α-Synuclein Oligomers Induce a Unique Toxic Tau Strain

Supplementary Information

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Atomic Force Microscopy
Recombinant as well as brain-derived oligomeric preparations were analyzed by AFM using a non-contact tapping method with a Multimode 8 AFM machine (Bruker, Billerica MA). Briefly, 10 µl of sample were applied onto a fresh-cleaved mica surface and allowed to adsorb. Mica was then washed with 100 µl of deionized water and air-dried. Analysis were performed in triplicates.

Electron Microscopy
Recombinant samples were adsorbed onto 200-mesh carbon and Formvar-coated grids (2 µL), air-dried, and washed for 1 min in distilled water. The samples were negatively stained with 2% uranyl acetate (Ted Pella Inc., Redding, CA) for 2 min and imaged with a Zeiss 10CR microscope (80 kV; Carl Zeiss, Oberkochen, Germany).

Thioflavin T Fluorescence
Tau aggregates were characterized as previously described (1). Briefly, 1 µl of 0.3 mg/ml protein sample and 250 µl of 5 µM ThT, 50 mM glycine buffer (pH 8.5) were combined. Fluorescence intensity values of samples were obtained. Thioflavin T (Sigma) binding was measured using a POLARstar OMEGA plate reader (BMG Labtechnologies, Melbourne, VIC, Australia) with 440-10 nm/520 nm excitation/emission filters set. Analysis were performed in triplicates.

Bis-ANS Fluorescence
1,1'-bis(anilino)-4-,4'-bis(naphthalene)-8,8'-disulfonate (Sigma), bis-ANS binding was measured using a POLARstar OMEGA plate reader (BMG Labtechnologies, Melbourne, VIC, Australia) with 355 nm/520 nm excitation/emission filters set and 1 µl of 0.3 mg/ml of protein sample, 250 µl of
10 μM bis-ANS and 100 mM glycine buffer (pH 7.4). Blank-corrected fluorescence intensity values of samples were used.

**Preparation of α-synuclein Oligomers**

α-synuclein oligomers were obtained by dissolving 0.5 mg of lyophilized protein in 200 μl of hexafluoroisopropanol (HFIP) and incubated at room temperature for 20 minutes. To this solution, 700 μl of dd H2O was added and a cap with holes was used to allow HFIP to evaporate. Sample was stirred at 500 RPM with a Teflon-coated micro stir bar for 48 hours inside the fume hood and subjected to size-exclusion chromatography using a LC-6AD Shimadzu high-performance liquid chromatography (HPLC) system fitted with a TSK-GEL G3000 SWXL (30 cm × 7.8 mm) column, Supelco-808541 (GE Healthcare). A purified fraction containing mostly homogenous population of α-synuclein oligomers was collected and used for experiments.

**Isolation of Oligomers From PD and PSP Brains**

Tau and α-synuclein oligomers were immunoprecipitated (IP) from PD and PSP cases using T22 (tau) followed by F8H7 (α-synuclein) antibodies as described previously (2). IP was carried out following manufacturer’s recommendations (Thermo Scientific Cat No 23600). Briefly, 1 mg of antibody was immobilized in 200 µl of amine-reactive resin for two hours at room temperature. Brain tissue was homogenized in freshly prepared ice-cold 1X PBS with protease inhibitor cocktail (Cat. 11836145001, Roche Diagnostics, IN, USA). The samples were centrifuged at 10,000 rpm for 10 min at 4°C. Brain lysates were pre-adsorbed in a control resin to eliminate nonspecific protein binding. Flow-through samples were incubated with the antibody-coupled resin with end-over-end mixing for 2 hours. Antibody-bound proteins were recovered using 0.1 M glycine (pH 2.8), adjusting the final pH to 7.0 by adding 1 M Tris-HCl (pH 8). Isolated fractions were subjected to Ultra Fast Liquid Chromatography (UFLC) purification and sterilized by filtration. The total protein concentration was measured with bicinchoninic acid protein assay (Micro BCA Kit, Pierce).
Samples were immediately applied onto nitrocellulose membrane to perform a dot blot analysis. Remaining samples were frozen and stored at -80°C.

**Characterization of Brain-derived Oligomers**

Immunoprecipitated oligomeric complexes isolated from PD and PSP human cases were characterized by multiple methods as previously described (2). AFM was performed to visualize the morphologies of oligomeric assemblies of isolated proteins. Immunoprecipitated brain derived oligomers were injected into a Shimadzu UFLC system fitted with a TSK-GEL G3000 SWXL (30 cm × 7.8 mm) column, Supelco-808541. PBS (pH 7.4) was used as the mobile phase with a flow rate of 0.5 ml/min. A gel filtration standard (Bio-Rad 51-1901) was used for calibrations.

