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***cis* p-tau: early driver of brain injury and tauopathy blocked by antibody**

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Online Content Methods, along with any Extended Data display items and source Data, are available in the online version of the paper; references unique to the sections appear only in the online paper.

Supplementary Information is available in the online version of the paper

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Abstract

Traumatic brain injury (TBI), characterized by acute neurological dysfunction, is one of the best known environmental risk factors for chronic traumatic encephalopathy (CTE) and Alzheimer's disease (AD), whose defining pathologic features include tauopathy made of phosphorylated tau (p-tau). However, tauopathy has not been detected in early stages after TBI and how TBI leads to tauopathy is unknown. Here we find robust *cis* p-tau pathology after sport- and military-related TBI in humans and mice. Acutely after TBI in mice and stress in vitro, neurons prominently produce *cis* p-tau, which disrupts axonal microtubule network and mitochondrial transport, spreads to other neurons, and leads to apoptosis. This process, termed "cistausis", appears long before other tauopathy. Treating TBI mice with *cis* antibody blocks cistausis, prevents tauopathy development and spread, and restores many TBI-related structural and functional sequelae. Thus, *cis* p-tau is a major early driver after TBI and leads to tauopathy in CTE and AD, and *cis* antibody may be further developed to detect and treat TBI, and prevent progressive neurodegeneration after injury.

Traumatic brain injury (TBI) is the leading cause of death and disability in children and young adults¹ and in the US, ~2.5 million people suffer TBI each year². Nearly 20% of the 2.3 million troops deployed by the military have sustained TBI³. Repetitive mild TBI (rmTBI), seen in contact sports, or even single moderate/severe TBI (ssTBI), seen in military blasts, may cause acute and potentially long-lasting neurological dysfunction, including the development of chronic traumatic encephalopathy (CTE)⁴⁻⁹. TBI is also an established environmental risk factor for Alzheimer's disease (AD)⁷⁻¹². However, no treatment is available to prevent CTE or AD.

CTE is characterized by neurofibrillary tangles made of hyperphosphorylated tau⁴⁻⁹. Such tangles are also a hallmark of AD and related neurodegenerative disorders, collectively termed tauopathies^{13, 14}. Tauopathy spreads in brains¹⁵⁻¹⁹ and is reduced by immunotherapy against tauopathy epitopes²⁰⁻²². However, since little tauopathy is detectable acutely or subacutely after TBI in humans and mice^{5, 7-9, 23-25}, whether tauopathy is a cause or consequence of post-traumatic neurodegeneration is unknown.

We have identified a unique proline isomerase, Pin1 that inhibits tauopathy in AD by converting the phosphorylated Thr231-Pro motif in tau (p-tau) from *cis* to *trans* in AD cell and mouse models²⁶⁻³⁴. In human AD, Pin1 is inhibited by multiple mechanisms^{27, 29, 35-37}, whereas the Pin1 genetic polymorphism that prevents its down-regulation is associated with delaying AD age of onset³⁸. In addition, Pin1 is located at a locus associated with late-onset AD³⁹, p-tau appears early in pretangle AD neurons⁴⁰ and its cerebrospinal fluid level correlates with memory loss in MCI and AD⁴¹. We have developed antibodies that

distinguish *cis* from *trans* p-tau and discovered that *trans* p-tau is physiological, promoting MT assembly, whereas the *cis* is early pathogenic, leading to tauopathy in AD⁴². Currently, it is unknown whether *cis* p-tau is present after TBI and if so, how to specifically eliminate it.

Robust *cis* p-tau in human CTE brains

We generated mouse monoclonal antibodies (mAbs), which, like our polyclonal antibodies⁴² were able to distinguish *cis* from *trans* tau. We identified a *cis* mAb clone, #113, and a *trans* mAb clone, #25, with no cross-reactivity (Extended Data Fig. 1a, b). Both clones reacted to a pT231-tau peptide, but not its non-phosphorylated counterpart (Extended Data Fig. 1a, b).

