The Variance in Phosphorylated, Insoluble α-Synuclein in Humans, Rats, and Mice is not Mainly Driven by Biological Sex

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Supplemental Methods

Human Brain Samples

Deidentified postmortem human olfactory bulb and amygdala tissues from the University of Miami Brain Endowment Bank and the Human Brain and Spinal Fluid Resource Center, VA West Los Angeles Healthcare Center were acquired from the Institutional Review Board (IRB)-approved repository at the National Institutes of Health NeuroBioBank. Samples were collected from 7 men and 6 women with diagnoses of Lewy body disorders, as well as 7 unaffected male and 6 unaffected female control subjects. Per the head of the Duquesne University IRB, specimen sampling did not require reapproval at Duquesne University. **Table S3** lists the demographic information provided for these 26 samples. Age and postmortem interval were not significantly different across groups, as shown in our previous report [2]. Images of amygdala dissections by a board-certified neuropathologist were provided for each UCLA subject and can be viewed in Figure S11 of our recent publication [12].

Animals

All animal experimentation was approved by the Duquesne University Institutional Animal Care and Use Committee (Protocols 1901-01, 2202-01, 2006-05, 1944, and 1903-04). CD-1 mice and Sprague Dawley rats were obtained from Charles River (Wilmington, MA) and Hilltop Lab Animals, Inc. (Scottdale, PA), respectively. Animals of both sexes were socially housed and bred in-house with *ad libitum* access to food and water in a 12:12 photoperiod.

Outbred CD-1 mice were used because they tend to display more genetic diversity and higher variability than inbred mouse strains such as C57, which can be an advantage when seeking to generalize lab results to wild populations [1, 8]. It is important to note that CD-1 mice are entirely different from CD1 receptor-deficient mice with knockout of Cd1d1/Cd1d2 loci [7]. Sprague-Dawley rats are also a genetically outbred strain. Colonies were refreshed approximately every six months with new breeders to minimize genetic drift.

Preformed α-Synuclein Fibrils and Stereotaxic Surgeries

Preformed fibrils were synthesized in Dr. Kelvin Luk's lab at the University of Pennsylvania from recombinant, wildtype mouse α-synuclein protein, as described previously (Polinski et al., 2018; Volpicelli-Daley et al., 2014). Per Dr. Luk, preformed fibril preparations contained ≤0.11 picograms lipopolysaccharides per microgram protein. Our protocol induces a mean fibril length of 39.8 nanometers and a median length of 35.2 nanometers by transmission electron microscopy (quantified from the original images of [10]).

Aliquots of stock α-synuclein fibrils (5 mg/mL) were stored at -80°C. For surgeries, fibrils were thawed, sealed with Parafilm, and sonicated at room temperature in an ultrasonic water bath

(Bransonic M1800, Branson Ultrasonics Corporation, Danbury, CT) for 60 minutes. After sonication, fibrils were maintained at room temperature and infused into the mouse or rat olfactory bulb/anterior olfactory nucleus (OB/AON), as confirmed with blue food dye prior to each set of surgeries (*e.g.*, **Figure S1a-b**). Any fibrils not infused directly into the brain within 4 hours of sonication were discarded. Our prior work has consistently demonstrated generation of limbic α-synucleinopathy *in vivo* using the abovementioned sonication parameters [2, 3, 10-12].

For surgeries, animals were anesthetized with 2-3% vaporized isoflurane, and the skull was stabilized in a stereotaxic frame (Stoelting, Wood Dale, IL). Forty-five CD1 mice were infused bilaterally into the OB/AON at eight months of age, or at 9-11 months of age with 5 μg preformed α-synuclein fibrils per hemisphere (n = 7 males and 7 females in the 8-month-old cohort, and n = 5 males and 5 females in the 9 to 11-month-old cohort) or an equivalent volume of sterile phosphate-buffered saline (1 μL PBS; n = 5 males and 6 females in the 8-month-old cohort, and n = 5 males and 5 females in the 9 to 11-month-old cohort). The 8-month-old mouse cohort was used for analyzing whole tissue extracts, whereas the 9 to 11-month-old mouse cohort was used for the ultracentrifugation and detergent-based extractions of the amygdala and piriform cortex (described below).

Mice were infused at the following coordinates, relative to Bregma: AP +4.0 mm, ML +/-1.0 mm, and DV -3.0 mm (from the skull surface). Stereotaxic coordinates were initially based on the mouse brain atlas of Franklin and Paxinos [5], but note that outbred CD1 mice tend to be larger than the C57 strain. Mice were perfused 12 or 6 weeks post-infusion, as indicated in the Figure Legends.

Thirty-two four-month-old rats received bilateral OB/AON infusions of 15 μg preformed α-synuclein fibrils (n = 10 males and 10 females) or 3 μL PBS (n = 5 males and 7 females) per hemisphere at the following coordinates, relative to Bregma: AP +5.0 mm, ML +/-1.5 mm, and DV -5.2 mm. Rats were sacrificed four months post-infusion.

All infusions were performed with a Hamilton syringe (80330, Hamilton, Reno, NV) connected to a motorized injection pump (Stoelting), at a rate of 0.25 μL/min, followed by a 2-3 minute rest period and gradual withdrawal of the needle. After surgery, animals were transferred to a heating pad and administered subcutaneous buprenorphine (0.05 mg/kg). Four percent lidocaine cream was also applied topically to the incision site for three days.

Mice and rats were anesthetized, euthanized by decapitation, and their brains divided along the midline. Hemibrains were dissected immediately for multiple limbic regions (OB/AON, piriform and entorhinal cortices, amygdala, and hippocampus). After dissection, tissues were frozen on dry ice and stored at -80 °C. Left hemispheres from rats were immersion-fixed in 4% formalin (Formal-Fixx; Cat. 9990244, Thermo Fisher Scientific, Waltham, MA) in 0.1 M phosphate buffer (PBS) for 10 days, followed by immersion in 30% sucrose in 10 mM phosphate buffered saline (PBS) for a minimum of two days until sectioning for immunostaining (described below).

Immunoblotting for Detection of Phosphorylated (pSer129) and Pan α-Synuclein

Although physiological roles for α-synuclein phosphorylation exist [13, 14], supraphysiological phosphorylation at serine 129 remains a sensitive indicator of Lewy body disease [4]. Human and rodent samples were weighed and prepared for SDS-PAGE in 1× cell lysis buffer (CLB; 20 µL CLB per mg tissue; 10× CLB purchased from Cell Signaling Technologies, 9803, Danvers, MA) supplemented with 10 mM sodium fluoride (S7920, Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride (PMSF; P7626, Sigma-Aldrich), 1% protease inhibitor cocktail (P8340, Sigma-Aldrich), and 1% phosphatase inhibitor cocktail (prepared in-house; contains 100 mM sodium fluoride, 100 mM sodium orthovanadate, and 200 mM imidazole).

A probe sonicator (Model XL2020, Misonix Inc., Farmingdale, NJ) was used to disrupt tissues (~30, 0.5 second pulses), and homogenates were then incubated in 4× Protein Sample Loading Buffer (928-40004, LI-COR, Lincoln, NE) with 10% fresh β-mercaptoethanol for 30 minutes at 37 °C (*i.e.*, samples were not boiled, to prevent *in vitro* formation of artificial protein aggregates).

To improve resolution when probing for α-synuclein, equal amounts of protein were loaded onto 12% polyacrylamide gels and separated with gel electrophoresis for >3 hours at 75V. Proteins were transferred onto Immobilon-FL PVDF membranes (IPFL0010, Millipore, Burlington, MA) and then dried for 10 minutes to prevent liftoff of proteins from the membrane.

