Supplemental Figures

Supplemental Figure Legends

Figure S1: Effectiveness of different antibody concentrations in blocking α-syn pff uptake. (**A**) WT primary neurons (DIV7) were treated with non-specific mouse IgG or different amounts of Syn211 or Syn303 antibodies 30 min before addition of hWT α-syn pffs. Neurons were sequentially extracted in 1% Triton-X100 and 2% SDS lysis buffer at 7 d post antibody and pff treatments. TX-soluble and TX-insoluble lysates were immunobloted with MAb 81A for p-α-syn and TUJ1 for neuronal tubulin as loading control. Syn211 and Syn303 reduced pff-induced insoluble aggregates, even when α-syn pff was at 3:1 molar excess to MAbs. (**B**) Bar graph summarizing quantitation of the TX-insoluble p-α-syn immunoblotting results of 4 independent experiments, each done in duplicate. *** $p < 0.001$.

Figure S2: Anti-Myc antibody effectively blocksh1-120myc pff uptake. (**A**) WT primary neurons were treated with MAb 9E10, an antibody against Myc tag, followed by addition of hWT or h1-120myc α-syn pffs. Immunoblotting of TX-soluble and TX-insoluble lysates for p-α-syn, mouse α-syn and TUJ1 showed that the anti-Myc antibody 9E10 was effective in reducing insoluble p-α-syn aggregates, as wlle as in preventing recruitment of endogenous mouse α-syn into these pathologic aggregates when h1-120myc pffs were used. (**B**) Bar graph summarizing quantitation of the TX-soluble and TX-insoluble mouse α-syn levels of 3 independent experiments, each done in duplicates. * $p < 0.05$, ** $p < 0.01$.

Figure S3: Anti-α-syn antibody treatment reduced α-syn pathology in WT neurons grown in triple chamber microfluidic devices. (**A**) WT primary neurons were seeded in triple chamber microfluidic devices. At DIV7, h1-120myc α-syn pffs were added to neurons in chamber 1. 7 days later, neurons in the device were fixed and stained with MAb 9E10 to detect Myc-tagged α-syn pffs. The Myc signal was only detected in chamber 1 to which pffs were added, suggesting that there was no leakage of α-syn pffs from chamber 1 to chamber 2 to chamber 3. (**B-D**) Propagation of pathologic p-α-syn (stained with MAb 81A) from neurons in chamber 1 to chamber 2 to chamber 3 at 7 (**B**) and 14 days (**C**) following transduction of hWT α-syn pffs. Syn211 or Syn303 treatment (added to chamber 2 neurons 30 min before addition of α-syn pffs) significantly reduced pathologic p-α-syn aggregates in the neurons in chambers 1 and 2 (**D**). This graph shows the quantitation of the results in C and although a reduction in pathology was also observed in chamber 3, the amount was too low for accurate quantification. Scale bars: 100 μm.

Figure S4: MAb Syn303 turnover in WT mice. At 1 d, 3 d, and 7 d following a single ip injection of Syn303 (10 mg/kg), WT mice (without pff treatment) were sacrificed; their plasma (**A**) and CSF (**B**) were collected to determine the level of circulating anti-α-syn antibodie using a direct ELISA with

mouse α-syn pff as coating material. (**C**) Ratio of anti-α-syn antibody detected in plasma vs CSF was roughly 0.1%. N = 4-5 mice per time point.

Figure S5: α-syn pathology was detected 7 days post α-syn pff inoculation in WT mice. At 7 d following a single intra-striatal injection of murine (mWT) α-syn pffs into WT mice, α-syn pathology, which was stained by MAb 81A (specific for for p-α-syn) and MAb Syn506 (specific for misfolded α-syn), was detected in the ipsilateral cortex (**A**) and ipsilateral SN (**B**). Scale bar: 100 µm.

