Stem Cell Reports, Volume *9*

Supplemental Information

Scalable Production of iPSC-Derived Human Neurons to Identify Tau-Lowering Compounds by High-Content Screening

Chao Wang, Michael E. Ward, Robert Chen, Kai Liu, Tara E. Tracy, Xu Chen, Min Xie, Peter Dongmin Sohn, Connor Ludwig, Anke Meyer-Franke, Celeste M. Karch, Sheng Ding, and Li Gan

Supplemental Figures and Legends

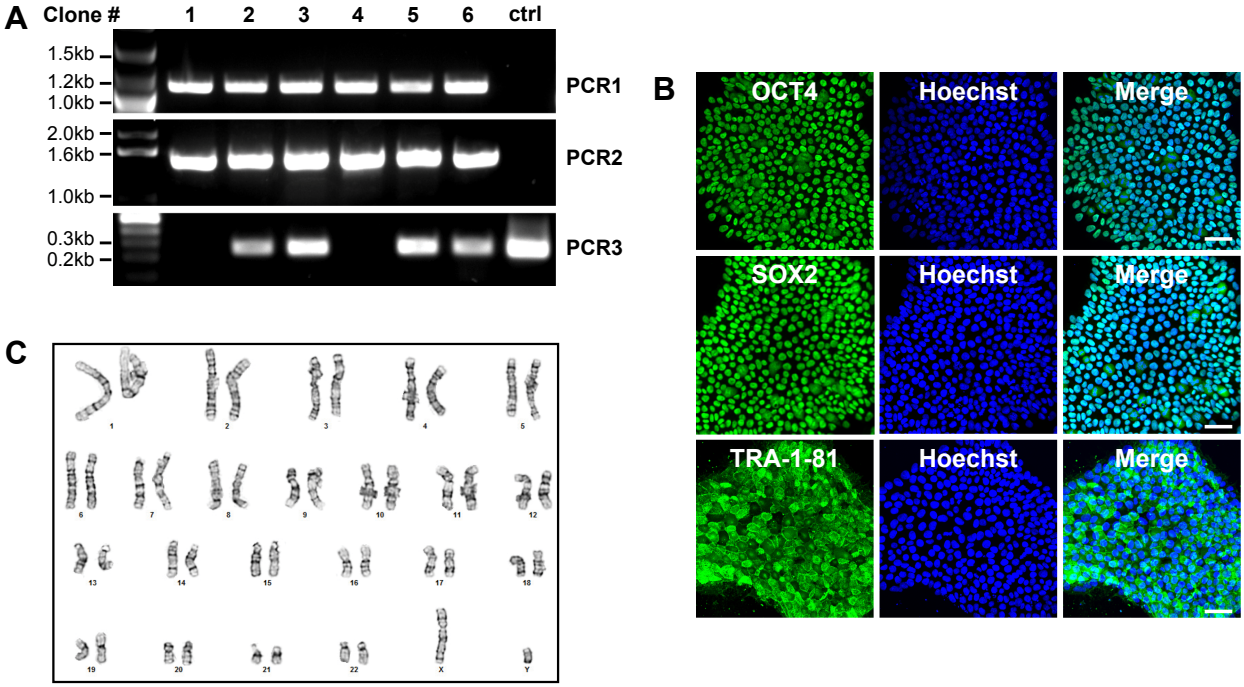

Figure S1. Characterization of i3N iPSC line. Related to Figure 1
(A) PCR1-3 results of six puromycin-resistant clones. The original WTC11 iPSC line served as control (ctrl).
(B) Representative images showing immunostaining of i^{3}N iPSC clone 1 for the pluripotency markers OCT4,SOX2, and TRA-1-81. Nuclei were labeled by Hoechst. Scale bar, 50 μm
(C) G-banded karyotype analysis of i^{3}N iPSC clone 1

