# Heat Shock Protein 70 as a Sex-Skewed Regulator of α-Synucleinopathy

## *Supplemental Files*

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# Supplemental Methods

1. **Primary neuron culture**: Primary hippocampal tissues from 0-2 day-old Sprague Dawley rats were harvested in ice-cold Hibernate A (HA; BrainBits, LLC., Springfield, IL). Tissue was washed twice in Hank's Balanced Salt Solution (HBSS; 14170-161, Invitrogen, Life Technologies) and incubated with 20 U/mL freshly prepared papain solution (LS 3126, Worthington Biochemical Corporation; Lakewood, NJ) at 37 °C and in 5% $CO_2$ for 30-60 minutes. Next, the tissue was incubated at room temperature with DNase solution (DN25, Sigma-Aldrich, St. Louis, MO), mechanically dissociated in 5 mL of HBSS, and filtered through a 40 μm nylon mesh cell strainer (22-363-547, Fisher-Scientific). Dissociated cells were then plated in NeuroBasal-A media with Glutamax (35-050-061, Fisher-Scientific), penicillin/streptomycin (15-140-122, Fisher-Scientific), B27 (17-504-044, Fisher-Scientific) and 10% fetal bovine serum (S11550H, Atlanta Biologicals), at a density of 60,000 cells per well in 96-well plates (3595, Corning, Corning, NY) or at a density of $2\times10^6$ cells per dish on 35 mm petri dishes (353001, Corning). Plates and dishes were coated with poly-D-lysine (66.7 μg/mL; P0899, Sigma-Aldrich) and laminin (6.7 μg/mL; 354232, Corning,) overnight prior to plating. After four hours, the plating media was exchanged for serum-free neuronal media (abovementioned plating media *without* the fetal bovine serum), as described (1).

2. **Immunocytochemistry, In-Cell Westerns, and Image Analyses**: On the day of assay, cells were fixed in 3% formalin (9990244, Fisher-Scientific) and 2% sucrose in 0.1 M phosphate buffer for 20 minutes at room temperature and washed three times (10 minutes per wash) with

10 mM phosphate-buffered saline (PBS). Nonspecific staining was impeded with 1) a 1:1 solution of LI-COR Odyssey block (927-40000, LI-COR Biosciences, Lincoln, NE) in 10 mM PBS, supplemented with 0.3% Triton X-100, for 30 minutes, or 2) the Intercept Blocking Buffer (927-70003, LI-COR Biosciences) supplemented with 0.3% Triton X-100, for 30 minutes. Next, cells were incubated overnight at 4 °C with the primary antibodies listed in **Table S1**, diluted in PBS and blocking buffer. On the following day, cells were washed three times with 10 mM PBS and incubated for 60 minutes with infrared secondary antibodies (for In-Cell Westerns) and visible-wavelength secondary antibodies (for microscopic analyses), as listed in **Table S2**. For nuclear staining, the marker DRAQ5 (0.5 μM; 62251, Thermo Scientific) or Hoechst 33258 (bisBenzimide, 5 μg/mL; B1155, Sigma-Aldrich) was applied in parallel with the secondary antibodies. Unbound secondary antibodies were washed off with three exchanges of 10 mM PBS, and the immunostained cells were scanned on an Odyssey infrared imager (9201-01, Odyssey Classic, LI-COR Biosciences). In-Cell Western analyses were then performed using Image Studio Lite Ver 5.2 software (LI-COR Biosciences). Higher-resolution images (including z-stacks) were captured with 20× and 40× objectives on an Olympus epifluorescent microscope (Olympus IX73, B&B Microscopes, Pittsburgh, PA). Z-stack images were deconvoluted using the Olympus cellSens software.

Experimental/control groups were processed in the same solutions and analyzed by blinded observers at identical settings. Omission of primary antibodies led to the expected loss of signal. Images were quantified in a blinded manner using the 'manual threshold' command

under the Count and Measure function on the cellSens software to record the numbers of Hoechst$^+$ cells or pSer129$^+$ inclusions. The same threshold values were maintained for all images from each experiment by the blinded observer. The Hoechst 33258 and DRAQ5 reagents stain all attached, formalin-fixed and permeabilized cells, regardless of viability status—as is evident from fragmented, dying profiles that are small in size and display highly condensed, intensely-fluorescent chromatin, although these are very few in number (2-5). Because our cell counts must reflect *viable* cells, our measurements exclude these dying Hoechst$^+$ nuclei with fragmented/condensed chromatin (<20 μm$^2$ in area, >600 in mean gray intensity). Cells found in large clumps that cannot be accurately resolved into individual cells (*i.e.,* >500 μm$^2$ in area) by the 'auto split' command in cellSens are also excluded. In the graphs, area fraction of inclusions, as determined in cellSens software, is described as "total area occupied by inclusions" to distinguish it from "average inclusion size".

To test the uptake of α-synuclein fibrils into neurons in male *vs.* female cultures, we used the 'manual threshold' command under the Count and Measure function to first generate ROIs, and cell clumps were split as described above. Next, the image for ATTO$_{647}$-conjugated α-synuclein fibrils from the same field of view was overlaid and thresholded to generate counts of total numbers of α-synuclein fibrils within the ROIs.

For studies testing the detergent-insoluble nature of pSer129$^+$ aggregates in our model, cells were fixed with formalin/sucrose (see above) in the presence of 1% Triton X-100 for 20 minutes at room temperature, followed by standard immunocytochemistry procedures,

as described above. Images were then captured on an epifluorescent microscope, as described above.

3. **Western Immunoblotting**: Whole cell lysates were scraped in 1× cell lysis buffer (9803S, Cell Signaling Technology, Danvers, MA), supplemented with 1% protease inhibitor cocktail (P8340, Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride (PMSF; P7626, Sigma-Aldrich), and 10 mM sodium fluoride (S7920, Sigma-Aldrich). Samples were sonicated for 20 pulses at 1 second each (Misonix Inc. Model XL2020 Farmingdale, NY) and protein content for the cell lysates was measured using the bicinchoninic acid assay (23225, Thermo Scientific). For immunoblotting on human postmortem samples, tissue was weighed and sonicated in 1× cell lysis buffer (20 µL buffer per mg tissue), as above. Samples were diluted in 4× Protein Loading Buffer (928-40004, LI-COR) supplemented with fresh β-mercaptoethanol. The cell lysis buffer that we use contains 1% Triton-X and we do not centrifuge lysates (with exception of data in **Fig. S3b-c**) in order to capture *all cell fractions* in SDS-PAGE. Equal amounts of protein were loaded onto polyacrylamide gels and transferred onto nitrocellulose (926-31092, LI-COR) or Immobilon-FL PVDF (IPFL0010, Millipore, Burlington, MA) membranes.

For total protein stain normalization, membranes were incubated for 90 s in the Revert 700 Total Protein Stain (926-11011, LI-COR) in methanol. Membranes were washed and imaged immediately on an Odyssey infrared imager. The Revert 700 Total Protein Stain was reversed using the Revert destaining solution, as per manufacturer instructions (926-11013, LI-COR). Next, membranes were washed three times in tris-buffered saline (TBS) and blocked for

60 minutes at room temperature in LI-COR Odyssey block (diluted 1:1 with TBS) or in undiluted Intercept Blocking Buffer (927-60003, LI-COR Biosciences). Membranes were incubated overnight at 4 °C with primary antibodies (**Table S1**) diluted in blocking buffer and TBS supplemented with 0.1% Tween-20. On the next day, unbound primary antibodies were washed off and membranes were incubated in secondary antibodies (listed in **Table S2**), diluted in the same solution as the primary antibodies, for 60 minutes at room temperature. Following six consecutive washes with TBS and 0.1% Tween-20, membranes were scanned on an Odyssey infrared imager and the fluorescent signal was quantified using Image Studio Lite Ver 5.2 (LI-COR Biosciences).

Phosphorylated and pan-α-synuclein was assessed by immunoblotting lysates from vehicle or fibril-treated mixed-sex cultures (**Fig. S3b-c**) that had been sequentially extracted, according to the method of Volpicelli-Daley *et al*. (1). Briefly, cells were scraped in 1× cell lysis buffer (containing 1% Triton-X 100), as above. Next, lysates were sonicated using a probe-sonicator as above, incubated on ice for ~30 minutes, and centrifuged at 100,000 × *g* at 4 °C for 30 minutes (Optima MAX-XP Ultracentrifuge, Beckman Coulter Life Sciences, Indianapolis, IN). The supernatant was collected as the Triton-X soluble fraction and the pellet was resuspended in fresh 1× cell lysis buffer (with 1% Triton-X 100). Following a second centrifugation at 100,000 × *g* at 4 °C for 30 minutes, the supernatant was collected as the 'Triton-X wash'. We did not observe any pSer129 or α-synuclein immunoreactivity in the wash fraction (not shown).

The pellet obtained after the second centrifugation was resuspended in TBS supplemented with 2% SDS and protease and phosphatase inhibitor cocktails, sonicated, and loaded as the Triton-X insoluble fraction. Proteins were subjected to SDS-PAGE, transferred onto PVDF membranes, and incubated with fixative containing 4% paraformaldehyde (9990244, Fisher-Scientific) and 0.01% glutaraldehyde (G7651, Sigma-Aldrich) in PBS on a shaker at room temperature for 30 minutes (6, 7). Standard immunoblotting procedures were then followed, as above.

4. **Perfusions & Sacrifice:** At indicated time-points post-surgery, anesthetized mice were intracardially perfused through the left ventricle with 50 mL of 0.9% saline supplemented with 20 units/mL heparin sodium salt (H3393, Sigma-Aldrich, St. Louis, MO), followed by 100 mL of 4% formalin (9990244, Thermo Scientific) in 0.1 M phosphate buffer. For the studies in **Fig. 7a-h** we began with 20 male mice, all receiving injections of α-synuclein fibrils in the OB/AON at 20-months of age, after which 10 mice received intranasal infusions of SSA1 (the yeast homologue of Hsp70) and the remaining 10 mice received intranasal PBS infusions, beginning 24 h after fibril-injections. Two mice from the PBS group and one mouse from the SSA1-infused group died prematurely; therefore, only 17 mice were perfused.

