SUPPLEMENTARY MATERIALS AND METHODS

Primary embryonic mouse cultures

The pregnant mouse was euthanized with CO_2 and *foeti* were extracted and decapitated. Skulls were opened and brain regions were dissected in ice-cold HBSS.

Hippocampal neuronal cultures

Hippocampi from E18 C57BL/6S mouse embryos were removed under a dissecting microscope. After 15 min of incubation at 37° C with 0.25% (wt/vol) trypsin (Sigma-Aldrich) dissolved in HBSS, the whole hippocampi were washed with HBSS to remove trypsin and then mechanically dissociated in plating medium (MEM, 1% N2 supplement (Gibco-Life Technologies), 10% horse serum (EuroClone-GE Healthcare), 3.3 mM glucose). Neurons, stained with vital dye (Trypan blue, Sigma-Aldrich), were counted by using a Burker chamber. Neurons for immunofluorescence analysis were plated in plating medium on 0.1 mg/ml poly-L-lysine (Sigma-Aldrich)-coated glass coverslips with paraffine dots. Once cells were attached, coverslips were placed upside down in a Petri dish containing a monolayer of cortical astroglial cells (1), prepared from E18 mouse embryonic cortices (see *Glial cultures*). Neurons for biochemical experiments were plated on 0.1 mg/ml poly-L-lysine-coated Petri dishes in Neurobasal medium (Neurobasal, 2% B27 supplement, 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 435 µg/ml GlutaMAX (Gibco-Life Technologies)); once attached, cells were maintained in Neurobasal medium without serum.

Glial cultures

Cortical astroglial cultures were prepared as in (2). Cortices from E18 C57BL/6S mouse embryos were removed under a dissecting microscope and collected. Cortices were minced with a blade into small pieces, washed in HBSS to remove the small debris and then trypsinized for 30 min at 37° C, in a solution containing 2.5% trypsin and 1 mg/ml DNAse (Sigma-Aldrich). Cortices were then washed with HBSS and mechanically dissociated. Cells were collected after a 1,000 x rpm centrifugation for 10 min, plated in glial medium (MEM medium, 10% horse serum, 0.6% glucose, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin) and maintained at 37° C in 5% CO_2 humidified atmosphere. One day before preparing hippocampal cultures, the glial medium was

removed and replaced with hippocampal medium (MEM, supplemented with 1% N2 supplement (Gibco-Life Technologies), 2 mM glutamine, 1 mM sodium pyruvate and 4 mM glucose).

Cortical neuronal cultures

Cortices from E18 C57BL/6S mouse embryos were isolated and prepared as in (*Glial cultures*). Cells were collected after a 1,000 x rpm centrifugation for 10 min, counted and plated in 3,5 cm diameter Petri dishes, previously coated with 0.01 mg/ml poly-L-lysine. Cells were cultured in Neurobasal medium containing 2% B27 supplement, 100 U/ml penicillin, 100 µg/ml streptomycin, 435 µg/ml GlutaMAX and maintained at 37° C in 5% CO_2 humidified atmosphere.

Primary skin fibroblast cultures

Ethic statement and patients

Primary fibroblasts were obtained by skin biopsies from 8 individuals, that include 4 healthy volunteers as control group and 4 patients affected by PD, 3 of which bearing Syn-duplication and one Syn-triplication (Table I). All patients were examined by movement disorder neurologists and clinical diagnosis of PD was established according to the UK Parkinson Disease Society Brain Bank (Hughes et al. 1992). The study was approved by the local ethics committee (Istituti Clinici di Perfezionamento, July 13th 2010) and all participants gave written informed consent.

Table I Phenotype and genotype characterization of investigated individuals.

	CODE	PHENOTYPE	GENOTYPE	SEX	AGE [a]	AGE OF ONSET [b]
CTRL	FFF0412011	HEALTHY		F	65	
	FFF0502011	HEALTHY		F	63	
	FFF0532011	HEALTHY		M	66	
	FFF0421991	HEALTHY		M	54	
PD	FFF0152009	AFFECTED	*SNCA* duplication	F	45	41
	FFF0252011	AFFECTED	*SNCA* duplication	F	51	48
	FFF0242011	AFFECTED	*SNCA* duplication	F	62	55
	ND27760	AFFECTED	*SNCA* triplication	F	55	50

[a] Age at time of skin biopsy and establishment of fibroblast cell line.

[b] Age at which the patient first noticed a PD-related symptom.

