Supplementary Materials for

Pathological α-Synuclein Transmission Initiates Parkinson-like Neurodegeneration in Non-transgenic Mice

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Materials and Methods

Animals

C57BL6/C3H F1, C57BL6/SJL F1, and CD1 mice were obtained from the Jackson Laboratories (Bar Harbor, ME). α-Syn knockout (*Snca*$^{-/-}$) and Snca$^{+/-}$ mice (*25*) were maintained on a C57BL6 background. All housing, breeding, and procedures were performed according to the NIH Guide for the Care and Use of Experimental Animals and approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Injection material and stereotaxic injections

Purification of recombinant α-Syn proteins and *in vitro* fibril assembly was performed as previously described (*20, 26-28*) using full-length wildtype mouse α-Syn (5 mg/mL). Assembly reactions were agitated in an Eppendorf orbital mixer (1,000 rpm at 37^{o}C) and PFFs harvested after 5d. Preparations were diluted in sterile PBS and sonicated briefly with a hand held probe (QSonica, Newtown, CT) before intracerebral injection. Mice between 2 and 3 months of age were anesthetized with ketamine hydrochloride (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and stereotaxically injected in one hemisphere with recombinant α-Syn fibrils (5 μg). Control animals received sterile PBS. A single needle insertion (co-ordinates: +0.2 mm relative to Bregma, +2.0 mm from midline) into the right forebrain was used to target the inoculum to the dorsal neostriatum (+2.6 mm beneath the dura; Fig.S1). Injections were performed using a 10 μL syringe (Hamilton, NV) at a rate of 0.1 μL per min (2.5 μL total per site) with the needle in place for $\geq$ 5 min at each target. Hippocampal (AP +2.5 mm, ML +2.0 mm, DV -1.8 mm) and cortical (AP +0.2 mm, ML +2.0 mm, DV -0.8 mm) injections were performed in a similar manner. Animals were monitored regularly following recovery from surgery, and sacrificed at various pre-determined time points (30, 90, or 180 dpi) by overdose with ketamine/xylazine. For histological studies the brain and spinal cord were removed after transcardial perfusion with PBS and underwent overnight postfixation in either neutral buffered formalin (Fisher Scientific) or 70% ethanol (in 150 mM NaCl, pH 7.4), before being processed and embedded in paraffin. For biochemical studies, tissues were immediately frozen after removal and stored at -80 ^{o}C until used.

Immunohistochemistry and mapping of α-syn pathology

Immunohistochemistry was performed on 6 μm thick serial sections as previously described (*29*). Primary antibodies used and working dilutions are detailed in Table S2. For histological and cell mapping studies, coronal sections were stained using 3'-diaminobenzidine (DAB; Vector Laboratories) as a chromogen. Immunoreactivity in double-labeled sections was revealed using appropriate fluorescent secondary antibodies conjugated to Alexa-fluor 488 or 594 (Invitrogen, Carlsbad, CA). Images were captured on a DP71 digital camera connected to a BX51 microscope (Olympus). Photoshop CS2 (Adobe Systems) was used to assemble montages. Fine mapping of pSyn pathology was performed by tracing all visible immunoreactive inclusions/cells and neurites at 20 x magnification from sections at multiple rostrocaudal levels corresponding to approximately +1.3, +0.26, -1.75, -3.0, and -6.0 mm relative to Bregma. Midbrain DA neurons belonging to the SN (A9), VTA (A10), and retrorubral field (A8) were quantified from TH-immunostained coronal sections through the entire extent of the midbrain (1:9 series). Immunoreactive neurons were counted at 10 x magnification following previously described criteria for each subgroup (*30*). Only intact neurons with visible nuclei were included.

DA neurons containing intracellular inclusions were quantified from an adjacent series double-labeled using antibodies against TH and pSyn. Densitometry was performed on immunostained sections (with hematoxylin counterstain omitted) from PFF-injected wt mice at three representative levels of the striatum. Images of rostral, commissural and caudal striatum (corresponding approximately to Bregma +1.5mm, +0.26mm, and -1.0mm, respectively) were analyzed using ImageJ (NIH).

Biochemical analysis

Dissected brain regions of interest were weighed and extracted in RIPA buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 5 mM EDTA, 1% NP40, 0.5% sodium doexycholate, and 0.1% SDS) containing protease and phosphatase inhibitors (Roche). Five mL of buffer was used per gram of tissue. Samples were sonicated and cleared at 100,000 x *g* for 30 min. Protein concentrations were determined using the BCA assay (Pierce) and samples (20 µg total protein) were separated on SDS-polyacrylamide gels (4-20% gradient) and transferred onto nitrocellulose membranes. Blots were blocked in 5% non-fat milk in TBS and probed using various primary antibodies (Table S2). Target antigens were detected using an Odyssey FC scanner (LiCor) following incubation with the appropriate infrared secondary antibodies.