After rehydration in methanol, membranes were fixed with 4% formalin (Formal-Fixx, 9990244, Fisher Scientific, Pittsburgh, PA) and 0.01% glutaraldehyde (G7651, Sigma-Aldrich) in 10 mM tris-buffered saline (TBS) for 15 minutes at room temperature [9, 15]. Membranes were washed once with water, and then LI-COR's Revert 700 Total Protein Stain Kit (926-11010, LI-COR) was applied. Briefly, membranes were incubated for 90 seconds in Revert 700 Total Protein Stain diluted 1:5 in methanol, washed twice with 6.7% glacial acetic acid and 30% methanol in water, and immediately scanned on an Odyssey Classic (Model 9201-01, LI-COR) or Odyssey M infrared imager (Model 3350-02, LI-COR). Revert Destaining Solution was then applied to membranes for 11 minutes to reverse the Total Protein Stain.

After removal of the REVERT stain, membranes were washed three times in TBS and blocked for one hour in undiluted Intercept TBS Blocking Buffer (927-60003, LI-COR). All membranes were incubated overnight at 4°C with primary antibodies diluted at concentrations listed in **Table S1** in a 3:1 solution of TBS Blocking buffer and TBS, supplemented with 0.1% Tween-20 (TBST). The following day, primary antibodies were washed off with three exchanges of TBST, and membranes were incubated for one hour at room temperature in secondary antibodies diluted at concentrations listed in **Table S2** in a 3:1 solution of TBS Blocking buffer and TBS, supplemented with 0.01% sodium dodecyl sulfate to minimize nonspecific binding. Membranes were then washed in TBST and scanned on the Odyssey Classic or Odyssey M.

Image Studio Lite (Version 5.2.5, LI-COR) was used to quantify protein levels from fluorescent signal, and total protein for each sample (*i.e.*, entire lane from top to bottom) was used as the loading control. Molecular masses were confirmed vis-à-vis the Precision Plus Protein Dual Color Standard (1610374, Bio-Rad, Hercules, CA), the Precision Plus Protein All Blue Standard (1610373, Bio-Rad), or the Chameleon 800 Pre-stained Protein Ladder (928-80000, LI-COR).

Full-length, brightened immunoblots are presented to show all potentially nonspecific antibody binding.

Sequential Extraction of Soluble and Insoluble α-Synuclein

Immunoblotting for phosphorylated and pan α-synuclein was also carried out on human, rat, and mouse lysates following sequential extraction, in accordance with the established protocol of Volpicelli-Daley *et al*. [17]. Briefly, tissues were probe sonicated in 1× cell lysis buffer (CLB; 30 µL CLB per mg tissue for rats and 80 µL CLB per mg tissue for mice; recipe as above without sodium fluoride) in TBS. Note that the 10× cell lysis buffer from Cell Signaling Technology (9803; Danvers, MA) contains 1% Triton X-100. After sonication, samples were centrifuged at 100,000 ×*g* for 30 minutes at 4°C (Sorvall mX 120+ Micro-Ultracentrifuge with the S55-A2 fixed angle rotor, Cat. 45865, Thermo Fisher Scientific). For the smaller-size mouse brain regions, we collapsed the amygdala with the piriform cortex of each animal, due to minimum spin volumes in our centrifuge.

The supernatant was collected and stored at -80°C as the Triton X-soluble fraction, and the pellet was resuspended in fresh 1× CLB. Samples were resonicated, until the pellet was completely dispersed (~20, 0.5 second pulses), and centrifuged a second time at 100,000 ×*g* for 30 minutes at 4°C. The supernatant was then collected and stored at -80°C as the Triton X wash fraction. Triton X-insoluble pellets were resuspended in TBS supplemented with 2% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 1% protease inhibitor, and 1% phosphatase inhibitor cocktail, resonicated, and stored at -80°C as the Triton X-insoluble fraction.

The protein concentration for each sample was measured with the bicinchoninic acid (BCA) Pierce Protein Assay Kit (23225, Fisher Scientific) or NanoDrop One^{C} spectrophotometer (Model NanoDrop One C, Thermo Fisher Scientific). Samples were prepared with 4× Laemmli Sample Buffer (1610747, Bio-Rad) supplemented with 10% fresh β-mercaptoethanol. Proteins were then subjected to the same immunoblotting procedures as described above.

Tyramide-Signal Amplified Immunostaining for Detection of pSer129^{+} Inclusions

Rat hemibrains were sectioned in the sagittal plane into 40 µm-thick slices on a freezing microtome (American Optical AO 860 Sliding Microtome, Rankin Biomedical Corporation, Holly, MI). A 1-in-6 series of free-floating brain sections was stored in cryoprotectant [6] at -20 °C until use.

Immunostaining for pSer129 was amplified in accordance with our previously developed protocol [3]. Briefly, rat brain sections were removed from cryoprotectant and washed 5 times in filtered 10 mM PBS with 0.01% Triton X-100 for 5 minutes/wash. Washed tissues were then permeabilized in 0.5% Triton X-100 in PBS for 10 minutes on a platform rocker at room temperature. Next, sections were washed with PBS and incubated in peroxidase quenching buffer (0.3% hydrogen peroxide in 10 mM PBS) for 15 minutes. After an additional three 5-minute washes with PBS, tissues were blocked in Intercept PBS Blocking Buffer (LI-COR; 927-70003) with 0.5% Triton X-

100 for one hour on a rocker at room temperature. Brain sections were then exposed to the primary antibody against pSer129 (ab51253, Abcam, Cambridge, UK) in 50% PBS Intercept Blocking Buffer / 50% 10 mM PBS, with 0.5% Triton X-100 overnight at 4 °C.

The next day, unbound primary antibodies were washed off, and sections were incubated in a solution containing 0.0025 µg/µL donkey horseradish peroxidase-tagged secondary antibodies (711-035-152, Jackson ImmunoResearch Labs, West Grove, PA) in 50% Intercept PBS Blocking Buffer / 50% 10 mM PBS, with 0.5% Triton X-100 for one hour at room temperature. Sections were washed again with PBS and incubated for 10 minutes at room temperature and protected from light in a solution of CF®543 Tyramide dye (92172, 0.002 mM, Biotium, Fremont, CA) diluted 1:500 in Tyramide Amplification Buffer (99832, Biotium) and supplemented with fresh 0.0015% hydrogen peroxide immediately prior to use. Tissues were washed three times with PBS, counterstained with Hoechst 33258 (bisBenzimide, 0.005 µg/mL; B1155, Sigma-Aldrich, St. Louis, MO) diluted 1:1000 in 50% Intercept PBS Blocking Buffer / 50% 10 mM PBS, with 0.3% Triton X-100 for one hour at room temperature and washed again three times.

Immunolabeled sections were mounted on glass Superfrost Plus Gold Microscope Slides (22-035813, Fisher Scientific), dried, and coverslipped with Krystalon mounting media (64969, EMD Chemicals, Gibbstown NJ). Rat brain sections were scanned on the Odyssey M (LI-COR) at 5-micron resolution for main **Figure 1b** and **Extended Figure S1c**. Infusion site images in **Figure 1a** and **Figure S1d-g** were captured with an epifluorescent microscope (Olympus IX73, B&B Microscopes, Pittsburgh, PA).