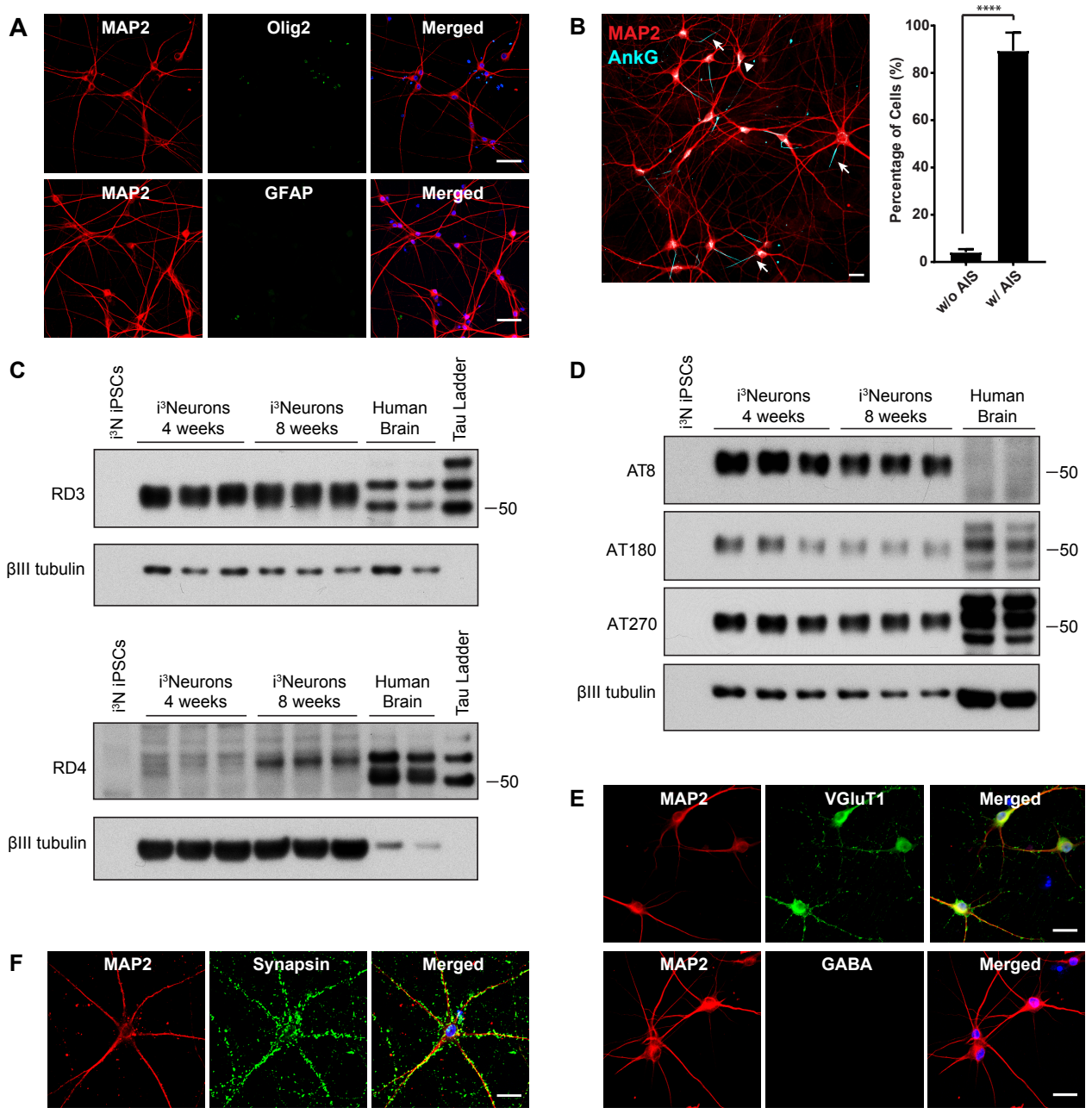

Figure S2. Characterization of i³Neurons. Related to Figure 1

(A) Representative images of immunocytochemical staining show no expression of the oligodendrocyte maker Olig2 or the astrocyte marker GFAP in mature 8-week-old i³Neurons. Nuclei were labeled by Hoechst. Scale bar, 50 μm.

(B) Representative image of immunocytochemical staining of AIS marker AnkG and dendritic marker MAP2 (left) and quantification of i³Neurons with or without AIS (right). Arrow, i³Neuron with AIS; arrowhead, i³Neuron without AIS. Scale bar, 20 μm. Values are means ± SEM. Data are from three independent culture, total N=331 neurons. ****p<0.0001, two-tailed unpaired student t-test.

