Original Article

α-Syn oligomers incubated with Parkinson’s disease plasma promote neuron damage

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Abstract: Alpha-Synuclein (α-Syn) aggregates represent the major component of Lewy bodies (LBs), a pathologic hallmark of Parkinson’s disease (PD). Current reports have assessed the toxicity of oligomeric α-Syn (o-α-Syn) mostly in vitro after the incubation with PBS, which leaves o-α-Syn non-phosphorylated and does not reflect actual physiologic conditions in PD patients. The present study aimed to assess the pathogenic role of o-α-Syn while addressing the above issues using o-α-Syn incubated with PD plasma. Several α-Syn oligomer types were prepared by incubating recombinant human α-Syn with phosphate-buffered saline (PBS), and plasma samples from normal controls (NS) and PD patients. O-α-Syn incubated with PD plasma (o-α-Syn-PD), moderately or highly phosphorylated at serine 129, induced cell death more substantially compared with the PBS and NS groups. PD plasma exhibited reduced PP2A activity and ceramide levels, promoting the phosphorylation of o-α-Syn. In agreement, ceramide addition alleviated o-α-Syn-PD cytotoxicity. In vivo, o-α-Syn-PD significantly reduced dopaminergic neurons in the substantia nigra and could be transferred to the cortex, hippocampus, and other parts of the brain. Mice administered o-α-Syn-PD exhibited significant PD-like dyskinesia changes in a short period of time. Finally, o-α-Syn-PD injection was associated with decreased GCase and PP2A activities in the mouse brain. The above findings provide novel insights into the effect of o-α-Syn on neurodegeneration in PD and dementia with LBs.

Keywords: α-synuclein, plasma, Parkinson’s disease, phosphorylation

Introduction

Parkinson’s disease (PD) is the second most common degenerative pathology of the nervous system following Alzheimer’s disease (AD). Pathologic changes in PD characterized by Lewy bodies (LBs) have been reported in the olfactory bulb, spinal cord, intramedullary plexus of the digestive tract, and other peripheral ganglia as early as at the dopaminergic neuron lesion stage [1].

The major component of LBs is fibrillar alpha-Synuclein (α-Syn), a 140-amino acid protein normally found in neurons in a monomeric form. Before forming fibrils, α-Syn aggregates into oligomers and protofibrils, which causes neurodegeneration [2]. Although many factors promote α-Syn aggregation in experimental studies, phosphorylation at serine (Ser) 129 may be the major factor under pathologic conditions; indeed, 90% of α-Syn in LBs is phosphorylated at Ser 129 compared with ≤4% in the normal brain [3]. In addition, in vitro studies revealed that phosphorylated α-Syn increases the formation of soluble oligomers [4, 5].

Various animal models have been established to demonstrate the neurotoxic effects of α-Syn aggregates, including a transgene that expresses an α-Syn mutant (tyrosine-to-cysteine mutant human α-Syn) with the tendency to aggregate, exhibiting age-dependent motor and cognitive impairment in a mouse model. Consistent with the time course of these behavioral changes, accumulation of α-Syn oligomers and neuronal degeneration occur in the parts of the nervous system responsible for motor and cognitive functions [6]. In recent years, scholars have artificially incubated human recombinant α-Syn in phosphate buffer saline (PBS) to generate oligomers or preformed fibrils, which were then injected into the striatum [7], olfactory mucosa [4], or digestive tract
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Previous studies have confirmed that α-Syn oligomers can diffuse between neurons, inducing their degeneration and death [8, 9].

As mentioned above, two studies have determined the neurotoxic effects of α-Syn aggregates. One of them utilized α-Syn formed by aggregation in vitro in PBS or Tris-HCl buffer, while the other employed a transfected cell or animal with an α-Syn mutant susceptible to aggregation. Although these two methods could demonstrate the neurotoxic effects of α-Syn oligomers to a certain extent, such α-Syn aggregates are not identical to the pathogenic ones found in PD patients. For example, the generation of these α-Syn oligomers did not involve phosphorylation, which is an important factor in the formation of α-Syn oligomers in PD patients. In addition, due to a lack of phosphorylation, the conformations and neurotoxic characteristics of the above synthetic α-Syn aggregates may differ from those of counterparts formed in PD patients.