Statistical Analyses

All statistical tests are specified within figure legends. Boxplots illustrate the median and interquartile ranges in all graphs, and each male and female subject or animal is depicted as an individual dot as the statistical unit. All statistical analyses were performed using GraphPad Prism (Version 9.5.1) with a two-tailed alpha threshold of 0.0500. For comparisons of normally distributed data, a two-way ANOVA was followed by the Bonferroni *post hoc* test. Log_{10} or square-root transformations were conducted on non-normally distributed or heteroscedastic data, and all raw values (before transformation) are also presented within **Supplemental Figures S5-S7**. Statistical interactions and main effects of biological sex or disease are shown below respective graphs.

Table S1: Primary Antibodies					
Antibody	Host	Company	Catalog/Clone #	Lot #	Dilution
Pan α-synuclein	Mouse	BD Biosciences	610786 [Clone 42]	0314065 2059868	1:1000 (1:500 for human/rat insoluble fraction)
Pan α-synuclein	Mouse	Abcam	Ab27766 [LB 509]	GR3174001-5	1:500
pSer129 α-synuclein	Rabbit	Abcam	Ab51253 [EP1536Y]	GR3232346-14 GR3378673-6 GR3378673-14 GR3378673-20 GR3378673-12	1:800 – 1:1000 (WB) 1:1500 (IHC)

Table S2: Secondary Antibodies				
Antibody and Host Species	Company	Catalog #	Lot #	Dilution
Donkey anti-Mouse 680	Jackson	715-625-151	138104	1:15k – 1:30k
Donkey anti-Rabbit 790	Jackson	711-655-152	136728, 153787	1:15k – 1:30k
Donkey anti-Rabbit HRP	Jackson	711-035-152	157399	1:320

Table S3. Demographics of NIH NeuroBioBank Human Samples (also available online)

Number	Age	Sex	C vs. D	PMI (h)	UCLA Neuropathological Diagnosis	Clinical Diagnosis
3298	79	M	Control	20.0	Recent cerebrum hypoxia	CA, prostate, Renal failure (acute), Diabetes Type I
3336	77	M	Control	15.5	Cerebrum hypoxia (intermediate)	Heart attack, CA, prostate
3401	82	M	Control	14.0	Minor aging changes	Congestive heart failure
3345	82	F	Control	12.9	Recent cerebrum hypoxia	CA, colon
3795	72	F	Control	14.0	Atherosclerosis (moderate), No evidence of metastatic carcinoma	CA, ovarian; CA, peritoneal
3924	79	F	Control	52.6	Multiple sclerosis (clinical)	Multiple sclerosis, MRSA (Methicillin resistant staphylococcus aureus), Seizures, Chronic obstructive pulmonary disease, Osteoporosis, Paraplegic, Hydrocephalus, Gastroesophageal reflux disorder
4002	82	M	Diseased	19.5	PD, Atherosclerosis	PD, Lewy Body disease, Depression, Dementia, Senile, Atherosclerosis, Sleep disorder, Hypothyroidism
4220	68	M	Diseased	13.8	AD (probable), Lewy body disease (cortical)	AD, LBD, Lewy Body disease (probable), Hypertension, Osteoarthritis
4365	71	M	Diseased	20.3	LBD	LBD, Depression, Hypertension, Diabetes Type II, Sleep disorder, Alcohol abuse
4150	82	F	Diseased	12.3	AD (probable), atherosclerosis (moderate)	AD, Organic brain syndrome (OBS), Dementia, Depression, Lewy Body disease, Ataxia, Transient Ischemic Attack (TIA), Renal failure (acute), Congestive heart failure, CA in lung
4155	79	F	Diseased	17.7	AD (definite), LBD	AD, Cognitive impairment, Depression, Dementia, Chronic obstructive pulmonary disease
4186	75	F	Diseased	15.2	AD (probable), LBD	LBD, PD, Vascular dementia (possible), AD, Hypothyroidism, Urinary tract infection, Depression, Psychosis, Obsessive compulsive disorder
Abbreviations: CA=Cancer, PD=Parkinson's disease, AD=Alzheimer's disease, LBD=Lewy Body Dementia						

Subject ID	Age	Sex	C vs. D	PMI (h)	Neuropathological Diagnosis (Univ. of Miami)	Race	Ethnicity	Frozen Hemisphere(s)
Hct15HAUA006	65	M	Control	21.8	Unaffected Control	White	Hispanic or Latino	Right
Hct15HBQA005	66	M	Control	6.83	Unaffected Control	White	Hispanic or Latino	Right
Hct15HBCA003	83	M	Control	25	Unaffected Control	White	Hispanic or Latino	Both
HctZZTA003	84	M	Control	15.5	Unaffected Control	White	Not Hispanic or Latino	Both
HBKVA003	74	M	Diseased	8.98	Dementia with Lewy Bodies	White	Not Hispanic or Latino	Both
BEB18025A002	79	M	Diseased	23.66	Dementia with Lewy Bodies	White	Not Hispanic or Latino	Right
HBKZA003	80	M	Diseased	28	Dementia with Lewy Bodies	White	Not Hispanic or Latino	Left
HBKTA003	81	M	Diseased	29	Dementia with Lewy Bodies	White	Not Hispanic or Latino	Left
HctZZHA003	65	F	Control	19.43	Unaffected Control	White	Not Hispanic or Latino	Both
HctZZCA003	82	F	Control	14.2	Unaffected Control	White	Not Hispanic or Latino	Both
HctZZVA003	86	F	Control	25.11	Unaffected Control	White	Not Hispanic or Latino	Both
HBJRA003	73	F	Diseased	7.71	Dementia with Lewy Bodies	White	Hispanic or Latino	Left
HBNMA003	79	F	Diseased	20.95	Dementia with Lewy Bodies	White	Not Hispanic or Latino	Both
HBNFA003	83	F	Diseased	25.08	Dementia with Lewy Bodies	White	Not Hispanic or Latino	Left

