Supplementary Data

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

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Supplemental method

Immunoblotting

Cells and brain samples were lysed and, if necessary, homogenized in lysis buffer (50 mM Tris, pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1.5 mM Na3VO4, 50 mM NaF, 10 mM sodium pyrophosphate and 10 mM sodium β-glycerophosphate, supplemented with a cocktail of protease inhibitors). Lysates were then centrifuged for 20 min at 4 °C 15000 rpm and protein concentration of the supernatant was measured with the Pierce BCA Protein Assay Kit (Part No. 23225). The supernatant was denatured at 95 °C in Laemmli buffer. After loading and running proteins in a SDS - PAGE gel, the samples were transferred to a nitrocellulose membrane. Membrane blocking and antibody staining were performed according to the primary antibody manufacturer's instructions. Primary antibodies to the following targets were used: alpha synuclein FL (Santa Cruz, Cat# SC69977) alpha-synuclein pS129 (LS bio, Cat# LS-C380861-1)); Tyrosine Hydroxylase (Santa Cruz, SC-25269; Abcam, Cat# ab112); netrin-1(Santa Cruz, Cat# SC20786 or SC-293197; Abcam, Cat# ab126729); netrin-3 (Abcam, Cat# ab185200); netrin-4 (Santa Cruz, Cat# SC365280) MAO-B(Abcam, Cat# ab133270) and cleaved caspase-3 (Cell signaling Cat# 9661).

Generation of α-Syn FL PFFs

Fibrils were prepared in reaction (500 μL per tube) containing 5 mg/mL of α-Syn FL and α-Syn FL (5 mg/mL) + NTN1 (5 mg/mL) protein monomers in PFF reaction buffer (50 mM Tris-HCL, 50 mM NaCl, pH 7.4). The monomer proteins were incubated for several days at 37 캜, with orbital shaking at 300 rpm until samples appeared cloudy. Usually, the reactions were subjected to 8-10 days shaking for the PFFs generation. PFFs were validated by Th-T in vitro assay.

Immunofluorescence

Free-floating slices were rinsed in PBS then permeabilized and blocked with PBS-BT (50 mM Tris-HCL, 150 mM NaCl, 3% bovine serum albumin (BSA), 0.1% Triton-X100, pH 7.4) blocking solution for 1 h. Afterwards, the sections were incubated with primary antibodies (see key resource table) in a 2% normal donkey serum (NDS) and 0.3% Triton X-100 PBS solution on a shaker overnight at 4 캜. The next day, sections were rinsed and incubated with corresponding secondary antibodies directly conjugated with fluorophores (1:2000 Alexa 594 and Alexa Fluor 488 conjugate from Jackson ImmunoResearch) for 2 h at room temperature. Finally, slices were rinsed in PBS and mounted (Sigma Aldrich, F4680).