High-performance liquid chromatography analysis

Dorsal striata were dissected from both hemispheres and homogenized in 0.2 M perchloric acid including 100 µM EDTA-2Na (1:10, w/v) at 0°C. After 10 min on ice, homogenates were centrifuged for 15 min at 3,000 x *g* at 4°C. The supernatant was mixed with 0.4 M sodium acetate buffer (pH 3.0; 1:2, v/v) and filtered through a 0.22 µm centrifugal filter (4 min, 14,000 x *g* at 4°C). Filtrates were stored at -80 °C prior to HPLC analysis. Monoamines DA, NA, 5-HT and their acidic metabolites DOPAC, HVA and 5-HIAA were determined by HPLC with electrochemical detection as described elsewhere (*31*). Briefly, the HPLC system consisted of a HTEC500 apparatus (Eicom, Kyoto, Japan), and a CMA/200 Refrigerated Microsampler (CMA Microdialysis, Stockholm, Sweden) equipped with a 20 µL loop operating at 4°C. The potential of the glassy carbon working electrode was set at + 450mV relative to the Ag/AgCl reference electrode. Separation was achieved using a 200 x 2.0 mm Eicompak CAX column (Eicom) protected with a guard column CAX-GC2/20 (Eicom). The mobile phase was a mixture of methanol and 0.1 M phosphate buffer (pH 6.0; 30:70, v/v) containing 40 mM potassium chloride and 0.13 mM EDTA-2Na. A separate HPLC system was used for determination of DOPAC, HVA and 5-HIAA, with separation achieved over a 150 x 3.0 mm Eicompak SC-5ODS column (Eicom). The working potential of the glassy carbon working electrode was set at + 750mV relative to the Ag/AgCl reference electrode. The mobile phase was a mixture of methanol and 0.1 M sodium acetate solution (pH 3.5; 16:84, v/v) containing octanesulfonic acid sodium salt (210 mg/L) and EDTA (5 mg/L). All chromatograms were recorded and integrated by use of the computerized data acquisition system Clarity (DataApex, Czech Republic).

Behavioral Analysis

To evaluate the effects of transmissible α-synuclein pathology on motor, cognitive learning and memory, and emotional behavior, mice were tested on a comprehensive behavioral test battery 1 week prior to sacrifice. The order of tests was randomized and all tests were conducted by an experimenter blinded to treatment group (J.C.). All tests were conducted between 08:00-

12:00 in the lights-on cycle. Mice were habituated to the testing room 1 hr before tests, and the apparatus were cleaned with 70% ethanol in between animals to minimize odor cues.

Rotarod

To assess motor learning, coordination, and balance, mice were tested on the Rotarod apparatus (MED-Associates). Each mouse was given a training session (four 5-min trials, 5 min apart) to acclimate them to the rotarod apparatus. During the test period (1 hr later), each mouse was placed on the rotarod with increasing speed, from 4 rpm to 40 rpm in 300 sec. The latency to fall off the rotarod within this time period was recorded. Each mouse received two consecutive trials and the mean latency to fall was used in the analysis as adapted from (*32*).

Wire Hang Test

The wire hang test of motor function was conducted by following a modified protocol previously described (*33*). The mice were placed on the top of a standard wire cage lid. The lid was lightly shaken to cause the animals to grip the wires and then turned upside down. The latency of mice to fall off the wire grid was measured and average values were computed from two trials (15 min apart). Trials were stopped if the mouse remained on the lid after 15 min.

Open Field

To assess general activity, locomotion, and anxiety, mice were tested in the open field as adapted from previously described (*34*). The San Diego Instruments' Photobeam Activity System Open Field (PAS-OF) apparatus was a square arena (16" x 16" x 15") with white Plexiglas walls and white floor that was evenly-illuminated to ~95 lx. Mice were individually placed in the center and allowed to freely explore for 10 min while the trial was videotaped. Subsequent video scoring was completed by an observer blind to treatment groups using the PAS-OF customized video software (San Diego Instruments). Number of beam breaks was automatically calculated by the tracking software as a direct measure of locomotion and activity.

Tail Suspension Test

The TST for depression-related behavior was conducted using methods based upon those previously described (*35*). The mouse was securely fastened with medical adhesive tape by the tip (1.5 cm) of the tail to a standard wire cage lid and suspended above the floor in a visually isolated field. Mobility was recorded during a single, 5 minute test period. The presence or absence of immobility, defined as the absence of limb movement, was recorded every 5 seconds to calculate an average % immobile during the 5-minute trial.

Elevated plus maze

To assess anxiety-related behavior, the EPM was conducted as previously described (*36*) using a maze modeled after San Diego Instruments elevated plus maze (model 7001-0336) with precise specifications (15" from floor, 11" arm length with 6" wall). Mice were allowed to explore the maze for 5 min and scored for % time spent in open arms.

Spontaneous Alternation Behavior

To measure hippocampal-dependent working memory deficits, mice were tested for SAB in a Y-maze modeled after San Diego Y maze Instrument (model 7001-0307) using a standard protocol (*36-38*). SAB during an 8 min test period score was calculated as the proportion of

alternations (an arm choice differing from the previous two choices) to the total number of alternation opportunities (total arm entries-2) (*39*). For example, if the mouse made following choices (C,B,A,B,C,B,A,C), its alternation opportunities: 8-2= 6 and SAB: 4/6= 67%.

