Supplemental Experimental Procedures

Development of caspase-6 cleaved tau neoepitope antibodies

Protein expression

Tau proteins (aa 1-441, aa 14-441, and aa 1-402) were expressed in *E. coli*. Human tau proteins were cloned into pET23a vectors with a 10x-histadine tag at the C-terminus for tau 1-441, a 6x-histadine tag at the N-terminus for tau 1-402, and Hisx6-smt3 N-terminal fusion for tau 14-441. *E. coli* strain BL21(DE3) Codon plus RP (Aligent) was transformed with the expression vectors and 5 mL cultures grown overnight in LB medium supplemented with 100 µg/ml carbenicillin. Overnight cultures were added to 1.5 L LB/carbenicillin medium and grown at 37C for ~3h to OD = 0.6-0.7. Tau expression was then induced with 1 mM IPTG at 37C for 4 hr. Cells were pelleted, lysed, and clarified by centrifugation. Soluble protein was then loaded onto a 1 mL cobalt talon resin gravity column. The column was washed with 20 mL 100 mM tris pH 8.0 and 150 mM NaCl. Tau protein was eluted with 5 mL 100 mM tris pH 8.0, 150 mM NaCl, and 500 mM imidazole. DTT was added to the eluant at a concentration of 1 mM. At this point tau 14-441 was cleaved with ULP1 in 100 mM tris pH 8.0, 150 mM NaCl, 500 mM imidazole, 2 mM DTT at a 1:100 molar ration overnight at 4C. The proteins were then concentrated to ~3 mL on a vivaspin turbo 10 kDa ultrafilter (Sartorius). The protein was then loaded onto a Superdex 200PG size exclusion column equilibrated and run with 100 mM phosphate buffer pH 7.5, 100 mM NaCl, and 1 mM DTT. Fractions containing clean tau were combined, concentrated to ~2 mg/ml, and flash frozen on liquid nitrogen.

ELISA analysis of neoepitope antibodies

The binding of neoepitope antibodies to antigenic tau peptides was evaluated by ELISA using 96-well microplates (Corning) coated with 100 µL of 1 µg/ml protein (recombinant full-length tau (R&D), tau 1-402, or BSA-tau peptide I (H^{14}AGTYGLGDRK^{24}C) and incubated overnight at 4C. The plates were washed 3 times with PBST and blocked with 1% BSA in PBST for 2h at 37C. Neoepitope antibodies were serially diluted in 6-fold dilutions, added to the ELISA plates, and incubated for 1h at 37C. Plates were washed three times with PBST and anti-mouse-HRP (Sigma; 1:5000; 100 µL/well) was added for 30 min at 37C, followed by additional PBST washes. 100 µL/well TMB substrate was used for 30 mins, followed by 50 µL/well 1N HCl, and absorbance at 450 nm read in a Molecular Devices Plus 384 plate reader.

Western blot analysis of neoepitope antibodies

The selectivity of neoepitope antibodies for caspase-cleaved tau was evaluated by western blot. Tau proteins were boiled in sample buffer at 90C for 5 mins and loaded onto a 4-12% gradient Bis-Tris polyacrylamide gel in denaturing buffer (NuPAGE MES-SDS Running Buffer). Samples and PageRuler Prestained Protein Ladder (ThermoFisher Scientific) were electrophoresed until the dye front reached the bottom of the gel (ca. 1h at 200 V). Samples were then transferred to PVDF membranes (Novex) using iBlot2 dry transfer (ThermoFisher Scientific). Membrane was then stained with revert 700 total protein stain and imaged on the LI-COR Odyssey

clx. Membranes were then blotted using the following procedure: membrane blocked with Intercept® TBS Blocking Buffer (LI-COR) for 1 hr and room temperature and washed three times with TBS/tween (5 mins/wash). Primary antibody in Intercept® TBS Blocking Buffer with 0.2% tween 20 was added and incubated with membrane overnight at 4C. Membrane was then washed three times in TBST (10 mins/wash), and secondary antibody added (anti-mouse IRDye® 800CW; LI-COR; 1:5000) and incubated for 1h at room temperature. Membrane was washed three times in 20 ml TBST (20 mins/wash). Blots were imaged on a LI-COR Odyssey clx Imaging System.

Analysis of antibody specificity

To evaluate the specificity of mAbD402 and mAbD13 to detect cleaved tau by immunofluorescence (IF), we performed antigen competition assays. mAbD402 and mAbD13 were preincubated with the peptides used as the immunogens for antibody generation (Table S1). Two immunohistochemistry experiments were run in duplicates in 96-well plates. In the primary antibody step, induced neurons (iNs) were pre-incubated with D402- or D13- immunogenic peptides, and the other two wells were untreated. All other experimental parameters remained constant. In each reaction, we also included wells in which the primary antibody was replaced by saline buffer as a negative control. We observed IF signal only in the wells incubated with the primary antibody in the absence of the cognate immunogenic peptide.

