

Neuroprotective effects of insulin-like growth factor-2 in 6-hydroxydopamine-induced cellular and mouse models of Parkinson's disease

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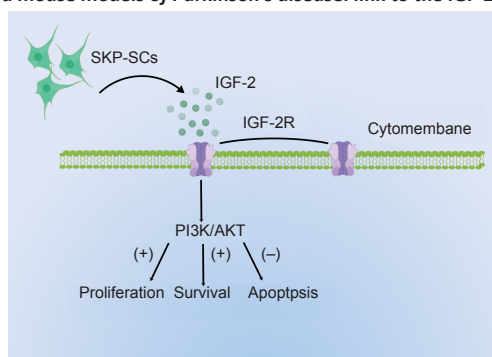
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Graphical Abstract

Neuroprotective effects of insulin-like growth factor-2 (IGF-2) in 6-hydroxydopamine (6-OHDA)-induced cellular and mouse models of Parkinson's disease: link to the IGF-2R/PI3K/AKT pathway



Abstract

Skin-derived precursor Schwann cells have been reported to play a protective role in the central nervous system. The neuroprotective effects of skin-derived precursor Schwann cells may be attributable to the release of growth factors that nourish host cells. In this study, we first established a cellular model of Parkinson's disease using 6-hydroxydopamine. When SH-SY5Y cells were pretreated with conditioned medium from skin-derived precursor Schwann cells, their activity was greatly increased. The addition of insulin-like growth factor-2 neutralizing antibody markedly attenuated the neuroprotective effects of skin-derived precursor Schwann cells. We also found that insulin-like growth factor-2 levels in the peripheral blood were greatly increased in patients with Parkinson's disease and in a mouse model of Parkinson's disease. Next, we pretreated cell models of Parkinson's disease with insulin-like growth factor-2 and administered insulin-like growth factor-2 intranasally to a mouse model of Parkinson's disease induced by 6-hydroxydopamine and found that the level of tyrosine hydroxylase, a marker of dopamine neurons, was markedly restored, α -synuclein aggregation decreased, and insulin-like growth factor-2 receptor down-regulation was alleviated. Finally, *in vitro* experiments showed that insulin-like growth factor-2 activated the phosphatidylinositol 3 kinase (PI3K)/AKT pathway. These findings suggest that the neuroprotective effects of skin-derived precursor Schwann cells on the central nervous system were achieved through insulin-like growth factor-2, and that insulin-like growth factor-2 may play a neuroprotective role through the insulin-like growth factor-2 receptor/PI3K/AKT pathway. Therefore, insulin-like growth factor-2 may be a useful target for Parkinson's disease treatment.

Key Words: 6-hydroxydopamine; α -synuclein; insulin-like growth factor-2 receptor; insulin-like growth factor-2; neurodegeneration; neuroprotection; Parkinson's disease; skin-derived precursor Schwann cells

Introduction

Parkinson's disease (PD) is the most common movement disorder and the second most common age-associated neurodegenerative disorder after Alzheimer's disease (Bloem et al., 2021; Tolosa et al., 2021). Patients with PD suffer from loss of neuromelanin-containing dopaminergic neurons in the substantia nigra pars compacta (Acharya and Kim, 2021). Once 50–80% of these neurons disappear, the patient's physical activities of daily living are significantly affected. In recent years, PD research has mainly focused on the mechanism of action of the main component of Lewy bodies, α -synuclein (α -syn) (Wong and Krainc, 2017; Mahul-Mellier et al., 2020; Zheng et al., 2021). The oligomers or aggregates that form when α -syn is overexpressed have toxic effects on cells and can cause mitochondrial dysfunction (Braak et al., 2003). However, the specific pathogenesis of PD is still unclear.

Skin-derived precursor (SKP) cells are a pluripotent source of adult stem cells that are capable of self-renewal and, when cultured appropriately, can differentiate into SKP Schwann cells (SKP-SCs) (Toma et al., 2005; Hunt et al., 2009; Liu et al., 2013). Previous studies have shown that SKP-SCs derived from rat skin precursor cells reduce 6-hydroxydopamine (6-OHDA)-induced dopaminergic neuron damage (Chen et al., 2020; Ma et al., 2021; Yan et al., 2022). However, there is little evidence that SKP-SCs develop into functional

neurons or form connections with neurons. The improved neuronal function seen in the presence of SKP-SCs is probably due to growth factors that they release, providing nourishment to host cells. In previous studies, we performed proteomic analysis of SKP-SCs conditioned medium (SKP-SCs-CM) and evaluated the literature to identify possible key growth factors secreted by SKP-SCs, including insulin-like growth factor (IGF)-2 (Chen et al., 2020; Ma et al., 2021; Yan et al., 2022); however, the specific regulatory mechanism by which IGF-2 promotes neuronal growth has not yet been fully elucidated.

IGFs such as IGF-1 and IGF-2, similar to insulin, are evolutionarily conserved peptides (Maki, 2010) that promote bone and muscle growth and differentiation (Agrogiannis et al., 2014). One study showed that IGF signal transduction is critical to neural development (O'Kusky and Ye, 2012). IGF-2 is widely expressed throughout the brain (Beletskiy et al., 2021). It has been found to be neuroprotective in Huntington's disease (García-Huerta et al., 2020), Alzheimer's disease (Mellott et al., 2014), amyotrophic lateral sclerosis (Allodi et al., 2016), and neuropsychiatric diseases (Steinmetz et al., 2018). However, there have been few studies regarding the role of IGF-2 in PD. IGF-2 is a soluble factor that exerts its effects by binding to its receptor, IGF-2 receptor (IGF-2R), which appears to be essential for growth, development, and lysosomal enzyme transport (El-Shewy and Luttrell, 2009). IGF-2R may also participate in the activation or removal of extracellular ligands, the

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activation of specific transduction proteins, the formation of lysosomes, and the separation and transport of lysosomal enzymes (O’Kusky and Ye, 2012). Studies have shown that, in Huntington’s disease, IGF-2 reduces the load of polyglutamine79 in cells by binding to IGF-2R, thereby exerting a protective effect (García-Huerta et al., 2020).

Based on the evidence described above, the purposes of this study were to verify whether IGF-2 is a key component of neuroprotective effect of SKP-SCs-CM and to further investigate the neuroprotective effect of IGF-2 and related pathways in PD.

Methods

Cell culture and drug treatments

Human neuroblastoma cells (SH-SY5Y, CRL-2266, RRID: CVCL_0019, American Type Culture Collection, Manassas, VA, USA) expressing tyrosine hydroxylase (TH) were used in the *in vitro* experiments in this study. The cells were cultured in Dulbecco’s modified Eagle medium/F12 (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin in a 5% CO₂ atmosphere. To promote differentiation, SH-SY5Y cells were cultured with 10 μM all-trans-retinoic acid (Sigma-Aldrich, St. Louis, MO, USA) for 7 days, with medium changes every other day (Cheung et al., 2009; Ma et al., 2021). Cells were pretreated for 12 hours with IGF-2 (Peprotech, Rocky Hill, NJ, USA) or SKP-SCs conditioned medium (SKP-SCs-CM), then exposed for 24 hours to 6-OHDA (Sigma-Aldrich). In the IGF-2 inhibition experiment, 0.1 μg/μL of anti-IGF-2 antibody (rabbit, Cat# A2086, RRID: AB_2764106, ABclonal, Wuhan, China) was added to the SKP-SCs-CM, which was then pre-incubated at 37°C for 30 minutes (Jeon et al., 2017; Son et al., 2019). A flowchart of the study design is shown in **Figure 1**.

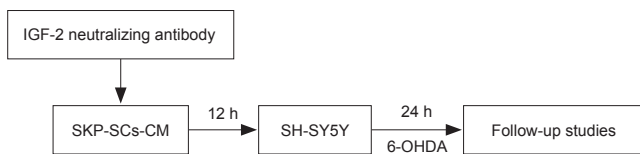


Figure 1 | Flow chart of *in vitro* experiments.

6-OHDA: 6-Hydroxidopamine; IGF-2: insulin-like growth factor-2; SKP-SCs-CM: skin-derived precursor Schwann cells conditioned medium.

Preparation of conditioned media

SKP-SCs isolated from the back skin of newborn green fluorescent protein–transgenic Sprague-Dawley rats were supplied by Nantong University (Wang et al., 2016; Zhu et al., 2018; Chen et al., 2020; Ma et al., 2021; Yan et al., 2022). The cells were cultured in Dulbecco’s Modified Eagle Medium/F12 (3:1, Gibco, Billings, MT, USA) containing 1% fetal bovine serum (Gibco), 2% N2 (Gibco), 5 μM forskolin (Gibco), 50 ng/mL heregulin-1β (Gibco), and 1% penicillin/streptomycin (Beyotime, Shanghai, China). High-purity cultures were obtained by serial passage. Cells at passages 18–20 were used in this study.

SKP-SCs were cultured at a concentration of 4×10^5 cells/cm² for 48 hours, after which the conditioned medium was collected by centrifugation at low speed (4000 × g) for 10–15 minutes and filtered using a 0.22-μm syringe filter. The filtered SKP-SCs-CM was then concentrated by ultrafiltration (Amicon Ultra-15, Millipore, Billerica, MA, USA) and stored at –80°C until use.

Animals

All experimental procedures were approved by the Animal Care and Use Committee of Nantong University (approval No. S20200420-802) on April 20, 2020, and were performed in accordance with Guide for the Care and Use of Laboratory Animals (8th edition). C57BL/6J mice (specific pathogen-free, 25–30 g, 10–12 weeks, adult, male) were reared under the following conditions: room temperature 22–24°C, relative humidity 60–65%, and photoperiod 12/12 hours light/darkness. Standard cages were used, and each cage contained no more than five mice. The mice were allowed to adapt to the environment for at least 1 week before the experiments were performed. After acclimatization, the mice were randomly allocated to the following groups: sham, IGF-2 + sham, 6-OHDA, and IGF-2 + 6-OHDA. There were eight mice in each experimental group.