Materials and methods

Patients

A total of 35 clinically diagnosed idiopathic PD patients (14 females and 21 males; age, 56.27 ± 14.23 years) attending an outpatient clinic in the Neurology Department of Xuanwu Hospital were assessed. The patients were diagnosed by a consultant neurologist based on the UK Parkinson’s Disease Society Brain Bank criteria for idiopathic PD [10]. Exclusion criteria were Parkinsonism, a history of brain surgery/deep brain stimulation, or any psychiatric illness. All participants provided informed consent for participation in the study, which was approved by the Ethics Committee of Xuanwu Hospital. Age and gender-matched healthy individuals were assessed as controls. The demographic features of PD patients and neurologically healthy subjects (n = 35 per group) are shown in Table 1. There were no differences between the patients and normal subjects with respect to age, education level, Montreal Cognitive Assessment (MoCA), or Beck Depression Inventory (BDI). For PD patients, mean L-dopa equivalent daily dose (mg), disease duration (year), Hoehn & Yahr (H&Y) stage, and UPDRS III scores were also recorded.

Table 1. Demographic data and clinical features of the study subjects

<table>
<thead>
<tr>
<th>No</th>
<th>Age, yr</th>
<th>Gender M/F</th>
<th>Education, yr</th>
<th>PD duration, yr</th>
<th>LEDD, mg</th>
<th>MoCA, mean ± SD (range)</th>
<th>UPDRS III</th>
<th>H&amp;Y stage median (25th, 75th percentile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>35</td>
<td>55.23 ± 13.81</td>
<td>21/14</td>
<td>16.67 ± 5.88</td>
<td>-</td>
<td>26.5 ± 1.4 (25-30)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PD</td>
<td>35</td>
<td>56.27 ± 14.23</td>
<td>21/14</td>
<td>15.33 ± 6.71</td>
<td>5.63 ± 4.3</td>
<td>568.33 ± 286.62</td>
<td>25.8 ± 1.5 (25-30)</td>
<td>35.84 ± 17.92</td>
</tr>
</tbody>
</table>

M = male, F = female, PD = Parkinson disease patients, LEDD = mean L-dopa equivalent daily dose, MoCA = Montreal Cognitive Assessment, UPDRS III = Unified Parkinson Disease Rating Scale, part III (motor), H&Y stage = Hoehn & Yahr stage.

Blood collection and plasma preparation

Blood samples were collected from cases and control patients in EDTA-coated vacuum tubes, and plasma was separated by centrifugation at 3000 × g for 20 min. Endogenous α-Syn in the obtained plasma was removed by affinity purification using an anti-α-Syn antibody prepared as described by Yu et al. [11]. Plasma samples were finally aliquoted and stored at -80°C until use.

Animals

Male C57BL/6 mice (7 weeks, 18-20 g) were supplied by Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed under a 12-h light/12-h dark cycle at 20-23°C with free access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committee of Capital Medical University (Approval No. SCXK-2019-007), and performed in strict accordance
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with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The median H&Y stage of PD patients was 3 (see Table 1). Therefore, plasma samples from H&Y stage 3 PD patients were used for preparing α-Syn-oligomers (o-α-Syn-PD), which were injected into the mouse striatum. α-Syn incubated with normal plasma was denoted o-α-Syn-NS, and PBS-incubated α-Syn oligomer as o-α-Syn-PBS. After SDS-PAGE and western blot identification, protein quantitation was performed, and protein concentration was adjusted to 2.5 µg/µL. The samples were then filtered and sterilized for later use.

Preparation and purification of recombinant human α-Syn

Recombinant α-Syn was prepared by transforming the pET-15b-NACP plasmid into *Escherichia coli* BL21 cells (both kindly provided by prof. Yu, Xuanwu Hospital, Capital Medical University), and purified by sequential ion-exchange chromatography, hydrophobic chromatography, and reverse-phase chromatography. The purity of the α-Syn protein was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), while its identity was confirmed by western blot with a monoclonal antibody (3D5) against o-α-Syn prepared as described by Yu et al. [11]. The protein concentration was determined with a bicinchonic acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA).

α-Syn incubation with plasma or PBS for α-Syn oligomer preparation

To prepare α-Syn oligomers, 100 µM of recombinant human α-Syn was dissolved in PBS (pH 7.0) or 1/3 PBS-diluted NS or PD plasma, and incubated at 37°C for 48 h with continuous shaking (1000 rpm) on an Eppendorf Thermomixer Comfort (2605271; Sigma-Aldrich, St. Louis, MO, USA). Immunofluorin chromatography was used to isolate α-Syn mixtures containing monomers, dimers, and oligomers from the plasma according to a previously described method [12]. The α-Syn oligomers formed in PBS (o-α-Syn-PBS) and plasma (o-α-Syn-NS or o-α-Syn-PD), respectively, were separated from the monomers and dimers using a Micro Protein Recovery kit (Sangon, Biotech, Shanghai, China).