For the studies in **Fig. 7i-k**, we began with 31 (all 5-month-old) male mice, out of which 7 mice received PBS injections in the OB/AON and the remaining 24 mice received injections of sonicated α-synuclein fibrils. Three mice developed dermatitis and/or an eye infection and had to be sacrificed prior to the COVID-19 pandemic lockdown; thus, unless otherwise stated,

a final count of 28 mice were available for the behavior testing in **Fig. 7i-k**, which were completed after our labs reopened following the pandemic lockdown. Note that there were no new eHsp70 exposures prior to collecting behavior data in **Fig. 7i-k**.

5. **Behavioral Analyses**: The buried/exposed pellet test was performed to assess olfaction in **Fig. 7c-d**. On the first day of this test, the latency to contact an exposed peanut placed on top of the bedding was measured, after allowing the mice to habituate to the testing arena (8). On the next day, the latency to contact an invisible peanut buried ~1 cm below the bedding was measured. No animals displayed a lack of motivation to contact the exposed peanut. Behavior videos from the exposed and buried pellet tests were analyzed by two independently trained, blinded observers and the combined average values from both observers are shown in **Fig. 7c-d**. Data from two animals on the buried pellet test had to be excluded due to technical problems with the video recording for one of the animals (as noted by both blinded observers) and the presence of leftover food on top of the bedding from the exposed pellet trial for the other animal (noted by 'observer 1'). As mentioned in the section on perfusions & sacrifice (above), three additional animals in this cohort had died by the time the olfaction test was performed.

The odor habituation/dishabituation test (8-10) in **Fig. 7k** was performed by first placing a small empty stainless-steel tealeaf holder in the animal's cage for 24 h prior to the test. On the day of testing, we measured the time spent contacting the same scented (lemon essence) cotton ball across 6 repetitive trials (50s each). The mice received a rest period of 5

mins in between each trial. We defined the duration of 'contact' as the time during which the animal's nose was oriented toward the scented tealeaf holder and was positioned less than ~1 cm from the tealeaf holder, but not if the animal, for example, tried to climb on top of the holder. Next, we measured the time spent contacting the essence in a 7$^{th}$ trial in which a novel (banana essence) odor was introduced. No animals were excluded from this test by the blinded observer.

Cognitive dysfunction in **Fig. 7j** was tested by the forced alternation test and by measuring the time spent in the novel arm of the Y-maze apparatus (11). Briefly, one arm of the Y-maze was blocked, and mice were first allowed to explore the remaining two arms for 5 mins in a 'sample trial'. After a rest period of 30 mins, mice were reintroduced into the Y-maze and allowed to explore all 3 arms in a 'retrieval trial'. Time spent in the novel vs. familiar arms within the first two minutes of the retrieval trial was measured on AnyMaze, as previous reports suggested that this timeframe has greater sensitivity to spatial preference for the novel arm (12, 13). One mouse that received intracerebral PBS infusions made less than three arm entries in the first minute of the retrieval trial and was excluded according to criteria described by others (11). To assess anxiety-related behaviors in **Fig. 7i**, animals were placed in an open field arena for 8 minutes and entries into the central vs. peripheral squares and distance traveled were analyzed as described by others (10, 14, 15), using AnyMaze software.

6. **Immunohistochemistry and histological analyses**: After cardiac perfusions with fixative, mouse brains were extracted, post-fixed in 4% formalin for 2 h, and then incubated for at least

48 h in 30% sucrose in 10 mM PBS. Next, brains were cut in the sagittal plane on a freezing microtome, collected as a 1-in-5 free-floating series, and stored in cryoprotectant at -20 °C (16). Cryoprotected tissue sections were washed thoroughly with three exchanges of 10 mM PBS and incubated in a 1:1 solution of LI-COR Odyssey block in 10 mM PBS with 0.3% Triton X-100 for 60 mins. Subsequently, tissue sections were incubated overnight at 4 °C with primary antibodies (diluted in the same blocking solution) listed in **Table S1**. The next day, sections were washed three times with PBS (10 mins per wash) and incubated for 60 minutes with the appropriate visible and/or infrared wavelength secondary antibodies, as listed in **Table S2**. After washing off the secondary antibodies with PBS, tissue sections were mounted on glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA) and coverslipped using Krystalon mounting medium (23-750008, Thermo Scientific). Images were captured on the Olympus epifluorescent microscope. Omission of primary antibodies led to expected loss of signal. For microscopic analyses in **Fig. 7**, all experimental/control tissues were processed by blinded investigators in the same solutions and analyzed at identical settings, in all sections that contained the horseshoe-shaped bulge of the AON into the rear of the olfactory bulb (2-4 sections in the random 1-in-5 sagittal section series). Blinded observers manually counted numbers of pSer129$^{+}$ inclusions (using the cell counter in ImageJ). Hoechst and NeuN counts were recorded using the threshold feature on the cellSens software, as described above. For these *in vivo* viability measurements, we excluded all thresholded objects that displayed areas below 20 μm$^2$ after auto-splitting cell clumps, as specified in the section on immunocytochemistry. For the initial staining shown in **Fig. 7e-g**, the OBs of two mice were

lost during processing. Further, the brain sections from three additional mice displayed uneven coverage by TrueBlack (23007, Biotium), a quencher of lipofuscin autofluorescence. Hence, the final count of the animals used for the histological data in **Fig. 7e-g** is 12. We repeated the immunostaining on another bin from the 1-in-5 sagittal series for the microglia activation marker, Iba1. For this latter immunostaining, we had a final count of 17 mice available as we did not employ TrueBlack and there were multiple sections containing the OB/AON available for the histological analyses. For the analyses shown in **Fig. 7h**, sections were scanned on the Odyssey infrared imager (9201-01, Odyssey Classic, LI-COR Biosciences) at 21 μm resolution, as also described in our prior work (17). Next, a blinded observer traced the boundaries of the OB/AON using Image Studio Lite Ver 5.2 (LI-COR Biosciences) and the levels of Iba1 signal were expressed as a function of the area of the traced region.

# Supplemental References


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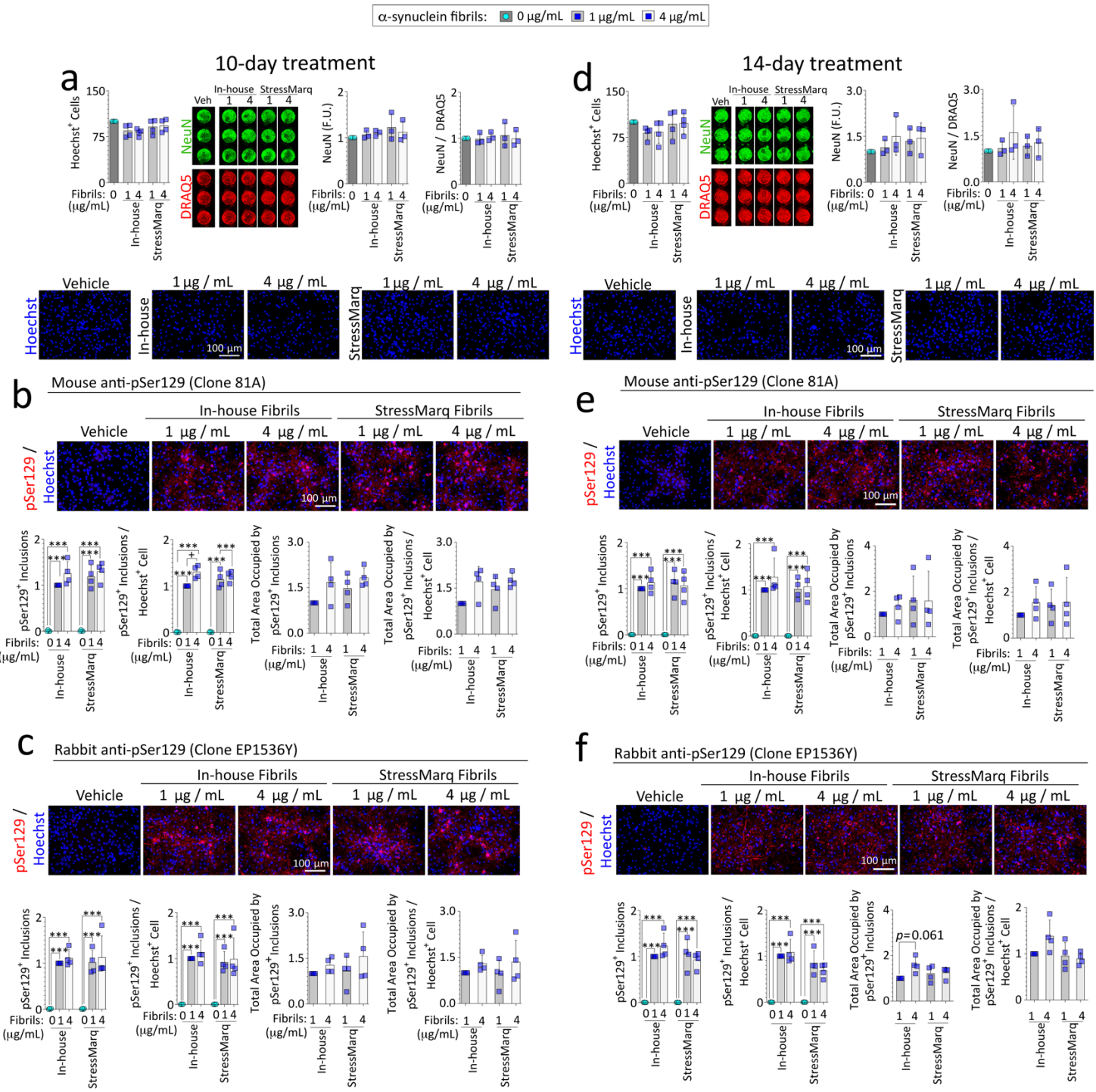