**Primary Cortical Cell Culture**

Cortical neurons from embryonic day 16 Htau mouse brain were plated and grown in Neurobasal medium supplemented with 2% B27 and l-glutamine at 3 × 10^6 cells/ml in a 24 well chamber, mounted on poly-d-lysine-coated glass coverslips (Corning, Inc.). Media changes were performed every 3–5 days. Cells were grown for 7–10 days *in vitro* before experiments. All experiments were performed in triplicates.

**Transfection**

CV-1 (African green monkey kidney) cells as well as YFP-tau and YFP-tubulin were kindly provided by Prof. George Bloom (University of Virginia, Charlottesville). As previously reported (3), cells were cultured in DMEM (Invitrogen) supplemented with 10% Cosmic Calf Serum (Hyclone) and 50 μg/ml gentamycin. Cells were transiently transfected using Lipofectamin 2000 (Invitrogen) with cDNAs for the longest human isoform of tau linked at their C terminals to YFP (CLONTECH Laboratories, Inc.); or YFP–tubulin (CLONTECH Laboratories, Inc.); Cells were cultured for 4 days after transfection and then exposed to tau oligomers (Tau/tauO, Tau/α-synO),
fibrils or seeds from α-synuclein. Three independent replications were performed for each experimental setting. Live cell imaging was performed as previously described (4) on an inverted microscope (Olympus) equipped with 40× 1.4 NA planapo and 25× 0.8 NA plan-neofluar objectives (Carl Zeiss MicroImaging, Inc.), a CARV spinning disk confocal head (BD Biosciences), an X-Cite 120 illuminator (EXFO Photonic Solutions), a cooled charge-coupled device (Orca ER; Hamamatsu), and cellSens imaging software (Olympus) for image acquisition.

**Cell Viability Assay**

Cell viability was determined in CV-1 cells as well as SH-SY5Y. SH-SY5Y cells were maintained in DMEM with 10 mM HEPES, 10% fetal bovine serum, 4 mM glutamine, penicillin (200 U/mL), and streptomycin (200 μg/mL) in 5% CO₂ at 37°C. The media was replaced every 2 days. Cells were plated at 10,000 cells/well in 96-well plates and differentiated in serum-free DMEM with N₂ supplement and 1 × 10⁻⁵ M all-trans retinoic acid before use. SH-SY5Y as well as CV-1 cells, were exposed to 1 μM of tau oligomers (Tau/tauO, Tau/α-synO) or tau fibrils. Briefly, media was removed, and tau oligomers (Tau/tauO, Tau/α-synO) or fibrils were exogenously added to cells in culture in fresh media without phenol red. After incubation for 6 h at 37°C, the cells were subjected to ATP assays using luminescence CellTiter Glo (Promega) according to the manufacturer’s directions. A minimum of four wells per experimental condition were analyzed in three independent experiments. One-way ANOVA followed by Tukey’s post hoc was used to analyze cell viability.

**Dot Blot Analysis**

Dot blot strips were prepared with nitrocellulose membranes. Briefly, 0.5–1.2 µl of each sample was applied onto the strips and blocked with 10% nonfat dried milk in 1X-TBST buffer, pH 7.4, overnight at 4°C. The strips were washed once with 1X-TBST buffer and incubated with Tau-13 (1:1000), T22 (1:500), TOMA (1:350) and A11 (1:700), for 1h at room temperature. The strips
were washed three times with 1X-TBST and then incubated with HRP-conjugated IgG anti-mouse secondary antibody (1:3000; GE Healthcare), or horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:3000; GE Healthcare), respectively, for 1h at room temperature. Finally, the membranes were washed three times with 1X-TBST and developed using ECL plus chemiluminiscence kit (GE Healthcare). Signal intensity of each dot was measured using Labworks 4.5 software (UVP).