Figure S6: IP injection of Syn303 at 7 days post α-syn pff inoculation reduced α-syn pathology in WT mice. (**A**) Schematic of experimental design. WT mice received a single intra-striatal injection of mWT α-syn pffs, followed by ip injections of 10mg/kg Syn303 or control IgG antibody at 7 d post pffs, then weekly for 8 wks. (**B, D, F**) α-syn pathology is visualized by staining with Syn506 MAb (specific for misfolded α-syn) in α-syn pff-injected WT mice following control IgG or Syn303 MAb treatment. Pathological α-syn is detected in neurons located in sites connected to the striatum such as the SNpc (**B**), ipsilateral and contralateral amygdala (**D, F**) but this pathology was significantly less in Syn303 compared to control IgG treated mice. (**C, E, G**) Quantification of Syn506-positive aggregates throughout the SNpc (**C**) and contralateral amygdala (**G**) showed significant reductions in LB-like α-syn inclusions, while there was a trend toward reduced pathology in the ipsilateral amygdala of Syn303-treated mice (**E**). N = 8 mice per treatment group. * $p < 0.05$, *** $p < 0.001$. Scale bars: 100 µm.

Figure S7: Analyses of motor learning and neuroinflammation following long-term α-syn pff inoculation and Syn303 treatments. (**A**) At 90 d and 180 d following mWT α-syn pffs inoculation, WT mice were tested on the rotarod task for motor learning. Latency to fall off the rotating rod was recorded: the longer the latency, the better the performance. Mice were impaired on this task at 180 d post α-syn pff inoculation; Syn303 treatment did not ameliorate this dysfunction. N = 15 mice per group. ns: not significant. (**B-E**) At 180 d post α-syn pff inoculation in WT mice, astrogliosis (**B-C**) and microgliosis (**D-E**) were seen in the SNpc following GFAP and Iba1 immunostaining, respectively. Arrowheads in (**D**) indicate Iba1-positive cells included in our manual counting. There were no marked differences in the extent of astro- and micro-gliosis in the ipsilateral vs contralateral SNpc of either control IgG or Syn303 treated mice. Scale bars: 100 µm.

Table S1: List of antibodies used

Antibody	Source/Reference	Host/Isotype	Dilution (ICC)	Dilution (IHC)	Antigen retrieval	Dilution (WB)
Syn211 (human α-syn)	(Giasson *et al.*, 2000; Giasson *et al.*, 2002)	Mouse (IgG1)	-	-	-	-
Syn303 (misfolded α-syn)	(Duda *et al.*, 2002)	Mouse (IgG1)	-	-	-	-
Syn202 (total α-syn)	(Giasson *et al.*, 2000)	Mouse (IgG2a)	1:500	-	-	-
Syn204 (total α-syn)	(Giasson *et al.*, 2000)	Mouse (IgG2a)				
81A (phospho α-syn)	(Waxman & Giasson, 2008; Volpicelli-Daley *et al.*, 2011)	Mouse (IgG2a)	1:3000	1:20,000	None	-
81A-HRP	(Volpicelli-Daley *et al.*, 2011; Tanik *et al.*, 2013)	Mouse (IgG2a)	-	-	-	1:1000
Mouse α-syn	(Volpicelli-Daley *et al.*, 2011)	Rabbit	-	-	-	1:1000
Syn506 (misfolded α-syn)	(Giasson *et al.*, 2003; Luk *et al.*, 2012b)	Mouse	-	1:20,000	Citrate buffer	-
LB509 (human α-syn)	(Giasson *et al.*, 2000)	Mouse (IgG1)	1:1000	-	-	-
Synapsin II	Epitomics, Burlingame, CA	Mouse	-	-	-	1:2000
SNAP 25	Synaptic Systems, Goettingen, Germany	Mouse	-	-	-	1:2000
9E10 (anti-Myc)	Santa Cruz	Mouse	1:500	-	-	-
K9JA (anti-tau)	Dako	Rabbit	1:3000	-	-	-
NeuN	Millipore	Rabbit	1:1000	-	-	-
Tyrosine hydroxylase	Pel-freeze	Rabbit	-	1:5000	Formic acid	-
Iba-1	Wako	Rabbit	-	1:1000	None	-
GFAP	DAKO	Rabbit	-	1:20,000	None	-

Supplemental Materials and Methods:

Primary Neuronal Cultures

Primary cultures were prepared from E16-E18 CD1 mouse brains. Hippocampi were dissected, dissociated with 20 μg/ml papain, and plated on poly-D-lysine coated coverslips (0.2 mg/ml in 50 mM borate buffer, pH 8.5) or dishes at 50,000 cells/cm^2 or 100,000 cells/cm^2, respectively. Neurons were maintained in neurobasal medium supplemented with 0.5% penicillin/streptomycin, 1% Glutamax, and 2% B27.