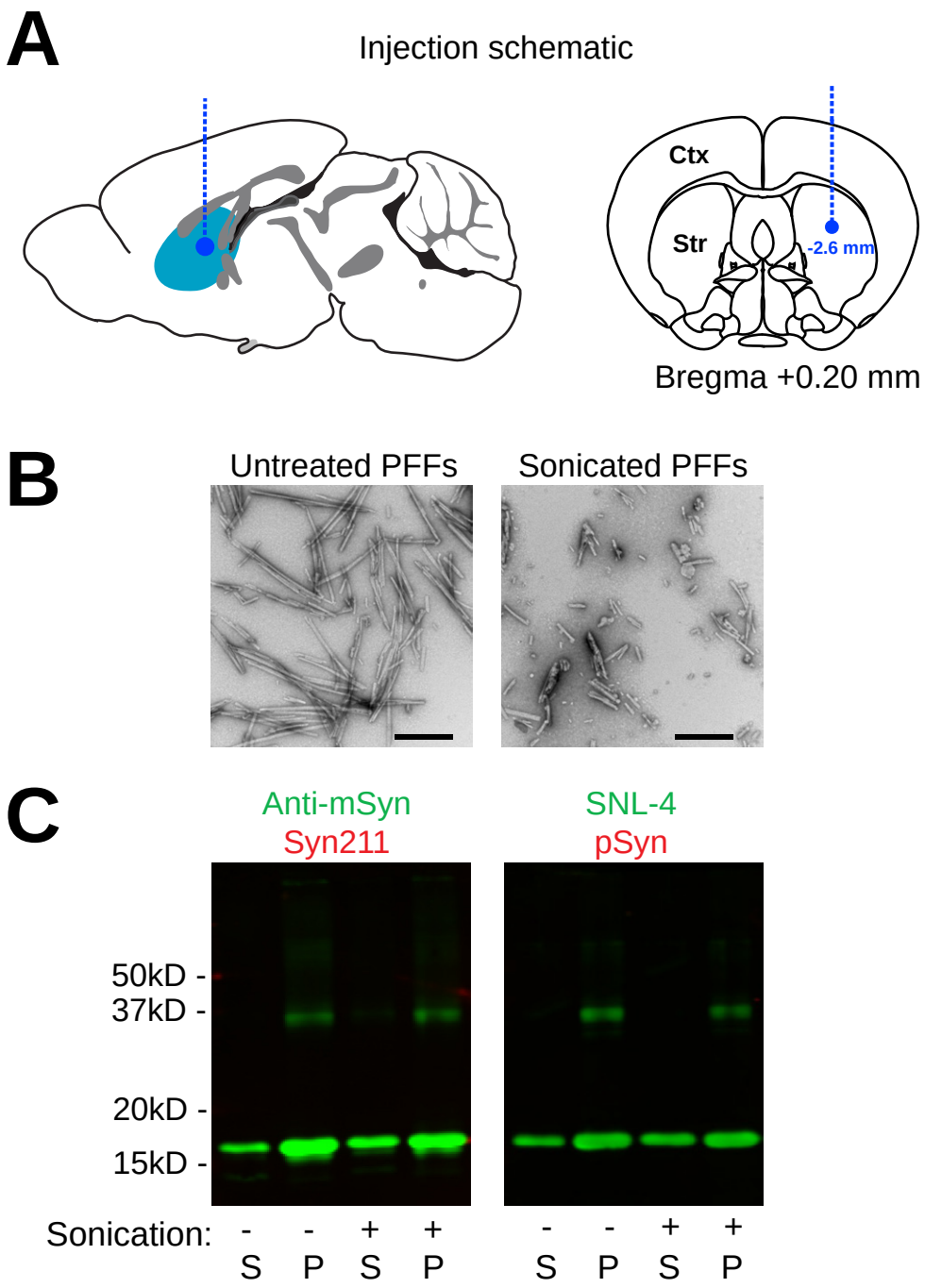

Fig. S1. Stereotaxic injection of mouse α-Syn PFFs. (***A***) Schematic diagram illustrating intra-striatal injections. Two to three month old wt mice received a single unilateral inoculation of synthetic PFFs assembled from recombinant mouse α-Syn protein (5 µg in 2.5 µL PBS). Injections were targeted to the right striatum at the position indicated. (***B***) Electron micrographs showing synthetic mSyn PFFs generated in vitro prior to and after sonication using the procedure described in "Materials and Methods". Mouse α-Syn PFFs share the same ultrastructural morphology and caliber as reported for those assembled from human α-Syn (*27*). Scale bars: 250 nm. (***C***) Solubility of synthetic mSyn PFFs. Untreated and sonicated fibrils were subjected to centrifugation (30 mins at 100,000 x *g*) and the resulting supernatant (S) and pellet (P) fractions analyzed using immunoblot. Blots were probed with antibodies specifically recognizing either mouse α-Syn (anti-mSyn), human α-Syn (Syn211), or α-Syn phosphorylated at Ser129 (pSyn), showing that sonication does not alter the solubility of synthetic mouse fibrils which are non-phosphorylated at Ser129. SNL4 is a rabbit polyclonal antibody that reacts with the N-terminal region of both murine and human α-Syn.

