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The couple of netrin-1/α-Synuclein regulates the survival of dopaminergic neurons via α-Synuclein disaggregation

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The abnormal accumulation and aggregation of the misfolded α-synuclein protein is the neuropathological hallmark of all α-synucleinopathies, including Parkinson's disease. The secreted proteins known as netrins (netrin-1, netrin-3, and netrin-4) are related to laminin and have a role in the molecular pathway for axon guidance and cell survival. Interestingly, only netrin-1 is significantly expressed in the substantia nigra (SN) of healthy adult brains and its expression inversely correlates with that of α-synuclein, which prompted us to look into the role of α-synuclein and netrin-1 molecular interaction in the future of dopaminergic neurons. Here, we showed that netrin-1 and α-synuclein directly interacted in pre-formed fibrils (PFFs) generation test, real time binding assay, and co-immunoprecipitation with neurotoxin treated cell lysates. Netrin-1 deficiency appeared to activate the dopaminergic neuronal cell death signal pathway via α-synuclein aggregation and hyperphosphorylation of α-synuclein S129. Taken together, netrin-1 can be a promising therapeutic molecule in Parkinson's disease. [BMB Reports 2023; 56(2): 126-131]

INTRODUCTION

The netrin family (netrin-1, netrin-3 and netrin-4) are secreted proteins that administer the migration and control the axonal guidance as a multifunctional plasticity cue (1, 2). During development, it is well expressed in neuroepithelial cells of the spinal cord (3). Netrin-1, -3, and -4 proteins are involved in the Laminin VI, Laminin V, and C345C domains, which are partially included in binding to the netrin receptors (UNC5) (4). Although netrin-1, -3, and -4 share protein structural domains, the brain regions where the proteins are expressed differ for different neuronal cell types (5, 6). Especially, netrin-1 protein is expressed in ventral tegmental area (VTA), substantia nigra (SN), and striatum (Str), which is related to the dopaminergic neurons (7, 8). Dopaminergic neuron is known to be specifically vulnerable to neurodegeneration in Parkinson's disease (PD) (9, 10). Patients with PD experience disabling motor symptoms as a result of the gradual degradation of dopaminergic neurons in the substantia nigra pars compacta (SNpc), which lowers their quality of life in general (11). Numerous PD molecular studies have been reported that α-synuclein aggregation and hyperphosphorylation trigger dopaminergic neuronal cell death and initiate the PD progression (12). Unfortunately, the etiology of the PD is still not clear, and current medications are only symptomatic and do not prevent the progression of the PD pathology. As a result, we looked into α-synuclein's direct interaction with netrin-1 in a specific dopaminergic neuron. Consequently, netrin-1 inhibits dopaminergic neuronal loss and motor dysfunctions by reducing α-synuclein aggregation and hyperphosphorylation. Given that netrin-1 expression in mature dopaminergic neurons and its well-known role in cell survival as a dependent secreted protein, we investigated the impact of netrin-1 in the fate of dopaminergic neurons.

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RESULTS

The netrin-1 is significantly reduced in the substantia nigra of PD patients

Overexpression of α-synuclein has been observed in the aging human substantia nigra brain region (SN) (13, 14) leading to Lewy body formation with highly phosphorylated α-synuclein S129 (15). To address importance of the netrin-1 in dopaminergic neurons, we confirmed the expression of netrin-1 in the substantia nigra (SN), which is related to the PD progression. We compared the mRNA expression of netrin-1 in the SN of healthy controls or age-matched PD patients using the Gene expression omnibus (GEO) dataset GDS2821. We observed that the expression of human netrin-1 was significantly reduced in the SN region of PD patients compared to an age-matched healthy control group (Fig. 1A, left). However, there are no differences between netrin-3 (Fig. 1A, middle) and netrin-4 in the mRNA expression of PD (Fig. 1A, right). Next, we used the α-synuclein gene ID in the reprogramming software system (GeneMANIA) to identify direct α-synuclein interactions with