**Fig. S1 Preformed α-synuclein fibril exposure elicits dense inclusions without cell loss in mixed-sex primary hippocampal cultures by 10 or 14 days.** Primary hippocampal cultures were exposed to 0, 1, or 4 µg/mL α-synuclein fibrils, synthesized at the University of Pennsylvania or procured from StressMarq Biosciences. Cultures were assayed 10 **(a-c)** or 14 days **(d-f)** after treatment initiation, on day-in-vitro 12 or 16, respectively. Counts of Hoechst⁺ cells **(a, d)** reveal no changes attributable to α-synuclein fibril source, concentration, or incubation period. Expression of neuron marker NeuN was analyzed by In-Cell Western analyses as a fraction of pan-nuclear DRAQ5 signal intensities **(a, d)**. To assess pSer129⁺ inclusion numbers, cultures were immunostained with two independent monoclonal antibodies in a side-by-side manner and imaged at the same settings: Clone 81A (BioLegend Ab 825701; **b, e**) and clone EP1536Y (Abcam ab51253; **c, f**). Blinded counts of pSer129⁺ inclusions and total area occupied by pSer129⁺ inclusions are shown alone or expressed as a fraction of Hoechst⁺ cell numbers **(b-c, e-f)**. Representative photomontages of pan-nuclear marker Hoechst (blue) and pSer129⁺ inclusions (red) are shown. Shown are mean + SD of 4 independent experiments, each in triplicate wells. *** $p \leq 0.001$ for 0 vs. 1 or 4 µg/mL α-synuclein fibrils; + $p \leq 0.05$ for 1 vs. 4 µg/mL α-synuclein fibrils, one-way ANOVA followed by Bonferroni *post hoc*

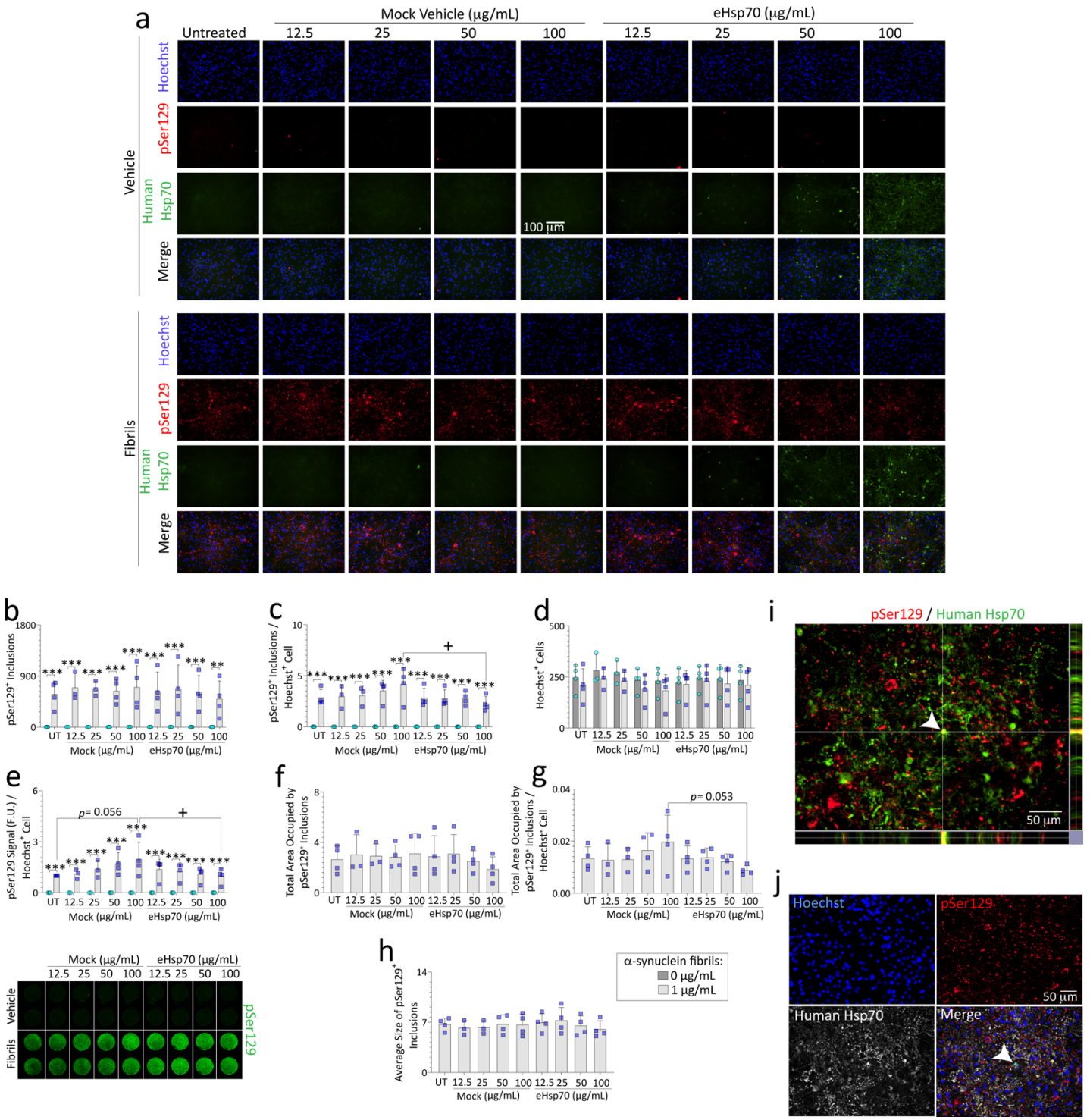


**Fig. S2 eHsp70 reduces pSer129⁺ inclusions in primary hippocampal cultures exposed to preformed α-synuclein fibrils.** Primary mixed-sex hippocampal cultures were exposed to 0 or 1 µg/mL α-synuclein fibrils for 10 d, and a mock-transfection vehicle control or exogenous Hsp70 (eHsp70) at 12.5, 25, 50, and 100 µg/mL. UT = untreated controls. Representative photomontage in **(a)** shows that the antibody raised against human Hsp70 protein does not cross-react with endogenous rodent Hsp70 in immunocytochemistry. Blinded measurements of pSer129⁺ inclusion counts are shown alone **(b)** or expressed as a fraction of Hoechst⁺ cell numbers **(c)**. Hoechst⁺ cell counts are in **d**. Total pSer129 protein was measured using In-Cell Westerns and expressed as a fraction of cell numbers **(e)**. The total area occupied by inclusions is shown alone **(f)** or as a fraction of Hoechst⁺ cell numbers **(g)**. The average size of inclusions is in **h**. **(i-j)** Representative z-stack images, all from the same field of view, of cells exposed in parallel to α-synuclein fibrils and 100 µg/mL eHsp70. The slice views in **i** show that eHsp70⁺ structures are largely pSer129-negative. Rare exceptions are indicated by arrowheads in **i-j**. The maximum intensity projection of the z-stack in **i** was deconvoluted in cellSens in **j**. Shown in **b-h** are the mean + SD of 3-4 independent experiments, each performed in triplicate wells. *** $p \leq 0.001$ for 0 vs. 1 µg/mL α-synuclein fibrils; + $p \leq 0.05$ for mock-transfection vehicle vs. eHsp70-treated cells; one or two-way ANOVA followed by Bonferroni *post hoc*

