

Supplementary Online Content

Woerman AL, Patel S, Kazmi SA, Oehler A, Freyman Y, Espiritu L, et al. Kinetics of human mutant tau prion formation in the brains of 2 transgenic mouse lines. *JAMA Neurol*. Published online October 23, 2017. doi:10.1001/jamaneurol.2017.2822

eAppendix. Supplemental Appendix

eFigure 1. Spontaneous Tau Prion Formation in PS19 Mice Is Highly Variable

eFigure 2. Detection of Spontaneous Tau Prion Formation Using Tau(4RD*LM)-YFP(1) Cells

eFigure 3. Spontaneous Tau Prion Formation Is Detectable Before 4 Weeks of Age in Tg(MAPT*P301S^{+/+}) Mice

eFigure 4. Tau Neuropathology in 6- and 24-Week-Old Tg(MAPT*P301S^{+/+}) Mice

eFigure 5. MC1 and HT7 Neuropathology Increases at 24 Weeks of Age in Tg(MAPT*P301S^{+/+}) Mice

eFigure 6. Astrogliosis in Tg(MAPT*P301S^{+/+}) Mice Increases in the Hindbrain at 24 Weeks of Age

eFigure 7. Measuring by Fluorescence per Cell, Spontaneous Tau Prion Formation Occurs by 8 Weeks of Age in Tg(MAPT*P301S^{+/-}) Mice

eFigure 8. Measuring by Percent Cells With Aggregates, Spontaneous Tau Prion Formation Occurs by 8 Weeks of Age in Tg(MAPT*P301S^{+/-}) Mice

eFigure 9. AT8 Neuropathology Is Delayed in Tg(MAPT*P301S^{+/-}) Mice

eFigure 10. Tg(MAPT*P301S^{+/-}) Mice Develop Minimal Tau Neuropathology by 24 Weeks of Age

eFigure 11. Astrogliosis Does Not Increase in Tg(MAPT*P301S^{+/-}) Mice by 24 Weeks of Age

eTable. Power Analysis Determination of Sample Size Needed to Detect Significant Extension in Survival

This supplementary material has been provided by the authors to give readers additional information about their work.

eAppendix. Supplemental Appendix

SUPPLEMENTARY MATERIALS AND METHODS

Mouse samples

Wild-type C57BL/6J, or B6/J, mice and PS19 mice¹ (sometimes referred to as P301S mice) were purchased from the Jackson Laboratory. B6-Tg(Thy1-MAPT*P301S)²⁵⁴¹² mice (sometimes referred to as the hTau.P301S model) were originally generated on a C57BL/6JxCBA/ca background and were subsequently backcrossed for eight generations with marker-assisted backcrossing to confirm the genetic background of the mice was close to 100% C57BL/6J. The mice were then intercrossed to generate homozygous animals. Homozygous B6-Tg(Thy1-MAPT*P301S), or Tg(MAPT*P301S^{+/+}), mice were maintained by intercrossing while the hemizygous P301S^{+/-} mice were generated by backcrossing homozygous animals with wild-type B6 mice. All animals were assessed for routine health daily and received two tests for neurological illness each week. Mice were euthanized using CO₂ followed by cervical dislocation. The brain from each mouse was then removed and bisected at the midline, with the left hemisphere snap-frozen for biochemical analysis and the right hemisphere fixed in 10% buffered formalin for neuropathology. Experiments were performed with approximately half males and half females, unless noted otherwise.

Inoculations

Two-month-old PS19 mice were anesthetized with isoflurane and inoculated with 30 μ L 1 \times DPBS or 1% control brain homogenate diluted in 5% bovine serum albumin into the right parietal lobe. All animals were then assessed twice a week and were euthanized, as described above, 160 days post inoculation (31 weeks of age).

Tau prion assay of PS19 mouse brains

A 10% brain homogenate was prepared using frozen brains from PS19 mice in calcium- and magnesium-free 1 \times DPBS. Brain homogenates were clarified at 1,000 \times g, and protein concentration was quantified using the bicinchoninic acid protein assay (BCA; Pierce). Concentrations were adjusted to 2 mg/mL and diluted 1:40 in 1 \times DPBS. Samples were incubated in 1.25% Lipofectamine 2000 (Thermo Fisher) for 1.5 h, and a final concentration of 78.75% OptiMEM (Thermo Fisher) was added. Samples were then incubated with Tau(4RD*LM)-YFP(2) cells³ for 3 d prior to imaging. Each sample was tested independently twice.

Immunohistochemistry

The formalin-fixed full brains from 31-week-old PS19 mice and half brains from 6-, 12-, 18-, and 24-week-old mice were cut coronally into four pieces, processed through graded alcohols, cleared with xylene, infiltrated with paraffin, and embedded. Once embedded, the tissue was cut into 8 μ m slices, collected on slides, deparaffinized, and used for immunohistochemistry. Primary antibody incubation was performed overnight at room temperature with AT8 (1:250 after autoclaving for 10 min; Thermo Fisher), MC1 (1:400 after autoclaving for 10 min; kindly provided by Peter Davies, Albert Einstein College of Medicine, Bronx, NY), HT7 (1:2,000 after steaming for 20 min; Thermo Fisher) or glial fibrillary acidic protein (GFAP; 1:500; Dako). Mouse on Mouse Blocking Reagent (Vector Laboratories) was used to block slides stained with mouse antibodies (AT8, MC1, and HT7). The Vectastain ABC peroxidase kit (Vector Laboratories) was used to detect bound antibody and was visualized using 3-3'-diaminobenzidine. The slides were counterstained using hematoxylin. Slides containing PS19 mouse brains were imaged on a Zeiss Axio Imager.A1. The B6/J and Tg(MAPT*P301S) slides were imaged using the Zeiss AxioScan.Z1.

Digital images of each slide were then analyzed using Zen Analysis software (Zeiss). For each antibody, a pixel intensity threshold was determined, and the same threshold was applied to all slides stained with that antibody. Regions of interest were drawn around the sensorimotor cortex, striatum, piriform cortex and amygdala, hippocampus, thalamus, hypothalamus, midbrain, and pons, and the percentage of positive pixels was determined in each region.

Proteinase K digestion

Brain homogenate from two 6-week-old and two 24-week-old male Tg(MAPT*P301S^{+/+}) mice were tested for tau resistance to proteinase K (PK) (Thermo Fisher). Nine volumes of the 10% brain homogenates were added to one volume of 10× detergent extraction buffer [5% (vol/vol) Nonidet P-40 (NP-40), 5% (wt/vol) sodium deoxycholate (DOC) in PBS], vortexed, and incubated on ice for 15 min. After samples were centrifuged at 1,000 × g for 5 min, the supernatant was collected and the protein was quantified by BCA. Samples were diluted to 1 mg of protein in a final volume of 400 μL 1× detergent buffer. PK was added to each sample for final concentrations of 0, 0.5, 1, 2.5, and 5 μg, and samples were digested at 37 °C for 30 min while shaking at 550 rpm in an Eppendorf ThermoMixer. PK digestion was terminated by adding phenylmethylsulfonyl fluoride (PMSF; Sigma) to a final concentration of 5 mM. Sarkosyl was added to each sample for a final concentration of 2%, and the samples were centrifuged at 100,000 × g for 1 h at 4 °C. The supernatants were removed from PK-digested samples by aspiration, and the pellets were resuspended in 1× NuPAGE lithium dodecyl sulfate (LDS) sample buffer with 2.5% (vol/vol) β-mercaptoethanol (BME; Sigma) and 1× NuPAGE Reducing Agent. For non-PK-digested samples, supernatant was diluted in an equal volume of 2× NuPAGE LDS sample buffer with 1× reducing agent. All samples were boiled for 10 min and analyzed by immunoblotting.

