Figures and figure supplements

Inert and seed-competent tau monomers suggest structural origins of aggregation

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Figure 1. Seeding activity of tau monomer in cells and in vitro. (A, B) FL Cys-Tau(2A) was labeled with Alexa488 and resolved by SEC (A), or was fibrillized in the presence of heparin, labeled with Alexa488, sonicated, and the assemblies resolved by SEC (B). The column was calibrated using standards of the indicated hydrodynamic radii. Color codes indicate the putative assembly units. (C) Tau assemblies were seeded into tau RD-CFP/YFP biosensor cells. M_i represents ‘inert’ monomer purified in (A), which had no seeding activity; M_s represents ‘seed-competent’ monomer purified in (B), which induced intracellular tau aggregation (p<0.001). (D) FL WT tau and FL Cys-Tau(2A) were similarly fibrillized, sonicated, and the fragments resolved by SEC. Seeding activity of each fraction was determined. M_s and larger assemblies of both forms of tau exhibited seeding activity, but not M_i. IFD = Integrated FRET Density. (E) Tau assemblies of n = 1, 2, 3 were passed through a 100kD size cutoff filter. Filtration had no effect on the M_s fraction, whereas it reduced seeding of assemblies of n = 2 or 3. (F) Tau fibrils, trimer, or monomer were used to induce fibrillization in vitro of full-length (0N4R) tau, measured by induced thioflavin fluorescence. M_i had no seeding activity, whereas M_s, trimer, and unfractionated fibrils had strong seeding activity. (G, H) Titration of assemblies was performed. (G) M_s exhibited an EC_50 of approximately 10 nM (monomer equivalent); (H) Dimer and trimer had similar potencies. Concentration is reflected as monomer equivalent. See Figure 1—source data 1.

DOI: https://doi.org/10.7554/eLife.36584.003
Figure 2. Analyses of M_i and M_s by CD and FCS. (A) CD spectra of M_i and M_s were similar. (B) FCS Diffusion times for M_i, M_s, dimer, trimer, and >10 mer, and the cross-correlation for M_i, M_s, dimer, trimer, and >10 mer were determined after labeling of fibrils with Alexa488, or double labeling additionally with tetramethylrhodamine prior to sonication. Table reflects the predicted diffusion time and the actual diffusion time. The variance between predicted vs. observed times is reported. (C–G) FCS for double-labeled tau assemblies. Cross correlation (CC) between the two dyes is indicated in grey lines. (H) Summary of FCS cross-correlation, including free dyes. Neither free dye, M_i, nor M_s showed any cross-correlation, indicating that single species predominate. All multimeric assemblies exhibited cross-correlation, indicating detection of both dyes within a single particle. See Figure 2—source data 1.

DOI: https://doi.org/10.7554/eLife.36584.005
Figure 3. Fidelity of SEC purification of assemblies. SEC fidelity was tested by isolating $M_i$ from fractions after fibril sonication. Remaining fractions were combined with $M_s$ and the mix was re-isolated by SEC. In Group 1, after the first isolation, the monomer fraction (which contains $M_s$) contained seeding activity. In Group 2, after the second purification by SEC, the monomer fraction (which contains $M_i$ spiked in) did not exhibit seeding activity. See Figure 3—source data 1.

DOI: https://doi.org/10.7554/eLife.36584.007
Figure 4. Heat denaturation of assemblies. (A–C) Heat-induced dissociation of assemblies. (A) The SEC fraction containing M₅ (B₅) was heated to 95°C for 3 hr and re-isolated by SEC prior to testing the FRET biosensor assay. No loss in seeding activity was observed. (B) When the SEC fraction containing trimer (B₈) was heated similarly, seeding activity shifted to fractions that contain dimer and monomer (B₇, B₅). (C) ~20 mer (A₅) was largely stable to heating, although some smaller seed-competent assemblies were liberated. (D–G) Various assemblies were subjected to heat denaturation at the indicated temperatures and times, followed by analysis of seeding activity in the FRET biosensor assay. Whereas ~10 mer and ~20 mer were relatively stable from 65–95°C, monomer, dimer and trimer showed temperature-dependent loss of seeding activity. (H) Plot of denaturation data for M₅ with multimodal regression curves superimposed. See Figure 4—source data 1.