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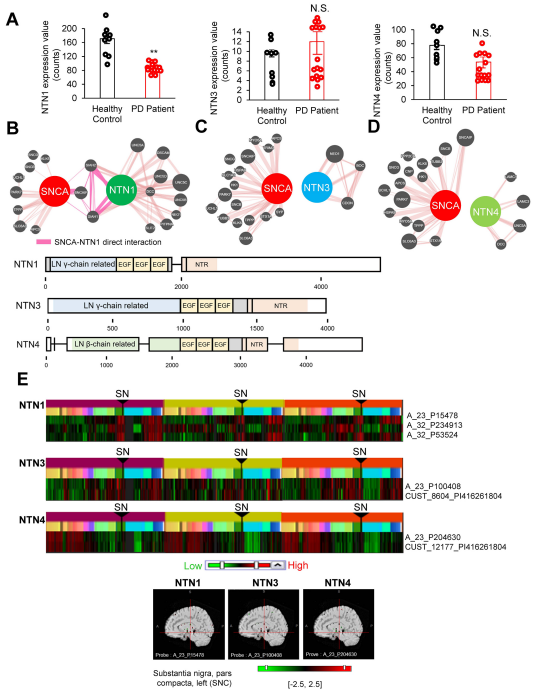

Fig. 1. Investigation of gene expression level among netrin-1, -3, and -4 from PD patients and non-PD (healthy control). (A) Netrin-1 (left), -3, (middle), and -4 (right) gene expression profiling by mRNA of SN from PD and healthy controls through GEO database profiling, accessed with GDS2821. Unpaired t-test, $^{**}P < 0.01$; N.S., not significant. (B-D) Human protein interaction map from (http://www.genemania.org). Each network was visualized with red line (physical interactions). (E) Netrin-1, -3, and -4 gene expression data from 3 healthy adults brains. Microarray data were come from Allen Human Brain Atlas. Codes at the right of the heatmap are probes used in the analysis. MRI images including expression level at SNc part, detected with the same probe, are below the microarray data.

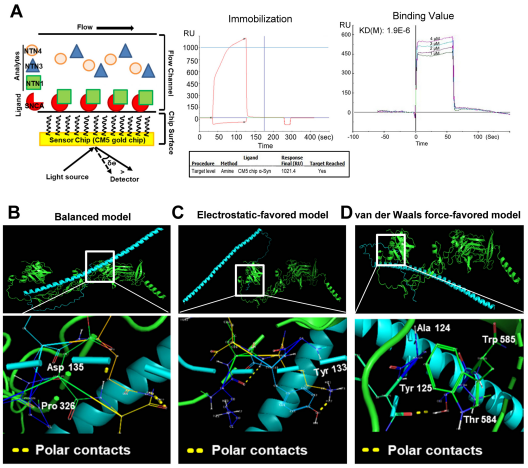

Fig. 2. Direct interaction between netrin-1 and α-synuclein. (A) Real time binding assay with SPR Biacore X100 using the CM5 chip as a binding ligand. *In vitro* test was conducted with purified hnetrin-1 and hα-synuclein recombinant proteins. (B-D) Predictive three-dimensional model of direct interaction structures between netrin-1 (PDB ID: AF-O95631-F1) and α-synuclein (PDB ID: AF-P37840-F1) by ClusPro 2.0 and PyMOL 2.5 software analysis. Balanced model, electrostatic-favored model, and van der Waals force-favored model showed the polar contacts between netrin-1 and α-synuclein.

the netrin family. According to the programming software analysis, netrin-1 interacted directly with α-synuclein (Fig. 1B). We observed that netrin-3 and netrin-4 were not involved in the α-synuclein molecular interactions (Fig. 1C, D). Moreover, we looked at the Allen Human Brain Atlas microarray database to determine the expression of netrin-1, -3, and -4 in PD and non-PD brain tissues in humans (Fig. 1E) and mice (Supplementary Fig. 1A-C). Interestingly, nerin-1 had the highest mRNA expression of all of them in the SN. However in the healthy control group, netrin-3 and netrin-4 expression was substantially lower than netrin-1 (Supplementary Fig. 1D). We isolated the 20 candidates and classified the subjects of netrin-1, -3, and -4 proteins according to their dynamic expression during neuronal cell survival signaling activation via predicted protein interaction reprogramming (Supplementary Fig. 1E). These results clearly presented the interactions of the netrin-1, -3 and -4 with α-synuclein molecules.