We determined antibody binding affinities using a Biacore assay. *cis* and *trans* mAbs specifically recognized the p-tau peptide (Fig. 1a, b), with their binding constants (K_D) being 0.27 and 42.1 nM, respectively (Table 1). Their IgG isotypes were IgG2b and IgG1, respectively (Extended Data Fig. 1c). Immunofluorescence (IF) and immunoblotting (IB) analyses showed robust *cis* signals in the soma and neurites, and *trans* signals in the soma in Tau-TG mice, but not in tau-null mice (Extended Data Fig. 1d, e) Thus, *cis* and *trans* mAb behave similarly to their polyclonal counterparts⁴².

Since the T231 tau phosphor-epitope is identical among species, we performed double IF with *cis* and *trans* mAbs on CTE brain tissues from 16 patients with a history of TBI exposure and 8 healthy controls⁶ (Supplemental Table 1). While *trans* mAb detected a few neurons in the soma in control and CTE brains, *cis* mAb detected no signal in control brains, but robust signals were observed in diffuse neurites in all CTE brains examined, with two typical patterns evident, distinguished by one with stronger *cis* p-tau signals, especially in soma (Fig. 1d, e and Extended Data Fig. 1f-h). *cis* mAb co-localized with AT180 (recognizing pT231-tau), T22 (tau oligomers⁴³), AT8 (early tangles), and AT100 and Alz50 (mature tangles), but *trans* mAb did not co-localize with T22 (Extended Data Fig. 2a, b). *cis* p-tau was more concentrated near blood vessels (Extended Data Fig. 2c), as expected⁶. *cis* p-tau co-localized diffusely with the axonal marker tau, but not the dendritic marker MAP2, in CTE brains (Fig. 1g, h and Extended Data Fig. 2d). Thus, *cis* p-tau localizes primarily to diffuse axons in CTE brains.

cis p-tau is earliest TBI tau epitope

To determine the temporospatial characteristics of *cis* p-tau induction after TBI, we used TBI mouse models induced by impact²⁵ and blast⁵, modeling sport- and military-related TBI, respectively. 48 h after impact TBI, *cis*, but not *trans*, p-tau was elevated in a severity-dependent manner, correlating with total tau (Fig. 2a, b and Extended Data Fig. 3a, b), and reflecting the stability of *cis* p-tau⁴². While single mild TBI (mTBI) moderately and transiently induced *cis* p-tau, which returned to the baseline by 2 weeks, single moderate/severe TBI (ssTBI) robustly and persistently induced *cis* p-tau, starting at 12 h and peaking at 48 h, but sustaining high levels over time (Fig. 2c, d and Extended Data Fig. 3c). Both repetitive mild TBI (rmTBI) and blast TBI also induced robust and persistent *cis* p-tau induction, with more profound effects in the latter (Fig. 2e, f and Extended Data Fig. 3c).

Robust *cis* p-tau was detected 48 hr after TBI without tau oligomers, aggregation or tangle epitopes (Extended Data Fig. 3d, e, and see results later). *cis* p-tau localized mainly to axons, but not dendrites in impact and blast TBI models (Fig. 2g-i), as in CTE brains (Fig. 1g, h and Extended Data Fig. 2d). *cis* p-tau expression was associated with axonal injury with marked disruption of MTs and mitochondria (MI), which was notably absent in dendrites (Fig. 2j), consistent with the fact that TBI mainly affects axons⁴⁴. Thus, robust *cis* p-tau is induced in axons acutely after impact and blast TBI long before other forms of p-tau appear.

***cis* p-tau spreads and is toxic after TBI**

Further analysis showed *cis* p-tau spreading in the brain after TBI. *cis* p-tau was mainly limited to the cortex from 24 h to 2 months after rmTBI, but 6 months later, robust *cis* p-tau was detected in the cortex and other brain regions including hippocampus (Fig. 3a-c). Marked *cis* p-tau spread from the cortex to the hippocampus and even to other side of the brain was also observed after blast TBI (data not shown). To examine whether *cis* p-tau might be neurotoxic, brain lysates prepared from rmTBI and sham mice 6 months post-injury were added to growing cultured neurons overnight. *cis*, but not *trans*, p-tau was readily detected in neurons treated with rmTBI lysates, but not when treated with sham controls (Fig. 3d). Compared with untreated or sham-treated controls, neurons treated with rmTBI lysates had much higher rates of apoptosis, which was rescued by immunodepletion of total tau or *cis*, but not *trans*, mAb (Fig. 3e). Thus, after impact and blast TBI, *cis* p-tau is robustly induced, spreads through the brain over time, and induces apoptosis that is blocked by *cis* mAb.