(C) Western blot analysis of 3R tau isoforms (RD3) and 4R tau isoforms (RD4) in i³N iPSCs and 4- and 8-week i³Neurons and healthy human brain. Neuron loading is determined by βIII tubulin.

(D) Western blot analysis of tau phosphorylation at Ser202/Thr205 (AT8), Thr231 (AT180) and Thr181 (AT270) in i³N iPSCs and 4- and 8-week i³Neurons and healthy human brain. Neuron loading is determined by βIII tubulin.

(E) Representative images of immunocytochemical staining show uniform expression of the glutamatergic neuronal marker vGlut1 and absence of GABA-positive inhibitory neurons in i³Neurons after 4 weeks of differentiation. Nuclei were labeled by Hoechst. Scale bar, 25 μm

(F) Representative images of immunocytochemical staining for the pre-synaptic marker synapsin-1 in mature 8-week-old i³Neurons. Nuclei were labeled by Hoechst. Scale bar, 25 μm

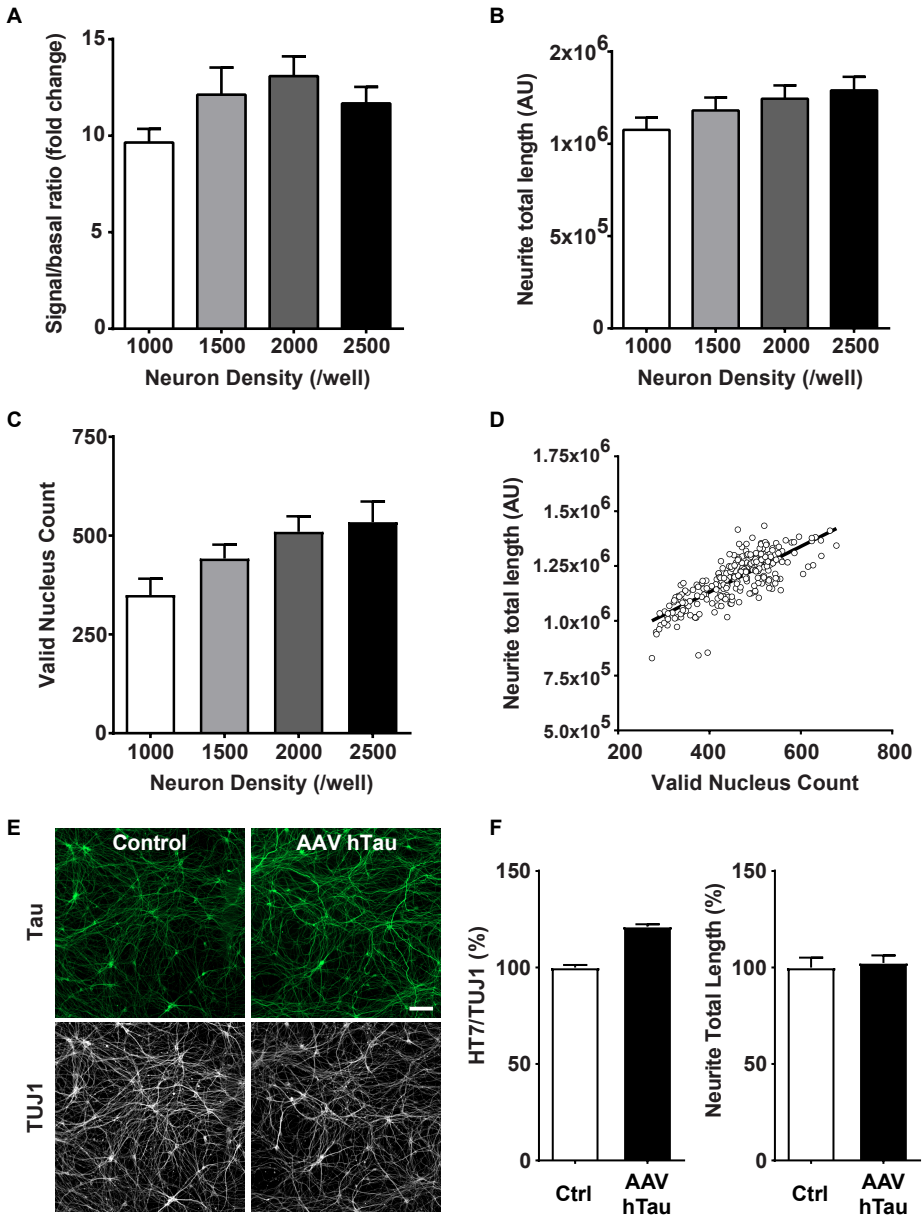

Figure S3. Optimization and validation of the tau HCS assay. Related to Figure 3

(A-D) Optimizing i^{3}Neuron density for HCS assay. Signal-to-background ratio **(A)**, neurite total length **(B)** and valid nucleus count **(C)** of high-content total tau assay were determined at seeding densities of 1000, 1500, 2000, and 2500 cells/well in 384-well plates. The signal-to-background ratio was calculated according to automated quantification of human tau signal (normalized to the TUJ1 signal) from control wells and background wells as described in Figure 3B. **(D)** Neurite total length is highly correlated with valid nucleus number. Pearson r=0.78. Results are from one experiment. For each density, N= 32 control wells and 32 background wells. Values are means ± SEM. AU: artificial unit.