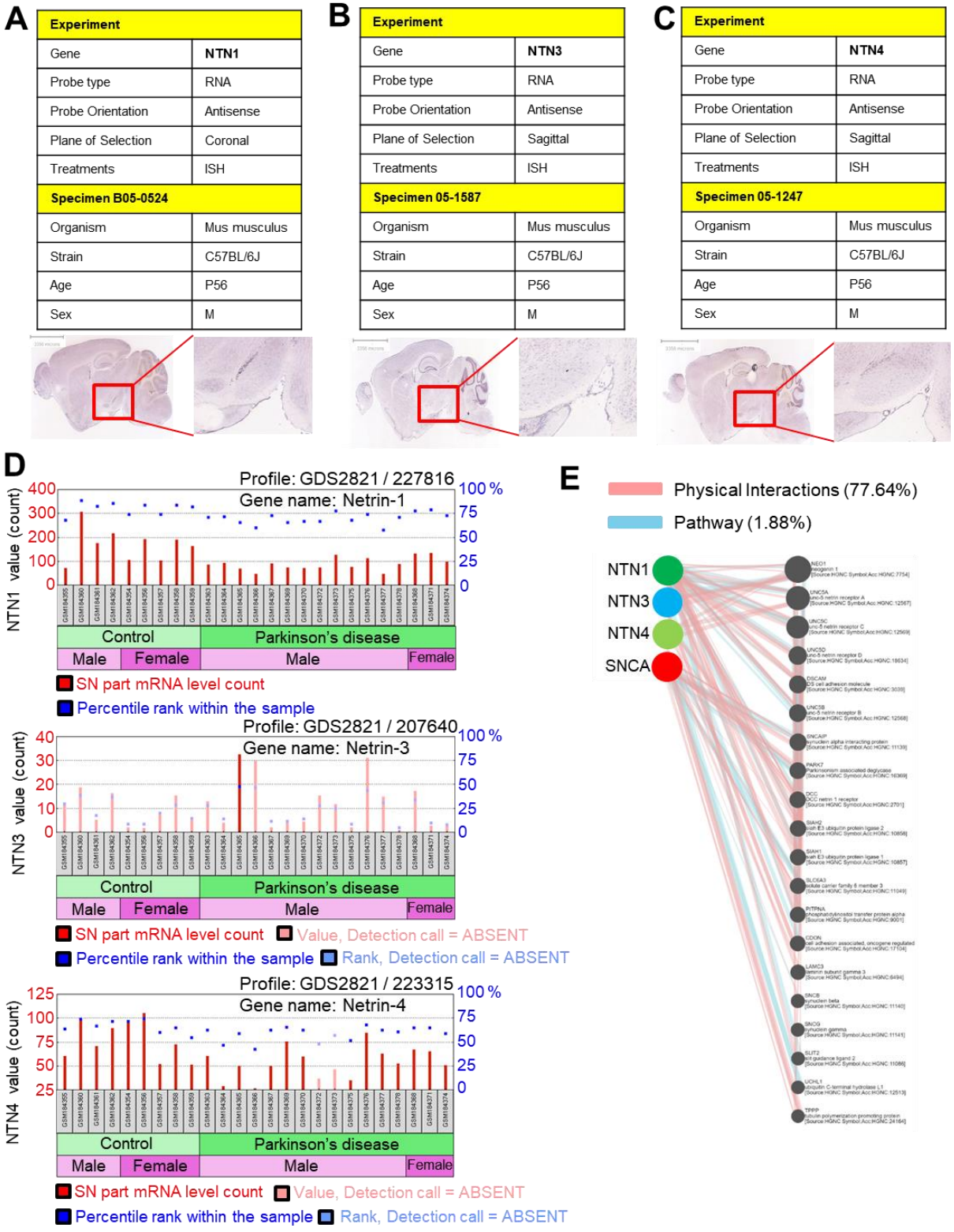
Transmission electron microscopy (TEM)

Electron microscopy images were produced from α-Syn FL and α-Syn FL + NTN1 after PFFs were generated. The samples (5 μL) were deposited on Formvar-coated 400 mesh copper grides, fixed with 0.5% glutaraldehyde, negative stained with 2% uranyl acetate (Sigma-Aldrich, Germany) and screened by CM-10 TEM.

Quantification and statistical analysis

All data are expressed as mean ± S.E.M. from three or more independent experiments. Representative morphological images obtained from at least 3 experiments with similar results were provided. Image J 1.47 software was used to analyze IF experiments, and Image Lab™ software for western blots analysis. The statistical analysis of results was performed using GraphPad5 (Prism) software. All data were tested for normal distribution in order to analyze results accordingly using parametric or non-parametric tests. To compare results between two groups, the Student's unpaired t-test was used. When more than two groups were compared, one-way ANOVA followed by Tukey post hoc test was applied. For repeated measures, a Repeated-Measures (RM) ANOVA or 2way ANOVA test was performed followed by Tukey multiple comparisons post hoc test. Assessments with $p < 0.05$ were considered significant.