Fibril transduction and Antibody Treatment in Primary Neurons

α-syn pffs made from recombinant full-length human and mouse α-syn as well as N- or C-terminal truncated α-syn, i.e. h32-140 and h1-120myc, were added to primary neurons as previously described (Volpicelli-Daley *et al.*, 2011). Briefly, 5 mg/ml pff stock was diluted to 0.1 mg/ml in sterile PBS, sonicated, and further diluted in conditioned neuronal media. Transduction of pffs was done when neurons were cultured for 7 – 10 days *in vitro* (DIV). For a 24-well tray, 1 µg/ml α-syn pffs were added. For 12-well and 6-well trays, 5 µg/ml pffs was added. For antibody treatment, neurons were treated with the anti-α-syn MAbs 30 min prior to α-syn pff addition. Unless otherwise noted, 6 µg/ml and 15 µg/ml of antibody were used in most immunocytochemical and biochemical experiments, respectively. Each experiment was performed in duplicates, and repeated 3-10 times.

Immunocyotchemistry

Neurons were fixed with 4% paraformaldehyde, 4% sucrose in PBS or 4% paraformaldehyde, 4% sucrose, and we also include 1% Triton X-100 to extract all soluble proteins. If neurons were fixed with 4% paraformaldehyde, permeabilization was performed by incubation in 3% BSA PBS containing 0.1% Triton-X. If neurons were fixed and the fixative included 1% Triton X-100, a subsequent permeabilization step was omitted. Coverslips were blocked in 3% BSA PBS for 1 h. Neurons were then incubated in blocking buffer containing primary antibodies (Table S1) overnight, followed by Alexa-Fluor conjugated secondary antibodies.

Two-stage IF was performed to distinguish between extracellular vs truly internalized pffs, as described (Volpicelli-Daley *et al.*, 2011). Briefly, live neurons were rinsed 5 times with cold neurobasal media containing 3% BSA, then stained with MAb LB509 (IgG1), which specifically recognizes human α-syn including α-syn pffs. Neurons were then fixed with 4% paraformaldehyde, permeabilized, and

subseauently incubated with human α-syn specific MAb Syn204 (IgG2a). Coverslips were mounted on FluoroGold reagent containing DAPI for nuclear counterstain. Epifluorescence images were obtained with a Nikon Eclipse microscope equipped with DS-Qi1MC-U3 camera.

Sequential Extraction and Immunoblotting

At either 7 or 14 d following antibody/pff treatments, neurons were extracted in Tris-buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.4) containing 1% Triton-X100, protease and phosphatase inhibitor cocktails. Neurons were sonicated and spun at 100,000 x g for 30 min at 4 °C. Pellets were washed, and re-suspended in 2% SDS in TBS. Protein concentration was determined by the Bradford protein assay (Pierce). Samples were separated on 5-20% gradient SDS-PAGE gels, and blotted with primary antibodies described in Table 1. The Odyssey ® Infrared imaging system was used to visualize and quantitate bands on blots for most of antibodies used except for 81A, which was conjugated to horseradish peroxidase (Pierce), and developed by enhanced chemiluminescence (Pierce).

LDH toxicity assay

LDH release assay (Promega, CytoTox 96® NonRadioactive Cytotoxicity Assay) was performed on conditioned media collected at 14 d post pff and antibody treatment; media were changed every 7 d. Media were spun at 13,300 x g for 5 min at 4 °C. 50 µl medium was incubated with 50 µl reconstituted substrate for 30 minutes at room temperature in darkness. Reaction was stopped by adding 50 µl stop solution, then absorbance was read at 490 nm using the SpectraMax plate reader. Background absorbance due to plain neuron medium was subtracted from all readings.

