Supporting Information

Holmes et al. 10.1073/pnas.1301440110

SI Text

Tau Fibril Entry Is an Active Process. Many studies have proposed that amyloid fibrils can directly permeabilize membranes, producing cellular toxicity and potentially providing a direct route to cell entry. We tested this possibility for tau fibrils with cultured cells. We administered tau repeat domain (RD) fibrils or monomer tagged with Alexa Fluor-488 dye to the medium of C17.2 cells, in conjunction with propidium iodide (PI) to indicate membrane disruption. Monomer equivalent of tau fibrils (150 nM) did not increase PI positivity, whereas 5% ethanol caused 72% of cells to score positive. Thus, we found no evidence for direct membrane permeabilization (Fig. S1 *A* and *B*). We tested for saturable uptake by incubating cells with increasing molar concentrations of RD fibrils for 30 min, harvesting the cells by trypsinization (to remove all extracellular tau), followed by Western blot for tau. Maximal RD fibril internalization occurred between 0.67 and 1.33 μM (Fig. S1 *C* and *D*), consistent with a saturable transport mechanism. We next tested for temperature dependence of tau uptake by comparing cells exposed at 37 °C vs. 4 °C. At 4 °C, a nonpermissive temperature for endocytosis, RD fibril uptake was virtually abolished (Fig. S1*E*). Additionally, ATP depletion by sodium azide and deoxyglucose decreased tau fibril uptake as measured by Western blot (Fig. S1*F*). Our data indicate that an active process of endocytosis is most likely.

Tau Fibril Uptake Is Independent of Clathrin or Caveolin. We excluded a role for clathrin- and caveolin-mediated endocytosis in tau fibril uptake. We first blocked clathrin-mediated endocytosis by siRNA knockdown of clathrin heavy chain (CHC) in cultured cells (Fig. S2*A*). CHC knockdown was confirmed by Western blot to monitor CHC protein levels (Fig. S2*B*). We observed robust tau aggregate uptake, even in the presence of significant CHC knockdown. This response was quantified by counting cells (Fig. S2 *C* and *D*). We next tested for colocalization of internalized tau aggregates with caveolin by staining cells with anti-caveolin antibody. We did not find any appreciable colocalization between tau and caveolin (Fig. S2*E*).

SI Materials and Methods

EM. To image tau RD fibrils directly, 5 μL of 4 μM tau RD fibril suspension was placed on a 3 × 3 mm chromo-sulfuric acid-cleaned, water-washed, and air-dried glass coverslip chip and allowed to rest for 5 min. The sample was rinsed once with distilled H_2O and immediately quick-frozen. To image tau fibrils associated with cells, C17.2 cells treated with 150 nM of tau RD fibrils were cultured on 3 × 3-mm glass coverslips, washed for 5 min in mammalian Ringer solution at 37 °C, and transferred to a dish of 2% glutaraldehyde in 100 mM NaCl, 30 mM Hepes, 2 mM $CaCl_2$, pH 7.2 (NaHCa), for 1 h at room temperature. Just before freezing, glass chips were rinsed in three exchanges of distilled H_2O and quick-frozen. Quick-freeze deep-etch EM was performed according to published protocol, with minor modifications (1). Before freezing, glass slips were rinsed with distilled H_2O and frozen by forceful impact against a pure copper block cooled to 4 K with liquid helium. Frozen samples were mounted in a Balzers 400 vacuum evaporator, etched for 20 min at −80 °C, and rotary-replicated with ∼3 nm platinum deposited from a 15° angle above the horizontal, followed by ∼10 nm stabilization film of pure carbon deposited from a 45° angle. Replicas were floated onto a dish of concentrated hydrofluoric acid and transferred through several rinses of distilled H_2O, all containing a loopful of Photo-Flo, picked up on Formvar-coated copper grids, and photographed with a JEOL 100CX transmission electron microscope with an attached AMT digital camera.