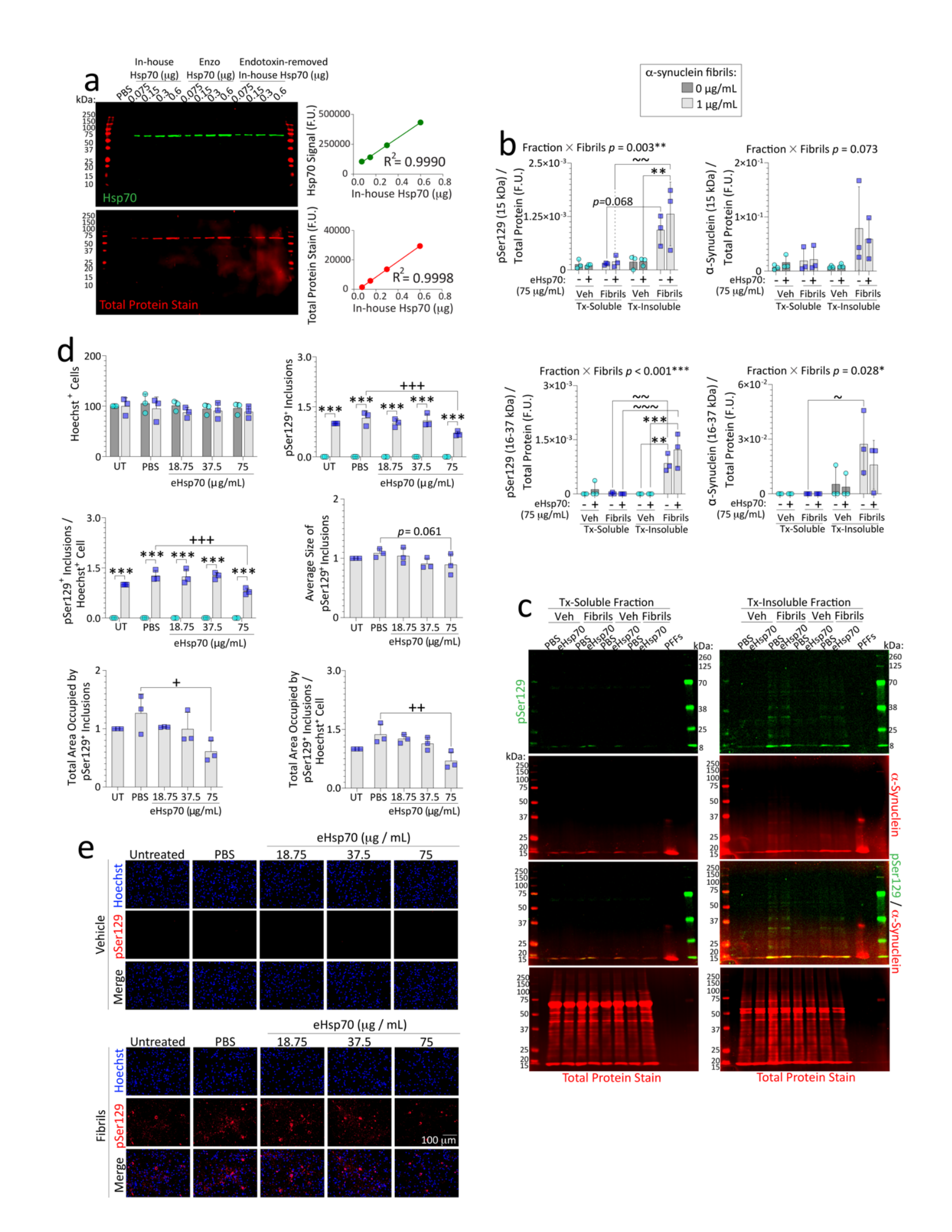
a
In-house Hsp70 (μg)
Enzo Hsp70 (μg)
Endotoxin-removed In-house Hsp70 (μg)
PBS 0.075 0.15 0.3 0.6 0.075 0.15 0.3 0.6 0.075 0.15 0.3 0.6
kDa:
Hsp70
Total Protein Stain
Hsp70 Signal (F.U.)
Total Protein Stain (F.U.)
R2= 0.9990
R2= 0.9998
In-house Hsp70 (μg)
α-synuclein fibrils:
0 μg/mL
1 μg/mL
b
Fraction × Fibrils p = 0.003**
Fraction × Fibrils p = 0.073
pSer129 (15 kDa) / Total Protein (F.U.)
α-Synuclein (15 kDa) / Total Protein (F.U.)
p=0.068
eHsp70: (75 μg/mL)
Veh Fibrils Veh Fibrils
Tx-Soluble Tx-Insoluble
Fraction × Fibrils p < 0.001***
Fraction × Fibrils p = 0.028*
pSer129 (16-37 kDa) / Total Protein (F.U.)
α-Synuclein (16-37 kDa) / Total Protein (F.U.)
c
Tx-Soluble Fraction
Tx-Insoluble Fraction
Veh Fibrils Veh Fibrils
PBS eHsp70 PBS eHsp70 PBS eHsp70 PBS eHsp70 PFFs
kDa:
pSer129
α-Synuclein
pSer129 / α-Synuclein
Total Protein Stain
d
Hoechst+ Cells
pSer129+ Inclusions
pSer129+ Inclusions / Hoechst+ Cell
Average Size of pSer129+ Inclusions
p= 0.061
Total Area Occupied by pSer129+ Inclusions
Total Area Occupied by pSer129+ Inclusions / Hoechst+ Cell
UT PBS 18.75 37.5 75
eHsp70 (μg/mL)
e
eHsp70 (μg / mL)
Untreated PBS 18.75 37.5 75
Vehicle
Fibrils
Hoechst
pSer129
Merge
100 μm

**Fig. S3 eHsp70 does not reduce nonionic detergent-insoluble α-synuclein levels, but diminishes pSer129$^+$ inclusions in primary hippocampal cultures exposed to preformed α-synuclein fibrils.** eHsp70 prepared in Brodsky lab (before and after endotoxin removal) or purchased from Enzo Life Sciences (certified endotoxin-low; < 50 EU/mg purified protein by the LAL test) was loaded on a gel. Immunoblotting was performed using the anti-human Hsp70 antibody and the Revert 700 Total Protein Stain **(a)** to test assay linearity and assess potential degradation products. **(b-c)** Primary mixed-sex hippocampal neuron cultures were exposed to 0 or 1 μg/mL α-synuclein fibrils for 10 d. In parallel, cells were also exposed to PBS or 75 μg/mL of the low-endotoxin eHsp70 preparation (Enzo). After 10 d of treatment, cell lysates were collected and subjected to centrifugation at 100,000 x *g* to obtain Triton-X soluble *vs.* insoluble fractions. Fractions were loaded on separate gels along with 0.15 μg of WT mouse α-synuclein preformed fibrils as a positive control **(b, c)**. Statistically significant intervariable interactions are written above graphs in **b**. Immunoblotting was performed using pSer129 and pan-α-synuclein antibodies **(b-c)**. Revert 700 Total Protein Stain was used as the loading control in **c**. **(d-e)** Primary mixed-sex hippocampal neuron cultures were exposed to 0 or 1 μg/mL α-synuclein fibrils for 10 d. In parallel, cells were also exposed to eHsp70 (Enzo). UT = untreated. Blinded measurements of Hoechst$^+$ cell numbers, pSer129$^+$ inclusion counts (expressed alone or as a fraction of Hoechst$^+$ cell numbers), average inclusion size, and total area occupied by inclusions (expressed alone or as a fraction of Hoechst$^+$ cell numbers) are shown in **d**. Representative photomontage of pSer129$^+$ inclusions and Hoechst-stained nuclei is shown in **e**. Shown are the mean + SD of 3 independent experiments. ** $p \leq 0.01$, *** $p \leq 0.001$ for 0 vs. 1 μg/mL α-synuclein fibrils; + $p \leq 0.05$, ++ $p \leq 0.01$, +++ $p \leq 0.001$ for vehicle vs. eHsp70; ~ $p \leq 0.05$, ~~ $p \leq 0.01$, ~~~ $p \leq 0.001$ for Tx-soluble vs. insoluble fraction, one, two, or three-way ANOVA followed by Bonferroni *post hoc*

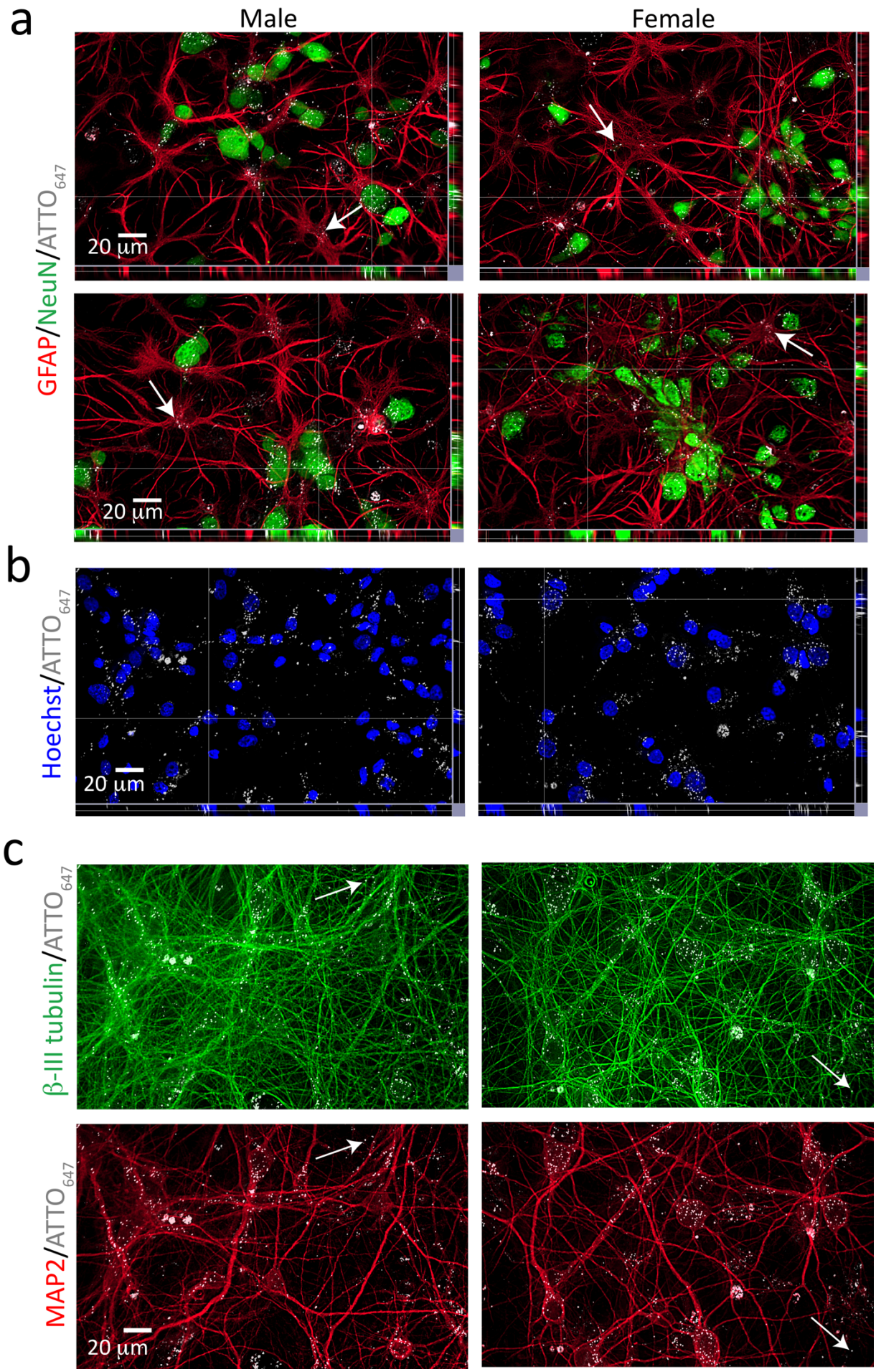
a
Male
Female
GFAP/NeuN/ATTO647
20 µm
20 µm
b
Hoechst/ATTO647
20 µm
c
β-III tubulin/ATTO647
MAP2/ATTO647
20 µm