Stress induces *cis* p-tau, blocked by mAb

To understand how *cis* p-tau induces apoptosis and spreads through the brain, we examined the *in vitro* response to neuronal stress, serum starvation or hypoxia. Both conditions induced *cis*, but not *trans*, p-tau (Fig. 4a and Extended Data Fig. 4a, d), well before tau aggregation (Extended Data Fig. 4e). The addition of stressed neuron lysates to neurons induced cell death, which was rescued by immunodepletion with *cis*, but not *trans*, mAb, as detected by the live/dead assay (Extended Data Fig. 4g). To examine whether *cis* p-tau is implicated in tau spreading, we generated SY5Y cells stably expressing GFP-tau or RFP-tau, and co-cultured them with or without stress. Without hypoxia, neurons continued to express either GFP-tau or RFP-tau, but rarely both proteins (Fig. 3f, and Extended Data Fig. 5b). However, consistent with *cis* p-tau spreading in TBI brains (Fig. 3a-c and Extended Data Fig. 5a), hypoxia induced *cis* p-tau (Extended Data Fig. 4d), and caused progressive tau spreading, which was prevented by *cis*, but not *trans*, mAb (Fig. 3f and Extended Data Fig. 5b). Moreover, serum-starved neurons released *cis*, but not *trans*, p-tau at 40 h before neuronal death at 72 h (Extended Data Fig. 4h). Similar patterns of *cis* p-tau spread and neurotoxicity were also observed in primary neurons and blocked by *cis*, but not *trans*, mAb (Fig. 3g, h, Extended Data Fig. 5c, d). Thus, toxic *cis* p-tau is induced and spreads after neuronal stress, similar to TBI.

***cis* mAb blocks cistauosis after stress**

Given the ability of *cis* mAb to block tau from spreading and inducing apoptosis, we examined whether *cis* or *trans* mAb could affect intracellular p-tau after stress. Indeed, *cis* mAb entered neurons and effectively blocked time-dependent *cis* p-tau induction, without affecting *trans* following serum starvation (Fig. 4a and Extended Data Fig. 4a) or hypoxia (Extended Data Fig. 4d). Conversely, *trans* mAb reduced *trans*, but not *cis*, p-tau (Fig. 4a), indicating that the two isomers are not readily interchangeable, as suggested in TBI (Fig. 2) or CTE (Fig. 1), and AD⁴².

Since Pin1 inhibition by downregulation^{27, 29}, C113 oxidization³⁵ and S71 phosphorylation^{36, 37} contributes to tauopathy in AD, we asked whether such Pin1 inhibition contributes to *cis* p-tau induction after stress. *cis* induction correlated highly with Pin1 downregulation after serum starvation and Pin1 C113 oxidization after hypoxia (Extended Data Fig. 6a, b). Pin1 S71 phosphorylation was also markedly elevated in TBI brains (Extended Data Fig. 6c). Moreover, Pin1 knockdown enhanced *cis* p-tau induction by hypoxia, which was eliminated by *cis* mAb (Extended Data Fig. 6d). Since Pin1 knockout induces p-tau accumulation only in old mice^{29, 32, 42} and stress activates Pro-directed kinases, increased tau phosphorylation may be also important for *cis* p-tau induction after stress or TBI.

Given the ability of *cis* mAb to ablate intracellular *cis* p-tau, we evaluated how *cis* mAb might enter neurons to remove *cis* p-tau. Tau mAbs enter neurons via Fc γ receptors⁴⁵ and mAbs trigger targeted protein degradation by the TRIM21-mediated proteasome pathway⁴⁶. Indeed, blocking Fc γ receptors prevented *cis* mAb from binding to or entering neurons (Extended Data Fig. 7a-c). Immunogold EM showed *cis* mAb on the outer cell surface and in intracellular vesicles (Extended Data Fig. 7d). TRIM21 knockdown⁴⁶, but not the autophagy inhibitor 3-MA, prevented *cis* mAb from ablating *cis* p-tau (Extended Data Fig. 7e-g). Thus, *cis* mAb likely enters neurons via Fc γ receptors to target *cis* p-tau degradation.

