the absorbance from control cells. The toxic effects of 6-OHDA to SH-SY5Y cells were also detected by measuring the leakage of the cytosolic enzyme lactate dehydrogenase (LDH) to the culture medium using a colorimetric LDH assay kit (KeyGen, China) according to the manufacturer's instructions (Dong et al. 2009). Briefly, after treatment of 6-OHDA, 20 μl of cell medium was added into basic solution to measure extracellular LDH activity, which could catalyze the conversion of lactate to pyruvate, which then reacted with 2,4-dinitrophenylhydrazine to give the brownish red color. The absorbance was measured at a wavelength of 440 nm by colorimetric assay, and the LDH leakage was expressed as the percentage versus control cells.

Double Immunofluorescent Labeling of TH and Frizzled-1

SH-SY5Y cells were seeded on sterile cover glasses. After attachment, they were incubated in 4 % paraformaldehyde for 15 min at room temperature. The fixed cells were permeabilized with 0.01 M phosphate-buffered saline (PBS) containing 0.1 % Triton X-100 for 20 min and then blocked with 10 % goat serum for 1 h at room temperature. After blocking, cells were incubated overnight at 4 °C with primary antibodies diluted in 10 % goat serum/

rabbit anti-Frizzled-1 (1:500 dilution, Bioworld Technology, USA) accompanied with mouse anti-tyrosine hydroxylase (TH) (1:600 dilution, Millipore, USA). Cells were washed three times with 0.01 M PBS for 5 min. Alexa Fluor 555-conjugated goat anti-mouse antibody (CST, USA) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, Inc., West Grove, PA, USA) were used as secondary antibody at a dilution of 1:1,000, and incubated for 2 h at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, CST, USA) for 45 s at room temperature before cells were examined under a confocal laser scanning microscope LEICA TCS SP5 MP (Leica, Heidelberg, Germany).

Flow Cytometric Detection of Apoptotic Cells

SH-SY5Y cell apoptosis was quantified by flow cytometry using FITC-conjugated annexin V and propidium iodide (PI) (Bai et al. 2009). The cells were seeded in six-well plates (5×10^5 cells/well) and treated with vehicle, 6-OHDA (100 μM), or 6-OHDA plus Wnt1 (50 ng/ml) for 24 h. Then, the cells were harvested, washed with cold PBS, and double-stained using an annexin V–FITC apoptosis detection kit (KeyGen, China). According to the manufacturer's instructions, cells resuspended in annexin V–FITC binding buffer were incubated with annexin V–FITC and PI for 10 min at room temperature in the dark. The number of apoptotic cells was evaluated by flow cytometry (BD, CA, USA). At least 10,000 cells were analyzed.

Measurement of MMP and Intracellular ROS Production

Changes in the mitochondrial membrane potential with various treatments in SH-SY5Y cells were measured by rhodamine 123 or 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) using flow cytometry as described before (Dong et al. 2009). Briefly, cells were treated with vehicle, 6-OHDA, and Wnt1 (50 ng/ml) plus 6-OHDA (100 μM) for 24 h and then incubated with rhodamine 123 (Sigma-Aldrich, USA) or DCFH-DA (Sigma-Aldrich, USA) in a final concentration of 10 μmol/l or 25 μmol/l, respectively, for 30 min at 37 °C. After washing twice with 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer saline (Invitrogen, USA), fluorescence was recorded at 488 nm excitation and 525 nm emission wavelengths.

Western Blot Analysis

Immunoblotting was performed in accordance with a standard procedure (Zhang et al. 2010; Luo et al. 2012). Briefly, cells in 100-mm dishes were washed twice with prechilled PBS followed by lysis with a Mammalian Cell Extraction Kit (BioVision, CA, USA) mixed with PhosSTOP (Roche, Basel, Switzerland). After measuring the protein concentrations with a BCA protein assay kit (Thermo Fisher Scientific Inc., IL, USA), cell lysates were separated by 8–12 % SDS-polyacrylamide gels and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). After transfer, the membranes were blocked with 5 % nonfat dry milk (Sigma-Aldrich, USA) in tris-buffered saline (20 mM Tris–HCl pH 7.6, 137 mM NaCl) containing 0.01 % Tween 20 (TBST) for 1 h at room temperature and then the membranes were probed with primary antibodies diluted according to the producers' datasheet at 4 °C overnight. The following primary antibodies were used: rabbit anti-β-catenin (1:1,000 dilution, Abcam, Cambridge, UK), mouse anti-TH (1:1,000 dilution, Millipore, USA), rabbit anti-Bcl-2 (1:1,000 dilution, CST, USA), rabbit anti-Bax (1:1,000 dilution, CST, USA), rabbit anti-p-GSK3β (Ser9) (1:1,000 dilution, CST, USA), rabbit anti-p-GSK3β (Tyr216) (1:1,000 dilution, CST, USA), rabbit anti-Bip/GRP78 (1:1,000 dilution, CST, USA), rabbit anti-p-Akt (Ser473) (1:1,000 dilution, Millipore, USA), mouse anti-β-actin (1:1,000 dilution, Millipore, USA), rabbit anti-CHOP (1:1,000 dilution, Santa Cruz Biotechnology, USA). Subsequently, membranes were washed with TBST three times followed by incubation with horseradish peroxidase (HRP)-labeled anti-mouse IgG or anti-rabbit IgG (KPL, MD, USA) secondary antibodies at room temperature for 1 h. After washing membranes with TBST for three times, proteins were detected with the SuperSignal® West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., IL, USA) and membranes were exposed to X-ray films (Fujifilm Corporation, Japan), which were scanned and analyzed using the Quantity One v4.62 for Windows software (Bio-Rad, CA, USA).

Statistical Analysis

Results were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test was used to compare differences between means in more than two groups. The level of significance was set at $P<0.05$. All the statistical analyses were performed with SPSS 12.0 for windows (SPSS Inc., Chicago, IL, USA).

Results

Expression of Frizzled-1 in SH-SY5Y Cells

Using double immunofluorescent labeling and Western blot, we tested the expression of Frizzled-1 and TH in SH-SY5Y cells and found that Frizzled-1, the membrane receptors of Wnt/β-catenin signal pathway, was expressed in dopaminergic SH-SY5Y cells (Fig. 1).