6-OHDA-induced lesions

The 6-OHDA-induced mouse model of PD was established as previously described (Heuer et al., 2012; Ma et al., 2021). The mice were treated with desipramine (25 mg/kg intraperitoneal, Sigma-Aldrich) 30 minutes before injection with 6-OHDA (Sigma-Aldrich) or vehicle to protect noradrenergic neurons. After anesthetization via intraperitoneal injection of 1.25% tribromoethanol (Aibei Biotechnology, Nanjing, China), the mice were restrained on a stereotaxic apparatus (Rayward Life Technologies, Shenzhen, China). 6-OHDA (8.0 μg/2 μL, dissolved in NaCl at 0.9%, supplemented with 0.02% ascorbic acid) was injected into the right striatum with a syringe pump at a rate of 0.5 μL/min in the following locations (relative to the bregma) (Heuer et al., 2012; Ma et al., 2021): anterior-posterior = +0.5 mm, medial-lateral = –2.0 mm, and dorsal-ventral = –3.0 mm. Control mice were injected with 2 μL of a 0.02% ascorbic acid solution.

IGF-2 administration

Starting from 3 days before surgery, mice in the IGF-2 + 6-OHDA group received IGF-2 250 ng/d (Novoprotein, Rocky Hill, NJ, USA) for 10 consecutive days. The specific methods of intranasal administration and drug concentrations used were described previously (Hanson et al., 2013; Ouchi et al., 2013; Mao et al., 2016; Yang et al., 2020). Briefly, the mouse’s head was immobilized with one hand and the mouse was placed in the supine position; then, a 2.5-μL pipette was used to introduce the IGF-2 solution alternately into both nostrils with the other hand. IGF-2 was dissolved in phosphate-buffered saline (PBS), and the total volume (25 μL) was delivered in 2.5-μL increments, alternating nostrils, every 5 minutes.

Apomorphine-induced rotation test

This test is commonly used to evaluate the progression of 6-OHDA-induced injury (Iancu et al., 2005; Gu et al., 2016). Briefly, apomorphine (Sigma-Aldrich, 0.5 mg/kg body weight) was injected into the neck subcutaneously 28 days after injury. As soon as the mouse has been injected, it is placed in a beaker with a 18.1-cm diameter and 5-L capacity. The number and direction of 360° rotations that the mouse makes in 30 minutes is recorded. When mice rotate to the uninjured side (i.e., the left side), in most cases they use the left hind limb as the fulcrum; the body then rotates to the left, and some mice may even turn over or perform a few small circular movements. Greater than or equal to seven rotations per minute was regarded as a successful *in vivo* successful model of PD.

Mouse serum and brain tissue collection

The mice were anesthetized with 1.25% tribromoethanol, after which tail vein blood was collected, and the serum was separated by centrifugation. The brain was isolated from each mouse, and midbrain tissue was harvested for western blot assay. For immunofluorescence studies, the deeply anesthetized mice were fixed in a supine position on a foam board, the chest cavity was opened (fully exposing the heart to enable the apex and the right atrial appendage to be identified), the infusion needle was inserted into the left ventricle first, cut the right atrial appendage and saline was infused slowly to clear the blood in the blood vessels. After the liver turned yellow, the infusion of normal saline was replaced with 4% paraformaldehyde. The sign of successful perfusion with 4% paraformaldehyde is that the mouse tail vibrates and tilts, and the whole body becomes rigid (Gage et al., 2012). After the end of perfusion, the whole brain was removed and placed in 4% paraformaldehyde, post-fixed for 24 hours, then placed in 20% and 30% sucrose for gradient dehydration. After dehydration, the brain tissue was embedded in optimal cutting temperature compound and frozen at –20°C for at least 20 minutes. After freezing, 8–10-μm serial sections were taken, and the tissue sections were mounted on clean slides.

Human serum collection

Participants signed informed consent forms, and the Affiliated Hospital of Nantong University approved the study (approval No. 2020-K047, July 9, 2020). The samples came from 19 patients diagnosed with moderate to advanced PD and 17 healthy controls. All PD patients met the clinical PD diagnostic criteria published by MDS in 2015 (Postuma et al., 2015). Exclusion criteria included the following: 1) patients with secondary Parkinson’s syndrome with a clear etiology (such as brain tumor, history of cerebrovascular disease, infection, trauma, non-PD dementia, poisoning, and other neurodegenerative diseases); 2) patients with definite mental illness; 3) patients with serious cardiovascular, cerebrovascular, liver, or kidney diseases; and 4) patients with serious diseases such as blood system diseases, systemic infectious diseases, and malignant tumors. Control subjects were healthy individuals who were matched for age and sex with the PD group and underwent routine physical examinations at the hospital (**Table 1**). 4 mL of fasting cubital median venous blood was taken from each subject in the morning and centrifuged at 3000 × g for 10 minutes to separate the serum, which was stored in aliquots at –80°C for later testing.

Table 1 | Comparison of general clinical data between the HC and PD groups

	HC (n = 17)	PD (n = 19)	t-value	P-value
Sex				
Male	11(65)	11(58)	–	–
Female	6(35)	8(42)	–	–
Age (yr)	63.24±4.93	62.37±5.48	0.511	0.932
Hoehn-Yahr stage	–	3–5		

Data in sex are expressed as number (percentage). Data in age are expressed as mean ± SD, and were analyzed by independent samples t-test. HC: Healthy controls; PD: Parkinson’s disease.

Enzyme-linked immunosorbent assay

To detect IGF-2 levels and explore possible changes in IGF-2 in PD, IGF-2 levels in human serum, mouse serum, and mouse midbrain tissue were determined using an enzyme-linked immunosorbent assay kit (Hengyuan, Shanghai, China) according to the manufacturer’s instructions. The absorbance at 450 nm was recorded by a microplate reader (BioTek, Covina, CA, USA).

Cell counting kit-8 assay

A cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was used according to the manufacturer’s instructions to detect cell viability. First, SH-SY5Y cells



were counted and diluted to the experimental concentration ($5 \times 10^3/100 \mu\text{L}$ per well), then seeded uniformly in 96-well plates. When the cells reached 60–70% confluency, the stimulating agent(s) and/or drug(s) were added for the appropriate amount of time, after which $10 \mu\text{L}$ of CCK-8 solution was added to each well. The 96-well plate was then reincubated for 2 hours, and the optical absorbance at 450 nm was determined by a microplate reader (BioTek).

5-Ethynyl-2'-deoxyuridine assay

The 5-ethynyl-2'-deoxyuridine (EdU) assay (Beyotime) was carried out to determine cell proliferation. For this experiment, we seeded SH-SY5Y cells into a sterile, glass-bottomed 24-well plate. When the cells reached 60–70% confluency, the stimulating agent(s) and/or drug(s) were added. The $2\times$ EdU working solution prepared in advance was preheated in an incubator and added to a 24-well plate in the same volume as the original medium, and the plates were incubated for another 2 hours. The culture medium with aspirated with a pipette, and cell fixative was added to fix the cells at room temperature. The prepared Click reaction mixture was added, and the cells were then protected from light and incubated at room temperature for 30 minutes. Nuclei were stained and cells were mounted with Hoechst 33342-containing anti-fluorescence quenching mounting solution. The cells were viewed and photographed using a fluorescence microscope (Olympus, Tokyo, Japan). The percentage of proliferating (EdU-positive) cells in the total cell population was determined after different treatments.

TdT-mediated dUTP nick-end labeling assay

We carried out this experiment strictly in accordance with the TdT-mediated dUTP nick-end labeling (TUNEL) assay apoptosis detection kit instructions (Beyotime) to assess apoptosis after various drug treatments. SH-SY5Y cells grown on glass slides were fixed with 4% paraformaldehyde at room temperature for 30 minutes, then permeabilized at room temperature for 5 minutes. Following washing with PBS, we added TUNEL detection solution to the cells, shielded the slides from light, and incubated then at 37°C for 1 hour. Finally, a fluorescence microscope was used to observe the cells. The percentage of apoptotic (TUNEL-positive) cells in the total cell population after the specified treatment was determined.

Western blot assay

We used Western blot assay to detect the protein levels of TH (a dopaminergic neuron marker), α -syn, IGF-2, IGF-2R, phosphorylated phosphatidylinositol 3 kinase (p-PI3K), and phosphorylated protein kinase B (p-AKT) after exposure of the cells to various stimuli. An appropriate amount of tissue or cell lysate solution was added to SH-SY5Y cells and mouse midbrain tissue, the supernatant was obtained by centrifugation. Next, $5\times$ loading buffer was added to the supernatant, and the solution was boiled. The boiled solutions were aliquoted into different Eppendorf tubes to avoid sample degradation caused by repeated freezing and thawing. Samples were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (stacking gel: 80 V , separating gel: 120 V) and transferred to specific membranes (0.2 or $0.45 \mu\text{m}$, Merck Millipore, Burlington, MA, USA) (transfer conditions: 250 mA , 60 – 180 minutes, on ice). Next, blocking was completed by immersion in 5% nonfat dry milk for 2 hours at room temperature. Then, the membranes were incubated with primary antibody overnight at 4°C , followed by incubations with secondary antibody (1:5000, Proteintech, Wuhan, China) for 2 hours at room temperature. Immunoreactive bands were visualized using a chemiluminescence reagent (Absin, Shanghai, China) and an Odyssey Infrared Imaging System (Li-Cor Bioscience, Lincoln, NE, USA). The following primary antibodies were used: β -actin (mouse, 1:1000, Cat# ab8226, RRID: AB_306371, Abcam, Cambridge Science Park, Cambridge, UK), IGF-2 (rabbit, 1:1000, Cat# A2086, RRID: AB_2764106, Abclonal), IGF-2R (rabbit, 1:1000, Cat# A13022, RRID: AB_2759869, Abclonal), TH (rabbit, 1:5000, Cat# 25859-1-AP, RRID: AB_2716568, Proteintech), α -syn (rabbit, 1:1000, Cat# ab51253, RRID: AB_869973, Abcam), p-PI3K (rabbit, 1:1000, Cat# ab182651, RRID: AB_2756407, Abcam), p-AKT (rabbit, 1:1000, Cat# ab38449, RRID: AB_722678, Abcam), total PI3K (rabbit, 1:1000, Cat# ab182651, RRID: AB_2756407, Abcam), and total AKT (rabbit, 1:1000, Cat# ab8850, RRID: AB_2112117, Abcam). The following secondary antibodies were used: goat anti-mouse IgG (1:5000, Cat# SA00001-1, RRID: AB_2722565, Proteintech) and goat anti-rabbit IgG (1:5000, Cat# SA00001-2, RRID: AB_2722564, Proteintech). The membranes were visualized with an enhanced chemiluminescence system (Beyotime). Images were analyzed using ImageJ v1.6.0 (National Institutes of Health, Bethesda, MA, USA) (Schneider et al., 2012).