**Fig. S4 Uptake of $ATTO_{647}$-conjugated α-synuclein fibrils by male and female primary hippocampal neurons.** Primary hippocampal neuron cultures harvested from male *vs.* female rat pups were exposed to 1 μg/mL of $ATTO_{647}$-conjugated α-synuclein fibrils for 4 d **(a-c)**. Representative photomontage in **a** shows preferential localization of $ATTO_{647}$-conjugated α-synuclein fibrils in $NeuN^+$ neurons. Arrows in **a** point to fewer instances of localization with $GFAP^+$ astroglia. Representative photomontage in **b** shows localization of $ATTO_{647}$-conjugated α-synuclein fibrils in $Hoechst^+$ nuclei of both male and female cultures. **(c)** Representative single frame extracted from z-stacks in **Fig. 2b**, with arrows pointing to $ATTO_{647}$-conjugated α-synuclein fibrils in those β-III $tubulin^+$ neurites that are MAP2-negative

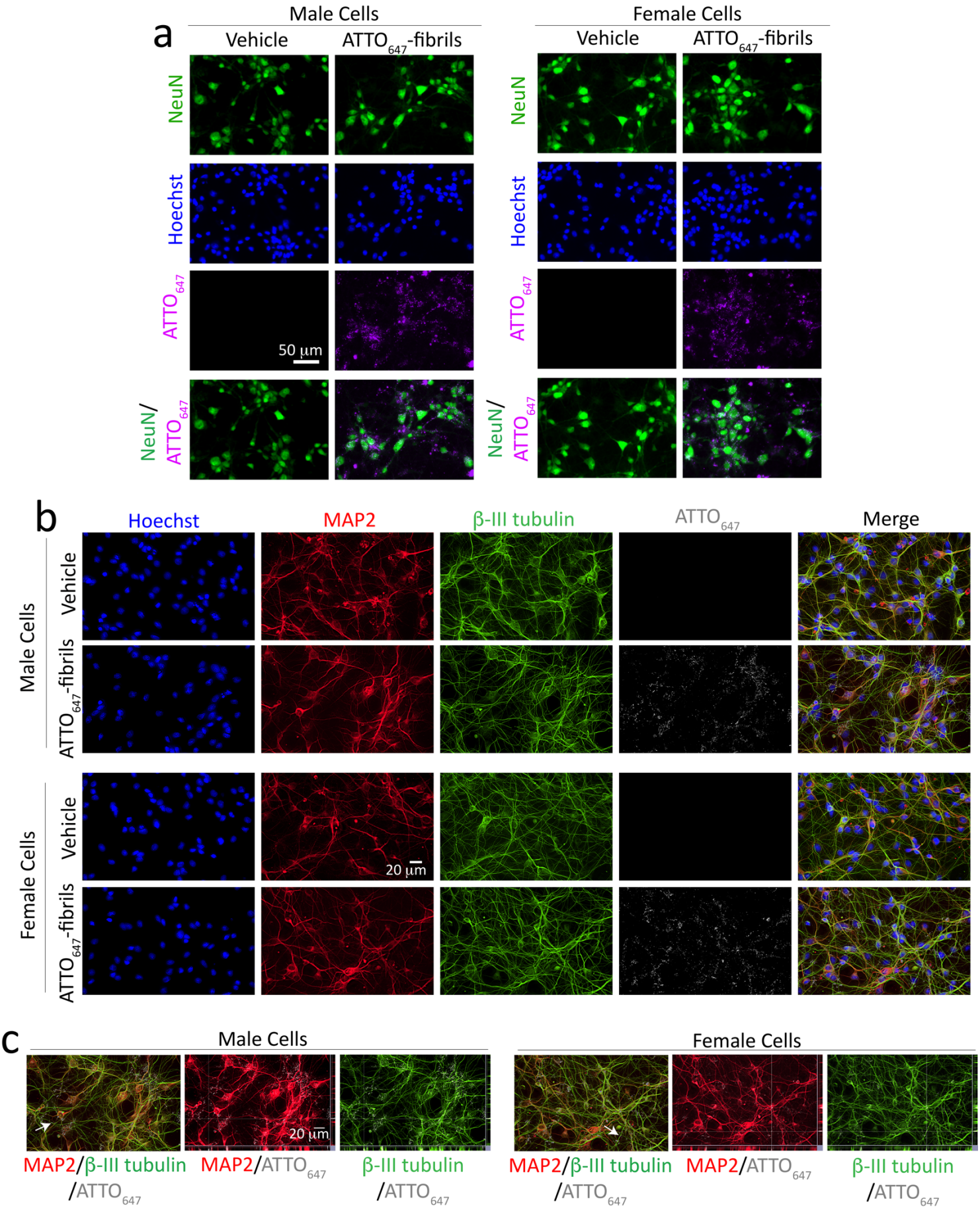


**Fig. S5 Uptake of ATTO$_{647}$-conjugated α-synuclein fibrils into male and female primary hippocampal neurons.** Primary hippocampal neuron cultures harvested from male *vs.* female rat pups were exposed to 0 or 1 µg/mL of ATTO$_{647}$-

conjugated α-synuclein fibrils **(a)** for 4 d. Representative photomontage in **a** shows $ATTO_{647}$-conjugated α-synuclein fibrils, $NeuN^+$ neurons, and $Hoechst^+$ nuclei, corresponding to data shown in **Fig. 2c**. Primary hippocampal cultures harvested from male *vs.* female rat pups were exposed to 0 or 1 μg/mL of $ATTO_{647}$-conjugated α-synuclein fibrils for 6 h. Representative photomontage in **b** shows $ATTO_{647}$-conjugated α-synuclein fibrils, $MAP2^+$ neurons, β-III $tubulin^+$ neurons, and $Hoechst^+$ nuclei. Arrows in the photomontage in **c** (same field of view as **b**) point to instances where $ATTO_{647}$-conjugated α-synuclein fibrils are present in those β-III $tubulin^+$ neurites that are MAP2-negative (MAP2 immunolabel was purposefully brightened in **c**)