(E,F) Increased human tau levels were detected by HCS assay. Representative high-content images of tau and βIII tubulin **(E)** and automated quantification of tau levels and neurite total length **(F)** from i^{3}Neurons infected with AAV full-length human tau. Scale bar, 100 μm. Results are from one experiment, N=16 control wells and AAV htau wells, values are means ± SEM. relative to control.

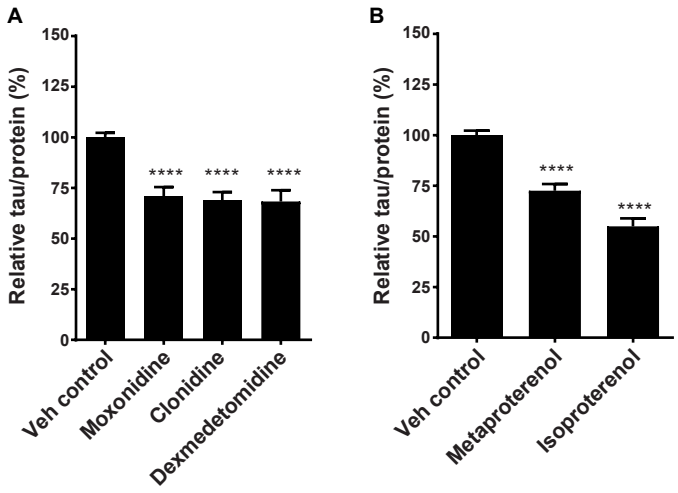

Figure S4 Activation of α- and β-AR reduces endogenous tau levels in i^{3}Neurons derived from independent i^{3}N iPSC line. Related to Figure 5

An independent iPSC line (F12486.13) was engineered to i^{3}N iPSC line at AAVS1 locus and differentiated to i^{3}Neurons using techniques described in Figure 1. 3-day incubation with α-AR agonists moxonidine (30 μM), clonidine (100 μM) and dexmedetomidine (100 μM) **(A)** or β-AR agonists metaproterenol (30 μM) and isoproterenol (30 μM) **(B)** significantly reduced endogenous tau levels in i^{3}Neurons. Human tau levels were quantified by HT7-Tau5 ELISA and normalized to protein level. Values are means ± SEM. relative to vehicle control. Data are from three independent experiments performed in triplicate, N=9 per treatment. ****P < 0.0001, STATA mixed model