To examine the functional significance of neuronal *cis* p-tau induction and elimination, we investigated whether *cis* p-tau might affect the MT network and function since *cis*, but not *trans*, p-tau loses its MT function⁴². Hypoxia not only induced *cis* p-tau (Extended Data Fig. 4d), but also caused MT collapse in neurites, an effect that was rescued by *cis*, but not *trans*, mAb (Fig. 4b and Extended Data Fig. 4b). Measuring MI movement along neurites showed that hypoxia stopped MT-based fast transport, but not actin-based slow movement (Fig. 4c and Supplementary Video 1, 2). This MI transport defect was restored by *cis* mAb, but not *trans* mAb (Fig. 4c and Supplementary Video 3), with the latter even causing neurite retraction (Supplementary Video 4), likely by *trans*-associated promotion of MT assembly⁴².

Serum starvation led to robust apoptosis by the time *cis* p-tau was highly induced, which was potently rescued by *cis*, but not, *trans* mAb, as detected by a live/dead assay (Fig. 4d, e), PARP cleavage (Fig. 4f and Extended Data Fig. 4c) and Annexin V FACS (Fig. 4g). Similar results were obtained with hypoxia (Extended Data Fig. 4f), even in primary neurons (Extended Data Fig. 5c, d). Thus, neuronal stress robustly induces *cis* p-tau, which disrupts

axonal MTs and organelle transport, spreads to other neurons and leads to apoptosis. These phenotypes, potently rescued by *cis* but not *trans* mAb, are here termed “cistauosis”.

To determine the importance of *cis* p-tau for neurotoxicity, we co-transfected neurons with GFP-tau or its T231A mutant and p25/Cdk5 phosphorylating tau on Th231 and others^{27, 47}. Co-expression of tau, but not its T231A mutant, with p25/Cdk5 increased *cis* p-tau, which was eliminated by *cis* mAb (Extended Data Fig. 8a). Importantly, most GFP-tau-, but not -tau^{T231A}-expressing cells were dead by 62 h, which was markedly blocked by *cis* mAb, but accelerated by *trans* mAb (Fig. 4h, i and Supplementary Videos 5, 6). Thus, *cis* p-tau is necessary and sufficient for p-tau to induce neurotoxicity.

***cis* mAb potently treats TBI and CTE**

To evaluate the efficacy of *cis* mAb in treating TBI *in vivo*, we showed that *cis* or *trans* mAb were detected in brains 3 days after peripheral administration (Extended Data Fig. 9a). After treating ssTBI mice with *cis* mAb or IgG isotype control for 2 weeks, *cis* mAb effectively eliminated *cis* p-tau induction, both with and without pre-treatment (Fig. 5a and Extended Data Fig. 9b, d), and also potently reversed post-TBI ultrastructural pathologies of axonal MTs and MIs (Fig. 5c and Extended Data Fig. 9f), defective cortical axonal long-term potentiation (Fig. 5d and Extended Data Fig. 9g), and even apoptosis (Extended Data Fig. 9c), which is observed after TBI even in humans⁴⁸.

To determine the impact of *cis* mAb on behavioral or functional outcomes after TBI, we treated ssTBI mice with *cis* mAb for 2 months. There was no difference in hippocampal-dependent spatial memory between IgG and *cis* mAb-treated TBI mice (Extended Data Fig. 9h). However, as *cis* p-tau was concentrated in the medial prefrontal cortex at this time point (Fig. 3a), we utilized the elevated plus maze, an innate anxiety/risk-taking paradigm that involves cortical circuitry⁴⁹ and is affected by TBI⁵⁰. All groups moved similar distances and times in the decision arm (Fig. 5e, f and Extended Data Fig. 10a-c). Sham mice stayed in the two closed or “safe” arms (Fig. 5e, f and Extended Data Fig. 10a and Supplementary Video 7), but all IgG-treated ssTBI mice strikingly displayed “risk-taking” behavior, exploring the two open or “aversive” arms (Fig. 5e, f and Extended Data Fig. 10a, b and Supplementary Video 8), consistent with disinhibition in a dysfunctional medial prefrontal cortex⁴⁹. By contrast, *cis* mAb-treated mice exhibited minimal risk-taking behavior, similar to sham mice (Fig. 5e, f and Extended Data Fig. 10a, and Supplementary Video 9).