Surface Plasmon Resonance. Surface plasmon resonance (SPR) experiments were performs on BIAcore 2000 SPR instrument (GE Healthcare-BIAcore). BIAcore sensor chip CM-5 (GE Healthcare) was activated by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and NHS in 1:1 ratio for 7 min. One flow cell was immobilized with RD tau fibrils (20 μg/mL in 10 mM sodium acetate, pH 4.0) on a Biacore CM-5 sensor chip at a flow rate of 5 μL/min. The remaining unbound area was deactivated by passage of 1 M ethanolamine, pH 8.5. One flow cell was used as a reference cell by activating and blocking with 1 M ethanolamine without any protein. Then, TAT peptide and HJ9.3 antibody were injected at the indicated concentrations in filtered, degassed 0.01 M Hepes buffer, 0.15 M NaCl, 0.005% surfactant P20, pH 7.4, at a flow rate of 10 μL/min. All the samples were run in duplicate. After each run with a single peptide or antibody concentration, the surface of the chip was regenerated by using 10 mM glycine, pH 1.7, to remove the bound RD tau fibrils. Data analysis was performed by using BIAevaluation software (GE Healthcare-BIAcore).

Lentiviral Transduction and Quantitative RT-PCR. *Ext1* shRNA viral vectors from the RNAi Consortium collection (1) were acquired from the Washington University RNAi Core (target sequence, CCCTTACTACTATGCTAATTT). *Ext1* and *luciferase* shRNA control viral vectors were used to produce lentiviral particles as described previously (2). Lentivirus containing media was concentrated 10× by using a LentiX Concentrator (Clontech), and 2 μL of virus suspension was added to mouse primary hippocampal neurons at 0 d in vitro (DIV) in a final volume of 200 μL. Neurons were treated with FL tau-488 fibrils or transferrin-488 on DIV 7 for 3 h before analysis by flow cytometry. Knockdown efficiency of *Ext1* shRNA was tested by using C17.2 neuronal cell lines grown in 24-well plates. A total of 5 μL of *Ext1* virus suspension was added in a final volume of 500 μL of complete media. RNA was harvested 7 d after transduction and purified by using the RNeasy kit (Qiagen). Transcript levels relative to GAPDH were determined by quantitative PCR (ΔCT method) by using an ABI Prism 7900HT sequence detection instrument (Applied Biosystems).

Preparation of α-Synuclein and Huntingtin Fibrils. Recombinant α-synuclein protein was produced in *Escherichia coli* by using previously described methods (3, 4). α-Synuclein protein was dialyzed overnight in 10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM DTT. Recombinant α-synuclein monomer (2 mg/mL) was incubated in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl for 72 h at 37 °C with shaking at 1,000 rpm in an Eppendorf Thermomixer R (Eppendorf North America, Hauppauge, NY; cat. 022670107) to induce fibrillization. Fibril concentration was calculated by centrifuging the fibril reaction mix at 15,000 × *g* for 15 min to separate fibrils from monomer. The concentration of α-synuclein monomer in the supernatant was determined in a BCA protein assay according to the manufacturer's instructions, using a BSA standard curve. The measured decrease in α-synuclein monomer concentration was used to determine the concentration of fibrils in the 72 h fibril reaction mixture.

Synthetic Htt(Q50), comprising the exon 1 fragment, was synthesized with fluorescein conjugated via the N-terminal primary amine by Fmoc chemistry at the Keck Biotechnology Resource Laboratory of Yale University. Crude peptide was solubilized in

formic acid and dialyzed into phosphate buffer solution. Fibrillization of Htt(Q50) occurred at room temperature for 24 h.

All aggregates were sonicated by using a Sonicator 3000 (Misonex) at a power of 3 for 30 s immediately before use.

1. Moffat J, et al. (2006) A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell* 124(6):1283–1298.
2. Sasaki Y, Vohra BP, Baloh RH, Milbrandt J (2009) Transgenic mice expressing the Nmnat1 protein manifest robust delay in axonal degeneration in vivo. *J Neurosci* 29 (20):6526–6534.
3. Giasson BI, et al. (2003) Initiation and synergistic fibrillization of tau and alpha-synuclein. *Science* 300(5619):636–640.
4. Huang C, Ren G, Zhou H, Wang CC (2005) A new method for purification of recombinant human alpha-synuclein in Escherichia coli. *Protein Expr Purif* 42(1): 173–177.