Netrin-1 and SNCA directly interact

Using purified recombinant proteins and an *in vitro* binding assay of SPR Biacore X100, we investigated the binding affinity of netrins and α-synuclein. Strikingly, real time binding assay demonstrated strong interaction between netrin-1 and α-synuclein (Fig. 2A). However, the SPR Biacore X100 analysis revealed no interaction between the recombinant netrin-3 and netrin-4 proteins on the α-synuclein-immobilized CM5 chip. Furthermore, we investigated the predictive interacting sites between netrin-1 and α-synuclein proteins. We obtained a three-dimensional structure model of both proteins, including the predictive interaction sites by ClusPro 2.0. As a result, three types of structures (balanced, electrostatically favored, and van der Waals force-favored) revealed polar contacts between netrin-1 and the 124th and 135th amino acids in the α-synuclein C-terminus (Fig. 2B-D). Quantitative RT-PCR (qRT-PCR) demonstrated that the mRNA augmentation of α-synuclein and MAO-B in SH-SY5Y cells by MPP^+ was inversely coupled with netrin-1 mRNA (Supplementary Fig. 2A). In addition, we infected AAV2-SNCA cells for 48 hours before treating SH-SY5Y cells and DIV-10 primary neurons with 200 ng of recombinant netrin-1 protein for 24 hours to see if recombinant netrin-1 regulates α-synuclein phosphorylation at residue S129 in the progression of SH-SY5Y or rat cortical neuronal (DIV 10) cell death. This recombinant netrin-1 effectively inhibited phosphorylated α-synuclein S129 and improved cell viability *in vitro* (Supplemental Fig. 2B-D).

Netrin-1 deprivation initiates α-synuclein aggregation in the primary dopaminergic neurons, inducing PD pathogenesis

We applied the 4th fibronectin domain of Deleted in Colorectal Cancer (DCC-4Fbn), which binds to netrin-1, in the primary dopaminergic neuronal cell to generate the netrin-1-deprived cellular model (8). TUNEL (Fig. 3C, D) and immunofluorescence (Fig. 3A, B) analyses clearly showed that blocking netrin-1 via DCC-4Fbn in primary dopaminergic neurons induced α-synuclein phosphorylation and DNA fragmentation. Consistent observations were made from primary dopaminergic neuronal cell lysates (Fig. 3E). In addition, overexpressed SNCA or deprived netrin-1 in dopaminergic neurons both increased the α-synuclein S129 phosphorylation and reduced the netrin-1 expression as shown in the co-immunofluorescence staining (Supplementary Fig. 3A, B) (16). MPP+ treatment reduced netrin-1 and tyrosine hydroxylase (TH) and inversely increased α-synuclein and phosphorylation on the S129 residue of α-synuclein in the insoluble fraction cell lysates, according to Western blotting. In addition, monoamine oxidase B (MAO-B), which is the major enzyme for dopamine metabolism and activated in PD, was escalated by MPP^+ in a dose-dependent manner. For netin-3 and netrin-4, we applied 120 μg of SH-SY5Y cell lysates due to the low expression in SH-SY5Y cells (Fig. 3F).