To examine the effects of *cis* mAb on tauopathy development and spread, and brain atrophy, hallmarks of CTE⁷⁻⁹, we treated ssTBI mice with *cis* mAb for 6 months. *cis* mAb effectively prevented tauopathy development and spread, as assayed by *cis* p-tau, tau oligomers, aggregation and tangle epitopes (Fig. 5a, b, g, h, and Extended Data Fig. 9d, e, 10d, e), and brain atrophy in the cortex and white matter (Fig. 5i, and Extended Data Fig. 10f). Thus, *cis* mAb not only eliminates *cis* p-tau and cistauosis, but also prevents tauopathy development and spread, restores LTP and behavioral defects, as well as prevents brain atrophy after TBI (Fig. 5j).

Discussion

Here, we used *cis* p-tau mAbs to demonstrate the presence of, and specifically eliminate, pathogenic *cis* p-tau in clinically relevant in vitro and in vivo models of sport- and military-related TBI. We found robust *cis* p-tau after sport- and military-related TBI in humans and mice, and in stressed neurons. Following TBI or neuronal stress, *cis* p-tau induces cistaurosis well before previously identified tauopathy is apparent. Treating TBI mice with *cis* mAb ablates *cis* p-tau and eliminates cistaurosis, prevents the development of widespread tauopathy and restores histopathological and many functional outcomes of TBI. Cistaurosis is an early precursor of previously described tauopathy and an early marker of neurodegeneration that can be blocked by *cis* mAb. We previously showed that *cis* p-tau plays an early pathological role in AD^{27-34, 42}. Our current data provide a direct link from TBI to CTE and AD, and suggest that cistaurosis is a common early disease mechanism in TBI, CTE and AD, and that *cis* p-tau and its mAb may be useful for early diagnosis, prevention and therapy for these devastating diseases (Fig. 5j).

ONLINE METHODS

Mouse mAb production

cis and *trans* mouse mAb were produced using the general strategy that we used to generate polyclonal *cis* and *trans* antibodies, as described⁴². Briefly, Balb/c female mice (2-3 months old) obtained from the Jackson Laboratories (Bar Harbor, ME) were immunized with 100 µg of pThr231-Homoproline (pThr231-Pip) tau peptide (CKKVAVVRpT(Pip)PKSPSSAK) that was coupled to KLH with N-terminal Cys mixed with complete Freund's adjuvant and boosted twice. The titration of antibody production was monitored using ELSA. When sufficient titration of antibody was produced, splenocytes were isolated and fused with SP2/0 myeloma cells to produce hybridoma cell lines, followed by screening for positive clones using ELISA for *cis* and *trans* mAbs. When positive clones were identified, they were subcloned by a limited dilution to generate single pure clones. mAbs were produced by injecting 2ml of 2.5×10^6 cells/ml hybridoma cells into nude mice intraperitoneally to collect ascites, followed by purifying mAbs from ascites using antibody purification kit (Pierce) and their specificity were fully characterized. All these animal experiments were approved by Beth Israel Deaconess Medical Center IACUC and complied with the NIH Guide for the Care and Use of Laboratory Animals.