Sequential detergent extraction

Aggregated tau was isolated from two 6-week-old and two 24-week-old Tg(MAPT*P301S^{+/+}) mice using the multidetergent extraction method described in detail in Watts et al.⁴ Briefly, 10% brain homogenate was exposed to increasingly stringent buffers to isolate aggregated tau prions. After the addition of each buffer, samples were sonicated in a water-bath sonicator for 5 min and centrifuged at 100,000 × g for 30 min at 4 °C. The supernatant was removed following each spin, and the resulting pellet was resuspended in the next buffer. For each step, 100 μL buffer was used. The buffers used, in order, were high salt [(HS); 750 mM NaCl and 5 mM EDTA (Thermo Fisher)]; high salt + Triton X-100 [(HST); 50 mM Tris•HCl, pH 7.5 (Thermo Fisher); 750 mM NaCl; 5 mM EDTA; and 1% (vol/vol) Triton X-100 (Sigma)]; radioimmunoprecipitation buffer [(RIPA); 50 mM Tris•HCl, pH 7.5; 150 mM NaCl; 5 mM EDTA; 1% (vol/vol) NP-40; 0.5% (wt/vol) DOC; and 0.1% (wt/vol) sodium dodecyl sulfate (SDS; Thermo Fisher)]; SDS [50 mM Tris•HCl, pH 7.5; 2% (wt/vol) SDS], and formic acid [(FA); 66% FA in ddH₂O]. Following FA, 80 μL of the supernatant was removed and placed in an Eppendorf tube where the acid was evaporated using a speed-vacuum centrifuge.

After HS, HST, RIPA, and SDS extractions, 60 μL of supernatant was added to 40 μL 4× NuPAGE LDS sample buffer while the FA pellet was resuspended in 40 μL 1× NuPAGE LDS sample buffer. Samples were diluted in 1× Dulbecco's phosphate-buffered saline (DPBS) (HS—1:30; HST—1:10; RIPA—1:2; SDS—1:2; FA—none) with 1× NuPAGE Reducing Agent and boiled for 10 min prior to immunoblotting.

Immunoblotting

All samples prepared for immunoblotting were loaded onto an 8% Bolt Bis-Tris gel (Thermo Fisher). SDS-PAGE was performed using the MES buffer system, and protein was transferred to polyvinylidene fluoride (PVDF) membranes using a semi-dry transfer. Membranes were blocked using blocking buffer [5% (wt/vol) nonfat milk in 1× Tris-buffered saline containing 0.05% (vol/vol) Tween 20 (TBST)] for 30 min at room temperature and then incubated in Tau12 primary antibody (1:10,000) overnight at 4 °C. Tau12 was isolated as described⁵. Membranes were washed three times with 1× TBST before incubating in horseradish peroxidase-conjugated secondary antibody (Bio-Rad) diluted in blocking buffer for 1 h at room temperature. Blots were again washed three times in 1× TBST and developed using the enhanced chemiluminescent detection system (GE Healthcare) for exposure to X-ray film.

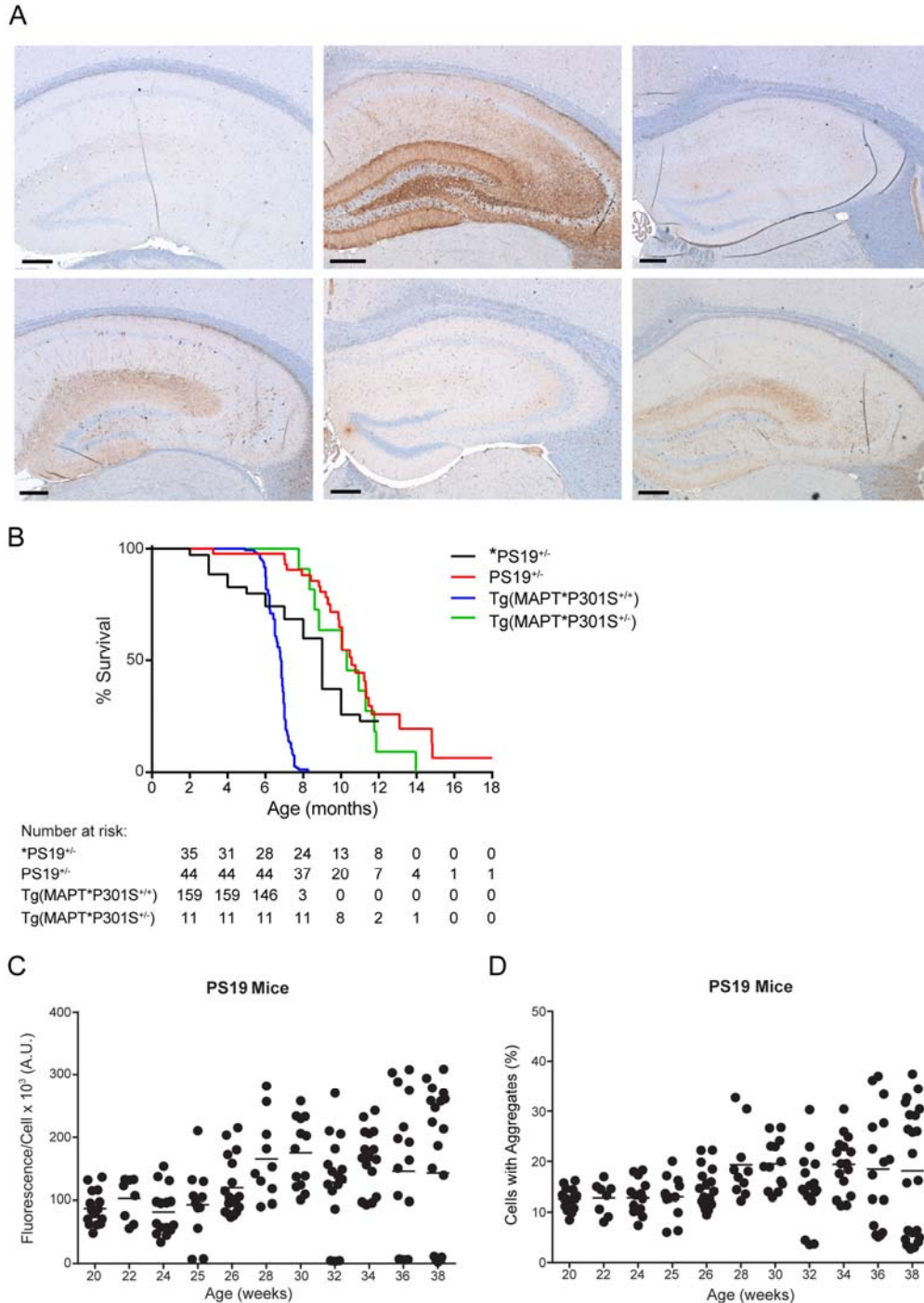
Statistical analysis

Data are presented as mean ± standard deviation. Data from the cell aggregation assay were analyzed by fitting nonparametric curves to the average data points collected from all mice for the B6/J, Tg(MAPT*P301S^{+/+}), and Tg(MAPT*P301S^{+/-}) mouse lines. The 95% confidence interval (CI) was determined, and significant differences between each mouse line were verified as the point at which the CIs no longer overlapped. Onset of neurological symptoms between female and male Tg(MAPT*P301S^{+/+}) mice was compared using a log-rank (Mantel-Cox) test. Mean and variance of survival time presented in eFigure 1 was calculated for each mouse line. These parameters