DOI: https://doi.org/10.7554/eLife.36584.009
Figure 5. Mₙ self-assembles. M₁ and Mₙ were incubated at 500 nM or with equivalent amounts (monomer equivalent) of dimer and trimer for various times prior to resolution by SEC. Assemblies were monitored by reading the absorbance of fractions using micro BCA assay. (A) M₁ showed no self-

Figure 5 continued on next page
association. (B) M₄ exhibited self-association over time. (C,D) Dimer and trimer were stable over time. (E,F) M₄ does not react with dimer or trimer to form larger assemblies. (G,H) M₄ reacts with dimer and trimer to form larger assemblies. See Figure 5—source data 1.

DOI: https://doi.org/10.7554/eLife.36584.011
Figure 6. Heparin induces transition from M<sub>i</sub> to M<sub>s</sub>. (A) Heparin treatment of FL WT tau was carried out for 15 min, 1 hr, or 4 hr. Samples were resolved by SEC, and fractions of various sizes were compared using the biosensor seeding assay. ‘Pre-SEC’ refers to the sample prior to fractionation. NT = monomer not treated with heparin. At 15 min, a small, but significant seeding activity was observed primarily in the monomer fraction. By 1 hr this signal was very strong, and comparable to the signal of M<sub>s</sub> derived from sonicated fibrils. (B) M<sub>s</sub> derived from 4 hr heparin exposure was heated at 95°C for different times, followed by analysis of seeding activity in the FRET biosensor assay. Seeding activity decayed over 24 hr. (C) Seeding efficiencies per nM of tau (monomer equivalent) of the various forms of M<sub>s</sub>, sonicated, or unsonicated fibrils were relatively similar. M<sub>i</sub> was sonicated identically to M<sub>s</sub>, followed by purification via SEC, but exhibited no seeding activity. Transfection of heparin failed to trigger intracellular aggregation (data not shown).

See Figure 6—source data 1.

DOI: https://doi.org/10.7554/eLife.36584.013
**Figure 6—figure supplement 1.** SDS-PAGE of tau after sonication or heparin treatment. (A) Two different FL WT tau preparations were sonicated or not, and 1.5 μg protein was then resolved by SDS-PAGE and coomassie stain. Sonication induced a small degree of protein fragmentation. (B) FL WT tau was exposed to heparin for 15 min, sufficient to induce conversion from M<sub>i</sub> to M<sub>s</sub>, followed by DSS crosslinking for the indicated time periods. 100 ng Protein was then resolved by SDS-PAGE and silver stain. No small fragments or higher-order crosslinked species were visible.

DOI: https://doi.org/10.7554/eLife.36584.014
Figure 7. Unique XL-MS patterns for different forms of $M_i$ and $M_s$. Tau monomers were prepared as described, heated at 95°C for 0, 3 or 24 hr, reacted with DSS, proteolyzed and analyzed by mass spectrometry to define intramolecular crosslinks. Diagrams represent crosslinks within the tau protein. Tau is shown in grey; RD is colored in red ($R1$), green ($R2$), blue ($R3$) and indigo ($R4$). Each diagram indicates consensus crosslinks present across replicates ($N = 3$) (green or red). Crosslinks uniquely observed within $M_s$ preparations are shown in red. Each sample was prepared, isolated by SEC, and then subjected XL-MS. (A) $M_i$: tau monomer not previously fibrillized; (B) $M_s$: fibril-derived tau monomer; (C) $M_s$: heparin-exposed tau monomer (0.25 hr or 1 hr). Crosslinks from aa150 to aa254-290 mark all forms of $M_s$ after exposure to 95°C for 0 hr, 0.25 hr and 3 hr, but not 24 hr. See Figure 7—source data 1 and 2.

DOI: https://doi.org/10.7554/eLife.36584.016
Frequency of crosslinks decreases with heat incubation. Heat denaturation of Mᵢ and Mₛ (fibril-derived and heparin treated for 0.25 hr, 1 hr) decreases the abundance of consensus crosslink pairs (A). Columns represent data after exposure to 95°C for 0 hr, 3 hr and 24 hr. See Figure 7—source data 2.

DOI: https://doi.org/10.7554/eLife.36584.017
Figure 8. AD brain contains seed-competent monomer. Tau from control and AD brains was immunoprecipitated and subjected to SEC. (A) SEC from control brain contained predominantly tau monomer. (B) SEC from AD brain contained a range of tau assembly sizes. (C) Tau monomer from control brain exhibited no seeding activity, whereas monomer from AD brain did, along with larger assemblies (p<0.001). Tau Unit refers to the putative number of molecules per assembly. LF = Lipofectamine control. (D) Tau KO mouse brain was spiked either with human tau M₅₅ or fibrils prior to dounce homogenization, immunopurification, and resolution by SEC. Samples spiked with M₅₅ exhibited monomer seeding activity, but not samples that had been spiked with fibrils. (E) AD-derived tau monomer was incubated for the indicated times prior to SEC and determination of seeding activity in each fraction. Larger seed-competent assemblies formed after 24 hr incubation at RT. (F, G) Three control and AD brains were homogenized, monomer isolated, and evaluated by XL-MS. Tau monomer from controls lacked the long-range crosslinks observed in M₅₅. AD-derived M₅₅ contained long-range crosslinks (aa150 to aa254-290) also observed in recombinant forms of M₅₅. See Figure 8—source datas 1 and 2.