ELISA assays

ELISA assays were performed using wild-type phosphorylated Thr231-Pro tau (KVAVVRpTPPKSPS), non-phosphorylated Thr231 tau (KVAVVRTPPKSPS), *cis* locked phosphorylated Thr231-Dmp tau (KVAVVRpT(5,5-dimethyl-L-proline)PKSPS) and *trans* lock phosphorylated Thr231-Ala tau (P232A)(KVAVVRpTAPKSPS) peptides, as described⁴². Briefly, peptides at various concentrations in 2,2,2-Trifluoroethanol (50 µl) were plated onto maxi soap ELISA plate and dried up at 37°C overnight. After blocking with buffer containing 5 % milk, 0.4 % bovine serum albumin and 0.05% Tween 20 in Tris-Buffered Saline, the *cis* or *trans* mAbs at various dilutions in 5% milk, 0.4% bovine serum albumin and 0.05% Tween 20 in Tris-Buffered Saline (50 µl) was loaded and incubated at

room temperature for 2 hours, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG in 5% milk, 0.4% bovine serum albumin and 0.05% Tween 20 in Tris-Buffered Saline (50 μ l) for 1 hour. The ELISA plates were washed 4 times with buffer containing 0.4% BSA and 0.05% Tween 20 in TBS after each step. The signals were detected by incubating with TMB substrate solution and were measured by Wallac 1420 software at 450 nm.

Surface plasmon resonance

Surface plasmon resonance experiments were performed on a BIAcore 3000 surface plasmon resonance instrument (GE Healthcare-BIAcore) as described by the manufacturer. Briefly, Biacore sensor chip CM-5 was activated by using EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) and NHS (N-hydroxysuccinimide) in a 1:1 ratio for 7 minutes. Anti-mouse IgG (Fc) (GE healthcare) was immobilized at pH5 on flow cells 1 and 2, followed by the capture of 3.7 μ g/ml of *cis* or *trans* mAb in 10 mM Sodium acetate with a flow rate of 5 μ l/min. Then all tau peptides were injected at different 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 50 μ l/min for 3 min on both flow cells 1 and 2 and allowed to dissociate for 10 min. All samples were run in duplicate. After each run with a single antibody concentration, the surface was totally regenerated by 10 mM Glycine pH 1.7 flow rate 10 μ l/min for 5 sec. Data analysis was performed by using BIAevaluation software (GE healthcare-BIAcore).

Immunoblotting analysis and immunodepletion experiment

Immunoblotting analysis was carried out as described⁴². Briefly, brain tissues or culture cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP 40, 0.1% SDS, 0.5% Nadeoxycholate, 50 mM NaF) containing proteinase inhibitors and then mixed with the SDS sample buffer and loaded onto a gel after boiling. The proteins were resolved by polyacrylamide gel electrophoresis and transferred to PVDF membrane. After blocking with 5% milk in TBST (10 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% Tween 20) for 1 hr, the membrane was incubated with primary antibodies (*cis* and *trans* mAbs), Tau5 (Biosource Camarillo, CA), α -tubulin (Sigma, St. Louis, MO) and β -actin antibodies (Sigma, St Louis, MO) in 5% milk in TBST overnight at 4°C. Then, the membranes were incubated with HRP-conjugated secondary antibodies in 5% milk in TBST. The signals were detected using chemiluminescence reagent (Perkin Elmer, San Jose, CA). The membranes were washed 4 times with TBST after each step. To deplete *cis* or *trans* p-tau from lysates, brain or cell lysates were mixed with the *cis* or *trans* mAb antibody at 425 μ g/ml in RIPA buffer containing proteinase inhibitors for 3 h at 4°C and then mixed with protein A/G Sepharose for 1 hours at 4°C, followed by collecting the supernatants for experiments. The supernatants were dialyzed against phosphate buffer saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) for overnight prior to cell culture application. Immunoblotting results were quantified using Quantity One from BioRad.

Sarkosyl extraction

Isolation of sarkosyl-insoluble and soluble fractions of cells and brain tissues was performed as described^{29, 32, 42}, with slight modifications. Briefly, whole brains of mice were