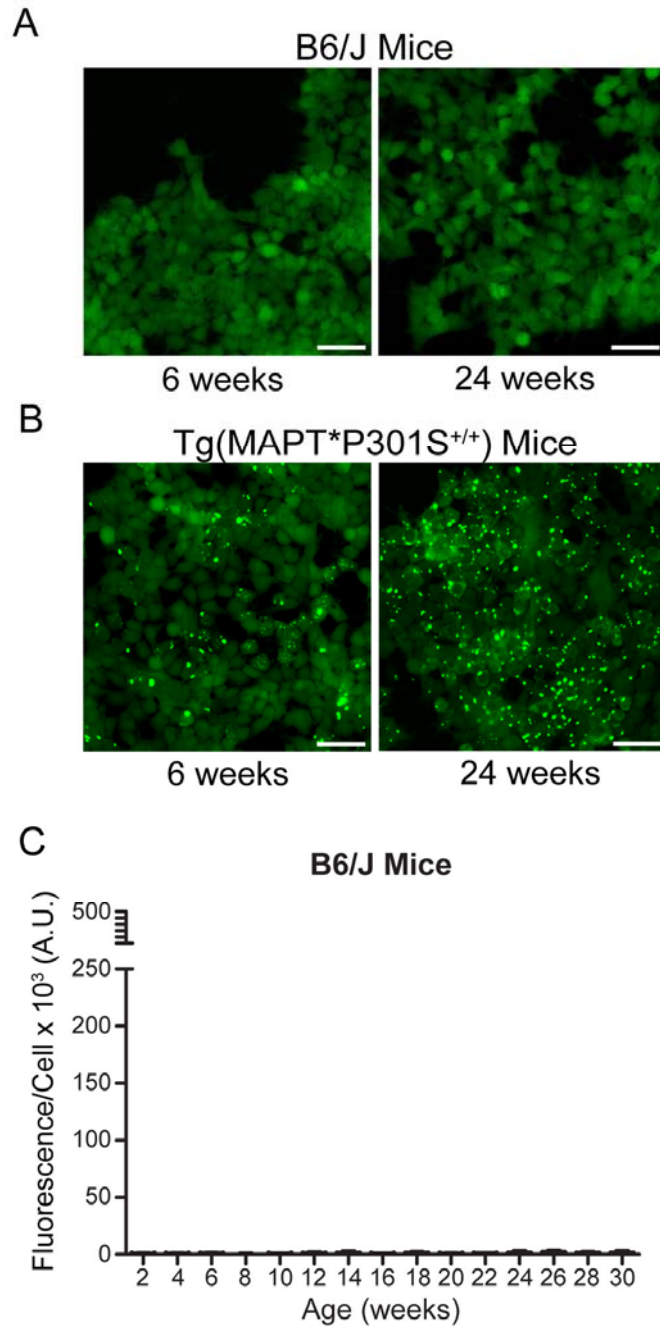
were then used to conduct a power analysis for a range of treatment effects with $\alpha=.05$ and a power of 80%. Statistical comparisons of neuropathology scores between B6/J and either Tg(MAPT*P301S^{+/+}) or Tg(MAPT*P301S^{+/-}) mice were performed using a two-tailed Student's *t* test with unequal variance. Significance was determined with a P value <.05.

SUPPLEMENTARY REFERENCES

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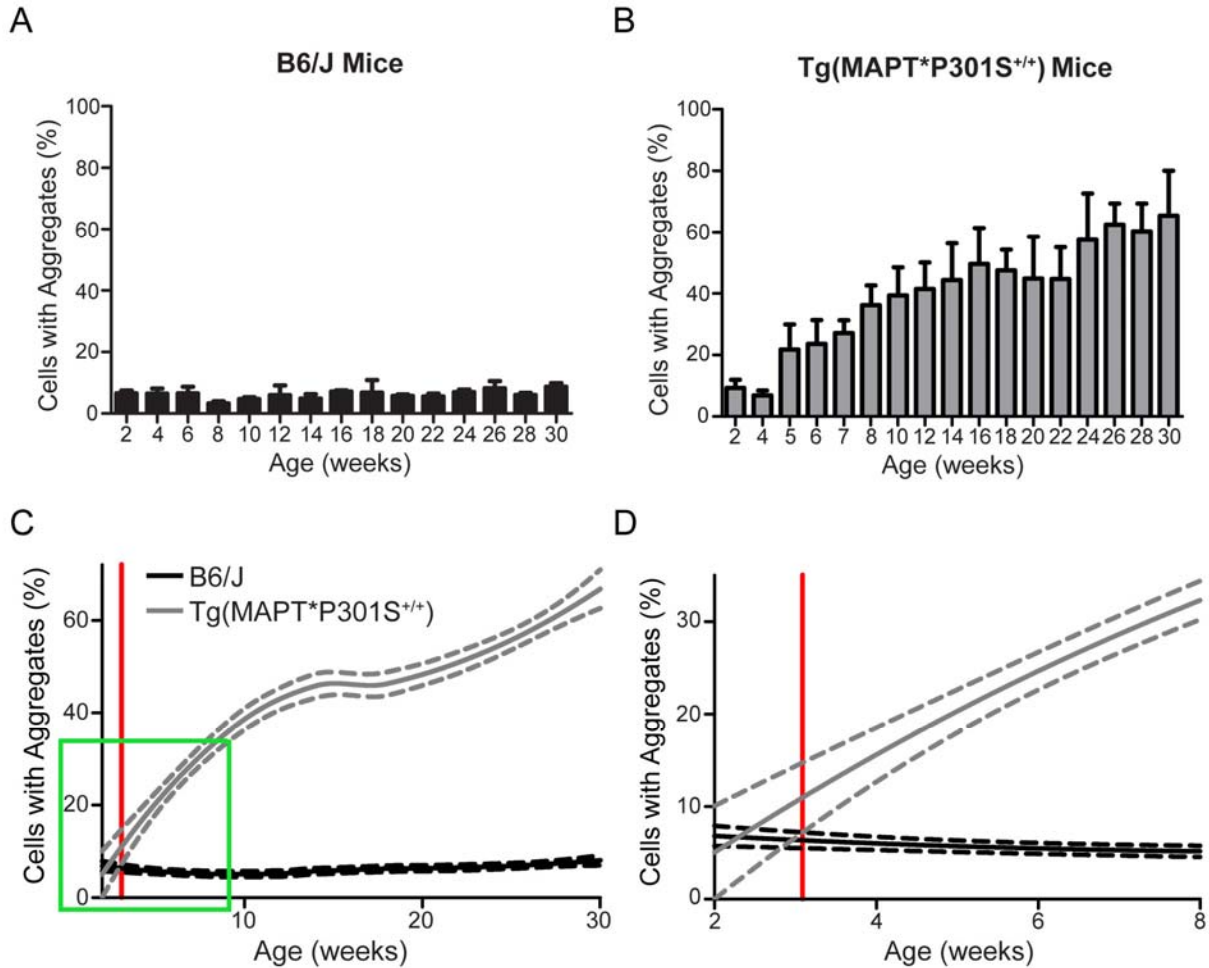