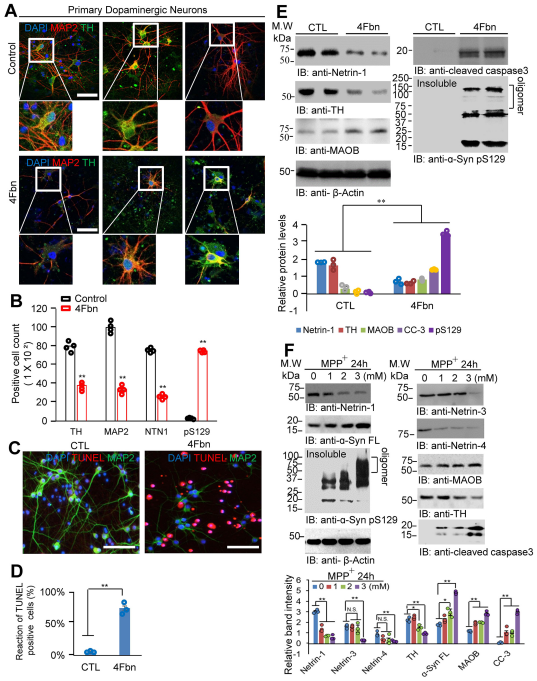

Fig. 3. Dopaminergic neuronal loss accompanied by the deprivation of netrin-1. (A) Co-immunofluorescence staining analysis upon DCC-4Fbn or control IgG treatment primary dopaminergic neurons conducted by TH/MAP2, MAP2/NTN1, and MAP2/pS129 (Scale bar, 50 μm). (B) Quantification bar graph of TH, MAP2, NTN1, and pS129 immunoreactive positive dopaminergic neuron cell numbers. Data are shown as mean + SEM. Statistical significance was determined by an unpaired t-test. N = 4 each group. **P < 0.01. (C) Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labelling (TUNEL) reactivity in primary dopaminergic neurons after control IgG and DCC-4Fbn treatments (Scale bar, 40 μm). (D) DNA fragmentation index (bar graph) expressed as a percentage of TUNEL positive dopamine (DA) neurons out of the total number of MAP2-positive neurons. N = 3 each group. Error bars represent the mean ± SEM. Statistical significance was conducted using a one-way ANOVA followed by post hoc Tukey test for multiple group comparison. **P < 0.01. (E) Immunoblotting assay of netrin-1, TH, MAO-B, cleaved caspase-3, α-Syn pS129 and β-actin levels in control IgG (N = 3) vs DCC-4Fbn treated cell lysates (N = 3). Band intensity quantification bar graph (right). (F) Immunoblot of SH-SY5Y cell lysates with treated neurotoxin MPP^+ in a dose-dependent manner (1-3 mM). Relative band intensity were calculated from the band densitometry software (Image J). Quantification of band intensity (right). N = 3 independent immunoblot assay. Error bars represent the mean ± SEM. Statistical significance was determined by Student's t-test. *P < 0.05; **P < 0.01; N.S., not significant.

Netrin-1 involves neurorestorative effects in cellular model of PD

Now, we investigated whether, inversely, a netrin-1 gain of function might protect or rescue the dopamine cells from cell death. To verify the levels of mRNA for α-synuclein and netrin-1 with or without netrin-1 reintroduction, we used qRT-PCR (Supplementary Fig. 4A, C). Cells were depleted of netrin-1 by treating them with MPP+ neurotoxin or DCC-4Fbn. Next, we applied the netrin-1 protein dose-dependently via qRT-PCR and immunoblotting. Reintroduced netrin-1 in dopaminergic cells induced TH activation and netrin-1 expression, as well as reductions in PD-related marker proteins, MAO-B, cleaved caspase-3, α-synuclein FL (Syn 204), and α-synuclein S129 (Supplementary Fig. 4B, D). In accordance with our previous result, we observed the DCF-DA (intracellular oxidative stress) signal reduction in netrin-1 treated with the DCC-4Fbn group only (Supplementary Fig. 4E, top) and obtained a similar observation in the TUNEL assay (Supplementary Fig. 4E, bottom). These results prove that netrin-1 is required for the maintenance and protection of dopaminergic neuron in adults.

Netrin-1 blocks α-synuclein aggregation via direct interaction in the pure *in-vitro* analysis

Thioflavin T (Th-T) α-synuclein fibrillization kinetics revealed that α-synuclein aggregated fibers formed in a reaction-day-dependent manner. The aggregation rate of α-synuclein + netrin-1 (1:1 ratio) mixture was significantly lower than that of α-synuclein alone (Fig. 4A, B). Anisotropic X-ray diffraction patterns from each sample showed the typical features of a cross-sub-structure, with axial inter-strand reflections at 4.5 for α-synuclein. XRD crystallographic structure analysis revealed that α-synuclein alone and the α-synuclein + netrin-1 mixture group had different x-ray count numbers. The α-synuclein + netrin-1 mixture group significantly reduced the total PFF intensity number from 5795.588 to 1990.044 (Fig. 4C, D). Furthermore, TEM revealed that the PFFs of α-synuclein + netrin-1 were much lower than those of the α-synuclein only group (Fig. 4E, left), and netrin-1 and α-synuclein monomer concentrations were adjusted by