Supplementary Figure



Supplementary Figure 1. Netrin-1 is deprived clearly in PD patient's SN part, showing the strongest molecular interactions with SNCA

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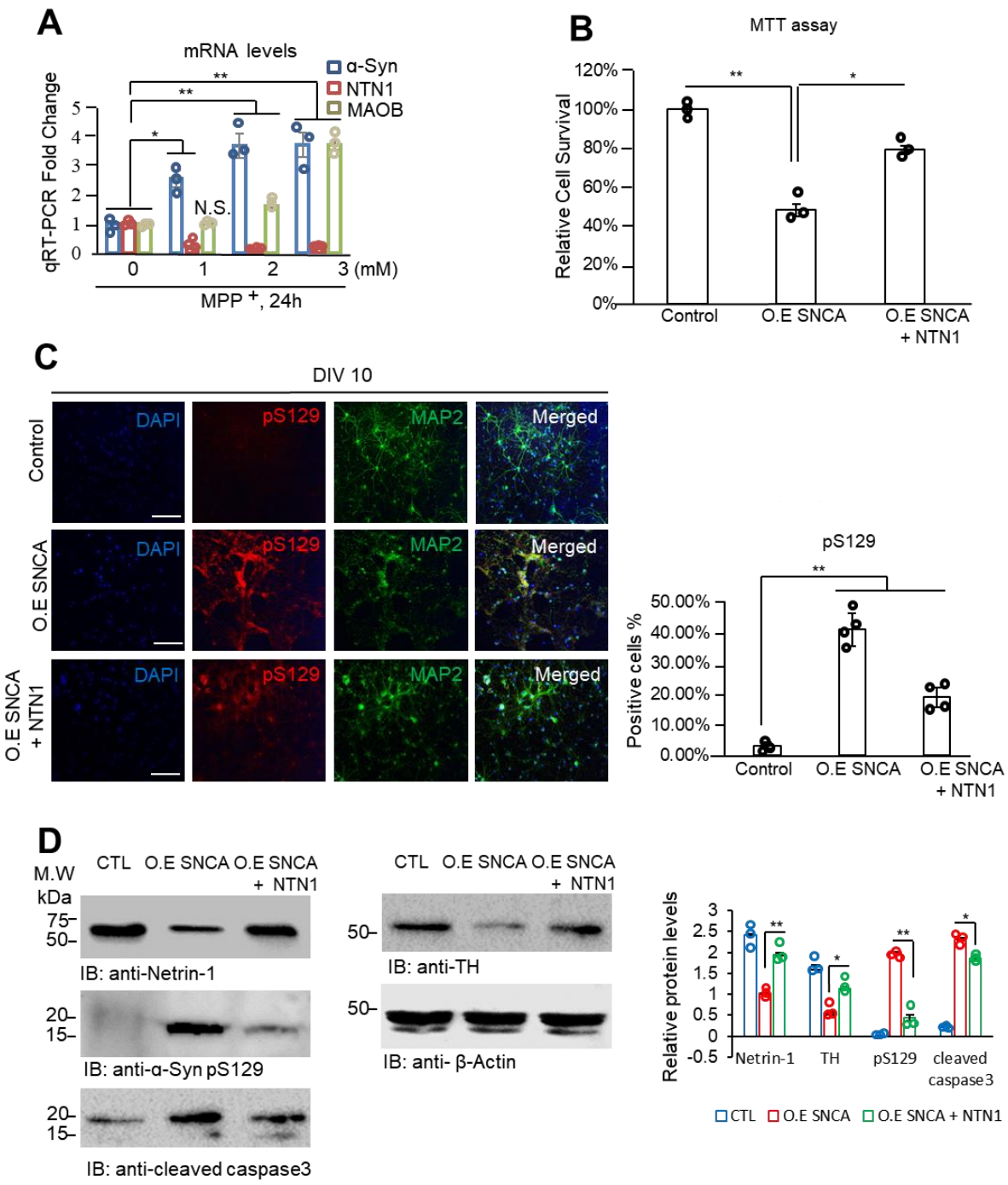
(A) In situ hybridization (ISH) images and visualized netrin-1 expressed regions in midbrain of C57BL/6J.

(B) ISH images and visualized netrin-3 expressed regions.

(C) ISH images and visualized netrin-4 expressed regions. Selected direction is Sagittal, and scale bars were shown identically as 3356 microns.

(D) Netrin-1, -3, and -4 expression profiling in SN part. GDS2821 is the code for accession, and each values were collected from 9 healthy controls and 16 PD patients.