Immunofluorescence

Immunofluorescence was used to detect the location of proteins expressed by the target genes or co-expression of proteins expressed by two target genes. We sequentially fixed SKP-SCs with ice-cold 4% paraformaldehyde and blocked them with QuickBlock™ blocking buffer (Beyotime). The cells were then incubated with the following primary antibodies for 2 hours at room temperature or overnight at 4°C : S100 (rabbit, 1:200, Cat# ab52642, RRID: AB_882426, Abcam), P75 (rabbit, 1:200, Cat# ab52987, RRID: AB_881682, Abcam), glial fibrillary acidic protein (GFAP; rabbit, 1:100, Cat# 3670T, RRID: AB_561049, CST, Danvers, MA, USA), IGF-2 (rabbit, 1:200, Cat# A2086, RRID: AB_2764106, Abclonal), TH (mouse, 1:50, Cat# sc-25269, RRID: AB_628422, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and $\beta 3$ tubulin (Tuj1; mouse, 1:50, Cat# sc-80005, RRID: AB_2210816, Santa Cruz Biotechnology). Then, the cells were incubated with immunofluorescent secondary antibody (1:1000, Alexa Fluor®-488; mouse, Cat# A11029, RRID: AB_2534088; Alexa

Fluor®-488; rabbit, Cat# A11034, RRID: AB_2576217; Alexa Fluor®-568; rabbit, Cat# A11036, RRID: AB_10563566, Invitrogen, Carlsbad, CA, USA) for 1 hour at 37°C or 2 hours at room temperature. From incubation with the immunofluorescent secondary antibody to final observation, all operations were performed in the dark. After final washes with PBS, a fluorescence quenching sealing tablet containing Hoechst 33342 was used to seal the slides. A fluorescence microscope (Olympus) was used to examine the cells and capture the images. For cellular immunofluorescence, five different fields were observed for each sample, and three samples were analyzed per group. For immunofluorescence analysis of brain tissue samples, three mice were randomly selected from each group, and at least five discontinuous sections were selected from each mouse for observation and analysis.

Ingenuity pathway analysis

Differentially expressed genes from substantia nigra tissues were determined from RNA sequencing (RNA-seq) data, which have been deposited in the China National Genebank DataBase (CNGBdb, <https://db.cngb.org/>) under Project ID accession number CNP0001036 (Yang et al., 2021). All targets were imported into Microsoft Excel (Microsoft Excel; version 2019; Microsoft Corporation, Redmond, WA, USA) to remove duplicate values. Ingenuity pathway analysis (IPA) was employed to predict canonical pathways and networks of compounds and targets based on known interactions between different proteins.

Statistical analysis

No statistical methods were used to predetermine sample sizes; however, our sample sizes are similar to those reported in previous publications (Gu et al., 2016; Ma et al., 2021). No animals or data points were excluded from the analysis. The evaluators were blinded to the assignments. Data are presented as mean \pm standard error of the mean (SEM). A two-tailed Student's *t*-test was used to compare two independent groups, and one-way analysis of variance followed by Tukey's *post hoc* analysis was used to compare the means of the experimental and control groups. Clinical sample data were compared using the independent samples *t*-test. Statistical analysis was conducted using GraphPad Prism 8.4.2 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Values of $P < 0.05$ were considered to be statistically significant.

Results

The neuroprotective effect of SKP-SCs is related to IGF-2 release

We recently demonstrated the neuroprotective effect of SKP-SCs (Chen et al., 2020; Ma et al., 2021; Yan et al., 2022). Conditioned medium from SKP-SCs can reduce the injury that 6-OHDA causes to SH-SY5Y cells, and direct transplantation of SKP-SCs can reduce 6-OHDA-induced dopaminergic neuron damage *in vivo* and *in vitro* by regulating autophagy. In our previous studies, we analyzed SKP-SCs-CM by mass spectrometry and identified the cytokine IGF-2 as being potentially related to the therapeutic effects of SKP-SCs. This factor is a member of the insulin polypeptide family, which is expressed at high levels in the brain and plays an instrumental role in brain development. In this study, we cultured SKP-SCs and confirmed their identity by immunofluorescence staining for the Schwann cell markers S100, P75, and GFAP (Figure 2A).

Next, we explored whether IGF-2 is involved in the neuroprotection mediated by SKP-SCs-CM. An IGF-2 neutralizing antibody was added to SKP-SCs-CM, which were then pre-incubated at 37°C for 30 minutes. Both the CCK-8 and the EdU results showed that the neuroprotective effect of SKP-SCs-CM was eliminated by pre-incubation with the neutralizing antibody. As shown in Figure 2B, compared with the 6-OHDA injury group, the SKP-SCs-CM pretreatment group exhibited significantly greater cell viability ($P < 0.01$). However, SKP-SCs-CM pre-incubated with the anti-IGF-2 neutralizing antibody was unable to protect SH-SY5Y cells from the damage induced by 6-OHDA. The EdU results were consistent with the CCK-8 results (Figure 2C and D). These results suggest that IGF-2 plays a crucial role in SKP-SCs-CM-mediated neuroprotection.

IGF-2 levels are increased in PD serum samples

Next, we sought to obtain a more in-depth understanding of the role of IGF-2 in this context. Because IGF-2 is a soluble secretory factor, its serum level is considered to be a biomarker for disease development and prognosis (Lewitt and Boyd, 2019). Therefore, we analyzed IGF-2 levels in peripheral serum samples from a mouse model of PD and patients with PD. First, we collected serum from mice in the 6-OHDA and sham groups. Statistical analysis of the enzyme-linked immunosorbent assay results showed that IGF-2 levels were higher in the serum samples of the 6-OHDA group compared with the sham group ($P < 0.01$; Figure 3A). Next, we examined the level of IGF-2 in the serum of PD patients. The results were consistent with those from the animal experiments ($P < 0.01$, vs. HC group; Figure 3B). In summary, these results indicate that the level of IGF-2 in the peripheral circulation is increased in PD.

IGF-2 promotes SH-SY5Y cell survival and proliferation

To determine whether IGF-2 is responsible for the observed neuroprotection, we pre-treated 6-OHDA-induced SH-SY5Y cells with recombinant IGF-2 at different concentrations (5, 25, 50, 100 ng/mL) and then measured cell viability by CCK-8 assay (Figure 4A). The results showed that IGF-2, when administered in a certain concentration range, inhibited the 6-OHDA-induced cytotoxicity. In particular, 50 ng/mL IGF-2 had a significant protective effect ($P < 0.05$, vs. 6-OHDA group).

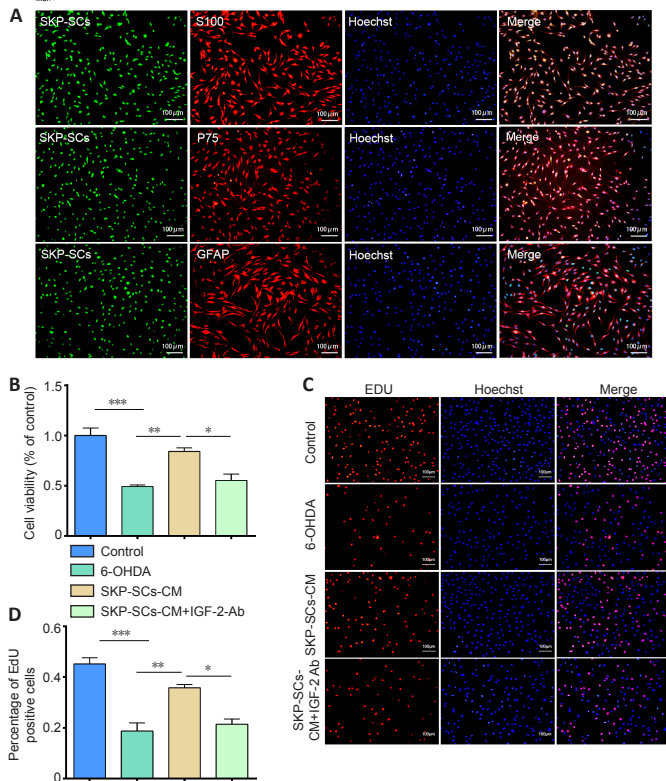


Figure 2 | Pretreatment with an anti-IGF-2 neutralizing antibody reduces the neuroprotective effect of SKP-SCs-CM on 6-OHDA-treated SH-SY5Y cells. (A) Characterization of SKP-SCs. S100 (red, Alexa Fluor-568), P75 (red, Alexa Fluor-568), and GFAP (red, Alexa Fluor-568) are expressed in green fluorescent protein-labeled SKP-SCs. (B) Cell counting kit-8 assay. SKP-SCs-CM was neutralized with an anti-IGF-2 antibody (0.1 $\mu\text{g}/\mu\text{L}$, preincubated for 30 minutes). SKP-SCs-CM or SKP-SCs-CM preincubated with anti-IGF-2 was applied to SH-SY5Y cells for 12 hours, followed by 24 hours of treatment with 50 μM 6-OHDA. (C, D) Proliferation of SH-SY5Y cells, as measured by EdU staining (red). Compared with the 6-OHDA group, there were more EdU-positive cells in SKP-SCs-CM group, but there were only a few EdU-positive cells in the SKP-SCs-CM + IGF-2-Ab group. Scale bars: 100 μm . Data are presented as mean \pm SEM. Each experiment was repeated five times. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way analysis of variance followed by Tukey's *post hoc* test). 6-OHDA: 6-Hydroxydopamine; CM: conditioned medium; EdU: 5-ethynyl-2'-deoxyuridine; GFAP: glial fibrillary acidic protein; IGF-2: insulin-like growth factor-2; IGF-2-Ab: IGF-2 antibody; SKP-SCs: skin-derived precursor Schwann cells; SKP-SCs-CM: skin-derived precursor Schwann cells conditioned medium.