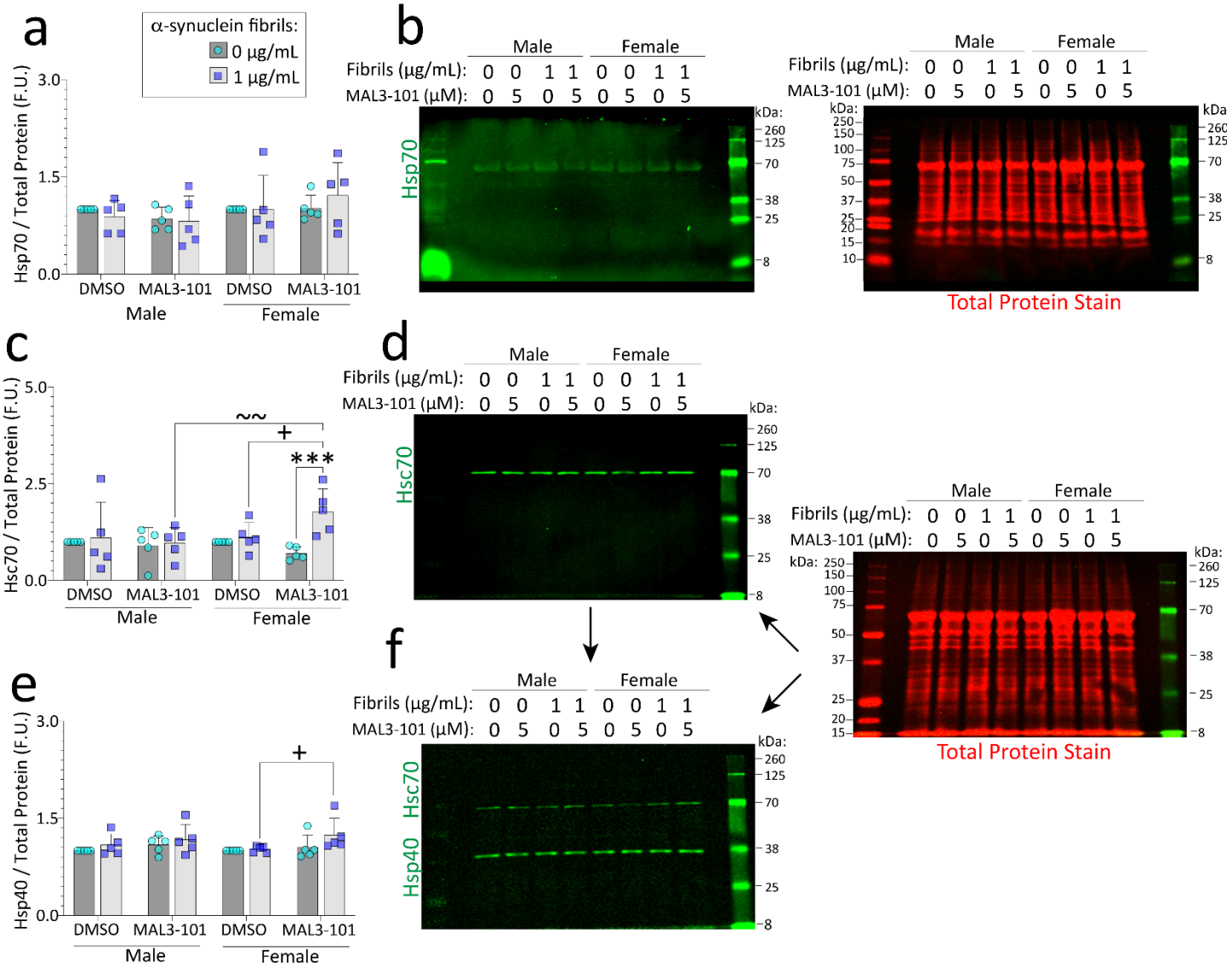


**Fig. S6 Inhibition of the chaperone function of endogenous Hsp70 elicits upregulation of Hsc70 and Hsp40 in female hippocampal cultures.** Primary hippocampal neuron cultures harvested from male *vs.* female rat pups were exposed to 0 or 1 µg/mL α-synuclein fibrils. In parallel, cells were also exposed to MAL3-101 (5 µM, **a-f**). Lysates were collected 72 h post-treatment and subjected to immunoblotting for Hsp70 **(a-b)**, Hsc70 **(c-d)**, and Hsp40 **(e-f)**. The quantification in the graphs is shown as a fold change of the values in the vehicle (DMSO-treated) wells. Full length-immunoblots are shown in panels **b**, **d**, and **f**. Revert 700 Total Protein Stain was used to correct for loading differences, and according to the manufacturer's instructions, was applied prior to blocking and incubation with primary antibodies. Note that the immunoblots in **d** and **f** were first probed for Hsc70 (shown in **d**) and then the same blot was re-probed for Hsp40 (not stripped; shown in **f**). Shown are the mean + SD of 5 independent experiments. *** $p \leq 0.001$ for 0 vs. 1 µg/mL α-synuclein fibrils; + $p \leq 0.05$ for 0 versus 5 µM MAL3-101; ~~ $p \leq 0.01$ for male versus female; three-way ANOVA followed by the Bonferroni *post hoc*

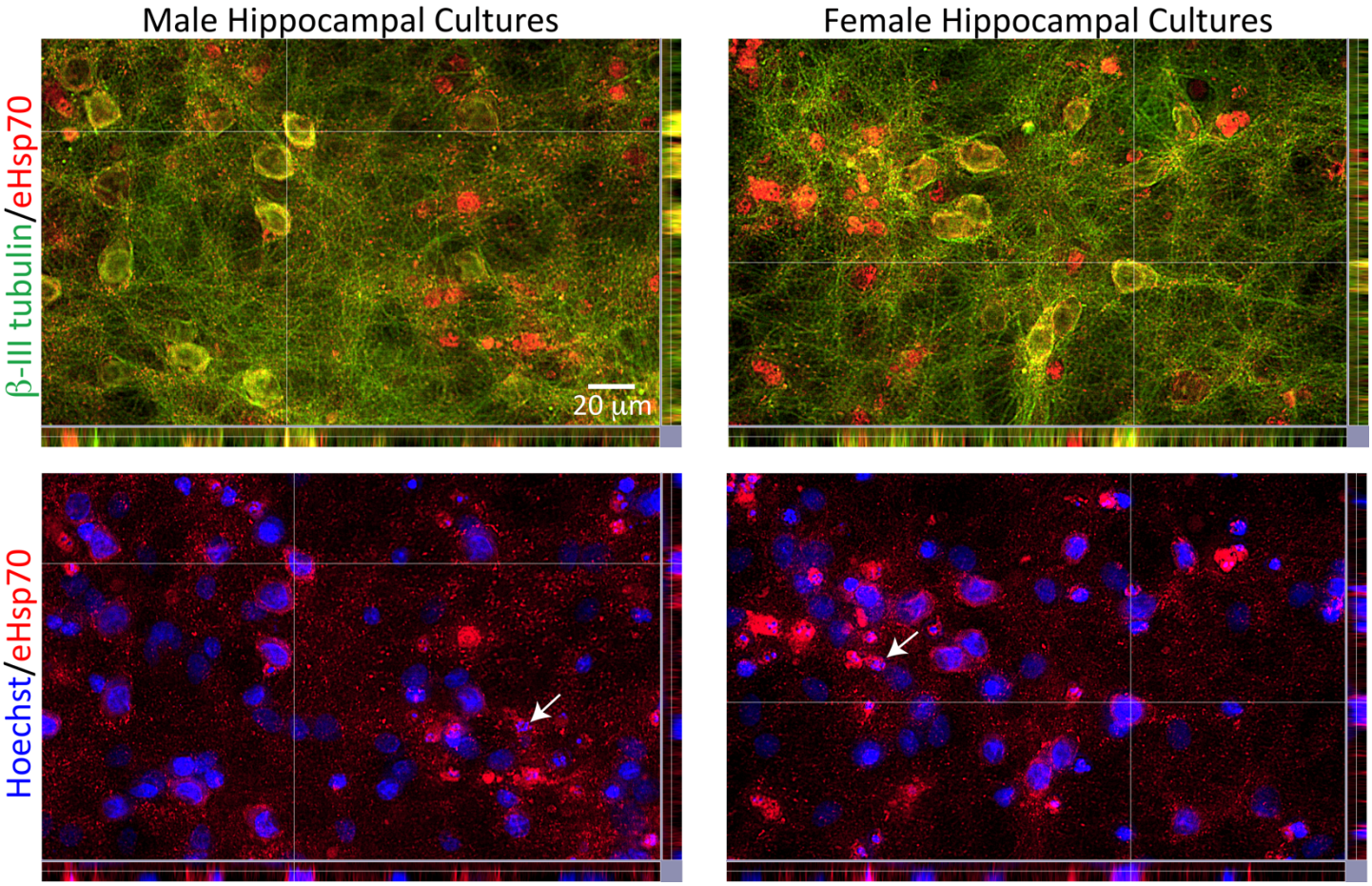


**Fig. S7 eHsp70 in male and female primary hippocampal cultures.** Primary hippocampal cultures harvested from male *vs.* female rat pups were exposed to preformed α-synuclein fibrils and eHsp70 for 10 d. Representative deconvoluted Z-stack slice shows eHsp70 in β-III tubulin$^+$ structures and surrounding Hoechst$^+$ nuclei in male and female cultures, including nuclei with shrunken appearance (arrows)

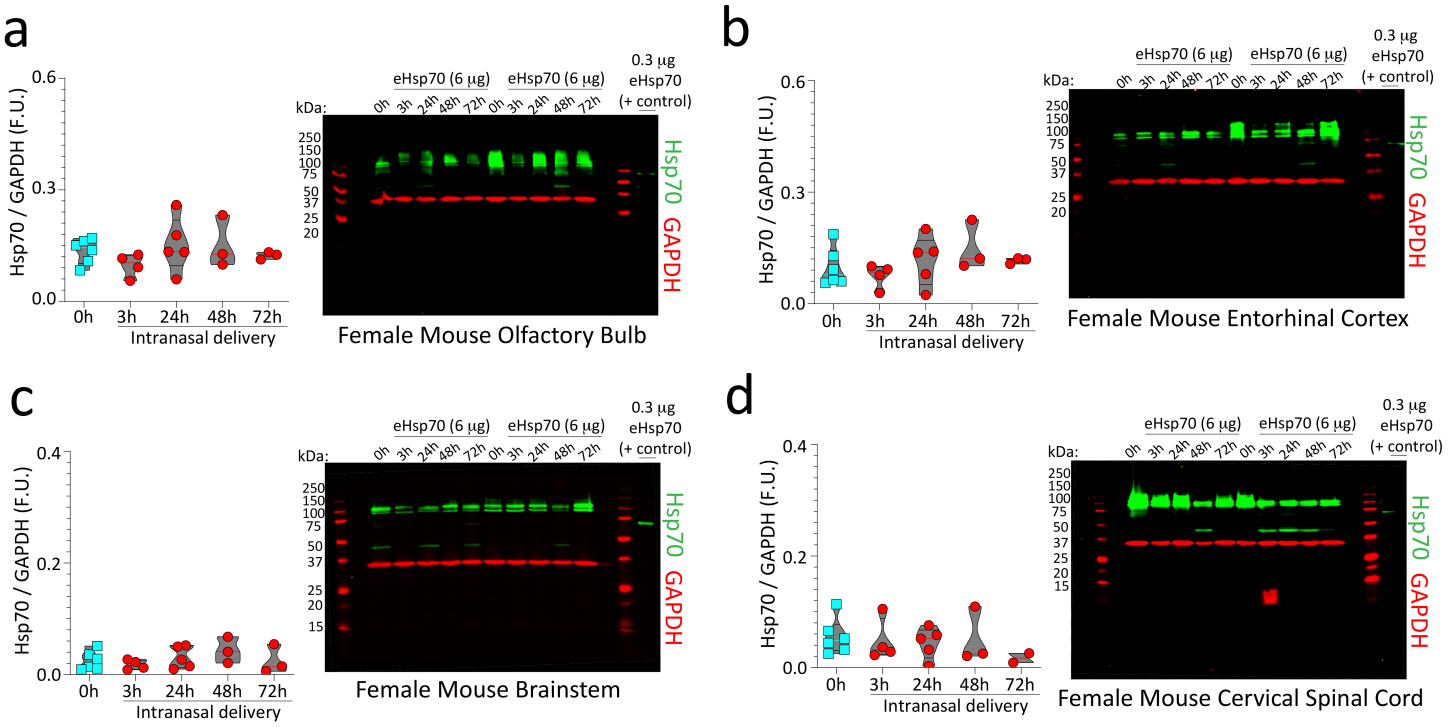


**Fig. S8 No evidence of eHsp70 uptake into the aged female mouse brain from the nasal cavities.** Aged (21-month-old) female mice were anesthetized with isoflurane and infused with PBS or low-endotoxin eHsp70 (6 µg; Enzo) behind the nares, after which the mice remained supine for 10 minutes and then returned to home cages to awaken. Brains were collected at 0 h (sham control), 3 h, 24 h, 48 h, and 72 h post-infusion and the olfactory bulb **(a)**, entorhinal cortex **(b)**, brainstem **(c)**, and cervical spinal cord **(d)** were subjected to SDS-PAGE and immunoblotting. As a positive control, recombinant eHsp70 protein was loaded in the last lane. Only the band at ~70 kDa was quantified in the violin plots. Higher bands may be aggregated eHsp70 or nonspecific, and the lower bands of ~44 kDa may be the cleaved ATPase domain. The spinal cord from one mouse in the 72 h group in **(d)** was lost during dissections. Data were analyzed by one-way ANOVA followed by the Bonferroni *post hoc*