N2A cell cultures and cross-linking experiments

Murine neuroblastoma Neuro2A (N2A) cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco-Life Technologies), supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin and 20 mM Hepes/KOH pH 7.2 at 37° C in a 5% CO_2 incubator. N2A cells were washed twice with the crosslinking buffer (0.1 M phosphate buffer, 0.15 M NaCl) and incubated in the presence or absence of 1 µM purified wt or A30P Syn and of the impermeable crosslinker 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP, Thermo Scientific-Pierce), at a final concentration of 1.5 mM (Bennet et al., 2000); reactions were carried out for 20 min at room temperature (RT) and then blocked with 20 mM Tris pH 7.4 for 15 min at 4° C. After washing with cold phosphate buffered saline (PBS, Gibco-Life Technologies), cells were collected in (50 mM Hepes/KOH pH 7.2, 150 mM NaCl, 5 mM EDTA pH 8.0, 1% Triton X-100, supplemented with protease and phosphatase inhibitors), deprived of nuclei by a 5 min cold centrifugation at 300 x g and subjected to IP with 2 µg anti-GRP78 antibody. Immunoprecipitated samples were then analyzed by SDS-PAGE and immunoblotting.

Immunofluorescence analysis

Cells were fixed with 4% paraformaldehyde solution, containing 120 mM phosphate buffer pH 7.4 and 4% sucrose. After washing with PBS, cells were incubated for 2 h at RT with primary antibodies, followed by 1 h incubation at RT with fluorescent secondary antibodies. Primary and secondary antibodies were diluted in goat serum dilution buffer, containing 15% goat serum (Gibco-Life Technologies), 450 mM NaCl, 0.1% Triton X-100, 20 mM phosphate buffer at pH 7.4, as in (3) and washes were performed in PBS. Coverslips were mounted in Shandon Immu-Mount (Thermo Scientific-Pierce, Rockford, IL) and maintained at -20° C.

SDS-PAGE, gel staining and blotting assays

Protein samples were diluted in Laemmli buffer (final concentration: 62.5 mM Tris pH 6.8, 1.5% SDS, 10% sucrose, 0.01% bromophenol blue, 5% β-mercaptoethanol) and subjected to SDS-PAGE analysis (Mighty Small Vertical Gel Electrophoresis units and Standard Vertical Gel Electrophoresis units, Hoefer, San Francisco, CA) (4). Broad range protein markers (Bio-Rad) were

used as standards to extrapolate the molecular weight of the analyzed protein samples. Coomassie staining of SDS-PAGE gels was performed by a 1 h staining at RT with Coomassie Brilliant Blue R.250 (Merck), as in (5). Silver staining was performed according to (6); (7). For blotting assays, protein samples subjected to SDS-PAGE were transferred (Trans-Blot cell, Bio-Rad) to nitrocellulose membranes (Whatman, Dessel, Germany), as previously described (8). Filters were blocked for 1 h at RT with 5% non-fat dry milk or 1% BSA in Tris-buffered saline containing 0.05% Tween 20 (TBST; 200 mM NaCl, 50 mM Tris/HCl, pH 7.4, 0.05% Tween 20), incubated for 2 h with primary antibodies at appropriate dilutions, washed five times for 5 min with TBST, incubated for 1 h at RT in a 1:10,000 dilution of the HRP-conjugated secondary anti-mouse or anti-rabbit antibodies in 5% non-fat dry milk in TBST. After 5 washes in TBST, nitrocellulose filters were finally developed by chemiluminescence with the ECL system (Amersham-GE Healthcare), according to the manufacturer's instructions. For the detection of biotin-labelled proteins, filters were blocked and then incubated with HRP-conjugated streptavidin (1:10,000) in 5% non-fat dry milk in TBST. Filters were finally developed with the ECL system. After detection with the ECL system, signals were scanned with a desktop scanner (Epson Expression 1680) at 600 dpi. Densitometric quantifications were performed with ImageJ software.

Two-dimensional gel electrophoresis, far Western blotting and mass spectrometry

Fourteen DIV hippocampal neurons were washed twice with cold HBSS and collected by scraping at 4° C in a buffer containing (120 mM K-Glu, 20 mM Hepes pH 7.2, 20 mM K-Acetate, 5 mM EGTA pH 8.0, supplemented with phoshatases and proteases inhibitors). Cells were mechanically homogenized by passing 10 times through a 27 ½ G needle of a syringe. Homogenates were deprived of nuclei by a 5 min cold centrifugation at 300 x g and total protein content was quantified by the bicinchoninic acid (BCA) colorimetric assay (9), using a BCA protein Assay kit (Thermo Scientific-Pierce), according to the manufacturer's instructions. In accordance to (7), samples containing 250 mg of total protein homogenates were purified with the two-dimensional Clean-Up Kit (Amersham-GE Healthcare), dissolved in rehydratation buffer (8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM DTT, 0.5% carrier ampholyte mixture, pH 3 – 10 and 0.02% bromophenol blue). Samples were applied to 13 cm polyacrilamide immobiline dry-gel (IPG) strips (pH range 3 – 10) (Amersham-Pharmacia Biotech, Piscataway, NJ). Isoelectrofocusing (IEF) in IPGphor (Amersham-Pharmacia Biotech) was stopped