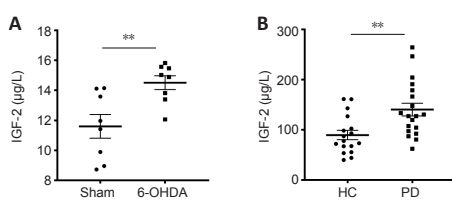


Figure 3 | IGF-2 levels are increased in serum from PD mice (A) and patients (B). IGF-2 serum concentrations were analyzed by enzyme-linked immunosorbent assay. Data are presented as mean \pm SEM (A: $n = 8/\text{group}$; B: HC, $n = 17$ and PD, $n = 19$). ** $P < 0.01$ (two-tailed Student's *t*-test). 6-OHDA: 6-Hydroxydopamine; HC: healthy controls; IGF-2: insulin-like growth factor-2; PD: Parkinson's disease.

Based on the CCK-8 results described above, we chose 50 ng/mL IGF-2 as the maximum drug concentration for subsequent experiments. Next, we tested cell proliferation by EdU assay. As illustrated in **Figure 4B and C**, the number of proliferating cells was significantly reduced after 6-OHDA treatment ($P < 0.001$, vs. control group), but there was no significant decrease in proliferation as a result of treatment with IGF-2. We then performed a TUNEL test to further verify the ability of IGF-2 to rescue 6-OHDA-induced cytotoxicity. Compared with the 6-OHDA group, there were fewer TUNEL-positive cells in the IGF-2 + 6-OHDA group ($P < 0.001$; **Figure 4D and E**).

Finally, we performed western blot analysis to determine the protein expression levels of TH and α -syn. The results showed that 6-OHDA treatment significantly decreased TH levels and increased α -syn levels ($P < 0.001$, vs. control group). Pretreatment with IGF-2 prevented the reduction in TH levels ($P < 0.01$, vs. 6-OHDA group) and reduced α -syn aggregation ($P < 0.001$, vs. 6-OHDA group; **Figure 4F-H**). In summary, IGF-2 has a protective effect in an *in vitro* 6-OHDA-induced model of PD.

The protective effect of IGF-2 on 6-OHDA-induced injury in mice

Next, we conducted *in vivo* experiments to verify our *in vitro* results. We treated C57BL/6J mice with 6-OHDA to mimic PD, administered IGF-2 intranasally, and performed an apomorphine-induced rotation experiment to evaluate the success of the model (**Figure 5A**). Twenty-eight days after 6-OHDA injection, the number of contralateral rotations had increased significantly ($P < 0.001$, vs. sham group), suggesting that 6-OHDA had damaged the dopaminergic system. Treatment with IGF-2 reduced the number of rotations induced by apomorphine ($P < 0.001$, vs. 6-OHDA group, **Figure 5B**). Next, the number of TH⁺ neurons in the substantia nigra was detected by immunofluorescence. In mice that had sustained injury with 6-OHDA, the number of TH⁺ neurons was significantly reduced ($P < 0.001$, vs. sham group). Administration of IGF-2 prevented the loss of TH⁺ neurons ($P < 0.05$, vs. 6-OHDA group, **Figure 5C and D**). Finally, we analyzed TH and α -syn protein levels. Consistent with the *in vitro* results, TH protein expression was significantly decreased after 6-OHDA treatment ($P < 0.01$), while α -syn protein expression was significantly increased ($P < 0.001$), and treatment with IGF-2 reversed these effects (**Figure 5E-G**). These findings suggest that IGF-2 has a neuroprotective effect *in vivo*.

Changes in IGF-2 and IGF-2R expression in *in vitro* and *in vivo* models of PD

To explore the specific mechanism by which IGF-2 exerts its neuroprotective effects, we first detected its localization in mouse midbrains by immunofluorescence double-label staining and showed that it was expressed in TH-positive cells, Tuj1-positive neurons, and GFAP-positive neurons (**Figure 6**). In addition, we measured IGF-2 protein expression levels. Both *in vivo* (**Figure 7A and B**) and *in vitro* (**Figure 7C and D**) experimental results indicated that IGF-2 expression was reduced after 6-OHDA treatment ($P < 0.001$, vs. sham group; $P < 0.01$, vs. control group). Considering that IGF-2 generally acts by binding to its receptors to transduce signals (Hawkes et al., 2006), we next asked whether IGF-2R expression changed during PD development. To test this, we performed western blot analysis of IGF-2R protein expression levels (**Figure 7E-J**) and found that, in the *in vivo* experiments, IGF-2R expression was dramatically reduced ($P < 0.01$, vs. sham group; **Figure 7E and H**). In parallel, we found in the *in vitro* experiments that the effect of 6-OHDA treatment on IGF-2R protein expression is time-dependent. As the duration of 6-OHDA injury increased, IGF-2R expression level gradually decreased ($P < 0.05$, vs. control group; **Figure 7F and I**). Next, we assessed the effect of treatment with IGF-2 on IGF-2R expression. As can be seen from **Figure 7G and J**, IGF-2 treatment prevented the 6-OHDA-induced down-regulation of IGF-2R in a time-dependent manner ($P < 0.05$, vs. 0 h group). The above results suggest that IGF-2 can inhibit 6-OHDA-induced neuronal damage in combination with IGF-2R. Given our previous observation regarding the increase in peripheral serum IGF-2 levels, we speculate that the lack of IGF-2 and its receptor in brain tissue may increase negative feedback increase in the periphery.

The PI3K/AKT pathway participates in the neuroprotective effect of IGF-2

PI3K/AKT is an important signaling pathway for neuron development, survival, and energy metabolism (Brunet et al., 2001). Therefore, to determine whether the PI3K/AKT pathway is involved in the neuroprotective effects of IGF-2, we assessed PI3K and AKT activity by western blot. The *in vitro* experimental results demonstrated that p-PI3K and p-AKT levels in the 6-OHDA group were significantly lower than those in the control group ($P < 0.001$), while IGF-2 pretreatment reversed this effect (**Figure 8A-C**).

To further confirm these results, we detected changes in phosphorylated and total PI3K and AKT protein levels in mouse brain tissue (**Figure 8D-F**). The results indicated that 6-OHDA inhibits PI3K and AKT activation, while treatment with IGF-2 activated the PI3K/AKT signaling pathway.

Finally, we analyzed the target molecules described above and their interactions using IPA software. The results are shown in **Figure 9**. Taken together, these results suggest that the PI3K/AKT pathway participates in the neuroprotective effect of IGF-2.

Discussion

In recent years, increasing evidence has shown that the protective effect of stem cells is related to the various substances they release (Tran and Damaser, 2015; Lin and Du, 2018; Wang et al., 2020). According to our previous studies, SKP-SCs can reduce the damage to dopaminergic neurons induced by 6-OHDA (Chen et al., 2020; Ma et al., 2021; Yan et al., 2022). In this study, we verified that IGF-2 is a key component by which SKP-SCs exert their protective effect and studied the neuroprotective effects of IGF-2 in 6-OHDA-induced cell and mouse models of PD.

SKP-SCs differentiate from SKPs when stimulated by the appropriate growth factors (Hunt et al., 2009), and have proven effective for treating acute and chronic nerve injuries (Khuong et al., 2014). A growing body of evidence has shown that transplanted cells can be therapeutically beneficial as a result of their pleiotropic properties, in that they directly replace damaged or lost cells and secrete paracrine factors, such as IGF-1 (Wang et al., 2014), IGF binding proteins (Jeon et al., 2017; Son et al., 2019) and stromal cell-derived factor-1 α (Wang et al., 2010). Our previous studies confirmed that SKP-SCs reduced the damage that 6-OHDA causes to dopaminergic neurons, and, through proteomic analysis of concentrated conditioned medium from SKP-SCs, we identified IGF-2 as a key component that may have a protective effect. The objectives of the current study were to further ascertain whether SKP-SCs play an effective therapeutic role by secreting IGF-2 and to determine whether this neurotrophic factor could be used for the treatment of PD.

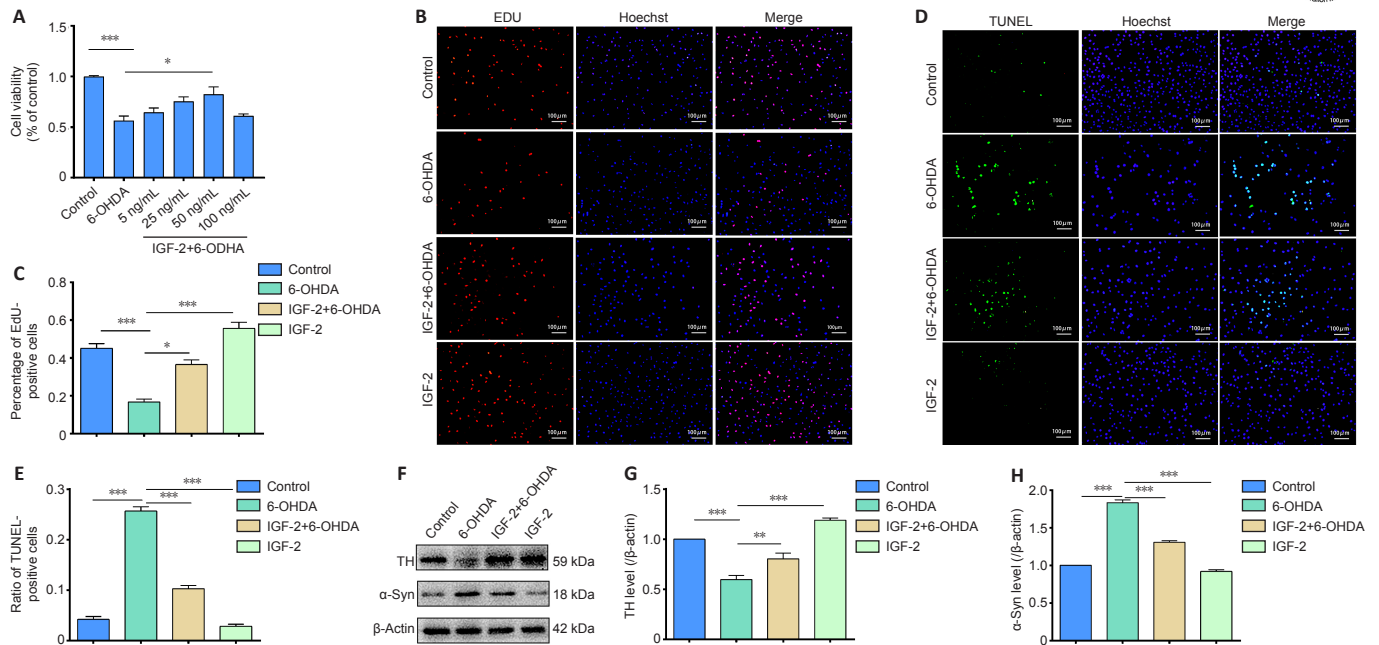


Figure 4 | IGF-2 pretreatment reduces the damage to SH-SY5Y cells induced by 6-OHDA.