Cytotoxic Effect of 6-OHDA on SH-SY5Y Cells

6-OHDA is a neurotoxin commonly used to lesion dopaminergic pathways and to generate experimental models for

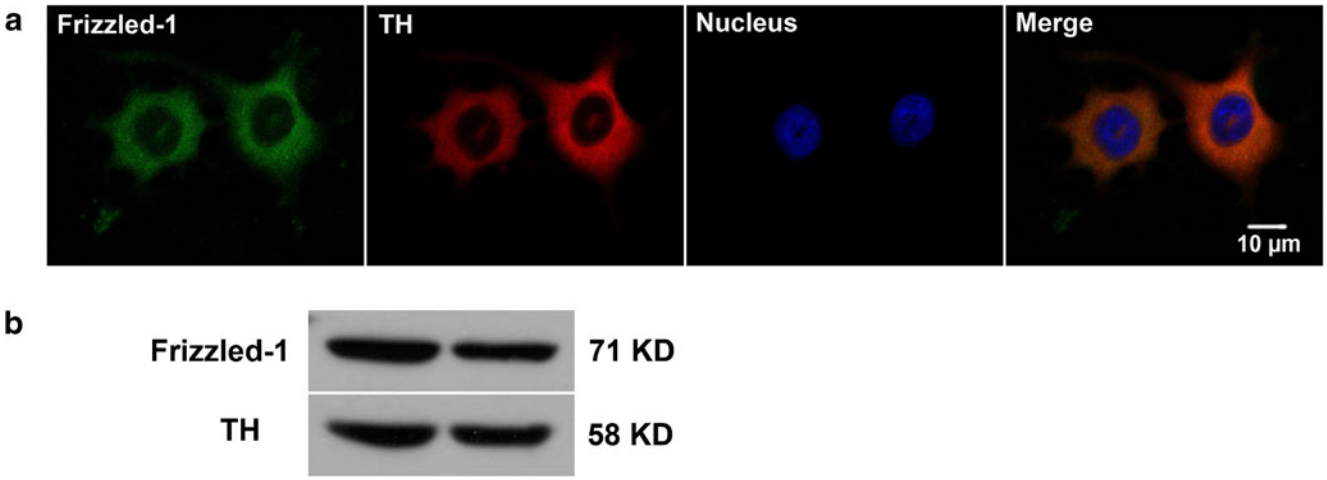

Fig. 1 Coexpression of Frizzled-1 and TH in SH-SY5Y cells. **a** Immunofluorescent labeling showed the expression of Frizzled-1 (*green*) and TH (*red*). **b** The expression of Frizzled-1 and TH were detected by Western blot. **a** Original magnification ×1,000

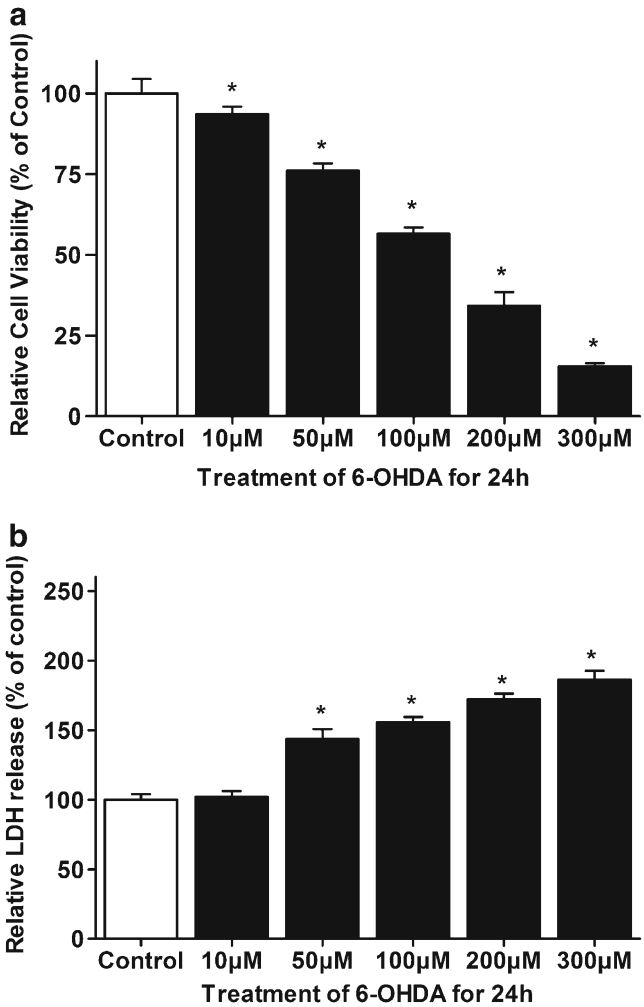

Fig. 2 Cytotoxic effect of 6-OHDA on SH-SY5Y cells. SH-SY5Y cells were treated with 0–300 μM 6-OHDA for 24 h, cell viability was measured by MTT assay (**a**) and LDH release assay (**b**), and expressed as percentage relative to vehicle group (n=6 per concentration point). All of the data were presented as mean ± SD from three independent experiments. *P<0.05 compared to the control

Parkinson disease. Treatment with 6-OHDA for 24 h caused a concentration-dependent reduction in cell viability (Fig. 2). Compared with controls, cell viability was 76.05±2.33 % at 50 μM 6-OHDA, 56.46±2.05 % at 100 μM, 34.19±4.29 % at 200 μM, and 15.57±0.90 % at 300 μM. Similar results were observed in the LDH release detection. A 100 μM 6-OHDA was chosen for further experiments.

6-OHDA Treatment Downregulates the Wnt/β-catenin Pathway

In a prior study, we found that 6-OHDA treatment reduced the expression of p-GSK3β (Ser9) and increased that of p-GSK3β (Tyr216) in a time-dependent manner (Li et al. 2011). β-Catenin is one of the downstream molecules of GSK3β in Wnt/β-catenin pathway and the level of β-catenin can reflect the activity of this pathway (Liu et al. 2011). SH-SY5Y cells were treated with 100 μM of 6-OHDA for 3, 6, 9, 12, and 24 h and then total protein was extracted and the level of β-catenin was determined by Western blot (Fig. 3a). Compared to vehicle control, 6-OHDA treatment initially caused a slight increase of the expression of β-catenin at 3 and 6 h following exposure to 6-OHDA, but the expression of β-catenin was decreased to 67.39±11.05 % at 9 h, 48.87±10.15 % at 12 h, and 32.24±29.96 % at 24 h (Fig. 3b). The result indicated that 6-OHDA treatment downregulated the Wnt/β-catenin pathway.

Wnt1 Attenuated 6-OHDA-Induced Cell Injury

We first tested the effect of Wnt1 on SH-SY5Y cells and found that treatment with Wnt1 at 1–100 ng/ml didn't obviously change the cell viability (Fig. 4a). Then, we investigated whether exogenous Wnt1 could attenuate the toxic effect of 6-OHDA on SH-SY5Y cells. Wnt1 protein (1–100 ng/ml) or

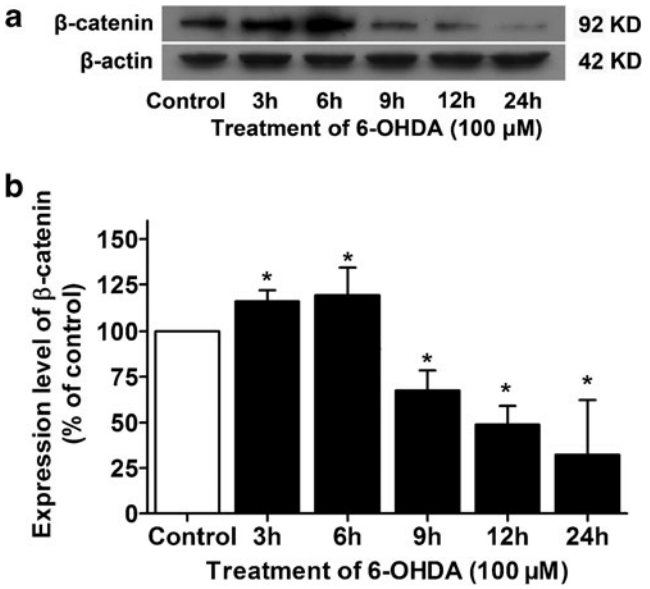

Fig. 3 6-OHDA treatment downregulates the protein level of β-catenin. **a** SH-SY5Y cells were treated with vehicle or 6-OHDA (100 μM) for 3, 6, 9, 12, and 24 h, and the protein level of β-catenin was detected by Western blot with β-actin as internal control. **b** The relative band intensities of β-catenin was measured by Quantity One software and normalized to the expression of β-actin in SH-SY5Y cells. All of the data were presented as mean ± SD from three independent experiments. *$P<0.05$ compared to the control