(E) Netrins/SNCA Physical interactions (Red line, 77.64%) and shared molecular signal pathways (Blue line, 1.88%).



Supplementary Figure 2. Comparison of cellular and neuronal viability between overexpressed SNCA group vs overexpressed SNCA + hNTN1

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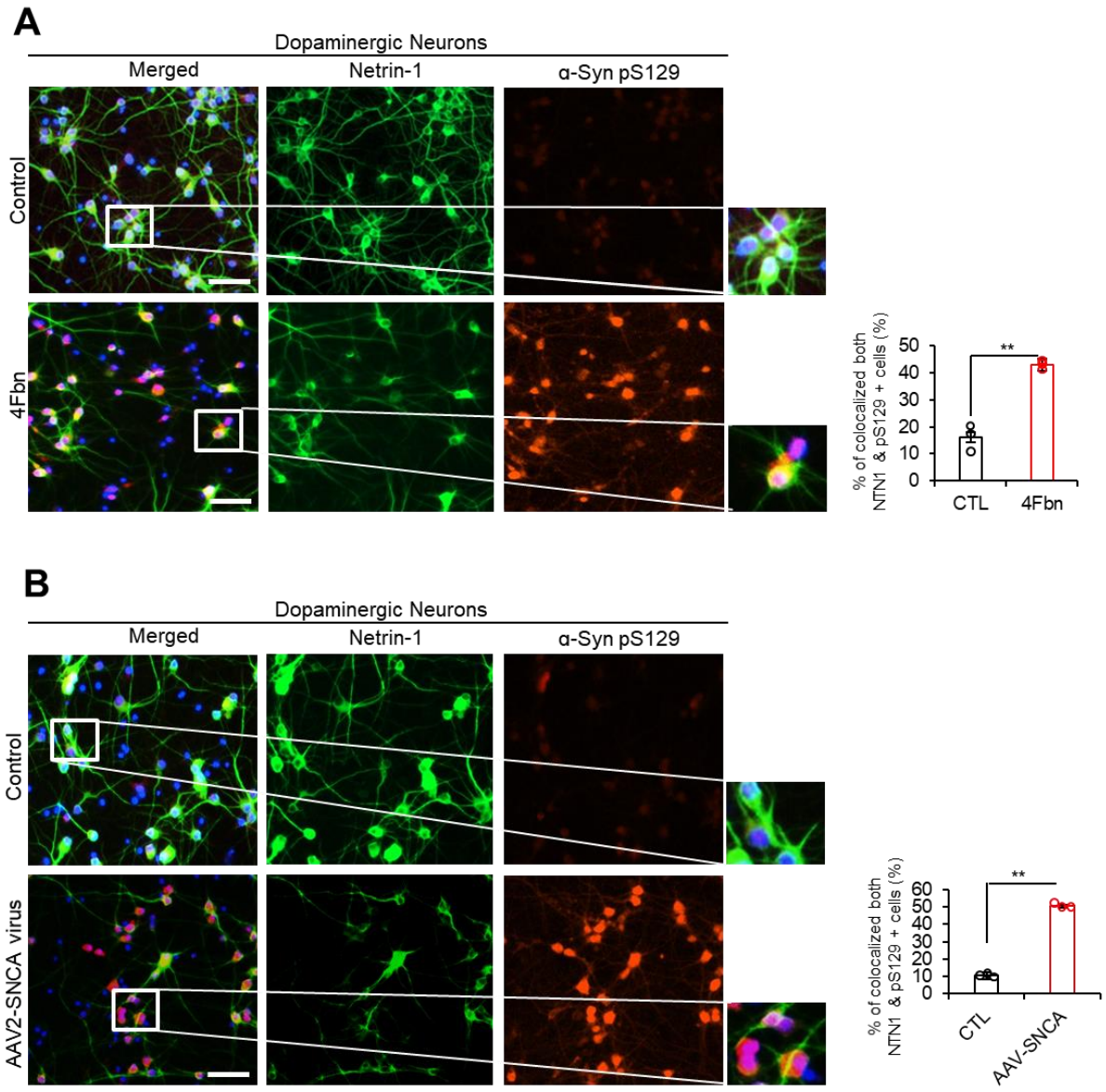
(A) qRT-PCR fold changes and mRNA levels of α-Syn, NTN1, and MAOB under the MPP^+ treated for 24 h, dose-dependently (0, 1, 2, 3 mM).