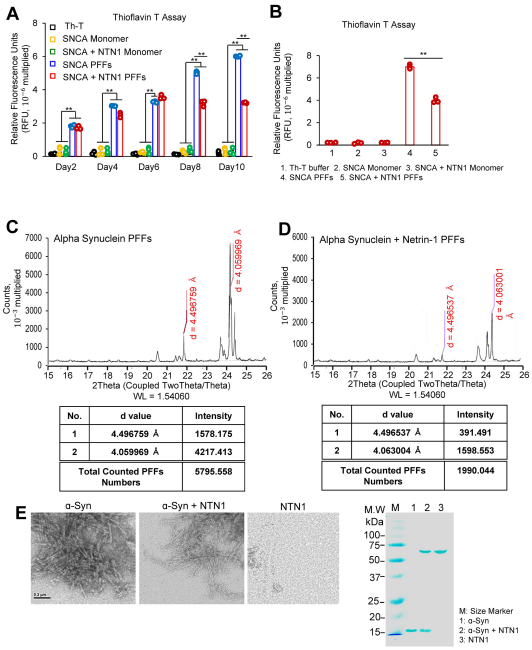

Fig. 4. Structural analyzes for pre-formed fibrils (PFFs) with verification netrin-1 as an inhibitor of α-Synuclein fibrilization. (A) Thioflavin T assay showed that different aggregation rates of purified hα-Syn, hα-Syn + hNTN1 combination at different day of PFFs generation reactions. (B) hα-Syn + hNTN1 complex aggregate and form fibrils much less than hα-Syn only group on reaction day 10. Results are shown as means ± SEM; N = 3 independent experiments; **P < 0.01 by two-tailed Student's t-test. (C) X-Ray diffraction pattern of partially aligned hα-Syn. (D) X-Ray diffraction pattern of partially aligned hα-Syn + hNTN1. (E) Negative stained TEM images of hα-Syn and hα-Syn + hNTN1 complex on day 10 (left). Coomassie blue staining results supported that each PFFs were generated under equal concentrations and ratios (right).

BSA assay to begin PFF reactions with equal amounts of protein (Fig. 4E, right).

DISCUSSION

The current data presented here support an essential and critical role of netrin-1 in the maintenance of mature nigral dopaminergic neurons. Notably, in the adult brain, netrin-1 is mainly expressed in the SNpc of brainstem. Dopaminergic neurons of these brain regions are predominantly impacted by Lewy pathology and neurodegeneration in PD. Targeting PD pathologies underlying the vulnerability of dopaminergic neurons by the specific interactions with α-synuclein might be one of the possible therapeutic approaches to delay or halt neurodegenerative disease progression. However, it is unclear whether the expression pattern of netrin-1 is extensively linked to the selective maintainability of dopaminergic neurons and how α-synuclein modulates dopaminergic neurodegeneration susceptibility. Hence, we revealed that depleting endogenous netrin-1 in the primary dopaminergic neuron. Brain-derived neurotrophic factor (BDNF), which is associated with neurodegeneration, induced such a massive cell loss of mature dopaminergic neurons by its depletion (7, 17-19), and the current study demonstrated a similar role of netrin-1 in the maintenance of dopaminergic neurons. Furthermore, using a real-time binding assay, we demonstrated the direct interaction between netrin-1 and α-synuclein, and predictive protein-protein interaction models support the binding affinity between netrin-1 and α-synuclein at the monomer stage. Considering that Ala 124-Asp 135 regions of α-synuclein interact with netrin-1, we suppose that netrin-1 can inhibit the hyperphosphorylation of α-synuclein via various physical interactions. Even with an equal concentration of starting monomer protein materials, the number of PFFs in the α-synuclein + netrin-1 complex was much lower than the number of PFFs in α-synuclein. These *in vitro* analyses support that the netrin-1 is a key molecule in α-synucleinopathies including PD. We also demonstrated that α-synuclein activation was associated with decreased netrin-1 mRNA and protein expression. Taken together, these findings strongly support the hypothesis that netrin-1 is a key molecule in the development of a therapeutic drug for Parkinson's disease or α-synucleinopathies.