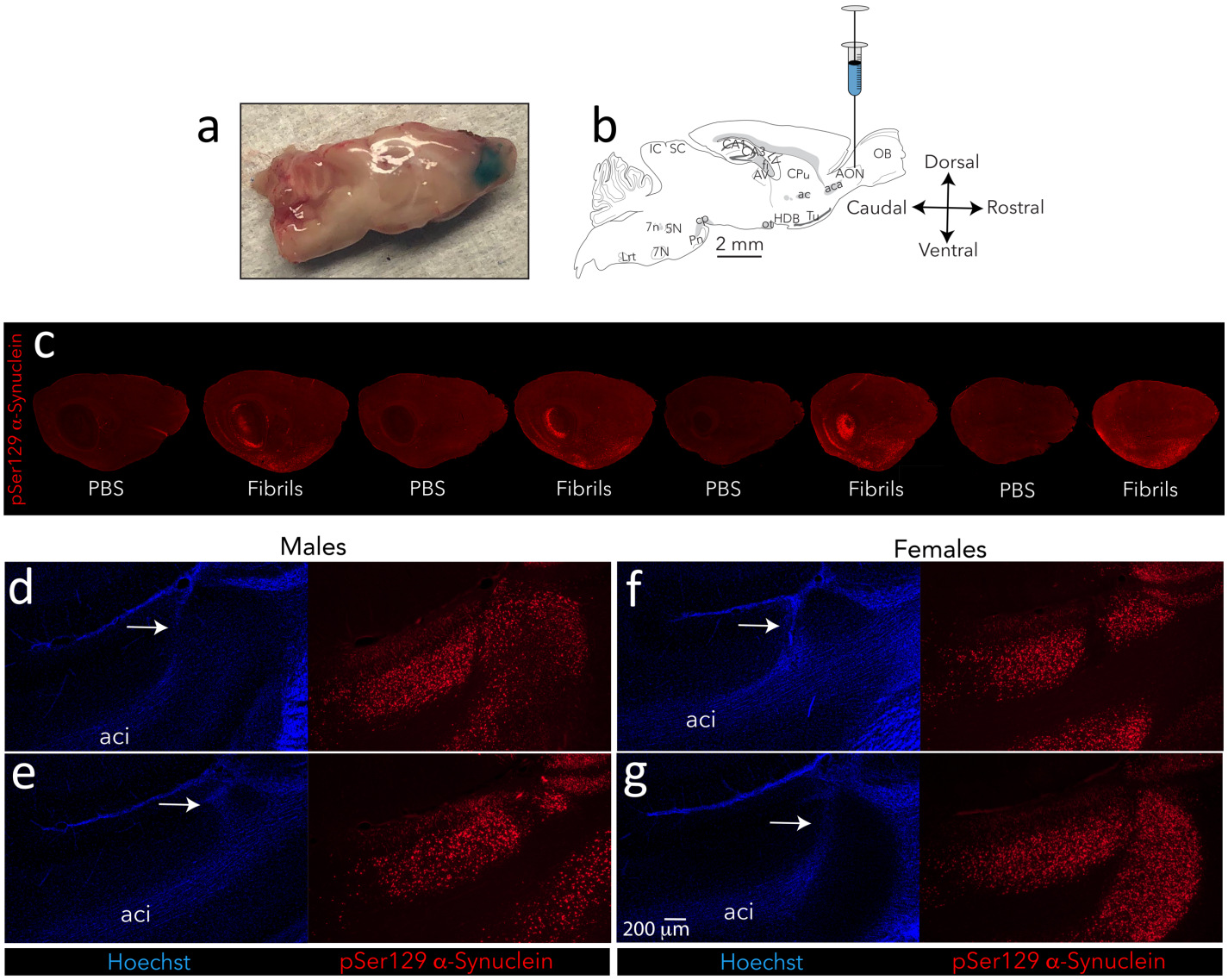


Figure S1: Verification of Rodent Injection Sites. Prior to commencing experiments on a rodent cohort, blue food dye was injected into spare animals of the same strain, bodyweight, age, and biological sex. (**a**) Medial view of freshly dissected CD1 mouse hemibrain immediately following *in vivo* stereotaxic infusions of 1.0 µL blue food dye through a burr hole in the skull. See illustration in **b** for anatomical orientation. The food dye has a substantial sphere of diffusion with an epicenter (darker blue) in the dorsal subdivision of the bulbar AON (roughly horseshoe shaped). Note that the sphere of diffusion varies depending on chemical structure, and therefore cannot be directly compared across reagents. Rather, it is the center of the infusion site that is important. Drawing in **b** is adapted from Figure 1A in Mason *et al.*, 2016 [10]. For abbreviations and AON subdivisions, please consult Supplemental File 1 of [10]. (**c**) Extension of main **Figure 1b**. The anterior olfactory nucleus (AON; dorsal subdivision) in the rear of the olfactory bulb (OB) was infused with 15 µg of sonicated, preformed fibrils in 3 µL of phosphate buffered saline (PBS) or an equivalent volume of vehicle in four-month-old male and female Sprague-Dawley rats. Four months later, one hemisphere was reserved for the biochemical assays in this report and the other hemisphere was immersion-fixed for histological processing of sagittal sections. Tyramide-amplified pSer129 immunostaining with clone EP1536Y is shown in panels **c-g**. Panel **c** was imaged at 5-micron resolution on the Odyssey M, which offers no scale bar. In panels **d-g**, pan-nuclear staining with the Hoechst reagent and pSer129^{+} structures in four different rats were captured on the Olympus IX73. Arrows in **d-g** point to the disturbance of tissue architecture and residual gliosis and/or necrosis from the needle track in the dorsal AON. The remainder of the OB is to the right of the images in **d-g**. The intrabulbar anterior commissure (aci) enters and bisects the AON at this sagittal level (~ 0.84 to 1.20 mm lateral from midline, per Figures 108 to 109 in Franklin and Paxinos' 2013 mouse brain atlas [5]).