Cell lines

Neuronal differentiation

All medium, reagents, and supplements for iPSC culture and differentiation were purchased from Invitrogen (ThermoFisher Scientific), and doxycycline, dimethylsulfoxide (DMSO), cytosine β -D-arabinofuranoside (Ara-C), and poly-D-lysine from Sigma. For pre-differentiation, iPSCs were incubated with doxycycline (2 µg/mL) for 3 days at a density of 2 x 10^6 cells/ well in six-well plates coated with Matrigel (Corning) in knockout Dulbecco's modified Eagle's medium (KO-DMEM)/ F12 medium containing N2 supplement, non-essential amino acids (NEAA), mouse laminin (0.2 mg/mL), brain-derived neurotrophic factor (BDNF, 10 ng/mL; Peprotech), neurotrophin-3 (NT3,10 ng/mL; Peprotech), and Y-27632 (Peprotech). The medium was changed daily and Y-27632 was removed from media after day 2. For maturation, precursor cells were dissociated, counted, and sub-plated in 24 or 96-well plates (50 or 25 x10^3 cells/ well, respectively) coated with poly-D-lysine (PDL) and laminin in maturation medium containing 50% DMEM/F12, 50% Neurobasal-A medium, 0.5 x B27 supplement, 0.5 x N2 supplement, GlutaMax, NEAA, mouse laminin (1 mg/mL), BDNF (10 ng/mL), and NT3 (10 ng/mL). Half of the medium was replaced on day 7 and again on day 14, and the medium volume was doubled on day 21. After that, one-third of the medium was replaced weekly.

Western blot protein analysis

iNs were harvested, washed in PBS, and lysed in N-PER buffer containing protease and phosphatase inhibitors (ThermoFisher Scientific). Lysates were sonicated using a water sonicator (Epigentek) for 5 min,

followed by centrifugation at 15,000 g for 15 min at 4°C. Total protein concentration was quantified using Bradford assay (Biorad) and a Gemini XPS microplate reader (Molecular Devices). Protein lysates were mixed with 4x Laemmli buffer and incubated at 95°C for 5 minutes. Western blot analyses were performed using Mini-PROTEAN Tetra system, 4-15% Mini-PROTEAN TGX Precast Protein Gels, dual-color precision plus protein standards (Biorad), or human recombinant tau protein ladder (Sigma). Gels were transferred onto PVDF membranes (Biorad) using standard procedures, followed by blocking in 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST) for 1 h, and overnight incubation with primary tau antibodies at 4°C (PHF-1, Gift from Dr. Peter Davies, mouse, 1:1000; TauC3 (D421), Invitrogen, AHB0061, mouse, 1:1000; mAb.D402, ChemPartner, clone 47G7B5, mouse, 1:1000; mAbD13, ChemPartner, clone 5G4-1C5, mouse, 1:1000; T18, Gift from Dr. Rakez Kayed, rabbit, 1:1000; MC1, Gift from Dr. Peter Davies, mouse, 1:1000; HT7, Invitrogen, MN1000, mouse, 1:1000; 3R Tau, Millipore, 05-803, mouse, 1:1000; 4R Tau, Millipore, 05-804, mouse, 1:1000). After three washes with TBST, membranes were incubated with corresponding IRDye fluorescent secondary antibodies in intercept blocking buffer for 1h and scanned using an Odyssey Clx imaging system (Li-cor). Protein band intensities (pixel mean intensity) were quantified using Image J gel analyzer (NIH) following normalization to GAPDH (Cell signaling Technologies, 14C10, rabbit, 1:1000) endogenous control bands. For native (non-denaturing) electrophoresis, SDS and methanol were omitted, and PVDF was replaced by nitrocellulose membrane (Biorad). Unstained protein standard and Coomassie G-250 (Invitrogen) were used for molecular weight estimation of the native proteins.

Immunocytochemistry and image analysis

Cells were fixed with 4% paraformaldehyde (Thermo Fisher Scientific) in PBS, directly in 96-well microplates (Greiner) for 15 min and blocked with 5% bovine serum albumin (Sigma) in PBS with 0.01% Triton X-100 for 1h at room temperature. Next, cells were incubated with primary antibodies overnight at 4°C (Active caspase-6 (cleaved at Asp179), Aviva Systems, OAAF05316, rabbit, 1:500; MAP2, Novus Biologicals, NB300213, chicken, 1:500; CTIP2, Abcam, ab20448, rabbit, 1:500; SATB2, Abcam, ab51502, mouse, 1:500; Vglut1, Invitrogen, 482400, Rabbit, 1:500; Nanog, Cell Signaling Technology, 4903T, Rabbit, 1:500; Oct4, Cell Signaling Technology, 2840T, Rabbit, 1:500; Sox2, Cell Signaling Technology, 3579T, Rabbit, 1:500; mAb.D402, ChemPartner, clone 47G7B5, mouse, 1:500; mAb.D13, ChemPartner, clone 5G4-1C5, mouse, 1:500). After three washes with PBS-T (0.05% Tween-20), cells were incubated with the corresponding secondary antibodies conjugated with Alexa Fluor 488, 555 or 647 (Invitrogen) for 1h at room temperature, followed by three washes with PBS-T, and 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) counterstaining for nuclei identification. Cells were stored submerged in PBS at 4°C until imaging. Multiplex immunofluorescent (IF) images were acquired directly from the 96-well plates using an IN Cell Analyzer 6500HS confocal imager (GE Healthcare) with a 20x/0.75 objective (Nikon Plan Apo).