MATERIALS AND METHODS

Primary cultured rat dopaminergic neurons, and cell lines

Animal care and handling was performed according to NIH animal care guidelines and Emory Medical School guidelines. Primary rat cortical neurons were cultured as previously described (20). All rats were purchased from the Jackson Laboratory. The protocol was reviewed and approved by the Emory Institutional Animal Care and Use Committee. SH-SY5Y cells were cultured in DMEM/F12 added with 10% FBS and penicillin (100 units/ml)-streptomycin (100 μg/ml) (all from Hyclone). Cells were incubated at 37°C in a humidified atmosphere of 5% CO2 (21).

Cell viability

Cell viability was measured colorimetrically using the CellTiterBlue (CTB, Promega, Madison, WI, USA) fluorescence-based assay. Cells were plated at a density of 1000 cells/well in 96-well plates (BD Biosciences, San Diego, CA, USA). Six different PFFs were directly introduced to each well after DIV 10 and then incubated for an additional 4 days. After incubation, 30 μl CTB reagent was added to each well and incubated at 37°C and 5% CO2 for 2.5-5 h. Fluorescence of the resorufin product was measured on a FluoDiaT70 fluorescence plate reader (Photon Technology International, Birmingham, NJ, USA). Wells that included vehicle but not protein served as the negative control (0% toxic), and wells containing 10% DMSO were the positive control (100% toxic). Results presented for viability experiments are an average of 3 experiments conducted independently on different days. Error bars represent the standard error of the mean (SEM).

Purification of human α-Syn FL

α-Syn FL cDNAs was subcloned into NdeI and HindIII restriction sites of the bacterial expression vector pRK172, and the proteins were expressed in Escherichia coli BL21 (DE3). Bacterial pellets were resuspended in high-salt buffer (0.75 M NaCl, 50 mM Tris, pH 7.4, 1 mM EDTA) containing a mixture of fresh protease inhibitors, sonicated at 25% power for 3 min in the ice (3 times), and centrifuged at 70,000 × g for 30 min. We used thrombin to remove His tag. After that, α-Syn FL proteins were dialyzed in FPLC buffer (50 mM NaCl, 50 mM Tris-HCl, pH 7.4) overnight at 8°C to change the buffer salt concentration. Proteins were filtered through a 0.22 μm syringe filter and injected into the FPLC machine (Superdex 200 column; GE Healthcare). The fractions were assayed for the presence of the α-Syn FL protein by SDS-PAGE followed by Coomassie Blue staininig (22).

Th-T assay

Thioflavin T stock solution was made by dissolving 16 mg Th-T to 50 ml PBS. Solution was filtered through a 0.2 μm syringe filter (Sigma-Aldrich, cat# T3516). The stock solution was diluted into the PBS to inject the working concentration (2.5 μl of 1 mM Th-T stock solution per each well of 96-well plate). The monomers and PFFs were added according to the working concentration (10 μM). The total volume per each well was fixed to 100 μl. The rest volume except for Th-T solutions and proteins was filled with phosphate buffer. Reaction plate was excited at 450 nm and emitted at 490 nm to measure the relative fluorescence units on the plate reader (Molecular Devices, SpectraMax iD3, California, USA). Measurements were conducted every 15 minutes.

Biacore X100 (real time binding assay)

The carboxylated dextran matrix of the sensor chip was activated by the injection of 60 μl of solution containing 0.2 M N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide and 0.05 M N-hydroxysuccinimide in water. We used the activation buffers from the kit, EDC + NHS. Immobilization step running time was 180s. Purified α-Syn FL proteins were immobilized at a concentration of 50 μg/ml in 10 mM sodium acetate (pH 4.0). 50 mM NaOH was employed for regeneration in 75 μl, maximum volume of deionized water and running buffer (HEPES + EDTA + P20) (BIAcore, GE Healthcare). The remaining binding sites were blocked with 1M ethanolamine (pH 8.5). Screening of purified netrin-1 proteins for binding to α-Syn FL was performed by injecting aliquots (120 μl) of samples (~100 μg protein/ml) onto the derivatized sensor chip. CM5 chip was used for regeneration step. Regeneration of sensor chip after each analysis cycle was performed by injecting 20 μl of 1.5 M glycine/HCl buffer (pH 3.5) and 6 M guanidinium HCl (pH 5.5).