Immunofluorescence

Immunofluorescence staining was performed in cells grown on coverslips as well as in paraffin tissue sections following standard procedures. Coverslips containing cells were fixed in methanol for 20 min at -20°C, while brain sections were deparaffinized, rehydrated, and washed in 0.01 M phosphate-buffered saline (PBS) three times (5 min each). After blocking in normal goat serum (Invitrogen) for 1 h, both tissue sections and cells were incubated overnight with primary antibodies. Samples were incubated directly in hybridoma supernatant containing F8H7 or incubated in T22, PSD95 (1:350; Abcam), Mapt2 (1:700; Abcam), AT180 (Thr231; 1:350; Thermoscientific), AT8 (1:350; Thermoscientific), Tau5 (1:350; Covance) or T22 (1:350). The next day, the sections were washed in PBS three times (10 min each) and then incubated with goat anti-mouse IgG Alexa Fluor 568 (1:700; Invitrogen) or goat anti-rabbit IgG Alexa Fluor 568 (1:700; Invitrogen) for 1h. The sections were then washed three times (10 min each) in PBS before incubation overnight with tubulin (1:350) or Tau5 (1:350). The next day, the sections were washed in PBS three times for 10 min each before incubation with goat anti-mouse IgG Alexa Fluor 488 (1:700; Invitrogen) for 1h. Sections were washed and mounted using Fluoromount G (Southern Biotech) mounting medium with DAPI (Invitrogen). The sections were examined using an epifluorescence microscope (Eclipse 800; Nikon) equipped with a CoolSnap-FX monochrome CCD camera (Photometrics) using standard Nikon FITC and DAPI filters. Images were analyzed using the Metavalue version 7.1 software (Molecular Devices). Confocal z-stack images were
acquired using Bio-Rad Radiance 2100 confocal system mounted on a Nikon Eclipse E800 microscope, using identical laser power, photomultiplier gain, and pinhole settings for each experiment. To build the z-stack 12 confocal planes/ 0.7µm thickness were captured. Experimenters quantifying immunoreactivity were blind to treatment conditions. Confocal images were taken using a Zeiss LSM-510 META confocal microscope with a 63 X 1.20 numerical aperture water immersion objective. Z-stack acquisition was carried out with 0.8-μm z-steps and prepared by using LSM 510 software. Thr231 quantification of immunofluorescence was performed using ImageJ software (NIH). Each channel was set to the same threshold across all experimental conditions and converted to a binary image. Background was subtracted and intensity to Thr231 was measured.

**Immunohistochemistry**

Immunohistochemistry was performed on paraffin sections. In brief, paraffin sections (8 µm) were deparaffinized and rehydrated. After blocking in normal goat serum for 1h, sections were incubated overnight with primary antibodies NeuN (1:250; Chemicon), AT8 (1:350; Thermo Fisher Scientific), LB509 (1:350; Abcam) and T22. The next day, the sections were washed in PBS three times (10 min each) and then incubated with biotinylated goat anti-mouse IgG (1:2000; Jackson Immuno Research) or biotinylated goat anti-rabbit IgG (1:2000; Jackson Immuno Research) for 1h. The sections were then washed three times (10 min each) in PBS and visualized using an ABC reagent kit (Vector Laboratories) according to the manufacturer’s recommendations. Finally, sections were counterstained with hematoxylin (Vector Laboratories) for nuclear staining and mounted.

**Stereology**

T22 and AT8-immunoreactive cells within the hippocampus and cortex, as well as Lewy body-like deposits immunoreactive to LB509 were quantified by an unbiased stereological sampling method.
based on optical fractionator stereological probe (Stereo Investigator, MBF Bioscience). A researcher blind to treatment groups analyzed the number of cells counted in a known uniformly random sample of a region of interest. One-way ANOVA was used to analyze treatment group differences using mean section thickness (T22, AT8, and LB509 cell counts) in each region. Accuracy of stereological estimates was evaluated using Gundersen’s smoothness classification m=1 coefficients of error (CEs).

Dendritic Spines Analysis
To assess the effects of oligomeric preparations on the number of mature synapses, primary cortical cells from Htau mice exposed to vehicle, Tau/tauO or Tau/α-synO were immunostained using PSD95 antibody. Dendrites were randomly imaged using identical laser power, photomultiplier gain, and pinhole settings for each experiment. All imaging experiments were performed on 5 cells per treatment and analyzed by a researcher blinded to experimental condition. Three independent replications were performed for each experimental setting. PSD95 puncta mean intensity was performed using ImageJ software (NIH, Bethesda, Maryland, USA). Each channel was set to the same threshold across all experimental conditions and converted to a binary image. Background was subtracted and intensity to PSD95 was determined. One-way ANOVA followed by Tukey’s post hoc was used to analyze the dendritic spine results.

Animals
All animals used here were males to control for changes in female hormone state that may impact cognitive data. Htau mice were bred at University of Texas Medical Branch (UTMB) free of enrichment to prevent any effect on behavioral test performance. Mice were housed at UTMB animal care facility and maintained according to US Department of Agriculture standards (12 h light/dark cycle, food and water ad libitum) in accordance with the Guide for the Care and Use of
Laboratory Animals (National Institutes of Health) and the Institutional Animal Care and Use Committee-approved procedures.