(A) Cell counting kit-8 assay. SH-SY5Y cells were pretreated with varying concentrations (5, 25, 50, or 100 ng/mL) of IGF-2 for 12 hours and then exposed to 6-OHDA (50 μM) for 24 hours. (B, C) EdU assay. Cells were pretreated with 50 ng/mL IGF-2 for 12 hours. The number of EdU-positive cells was significantly reduced after 6-OHDA treatment, but there was no decrease after treatment with IGF-2. (D, E) TUNEL assay. Compared with the 6-OHDA group, there were fewer TUNEL-positive cells in the IGF-2 + 6-OHDA group. Scale bars: 100 μm. (F–H) Western blot analyses of TH and α-syn expression. Relative protein expression was normalized to β-actin expression in the control group. Data are presented as mean ± SEM. Each experiment was repeated at least three times. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (one-way analysis of variance followed by Tukey's *post hoc* test). 6-OHDA: 6-Hydroxydopamine; EdU: 5-ethynyl-2'-deoxyuridine; IGF-2: insulin-like growth factor-2; TH: tyrosine hydroxylase; TUNEL: TdT-mediated dUTP nick-end labeling; α-syn: α-synuclein.

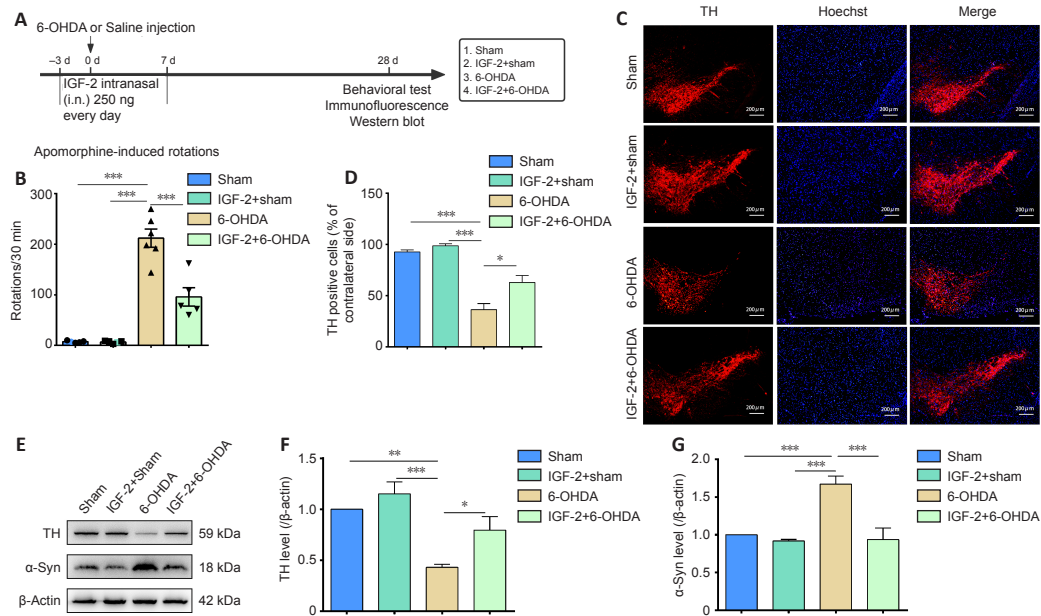


Figure 5 | Effect of intranasal IGF-2 administration on 6-OHDA-induced injury in mice.

(A) Experimental protocol. (B) The effects of unilateral striatal injection of 6-OHDA and intranasal injection of IGF-2 were assessed by the apomorphine-induced rotation test. (C, D) Representative images of immunofluorescence staining of the substantia nigra pars compacta of 6-OHDA-injured mice showing that IGF-2 treatment decreased TH+ cell (red, Alexa Fluor-568) loss. A reduction in the number of dopaminergic neurons compared with the sham group was observed following 6-OHDA injury. Intranasal administration of IGF-2 attenuated this effect, as indicated by greater numbers of dopaminergic neurons compared with the 6-OHDA group. Scale bars: 200 μm. (E–G) Western blot of TH and α-syn expression. Relative protein expression was normalized to β-actin expression in the control group. Data are presented as mean ± SEM (*n* ≥ 3). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (one-way analysis of variance followed by Tukey's *post hoc* test). 6-OHDA: 6-Hydroxydopamine; IGF-2: insulin-like growth factor-2; TH: tyrosine hydroxylase; α-syn: α-synuclein.

We found that adding an anti-IGF-2 antibody to SKP-SCs-CM inhibited the neuroprotective effects of SKP-SCs-CM *in vitro*. Based on this, we searched the protein interaction database and related literature and discovered that IGF-2 expression decreased significantly in the late disease stage in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced mouse model of PD. This prompted us to further investigate whether IGF-2 has a definite protective effect in the context of PD. We first explored possible changes in IGF-2 expression in peripheral serum samples from a mouse model of PD and patients with PD. Surprisingly, IGF-2 expression was increased in the peripheral serum in both cases, which led us to explore this phenomenon in more detail.

IGF-2 can traverse the blood-brain barrier and play an endocrine role, and is highly expressed in the nervous system during early fetal development (Beletskiy et al., 2021). Increasing numbers of studies have shown that IGF-2 is a neuroprotective agent. Exogenous low-dose IGF-2 ameliorates negative age-related changes in rats by reducing oxidative damage, increasing antioxidant enzyme activity, and increasing mitochondrial function (Castilla-Cortázar et al., 2011). Intracerebroventricular injection of IGF-2 can rescue partial damage caused by occlusion of large intracerebral arteries and play a critical part in brain recovery after stroke (Vafaei et al., 2020). In amyotrophic lateral sclerosis, IGF-2 can inhibit glycogen synthase kinase through the PI3K/AKT pathway, promote the retention of motor neurons, and induce nerve

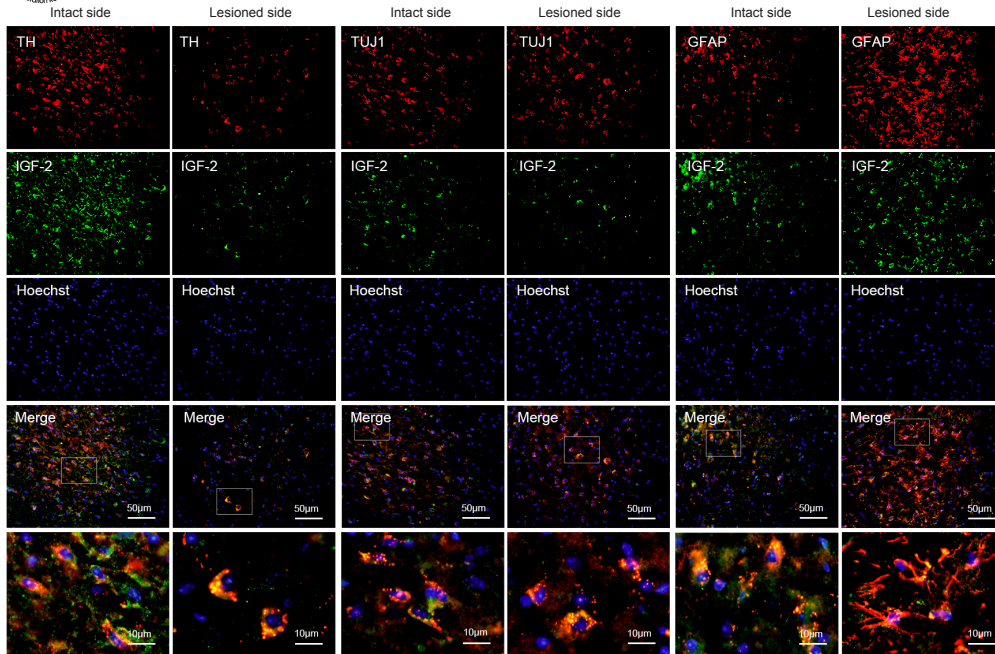


Figure 6 | Double immunofluorescence staining for IGF-2 in the midbrain region of a mouse model of PD.

Midbrains from the ipsilateral and contralateral sides of mice with 6-OHDA-induced lesions were subjected to immunohistochemistry. IGF-2 (green, Alexa Fluor-488) was expressed in TH-positive cells (red, Alexa Fluor-568), Tuj1-positive neurons (red, Alexa Fluor-568), and GFAP-positive neurons (red, Alexa Fluor-568). Compared with the intact side, there were fewer TH-positive neurons and Tuj1-positive neurons and more GFAP-positive neurons on the lesioned side. Scale bars: 50 μ m (upper four rows) and 10 μ m (lowest row). GFAP: Glial fibrillary acidic protein; IGF-2: insulin-like growth factor-2; PD: Parkinson's disease; TH: tyrosine hydroxylase; TUJ1: β 3 tubulin.