Supplemental Table

Table S1. Real-time qPCR primers list. Related Figure 2

Gene Name	Description	Forward	Reverse
ANK2	adaptor protein, VGSC initiation zone	AGC ACT CTT TCC CCA AAC TC	TCT CTG ATG TTT CTG TTC CTG G
TUBB3	pan-neuronal	TTT GGA CAT CTC TTC AGG CC	TTT CAC ACT CCT TCC GCA C
MAP2	pan-neuronal	CAG GAG ACA GAG ATG AGA ATT CC	CAG GAG TGA TGG CAG TAG AC
NCAM1	pan-neuronal	GAC ATC ACC TGC TAC TTC CTG	GGC TCC TTG GAC TCA TCT TTC
DCX	pan-neuronal	TCA GGG AGT GCG TTA CAT TTA C	GTT GGG ATT GAC ATT CTT GGT G
RBFOX3	pan-neuronal	GTA GAG GGA CGG AAA ATT GAG G	CAT AGA ATT CAG GCC CGT AGA C
ELAVL1	pan-neuronal	AGA CGC CAA CTT GTA CAT CAG	ATA AAC GCA ACC CCT CTG G
GLUR1/GRIA1	AMPA receptors	TGA TGG AAA ATA CGG AGC CC	CTT CCC GGA CCA AAG TGA TAG
GLUR2/GRIA2	AMPA receptors	TCT CTG GTT TTC CTT GGG TG	CAG TCA GGA AGG CAG CTA AG
GRIA3	AMPA receptors	GTC TTT GGT TTT CCT TGG GTG	CAG CGA GAT TGG CAG TAT AGG
GRIA4	AMPA receptors	CAA GGA GAG GAA ATG CTG GG	ACG TCC ATA GTG GTC AAA CTG
NR1/GRIN1	NMDA receptors	GAG AAG GAG AAC ATC ACC GAC	GTC CCC ATC CTC ATT GAA CTC
NR2A/GRIN2A	NMDA receptors	CGC TGT CAT ATT CCT GGC TAG	GCA CTG TCC CAA ATC GAA AAG
NR2B/GRIN2B	NMDA receptors	CGT GGC TGT CTT TGT CTT TG	ACC CCA GAG CAA CCA AAT AG
VGLUT1/SLC17A7	glutamate related, glutamate transport	TCA ATA ACA GCA CGA CCC AC	TCC TGG AAT CTG AGT GAC AAT G
VGLUT2/SLC17A6	glutamate related, glutamate transport	TGG TCG TTG GCT ATT CTC ATA C	ATA CTG GCA TAT CTT GGA GCG
CAMK2A	glutamate related, glutamate transport	CAG TTC CAG CGT TCA GTT AAT G	TTC GTG TAG GAC TCA AAA TCT CC
GABRA2	GABA Receptor	TTA GCC AGC ACC AAC CTG	TCG TCA AGA TCA GGG CAA AAG
GABRB1	GABA Receptor	TGC TGG AAA TAC ATG GTG AGT G	AGT GGA CAG ATT GCT CAA AGG
GAD65/GAD2	GABA Synthesis	CAC CAT CTC ATA TCC TTC TCG G	AAA CTA TGG CTG ATG TGG AGG
GAD67/GAD1	GABA Synthesis	CAC TCA CAA GGC GAC TCT TC	GAC CCC AAT ACC ACT AAC CTG
VGAT/SLC32A1	GABA Transporter	AGA TGA TGA GAA ACA ACC CCA G	CAC GAC AAG CCC AAA ATC AC
SYN1	pre-synaptic	CCC CAA TCA CAA AGA AAT GCT C	ATG TCC TGG AAG TCA TGC TG
PSD95/DLG4	post-synaptic	AGT CAG AAA TAC CGC TAC CAA G	CCG TTC ACC TGC AAC TCA TAT C
SHANK3	post-synaptic	ATC AAG ACC CAC AAA GAC TCG	CCC TTC AGG TTG ATA TCG CTG
KCNC1	potassium channel,Kv3 type/shaw related fast spiking	TCT TCG AGG ACC CGT ACT C	CTC GAT CTC CGT CTT GTT CAC