vehicle was added to the cultures 20 min prior to 6-OHDA. Cells pretreated with Wnt1 were partially protected against 6-OHDA toxicity (Fig. 4b). Treatment with 100 μM 6-OHDA for 24 h decreased the cell viability to 54.84±2.94 % compared with control group. However, when cells were pretreated with Wnt1, the reduction of cell viability was ameliorated. Specifically, the level of cell viability increased to ~67 % of the control value when 5 ng/ml of Wnt1 was used and to ~73 % when 10 ng/ml or higher concentrations of Wnt1 was added. Similarly, pretreatment of 10, 50, and 100 ng/ml Wnt1 could significantly inhibit LDH release induced by 6-OHDA (Fig. 4c).

The effects of Wnt1 on SH-SY5Y cells were further examined for the presence of apoptotic cells using FITC–Annexin–V/PI staining assay (Fig. 5). Annexin V has a strong affinity for phosphatidylserine (PS) that translocates from the inner surface of the plasma membrane to the cell surface upon initiation of apoptosis and is widely used as a marker of apoptosis. When used in combination with PI, apoptotic cells can easily be differentiated from necrotic and living cells. Our result showed that the percentage of apoptosis cells in the control group was 4.31±1.32 %, while that in 6-OHDA-treated cells was 26.03±3.30 %. However, pretreatment with Wnt1 could reduce the 6-OHDA-induced apoptosis to 16.43±2.08 %. Treatment with Wnt1 alone did not show obvious change of apoptosis compared with control.

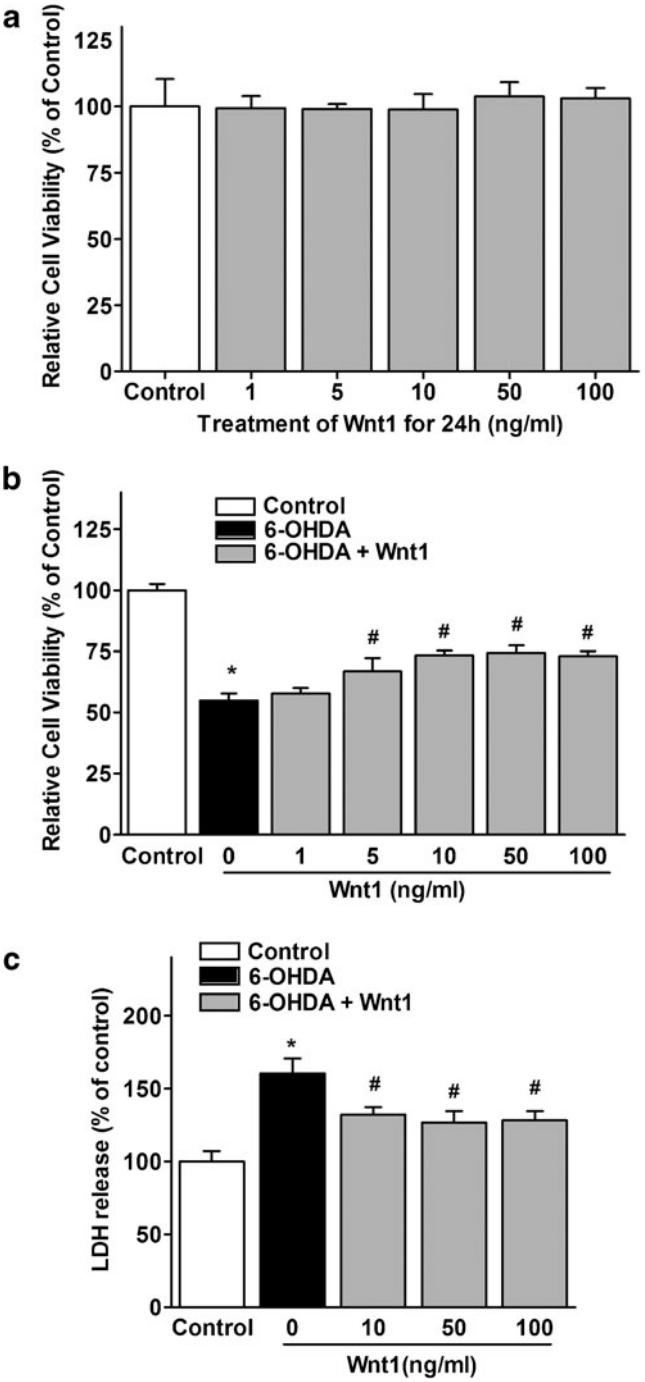

Fig. 4 Effects of Wnt1 on SH-SY5Y cells. **a** SH-SY5Y cells were treated with 0–100 ng/ml Wnt1 for 24 h, and cell viability was measured by MTT assay. **b** SH-SY5Y cells were pretreated with Wnt1 (5–100 ng/ml) prior to 6-OHDA (100 μM) treatment for 24 h and cell viability was assessed using the MTT assay. **c** SH-SY5Y cells were pretreated with Wnt1 (10, 50, and 100 ng/ml) prior to 6-OHDA (100 μM) treatment for 24 h and LDH assay was performed to determine the degree of cell injury. The data are expressed as percentage relative to vehicle group ($n=6$ per group) and presented as mean ± SD from three independent experiments. *$P<0.05$ compared to the control, #$P<0.05$ compared to 6-OHDA treated group