homogenized by polytron in 10 volumes of buffer H (10 mM Tris-HCl [pH 7.5] containing 0.8M NaCl, 1 mM EGTA, and 1 mM dithiothreitol). The cell extraction was also performed with a convenient amount of buffer H (200 μ l / 35 mm culture dish) and sonication. The samples were spun at 100,000 \times g for 30 min at 4°C. Another 2 ml of buffer H was added to the pellet and the samples were homogenized again by polytron, incubated in 1% Triton X-100 at 37°C for 30 min. Following the incubation, the samples were spun at 100,000 \times g for 30 min at 4°C, the pellet was homogenized by polytron on 1 ml of buffer H and was then incubated in 1% sarkosyl at 37°C for 30 min and spun at 100,000 \times g for 30 min at 4°C. The supernatant was then collected (sarkosyl-soluble fraction). Detergent-insoluble pellets were extracted in 100 μ l of urea buffer (8 M urea, 50 mM Tris-HCl [pH 7.5]), sonicated, and spun at 100,000 \times g for 30 min at 4°C. The supernatant was then collected (sarkosyl-insoluble fraction). The protein concentrations of extracts were determined by BCA assay (Thermo Scientific). Sarkosyl-insoluble and -soluble fractions were run on SDS-PAGE gels.

Immunostaining Analysis

The primary antibodies used were *cis* and *trans* mAb, tau tangle-related mAbs AT180, AT8, AT100 (all from Innogenetics, Alpharetta, GA), oligomeric tau T22 polyclonal antibodies (EMD Millipore, Billerica, MA), PHF1 and Alz50 (gifts from Dr. Peter Davies), anti-tau rabbit mAb (E178, Abcam) and anti-neurofilament mouse mAb (SMI-312, IgG1, Abcam) for labeling axons, and anti-MAP2 mAb (SMI-52, IgG1, Abcam) for labeling dendrites. Immunofluorescence staining of mouse and human brains was done essentially as described^{29, 32, 42}. After treatment with 0.3 % hydrogen peroxide, slides were briefly boiled in 10 mM sodium citrate, pH 6.0, for antigen enhancement. The sections were incubated with primary antibodies overnight at 4°C. Then, biotin-conjugated secondary antibodies (Jackson ImmunoResearch), streptavidin-conjugated HRP (Invitrogen) were used to enhance the signals. For double immunofluorescence staining, the sections were also incubated with and Alexa Fluor 488 or 568 conjugated isotype-specific secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Manufacturer-supplied blocking buffer (Invitrogen) was used for each reaction. The sections were washed 4 times with TBS after each step. Labeled sections were visualized with a Zeiss confocal microscope. The gain of confocal laser was set at the level where there are no fluorescence signals including autofluorescence in sections without primary antibody but with secondary antibody. Immunostaining images and their colocalization were quantified using Volocity 6.3 from Perkin Elmer and Fiji/ImageJ Coloc 2, respectively.

Electronic microscopy

Sham and TBI mouse models treated with either control IgG or *cis* mAb were perfused with a fixative solution, a mixture of 15% picric acid (13% saturated solution; Sigma, St. Louis, MO, USA), 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), and 0.1% glutaraldehyde (EM grade 50% solution; Electron Microscopy Sciences) dissolved in PEM buffer (0.1 M PIPES, pH 7.2, 1 mM EGTA, 1 mM MgCl₂). Perfused brains were removed, sliced and kept in the same fixative for further 4 hours at 4°C. The samples were processed for electron microscopic observation as described^{51, 52}. Specimens were examined with a JEM-1010 transmission electron microscope (JEOL). For immunogold staining, SY5Y cells were treated with *cis* mAb for 18 hr, trypsinized and collected by

centrifugation, followed by fixation with 4% PFA. Samples were dehydrated with ethanol, processed for LR white resin embedding and sectioning, followed by gold staining as described⁵³.

Human brain specimens

Fixed human brain tissue from the frontal cortex of individuals with neuropathologically verified CTE was provided from the VA-BU-SLI Brain Bank of the Boston University Alzheimer's Disease Center CTE Program, including 16 patients with a history of exposure to TBI and 8 age-matched healthy controls (Supplementary Table 1)⁶. Next of kin provided written consent for participation and brain donation. Institutional review board approval for brain donation was obtained through the Boston University Alzheimer's Disease Center, CTE Program, and the Bedford VA Hospital. Institutional review board approval for neuropathological evaluation was obtained through Boston University School of Medicine⁶. Our studies on human samples have been approved by our Institutional Review Boards at Boston University and Beth Israel Deaconess Medical Center

Transgenic overexpression and knockout mice

Tau-Tg mice⁵⁴ and Tau knockout mice⁵⁵ (Jackson laboratory) in the C57BL/6 background were generated, as described^{29, 32, 42}. Animal care and use for the experiments have been approved by Institutional Animal Care and Use Committees at Beth Israel Deaconess Medical Center.