Stereotactic injection

Mice were anaesthetized by peritoneal injection of 4 ml/kg of 6% chloral hydrate. Purified α-Syn oligomers were injected into the striatum at the following stereotactic coordinates: -0.8 mm from the bregma, 2.0 mm to the left of the midline, and 3.0 mm below the subdural matter. Purified α-Syn oligomers (2 µl) was injected into experimental mice while control groups received equal volumes of physiological saline.

Cell viability assay

Cell viability was estimated using the MTT formazan colorimetric assay. 20 µL of MTT solution (5 mg/mL in PBS) was added to each well of 96-well plates and incubated in a humidified incubator with 5% CO₂ at 37°C for 4 h. The medium was removed, followed by addition of 100 µL of DMSO. The plates were centrifuged at 40,000 rpm for 10 min. The optical density of the formazan product in solution was measured at 490 nm on a microplate reader (Mutiskan MK3, Thermo Scientific, Waltham, MA, USA). 5 µM C₂-ceramide (Sigma, 860644P) was added to PD plasma for 8 h to detect cell viability in the o-α-Syn-PD group.

Western blot analysis of α-Syn phosphorylation and oligomerization

The mouse brain was removed 60 days after α-Syn oligomer injection and immediately placed in chilled artificial cerebral spinal fluid (ACSF, in mM: NaCl 125.0, KCl 2.5, CaCl₂ 2.0, NaHCO₃ 26.0, NaH₂PO₄ 1.25, MgCl₂ 1.0, glucose 5.0, pH 7.4) bubbling with 95% O₂ and 5% CO₂. Brain tissues from the indicated regions were collected and lysed with a buffer containing Tris-Cl (50 mM, pH 7.5), NaCl (150 mM), EGTA (5 mM), EDTA (5 mM), SDS (2% w/v), and a protease inhibitor cocktail (Roche, Basel, Switzerland). The lysates were centrifuged at 12000 × g for 30 min at 4°C, and the resulting supernatants were used as whole-cell homogenates. Western blot was performed as described by Cheng. et al. [13]. Briefly, samples (20 µg proteins/lane) were separated by 10% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), and incubated at 4°C overnight with respective primary antibodies, including anti-3D5 anti-α-Syn (1:5000) and anti-pS α-Syn (1:5000 Santa Cruz Biotechnology, Dallas, TX,
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USA) antibodies followed by a 1 h reaction at room temperature with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:5000) (Santa Cruz Biotechnology). Immunoreactivity was visualized using ECL reagents. All western blot experiments were repeated at least five times.

Detection of α-Syn oligomerization and phosphorylation by enzyme-linked immunosorbent assay (ELISA)

Levels of α-Syn oligomers formed in PBS and NS/PD plasma or brain tissues were measured by a previously described ELISA method [13]. Briefly, non-biotinylated and biotinylated 3D5 mouse monoclonal antibodies [11] were used for capture and detection, respectively. After immunoreaction, incubation was performed with ExtrAvidin alkaline phosphatase (E-2636; Sigma-Aldrich, St. Louis, MO, USA) followed by a reaction with p-nitro-phenyl phosphate (N1891; Sigma-Aldrich). Absorbance at 405 nm was read on a Multiskan MK3 microplate reader (Thermo Scientific, UT, USA). To detect phosphorylated α-Syn, an anti-pS α-Syn polyclonal antibody (Santa Cruz Biotechnology) was used as a capture antibody. The remaining steps were the same as those described for the detection of α-Syn oligomers.

Ceramide assessment in plasma

Plasma ceramide levels were measured by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) (ProteinChip System 4000 mass spectrometer, enterprise version; Bio-Rad). A plasma sample (10 µl) was spotted onto a reversed phase H50 (hydrophobic surface) ProteinChip array in 5% acetonitrile and 0.05% trifluoroacetic acid, and incubated in a humid environment for 20 min at room temperature. This was followed by washing with 10 µl of 5% acetonitrile and 0.05% trifluoroacetic acid. After drying, 0.5 µl of a saturated sinapinic acid solution in 50% acetonitrile (v/v) and 0.5% trifluoroacetic acid (v/v) was applied to the spots, which were given time to dry prior to analysis on a ProteinChip reader (Ciphergen Biosystems, Surrey, UK). Calibration was performed in parallel using protein standards with molecular masses ranging from 1084.247 Da (vasopressin) to 1570.600 Da (fibrinogen peptide). The ceramide peak at 422.9 m/z was identified by ceramide pre-absorption with anti-ceramide monoclonal antibody (C8104, Sigma-Aldrich); 10 µg of antibody was added to 200 µL of plasma, followed by overnight incubation at 4°C. The antibody-ceramide complex was removed using Protein G Sepharose Fast Flow (P3296; Sigma-Aldrich), and 10 µL of the ceramide-depleted plasma sample was spotted onto the H50 ProteinChip Array. Data were processed with the ProteinChip Data Manager Software. Duplicate samples were averaged before data analysis.