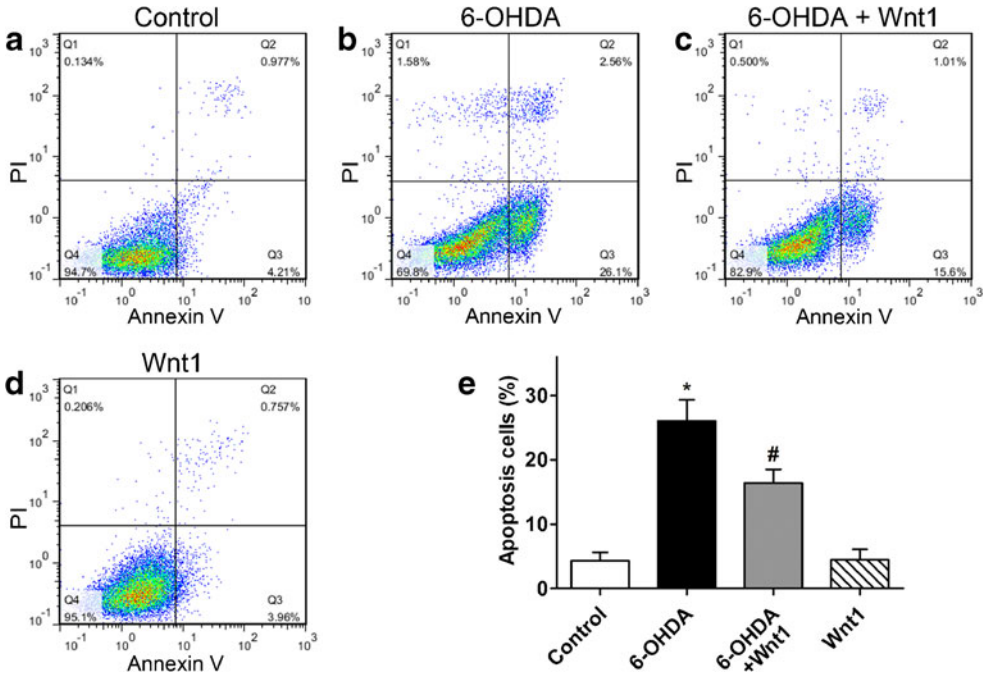

Fig. 5 Effect of Wnt1 on 6-OHDA-induced apoptosis in SH-SY5Y cells measured by flow cytometry. SH-SY5Y cells were treated with vehicle, 6-OHDA (100 μM), Wnt1 (50 ng/ml) 20 min prior to 6-OHDA, or Wnt1 alone for 24 h, and a combination of annexin V–FITC and PI staining was performed followed by flow cytometry assay. **a**, **b**, **c**, and **d** Representative set of flow cytometric two-parameter dot plots in corresponding groups. The *lower right quadrant* which shows annexin V–FITC positive and PI negative represents early apoptotic cells. The *upper right quadrant* which shows both annexin V–FITC and PI positive represents cells in the end stages of apoptosis and necrosis. **e** The percentage of cells under early apoptosis (annexin V–FITC-positive and PI-negative cells). Data were presented as mean ± SD from three independent experiments. *$P<0.05$ compared to the control, #$P<0.05$ compared to 6-OHDA-treated group

Wnt1 Attenuated 6-OHDA-Induced Loss of TH

As an important rate-limiting enzyme in the production of dopamine, TH was found to be downregulated by many studies both in vitro and in vivo (Tiong et al. 2010; L'Episcopo et al. 2011a). Our results also demonstrated that 100 μM 6-OHDA treatment for 24 h induced a significant decrease of TH protein level in SH-SY5Y cells. However, this effect was reversed by pretreatment of Wnt1 (10–100 ng/ml) in a concentration-dependent manner, suggesting the protective effect of Wnt1 against 6-OHDA-induced dopaminergic cell injury (Fig. 6).

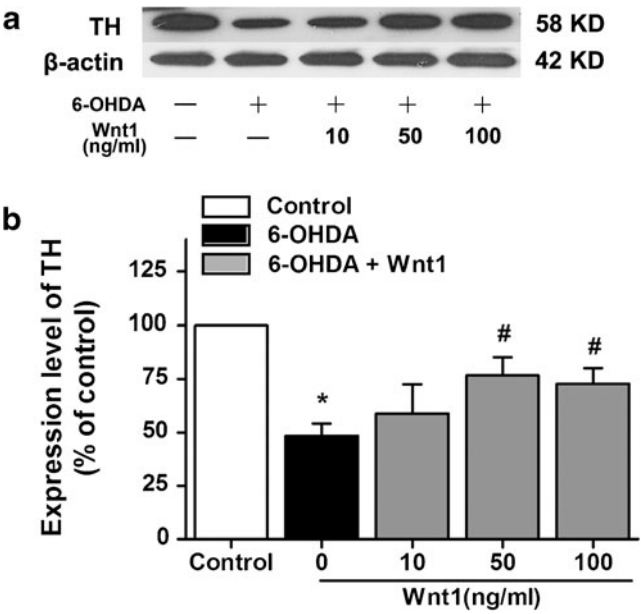

Fig. 6 Effects of Wnt1 on 6-OHDA-induced TH loss. **a** SH-SY5Y cells were treated with vehicle, 6-OHDA (100 μM), or Wnt1 (10, 50, and 100 ng/ml) 20 min prior to 6-OHDA for 24 h, and the protein levels of tyrosine hydroxylase (TH) were detected by Western blot with β-actin as internal control. **b** The relative band intensities of TH were measured by Quantity One software and normalized to the expression of β-actin. Data were presented as mean ± SD of three independent experiments. *$P<0.05$ compared to the control, #$P<0.05$ compared to 6-OHDA-treated group

Wnt1 Antagonized 6-OHDA-Induced MMP Decrease and Intracellular ROS Production

Because mitochondria are involved in a variety of key events in apoptosis, markers of mitochondria function, such as mitochondrial membrane potential, are often used to monitor apoptosis. The present study showed that there was a significant reduction of mitochondrial transmembrane potential (MMP) in 6-OHDA-treated SH-SY5Y cells. However, a partial restoration of MMP was observed in cells treated with Wnt1 (50 ng/ml) (Fig. 7a and c). Moreover, since reactive oxidative species (ROS) elevation is believed to

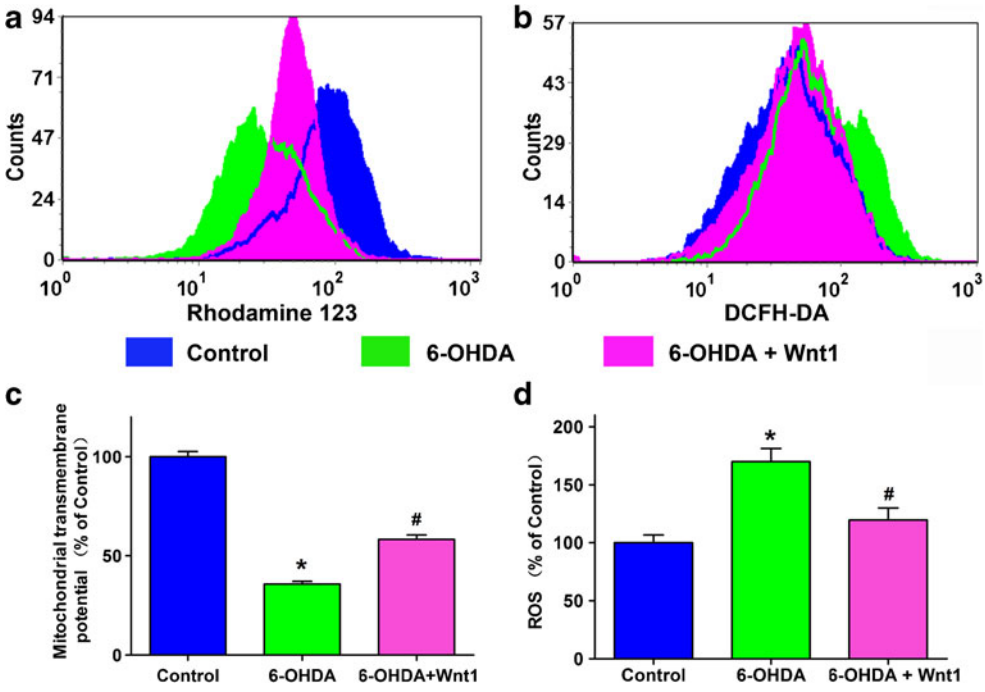

Fig. 7 Mitochondrial transmembrane potential (MMP) and intracellular ROS production assessed by flow cytometry. SH-SY5Y cells were treated with vehicle, 6-OHDA (100 μM), or Wnt1 (50 ng/ml) 20 min prior to 6-OHDA for 24 h, and MMP (**a**) and intracellular ROS (**b**) were detected by a fluorometric technique. Results of MMP (**c**) and intracellular ROS (**d**) are expressed as relative fluorescent intensity. Data were presented as mean ± SD from three independent experiments. *$P<0.05$ compared to the control, #$P<0.05$ compared to 6-OHDA-treated group

initiate a neurotoxic cascade induced by 6-OHDA (Hwang and Chun 2012), we further examined if Wnt1 could inhibit 6-OHDA-induced cell apoptosis by suppressing ROS production. As shown in Fig. 7b and d, 6-OHDA treatment alone for 24 h induced about 1.7-fold increase in ROS level compared with the control group, whereas pretreatment with Wnt1 (50 ng/ml) exhibited only a near ~1.2-fold increase relative to control.