Microfluidic Chambers

Microfluidic devices with 3 somal compartments connected by a series of microgrooves were obtained from Xona Microfluidic (TCND500, Temecula, CA). Glass coverslips (Corning Inc) were sonicated in water bath to remove impurities, sterilized in 70% ethanol, and coated in 0.4 mg/ml poly-d-lysine in borate buffer (50 mM, pH8.5). Coverslips were allowed to air dry completely before being affixed to inside the chambers of the neuronal microfluidic devices. Approximately 120,000 neurons were plated per chamber in neurobasal media supplemented with 1% Glutamax, 2% B27, and 0.5% penicillin/streptomycin. At DIV7, 3 ug of α-syn antibodies were added to chamber 2 at 30 min before addition of 0.5 ug α-syn pffs to chamber 1. To control for direction of flow, a 75 µl difference in media volume was maintained between chamber 1 and 2 and between change 2 and 3 according to the manufacturers' instructions. Neurons were fixed 7 or 14 d post treatment and transduction using 4%

paraformaldehyde, 4% sucrose in PBS. Devices were then removed from cover glass for immunocytochemistry (see section above). Images were taken using Nikon Eclipse microscope equipped with DS-Qi1MC-U3 camera or Leica DMI6000 microscope. All images were captured at same exposure without additional manipulations. Images were converted to grayscale and thresholded using Image J (NIH). The same thresholdings were applied to each biological and technical replicate (n = 3 for all experiments). Area occupied in each image was analyzed by the "Analyze Particle" function in Image J.

Stereotaxic Injections of α-syn pffs and Antibody Treatment in WT Mice:

All surgical procedures were performed as previously described (Luk *et al.*, 2012a; Luk *et al.*, 2012b). Briefly, mice of either gender were anesthetized with a mixture of ketamine/xylazine, and placed on a stereotaxic frame. Mice were kept at 37°C via a heating pad. Stock mouse α-syn pffs weres thawed from vials stored at –80 °C, diluted to 2 µg/µl in sterile PBS, and sonicated with 20 pulses of 10 sec each. Next, 5 ug pff in in a total volume of 2.5 µl were unilaterally injected into the dorsal striatum of 2–3 mo old WT C57BL/C3H mice (AP: +0.2 mm, ML: +2 mm, and DV: -2.6 mm). Mice were monitored during and after surgery; they became ambulatory within an h post surgery. Either immediately or at 7 d post pff injection, animals were treated with the Syn 303 MAb or the control IgG1 antibody at 30 mg/kg or 10 mg/kg via ip injections, respectively. The mice received weekly ip injections of antibodies until they were sacrificed at predetermined periods.

Immunohistochemistry

Mice were sacrificed at 30 d, 60 d, and 180 d post α-syn pff injection by overdose with ketamine/xylazine, followed by transcardial perfusion with PBS. Brains and spinal cords were extracted, fixed overnight in 70% Ethanol in 150 mM NaCl, and embedded in paraffin. 8 µm coronal sections were collected through the entire CNS (brain, brain stem, and spinal cord) and stained for immunohistochemistry (IHC) using 3'-diaminobenzidine (DAB; Vector Laboratories) as a chromogen. For assessment of pathologic α-syn aggregates, every 10th section was stained with Syn 506 against misfolded α-syn. Numbers of Syn 506-positive cells in the SNpc, ipsilateral and contralateral amygdala were quantified in a blinded manner, by two independent raters. Both intra-rater and inter-rater reliability were ≥ 90% (data not shown). For assessment of dopaminergic SNpc neurons, every 10th section was stained by IHC with tTH. Images were taken using a DP71 digital camera connected to BX51 microscope. Images were converted to grayscale and thresholded using the Max Entropy function in Image J (NIH). Numbers of TH-positive cells in the SNpc in 9 mice were manually counted or

estimated with the "Analyze Particle" function in Image J. We obtained a good correlation between the numbers of SNpc TH-positive neurons from manual counting and the Image J method (Pearson's r = 0.81). Therefore, SNpc TH-positive neurons for all experimental mice were estimated using the Image J method. Every 20th section was stained with GFAP and Iba1 antibodies for astrocytes and microglia, respectively. Estimation of numbers of GFAP-positive cells in the SNpc was carried similar to the TH-positive cells using the Image J method. Iba1-positive cells as defined by those with darkly-stained cell bodies were manually counted under 40x magnification.