Immunohistochemistry

Brain tissue sections were prepared as previously described [14]. They were cut into 20-µm sections on a cryostat for immunohistochemistry. The sections were blocked with 5% normal goat serum, and incubated sequentially with anti-TH monoclonal antibody (1:10000), biotinylated goat anti-mouse IgG (1:500), and horseradish-peroxidase-conjugated streptavidin. The bound peroxidase was subsequently revealed using a solution containing diaminobenzidine, hydrogen peroxide, and nickel ammonium sulfate. The images were captured under a microscope (Leica, Wetzlar, Germany). On average, 50 neurons were imaged per treatment condition, and individual images were analyzed with the Image J software.

Protein phosphatase 2A (PP2A) activity assay

PP2A activity in brain homogenates was measured as previously described [15] using a PP2A Colorimetric Assay Kit (GenMed Scientifics Inc., Arlington, MA, USA). Protein levels in supernatants were determined by the Bradford assay (GMS 30030.1; GenMed Scientifics Inc.) and normalized to 5 mg/mL.

GCase activity assay

GCase activity was determined using the QuantiChrom β-Glucosidase Assay Kit (DBGD-100; BioAssay Systems Inc., Hayward, CA, USA). Distilled water (20 µL) was added to 2 wells of a clear bottom 96-well plate; 200 µL of either distilled water or calibrator was then added to the wells for a total volume of 220 µL. Samples (20 µL) were loaded in the remaining wells, and 200 µL of working reagent was added to each sample to a final reaction volume of 220 µL. The solutions were mixed by
briefly tapping the plate, and optical density at 405 nm was measured immediately and at 20 minutes (t¼ 20 minutes) on a plate reader. The data were used to calculate the GCase activity of the sample (U/L) based on the hydrolysis of 1-mM substrate per minute by 1 unit of enzyme at pH 7.0.

Measurement of ceramide levels

Plasma C₂-ceramide levels were measured with a human ceramide ELISA kit (DRE126-57; Rapidbio Biosource, West Hills, CA, USA) according to the instructions provided by the manufacturer.

Pole test

According to Ogawa et al. [16], a straight wooden rod (diameter, 0.8 cm; height, 60 cm) was made. A small wooden ball was placed on the top of the rod, which was covered with gauze to prevent mice from slipping. Mice were placed facing upwards at the top of the rod; then, the time at which the animal began to move down the rod and that at which the animal reached the bottom were evaluated. Each detection interval was 1 minute, and a total of 5 detections were averaged.

Grid test

According to Kuribara et al. [17], a hanging metal rod (1.5 mm diameter horizontal wire) was made and placed horizontally, 30 cm from the ground. A cover was placed 1 cm above the rod to prevent mice from sitting. In this experiment, each mouse was suspended from the metal rod. A mouse using both hind paws scored 3 points, and one utilizing one hind paw had 2 points. If both hind paws were unable to grasp the rod, the animal scored 1 point. A total of 5 measurements were averaged for statistical analysis.

Roller test

This task is commonly used to detect motion coordination. It requires the animal to maintain balance and continuous movement on rollers. A Rotamex-5 mouse electric spindle instrument (Columbus Instruments, Columbus, OH, USA; diameter of 6 cm, rotation speed of 20 r/min) was employed. The average value was calculated after 20 consecutive measurements [18].

Beam test

For this task, a 105 cm × 4 cm × 3 cm wooden pole was positioned 80 cm above the ground, with both ends supported by a wooden frame. One end of the wooden pole was the starting area (20 cm), and the other was the ending area leading to a mouse cage. During the experiment, the mouse was placed in the starting area facing the direction of the squirrel cage, and a timer was started. The time it took for the mouse to walk through the starting area (latency period) and that required to walk through the entire balance bar were recorded. The maximum allowable time for the latency period was 1 min. The maximum allowable time for walking across the bar was 2 min [19]. The mice were trained three times before starting the actual experiment. Three tests were performed and data were averaged.