Wnt1 Inhibited 6-OHDA-Induced Changes in the Protein Levels of Bax and Bcl-2

Studies have revealed that oxidative stress can cause apoptosis. Several protein families, for example, the Bcl-2 family of proteins, are considered to be specifically involved in regulating programmed apoptotic cell death. The balance between Bax and Bcl-2 plays a critical role in maintaining cell integrity and maintain the mitochondria function (Tsujimoto 2002). In this study, the Bcl-2 and Bax protein levels were detected by Western blot and the Bax/Bcl-2 ratio was analyzed (Fig. 8). After treatment with 6-OHDA for 24 h, Bax/Bcl-2 ratio was significantly increased to 1.51-fold compared with control, while pretreatment of Wnt1 at 50 and 100 ng/ml could attenuated these changes.

Wnt1 Antagonized 6-OHDA-Induced ER Stress

Our previous study has showed that ER stress was involved in 6-OHDA-induced apoptosis in SH-SY5Y cells (Luo et al. 2012). ER stress is characterized by the activation of a series

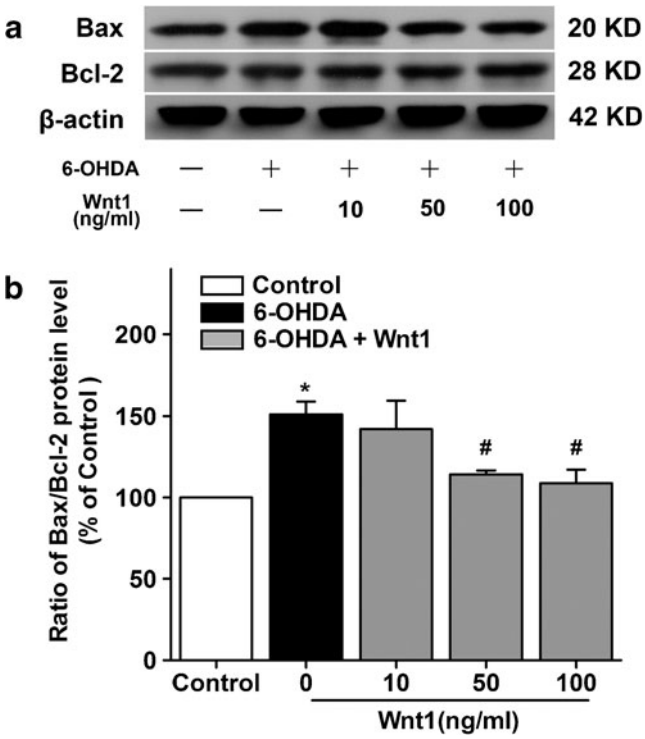

Fig. 8 Effects of Wnt1 on 6-OHDA-induced Bax and Bcl-2 protein expression. **a** SH-SY5Y cells were treated with vehicle, 6-OHDA (100 μM), or Wnt1 (10, 50, and 100 ng/ml) 20 min prior to 6-OHDA for 24 h, and the protein levels of Bax and Bcl-2 were detected by Western blot with β-actin as internal control. **b** The relative band intensities of Bax and Bcl-2 were measured by Quantity One software and normalized to the expression of β-actin. The ratio of Bax/Bcl-2 was calculated and each value was represented as mean ± SD of three independent experiments. *$P<0.05$ compared to the control, #$P<0.05$ compared to 6-OHDA-treated group

of signal transduction molecules such as ER luminal binding protein (Bip/Grp78) and transcription factor C/EBP homologous protein (CHOP) (McCullough et al. 2001; Rao et al. 2002). To investigate whether the protective effect of Wnt1 was related to a reduction in ER stress, Western blot was used to detect the expression of Bip/GRP78 and CHOP (Fig. 9). Treatment of 100 μM 6-OHDA for 24 h could elevate the levels of Chop and Bip/GRP78 to 513.69±97.04 % and 305.97±26.16 %, respectively, compared with control, which indicated the occurrence of ER stress, while Wnt1 at a high concentration (100 ng/ml) could attenuate these changes.

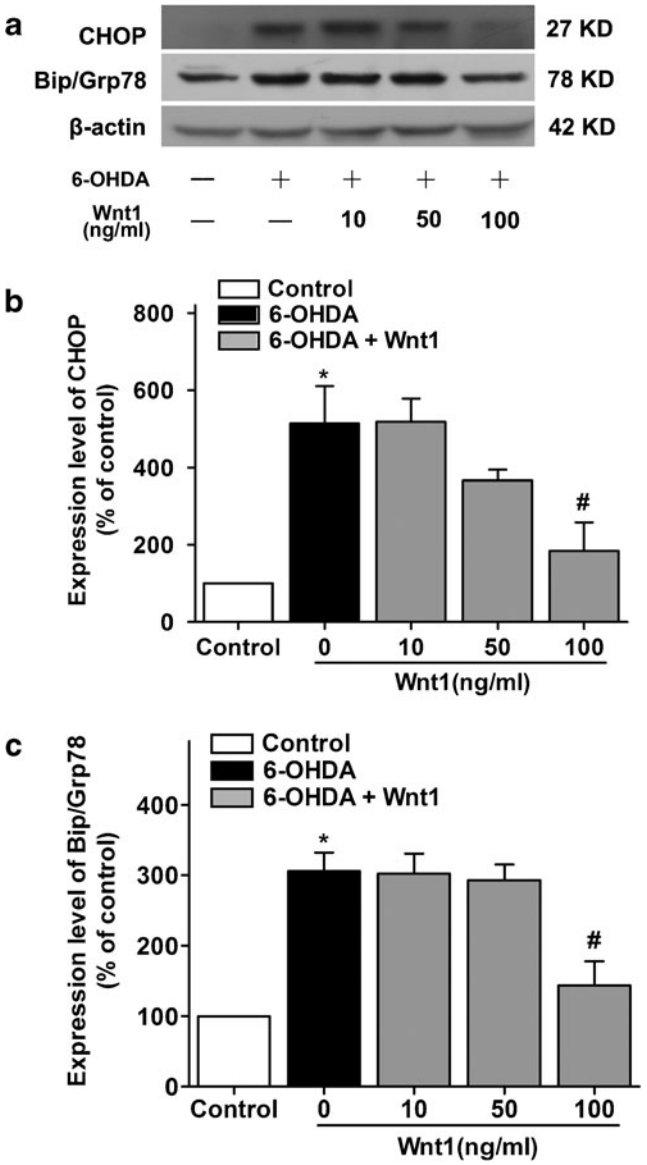

Fig. 9 Effect of Wnt1 on 6-OHDA-induced ER stress. **a** SH-SY5Y cells were treated with vehicle, 6-OHDA (100 μM), or Wnt1 (10, 50, and 100 ng/ml) 20 min prior to 6-OHDA for 24 h, and the protein levels of CHOP and Bip/Grp78 were detected by Western blot with β-actin as internal control. The relative band intensities of CHOP (**b**) or Bip/GRP78 (**c**) were measured by Quantity One software and normalized to the expression of β-actin in SHSY5Y cells. Data were presented as mean ± SD of three independent experiments. *$P<0.05$ compared to the control, #$P<0.05$ compared to 6-OHDA-treated group