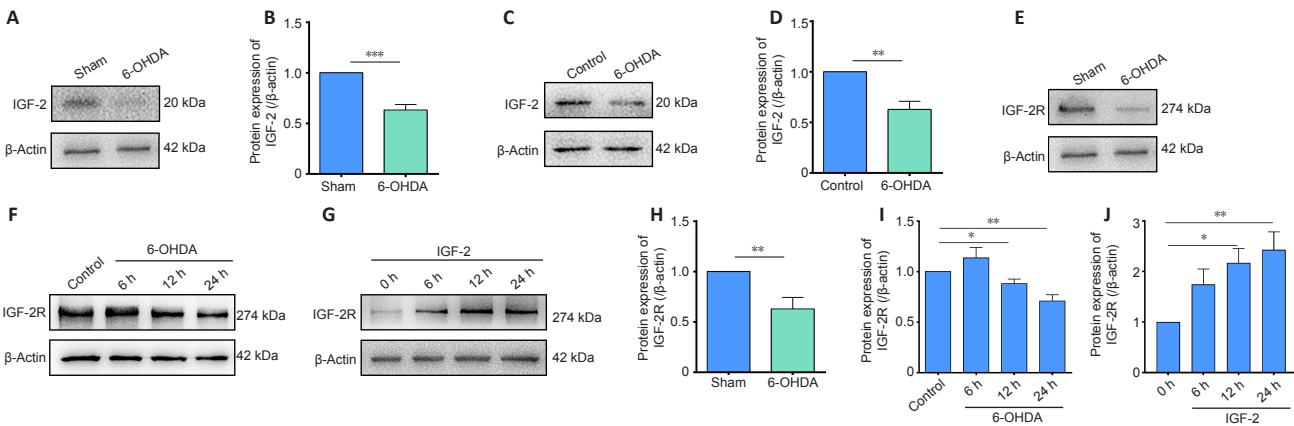


Figure 7 | Changes in IGF-2 and IGF-2R expression in PD models.

(A, B) In PD model mice, IGF-2 protein expression was reduced. (C, D) In SH-SY5Y cells, IGF-2 protein expression in the 6-OHDA group was reduced. (E, H) IGF-2R protein expression was also decreased in PD model mice. (F, I) Time-dependent immunoblotting study of the effect of 6-OHDA on IGF-2R expression in SH-SY5Y cells. (G, J) Effect of IGF-2 pretreatment on IGF-2R expression in SH-SY5Y cells subjected to 6-OHDA-induced injury. Western blot results showing the effect of different durations of IGF-2 pretreatment on IGF-2R expression 24 hours after 6-OHDA injury. All data are presented as mean \pm SEM ($n \geq 3$). * $P < 0.05$, ** $P < 0.01$ (one-way analysis of variance followed by Tukey's *post hoc* test). 6-OHDA: 6-Hydroxydopamine; IGF-2: insulin-like growth factor-2; IGF-2R: insulin-like growth factor-2 receptor; PD: Parkinson's disease.

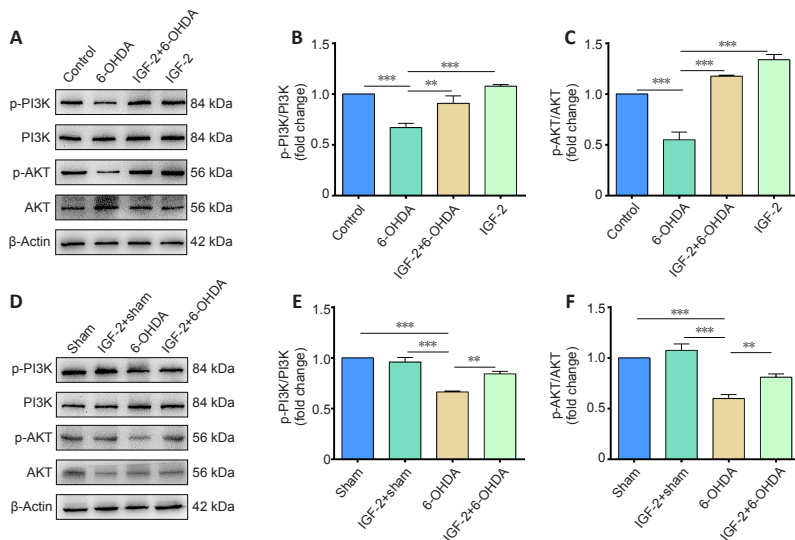


Figure 8 | PI3K and AKT are activated in SH-SY5Y cells and mice pretreated with IGF-2.

SH-SY5Y cells and mice were pretreated with IGF-2 followed by 6-OHDA, and p-PI3K/PI3K and p-AKT/AKT levels were detected by western blot. (A-C) Bands and quantitative analysis of p-PI3K/PI3K and p-AKT/AKT expression in SH-SY5Y cells. (D-F) Bands and quantitative analysis of p-PI3K/PI3K and p-AKT/AKT mouse brain tissue. Data are presented as mean \pm SEM ($n \geq 3$). ** $P < 0.01$, *** $P < 0.001$ (one-way analysis of variance followed by Tukey's *post hoc* test). Protein expression fold changes are expressed relative to control (B and C) or sham (E and F). 6-OHDA: 6-Hydroxydopamine; AKT: protein kinase B; IGF-2: insulin-like growth factor-2; PI3K: phosphatidylinositol 3 kinase; p-AKT: phosphorylated protein kinase B; p-PI3K: phosphorylated phosphatidylinositol 3 kinase.

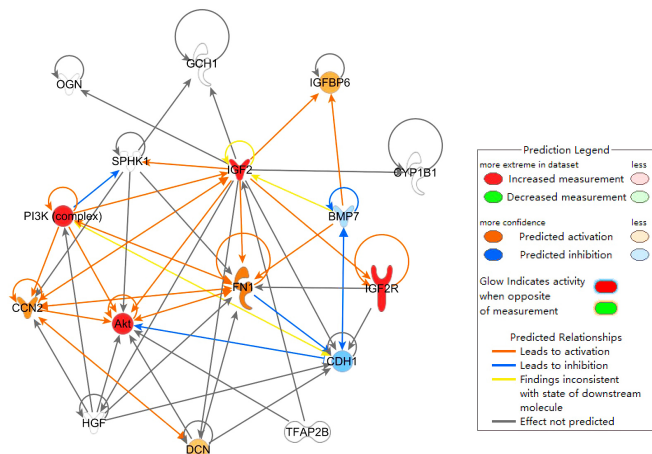


Figure 9 | Protein-protein interaction network in PD mice. The protein-protein interactions between the differentially expressed molecules and IGF-2 were analyzed using Ingenuity Pathway analysis software. Red indicates an increased interaction; green indicates a decreased interaction. Orange indicates predicted activation; blue, indicates predicted inhibition. Orange lines indicate predicted activation; blue lines indicate predicted inhibition; yellow lines indicate findings inconsistent with the state of the downstream molecule; gray lines indicate no predicted effect. IGF-2: Insulin-like growth factor-2; PD: Parkinson's disease.

regeneration (Allodi et al., 2016). Recently, studies have demonstrated the physiological functions of IGF-2 in cognition, memory, and learning. Chen et al. (2011) first demonstrated that IGF-2 administration in rats can significantly improve memory retention and prevent memory loss. Several later studies confirmed that IGF-2 can enhance memory (Stern et al., 2014; Lee et al., 2015; Yu et al., 2020). Taken together, these studies show that IGF-2 can play a therapeutic role in multiple neurological diseases, including neurodegenerative diseases.

However, few studies have investigated the ameliorating effects of IGF-2 on neuronal injury in PD, and even fewer studies have addressed this topic in the 6-OHDA-induced mouse model of PD. Martín-Montañez et al. (2021) described the protective effects of IGF-2 in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neuronal culture and animal models of PD. Their experiments revolved around oxidative stress and mitochondrial function. Although the experimental models and methods they used and the specific research mechanisms studied were different from ours, the results point to a similar conclusion: that IGF-2 is neuroprotective in PD models. In addition, owing to abundant evidence that autophagy is linked to PD progression (Zhang et al., 2015; Bonam et al., 2021; Lizama and Chu, 2021), Sepúlveda et al. (2022) explored possible changes in the expression of autophagy-related genes and IGF-2 in PD. Their results showed that PD patients had altered levels of IGF-2, as well as autophagy-related genes, and that these changes in expression were correlated (Sepúlveda et al., 2022). The IGF-2 levels they measured in PD patients' plasma were significantly lower than those detected in the control subjects' plasma. This contradicts our results showing elevated levels of IGF-2 in the serum of patients and a 6-OHDA-induced mouse model of PD, and indeed results from many previous studies showing that IGF-1 is elevated in PD patients' serum (Godau et al., 2010, 2011; García-Huerta et al., 2020). Our hypothesis is that these discrepancies are related to variations in geographical, clinical, and ethnic factors. In addition, experimental results can also be affected by sample size and detection technique.

We found that, *in vitro*, the neuroprotective effect of IGF-2 is dose-dependent, and that treatment with the appropriate concentration of IGF-2 can confer resistance to the cytotoxic and apoptotic effects of 6-OHDA. In addition, intranasal administration of recombinant IGF-2 to a mouse model of PD reversed the 6-OHDA-induced behavioral motor deficits, DA neuron loss, and α -syn aggregation. Intranasal administration has been gradually adopted in recent years because of its many advantages. First, unlike intravenous administration and direct intraventricular injection, it is a non-invasive approach. Second, intranasal delivery delivers the drug along the olfactory and trigeminal nerves, a route that bypasses the blood-brain barrier, allowing direct delivery of exogenous substances into the brain (Lochhead and Thorne, 2012; Aly and Waszczak, 2015; Lochhead et al., 2019; Long et al., 2020; Wu et al., 2021).

To explore the mechanism underlying the effects described above, we detected IGF-2 localization in the mouse midbrain and found that it was expressed in dopaminergic neurons and surrounding glial cells and neurons. Western blot results suggested that, both in mouse midbrain and in SH-SY5Y cells, IGF-2 expression is reduced after 6-OHDA-induced injury, unlike the increase in IGF-2 expression seen in the peripheral serum of PD patients and the PD mouse model. Because IGF-2 generally binds to its receptor to exert its effects, we assessed the expression of its receptor IGF-2R in the PD mouse model and found that it was reduced compared with that in control mice. Furthermore, *in vitro* results illustrated that IGF-2R expression decreased gradually as 6-OHDA injury time increased, and that treatment with IGF-

2 rescued this effect. This result is consistent with the study by Martín-Montañez et al. (2021). We speculate that the neuroprotective effect of IGF-2 in PD may be mediated by IGF-2R. However, it remains unclear why IGF-2 expression increases in the peripheral serum but decreases in the central brain tissue. A review of the literature showed that there are many precedents for similar apparent contradictions in neurological diseases. For example, insulin and IGF-1 expression are decreased in the brain and increased in the peripheral serum of Alzheimer's disease patients, and administering insulin or IGF-1 can improve cognition and regulate β -amyloid (Craft et al., 1998; Hoyer, 2004; Horvath et al., 2020; Selles et al., 2020). Similarly, in PD, IGF-1 function is impaired, but IGF-1 expression in the circulation increases (Kao, 2009; Picillo et al., 2013; Fan et al., 2018). We speculate that, during the chronic injury that occurs during PD, IGF-2 and IGF-2R production in the central nervous system decreases, resulting in an increase in negative feedback from IGF-2 in the peripheral serum. However, balance is not completely restored, possibly because the amount of IGF-2 produced in the periphery is insufficient, and the efficiency with which it crosses the blood-brain barrier is inadequate to rescue expression in the brain tissue. At the same time, reduced IGF-2R expression prevents IGF-2 from exerting its effects. Thus, administration of IGF-2 at an early stage of the disease could prevent the extreme deficiency of IGF-2 and IGF-2R in brain tissue. Moreover, intranasal administration bypasses the blood-brain barrier, so, compared with subcutaneous injection or intravenous injection, small doses of exogenous IGF-2 administered intravenously could be a potent promoter of neuronal cell survival.