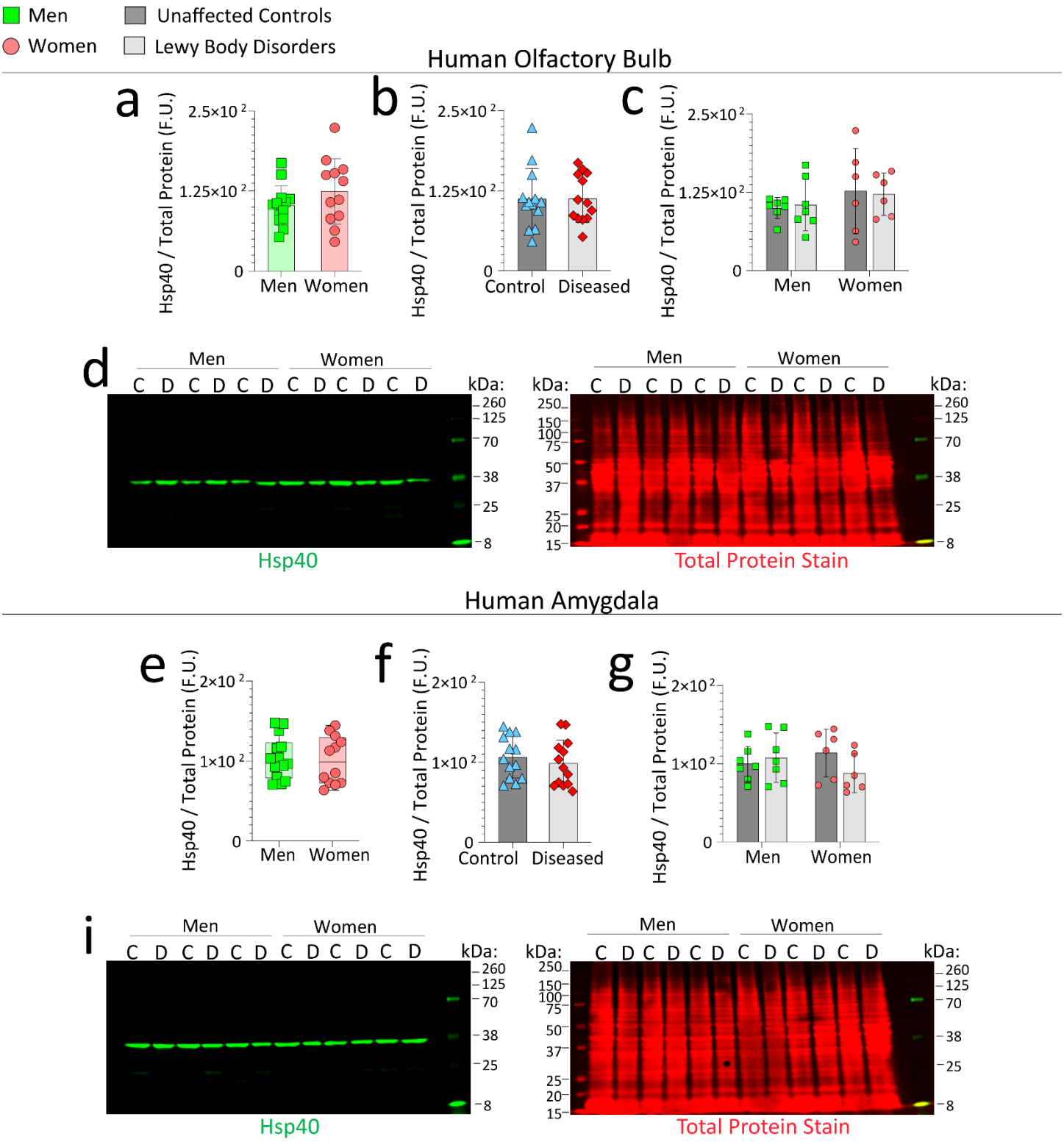


**Fig. S9 Biological sex and disease do not impact Hsp40 expression in the human OB or amygdala.** Postmortem olfactory bulb (OB) and amygdala tissues were obtained from men and women with Lewy body disorders and age-matched control subjects via NIH NeuroBioBank (UCLA and University of Miami) and subjected to immunoblotting. Values from control and diseased subjects were combined to test the impact of biological sex on Hsp40 levels in the OB in **a** and amygdala in **e**. Values from male and female subjects were combined to test the impact of disease on Hsp40 levels in the OB in **b** and amygdala in **f**. Two-way ANOVA graphs showing the individual impacts of sex and disease on Hsp40 levels in the OB **(c)** and amygdala **(g)**. Total Protein Stain was used as a loading control. Data in panels **a-b** and **f** were analyzed by the two-tailed unpaired Student's *t*-test. Data in panel **e** were analyzed by the Mann-Whitney U. Data in panels **c** and **g** were analyzed by two-way ANOVA followed by the Bonferroni *post hoc*

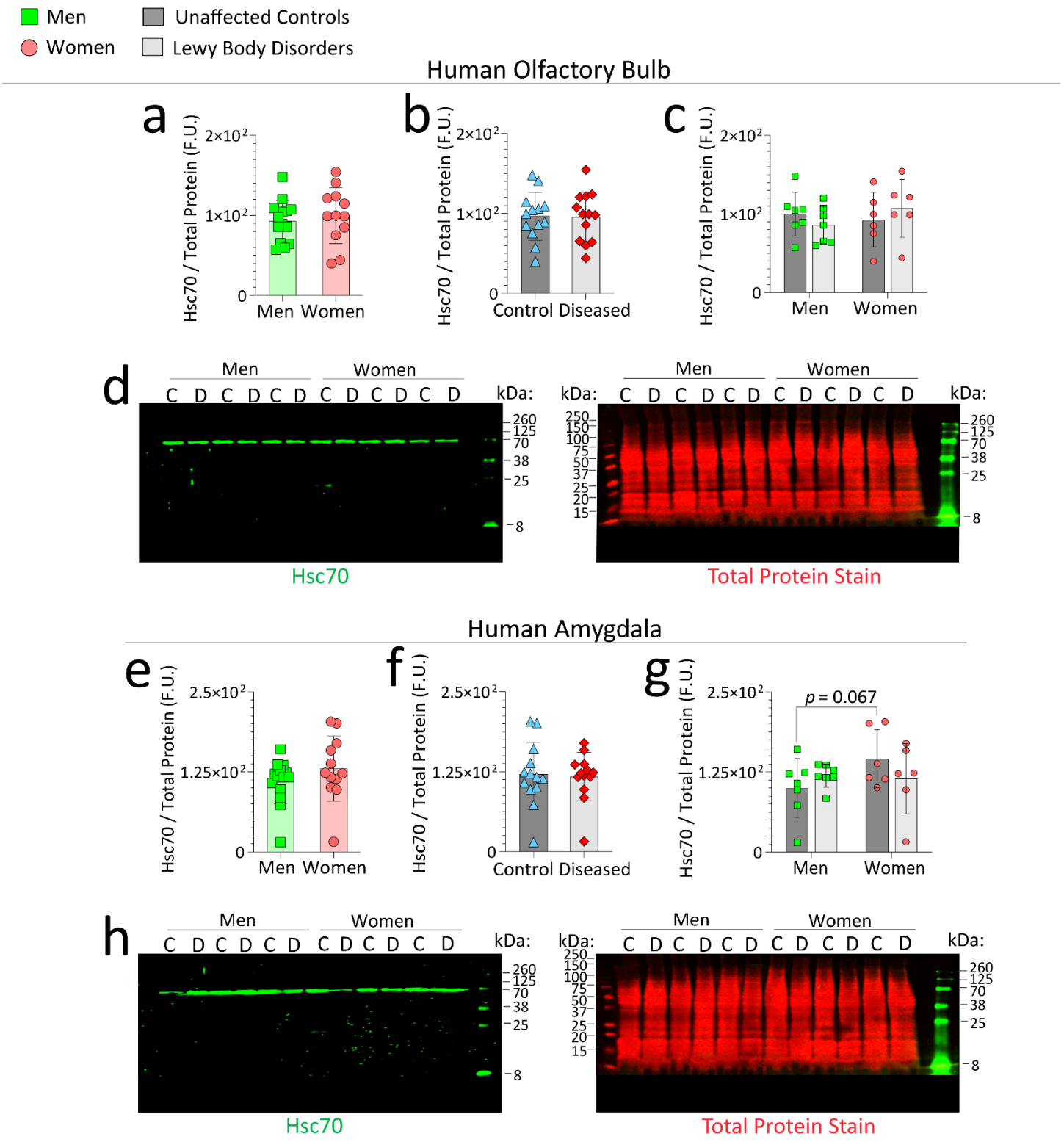


**Fig. S10 Biological sex and disease do not impact Hsc70 expression in the human OB or amygdala.** Postmortem olfactory bulb (OB) and amygdala tissues were obtained from men and women with Lewy body disorders and age-matched control subjects via NIH NeuroBioBank (UCLA and University of Miami) and subjected to immunoblotting. **(a, e)** Control and diseased subject data were combined to test the impact of biological sex on Hsc70 levels in the OB and amygdala. **(b, f)** Male and female subject data were combined to test the impact of disease on Hsc70 levels in the OB and amygdala. Two-way ANOVA graphs showing the impacts of sex and disease on Hsc70 levels in the OB **(c)** and amygdala **(g)**. Total Protein Stain was used to control for loading errors. Note that the blots in **d** and **h** were first probed for the Hsp70 cofactor Bag1 (not shown) and then reprobed for Hsc70. Panels **a-b** and **e-f** were analyzed by two-tailed, unpaired Student's *t*-test. Panels **c** and **g** analyzed by two-way ANOVA followed by Bonferroni *post hoc*