DOI: https://doi.org/10.7554/eLife.36584.020
Figure 8—figure supplement 1. Different brain homogenization methods yield similar crosslink patterns. A single AD brain sample was homogenized using four different methods: (A) Dounce homogenization; (B) Pulse sonication; (C) Mechanical homogenization; (D) Mechanical homogenization followed by pulse sonication. Diagrams represent crosslinks within the FL tau protein. RD is colored in red (R1), green (R2), blue (R3) and indigo (R4). High confidence XL-MS crosslinks are shown as light green lines; crosslinks found in Mₘ are shown in red. See Figure 8—source data 1 and Figure 8—source data 2.

DOI: https://doi.org/10.7554/eLife.36584.021
Figure 9. Models of $M_i$ and $M_s$ suggest differences in the R1R2 and R2R3 regions. XL-MS identified pairs were used as restraints in Rosetta to create structural models of discrete tau domains. (A) Schematic highlighting the region of the RD encoding structural differences between $M_i$ and $M_s$. Tau RD is colored in red ($R1$), green ($R2$), blue ($R3$) and indigo ($R4$); N- and C-terminal portions of tau are shown in grey. Fragments of interest are shown with their position in the RD. (B) recombinant $M_i$; (C) fibril-derived $M_s$; (D) Control $M_i$ and (E) AD-derived $M_s$. Regions surrounding the R1R2 and R2R3 are indicated, highlighting two amyloid-forming sequences, VQIINK (green spheres) and VQIVYK (blue spheres). In both forms of $M_i$ VQIINK and VQIVYK are associated with flanking amino acids in hairpin structures. In both forms of $M_s$ the VQIINK and VQIVYK sequences are presented at the protein surface. See Figure 9—source data 1.

DOI: https://doi.org/10.7554/eLife.36584.024
Figure 9—figure supplement 1. Energetics of Rosetta structural ensembles. The ensembles are shown as a distribution of total energy of each model and radius of gyration for recombinant M<sub>i</sub> (A), recombinant M<sub>s</sub> (B), control brain-derived M<sub>i</sub> (C) and AD-derived M<sub>s</sub> (D). Figure 9—source data 2.
DOI: https://doi.org/10.7554/eLife.36584.025
Figure 10. Proteolysis of Mᵢ and Mₛ reveals distinct patterns. (A) Mᵢ and Mₛ were prepared as technical triplicates (N = 3), isolated by SEC, and passed through a 100kD filter immediately prior to exposure to trypsin for 1, 5, 10, 30, 60 and 120 min. Samples were analyzed by mass spectrometry and Figure 10 continued on next page.
kinetic profiles were generated for peptides present at each time point. (B) Tau RD is colored in red (R1), green (R2), blue (R3) and indigo (R4). Identified peptides are shown with their position in the RD. (C–H) Kinetic profiles are indicated for peptides that were more abundant in Mi (C, F), Ms (D, G) or equal in Mi and Ms (E, H). Mi and Ms kinetic profiles are shown in blue and black, respectively. Fragments enriched in Mi or Ms were mapped onto corresponding regions in the structural models (I, J). The models are shown as cartoons colored in red (R1), green (R2) and blue (R3). Cleavage sites are indicated by arrows for Mi (blue) and Ms (black). See Figure 10—source data 1.

DOI: https://doi.org/10.7554/eLife.36584.028
Figure 10—figure supplement 1. Proteolysis reveals localized differences between M_i and M_s. The medians of the averaged (N = 3) kinetic profiles were compared as ratios for M_i and M_s. The data were compared to the mean of all ratios (red line) and standard deviation (dotted grey line). Peptides within the RD that are enriched in M_i or M_s are shown as colored dots according to location in the RD and labeled with N-term and C-term peptide positions. As a reference the tau RD is colored in red (R1), green (R2), blue (R3) and indigo (R4). Identified peptides are shown with their position in the RD. See Figure 10—source data 1 and 2.

DOI: https://doi.org/10.7554/eLife.36584.029