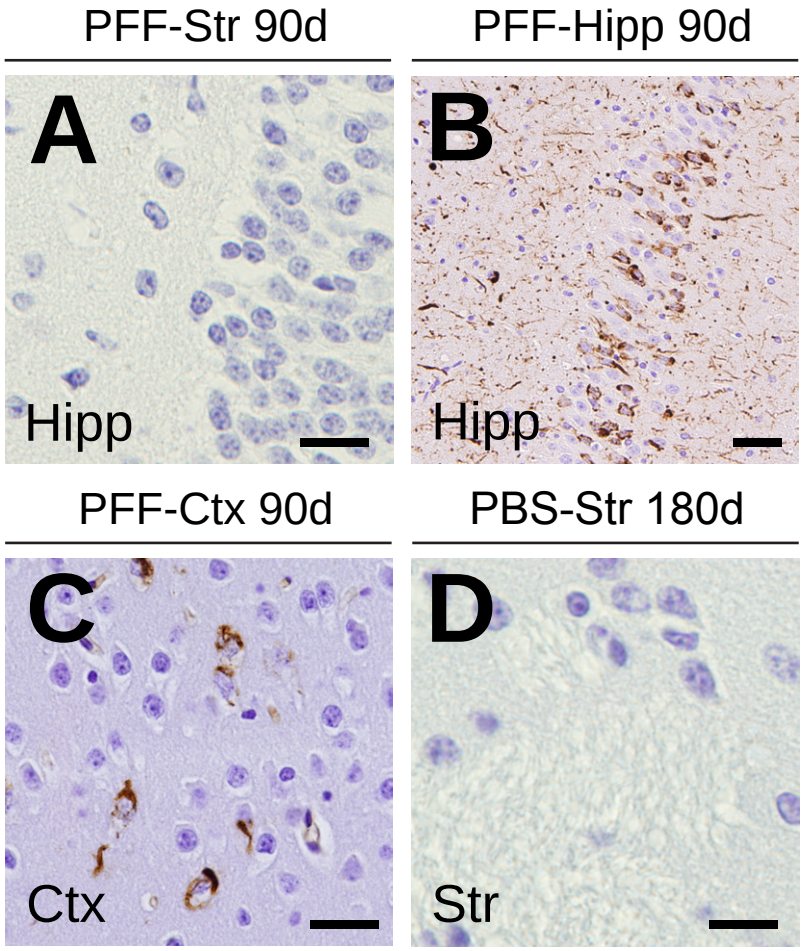

Fig. S2. Multiple neuronal populations are permissive to pathologic α-Syn transmission. (***A***) pSyn immunostaining of hippocampal neurons in a wt mouse 90 d following inoculation of mouse α-Syn PFFs into the dorsal striatum. The hippocampus ipsilateral to the injection site is shown. Note the absence of pSyn. (***B***) Cytoplasmic and neuritic pSyn pathology in the hippocampus of a wt mouse that received a single PFF injection to this region and sacrificed after 90 d. The region adjacent to the injection site is shown. (***C***) Intraneuronal pSyn accumulations in neocortical neurons 90 d after injection into the somatosensory cortex. (***D***) Lack of striatal α-Syn pathology in wt mouse 180 d after injection with PBS. Scale bars: 20 µm (***A,C,D***), 40 µm (***B***).