eFigure 1. Spontaneous tau prion formation in PS19 mice is highly variable. (A) Tau neuropathology in the hippocampus of 31-week-old PS19 mice was detected with the AT8 monoclonal antibody (scale bars = 200 μ m). (B) Kaplan-Meier plot represents onset of neurological signs in tauopathy mice. Black line (*PS19 mice) shows disease onset as initially reported in PS19 mice ($n=35$)¹, which is delayed in the mice assessed here (red line; $n=44$). For comparison, the Kaplan-Meier plots for the homozygous Tg(MAPT*P301S^{+/+}) mice (blue line; $n=159$) and hemizygous Tg(MAPT*P301S^{+/-}) mice (green line; $n=11$) are also shown. (C & D) Brain homogenate from PS19 mice was clarified, standardized to 2 mg/mL total protein, and diluted 1:30 in 1 \times DPBS before incubating with Tau(4RD*LM)-YFP(2) cells for 3 days before imaging. Infected cells were quantified by (C) dividing the total fluorescence in each image by the number of living cells ($\times 10^3$ A.U.) and by (D) quantifying the percentage of cells containing aggregates. Black line indicates mean for each time point. Each mouse sample was tested in two independent preparations, and data are shown as mean of the two measurements. Black line in (B) reprinted from Yoshiyama et al. *Neuron* (2007) 53: 337–351.

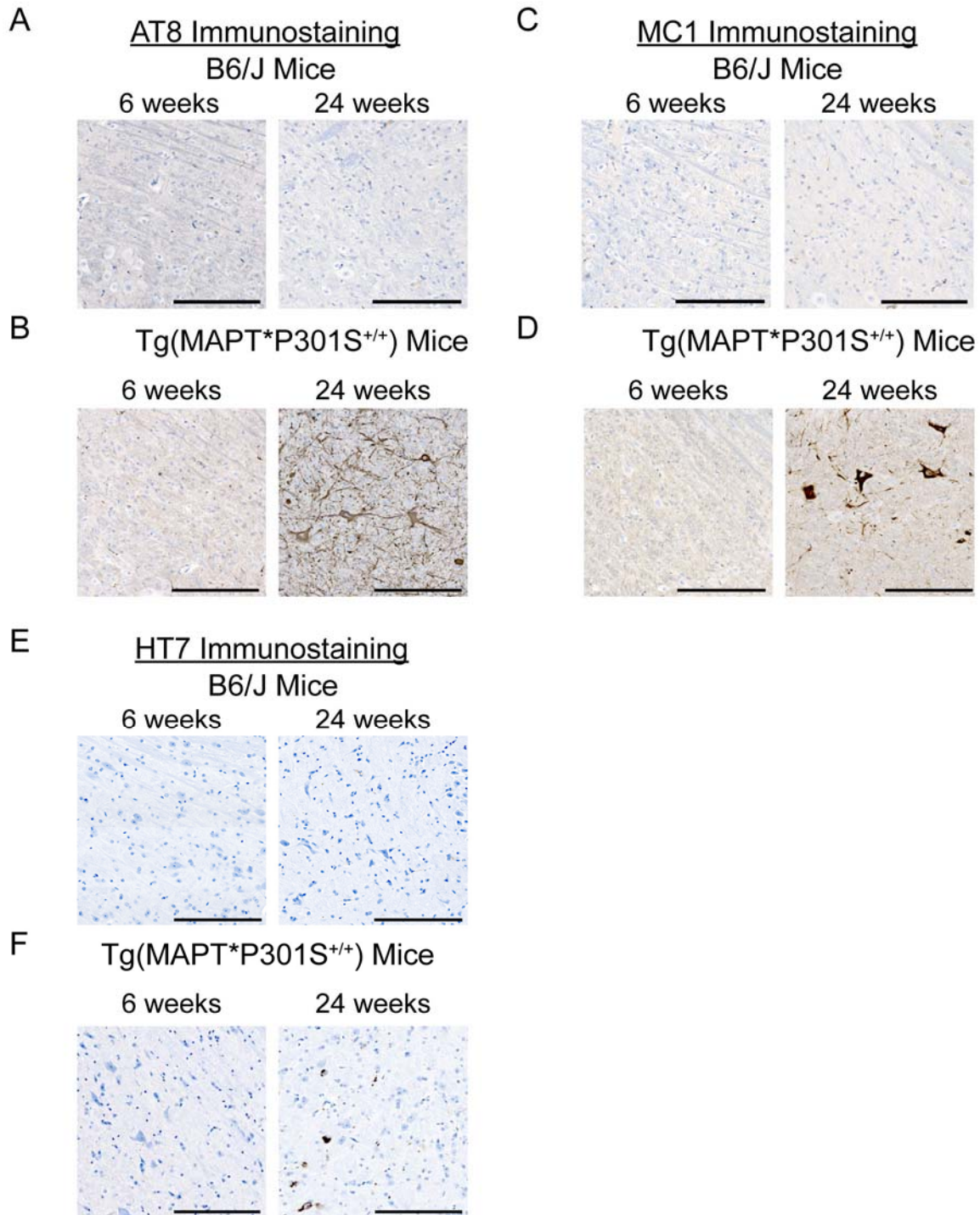


eFigure 2. Detection of spontaneous tau prion formation using Tau(4RD*LM)-YFP(1) cells.

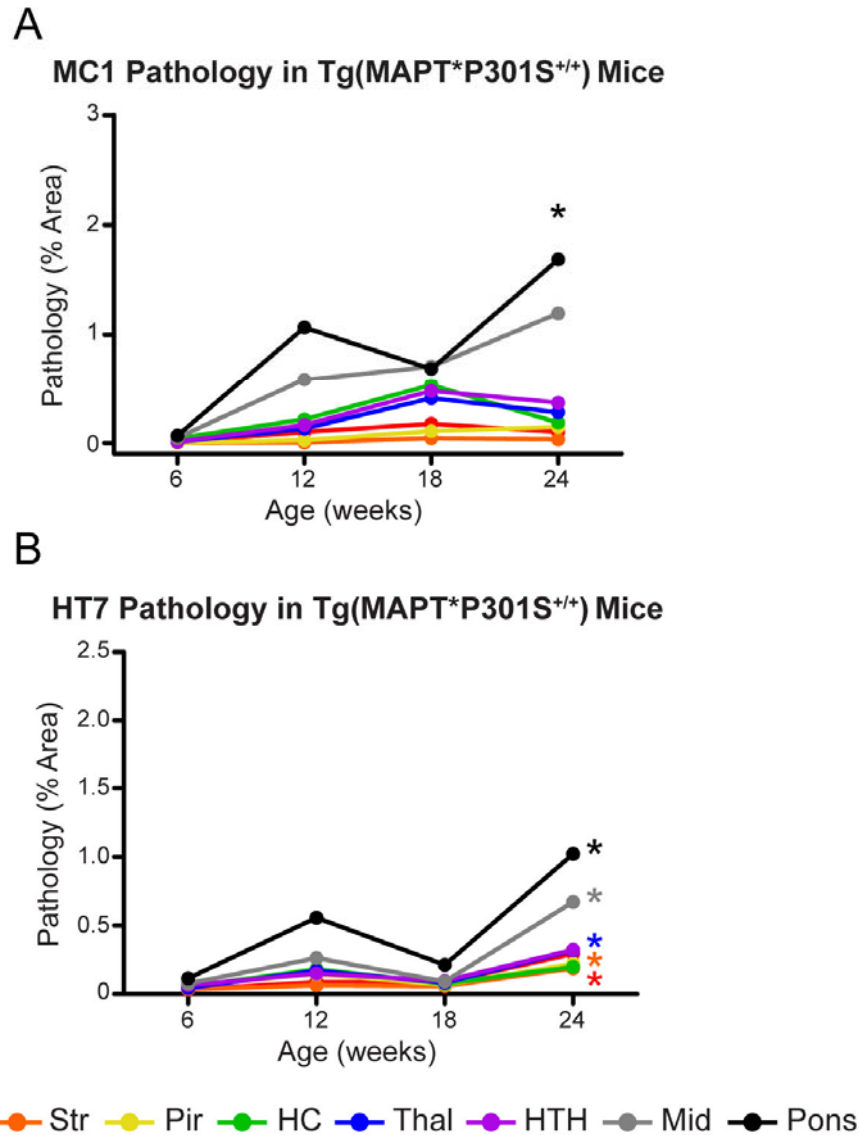
Tau prions were isolated from B6/J and Tg(MAPT*P301S^{+/+}) mouse brain homogenates using sodium phosphotungstic acid. Protein pellets were diluted 1:4 in 1× DPBS and incubated with Tau(4RD*LM)-YFP(1) cells for 4 days before imaging. (A & B) Representative images of Tau(4RD*LM)-YFP(1) cells incubated with phosphotungstic acid–precipitated protein aggregates from 6- and 24-week-old B6/J (A) and Tg(MAPT*P301S^{+/+}) (B) mice are shown (scale bars = 50 μm). (C) After incubating with protein isolated from B6/J mice, infected cells were quantified by dividing the total fluorescence in each image by the number of living cells (×10³ A.U.). The time course of spontaneous tau prion formation was quantified by testing samples from mice 2 to 30 weeks of age. Data shown as mean ± standard deviation for each time point.