| Table S1: Primary Antibodies for Western Immunoblotting | | | | | |
|---|---|---|---|---|---|
| Antibody | Source | Company | Catalog # | Lot # | Dilution |
| Hsp70 (anti-human) | Mouse | Enzo Life Sciences | ADI-SPA-8134-D | 09010908 | 1:1000 |
| Hsp70 (anti-rat) | Rabbit | Millipore | AB9920 | 2278553 | 1:1000 |
| Hsp40 (anti-human / rat) | Rabbit | Cell Signaling Technology | 4868S | 2 | 1:1000 |
| Hsc70 (anti-human / rat) | Rat | Enzo Life Sciences | ADI-SPA-815-D | 1205144 | 1:1000 |
| α-synuclein | Mouse | BD Biosciences | 610786 | 8249521 | 1:1000 |
| pSer129 (clone EP1536Y) | Rabbit | Abcam | ab51253 | GR3317474-4 | 1:1000 |
| GAPDH | Rabbit | Cell Signaling Technology | 2118S | 8 | 1:5k-10k |

| Table S1 Continued: Primary Antibodies for Immunocytochemistry / Immunohistochemistry | | | | | |
|---|---|---|---|---|---|
| Antibody | Source | Company | Catalog # | Lot # | Dilution |
| β-III tubulin | Rabbit | Cell Signaling Technology | 5568 | 4 | 1:1000 |
| β-III tubulin | Chicken | Aves Labs | TUJ | TUJ89947984 | 1:500 |
| MAP2 | Mouse | Sigma-Aldrich | M9942 | 018M4785V | 1:1k-2k |
| pSer129 (clone 81A) | Mouse | Gift from Dr. Kelvin Luk, University of Pennsylvania | | | 1:5000 |
| | Mouse | BioLegend | 825701 | B230550 | 1:1000 |
| pSer129 (clone EP1536Y) | Rabbit | Abcam | ab51253 | GR3317474-4 | 1:1000 |
| NeuN | Guinea Pig | Millipore | ABN90 | 2834791 | 1:3k (ICC); 1:6k (IHC) |
| Hsp70 (anti-human) | Mouse | Enzo Life Sciences | ADI-SPA-8134-D | 09010908 | 1:1000 |
| Hsp70 (anti-mouse / rat) | Mouse | Calbiochem | 386032 | D00141952 | 1:1000 |
| GFAP | Mouse | Cell Signaling Technology | 3670S | 5 | 1:1000 |
| Iba1 | Rabbit | Wako | 019-19741 | PTR2404 | 1:500 |

| Table S2: Secondary Antibodies | | | | |
|---|---|---|---|---|
| Antibody | Company | Catalog # | Lot # | Dilution |
| Donkey anti-Mouse 546 | Invitrogen | A10036 | 1832039 | 1:700 (ICC / IHC) |
| Donkey anti-Mouse 790 | Jackson ImmunoResearch | 715-655-151 | 133569 | 1:700 (ICC); 1:30k (WB) |
| Donkey anti-Rabbit 555 | Invitrogen | A31572 | 2088692 | 1:700 (ICC / IHC) |
| Donkey anti-Rabbit 790 | Jackson ImmunoResearch | 711-655-152 | 136728 | 1:700 (ICC/IHC); 1:30k (WB) |
| Donkey anti-Rabbit 488 | Jackson ImmunoResearch | 711-545-152 | 130816 | 1:700 (ICC) |
| Donkey anti-Mouse 488 | Jackson ImmunoResearch | 715-545-150 | 130996 | 1:700 (ICC / IHC) |
| Donkey anti-Mouse 680 | Jackson ImmunoResearch | 715-625-151 | 138104 | 1:700 (ICC); 1:30k (WB) |
| Donkey anti-Rabbit 680 | Jackson ImmunoResearch | 711-625-152 | 135980 | 1:700 (ICC) |
| Donkey anti-Rat 790 | Jackson ImmunoResearch | 712-655-150 | 106408 | 1:30k (WB) |
| Donkey anti-Chicken 488 | Jackson ImmunoResearch | 706-545-155 | 130357 | 1:700 (ICC) |
| Donkey anti-Guinea Pig 680 | Jackson ImmunoResearch | 706-625-148 | 106246 | 1:700 (ICC) |
| Donkey anti-Guinea Pig 647 | Jackson ImmunoResearch | 706-605-148 | 123960 | 1:700 (ICC / IHC) |
| Donkey anti-Guinea Pig 488 | Jackson ImmunoResearch | 706-545-148 | 127887 | 1:700 (ICC) |
| Donkey anti-Guinea Pig 790 | Jackson ImmunoResearch | 706-655-148 | 106036 | 1:700 (ICC) |
| Donkey anti-Mouse 800 | LI-COR | 926-32212 | | 1:2k (ICC) |
| Donkey anti-Rabbit 680 | LI-COR | 926-68073 | | 1:20k (WB) |
| Goat anti-Mouse 800 | LI-COR | 926-32210 | | 1:2k (ICC); 1:20k (WB) |
| Goat anti-Mouse 488 | Life Technologies | A11001 | | 1:2k (ICC) |
| Goat anti-Rabbit 555 | Life Technologies | A21429 | | 1:1k (ICC) |
| Goat anti-Mouse 555 | Life Technologies | A21424 | | 1:1k (ICC) |

**Table S3**: Patient Characteristics as per UCLA and University of Miami Brain Banks

**UCLA**

| Number | Age at Death (years) | Gender | Control or Diseased | Clinical Diagnoses | Additional Neuropathological Diagnoses |
|---|---|---|---|---|---|
| 1 | 82 | M | C | CHF | Unaffected Control, Normal Aging |
| 2 | 79 | M | C | CA (prostate), ARF, Diabetes Type I | Unaffected Control, Hypoxia (recent, cerebrum) |
| 3 | 77 | M | C | CA (prostate), Heart Attack | Unaffected Control, Hypoxic Changes (intermediate, cerebrum) |
| 4 | 82 | F | C | CA (colon) | Unaffected Control, Hypoxia (recent, cerebrum) |
| 5 | 72 | F | C | CA (ovarian), CA (peritoneal) | Unaffected Control, Atherosclerosis |
| 6 | 79 | F | C | MS (clinical only), MRSA, Seizure Disorder, COPD, Osteoporosis, Paraplegic, Hydrocephalus, GERD | Unaffected Control |
| 7 | 82 | M | D | PD, LBD, Depression, Dementia (senile), Atherosclerosis, Sleep Disorder, Hypothyroidism | PD, Atherosclerosis |
| 8 | 68 | M | D | AD, Dementia (Lewy body), HTN, LBD (probable), Osteoarthritis | AD (probable), Cortical LBD |
| 9 | 71 | M | D | Dementia (Lewy body), Depression, HTN, Diabetes Type II, Sleep Disorder, Alcohol Abuse | Dementia (Lewy body) |
| 10 | 82 | F | D | AD, OBS, Dementia, Depression, LBD, Ataxia, TIA, ARF, CHF, CA (lung) | AD (probable), Atherosclerosis (moderate) |
| 11 | 79 | F | D | AD, Cognitive Impairment, Depression, Dementia, COPD | AD, Dementia (Lewy body) |
| 12 | 75 | F | D | Dementia (Lewy body), PD, Vascular Dementia (possible), Hypothyroidism, Chronic UTI, Dementia, Depression, Psychosis, OCD, AD | Dementia (Lewy body), AD (probable) |

**U. Miami**

| Number | Age at Death (years) | Gender | Control or Diseased | Brain Type |
|---|---|---|---|---|
| 1 | 65 | M | C | Unaffected Control |
| 2 | 66 | M | C | Unaffected Control |
| 3 | 83 | M | C | Unaffected Control |
| 4 | 84 | M | C | Unaffected Control |
| 5 | 65 | F | C | Unaffected Control |
| 6 | 82 | F | C | Unaffected Control |
| 7 | 86 | F | C | Unaffected Control |
| 8 | 74 | M | D | Dementia with Lewy Bodies |
| 9 | 79 | M | D | Dementia with Lewy Bodies |
| 10 | 80 | M | D | Dementia with Lewy Bodies |
| 11 | 81 | M | D | Dementia with Lewy Bodies |
| 12 | 73 | F | D | Dementia with Lewy Bodies |
| 13 | 79 | F | D | Dementia with Lewy Bodies |
| 14 | 83 | F | D | Dementia with Lewy Bodies |

Abbreviations: CHF=Congestive Heart Failure, CA=Cancer, MS=Multiple Sclerosis, MRSA=Methicillin-Resistant Staphylococcus Aureus, COPD=Chronic Obstructive Pulmonary Disease, GERD=Gastroesophageal Reflux Disease, ARF=Acute Renal Failure, PD=Parkinson's disease, LBD=Lewy Body Disease, AD=Alzheimer's disease, HTN=Hypertension, OBS=Organic Brain Syndrome, TIA=Transient Ischemic Attack, UTI=Urinary Tract Infection, OCD=Obsessive Compulsive Disorder