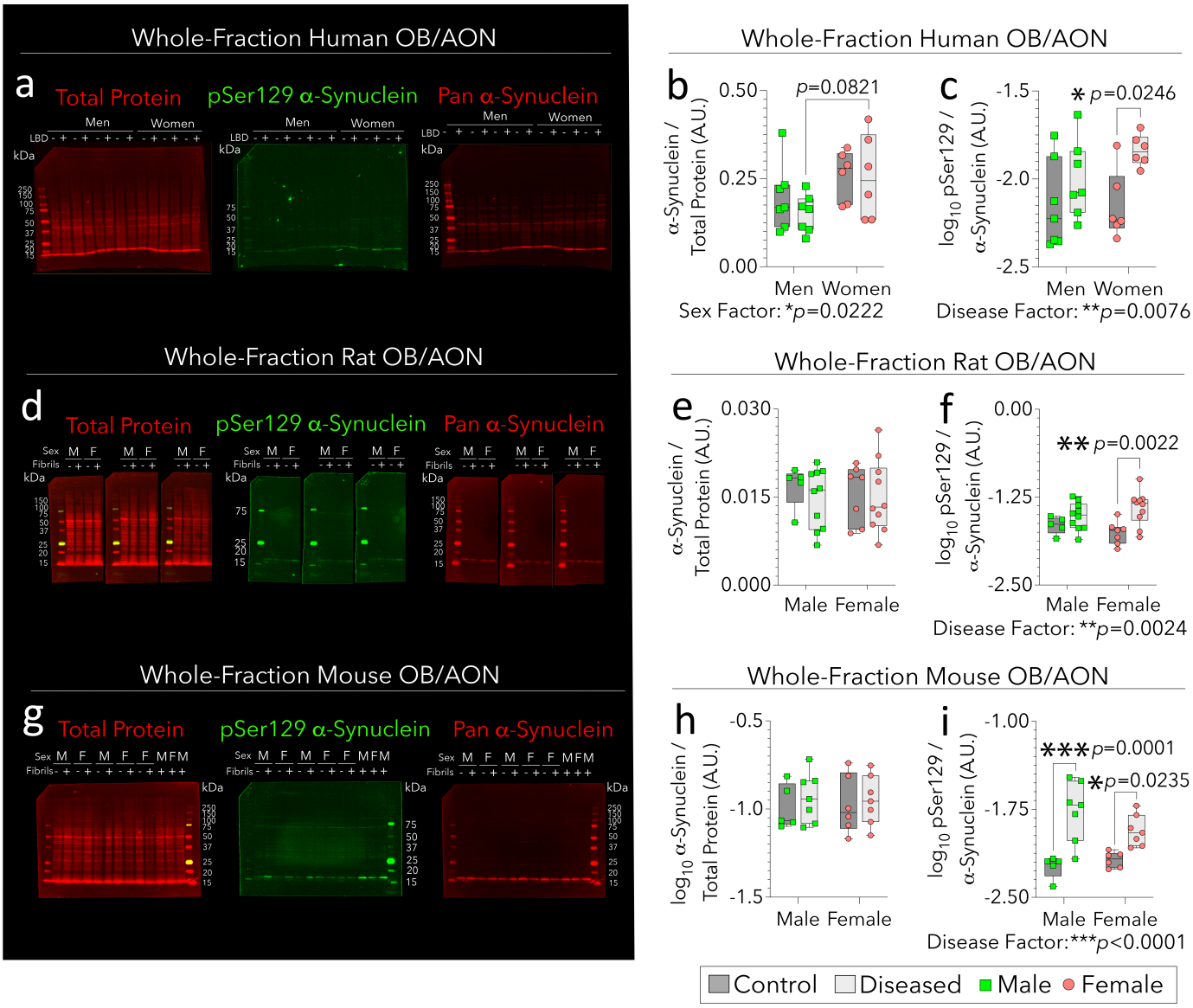


Figure S2: Whole-fraction bulbar α-synucleinopathy in humans, rats, and mice as a function of biological sex. (**a-c**) Postmortem olfactory bulb tissues from men and women with or without a diagnosis of Lewy body disorders were provided by the UCLA and University of Miami Brain Banks (demographics available in **Table S3** and dissection photos available in Figure S11 of Miner *et al.*, 2022 [12]). (**d-f**) Four-month-old male and female rats were infused bilaterally in the olfactory bulb/anterior olfactory nucleus (OB/AON) with sonicated α-synuclein fibrils (15 µg/hemisphere) or an equivalent volume of PBS (3 µL). After a four-month survival period, the OB/AON was dissected. (**g-i**) Eight-month-old male and female mice were infused bilaterally in the OB/AON with α-synuclein fibrils (5 µg/hemisphere) or an equivalent volume of PBS (1 µL). Mice were sacrificed three months later, and brains were dissected for OB/AON tissues. Pan α-synuclein and phosphorylated α-synuclein (pSer129) levels in human and rodent whole-fraction OB tissues were determined by SDS-PAGE/immunoblotting and expressed as a fraction of (**b, e, h**) total protein levels (REVERT 700 nm stain from LI-COR) or (**c, f, i**) pan α-synuclein, respectively. (**a, d, g**) Full-length immunoblots depicting total protein, pSer129, and pan α-synuclein in the human, rat, and mouse whole-fraction OB/AON. Remaining lanes on the blots in panel **d** were not part of this project and are thus not shown. Note that monomeric α-synuclein typically migrates at ~17 kDa in denaturing gels [16]. Boxplots illustrate the median and interquartile ranges, and every individual subject/animal is illustrated as a colored dot as the statistical unit. Non-Gaussian raw data are shown before $\log_{10}$-transformation in **Fig. S5-S7**. LBD = Lewy body disorder; A.U. = arbitrary units. Two-tailed, multiplicity-adjusted *p* values per two-way ANOVA/Bonferroni are shown above indicated comparisons. Main statistical effects are listed below respective graphs. No intervariable interactions were observed for disease *x* sex.

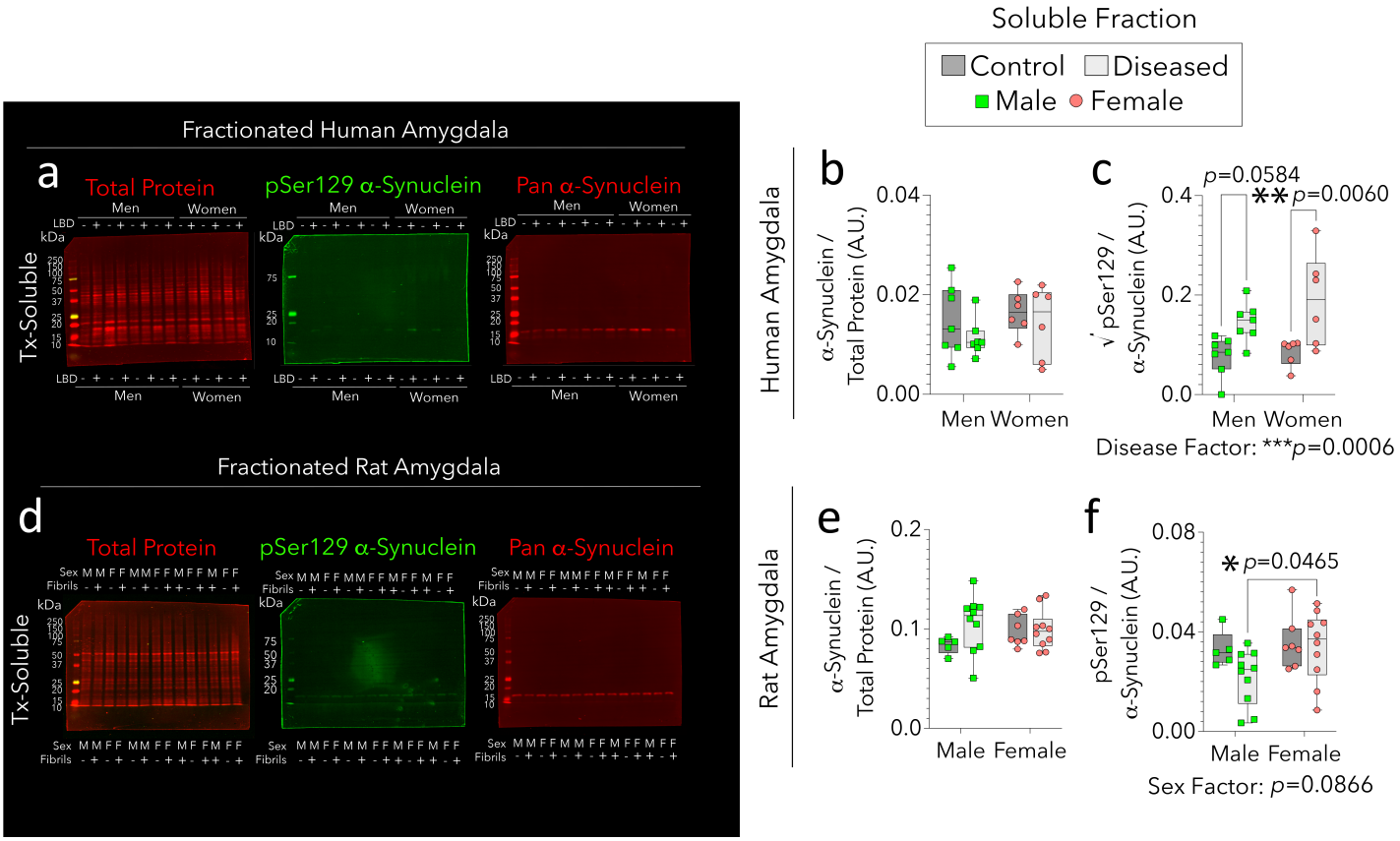
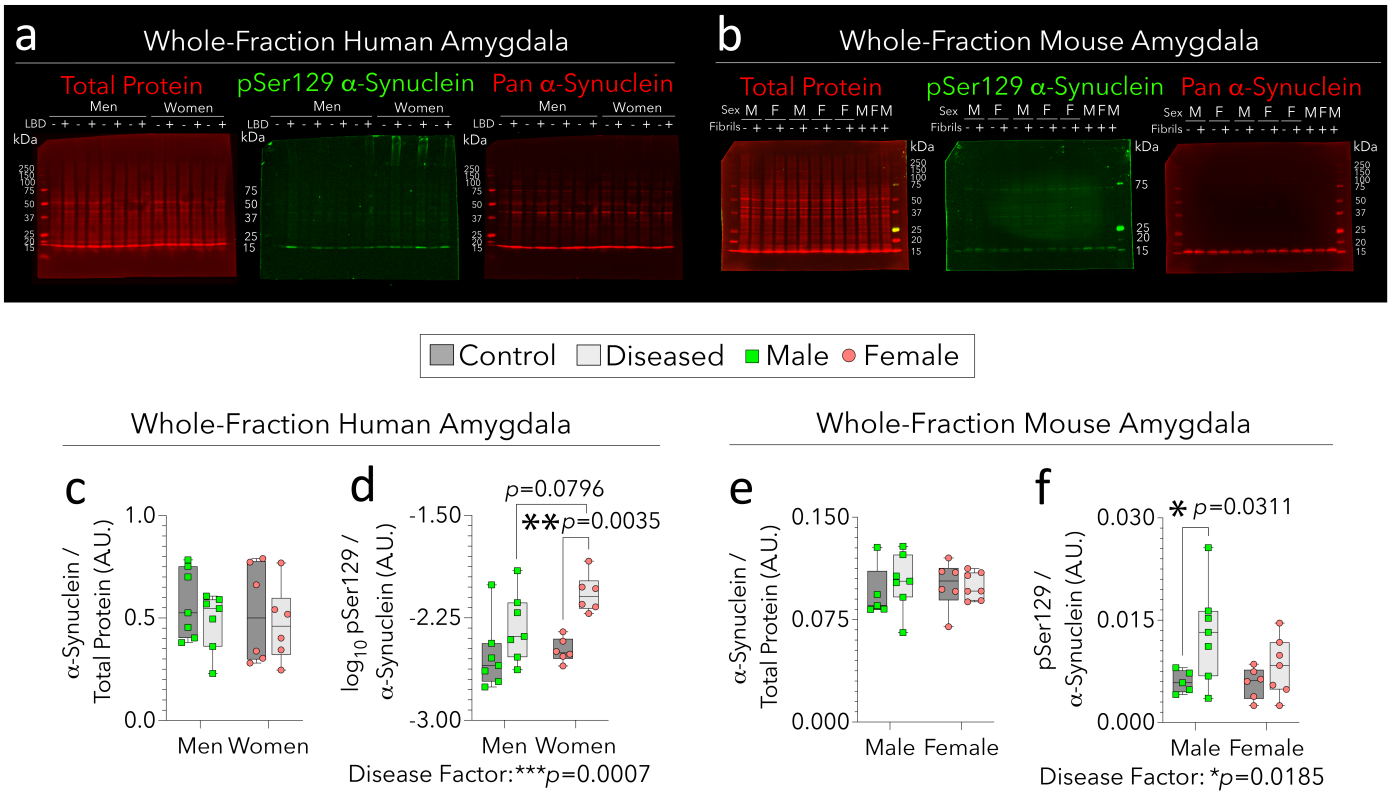