Statistical analysis

Statistical analysis was performed using the SPSS 19.0 software. Measurement data are mean ± standard deviation (SD). Comparisons between the two groups were performed by independent samples t-test. Comparisons of multiple groups were performed by one-way ANOVA, followed by post-hoc Tukey test. P<0.05 was considered statistically significant.

Results

α-Syn oligomers generated in plasma samples from PD patients are prone to phosphorylation

Recombinant human α-Syn was expressed in bacteria and purified by HPLC. Then, western blotting and SDS-PAGE were performed to identify recombinant human α-Syn monomer with high purity (Figure 1A). After incubation of α-Syn for 48 h with PD plasma, immunoaffinity chromatography was performed to purify α-Syn oligomers, mainly including dimers, trimers, and tetramers (Figure 1B, 1C). 3D5 and anti-pS α-Syn were used as primary antibodies in western blotting. The obtained α-Syn oligomers were not disassembled by 1% Triton X-100 or 2% SDS, and remained stable (Figure 1D). In addition, the levels of the oligomers formed in PD plasma were higher than those generated in PBS (Figure 1E). Moreover, the α-Syn oligomers purified from the PD group had higher phosphorylation levels than those generated in NC.
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cell viability was assessed. Notably, in the o-α-Syn-NS group, cell viability was approximately 30% lower than that of the control group. In addition, the viability of cells was significantly lower in the o-α-Syn-PD group compared with the o-α-Syn-NS group (Figure 2A). Meanwhile, cell viability decreased in a time- and dose-dependent manner in the o-α-Syn-PD group (Figure 2B, 2C). Flow cytometry indicated that the total number of apoptotic cells in the o-α-Syn-PD group was approximately 14.3% and 7.6% elevated compared with those of the control and o-α-Syn-NS groups, respectively (Figure 2D, 2E).

Decreased PP2A activity leads to α-Syn hyperphosphorylation and oligomerization through ceramide level reduction in PD plasma

WB demonstrated that o-α-Syn and pS-α-Syn amounts were increased significantly in α-Syn incubated with PD plasma compared with the NS plasma group (Figure 3A, 3B). SELDI-TOF-MS analysis of plasma C2-ceramide identified a peak at 422.9 that disappeared in the presence of an antibody targeting naturally occurring ceramide (Figure 3C, 3D). The C2-ceramide peak was lower in PD plasma compared with the NS group (n = 35, P<0.01) (Figure 3E). Meanwhile, in agreement with C2-ceramide changes (Figure 3F), levels of GCase and PP2A activities were lower in PD plasma compared with NS plasma (Figure 3G, 3H). However, addition of C2-ceramide, a PP2A activator, could reverse PP2A activity but not GCase activity in PD plasma. Furthermore, we assessed whether changes in PP2A activity and C2-ceramide levels would mediate the increased α-Syn neurotoxicity of plasma from

Figure 1. α-Syn oligomers generated in plasma samples from PD patients are prone to phosphorylation. A. Recombinant human α-Syn monomer showing high purity as assessed by western blotting and SDS-PAGE. B. C. Immunoaffinity chromatography was performed to purify α-Syn dimers, trimers and tetramers. D. α-Syn oligomers remain stable after treatment with 1% Triton X-100 or 2% SDS. Ser 129: phosphorylated α-Syn at serine 129. o-α-Syn: α-Syn oligomers. m-α-Syn: α-Syn monomers. E. The levels of the oligomers formed in PD plasma (3) were higher than those generated in PBS (1) or NC plasma (2). F. α-Syn oligomers were more phosphorylated in PD plasma (3) than the NC group (2); no phosphorylated o-α-Syn was found in the PBS group (1).

plasma, while phosphorylated o-α-Syn was absent in the PBS group as expected (Figure 1F). These results suggested o-α-Syn is prone to phosphorylation in PD plasma.

α-Syn oligomers incubated in PD plasma induce MES23.5 neuron damage

To investigate differences in cytotoxic effects among α-Syn oligomers incubated with different plasma types on MES23.5 neurons, neuron viability was assessed. Notably, in the o-α-Syn-NS group, cell viability was approximately 30% lower than that of the control group. In addition, the viability of cells was significantly lower in the o-α-Syn-PD group compared with the o-α-Syn-NS group (Figure 2A). Meanwhile, cell viability decreased in a time- and dose-dependent manner in the o-α-Syn-PD group (Figure 2B, 2C). Flow cytometry indicated that the total number of apoptotic cells in the o-α-Syn-PD group was approximately 14.3% and 7.6% elevated compared with those of the control and o-α-Syn-NS groups, respectively (Figure 2D, 2E).