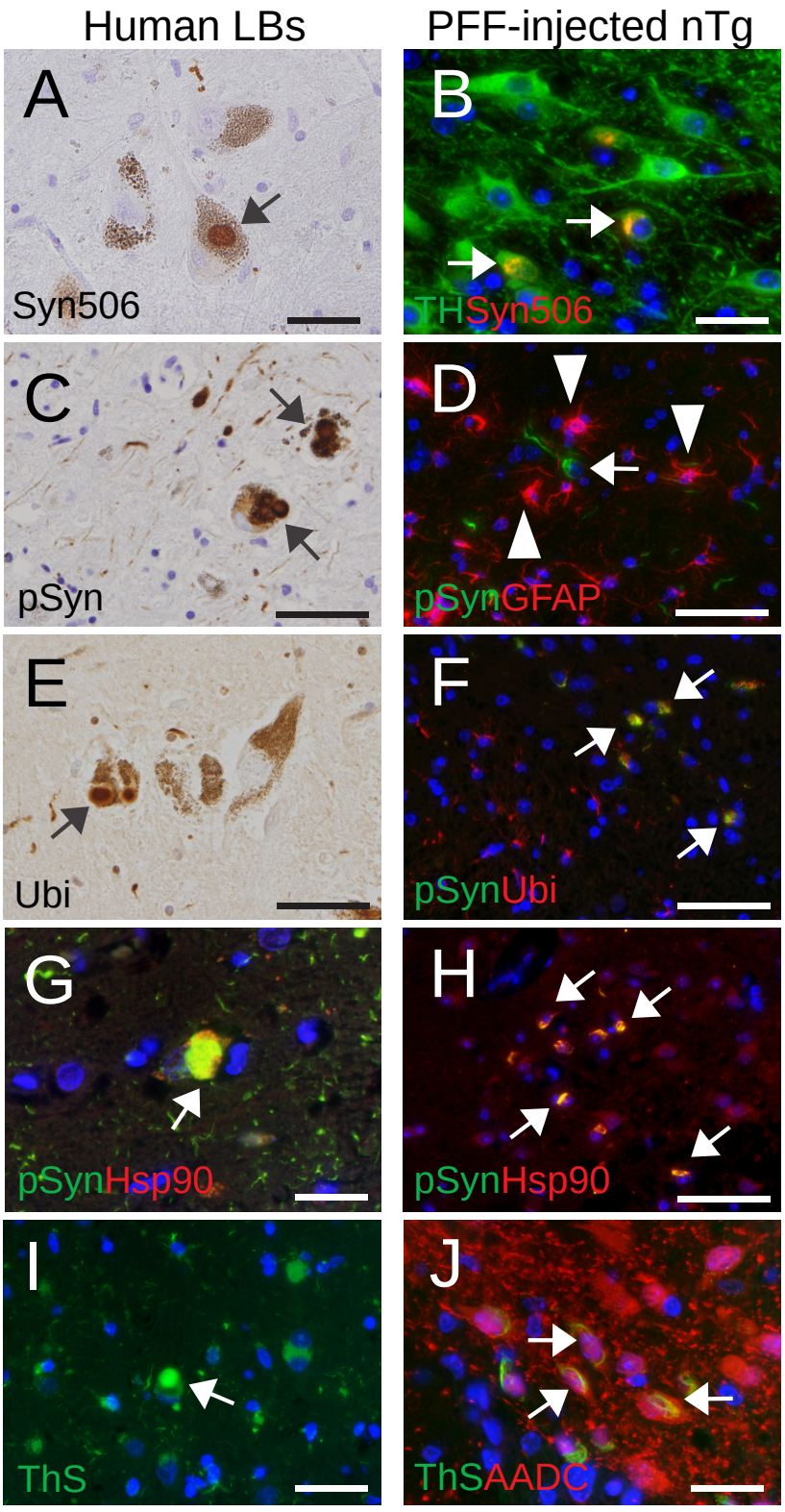

Fig. S3. PFF-seeded intraneuronal inclusions share common features with human LBs/LNs. Sections of substantia nigra from human PD brains (left) or C57BL6/C3H mice that received a single intrastriatal injection of mouse α-SynPFFs (right) were stained using various markers for Lewy pathology. As with human LBs, intraneuronal inclusions in the SNpc of PFF-treated mice show strong immunoreactivity towards antibodies recognizing disease-specific conformations of α-Syn (Syn506; ***A,B***), pSyn (***C,D***), ubiquitin (Ubi; ***E,F***), and the molecular chaperone heat shock protein 90 (Hsp90; ***G,H***), as indicated by arrows. PFF-seeded inclusions in mice associated with astrocytes, but did not colocalize with GFAP staining (arrowheads in ***D***). Both human LBs and PFF-seeded inclusions in mice were double-labeled with Thioflavin S (ThS) and L-aromatic amino-acid decarboxylase (AADC), indicating the presence of amyloid fibrils in DA neurons (***I,J***). Scale bars: 20 μm (***A,G,I,J***) and 50 μm (***B-F,H***).