The PI3K/AKT pathway performs an indispensable role in maintaining cell survival and inhibiting apoptosis (Brunet et al., 2001). AKT activates the pathway by promoting the binding of upstream growth factors to their receptors. Our results indicate that this pathway plays a key role in the protective effect of IGF-2 in PD models. After 6-OHDA treatment, p-AKT and p-PI3K levels decreased, and treatment with IGF-2 reversed these changes. This suggests that IGF-2 enhances AKT and PI3K phosphorylation through IGF-2R signaling, thereby activating the anti-apoptotic PI3K/AKT signaling pathway.

Although our results are significant, the study had some limitations. Because we only collected data at a single time point, we cannot draw any conclusions about the effect of IGF-2 treatment over time. In addition, we did not explore whether IGF-binding protein are involved in the IGF-2 signaling changes observed in PD. Therefore, the results should be interpreted with caution. We speculate that IGF-2 may play a neuroprotective effect in PD through IGF-2R/PI3K/AKT signaling. Finally, only male mice were used in our study, as male mice are more resistant and recover better than female mice after injury; indeed, almost all published studies 6-OHDA-induced PD also used male mice.

Despite these limitations, this study clearly demonstrated the effect of IGF-2 on 6-OHDA-induced models PD and characterized changes in IGF-2 expression in PD. Our results suggest that IGF-2 has many effects, such as promoting neuronal survival, inhibiting apoptosis, and degrading toxic protein aggregates. Upregulating IGF-2 in the brain may be an important avenue for therapeutic development in the future. Based on the available literature and the current study, we believe that targeting IGF-2 expression could benefit patients with PD in the future.

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Conflicts of interest: The authors declare that they have no conflicts of interest.

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References

Acharya S, Kim KM (2021) Roles of the functional interaction between brain cholinergic and dopaminergic systems in the pathogenesis and treatment of schizophrenia and Parkinson's disease. *Int J Mol Sci* 22:4299.
 Agrogiannis GD, Sifakis S, Patsouris ES, Konstantinidou AE (2014) Insulin-like growth factors in embryonic and fetal growth and skeletal development (Review). *Mol Med Rep* 10:579-584.
 Allodi I, Comley L, Nichterwitz S, Nizzardo M, Simone C, Benitez JA, Cao M, Corti S, Hedlund E (2016) Differential neuronal vulnerability identifies IGF-2 as a protective factor in ALS. *Sci Rep* 6:25960.
 Aly AE, Waszczak BL (2015) Intranasal gene delivery for treating Parkinson's disease: overcoming the blood-brain barrier. *Expert Opin Drug Deliv* 12:1923-1941.
 Beletskiy A, Chesnokova E, Bal N (2021) Insulin-like growth factor 2 as a possible neuroprotective agent and memory enhancer: its comparative expression, processing and signaling in mammalian CNS. *Int J Mol Sci* 22:1849.