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Figure 2. α-Syn oligomers incubated in PD plasma induce MES23.5 neuron damage. (A) Reduced cell viability in the o-α-Syn-NS group versus the control and o-α-Syn-NS groups. *P<0.05 vs. control group; **P<0.01 vs. control group; #P<0.05 vs. o-α-Syn-NS group. (B, C) Decreases in cell viability in a time- and dose-dependent manner in the o-α-Syn-PD group. (D, E) Promotion of cell apoptosis in the o-α-Syn-PD group compared with the control and o-α-Syn-NS groups, as shown by flow cytometry (D, E). *P<0.05 vs. control group; **P<0.01 vs. control group; #P<0.05 vs. o-α-Syn-NS group.
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PD patients (Figure 3I). The o-α-Syn-PD group showed significantly decreased cell viability compared with the o-α-Syn-PBS and o-α-Syn-NS groups. However, C2-ceramide (5 μM, 8 h), an activator of PP2A, significantly attenuated the cell death induced by α-Syn oligomers incubated in PD plasma (Figure 3I). This suggested that α-Syn underwent phosphorylation in PD plasma by PP2A activity and ceramide level reduction, causing oligomer formation and enhancing cell death.

**PD plasma-incubated α-Syn oligomers inhibit dopaminergic neurons in the substantia nigra in the mouse brain**

A total of 60 days after striatum injection, immunohistochemical detection showed a
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decrease in the number of TH-positive neurons (TH-ir) in the substantia nigra of o-α-Syn-PD treated mice, while the o-α-Syn-NS and o-α-Syn-PBS groups showed no significant inhibition of TH-ir neurons (immunohistochemistry) in the o-α-Syn-NS and o-α-Syn-PBS groups. (A) Representative micrographs. (B) Quantitation of A. **P<0.01 vs. Control Group; #P<0.05 vs. o-α-Syn-NS group. (C, D) Tissue samples were from control animals, and mice injected with o-α-Syn-PBS, o-α-Syn-NS, or o-α-Syn-PD for detecting o-α-Syn by Western blot (C) and ELISA (D). β-actin was used as the loading control. (C) and (D) Various CNS regions, including the Str (striatum), Frc (frontal cortex), Hip (hippocampus) and Mes (mesencephalon), were assessed. n = 5. *P<0.05 vs. Control Group; #P<0.05 vs. o-α-Syn-NS group. (E, F) Tissue samples were from control mice, and animals injected with o-α-Syn-PBS, o-α-Syn-NS, or o-α-Syn-PD for detecting pS-α-Syn by western blot (E) and ELISA (F). β-actin is the loading control. Various CNS region, including the Str (striatum), Frc (frontal cortex), Hip (hippocampus) and Mes (mesencephalon) were assessed. n = 5. *P<0.05 vs. Control Group; #P<0.05 vs. o-α-Syn-NS group.

Figure 4. Distribution of PD plasma-incubated α-Syn oligomers damage dopaminergic neurons in the mouse brain. Fewer TH-positive neurons (TH-ir) neurons found in the substantia nigra of o-α-Syn-PD treated mice (injection into the striatum) and no significant inhibition of TH-ir neurons (immunohistochemistry) in the o-α-Syn-NS and o-α-Syn-PBS groups. (A) Representative micrographs. (B) Quantitation of A. **P<0.01 vs. Control Group; #P<0.05 vs. o-α-Syn-NS group. (C, D) Tissue samples were from control animals, and mice injected with o-α-Syn-PBS, o-α-Syn-NS, or o-α-Syn-PD for detecting o-α-Syn by Western blot (C) and ELISA (D). β-actin was used as the loading control. (C) and (D) Various CNS regions, including the Str (striatum), Frc (frontal cortex), Hip (hippocampus) and Mes (mesencephalon), were assessed. n = 5. *P<0.05 vs. Control Group; #P<0.05 vs. o-α-Syn-NS group. (E, F) Tissue samples were from control mice, and animals injected with o-α-Syn-PBS, o-α-Syn-NS, or o-α-Syn-PD for detecting pS-α-Syn by western blot (E) and ELISA (F). β-actin is the loading control. Various CNS region, including the Str (striatum), Frc (frontal cortex), Hip (hippocampus) and Mes (mesencephalon) were assessed. n = 5. *P<0.05 vs. Control Group; #P<0.05 vs. o-α-Syn-NS group.