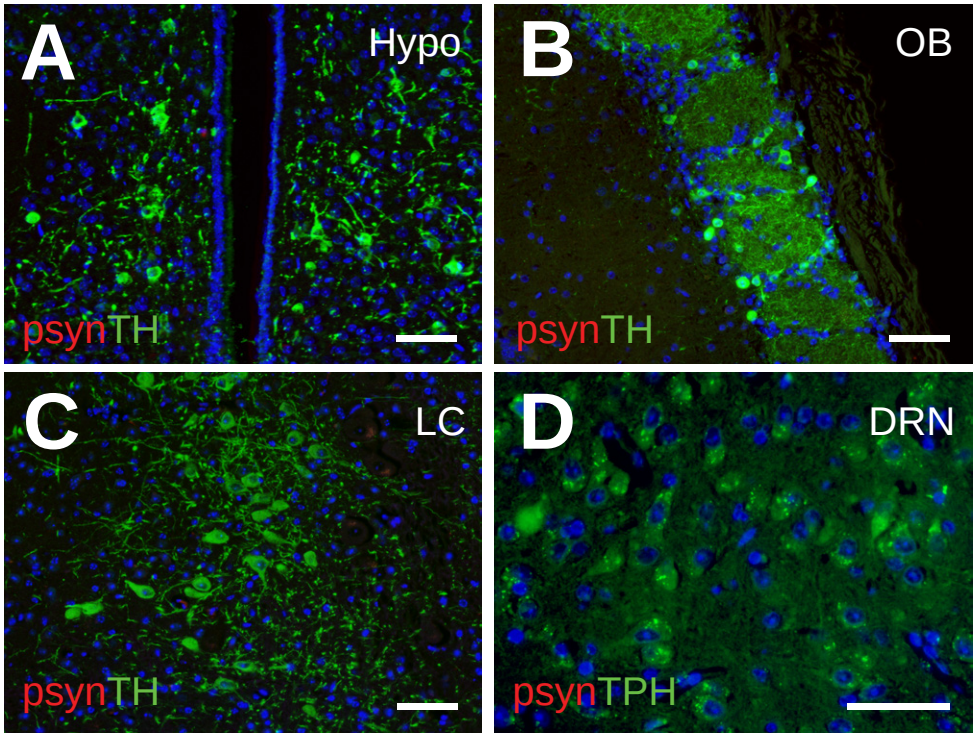

Fig. S4. Lack of LN/LB pathology in multiple monoaminergic populations. Double-immunostaining for pSyn and either tyrosine hydroxylase (TH) or tryptophan hydroxylase (TPH) in (***A***) arcuate neurons of the hypothalamus (Hypo), (***B***) glomerular interneurons in the olfactory bulb (OB), (***C***) locus coeruleus (LC) and (***D***) the dorsal Raphe nucleus (DRN) of mice that received intrastriatal mouse α-SynPFF injections. Animals were sacrificed at 180 d following injection. Note the absence of pSyn immunoreactivity in these regions. Scale bars: 20 μm.