Wnt1 Activated Wnt/β-catenin Pathway

To certify the activation effect of exogenous Wnt1 on Wnt/β-catenin pathway, Western blot was used to detect the expression of p-GSK3β (Ser9), p-GSK3β (Tyr216), and β-catenin in SH-SY5Y cells treated with 6-OHDA and/or Wnt1. We found that treatment of 100 μM 6-OHDA for 24 h could increase the p-GSK3β (Tyr216) level to 214.86±14.39 % and decrease the p-GSK3β (Ser9) and β-catenin levels to 52.05±9.79 % and 40.47±22.77 %, respectively, compared with control group. Pretreatment with 10, 50, and 100 ng/ml Wnt1 could reverse these changes induced by 6-OHDA treatment (Fig. 10a, b, d, and e).

Wnt1 Reversed 6-OHDA-Induced p-Akt (Ser473) Downregulation

Several studies have documented that Wnt can rely upon PI3K/Akt activation to support cell survival. We found that the protein level of p-Akt (Ser473), the active form of Akt, in 6-OHDA-treated group were decreased to 32.67±7.61 % compared with the control group, while that in cells pretreated with 10, 50, and 100 ng/ml Wnt1 were 68.39±16.21 %, 63.32±17.4 %, and 72.42±16.03 %, respectively (Fig. 10c and e), which suggested that treatment of Wnt1 could reverse the downregulation of PI3K/Akt pathway by 6-OHDA treatment.

Discussion

This study investigated the protective effects of exogenous Wnt1 on a cellular model of PD. Our present study demonstrated that canonical Wnt/β-catenin pathway was inhibited after treatment of 6-OHDA for 24 h, evidenced by the decreased β-catenin level and increased GSK3β activity (upregulation of p-GSK3β (Tyr216) which is the active form of GSK3β and downregulation of p-GSK3β (Ser9) which is the inactive form). Moreover, we also found that upregulation of Wnt/β-catenin pathway by exogenous Wnt1 could attenuate 6-OHDA-induced neurotoxicity and TH loss in SH-SY5Y cells through restoration of mitochondria transmembrane potential, reducing ROS production as well as inhibiting ER stress, indicating mechanisms of regulating mitochondria and ER functions involved.

SH-SY5Y cell line was chosen in this study for its expression of TH, which was considered as a dopaminergic cell line and used to simulate DAergic neurons (Takahashi et al. 1994). Moreover, our immunofluorescent result disclosed that Frezzled-1, the membrane receptor of Wnt/β-catenin pathway,

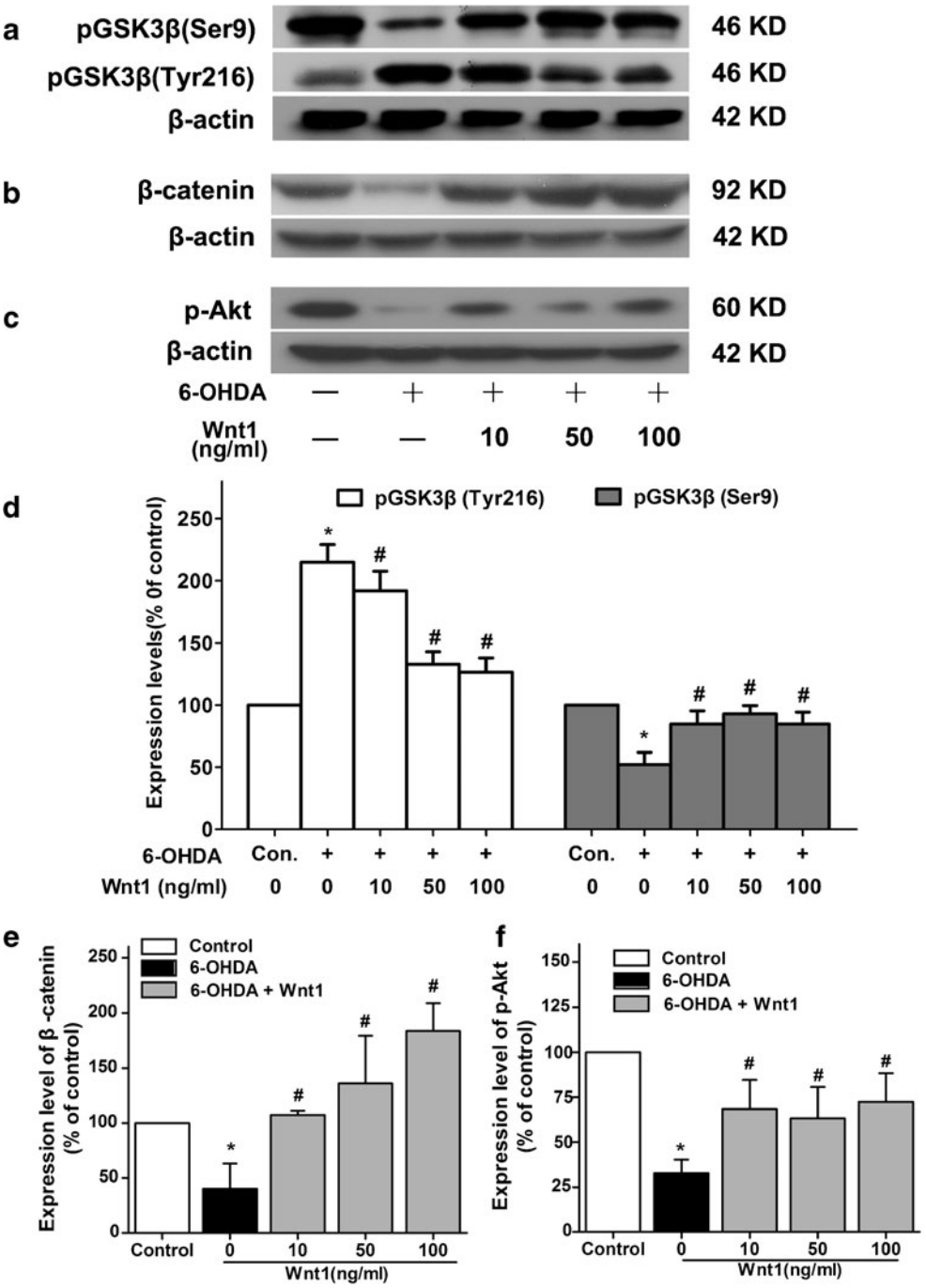

Fig. 10 Changes of related signal proteins in the cytoprotective effect of Wnt1 in SH-SY5Y treated with 6-OHDA. SH-SY5Y cells were treated with vehicle, 6-OHDA (100 μM), or Wnt1 (10, 50, and 100 ng/ml) 20 min prior to 6-OHDA for 24 h, and the protein levels of two phosphorylation forms of GSK3β (**a**), β-catenin (**b**), and p-Akt (Ser473) (**c**) were detected by Western blot with β-actin as internal control. The relative band intensities of the two forms of GSK3β (**d**), β-catenin (**e**), and p-Akt (Ser473) (**f**) were measured by Quantity One software and normalized to the expression of β-actin in SHSY5Y cells. Data were presented as mean ± SD of three independent experiments. *$P<0.05$ compared to the control, #$P<0.05$ compared to 6-OHDA-treated group