(B) Cell survival counts were calculated through MTT assay, netrin-1 elevated relative cell survival values than overexpressed SNCA only.

(C) Co-immunofluorescence staining of α-Syn pS129 (red) /MAP2 (green). Scale bar, 20 μm.

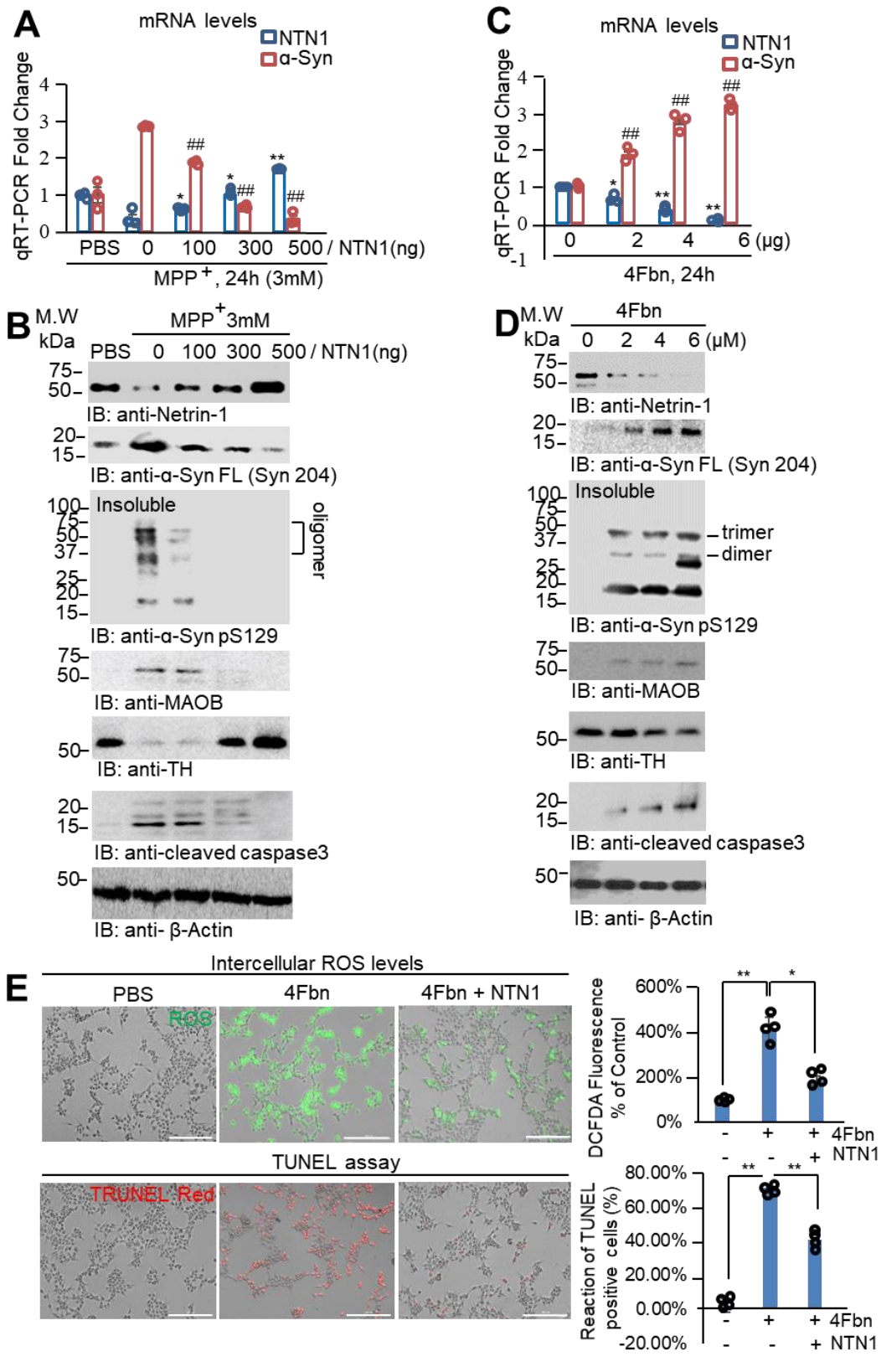
(D) Immunoblot on CTL, overexpressed-SNCA, and overexpressed-SNCA + hNTN1 200 ng treated DIV 10 lysates showing the modulation of netrin-1, α-Syn pS129, cleaved caspase-3, and TH levels. N=3 each group. Unpaired t-test $*P < 0.05$; $**P < 0.01$.