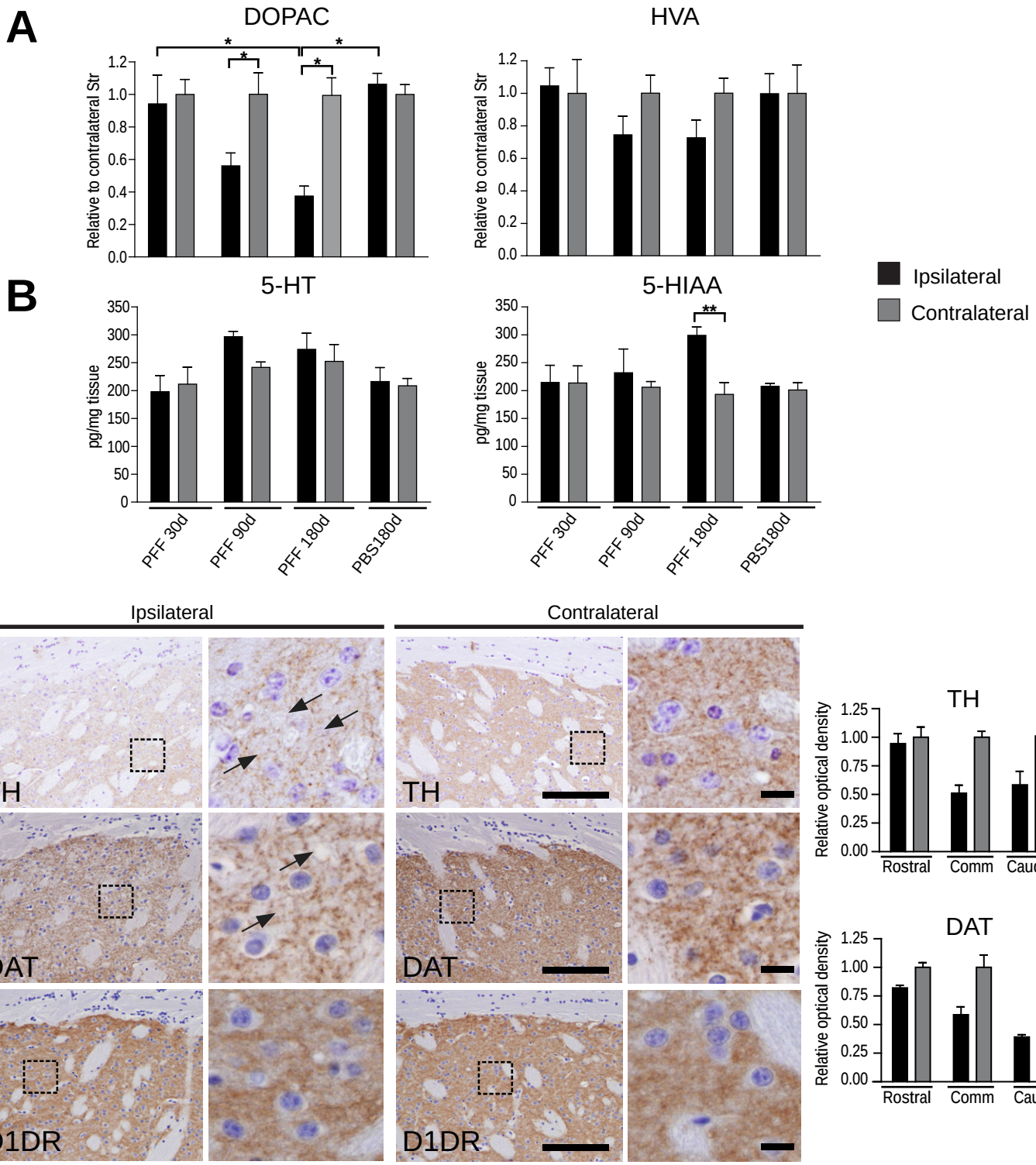

Fig.S5. α-Syn pathology induces loss of dopamine input to the striatum. (***A***) Levels of DA metabolites 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in dorsal striata of wt mice treated with mouse α-SynPFFs as determined by HPLC at the indicated time points. DOPAC and HVA levels showed a time-dependent reduction consistent with the progressive loss of SNpc neurons and decrease in striatal DA following PFF inoculation. Data show mean +/- SD (*N* = 3-5 animals per group). Groups were compared using one-way ANOVA (P=0.0005) and Tukey post-hoc test (*P<0.01). (***B***) Corresponding concentrations of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in the samples from (***A***) showing increases in serotonin that accompanies loss of striatal DA. One-way ANOVA (P=0.01);**, P<0.05. (***C***) Immunohistochemistry for tyrosine hydroxylase (TH), dopamine transporter (DAT), and D1 dopamine receptor (D1DR) in dorsal striatum of mice injected with mouse Syn fibrils and sacrificed at 90 dpi. TH and DAT immunoreactivity are visibly reduced in dorsal striatal regions ipsilateral to the injection site (top and middle panels). Areas delineated by boxes are shown at high magnification in insets. Arrows indicate reductions in the density of dopamine terminals, consistent with biochemical findings (Fig.3C). No similar decrease was observed for D1DR in striatal projection neurons (bottom panel). Scale bars: 70 µm (***C***), 10 µm (insets). Optical density measurements of striatal TH and DAT in dorsal striatum at 3 rostrocaudal levels are shown on the right. Data are mean+/- SD (*N* = 3 per group).

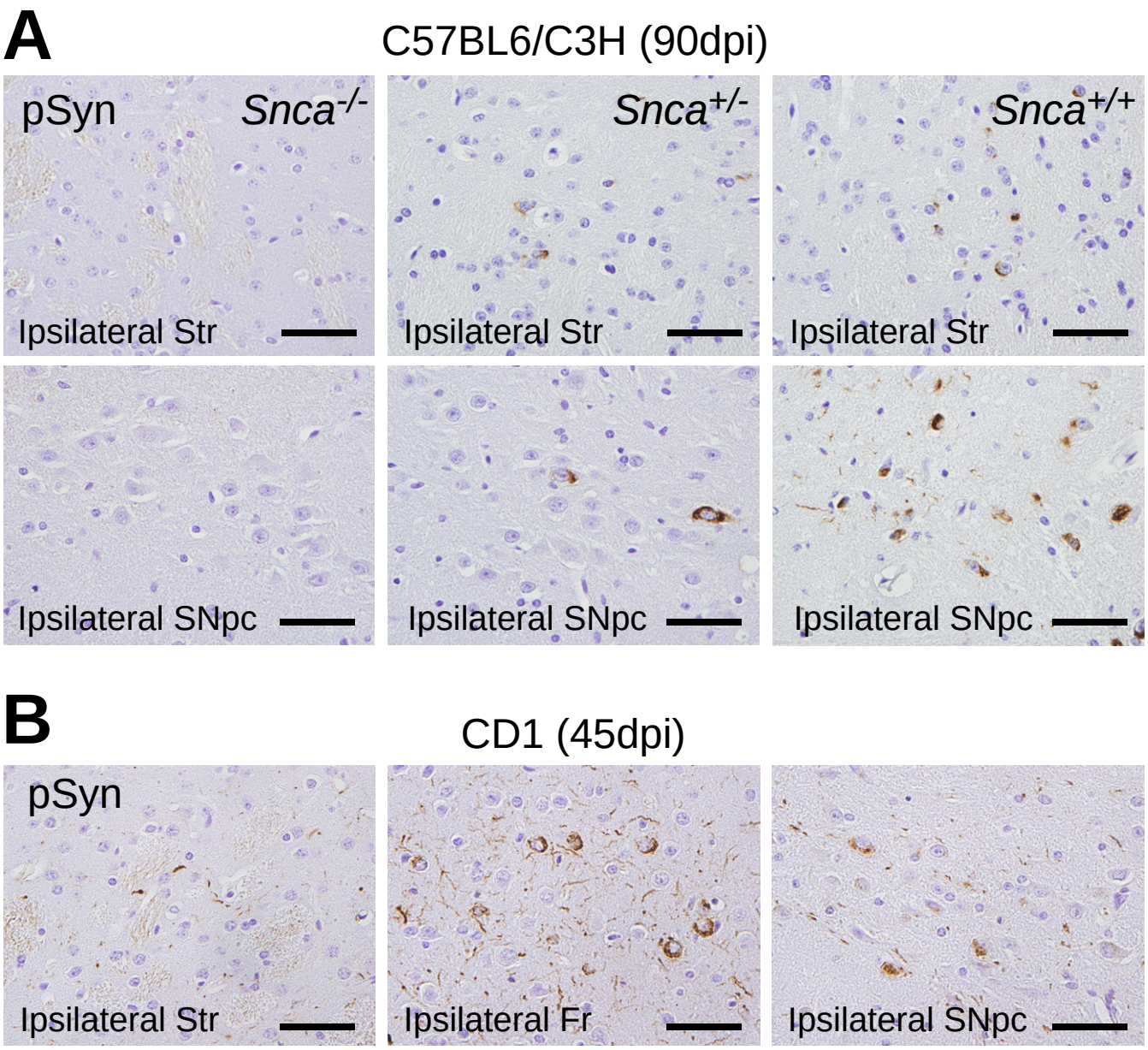

Fig. S6. Mouse α-Syn fibrils induce α-Syn pathology in multiple mouse strains. (***A***) Striatal (Str) and substantia nigra (SNpc) pSyn pathology in *Snca*$^{-/-}$, *Snca*$^{+/-}$ and wt C57BL6/C3H (*Snca*$^{+/+}$) mice injected with mouse α-Syn PFFs. Animals were sacrificed 90 d post-injection. In contrast to wt mice that developed robust striatal and SNpc pathology (right), no α-Syn accumulation was detectable in α-Syn null mice (left), whereas heterozygous animals exhibited an intermediate level of pathology (middle), suggesting that the severity and distribution of pathology is determined in part by α-Syn expression levels. (***B***) Pathological α -Syn inclusions revealed by pSyn immunohistochemistry in the striatum, frontal cortex (Fr), and SNpc of CD1 mice 45 d following a single intrastriatal injection with mouse α-SynPFFs. Scale bars: 50 μm.