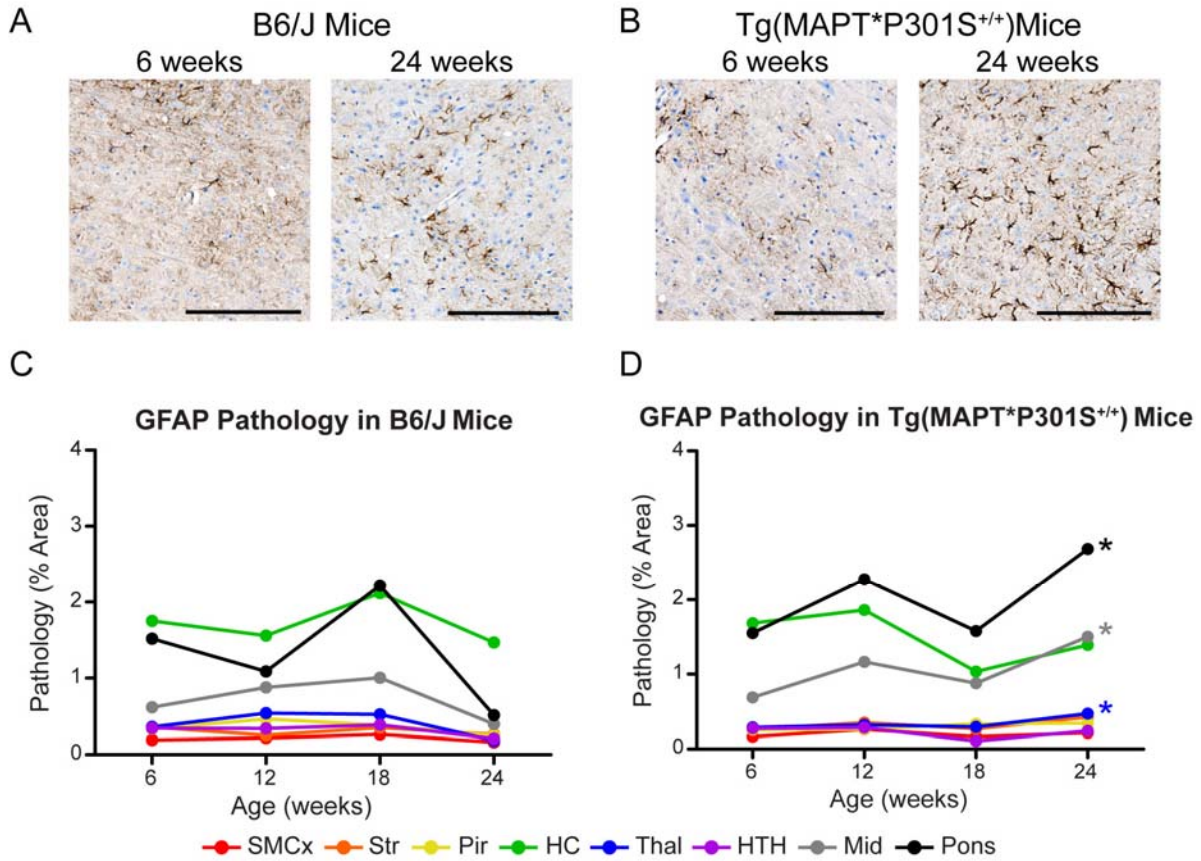
eFigure 3. Spontaneous tau prion formation is detectable before 4 weeks of age in Tg(MAPT*P301S^{+/+}) mice. Tau prions were isolated from B6/J and Tg(MAPT*P301S^{+/+}) mouse brain homogenates using sodium phosphotungstic acid. Protein pellets were diluted 1:4 in 1× DPBS and incubated with Tau(4RD*LM)-YFP(1) cells for 4 days before imaging. (A & B) Infected cells were quantified by determining the percentage of living cells containing aggregates after incubation with B6/J (A) or Tg(MAPT*P301S^{+/+}) (B) mouse samples. Samples were tested from mice 2 to 30 weeks of age, quantifying the time course of spontaneous tau prion formation in the mice. Data shown as mean ± standard deviation for each time point. (C) Nonparametric curves were fit to the data collected from each B6/J mouse (solid black line) and each Tg(MAPT*P301S^{+/+}) mouse (solid gray line). The 95% confidence interval (CI) was calculated for both mouse lines (dotted lines), and the age at which the two 95% CIs no longer overlapped was determined (3.1 weeks old; red line). Inset (green box) (C) magnified in (D).