Behavioral assessments:

Behavioral tests were given 3 d after the last ip injections at 30 d, 90 d, and 180d periods to avoid stress. Mice were allowed to acclimatize to testing environment 1 d before the actual tests. Only 1 test was performed/d to avoid exhaustion. All behavioral tests were performed by an investigator blinded to treatment conditions in the following order: wire hang, then rotarod, as described (Luk *et al.*, 2012a).

Wire hang test

To assess grip strength and motor coordination, the wire hang test was performed with slight modifications from (Luk *et al.*, 2012a). Briefly, mice were placed on top of a standard wire cage lid. Lid was gently agitated and turned upside down to cause the animals to grip the wires. The latency of mice to fall off the grid was recorded and averaged over two trials (15 min apart). Trials were stopped if mice remained on lid for over 10 min.

Rotarod test

To assess motor learning and balance, the rotarod test was performed as described (Luk *et al.*, 2012a). Mice were first trained on a rotating rod (MED-Associates Rotarod Apparatus) which accelerated from 4 rpm to 40 rpm in 5 min. Two training sessions were given, followed by 1 h rest, and then two trial sessions. Latency to fall was recorded and averaged over two testing trials.

Supplemental References

Duda, J.E., Giasson, B.I., Mabon, M.E., Lee, V.M. & Trojanowski, J.Q. (2002) Novel antibodies to synuclein show abundant striatal pathology in Lewy body diseases. *Annals of neurology*, **52**, 205-210.

Giasson, B.I., Duda, J.E., Quinn, S.M., Zhang, B., Trojanowski, J.Q. & Lee, V.M. (2002) Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. *Neuron*, **34**, 521-533.

Giasson, B.I., Forman, M.S., Higuchi, M., Golbe, L.I., Graves, C.L., Kotzbauer, P.T., Trojanowski, J.Q. & Lee, V.M. (2003) Initiation and synergistic fibrillization of tau and alpha-synuclein. *Science (New York, N.Y*, **300**, 636-640.

Giasson, B.I., Jakes, R., Goedert, M., Duda, J.E., Leight, S., Trojanowski, J.Q. & Lee, V.M. (2000) A panel of epitope-specific antibodies detects protein domains distributed throughout human alpha-synuclein in Lewy bodies of Parkinson's disease. *Journal of neuroscience research*, **59**, 528-533.

Luk, K.C., Kehm, V., Carroll, J., Zhang, B., O'Brien, P., Trojanowski, J.Q. & Lee, V.M. (2012a) Pathological alpha-synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice. *Science (New York, N.Y*, **338**, 949-953.

Luk, K.C., Kehm, V.M., Zhang, B., O'Brien, P., Trojanowski, J.Q. & Lee, V.M. (2012b) Intracerebral inoculation of pathological alpha-synuclein initiates a rapidly progressive neurodegenerative alpha-synucleinopathy in mice. *J Exp Med*, **209**, 975-986.

Tanik, S.A., Schultheiss, C.E., Volpicelli-Daley, L.A., Brunden, K.R. & Lee, V.M. (2013) Lewy body-like alpha-synuclein aggregates resist degradation and impair macroautophagy. *The Journal of biological chemistry*, **288**, 15194-15210.

Volpicelli-Daley, L.A., Luk, K.C., Patel, T.P., Tanik, S.A., Riddle, D.M., Stieber, A., Meaney, D.F., Trojanowski, J.Q. & Lee, V.M. (2011) Exogenous alpha-Synuclein Fibrils Induce Lewy Body Pathology Leading to Synaptic Dysfunction and Neuron Death. *Neuron*, **72**, 57-71.

Waxman, E.A. & Giasson, B.I. (2008) Specificity and regulation of casein kinase-mediated phosphorylation of alpha-synuclein. *Journal of neuropathology and experimental neurology*, **67**, 402-416.