**Intracerebroventricular Injection (ICV)**

One-month-old Htau mice were anesthetized with ketamine (80–100 mg/kg, I.P.) and xylazine (10 mg/kg, I.P.) and placed in a stereotaxic apparatus (Motorized Stereotaxic StereoDrive; Neurostar). For each mouse, the scalp was shaved, an incision was made through the midline to expose the top of the skull, and the bregma was located. A hole was drilled into the skull at −2.06 mm caudal to the bregma and 1.7 mm lateral to the midline at a depth of 2.5 mm. A 5.0 μl Hamilton syringe was used to inject 0.5 μg of PD-α-synO/tauO (n=8), PSP-tauO (n=8) or equal volume of PBS (n=8) into the hippocampus and wild-type mice into both sides at a rate of 0.5 μl/min. The incision was closed using Vet-Bond and the mice were placed on an isothermal pad at 37°C and continuously monitored after surgery until recovery.

**Y-maze**

The Y maze test was performed as we previously published (5). Briefly, animals were placed in a symmetrical Y-shaped maze (San Diego Instruments). Arms were randomly designated A, B, or C. Each mouse (n=8/ per group) was placed in an arm facing the center (arm A) and allowed to explore the maze for 8 min. The number of arms entered and the sequence of entries was recorded. A correct alternation occurred when the animal moved from the arm in which it began to the other two arms without retracing its steps (i.e., ABC or ACB). Spontaneous alternation, expressed as a percentage, was calculated by dividing the number of entries into all 3 arms on consecutive choices (correct choices) by the total number of arm entries subtracted by 2, then multiplying the quotient by 100. A high spontaneous alternation rate is indicative of sustained working memory.
**Grip Meter**

Grip strength was measured using the BIOSEB grip strength test. Mice were gently lowered onto a grid, allowing the forepaws alone to grip the grid (n=8/ per group). The mouse was then slowly pulled until the animal released its grip. This procedure was repeated 5 times for hindlimbs. The maximal grip strength value is displayed when the animal releases the grid. The grid was wiped out with 70% alcohol between animals.

**Tissue Harvesting**

Following behavioral testing, animals were euthanized with CO₂ and brains were collected. The left hemisphere was embedded in paraffin and sectioned. The right hemisphere was immediately processed for biochemical analysis. Briefly, frozen brains were diced and homogenized in PBS with a protease inhibitor mixture (Roche) and 0.02% NaN₃ using a 1:3 (w/v) dilution. Samples were then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatants were divided into aliquots, snap-frozen, and stored at -80°C until use (5). The pellet containing PBS insoluble material, was resuspended in 70% formic acid, mechanically dissociated, and centrifuged at 14,000g for 90 minutes at 4°C. The supernatants were collected as formic acid soluble and stored at -80°C for further analysis (6).

**Western Blot Analysis**

A total of 20–25 µg of protein were loaded on precast NuPAGE 4–12% Bis-Tris Gels for SDS-PAGE (Invitrogen) and subsequently transferred onto nitrocellulose membrane. After blocking overnight at 4°C with 10% non-fat dried milk, membranes were probed for 1h at room temperature with T22 (1:500), Tau 5 (1:1000; Covance), active-Caspase-3 (1:1000) and actin (1:1000; Thermo Fisher Scientific), and then diluted in 5% nonfat dried milk. T22 and active-Caspase 3, were detected with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:3000; GE Healthcare). Tau 5, and actin antibody detection was done with HRP-conjugated IgG anti-mouse secondary
antibody (1:3000; GE Healthcare). ECL Plus (GE Healthcare) was used for signal generation. For protein quantification, the densitometry of each band was measured using Labworks 4.5 software (UVP), and normalized with actin. Quantitative data display average values from independent experiments.