at 75,000-90,000 VoltHours. Second dimension runs were performed using a Standard Vertical Gel Electrophoresis unit (Hoefer). After IEF, strips were soaked first in equilibration buffer containing 6 M urea, 2% SDS, 50 mM Tris pH 6.8, 30% glycerol, 2% DTT, then in equilibration buffer containing 3% iodoacetamide and traces of bromophenol blue. Strips were then applied onto 10% polyacrilamide gels, run at 100 V for ~ 7 h, and either silver stained or transferred onto nitrocellulose membranes for far Western blotting experiments. According to (7), ImageMaster 2D software was used to calculate the isoelectric point (pI) of a spot from its position in a gel and to estimate the number of specific modifications corresponding to a defined difference in the pI. Isolated protein spots were excised from 2D gels stained with silver staining, reduced and alkylated as described in (6). By sequential proteolytic or chemical cleavages, as seen in (7), digestion products were obtained and loaded on the MALDI target; MALDI-time of flight (TOF) mass measurements were performed on a Voyager-DE STR TOF mass spectrometer (Applied Biosystems, Framingham, MA). Internally calibrated spectra were processed via the Data Explorer software. For far Western blotting assays, 2D-separated gels were transferred to nitrocellulose filters with the probe or bait proteins, Syns (10, 11). Membranes were blocked for 1 h at RT with 5% non-fat dry milk and incubated ON at 4° C on a rocking platform with 1% BSA in TBST solution containing 1 μM purified wt or A30P Syn on a rocking platform; for control experiments, filters were blocked with 1% BSA solution in TBST. After washing in TBST, filters were processed for immunoblotting with anti-Syn primary antibody and developed by chemiluminescence.

Toxicity assays

Cell vitality assay was performed on 2 DIV neurons and on 14 DIV neurons, exposed or not to wt or A30P Syns for 4 h and 2, 7, and 14 days, respectively. Neurons were co-stained with HOECHST and sytox orange fluorescent compounds for 20 min at 37° C in KRH buffer solution. Neurons were then imaged at an Axiovert 135 inverted microscope and cell mortality was evaluated as percentage of the ratio between the number of dead cells (nuclei stained by sytox orange) and the total number of cells (nuclei stained by HOECHST).

5,5’,6,6’-tetrachloro-1,1’,3,3’tetraethilbenzimidazolocarbocyanine iodide (JC-1) assay (Molecular Probes-Life Technologies) was used to measure mitochondrial potential according to the manufacturer instructions. JC-1 assay was performed on neurons treated or not with 1 μM Syns for 2, 7 and 14 days. Confocal images were acquired using Leica TCS SP5 microscope using a 63 x

objective with 3 x digital magnification (Leica Microsystems, Wetzlar, Germany). JC-1 monomers were acquired with excitation at 488 nm and emission at 540 nm. JC-1 aggregates were acquired with excitation at 561 nm and emission at 630 nm. Ratios of the intensity of JC-1 aggregates to the intensity of JC-1 monomers in cell bodies were measured using ImageJ.

Atomic force microscopy

AFM probe functionalization

Polystirene beads (4 μm of diameter, Polysciences Inc., USA) were mounted on silicon tipple cantilevers TL1 (Nanosensors, Switzerlad), with nominal spring constant of 0.03 N/m. Probes were coated with 50 nm gold particles via sputtering technique and immediately incubated for 60 min in a chloroform solution containing 5 mg/ml NHS-PEG-PDP (Polypure, Oslo, Norway). The disulfide bond of the PDP terminal group reacts with the gold surface of the beads, forming a stable bond (12). AFM probes were accurately washed in chloroform to remove weakly bound molecules. Cantilevers were incubated ON at 4° C with a 2 mg/ml solution containing the appropriate secondary antibody. The same functionalization procedure was applied to commercially available gold-coated AFM probes (NPG, Bruker, USA), with a nominal spring constant of 0.06 N/m and typical apex radius of curvature of 30 nm. Bare chips were washed in chloroform to remove oils and gross contaminants and then exposed for 20 min to a UV-ozone cleaner, in order to remove organic and other surface contaminants before the functionalization procedure (12, 13).

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