Distribution of α-Syn oligomers incubated in PD plasma

Western blotting and ELISA showed that no o-α-Syn was detected in the control group at 60
Figure 5. PD plasma-incubated α-Syn oligomers reduce GCase activity, ceramide levels, and PP2A activity in the mouse brain and cause PD-like behavioral changes in mice. (A-D) At 60 days after striatum injection, GCase activities, ceramide levels, and PP2A activities were assessed in the Str (striatum) (A), Frc (frontal cortex) (B), Hip (hippo-
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campus) (C) and Mes (mesencephalon) (D) in the control, α-Syn-PBS, o-α-Syn-NS, and o-α-Syn-PD groups. *P<0.05 vs. Control Group; #P<0.05 vs. o-α-Syn-NS group. (E) Mean scores in the pole test in C57BL/6 mice. The o-α-Syn-PD showed decreased scores than the control and o-α-Syn-NS groups at 60 days after injection. n = 4, *P<0.05 vs. Control Group; #P<0.05 vs. o-α-Syn-NS group. (F) Mean scores in the suspension test assessed by C57BL/6 mice. n = 4, *P<0.05 vs. Control Group; #P<0.05 vs. o-α-Syn-NS group. (G) Balance times in the rotarod test assessed by C57BL/6 mice. n = 4, *P<0.05 vs. Control Group; #P<0.05 vs. o-α-Syn-NS group. (H) Spent times in the beam test in C57BL/6 mice. 60 days following injection, the times spent by the o-α-Syn-PBS and o-α-Syn-NS groups were increased; the time that the o-α-Syn-PD group spent through the beam was increased significantly compared to before injection, with significant differences with o-α-Syn-NS group. n = 4, *P<0.05 vs. Control Group; #P<0.05 vs. o-α-Syn-NS group.

days after striatum injection. O-α-Syn in the brain striatum of mice in each experimental group was elevated after the injection of oligomers, and there were no differences among groups. In the o-α-Syn-PD group, o-α-Syn was detected in the hippocampus, midbrain and frontal lobe on the 60th day after injection, with significantly increased contents compared with the o-α-Syn-NS and o-α-Syn-PBS groups (Figure 4C, 4D). These findings suggested that o-α-Syn incubated in PD plasma exhibited stronger stability and was easier to spread. No pS-α-Syn was detected in any brain area in the control or o-α-Syn-PBS group. In the o-α-Syn-PD group, the striatum, hippocampus, and midbrain all contained pS-α-Syn, whose amounts were higher compared with the o-α-Syn-NS group (Figure 4E, 4F). This result suggested that α-Syn oligomers with higher phosphorylation levels were more rapidly aggregated and transported to brain regions in mice after incubation with PD plasma.

PD plasma-incubated α-Syn oligomers reduce GCase activity, ceramide levels, and PP2A activity in the mouse brain

At 60 days after striatum injection, the o-α-Syn-PD group showed lower GCase activities, ceramide levels, and PP2A activities in the striatum and midbrain compared with the o-α-Syn-NS group (Figure 5A, 5D). Meanwhile, there were no differences in GCase activity, ceramide levels, and PP2A activity between the o-α-Syn-PD and o-α-Syn-NS groups in the frontal lobe and hippocampus (Figure 5B, 5C). The above results suggested that o-α-Syn incubated with PD plasma caused alterations in intrinsic enzymes regulating α-Syn phosphorylation after striatum injection.

PD plasma-incubated-Syn oligomer injection into the striatum causes PD-like behavioral changes in mice

Seven-week-old C57BL/6 mice were randomized, and α-Syn oligomers incubated in PD plasma were injected into the striatum for neurotoxicity observation in vivo. At 60 days following injection, rod climbing, suspension, roller, and balance rod experiments were performed to evaluate the exercise abilities of mice. 60 days after injection, the o-α-Syn-NS group showed slight tremors, reduced movement, individual hindlimb opening, and vertical hair changes. Behavioral experiments showed that the animals had longer rod climbing time and weaker suspension abilities, and required shorter and prolonged times to maintain balance on the roller and to achieve balance on the rod, respectively (Figure 5E-H). Compared with the control and normal human plasma groups, the o-α-Syn-PD group showed significantly decreased motor capacity, markedly increased tremors, and stiff limbs. Individual mice had PD-like dyskinesia with gait instability and slow responses (Figure 5E-H).

Discussion

In the present study, we found that exogenous α-Syn incubated with PD plasma forms oligomers, which promote neuronal cell death upon incubation with PD plasma. At 60 days after injection with purified o-α-Syn incubated in PBS, NS plasma and PD plasma, the amounts of substantia nigra TH neurons were significantly lower in the PD group compared with the two remaining groups, and mice treated with o-Syn-PD exhibited more PD-like symptoms compared with the remaining groups. We further demonstrated that o-α-Syn incubated with PD plasma was more easily transmitted to the hippocampus, frontal lobe, and midbrain compared with the other two groups. Moreover, the PD plasma-derived oligomer exhibited a high stability and inhibited PP2A and GCase activities in four different brain regions.