Figure S3: Nonionic detergent-soluble α-synuclein fraction in the amygdala of humans and outbred rats as a function of biological sex. (a-c) Postmortem amygdalar tissues from men and women diagnosed with or without Lewy body disorders were provided by the UCLA and University of Miami Brain Banks (demographics available in **Table S3**, and dissection photos available in Figure S11 of Miner *et al.*, 2022 [12]). (**d-f**) Four-month-old male and female rats were infused bilaterally in the olfactory bulb/anterior olfactory nucleus (OB/AON) with sonicated α-synuclein fibrils (15 µg/hemisphere) or an equivalent volume of PBS (3 µL). After a four-month survival period, the amygdala was dissected. Nonionic-detergent soluble fractions extracted from human and rodent tissues were probed for pan α-synuclein and phosphorylated α-synuclein (pSer129) by SDS-PAGE immunoblotting and expressed as a fraction of (**b, e**) total protein levels (REVERT 700 nm stain from LI-COR) or (**c, f**) pan α-synuclein, respectively. The soluble fraction of mouse tissues was highly diluted for ultracentrifugation and did not run well on SDS-PAGE after being processed through protein concentrators (not shown here). Non-Gaussian raw data are shown before $\log_{10}$ or square-root transformation in **Figures S5-S7**. A.U. = arbitrary units. Boxplots illustrate the median and interquartile ranges, and every individual subject/animal is illustrated as a colored dot as the statistical unit. Two-tailed, multiplicity-adjusted *p* values per two-way ANOVA/Bonferroni are shown above indicated comparisons. Main statistical effects are listed below respective graphs. No intervariable interactions were observed for disease *x* sex. Blots shown in this figure were captured at the same settings as the ones illustrated in main **Figure 1**, on the Odyssey M.



Figures S4: Whole-fraction amygdalar α-synucleinopathy in humans and outbred mice as a function of biological sex. (a, c-d) Postmortem limbic tissues from men and women with or without a diagnosis of Lewy body disorders were provided by the UCLA and University of Miami Brain Banks (demographics available in **Table S3**, and amygdala dissection photos available in Figure S11 of Miner *et al.*, 2022 [12]). (**c-d**) Pan α-synuclein and phosphorylated α-synuclein (pSer129) levels in human whole-fraction amygdalar tissues were determined by SDS-PAGE/immunoblotting and expressed as a fraction of (**c**) total protein levels (REVERT 700 nm stain from LI-COR) or (**d**) pan α-synuclein, respectively. (**b, e-f**) Eight-month-old male and female mice were infused bilaterally in the olfactory bulb/anterior olfactory nucleus (OB/AON) with sonicated α-synuclein fibrils (5 µg/hemisphere) or an equivalent volume of PBS (1 µL). After a three-month survival period, brains were dissected, and pan α-synuclein and pSer129 levels in whole-fraction amygdalar tissues were determined by SDS-PAGE/immunoblotting and expressed as a fraction of (**e**) total protein or (**f**) pan α-synuclein, respectively. (**a-b**) Full-length immunoblots depicting total protein, pSer129, and pan α-synuclein in the human and mouse whole-fraction amygdala. Note that monomeric α-synuclein typically migrates at ~17 kDa in denaturing gels [16]. Non-Gaussian raw data in **d** are shown before $\log_{10}$-transformation in **Fig. S5c**. LBD = Lewy body disorder; A.U. = arbitrary units. Boxplots illustrate the median and interquartile ranges, and every individual subject/animal is illustrated as a colored dot as the statistical unit. Two-tailed, multiplicity-adjusted *p* values per two-way ANOVA/Bonferroni are shown above indicated comparisons. Main statistical effects are listed below respective graphs. No intervariable interactions were observed for disease *x* sex.