KCNC3	potassium channel,Kv3 type/shaw related fast spiking	GAC AAG AGC CCC ATC ACG	GCT TAC GCC AGT CTT GGG
RELN	telencephalic	TCT ACC TTC CAC TCT CCA CC	ATC ACA AAT CCC TCG TCC TG
SOX5	telencephalic	TCA TGG TGT GGG CTA AAG ATG	TGG CTG TTT CTC TAG GTT TGT C
BRN2/POU3F2	telencephalic	AAA GTA ACT GTC AAA TGC GCG	GCT GTA GTG GTT AGA CGC TG
OTX2	telencephalic	CCA GAC ATC TTC ATG CGA GAG	GGC AGG TCT CAC TTT GTT TTG
LHX2	telencephalic	GGT CTT CCC TAC TAC AAT GGC	GTC GTT TTC GTT GCA GCT TAG
ROR-BETA/RORB	telencephalic	GCA GAC CCA CAC CTA TGA AG	TCC ATG AAG CCT GTT ATC CG
TBR1	telencephalic	GGA GCT TCA AAT AAC AAT GGG C	GAG TCT CAG GGA AAG TGA ACG
CUX1	cortical(layer 2/3)	TCA CCT CTT CAT AGT CAG CCT	CAG CCA GAT CTC ACA GCT TG
SATB2	cortical	CCT TAC GCA GAA TCT CAG ACA A	CCA GAT ATC TAC CAG CAA GTC AG
CTGF	cortical	GCT CGG TAT GTC TTC ATG CTG	GAA GCT GAC CTG GAA GAG AAC
TLE4	cortical	GAT GAG GAT TCT TTG GAC TGG A	GTG CTG TCG CTC AAG TTT G
CTIP2/BCL11B	cortical	GTT GTG CAA ATG TAG CTG GAA	GAA GAT GAC CAC CTG CTC TC
CLIM1/LDB2	cortical	ACT TCT GAA ACA AGC AGG TCT	CAA ACT TCA CCC TCA ACT ACC T
NR2F1	cortical	AGA TGT AGC CGG ACA GGT AG	CTC AAG AAG TGC CTC AAA GTG
BHLHB5/BHLHE22	cortical	CAT ACT CCA TCC CAA CTC CAC	GAG GCA AGA ACT GAG GAG AAG
DIAPH3	cortical	GTA GCG TTG TTT TCT GAT CTG C	GCT GGA ACT TGT ATT GCT AAT GG
FEZF2	cortical	GTC AGC TTG TGG TTC TTG TAG T	ACG CTC AAC ACG CAT ATC C
FOG2/ZFPM2	cortical	ATG GCC TTC GTA GTT GTA CAC	TGG TTG CTG GAT GTG ACT TG
OTX1	cortical	ACC ACG CAG TCC TTC AG	GAT CCA GGT AGA TGG TGA ACG
PAX6	NPC marker	GCC CTC ACA AAC ACC TAC AG	TCA TAA CTC CGC CCA TTC AC
NESTIN/NES	NPC marker	TGC GGG CTA CTG AAA AGT TC	GGC TGA GGG ACA TCT TGA G
GFAP	Glia	GAA GCT CCA AGA TGA AAC CAA C	CCT CCA GCG ATT CAA CCT TT
OLIG2	Glia	AGA TAG TCG TCG CAG CTT TC	GTT CTC CCC TGA GGC TTT TC
MBP	Glia	TCC TCT CCC CTT TCC CTG	CCC AAG ATG AAA ACC CCG TAG
NGN2/NEUROG2	neural-specific bHLH transcription factor	TAC CTC CTC TTC CTC CTT CA	GAC ATT CCC GGA CAC ACA C
GAPDH	internal ctrl	GGC CAT CCA CAG TCT TCT G	TCA TCA GCA ATG CCT CCT G

Supplemental Experimental Procedures

Generating and selecting integrated, isogenic, and inducible neurogenin-2 (i^3N) iPSC clones. The original pUCM donor vector, which contains AAVS1 homology arms and the Tet-On 3G system, and a TALEN nuclease expression pair that targets the AAVS1 locus (kind gifts from Dr. Bruce Conklin, Gladstone Institutes) have been characterized. Hemagglutinin-tagged mouse Ngn2 (a kind gift from Dr. Thomas C. Südhof, Stanford University) was subcloned downstream of TRE3G (tet response element) in the pUCM vector (pUCM-Ngn2) with In-Fusion Cloning kits (Clontech Laboratories). A reverse tetracycline-controlled transactivator (rtTA3G, driven by the CAG promoter) and mouse Ngn2, were arranged in a tail-to-tail orientation. A splicing acceptor-linked puromycin in the pUCM vector facilitates selection of clones in which the donor plasmid is integrated at the AAVS1 locus. A well-characterized human iPSC line with a wildtype genetic background (WTC11) and another independent wildtype human iPSC line F12486.13 were used to generate Ngn2-integrated clones by a DNA-In Stem transfection reagent (MTI-GlobalStem). The iPSC lines were maintained on growth-factor reduced Matrigel (Corning) and fed every other day with Essential 8 medium (E8 medium).