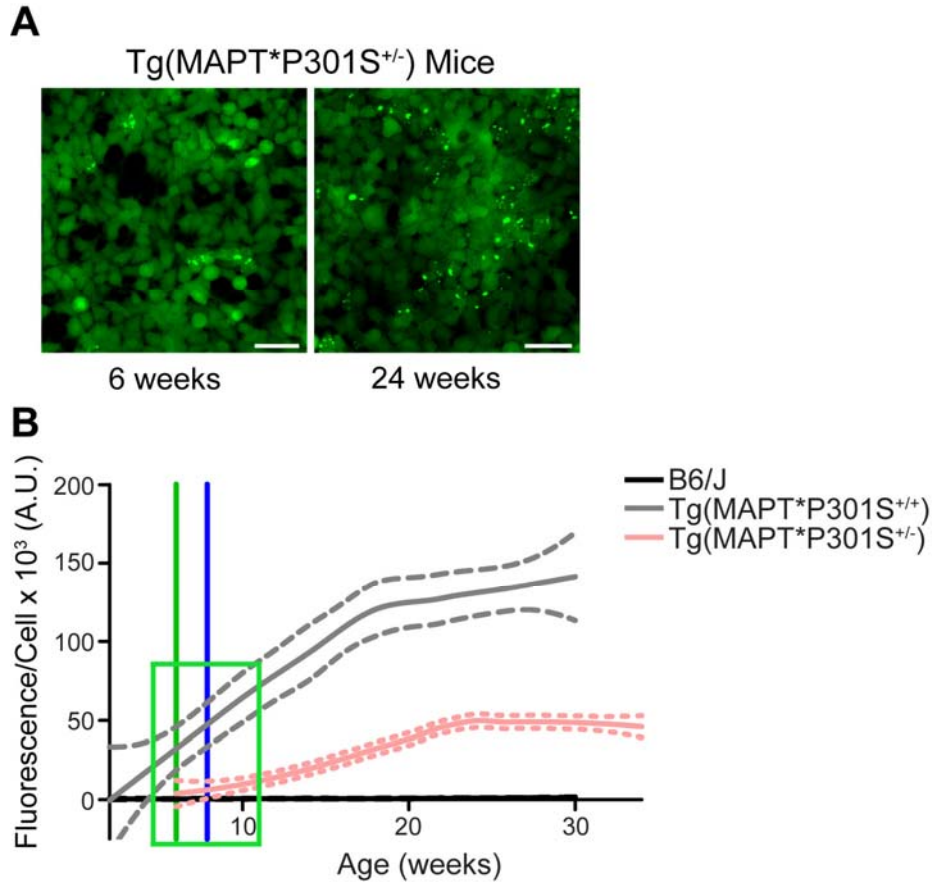
eFigure 4. Tau neuropathology in 6- and 24-week-old Tg(MAPT*P301S^{+/+}) mice. Tau aggregates were detected in 6- and 24-week old B6/J and Tg(MAPT*P301S^{+/+}) mice using the antibodies AT8 (A & B), MC1 (C & D), and HT7 (E & F) in the Pons. Representative images of immunostaining in the Pons of 6- and 24-week old B6/J (A, C, & E) and Tg(MAPT*P301S^{+/+}) mice (B, D, & F). Scale bars = 200 μ m.



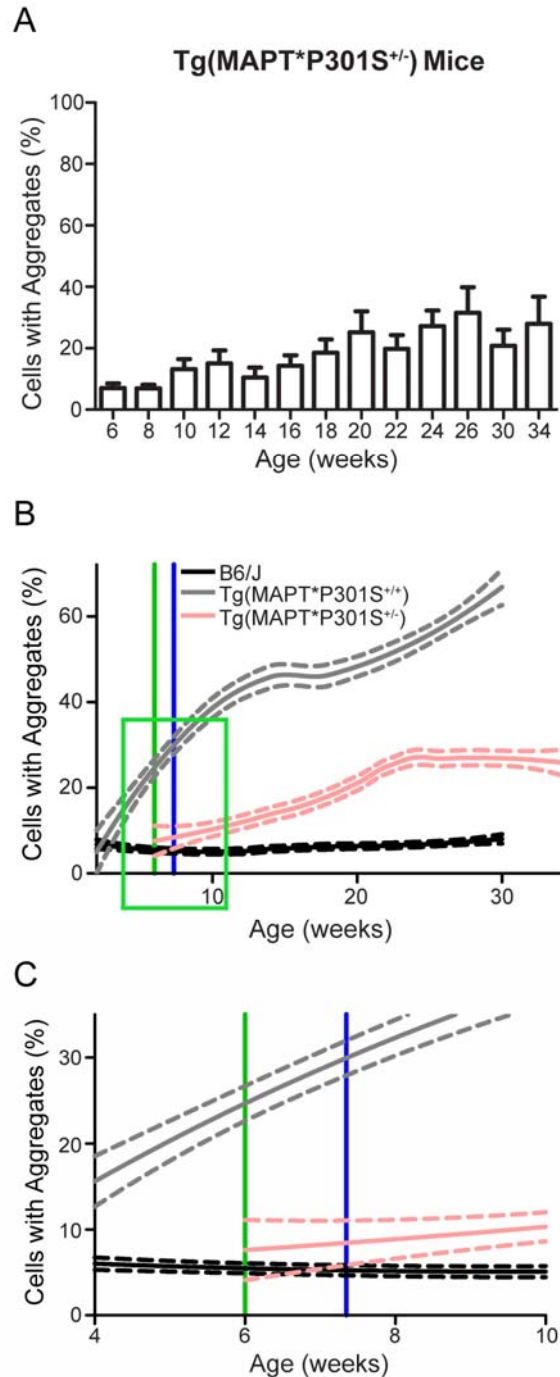
eFigure 5. MC1 and HT7 neuropathology increases at 24 weeks of age in Tg(MAPT*P301S^{+/+}) mice. Tau aggregates were detected in 6-, 12-, 18-, and 24-week old Tg(MAPT*P301S^{+/+}) mice using the antibodies MC1 (A) and HT7 (B) in the sensorimotor cortex (SMCx), striatum (Str), piriform cortex and amygdala (Pir), hippocampus (HC), thalamus (Thal), hypothalamus (HTH), midbrain (Mid), and Pons. (A) Time course of MC1 staining in Tg(MAPT*P301S^{+/+}) mice. MC1 pathology was significantly increased in all brain regions in Tg(MAPT*P301S^{+/+}) mice (SMCx, P<.001; Str, P<.001; Pir, P<.001; HC, P=.01; Thal, P=.002; HTH, P<.001; Mid, P<.001; Pons, P<.001) at 24 weeks of age compared with 6-week-old animals. Data shown as mean. (B) Time course of HT7 staining in Tg(MAPT*P301S^{+/+}) mice. HT7 pathology significantly increased in five brain regions in the Tg(MAPT*P301S^{+/+}) mice at 24 weeks compared with the 6-week-old mice (SMCx, P=.04; Str, P=.04; Thal, P=.01; Mid, P=.005; Pons, P=.01). Data shown as mean. *=P<.05.