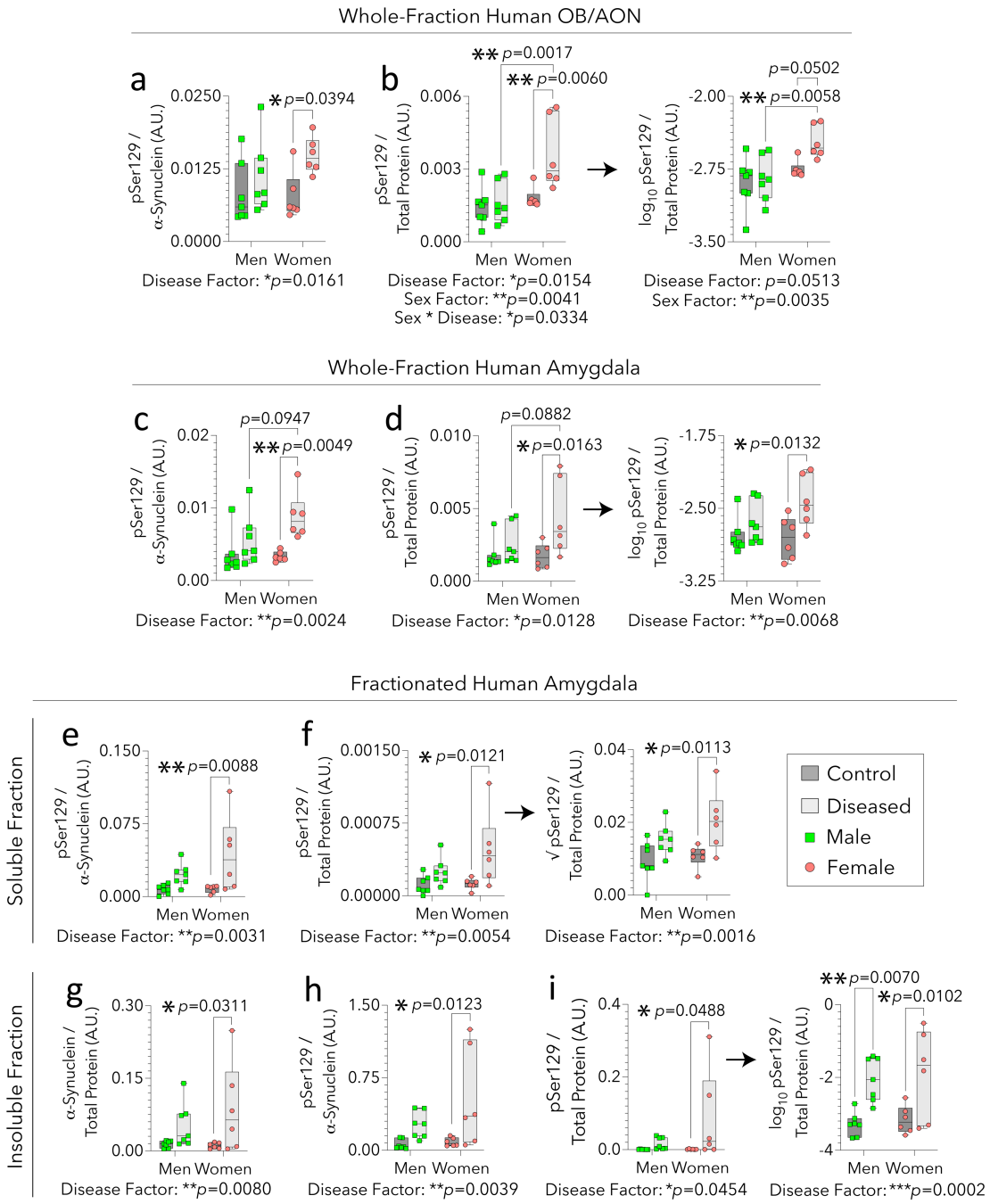


Figure S5: α-Synucleinopathy in whole and fractionated limbic tissues of humans as a function of sex. Postmortem limbic tissues from men and women with or without a diagnosis of Lewy body disorders were provided by the UCLA and University of Miami Brain Banks (demographics available in **Table S3**, and dissection photos available in Figure S11 of Miner *et al.*, 2022 [12]). Phosphorylated α-synuclein (pSer129) in human whole-fraction (**a-b**) OB/AON and (**c-d**) amygdalar tissues was determined by SDS-PAGE/immunoblotting and expressed as a fraction of (**a, c**) pan α-synuclein or (**b, d**) total protein levels (REVERT 700 nm stain from LI-COR). (**e-i**) Nonionic detergent-soluble and insoluble fractions extracted from amygdalar human tissues were probed for pan α-synuclein and pSer129 and expressed as a fraction of (**f, g, i**) total protein or (**e, h**) pan α-synuclein. Non-Gaussian data in **b, d, f** and **i** were log-transformed or square-root transformed (arrow). A.U. = arbitrary units. Boxplots illustrate the median and interquartile ranges, and every individual subject is illustrated as a colored dot as the statistical unit. Two-tailed, multiplicity-adjusted *p* values per two-way ANOVA/Bonferroni are shown above indicated comparisons. Main statistical effects and intervariable interactions for disease *x* sex are listed below respective graphs.