The day before transfection, human iPSCs were dissociated into single cell with Accutase and plated at 1.0-1.5x10^6 cells per six-well dish in E8 medium containing the Rho-associated kinase inhibitor Y-27632 (10 μM, Cayman Chemical). For transfection, pUCM-Ngn2 donor vector (2.5 μg) and the TALEN pair (1.25 μg each) were incubated with DNA-In Stem reagent (10 μl) and OptiMEM (250 μl, Invitrogen) for 10 min and added drop-wise to iPSCs. The day after transfection, cells were dissociated with Accutase and plated at 1:6 to 1:12 on six-well dishes. Selection with puromycin (0.1–0.3 μg/ml) in E8 medium plus Y-27632 started 24–48 hours after transfection and continued for ~10 days until stable colonies formed. Parallel control transfections with donor vector but not TALEN pairs facilitated empirical determination of optimal puromycin concentrations for clonal selection. Individual colonies were picked with a P200 pipette tip under an EVOS FL microscope (Invitrogen). Genomic DNA from expanded clones was purified with DNeasy Blood & Tissue Kit (Qiagen) and genotyped with the Q5 High-Fidelity PCR system (NEB) and the following primers: PCR1 (forward: 5′-CTGCCGTCTCTCTCCTGAGT-3′; reverse: 5′-GGGCTTGTACTCGGTCATCT-3’); PCR2 (forward: 5′-GCAGCATCAGTACCTCCTCT-3′; reverse: 5′-AAAAGGCAGCCTGGTAGACA-3′); PCR3 (forward: 5′-CGGTTAATGTGGCTCTGGTT-3′; reverse: 5′-AGGATCCTCTCTGGCTCCAT-3′). Selected integrated Ngn2 clones were frozen in 90% fetal bovine serum (HyClone) and 10% DMSO. Culture medium were routinely tested for mycoplasma contamination.

Electrophysiology. i^3Neurons were submerged in external solution containing 140 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM $MgCl_2$, and 2.5 mM $CaCl_2$, pH 7.4. Patch pipette resistance was 3–5 MΩ. Current clamp recordings of action potentials were done with internal solution containing 135 mM K-gluconate, 10 mM HEPES, 0.1 mM EGTA, 5 mM KCl, 2 mM $MgCl_2$, 4 mM Mg-ATP, and 0.3 mM Na-GTP, pH 7.3. Picrotoxin (100 μM), CNQX (10 μM), and D-AP5 (100 μM) were added to the external solution to block synaptic inputs. The neuron was held at –60 mV, and current was injected in incremental steps of 500-ms duration. Voltage clamp recordings of excitatory postsynaptic currents were done with internal solution containing 120 mM $CsCl_2$, 10 mM HEPES, 10 mM EGTA, 5 mM NaCl, 1 mM $MgCl_2$, 4 mM Mg-ATP, 0.3 mM Na-GTP, and 10 mM QX-314, pH 7.2. AMPA receptor-mediated postsynaptic currents were blocked with CNQX (10 μM). Recordings were done with a Multiclamp 700B amplifier (Molecular Devices), digitized at 10 kHz, and acquired with WinLTP software (version 1.11b, University of Bristol).

Western Blot Analysis i^3N iPSCs and 4- and 8-week i^3Neurons and healthy human brain were homogenized and sonicated at 4 °C in N-PER™ Neuronal Protein Extraction Reagent (87792, Thermo Fisher) containing protease inhibitor cocktail and phosphatase inhibitor cocktail 2 & 3 (Sigma). Designed amounts of protein (by BCA assay) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. After blocking, membranes were labeled with mouse anti-3-repeat isoform or 4-repeat isoform tau (RD3, RD4, Millipore), mouse anti-phospho-Ser202/Thr205 tau (AT8, Fisher), phospho-Thr231 tau (AT180, Fisher), phospho-Thr181 tau (AT270, Fisher), rabbit anti-βIII tubulin (ab68193, Abcam) overnight, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG antibody (Millipore). Bands were visualized by enhanced chemiluminescence.