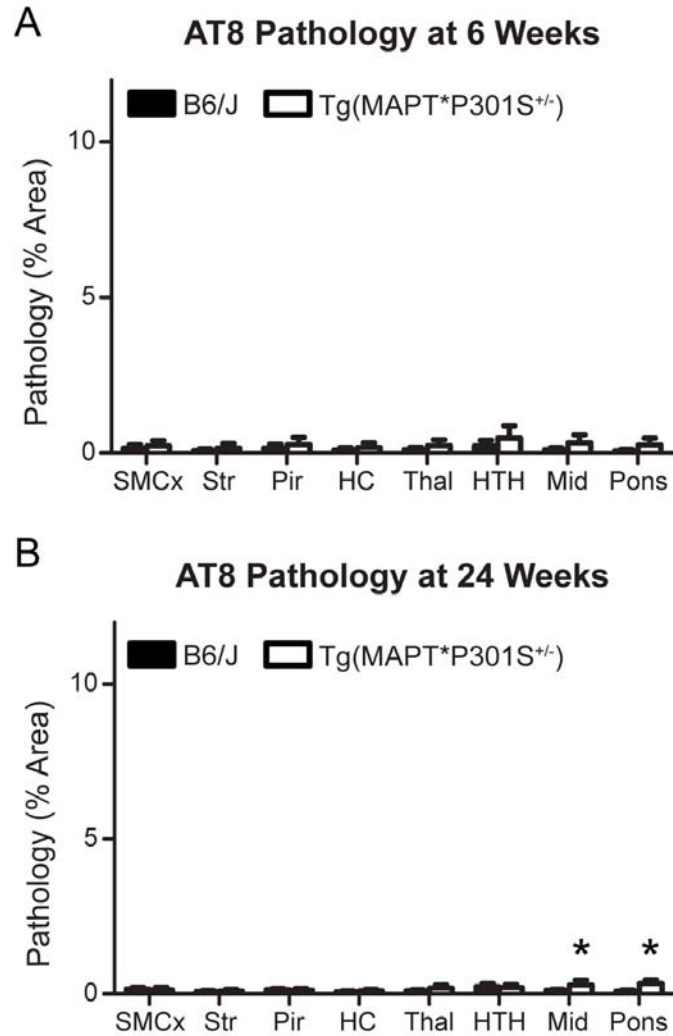
eFigure 6. Astroglial pathology in Tg(MAPT*P301S^{+/+}) mice increases in the hindbrain at 24 weeks of age. GFAP immunostaining was measured in the sensorimotor cortex (SMCx), striatum (Str), piriform cortex and amygdala (Pir), hippocampus (HC), thalamus (Thal), hypothalamus (HTH), midbrain (Mid), and Pons of 6-, 12-, 18-, and 24-week-old B6/J and P301S^{+/+} mice. (A & B) Representative images of astroglial pathology in the Pons of 6- and 24-week-old B6/J (A) and Tg(MAPT*P301S^{+/+}) mice (B) (scale bars = 200 μ m). (C & D) Time course of GFAP staining in B6/J (C) and Tg(MAPT*P301S^{+/+}) mice (D). The amount of astroglial pathology in the B6/J mouse brains remained consistent between 6 and 24 weeks of age; however, GFAP pathology significantly increased in the Thal (P=.03), Mid (P=.002), and Pons (P=.005) in the 24-week-old Tg(MAPT*P301S^{+/+}) mice compared with the 6-week-old animals. Data shown as mean. *=P<.05.



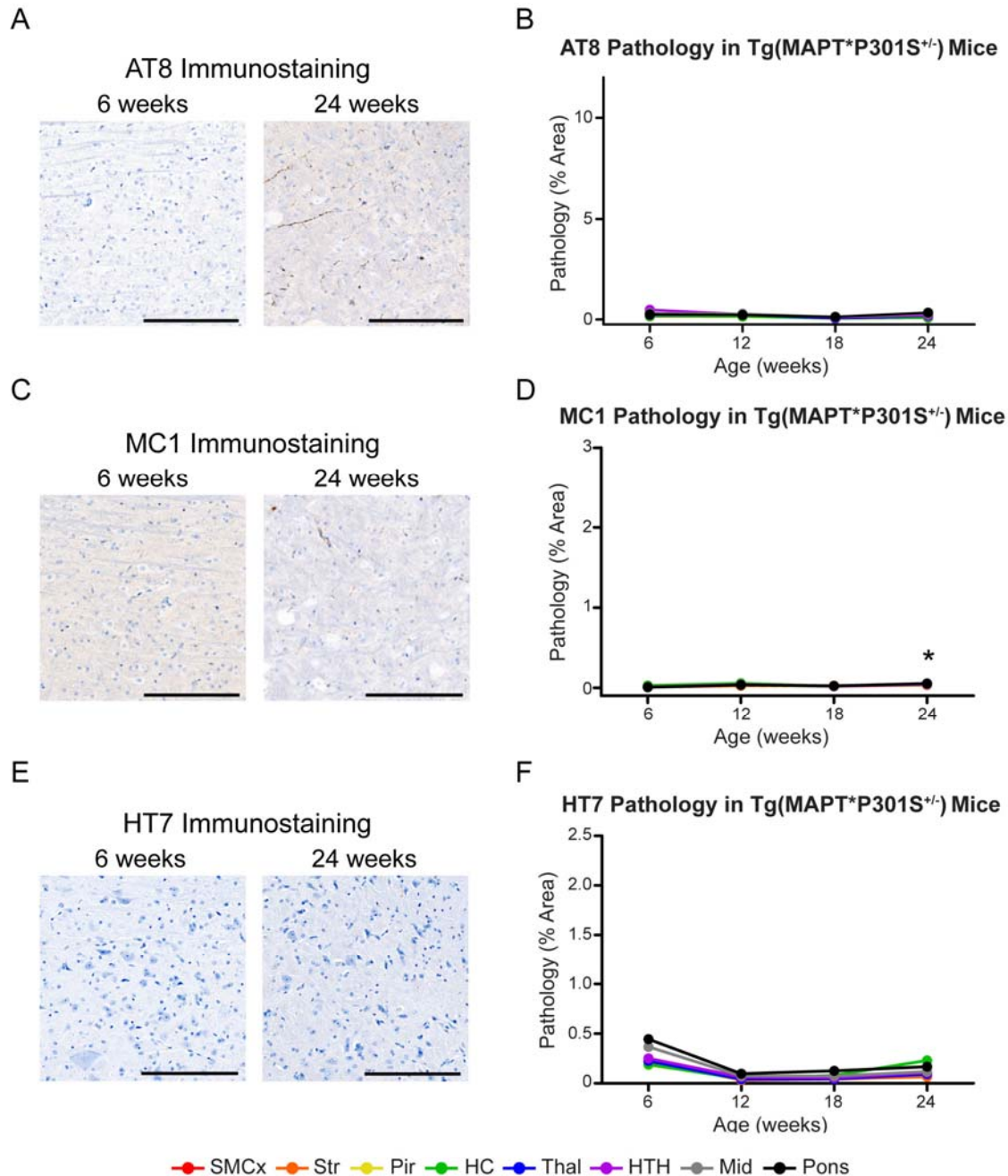
eFigure 7. Measuring by fluorescence per cell, spontaneous tau prion formation occurs by 8 weeks of age in Tg(MAPT*P301S^{+/-}) mice. Tau prions were isolated from 6- to 34-week-old Tg(MAPT*P301S^{+/-}) mice by precipitating the samples with sodium phosphotungstic acid. Protein pellets were diluted 1:4 in 1× DPBS and incubated with Tau(4RD*LM)-YFP(1) cells for 4 d. Tau prions were quantified from each animal by averaging three independent preparations tested in the cell assay. (A) Representative images of Tau(4RD*LM)-YFP(1) cells after incubation with tau prions from 6- and 24-week-old Tg(MAPT*P301S^{+/-}) mice are shown (scale bars = 50 μm). (B) Cell infection was measured by the total fluorescence in each image divided by the number of cells (×10³ A.U.). A nonparametric curve was fit to the data collected from Tg(MAPT*P301S^{+/-}) mice (solid pink line), and the 95% CI was calculated (dotted lines). These data were compared with curves generated from the B6/J (black lines) and Tg(MAPT*P301S^{+/+}) (gray lines) mouse data. The age at which the 95% CI for the Tg(MAPT*P301S^{+/-}) mice no longer overlapped with the 95% CI for the Tg(MAPT*P301S^{+/+}) mice occurred prior to 6 weeks (green line), and the age at which the Tg(MAPT*P301S^{+/-}) data no longer overlapped with the B6/J data occurred at 7.9 weeks (blue line). Inset (green box) shown in Fig. 5G.



eFigure 8. Measuring by percent cells with aggregates, spontaneous tau prion formation occurs by 8 weeks of age in Tg(MAPT*P301S^{+/-}) mice. Tau prions were isolated from 6- to 34-week-old Tg(MAPT*P301S^{+/-}) mice by precipitating samples with sodium phosphotungstic acid. Protein pellets were diluted 1:4 in 1× DPBS and incubated with Tau(4RD*LM)-YFP(1) cells for 4 d. (A) Tau prions were quantified from each animal by determining the percentage of cells containing aggregates, and each mouse was tested using three independent preparations. Data shown as mean ± standard deviation for each time point. (B) A nonparametric curve was fit to the data collected from the Tg(MAPT*P301S^{+/-}) mice (solid pink line), and the 95% CI was calculated (dotted lines). These curves were compared with curves generated from the B6/J (black lines) and Tg(MAPT*P301S^{+/+}) (gray lines) mouse data. The age at which the 95% CI for the Tg(MAPT*P301S^{+/-}) mice no longer overlapped with the homozygous mice occurred prior to 6 weeks (green line). The age at which the Tg(MAPT*P301S^{+/-}) and B6/J 95% CIs no longer overlapped occurred at 7.3 weeks (blue line). Inset (green box) (B) shown in (C).