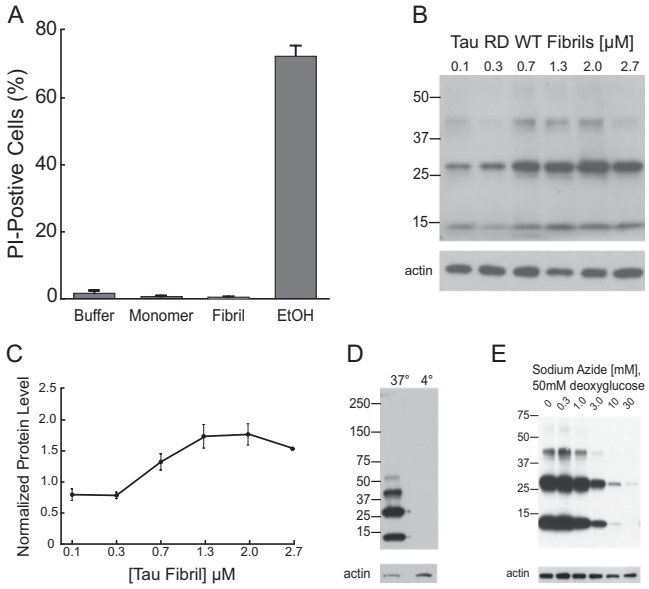


Fig. S1. Recombinant tau RD fibrils are actively internalized by C17.2 neural cells in culture. (*A*) Tau RD fibrils do not disrupt the plasma membrane. Cells were incubated with 150 nM Tau RD-488 fibrils, monomer, buffer, and PI for 3 h. Cells were incubated for the same period with 5% ethanol as a positive control for PI staining. The percentage of PI-positive cells was quantified by direct counting via microscopy ($n = 4$ replicates per experiment, 100 cells counted per replicate). (*B*) Tau RD fibril internalization is saturable. Cells were incubated with tau RD-HA fibrils at the indicated concentrations for 30 min. Trypsinized cell lysates were loaded onto a gradient gel and tau RD fibrils were probed with an anti-HA antibody. (*C*) Quantification of data from *B* from two separate Western blots. Error bars show SEM. (*D*) Tau RD fibril uptake is temperature-dependent. Tau RD fibrils were added to cells at 150 nM and were incubated at 37 °C or 4 °C for 1 h. (*E*) Tau RD fibril uptake requires ATP. Cells were pretreated at the indicated concentrations of sodium azide and 50 mM deoxyglucose before a 1-h application of 150 nM tau RD fibrils.