HCS assay to determine total tau levels. After pre-differentiation, i^3N precursor cells were placed in PDL pre-coated 384-well plates (781946, Greiner) at a density of 2000 cells/well in 50 μl of maturation medium. To simplify the procedure and reduce variability and staining background, no mouse glia were added. To reduce disturbance to

cells and the possibility of contamination, the medium was not changed; instead, fresh maturation medium (25 µl/well) was added with electronic multichannel pipettes on days 7 and 14. On day 18, extra medium was removed with an EL406 Washer/Dispenser (BioTek) to ensure that 50 µl of medium was left in each well. For validation of siRNA or known tau-lowering compounds, stock concentrations of human tau Accell siRNA (GE Dharmacon), salicylate, YM-01, and methylene blue (kind gifts from Dr. Jason E. Gestwicki, University of California, San Francisco) were adjusted to ensure that each well needed 5 µl of reagents to reach the desired final concentration. For HCS, 1280 compounds from LOPAC library were dissolved in DMSO at 10 mM stock concentration and distributed to three and one-half 384-well plates. Each plate contained two columns (32 wells) of DMSO controls, which were used as control wells and background wells (n = 16/each). DMSO or compounds (50 pl) were added with a BioMek FX workstation (Beckman Coulter) to reach a final concentration of 10 µM. Cells were incubated for 3 days (LOPAC), 7 days (siRNA), or 24–72 hours (salicylate, YM-01, and methylene blue) at 37 °C in 5% CO_2.

The immunostaining protocol was similar to that described in Experimental Procedures, but with two modifications: 1% bovine serum albumin (IgG-free and protease-free, Jackson ImmunoResearch) was used to reduce cost, and the permeabilization and blocking steps were combined to reduce disturbance to cells. HT7 antibody was used to detect human tau and TUJ1 antibody was used to label βIII tubulin in neurites. Nuclei were labeled with Hoechst (H3570, Invitrogen). Reagents were added with electronic multichannel pipettes, and all washing steps were done with an automated EL406 Washer/Dispenser. Images from individual wells were acquired with a fully automated ArraySan XTi high-content system (Thermo Fisher) and a 10x objective, which provides sufficient resolution to distinguish neurites. Nine fields encompassing the total well area were captured. Images were auto-quantified with the neuronal profiling module of Cellomics software (Thermo Fisher). TUJ1 immunostaining identified all of the neurites labeled with HT7 (**Figure 3B**), which was used to define the quantification area. Background of the assay was quantified in the absence of HT7 antibody (**Figure 3B**). Multiple cellular and well neuronal features were auto-quantified with Cellomics software, including valid nucleus count (quantification of viable cell number based on nuclei size and intensity), neurite total length (based on TUJ1 staining), and total tau levels (defined by HT7 intensity normalized to TUJ1 intensity).

Due to the lack of a non-toxic tau-lowering compound, Accell tau siRNA is used as positive control and control siRNA is used as negative control (N=42). Assay reproducibility and robustness is evaluated by Z'-factor calculated as follows, where μ_{c+} and σ_{c+} are the mean and standard division of positive control; μ_{c-} and σ_{c-} are the mean and standard division of negative control.

$$Z' = 1 - 3\frac{(\sigma_{c+} + \sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}$$

Computed on a plate-by-plate basis, Z-score for raw value of x_i is defined as

$$Z_i = \frac{x_i - \mu_c}{\sigma_c}$$

where μ_c is the mean of all sample values on a plate and σ_c is the standard division of these values.

Human tau and βIII tubulin ELISA High-binding ELISA plates (Costar) were coated with capture antibodies (2 µg/ml) overnight at 4°C and washed with Dulbecco's phosphate-buffered saline (DPBS). Nonspecific binding was blocked with 3% bovine serum albumin (protease free, fraction V; Roche Biochemicals) (w/v) in DPBS for 2–4 hours with gentle shaking. Diluted standards or samples (50 µl) were added to each well in a final assay buffer containing 0.3% bovine serum albumin and 0.1% Tween-20 (v/v) in DPBS. After 1 h of incubation at room temperature, detection antibodies (50 µl) diluted in final assay buffer were added and incubated overnight at 4°C. The plates were washed with DPBS containing 0.05% Tween-20 and developed with alkaline phosphatase substrate. Chemiluminescence was measured with a SpectraMax M5 (Molecular Devices).

The limit of quantification was defined as the value calculated from the background signal plus three standard deviations.