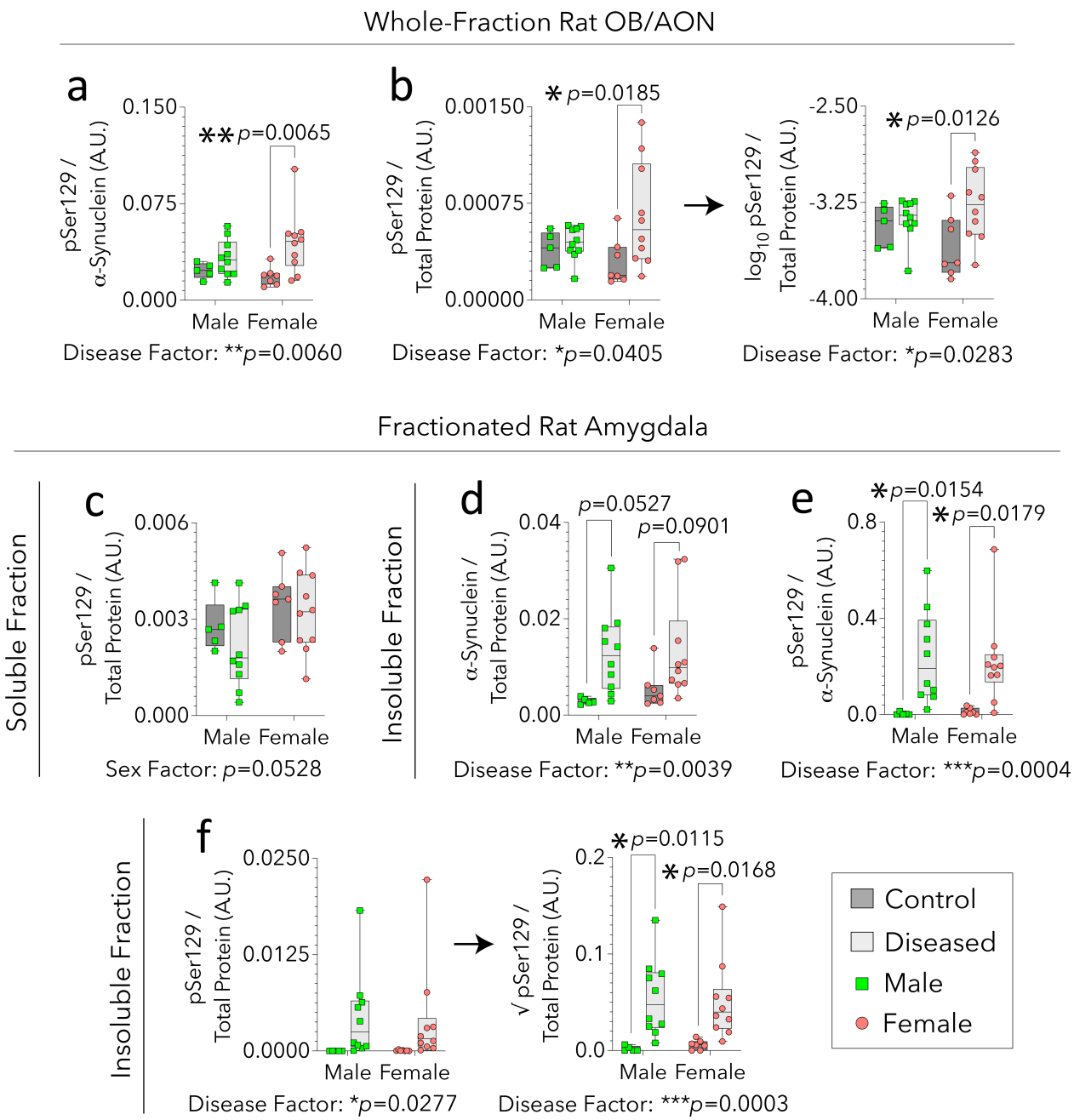


Figure S6: α-Synucleinopathy in whole and fractionated limbic tissues of outbred rats as a function of sex. Four-month-old male and female rats were infused bilaterally in the olfactory bulb/anterior olfactory nucleus (OB/AON) with sonicated, preformed α-synuclein fibrils (15 µg/hemisphere) or an equivalent volume of PBS (3 µL). (**a-b**) After a four-month survival period, brains were dissected, and phosphorylated α-synuclein (pSer129) levels in whole-fraction OB/AON tissues were determined by SDS-PAGE/immunoblotting and expressed as a fraction of (**a**) pan α-synuclein or (**b**) total protein levels (REVERT 700 nm stain from LI-COR). (**c-f**) Nonionic detergent-soluble and insoluble fractions extracted from amygdalar rat tissues were probed for pan α-synuclein and pSer129 and expressed as a fraction of (**c-d, f**) total protein or (**e**) pan α-synuclein. Non-Gaussian data in **b** and **f** were log-transformed or square-root transformed (arrow), respectively. A.U. = arbitrary units. Boxplots illustrate the median and interquartile ranges, and every individual subject/animal is illustrated as a colored dot as the statistical unit. Two-tailed, multiplicity-adjusted *p* values per two-way ANOVA/Bonferroni are shown above indicated comparisons. Main statistical effects are listed below respective graphs. No intervariable interactions were observed for disease *x* sex.

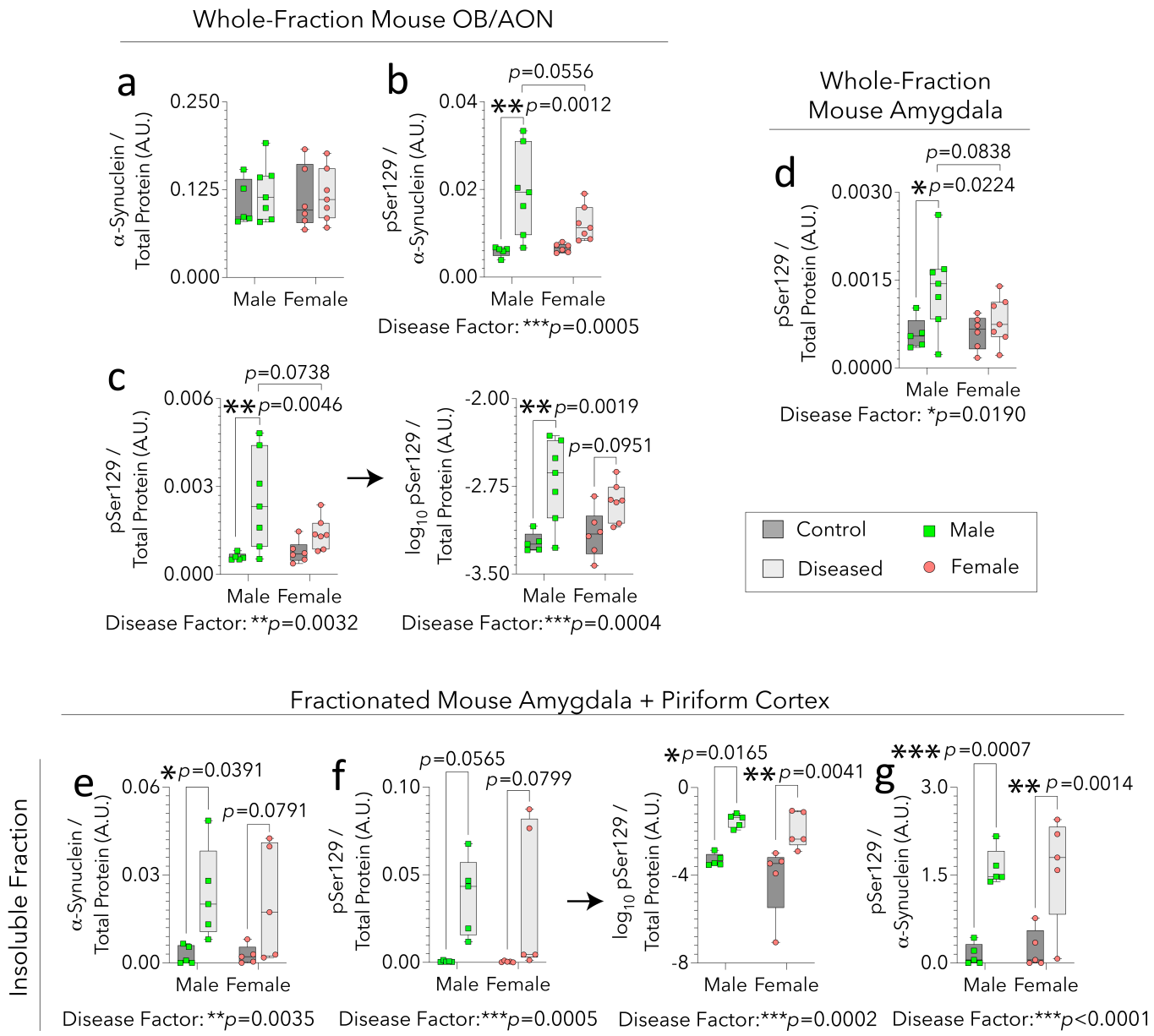


Figure S7: α-Synucleinopathy in whole and fractionated limbic tissues of outbred mice as a function of sex. Eight-month-old male and female mice were infused bilaterally in the olfactory bulb/anterior olfactory nucleus (OB/AON) with sonicated, preformed α-synuclein fibrils (5 µg/hemisphere) or an equivalent volume of PBS (1 µL). (**a-d**) After a three-month survival period, brains were dissected, and pan α-synuclein and phosphorylated α-synuclein (pSer129) levels in whole-fraction OB/AON and amygdalar tissues were determined by SDS-PAGE/immunoblotting and expressed as a fraction of (**b**) pan α-synuclein or (**a, c-d**) total protein levels (REVERT 700 nm stain from LI-COR). (**e-g**) Male and female mice were infused bilaterally in the OB/AON with sonicated α-synuclein fibrils (5 µg/hemisphere) or PBS (1 µL) at 9-11 months of age. Six weeks later, soluble and nonionic detergent-insoluble fractions were extracted from the amygdala combined with the piriform cortex (see Supplemental Methods), probed for pan α-synuclein and pSer129, and expressed as a fraction of (**e, f**) total protein or (**g**) pan α-synuclein. The soluble fraction of these mouse tissues was highly diluted for ultracentrifugation and did not run well on SDS-PAGE after being processed through protein concentrators (not shown here). Non-Gaussian data in **c** and **f** were $\log_{10}$-transformed (arrow). A.U. = arbitrary units. Boxplots illustrate the median and interquartile ranges, and every individual subject/animal is illustrated as a colored dot as the statistical unit. Two-tailed, multiplicity-adjusted *p* values per two-way ANOVA/Bonferroni are shown above indicated comparisons. Main statistical effects are listed below respective graphs. No intervariable interactions were observed for disease *x* sex.

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