**Statistical Analysis**

Each experimental condition was repeated at least three times. Behavior and stereology analyses were performed by an experimenter blinded to treatment conditions. Statistical analyses were carried out using Prism 5.0 (GraphPad). For three group comparisons, a parametric one-way ANOVA test was used. To correct for multiple comparison, Bonferroni and Tukey’s *post hoc* test were used. For two group comparisons, Student’s *t*-test analysis was conducted. For all test, significance was set at *p* ≤ 0.05. Data is presented as mean ± SEM.
Supplementary Figure S1. Seeds of α-synuclein enhance tau oligomer toxicity. (A-C) Representative Western blot of monomeric tau seeded with preformed tau or α-synuclein.
oligomers, probed with T22 (A) or Tau5 (B) antibodies (Multiple t-test non-parametric Tau 5, \( *p<0.01, n=3, df = 4; \) T22, \( p <0.2, n=3, df = 4; \) C). (D) Representative live cell images of CV-1 cells transfected with YFP-control plasmid and YFP-tau plasmid containing full-length tau, treated with the equivalent amount of α-synuclein oligomers seeds. (E) Cell viability of CV-1 cells transfected with YFP-Control or YFP-tau exposed to α-synuclein oligomers seeds confirmed lack of toxicity. (F-I) Viability assay of SH-SY5Y cells treated with PBS (Vehicle; white bar), tau seeded with tau oligomers (light gray bar; F), or α-synuclein oligomers (dark gray bar; G) and heparin (black bar; H). The graph represents the relative luminescence units (RLU) of CellTiter Glo to cellular ATP. ****\( p<0.0001; ***p<0.004, n=4 \) independent experiments. Bars represent the mean and SEM; One-way ANOVA, Tukey’s multiple comparisons test.
Supplementary Figure S2. (A-C) Representative live cell images of CV-1 cells transfected with YFP-tau plasmid exposed to PBS (Vehicle; A), tau seeded with preformed tau oligomers (B) or α-synuclein oligomers (C). Tau induced by oligomeric α-synuclein seeds retains its toxicity but not tau seeded with tau oligomers, as demonstrated by the relative luminescence units (RLU) of CellTiter Glo to cellular ATP (D). Bar represents the mean, error bars the SEM *p<0.03, **p<0.006, One-way ANOVA Tukey’s multiple comparisons test. (E-M) Representative confocal images of cells immunostained with T22 (red; E,H,K), Tau 5 (green; F,I,L) and merged (yellow, G,J,M). Scale bar 20 µm.
Supplementary Figure S3. Parkinson’s disease brain-derived oligomeric complexes of α-synuclein/tau (PD α-synO/tauO) exacerbate behavioral deficits and increase tau oligomers levels in Htau mice. Behavior analysis of Htau mice receiving ICV injections of vehicle (n=8), PSP brain-derived tau oligomers (PSP-tauO; n=8) or PD α-synO/tauO (n=8) was conducted 7 and 14 months post-injections. (A, B) Hindlimb grip strength (*p<0.02) and spatial memory (*p<0.02; Y-maze) is impaired at 7 months post injection with PD α-synO/tauO compared to Vehicle. Error bar, SEM, one-way ANOVA, Bonferroni post hoc comparisons test. (C, D) At fourteen months post-injection, all mice showed declining strength and spatial memory, as depicted by the reduced grip strength (***p<0.005; *p<0.01) and percent of alternations (*p<0.01). Behavioral deficits in PD α-synO/tauO were prominent compared to vehicle. Error bar, SEM, One-way ANOVA, Bonferroni’s post hoc comparisons test. (E, F) ELISA analysis of brain fractions (E) PBS soluble and (F) formic acid soluble (FA), from mice injected with vehicle, PSP-tauO or PD α-synO/tauO using T22 and PHF-13 antibodies. Tau oligomers levels are elevated in PD α-synO/tauO compared to PSP-tauO or vehicle (*p< 0.03, n=3). PBS insoluble material extracted with formic acid increased in mice receiving PSP-tauO, compared to PD α-synO/tauO or vehicle (*p< 0.04, n=3). Error bar, SEM, One-way ANOVA, Tukey’s post hoc comparisons test.
**Supplementary Table S1.** Summary of cases examined in the present study.

| Patient | Diagnosis | Age | Gender | PMI (hours) |
|---------|-----------|-----|--------|-------------|
| 1       | Cntrl     | 73  | Female | 9           |
| 2       | Cntrl     | 53  | Female | 5           |
| 3       | Cntrl     | 68  | Male   | 14          |
| 4       | Cntrl     | 87  | Female |             |
| 4       | PSP       | 72  | Male   | 11          |
| 5       | PSP       | 73  | Male   | 8.5         |
| 6       | PSP       | 84  | Male   | 4.5         |
| 7       | PSP       | 79  | Male   | 12          |
| 8       | PD        | 72  | Female | 20          |
| 9       | PD        | 80  | Female | 4           |
| 10      | PD        | 86  | Male   | 11          |
| 11      | PD        | 61  | Female | 9           |

Cntrl, control; PSP, Progressive supranuclear palsy; PD, Parkinson’s disease; PMI, postmortem interval.
Supplementary References

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