was expressed in SH-SY5Y cells, which indicated that Wnt1 might exert effects in this cell line. As an endogenous oxidative metabolite of dopamine, 6-OHDA has been found to be taken up by the plasma membrane dopamine transporter. Once in the cytoplasm, the cytotoxicity of 6-OHDA has been thought to be based primarily on the damage of dopaminergic neurons by quite similar mechanisms that have been proposed for patients with PD. For example, 6-OHDA inhibits mitochondrial Complex I, produce large amount of free radicals, induce cell death, and has been widely used to study the neurodegenerative process in PD (Blum et al. 2000; Soto-Otero et al. 2000; Li et al. 2011). Moreover, recent studies have declosed the involvement of ER stress in 6-OHDA-induced apoptosis in SH-SY5Y cells (Chen et al. 2004; Luo et al. 2012). A series of studies have indicated that Wnt signal may be inhibited by oxidative stress and ER stress in various disease models (Song et al. 2002; Chen et al. 2004; Almeida et al. 2009; Chen et al. 2010). Our present data also showed the downregulation of Wnt/β-catenin pathway after treatment of 6-OHDA.

Wnt1 is a cysteine-rich glycosylated protein which has been shown to protect against ischemic cortical injury (Chong et al. 2010), attenuate neuronal death during amyloid

exposure (Chong et al. 2007), and promote neuronal cell and astrocyte crosstalk as a mechanism of neuroprotection in PD models (L'Episcopo et al. 2011b). A recent study by L'Episcopo et al. showed that exogenous Wnt1 exert robust neuroprotective effects against caspase-3 activation, loss of TH^+ neurons, and [^{3}H]dopamine uptake induced by DA-specific insults, including serum and growth factor deprivation, 6-OHDA and MPTP/MPP^+ (L'Episcopo et al. 2011a). L'Episcopo et al. also found the β-catenin protein acts as a prosurvival factor for mesencephalic TH^+ neurons (L'Episcopo et al. 2011a). Our present study confirmed that exogenous Wnt1 could increase the β-catenin protein level, which might contribute to the protective effect. Moreover, our previous study uncovered that downregulation of GSK3β, also a central component of the Wnt/β-catenin pathway, could attenuate 6-OHDA-induced neuronal death and apoptosis (Li et al. 2011). The protective effects by inhibiting GSK3β might link with the attenuation of cell stress including oxidative stress and ER stress (Chen et al. 2004; Li et al. 2011). This study also found that exogenous Wnt1 could inhibit the activity of GSK3β by increasing the phosphorylation at site Ser9 and decreasing the phosphorylation at site Tyr216.

Because the mitochondrial dysfunction was considered a key factor in PD onset, we measured Bax and Bcl-2 levels, and MMP and ROS production. Bax and Bcl-2 proteins have a role in apoptotic signal transduction by regulating the permeability of the mitochondrial membrane (Oltvai et al. 1993). MMP, an important factor in apoptosis, is disrupted by ROS and it interacts with Bcl-2 family proteins. Bcl-2, as an antiapoptotic protein on the outer mitochondrial membrane, could stabilize membrane permeability and preserve mitochondrial integrity, while Bax is a proapoptotic protein which could influence membrane permeability by inducing cytochrome *c* release from the mitochondria into the cytosol, which subsequently leads to apoptosis (Vander Heiden and Thompson 1999). The present study showed that Wnt1 treatment could eliminate ROS production, stabilize MMP, and regulate Bax and Bcl-2 expressions.

Wnt1 may also exert a cytoprotective effect through PI3K and Akt pathways (Chong et al. 2002, 2010). PI3K and especially Akt are central to the regulation of cell growth and survival throughout the body (Chong et al. 2002, 2011). Akt, which is also known as protein kinase B (PKB), is a key molecule in growth factor signaling pathways mediating neuronal survival in both development and disease in multiple paradigms, including resistance against oxidative insults in the brain (Johnson-Farley et al. 2006; Rodriguez-Blanco et al. 2008). Moreover, many studies also found the involvement of Akt in regulating ER and its crosstalk with mitochondrial function (Koo et al. 2012; Liu et al. 2012). Our data showed that Wnt1 could reverse the downregulation of p-Akt (Ser473) which is the active form of Akt caused by 6-OHDA treatment and clearly suggested the mediation of the PI3K/Akt pathway in the protective effect of Wnt1 on 6-OHDA-induced injury in SH-SY5Y cells.

Considering the involvement of Wnt/β-catenin pathway in cell proliferation (Huang et al. 2008; Sklepkiewicz et al. 2011), there comes another question as to whether the protective effect of Wnt1 is due to its proliferative instead of anti-apoptotic effect. The present data showed that solely Wnt1 treatment at a concentration range of 1–100 ng/ml for 24 h didn't obviously change the cell viability by MTT assay which is often used in the detection of cell proliferation. This in vitro study indicates that within certain concentration ranges, Wnt1 mainly exerts its cytoprotective effect in SH-SY5Y cells.

In conclusion, we found that the expression level of β-catenin decreased after 6-OHDA treatment in SH-SY5Y cells and activation of Wnt/β-catenin pathway by treatment of exogenous Wnt1 could attenuate 6-OHDA-induced neurotoxicity. These results add further evidence to explore a new therapeutic target in treating PDs.

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References

Almeida M, Ambrogini E et al (2009) Increased lipid oxidation causes oxidative stress, increased peroxisome proliferator-activated receptor-gamma expression, and diminished pro-osteogenic Wnt signaling in the skeleton. J Biol Chem 284:27438–27448

Andersen JK (2004) Oxidative stress in neurodegeneration: cause or consequence? Nat Med 10(Suppl):S18–S25

Bai Y, Meng Z et al (2009) An Ang1–Tie2–PI3K axis in neural progenitor cells initiates survival responses against oxygen and glucose deprivation. Neuroscience 160:371–381

Blum D, Torch S, Nissou MF, Benabid AL, Verna JM (2000) Extracellular toxicity of 6-hydroxydopamine on PC12 cells. Neurosci Lett 283:193–196

Castelo-Branco G, Wagner J et al (2003) Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a. Proc Natl Acad Sci U S A 100:12747–12752

Castelo-Branco G, Rawal N et al (2004) GSK-3beta inhibition/beta-catenin stabilization in ventral midbrain precursors increases differentiation into dopamine neurons. J Cell Sci 117:5731–5737

Chen G, Bower KA et al (2004) Glycogen synthase kinase 3beta (GSK3beta) mediates 6-hydroxydopamine-induced neuronal death. FASEB J 18:1162–1164

Chen JR, Lazarenko OP et al (2010) A role for ethanol-induced oxidative stress in controlling lineage commitment of mesenchymal stromal cells through inhibition of Wnt/beta-catenin signaling. J Bone Miner Res 25:1117–1127