- Bloem BR, Okun MS, Klein C (2021) Parkinson's disease. *Lancet* 397:2284-2303.
- Bonam SR, Tranchant C, Muller S (2021) Autophagy-lysosomal pathway as potential therapeutic target in Parkinson's disease. *Cells* 10:3547.
- Braak H, Del Tredici K, Rub U, de Vos RA, Jansen Steur EN, Braak E (2003) Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging* 24:197-211.
- Brunet A, Datta SR, Greenberg ME (2001) Transcription-dependent and-independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr Opin Neurobiol* 11:297-305.
- Castilla-Cortázar I, García-Fernández M, Delgado G, Puche JE, Sierra I, Barhoum R, González-Barón S (2011) Hepatoprotection and neuroprotection induced by low doses of IGF-II in aging rats. *J Transl Med* 9:103.
- Chen DY, Stern SA, Garcia-Osta A, Saunier-Rebori B, Pollonini G, Bambah-Mukku D, Blitzer RD, Alberini CM (2011) A critical role for IGF-II in memory consolidation and enhancement. *Nature* 469:491-497.
- Chen Y, Shen J, Ma C, Cao M, Yan J, Liang J, Ke K, Cao M, Xiaosu G (2020) Skin-derived precursor Schwann cells protect SH-SY5Y cells against 6-OHDA-induced neurotoxicity by PI3K/AKT/Bcl-2 pathway. *Brain Res Bull* 161:84-93.
- Cheung YT, Lau WK, Yu MS, Lai CS, Yeung SC, So KF, Chang RC (2009) Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. *Neurotoxicology* 30:127-135.
- Craft S, Peskind E, Schwartz MW, Schellenberg GD, Raskind M, Porte D, Jr. (1998) Cerebrospinal fluid and plasma insulin levels in Alzheimer's disease: relationship to severity of dementia and apolipoprotein E genotype. *Neurology* 50:164-168.
- El-Shewy HM, Luttrell LM (2009) Insulin-like growth factor-2/mannose-6 phosphate receptors. *Vitam Horm* 80:667-697.
- Fan D, Alamri Y, Liu K, MacAskill M, Harris P, Brimble M, Dalrymple-Alford J, Prickett T, Menzies O, Laurenson A, Anderson T, Guan J (2018) Supplementation of blackcurrant anthocyanins increased cyclic glycine-proline in the cerebrospinal fluid of Parkinson patients: potential treatment to improve insulin-like growth factor-1 function. *Nutrients* 10:714.
- Gage GJ, Kipke DR, Shain W (2012) Whole animal perfusion fixation for rodents. *J Vis Exp*:3564.
- García-Huerta P, Troncoso-Escudero P, Wu D, Thiruvalluvan A, Cisternas-Olmedo M, Henriquez DR, Plate L, Chana-Cuevas P, Saquel C, Thielens P, Longo KA, Geddes BJ, Lederkremer GZ, Sharma N, Shenkman M, Naphade S, Sardi SP, Spichiger C, Richter HG, Court FA, et al. (2020) Insulin-like growth factor 2 (IGF2) protects against Huntington's disease through the extracellular disposal of protein aggregates. *Acta Neuropathol* 140:737-764.
- Godau J, Herfurth M, Kattner B, Gasser T, Berg D (2010) Increased serum insulin-like growth factor 1 in early idiopathic Parkinson's disease. *J Neurol Neurosurg Psychiatry* 81:536-538.
- Godau J, Knauer K, Weber K, Brockmann K, Maetzler W, Binder G, Berg D (2011) Serum insulinlike growth factor 1 as possible marker for risk and early diagnosis of Parkinson disease. *Arch Neurol* 68:925-931.
- Gu XS, Wang F, Zhang CY, Mao CJ, Yang J, Yang YP, Liu S, Hu LF, Liu CF (2016) Neuroprotective effects of paeoniflorin on 6-OHDA-lesioned rat model of Parkinson's disease. *Neurochem Res* 41:2923-2936.
- Hanson LR, Fine JM, Svitak AL, Faltsek KA (2013) Intranasal administration of CNS therapeutics to awake mice. *J Vis Exp*:4440.
- Hawkes C, Jhamandas JH, Harris KH, Fu W, MacDonald RG, Kar S (2006) Single transmembrane domain insulin-like growth factor-II/mannose-6-phosphate receptor regulates central cholinergic function by activating a G-protein-sensitive, protein kinase C-dependent pathway. *J Neurosci* 26:585-596.
- Heuer A, Smith GA, Lelos MJ, Lane EL, Dunnett SB (2012) Unilateral nigrostriatal 6-hydroxydopamine lesions in mice: I: motor impairments identify extent of dopamine depletion at three different lesion sites. *Behav Brain Res* 228:30-43.
- Horvath A, Salman Z, Quinlan P, Wallin A, Svensson J (2020) Patients with Alzheimer's disease have increased levels of insulin-like growth factor-I in serum but not in cerebrospinal fluid. *J Alzheimers Dis* 75:289-298.
- Hoyer S (2004) Glucose metabolism and insulin receptor signal transduction in Alzheimer disease. *Eur J Pharmacol* 490:115-125.
- Hunt DP, Jahoda C, Chandran S (2009) Multipotent skin-derived precursors: from biology to clinical translation. *Curr Opin Biotechnol* 20:522-530.
- Iancu R, Mohapel P, Brundin P, Paul G (2005) Behavioral characterization of a unilateral 6-OHDA-lesion model of Parkinson's disease in mice. *Behav Brain Res* 162:1-10.
- Jeon HJ, Park J, Shin JH, Chang MS (2017) Insulin-like growth factor binding protein-6 released from human mesenchymal stem cells confers neuronal protection through IGF-1R-mediated signaling. *Int J Mol Med* 40:1860-1868.
- Kao SY (2009) Rescue of alpha-synuclein cytotoxicity by insulin-like growth factors. *Biochem Biophys Res Commun* 385:434-438.
- Khuong HT, Kumar R, Senjaya F, Grochmal J, Ivanovic A, Shakhbazau A, Forden J, Webb A, Biernaskie J, Midha R (2014) Skin derived precursor Schwann cells improve behavioral recovery for acute and delayed nerve repair. *Exp Neurol* 254:168-179.
- Lee Y, Lee YW, Gao Q, Lee Y, Lee HE, Ryu JH (2015) Exogenous insulin-like growth factor 2 administration enhances memory consolidation and persistence in a time-dependent manner. *Brain Res* 1622:466-473.
- Lewitt MS, Boyd GW (2019) The role of insulin-like growth factors and insulin-like growth factor-binding proteins in the nervous system. *Biochem Insights* 12:1178626419842176.
- Lin L, Du L (2018) The role of secreted factors in stem cell-mediated immune regulation. *Cell Immunol* 326:24-32.
- Liu GY, Yang LY, Li WY, Zheng JK (2013) Phenotypes and characteristics of human skinderived precursors. *Zhongguo Zuzhi Gongcheng Yanjiu* 17:6495-6500.
- Lizama BN, Chu CT (2021) Neuronal autophagy and mitochondria in Parkinson's disease. *Mol Aspects Med* 82:100972.
- Lochhead JJ, Thorne RG (2012) Intranasal delivery of biologics to the central nervous system. *Adv Drug Deliv Rev* 64:614-628.
- Lochhead JJ, Kellohen KL, Ronaldson PT, Davis TP (2019) Distribution of insulin in trigeminal nerve and brain after intranasal administration. *Sci Rep* 9:2621.
- Long Y, Yang Q, Xiang Y, Zhang Y, Wan J, Liu S, Li N, Peng W (2020) Nose to brain drug delivery- a promising strategy for active components from herbal medicine for treating cerebral ischemia reperfusion. *Pharmacol Res* 159:104795.
- Ma C, Zhang W, Wang W, Shen J, Cai K, Liu M, Cao M (2021) SKP-SCs transplantation alleviates 6-OHDA-induced dopaminergic neuronal injury by modulating autophagy. *Cell Death Dis* 12:674.
- Mahul-Mellier AL, Burtscher J, Maharjan N, Weerens L, Croisier M, Kuttler F, Leleu M, Knott GW, Lashuel HA (2020) The process of Lewy body formation, rather than simply alpha-synuclein fibrillization, is one of the major drivers of neurodegeneration. *Proc Natl Acad Sci U S A* 117:4971-4982.
- Maki RG (2010) Small is beautiful: insulin-like growth factors and their role in growth, development, and cancer. *J Clin Oncol* 28:4985-4995.
- Mao YF, Guo Z, Zheng T, Jiang Y, Yan Y, Yin X, Chen Y, Zhang B (2016) Intranasal insulin alleviates cognitive deficits and amyloid pathology in young adult APPswe/PS1dE9 mice. *Aging Cell* 15:893-902.
- Martin-Montañez E, Valverde N, Ladrón de Guevara-Miranda D, Lara E, Romero-Zerbo YS, Millon C, Boraldi F, Ávila-Gámiz F, Pérez-Cano AM, Garrido-Gil P, Labandeira-Garcia JL, Santin LJ, Pavia J, García-Fernández M (2021) Insulin-like growth factor II prevents oxidative and neuronal damage in cellular and mice models of Parkinson's disease. *Redox Biol* 46:102095.
- Mellott TJ, Pender SM, Burke RM, Langley EA, Blusztajn JK (2014) IGF2 ameliorates amyloidosis, increases cholinergic marker expression and raises BMP9 and neurotrophin levels in the hippocampus of the APPswePS1dE9 Alzheimer's disease model mice. *PLoS One* 9:e94287.
- O'Kusky J, Ye P (2012) Neurodevelopmental effects of insulin-like growth factor signaling. *Front Neuroendocrinol* 33:230-251.
- Ouchi Y, Banno Y, Shimizu Y, Ando S, Hasegawa H, Adachi K, Iwamoto T (2013) Reduced adult hippocampal neurogenesis and working memory deficits in the Dgcr8-deficient mouse model of 22q11.2 deletion-associated schizophrenia can be rescued by IGF2. *J Neurosci* 33:9408-9419.
- Picillo M, Erro R, Santangelo G, Pivonello R, Longo K, Pivonello C, Vitale C, Amboni M, Moccia M, Colao A, Barone P, Pellicchia MT (2013) Insulin-like growth factor-1 and progression of motor symptoms in early, drug-naïve Parkinson's disease. *J Neurol* 260:1724-1730.
- Postuma RB, Berg D, Stern M, Poewe W, Olanow CW, Oertel W, Obeso J, Marek K, Litvan I, Lang AE, Halliday G, Goetz CG, Gasser T, Dubois B, Chan P, Bloem BR, Adler CH, Deuschl G (2015) MDS clinical diagnostic criteria for Parkinson's disease. *Mov Disord* 30:1591-1601.
- Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9:671-675.
- Selles MC, Fortuna JTS, Zappa-Villar MF, de Faria YPR, Souza AS, Suemoto CK, Leite REP, Rodriguez RD, Grinberg LT, Reggiani PC, Ferreira ST (2020) Adenovirus-mediated transduction of insulin-like growth factor 1 protects hippocampal neurons from the toxicity of Aβ oligomers and prevents memory loss in an Alzheimer mouse model. *Mol Neurobiol* 57:1473-1483.
- Sepúlveda D, Grunenwald F, Vidal A, Troncoso-Escudero P, Cisternas-Olmedo M, Villagra R, Vergara P, Aguilera C, Nassif M, Vidal RL (2022) Insulin-like growth factor 2 and autophagy gene expression alteration arise as potential biomarkers in Parkinson's disease. *Sci Rep* 12:2038.
- Son JW, Park J, Kim YE, Ha J, Park DW, Chang MS, Koh SH (2019) Glia-like cells from late-passage human MSCs protect against ischemic stroke through IGFBP-4. *Mol Neurobiol* 56:7617-7630.
- Steinmetz AB, Stern SA, Kohtz AS, Descalzi G, Alberini CM (2018) Insulin-like growth factor II targets the mTOR pathway to reverse autism-like phenotypes in mice. *J Neurosci* 38:1015-1029.
- Stern SA, Kohtz AS, Pollonini G, Alberini CM (2014) Enhancement of memories by systemic administration of insulin-like growth factor II. *Neuropsychopharmacology* 39:2179-2190.
- Tolosa E, Garrido A, Scholz SW, Poewe W (2021) Challenges in the diagnosis of Parkinson's disease. *Lancet Neurol* 20:385-397.
- Toma JG, McKenzie IA, Bagli D, Miller FD (2005) Isolation and characterization of multipotent skin-derived precursors from human skin. *Stem Cells* 23:727-737.
- Tran C, Damaser MS (2015) Stem cells as drug delivery methods: application of stem cell secretome for regeneration. *Adv Drug Deliv Rev* 82:83-111.
- Vafaei F, Zarifkar A, Emamghoreishi M, Namavar MR, Shirzad S, Ghazavi H, Mahdaviadeh V (2020) Insulin-Like Growth Factor 2 (IGF-2) Regulates neuronal density and IGF-2 distribution following hippocampal intracerebral hemorrhage. *J Stroke Cerebrovasc Dis* 29:105128.
- Wang F, Yasuhara T, Shingo T, Kameda M, Tajiri N, Yuan WJ, Kondo T, Baba T, Tayra JT, Kikuchi Y, Miyoshi Y, Date I (2010) Intravenous administration of mesenchymal stem cells exerts therapeutic effects on parkinsonian model of rats: focusing on neuroprotective effects of stromal cell-derived factor-1alpha. *BMC Neurosci* 11:52.
- Wang HK, Wang YX, Xue CB, Li ZM, Huang J, Zhao YH, Yang YM, Gu XS (2016) Angiogenesis in tissue-engineered nerves evaluated objectively using MICROFIL perfusion and micro-CT scanning. *Neural Regen Res* 11:168-173.
- Wang J, Hu WW, Jiang Z, Feng MJ (2020) Advances in treatment of neurodegenerative diseases: perspectives for combination of stem cells with neurotrophic factors. *World J Stem Cells* 12:323-338.
- Wang X, Zhao Z, Gong J, Zhou S, Peng H, Shatara A, Zhu TZ, Meltzer R, Du Y, Gu H (2014) Adipose stem cells-conditioned medium blocks 6-hydroxydopamine-induced neurotoxicity via the IGF-1/PI3K/AKT pathway. *Neurosci Lett* 581:98-102.
- Wong YC, Kraic D (2017) alpha-synuclein toxicity in neurodegeneration: mechanism and therapeutic strategies. *Nat Med* 23:1-13.
- Wu H, Zhou Y, Wang Y, Tong L, Wang F, Song S, Xu L, Liu B, Yan H, Sun Z (2021) Current state and future directions of intranasal delivery route for central nervous system disorders: a scientometric and visualization analysis. *Front Pharmacol* 12:717192.
- Yan JN, Zhang HY, Li JR, Chen Y, Jiang YC, Shen JB, Ke KF, Gu XS (2022) Schwann cells differentiated from skin-derived precursors provide neuroprotection via autophagy inhibition in a cellular model of Parkinson's disease. *Neural Regen Res* 17:1357-1363.
- Yang L, Zhang X, Li S, Wang H, Zhang X, Liu L, Xie A (2020) Intranasal insulin ameliorates cognitive impairment in a rat model of Parkinson's disease through Akt/GSK3β signaling pathway. *Life Sci* 259:118159.
- Yang W, Hao W, Meng Z, Ding S, Li X, Zhang T, Huang W, Xu L, Zhang Y, Yang J, Gu X (2021) Molecular regulatory mechanism and toxicology of neurodegenerative processes in MPTP/probenecid-induced progressive Parkinson's disease mice model revealed by transcriptome. *Mol Neurobiol* 58:603-616.
- Yu XW, Pandey K, Katzman AC, Alberini CM (2020) A role for CIM6P/IGF2 receptor in memory consolidation and enhancement. *eLife* 9:e54781.
- Zhang H, Duan C, Yang H (2015) Defective autophagy in Parkinson's disease: lessons from genetics. *Mol Neurobiol* 51:89-104.
- Zheng H, Shi C, Luo H, Fan L, Yang Z, Hu X, Zhang Z, Zhang S, Hu Z, Fan Y, Yang J, Mao C, Xu Y (2021) alpha-Synuclein in Parkinson's disease: does a prion-like mechanism of propagation from periphery to the brain play a role? *Neuroscientist* 27:367-387.
- Zhu C, Huang J, Xue C, Wang Y, Wang S, Bao S, Chen R, Li Y, Gu Y (2018) Skin derived precursor Schwann cell-generated acellular matrix modified chitosan/silk scaffolds for bridging rat sciatic nerve gap. *Neurosci Res* 135:21-31.