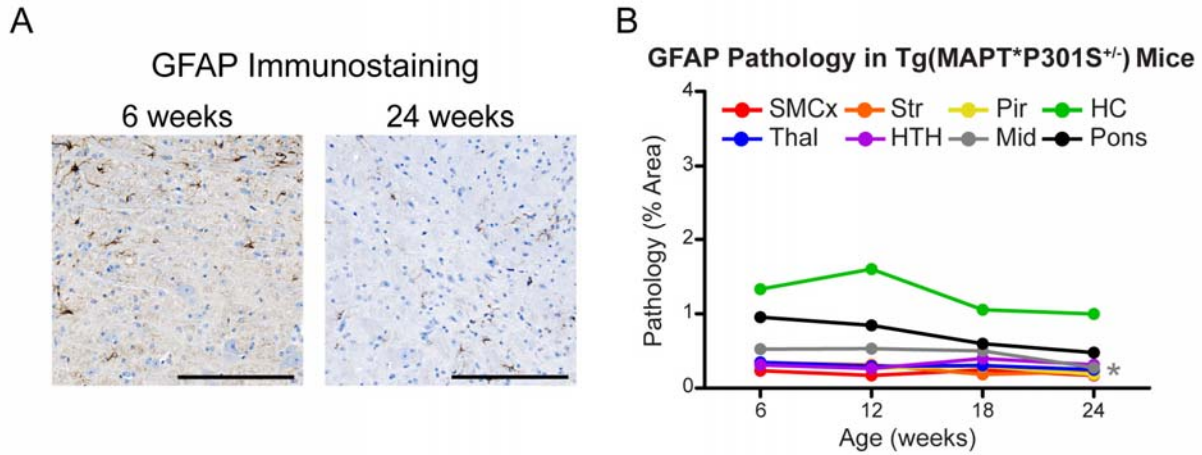


eFigure 9. AT8 neuropathology is delayed in Tg(MAPT*P301S^{+/-}) mice. AT8 neuropathology was measured in the sensorimotor cortex (SMCx), striatum (Str), piriform cortex and amygdala (Pir), hippocampus (HC), thalamus (Thal), hypothalamus (HTH), midbrain (Mid), and Pons of 6-, 12-, 18-, and 24-week-old Tg(MAPT*P301S^{+/-}) mice. (A & B) AT8-positive inclusions in 6-week-old (A) and 24-week-old B6/J and Tg(MAPT*P301S^{+/-}) mice (B) were compared. (A) No significant differences were detected in 6-week-old mice. (B) AT8-positive inclusions were significantly increased in the Mid and Pons ($P < .001$) of 24-week-old Tg(MAPT*P301S^{+/-}) mice compared with 24-week-old B6/J animals. *= $P < .001$. Data shown as mean \pm standard deviation.



eFigure 10. Tg(MAPT*P301S^{+/-}) mice develop minimal tau neuropathology by 24 weeks of age.

AT8, MC1, and HT7 immunostaining was measured in the sensorimotor cortex (SMCx), striatum (Str), piriform cortex and amygdala (Pir), hippocampus (HC), thalamus (Thal), hypothalamus (HTH), midbrain (Mid), and Pons of 6-, 12-, 18-, and 24-week-old Tg(MAPT*P301S^{+/-}) mice. (A) Representative micrographs of AT8 neuropathology in the Pons of 6- and 24-week-old Tg(MAPT*P301S^{+/-}) mice (scale bars = 200 μ m). (B) Time course of AT8 neuropathology in Tg(MAPT*P301S^{+/-}) mice. No differences in AT8 immunoreactivity were seen between 6- and 24-week-old Tg(MAPT*P301S^{+/-}) mice. Data shown as mean. (C) Representative micrographs of MC1 neuropathology in the Pons of 6- and 24-week-old Tg(MAPT*P301S^{+/-}) mice (scale bars = 200 μ m). (D) Time course of MC1 neuropathology in Tg(MAPT*P301S^{+/-}) mice. MC1 immunoreactivity was increased in 7 of the 8 brain regions analyzed at 24 weeks of age compared with 6 weeks (SMCx, $P=0.002$; Str, $P<0.001$; Pir, $P=0.03$; Thal, $P<0.001$; HTH, $P=0.002$; Mid, $P<0.001$; Pons, $P<0.001$). Data shown as mean. (E) Representative micrographs of HT7 neuropathology in the Pons of 6- and 24-week-old Tg(MAPT*P301S^{+/-}) mice (scale bars = 200 μ m). (F) Time course of HT7 neuropathology in Tg(MAPT*P301S^{+/-}) mice. No differences in HT7 immunoreactivity were seen between 6- and 24-week-old Tg(MAPT*P301S^{+/-}) mice. Data shown as mean. *= $P<0.05$.



eFigure 11. Astrogliosis does not increase in Tg(MAPT*P301S^{+/-}) mice by 24 weeks of age. GFAP neuropathology was measured in the sensorimotor cortex (SMCx), striatum (Str), piriform cortex and amygdala (Pir), hippocampus (HC), thalamus (Thal), hypothalamus (HTH), midbrain (Mid), and Pons of 6-, 12-, 18-, and 24-week-old Tg(MAPT*P301S^{+/-}) mice. (A) Representative micrographs showing GFAP neuropathology in the Pons of 6- and 24-week-old Tg(MAPT*P301S^{+/-}) mice (scale bars = 200 μ m). (B) Time course of GFAP neuropathology in Tg(MAPT*P301S^{+/-}) mice. Astrogliosis was only increased in the Mid (P=.04) of 24-week-old Tg(MAPT*P301S^{+/-}) mice compared with 6-week-old animals. Data shown as mean. *P<.05.

eTable. Power analysis determination of sample size needed to detect significant extension in survival.

Percent increase in survival	Sample size required			
	*PS19 [†]	PS19	Tg(MAPT*P301S ^{+/+})	Tg(MAPT*P301S ^{+/-})
5%	302	182	16	106
10%	152	92	8	54
15%	102	62	6	36
20%	76	46	4	28
25%	62	38	4	22
30%	52	32	4	18
35%	44	26	4	16
40%	38	24	4 [‡]	14
45%	34	22	4 [‡]	12
50%	32	20	4 [‡]	12

Power analysis performed for a two-tailed t-test, $\alpha=.05$, and a power of 80%. Reported sample size indicates total number of mice split evenly between control and treatment groups.

[†]Derived from Kaplan-Meier plot reported in¹.

[‡]Power analysis actually indicated n=2, but statistical analysis requires a minimum n=4.