Chong ZZ, Kang JQ et al (2002) Erythropoietin is a novel vascular protectant through activation of Akt1 and mitochondrial modulation of cysteine proteases. Circulation 106:2973–2979

Chong ZZ, Li F et al (2007) Cellular demise and inflammatory microglial activation during beta-amyloid toxicity are governed by Wnt1 and canonical signaling pathways. Cell Signal 19:1150–1162

Chong ZZ, Shang YC et al (2010) Wnt1 neuroprotection translates into improved neurological function during oxidant stress and cerebral ischemia through AKT1 and mitochondrial apoptotic pathways. Oxidative Med Cell Longev 3:153–165

Chong ZZ, Hou J et al (2011) EPO relies upon novel signaling of Wnt1 that requires Akt1, FoxO3a, GSK-3beta, and beta-catenin to foster vascular integrity during experimental diabetes. Curr Neurovasc Res 8:103–120

De Ferrari GV, Chacon MA et al (2003) Activation of Wnt signaling rescues neurodegeneration and behavioral impairments induced by beta-amyloid fibrils. Mol Psychiatry 8:195–208

Dexter DT, Carayon A et al (1991) Alterations in the levels of iron, ferritin and other trace metals in Parkinson's disease and other neurodegenerative diseases affecting the basal ganglia. Brain 114 (Pt 4):1953–1975

Dong J, Song N et al (2009) Ghrelin antagonized 1-methyl-4-phenylpyridinium (MPP(+))-induced apoptosis in MES23.5 cells. J Mol Neurosci 37:182–189

Huang CL, Liu D et al (2008) Wnt1 overexpression promotes tumour progression in non-small cell lung cancer. Eur J Cancer 44:2680–2688

Hwang CK, Chun HS (2012) Isoliquiritigenin isolated from licorice *Glycyrrhiza uralensis* prevents 6-hydroxydopamine-induced apoptosis in dopaminergic neurons. Biosci Biotechnol Biochem 76:536–543

Imai Y, Soda M et al (2001) An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. Cell 105:891–902

Johnson-Farley NN, Travkina T et al (2006) Cumulative activation of akt and consequent inhibition of glycogen synthase kinase-3 by brain-derived neurotrophic factor and insulin-like growth factor-1 in cultured hippocampal neurons. J Pharmacol Exp Ther 316:1062–1069

Koo HJ, Piao Y et al (2012) Endoplasmic reticulum stress impairs insulin signaling through mitochondrial damage in SH-SY5Y cells. Neurosignals

L'Episcopo F, Serapide MF et al (2011a) A Wnt1 regulated Frizzled-1/beta-catenin signaling pathway as a candidate regulatory circuit controlling mesencephalic dopaminergic neuron-astrocyte crosstalk: therapeutical relevance for neuron survival and neuroprotection. Mol Neurodegener 6:49

L'Episcopo F, Tirolo C et al (2011b) Reactive astrocytes and Wnt/beta-catenin signaling link nigrostriatal injury to repair in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. Neurobiol Dis 41:508–527

Li F, Chong ZZ et al (2006) Winding through the WNT pathway during cellular development and demise. Histol Histopathol 21:103–124

Li Y, Luo F et al (2011) Knockdown of glycogen synthase kinase 3 beta attenuates 6-hydroxydopamine-induced apoptosis in SH-SY5Y cells. Neurosci Lett 487:41–46

Lin S, Cheng M et al (2009) Norrin attenuates protease-mediated death of transformed retinal ganglion cells. Mol Vis 15:26–37

Liu J, Ding X et al (2011) Enhancement of canonical Wnt/beta-catenin signaling activity by HCV core protein promotes cell growth of hepatocellular carcinoma cells. PLoS One 6:e27496

Liu Z, Zhang HM et al (2012) The immunity-related GTPase Irgm3 relieves endoplasmic reticulum stress response during coxsackievirus B3 infection via a PI3K/Akt dependent pathway. Cell Microbiol 14:133–146

Luo F, Wei L et al (2012) HtrA2/Omi is involved in 6-OHDA-induced endoplasmic reticulum stress in SH-SY5Y cells. J Mol Neurosci 47:120–127

Mazemondet O, Hubner R et al (2011) Quantitative and kinetic profile of Wnt/beta-catenin signaling components during human neural progenitor cell differentiation. Cell Mol Biol Lett 16:515–538

McCullough KD, Martindale JL et al (2001) Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 21:1249–1259

Nusse R (1999) WNT targets. Repression and activation. Trends Genet 15:1–3

Oltvai ZN, Milliman CL et al (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74:609–619

Rao RV, Peel A et al (2002) Coupling endoplasmic reticulum stress to the cell death program: role of the ER chaperone GRP78. FEBS Lett 514:122–128

Rodriguez-Blanco J, Martin V et al (2008) Intracellular signaling pathways involved in post-mitotic dopaminergic PC12 cell death induced by 6-hydroxydopamine. J Neurochem 107:127–140

Schapira AH, Gegg M (2011) Mitochondrial contribution to Parkinson's disease pathogenesis. Parkinsons Dis 2011:159160

Schapira AH, Cooper JM et al (1989) Mitochondrial complex I deficiency in Parkinson's disease. Lancet 1:1269

Sklepkiewicz P, Schermuly RT et al (2011) Glycogen synthase kinase 3beta contributes to proliferation of arterial smooth muscle cells in pulmonary hypertension. PLoS One 6:e18883

Song L, De Sarno P et al (2002) Central role of glycogen synthase kinase-3beta in endoplasmic reticulum stress-induced caspase-3 activation. J Biol Chem 277:44701–44708

Soto-Otero R, Mendez-Alvarez E et al (2000) Autoxidation and neurotoxicity of 6-hydroxydopamine in the presence of some antioxidants: potential implication in relation to the pathogenesis of Parkinson's disease. J Neurochem 74:1605–1612

Takahashi T, Deng Y et al (1994) Uptake of a neurotoxin-candidate, (R)-1,2-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline into human dopaminergic neuroblastoma SH-SY5Y cells by dopamine transport system. J Neural Transm Gen Sect 98:107–118

Tiong CX, Lu M et al (2010) Protective effect of hydrogen sulphide against 6-OHDA-induced cell injury in SH-SY5Y cells involves PKC/PI3K/Akt pathway. Br J Pharmacol 161:467–480

Tsujimoto Y (2002) Bcl-2 family of proteins: life-or-death switch in mitochondria. Biosci Rep 22:47–58

Vander Heiden MG, Thompson CB (1999) Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? Nat Cell Biol 1: E209–E216

Vidya Priyadarsini R, Senthil Murugan R et al (2012) Aberrant activation of Wnt/beta-catenin signaling pathway contributes to the sequential progression of DMBA-induced HBP carcinomas. Oral Oncol 48:33–39

Weinreb O, Amit T et al (2006) Involvement of multiple survival signal transduction pathways in the neuroprotective, neurorescue and APP processing activity of rasagiline and its propargyl moiety. J Neural Transm Suppl 70:457–465

Yamaguchi TP (2001) Heads or tails: Wnts and anterior-posterior patterning. Curr Biol 11:R713–R724

Zhang Z, Cao X et al (2010) DNA polymerase-beta is required for 1-methyl-4-phenylpyridinium-induced apoptotic death in neurons. Apoptosis 15:105–115