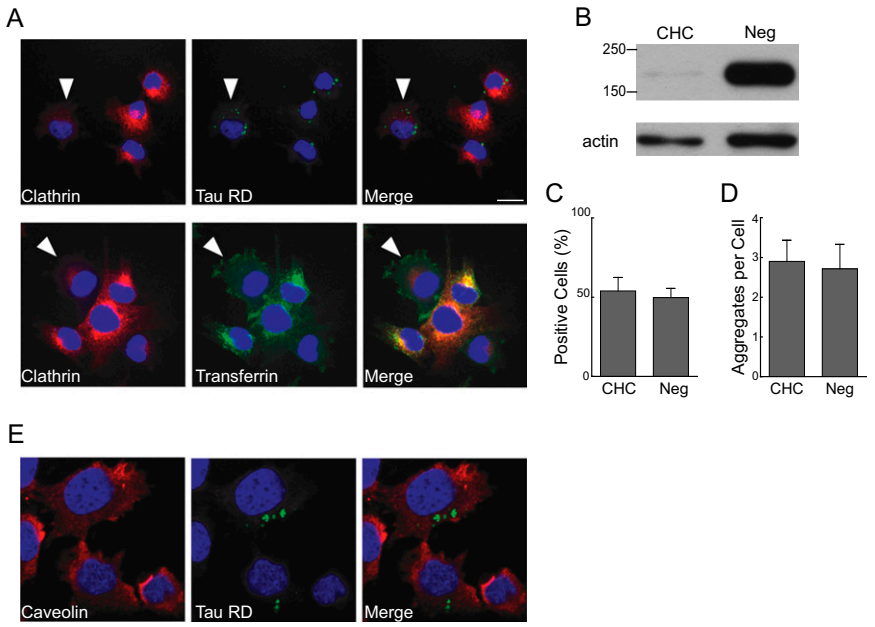


Fig. S2. Tau RD fibril internalization is independent of clathrin- and caveolin-mediated endocytosis. (*A*) CHC is not required for tau RD fibril internalization. C17.2 cells were transfected separately with anti-CHC or AllStars Negative Control siRNA for 24 h and then treated with 150 nM tau RD-488 fibrils (green) or 25 μg/mL Tfn-488 (green) for 1 h. Arrows designate CHC-depleted cells as measured by anti-CHC immunostain (red). (Scale bar: 20 μm.) (*B*) Cells transfected with CHC or negative control siRNA were harvested and immunoblotted with mouse anti-CHC antibody. (*C* and *D*) Cells from *A* were counted and scored for the presence or absence of tau RD aggregates (*C*) and the number of aggregates per cell (*D*). Two hundred cells were counted per experiment, and the graphs represent the average of two independent experiments. Error bars show SEM. (*E*) Cells were treated with 150 nM tau RD-488 fibrils for 1 h, washed with PBS solution, and stained for caveolin-1. No colocalization was observed.

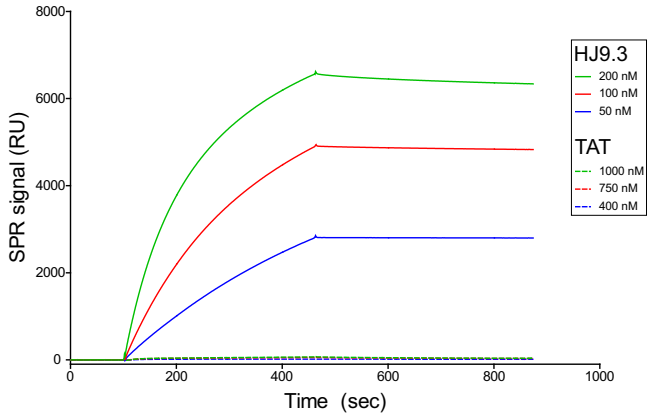


Fig. S3. Overlay of representative SPR sensorgrams resulting from the injection of TAT (dashed lines) or anti-tau antibody HJ9.3 (solid lines) at the indicated concentrations over immobilized tau RD fibrils. RU, resonance units. TAT exhibits no detectable binding to tau RD fibrils.

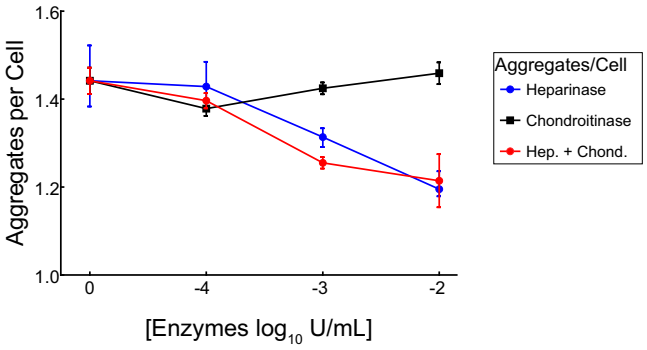


Fig. S4. Average number of aggregates per cell for tau fibril internalization in the presence of heparinase III or chondroitinase AC as measured by automated microscopy analysis. Approximately 40,000 cells were analyzed for each condition, run in duplicate. Error bars show SEM.