Table S1. Summary of histological findings.

Mouse strain	Inoculum	Injection Site	Duration	*N*	Str	Fr	SNpc	Amyg	Hipp
C57BL6/C3H	α-Syn PFF	Str	3 d	4	-	-	-	-	-
C57BL6/C3H	α-Syn PFF	Str	14 d	2	+		-	+	-
C57BL6/C3H	α-Syn PFF	Str	30 d	9	++	++	+	++	-
C57BL6/C3H	α-Syn PFF	Str	90 d	10	++	+++	+++	+++	-
C57BL6/C3H	α-Syn PFF	Str	180 d	11	++	+++	+++	+++	-
C57BL6/C3H	α-Syn PFF	Ctx	180 d	3	++	+++	-	+	-
C57BL6/C3H	α-Syn PFF	Hipp	180 d	3	-	+	-	+	+++
C57BL6/C3H	α-Syn PFF	Str+Ctx	90 d	2	++	+++	+++	+++	-
C57BL6/C3H	α-Syn PFF	Str+Ctx	180 d	4	++	+++	+++	+++	-
C57BL6/C3H	PBS	Str	90 d	4	-	-	-	-	-
C57BL6/C3H	PBS	Str	180 d	8	-	-	-	-	-
C57BL6/C3H	α-Syn mono	Str	50 d	1	-	-	-	-	-
C57BL6/C3H	α-Syn mono	Str	90 d	1	-	-	-	-	-
C57BL6/C3H	α-Syn mono	Str	160 d	3	-	-	-	-	-
CD1	α-Syn PFF	Str	45 d	3	+	+	+	+	-
Snca$^{+/-}$	α-Syn PFF	Str	90 d	4	+	+	-	+	-
Snca$^{+/-}$	α-Syn PFF	Str	180 d	4	+	+	+	++	-
Snca$^{-/-}$	α-Syn PFF	Str+Ctx	180 d	4	-	-	-	-	-
C57BL6/SJL	α-Syn PFF	Str+Ctx	180 d	4	++	++	++	+++	-

Table S1. Summary of experimental animals used in this study. Mice bred on various backgrounds received a single stereotaxic injection in the region indicated. Pathology as observed in pSyn immunostained coronal sections was graded using a scale ranging from no detectable pathology (-), to mild (+), moderate (++), or severe (+++) neuritic and cytoplasmic inclusions in the nuclei shown. Abbreviations: Amyg (amygdala), Ctx (cortex), Fr (frontal cortex), Hipp (hippocampus), mono (monomeric), PFF (pre-formed fibrils), SNpc (substantia nigra pars compacta), Str (striatum).

Table S2. Antibodies used in this study

Antibody	Source / Reference	Host	Dilution (IHC)	Antigen Retrieval	Dilution (WB)
α-Synuclein					
Syn 303	(*40*)	Mse	1:7,000	Formic acid*	1:2,000
Syn 506	(*41, 42*)	Mse	1:20,000	Citrate#	-
Mouse α-Syn	(*20*)	Rab	-	-	1:500
Hyperphosphorylated α-Syn (pSyn)					
pSyn 6.2	(*20*)	Rb	1:1,000	None	-
$Syn^{pSer129}$	(*43*)	Mse	1:20,000	None	-
CNS markers					
Tyrosine Hydroxylase (TH)	Sigma	Mse	1:5,000	Formic Acid	-
TH	Pel-freeze	Rb	-	-	1:2,000
Aromatic L-amino acid decarboxylase (AADC)	Abcam	Rb	1:5,000	Citrate	-
Tryptophan hydroxylase (TPH)	Sigma	Mse	1:5,000	None	-
D1 dopamine receptor (D1DR)	Sigma	Rat	1:1,000	Citrate	1:1,000
D2 dopamine receptor (D2DR)	Millipore	Rb	-	-	1:500
Dopamine transporter (DAT)	Millipore	Rat	1:1,000	Citrate	1:1,000
Miscellaneous					
Ubiquitin (1510)	Millipore	Mse	1:25,000	Formic Acid*	-
Glial fibrillary acidic protein (GFAP)	Dako	Rb	1:1,000	None	-
Heat shock protein 90 (Hsp90)	Stressgen	Rat	1:1,000	None	-

Abbreviations: mse (mouse); Rb (rabbit)
* Formic acid treatment: 88% formic acid for 5 mins at room temperature.
# Citrate treatment: 0.1% Antigen unmasking solution (Vector Laboratories) for 15 mins at 99^{o}C.