X-ray diffraction (XRD)

Dried stock samples were prepared for X-ray fiber diffraction by vortexing α-Syn FL and α-Syn FL + NTN1 PFFs for 30 min at RT. The PFFs were lyophilized for 3 days using a freeze-drying machine for X-ray diffraction. The sample-to-detector distance was 300 mm, with an exposure time of 30 sec. Diffraction patterns were converted to Tiff files using the program fit-2d (Hammersley/ESRF) and radially integrated to generate one-dimensional scattering patterns using Matlab code. The position of maximum intensity was used to determine the position of anionic reflections and the difference in position of either side of the pattern was used to assess the error in the position of the reflection. The variation in X-ray intensity for the reflections at α-Syn FL PFFs 4.49 Å and 4.05 Å ; α-Syn FL + NTN1 PFFs 4.49 Å and 4.06 Å (fibril reaction buffer: 50 mM Tris-HCl, 50 mM NaCl, pH 7.0). Moreover, we determined X-ray intensity using the same Matlab code. A calibrant of high density polyethylene (HDPE) was used to determine the position of the beam sample-to-detector distance and pixel size.

TUNEL assay

Dopamine neuron death was detected with an *in-situ* cell death detection kit TMR Red (Roche, Cat# 12156792910). The apoptotic index was expressed as the percentage of TUNEL positive neurons out of the total number of TH-positive neurons.

Mouse midbrain primary neuron culture

Dopamine primary neurons were prepared from the ventral midbrain of embryonic mice on day 13 of gestation. Ventral midbrain was isolated and dissected in ice-cold Dulbecco's medium + 0.2% BSA. Fragments were dissected into tiny pieces and collected in 2 ml vials. Then, pieces were washed three times in HBSS (Ca^{2+} and Mg^{2+} free) medium. Pieces were dissociated in HBSS containing 0.01% trypsin for 20 min at 37°C. Cells were dissociated by soft trituration in FBS medium containing 1 μg/ml of DNase I. After trituration, cells were washed in DA neuron culture medium (DMEM F12, N2 1X, 0.36% D-(+)-Glucose (wt/vol), primocin 100 μg/ml) and plated on 96-well plates coated with poly-L-ornithine at a density of 5 × 10^4 cells/well and let to adhere for 1 hour. Then, cells were treated with netrin-1 or GDNF. The treatment was renewed every two days for four days.

Netrin-1 gene-expression profiling dataset

Netrin-1, -3 and -4 gene expression profiling in the SN of PD patients was conducted using a gene dataset available on the GEO repository (GDS2821 accession number). We selected this dataset because the corresponding study (23) included a large number of participants, 9 control patients and 16 PD patients, and very strict RNA quality control criteria were used. Gene expression profiling was done using Affymetrix Human Genome U133 Plus 2.0 GeneChip arrays. We analyzed probe set data for NTN1, NTN3, and NTN4 in the substantia nigra and selected NTN1 probe set on the criterion of "present" (detectable) call. GeneMANIA software was used to investigate actual molecular interactions and common pathway among netrin family and α-Synuclein. Data were collected from primary

studies found in various databases, including BioGRID, PathwayCommons, Reactome and BioCyc (24, 25).

Protein-protein interaction prediction analysis

Protein-protein interaction was analyzed and visualized by ClusPro 2.0 (https://cluspro.bu.edu/) and PyMOL 2.5 (https://pymol.org/2/) software. Protein Data were collected Protein Data Bank (PDB, USA) and PDB files for netrin-1 and α-Synuclein were entered into receptor protein and ligand protein. Binding residues are presented as the technical preset mode and modified with an extensive binding atomic groups per one residue. Polar contacts, inter-chain contacts, and pi interactions were investigated at the target amino acid regions (26, 27).

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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