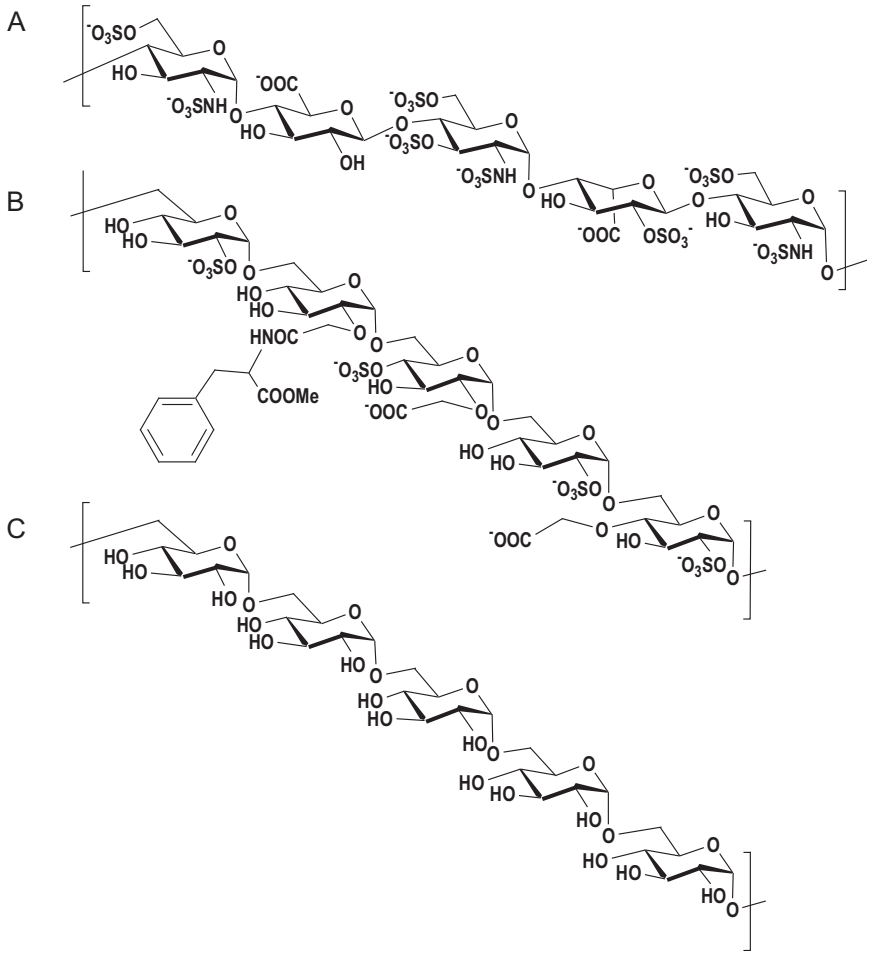
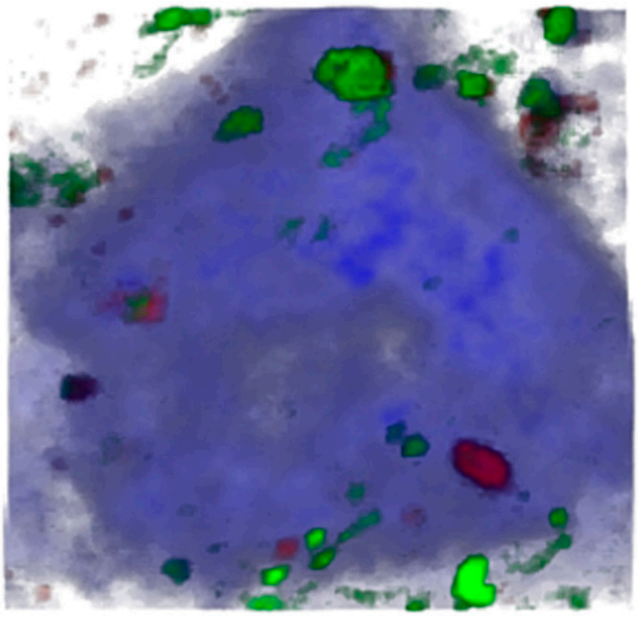


Fig. S5. Schematic structure of heparin, heparin mimetic F6, and polymeric dextran. (*A*) Representative heparin fragment consisting of repeating disaccharide units of D-glucosamine and D-glucuronic acid or L-glucuronic acid. The amounts and distribution of sulfate groups are highly diverse. (*B*) Representative fragment of the heparin mimetic F6 derived from dextran T5 (molecular weight, 5,000 Da). A total of 70% of the F6 glucosidic units contains a sulfate group, 61% a carboxymethyl group, and 15% a phenylalanine methyl ester group. F6 was synthesized, analyzed, and structurally modeled as in the study of Papy-Garcia et al. (1). (*C*) Polymeric dextran.

1. Papy-Garcia D, et al. (2005) Nondegradative sulfation of polysaccharides. Synthesis and structure characterization of biologically active heparan sulfate mimetics. *Macromolecules* 38(11):4647–4654.



Movie S1. Rotating 3D projection of tau aggregates and transferrin internalized into a neuron. Blue indicates NeuN, red indicates tau aggregates, and green indicates transferrin.

Movie S1