The reason behind the significant difference in the cytotoxicity of α-Syn oligomers incubated with PD plasma compared with the NS plasma
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and PBS groups remains unclear. However, α-Syn phosphorylation at Ser 129, which further determines α-Syn conformation, might play a role. Indeed, C₂-ceramide, an activator of PP2A, significantly attenuated the cell death induced by α-Syn oligomers incubated with PD plasma. In support of this notion, both our present and previous findings show that recombinant human α-Syn incubated in NS and PD plasmas is differentially phosphorylated at Ser 129 [20]. Elevated α-Syn oligomer phosphorylation in PD plasma exerted more potent effects on cell death compared with the reduced amounts of phosphorylated α-Syn oligomers found in NC plasma and non-phosphorylated α-Syn oligomers detected in PBS, although the same concentrations of different α-Syn oligomers were used.

The molecule in PD plasma causing α-Syn hyperphosphorylation remains undefined. Nevertheless, it is known that C₂-ceramide reduces α-Syn phosphorylation and oligomerization by activating PP2A [21]. We further demonstrated that decreased PP2A activity in PD plasma resulted from reduced endogenous ceramide levels, leading to elevated α-Syn phosphorylation and oligomerization. These results corroborated a previous study reporting a reduction in ceramide levels in the brains of PD patients [22]. The alterations in PP2A activity and ceramide levels found in plasma likely reflect changes in the brains of PD patients.

At 60 days after o-α-Syn injection in the striatum, the oligomers were transmitted to the mouse midbrain, frontal lobe and hippocampus. But the underlying mechanism remains unclear. On the one hand, under normal conditions, α-Syn exists at the synaptic terminals and can be reversely transported to axons; therefore, after 60 days human-derived α-Syn oligomers can be detected in the mouse midbrain. A study showed that synthetic α-Syn fibrils injected into adult wild-type marmoset brains (caudate nucleus and/or putamen) result in abundant α-Syn appearance within only three months post-injection and robust formation of Lewy body-like inclusions in TH-positive neurons. This strongly suggests a retrograde spreading of abnormal α-Syn from striatum to substantia nigra. On the other hand, the prion-like propagation theory, which states that α-Syn can propagate from neurons to neurons, could explain o-α-Syn transmission. When α-Syn oligomers cause neuron death in the brain, the dead cells release o-α-Syn and are taken up again by other neurons. A brilliant study showed that after 14 years of transplantation into the striatum of an individual with PD, grafted nigra neurons have Lewy body-like inclusions, supporting the notion that α-Syn can spread from neurons to neurons in the brain [9].

We found that α-Syn oligomers incubated in PD plasma exhibited more stability. One reason is that hyperphosphorylated o-α-Syn can inhibit the cell protease degradation system. On the other hand, it may be related to changes in GCase activity in neurons. Previous studies have revealed a bidirectional positive feedback loop between α-Syn and GCase [23]. On the one hand, loss of GCase activity leads to the accumulation of GlcCer, which stabilizes α-Syn oligomers; on the other hand, α-Syn such accumulation blocks the transport of GCase from the endoplasmic reticulum to lysosomes, resulting in lysosomal GCase depletion and o-α-Syn-PD stability.

However, it remains unknown how α-Syn inhibits GCase activity. One possibility is that α-Syn oligomers have a higher binding affinity with GCase and can therefore modulate its enzymatic activity to a greater extent in isolated lysosomes. The co-localization of α-Syn and GCase in LBs indicates that aggregated α-Syn can tightly bind to GCase [24].

The limitations of this study should be mentioned. First, the conformation of pathogenic α-Syn remains unknown. In addition, this was a combination of in vitro and mouse experiments, and whether these results could be repeated in humans is not known. Therefore, further studies are required to confirm these findings.

In summary, o-α-Syn-PD causes neuron cell death in vitro and in vivo. Meanwhile, α-Syn oligomers formed in PBS are not phosphorylated and differ from those generated in NC and PD plasma, which are moderately and highly phosphorylated at Ser 129, a key phosphorylation site of α-Syn in PD patients. Overall, this study provides new insights into building new PD animal models, which could use pathogenic α-Syn oligomers formed in PD plasma.
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Disclosure of conflict of interest

None.

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