Supplementary Figure 3. Netrin-1 depletion in dopaminergic neurons increases α-Syn pS129 formation

(A) Co-immunofluorescence staining with netrin-1 and pS129 validated that netrin-1 deprivation in 4Fbn-treated neuron contributed to the neuronal death.

(B) In AAV6-SNCA virus injected neurons, toxic α-synuclein (α-Syn pS129) levels were highly increased compared to the control group.



Supplementary Figure 4. Neurorestorative effect measurements of netrin-1 in dopamine cell model

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(A) SH-SY5Y cells were treated with 0-500 ng of recombinant netrin-1 protein under neurotoxin stress for 24 h. Netrin-1 and α-Syn mRNA levels conducted by qRT-PCR. N=3independently experiments. 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. $*P < 0.05$; $**P < 0.01$ (NTN1); $\#\#P < 0.01$ (α-Syn).

(B) Immunoblot showed the netrin-1, α-Syn FL, insoluble α-Syn pS129, MAOB, TH, cleaved caspase-3, and β-actin levels.

(C) DCC-4Fbn dose-dependently (0-6 μg) reduced netrin-1 mRNA level. N=3 independently experiments. 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. $*P < 0.05$; $**P < 0.01$ (NTN1); $\#\#P < 0.01$ (α-Syn).

(D) Immunoblot results identical with (B), PD marker protein (α-Syn FL, insoluble α-Syn pS129, MAOB, TH, and cleaved caspase-3).

(E) Representative images for DCF-DA (intracellular ROS) dye positive cell intensity in DCC-4Fbn vs DCC-4Fbn + NTN1 protein treated group (upper). DCF-DA fluorescence intensity quantification bar graph on the upper right panel. TUNEL assay representative images (bottom left) and quantification bar graph (bottom right) showed the recombinant netrin-1 protein neurorestorative effect. N=4 independently experiments (Scale bar, 20 μm). 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. $*P < 0.05$; $**P < 0.01$.

Supplementary Table 1. Abbreviations

Abbreviations

AAV: Adeno-associated Virus

BDNF: Brain-derived Neurotrophic Factor

DA: Dopaminergic

DCC-4Fbn: 4th fibronectin domain of Deleted in Colorectal Cancer

FL: Full Length

GDNF: Glial cell-derived Neurotrophic Factor

GEO: Gene Expression Omnibus

GST: Glutathione S-Transferase

HDPE: High Density Polyethylene

hNTN1: human NTN1

HPC: Hippocampus

IF: Immunofluorescence

KO: Knock-out

LAL: Limulus Amebocyte Lysate

MAO-B: Monoamine Oxidase B

MAP2: Microtubule-associated Protein 2

MPP^+: 1-methyl-4-phenylpyridinium

NTN1: Netrin-1

NTN3: Netrin-3

NTN4: Netrin-4

PD: Parkinson's Disease

PFFs: Pre-formed fibrils

pS129: Phosphorylated α-Synuclein at the residue S129

qRT-PCR: Quantitative RT-PCR

SN: Substantia Nigra

SNpc or SNc: Substantia Nigra pars compacta

Tg: Transgenic

TH: Tyrosine Hydroxylase

Th-T: Thioflavin-T

VTA: Ventral Tegmental Area

WT: Wild-type

α-Syn: α-Synuclein