Cell culture

Neuronal cell lines including SH-SY5Y, PC12, H4 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. The media were supplemented with 100 Units/ml penicillin/streptomycin. PC12 cells were differentiated with NGF (50 ng/ml) and cultured for 2 days before stress. SY5Y cells (2.5×10^5 per ml) were transiently co-transfected with 2.5 μ g GFP-tau, 2.5 μ g Cdk5 and 2.5 μ g p25 with Lipofectamine 2000 (Invitrogen) as described⁴⁰. Cells were treated with *cis* or *trans* mAbs at 85 μ g/ml once 4 hours after transfection until observation.

Cell viabilities were examined using Live & Dead cell assay kit (Abcam) according to the manufacturer. For apoptosis assay, cells were trypsinized and suspended in a binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 2.5 mM CaCl₂), stained with Annexin V (Biolegend #640912) for 15 minutes and subjected for flow cytometry. Brain or cell extracts were applied to culture SY5Y cells for 18 hours and the cell viabilities were examined as described earlier. We performed the cell or brain lysate extraction using RIPA buffer but efficiently dialyzed the extracts against PBS for 48 hours dialysis with 4 changes of buffer.

Stably overexpressing RFP or GFP-tau SY5Y cells were routinely generated. Briefly, the plasmids pcDNA3.1-tau-RFP and -GFP constructed through restriction sites and were transfected into SY5Y cells via Lipofectamine 2000. Cells stably expressing tau selected with G418). Equal number of GFP- and RFP-tau expressing cells (2.5×10^5 per ml) were cocultured, treated or untreated with either *cis* or *trans* mAbs at concentration of 170 μ g/ml for 18 hours before moving into hypoxia chamber.

To testing cell or brain lysates, accordingly, we performed the extraction using RIPA buffer but efficiently dialyzed the extracts against PBS with a cocktail of protease inhibitors at 4°C for 48 hours dialysis with 4 changes of the buffer. After dialysis, we examined *cis* tau concentration and conformation with IB and applied the amount of dialyzed lysates similar to the original lysates to culture dishes. SY5Y cells were treated with 3-Methyladenine at concentration of 5 mM for 24 hours.

Primary neurons were prepared from 17-day-old embryonic mouse brain cerebral cortex of either sex. Neurons were seeded on pre-coated culture dishes (2×10^5 per ml). The medium was then changed to neurobasal medium supplemented with B27 (Invitrogen) and 1 mM L-glutamine as described⁵². Neurons were infected with lentivirus coding for either GFP- or RFP-Tau for 72 hours.

Mitochondrial transport assay

PC12 cells were differentiated with NGF (Cell Signaling) at 50 ng/ml and cultured for 2 days before stress. They were treated with *cis* or *trans* mAbs at 85 µg/ml for 18 hours and transferred into hypoxia chamber for 48 hr more. Then, mitochondria were stained using Mitotracker Green FM (Life technologies) according to manufacturer and observed with a Zeiss confocal microscope for 30 min using an incubation chamber with 5% CO₂ at 37°C. Fluorescent images of labeled mitochondria in the longest process of each PC12 cell were acquired at intervals of 5 s over a period of 30 min. Individual mitochondrial movements were analyzed with ZEN 2008 software (Zeiss). Differences in the position of each mitochondrion between two frames during each 5 s interval were exported to Excel, and they were classified and scored as stationary, fast (>0.05 µm/s) or slow (<0.05 µm/s) movements.

Traumatic brain injury

The mouse mild TBI model was used as previously described^{25, 56}. Briefly, male C57BL/6 mice (2-3 months old) obtained from the Jackson Laboratories (Bar Harbor, ME) were randomized to undergo injury or sham-injury. The mice were anesthetized for 45 seconds using 4% isoflurane in a 70:30 mixture of air:oxygen. Anesthetized mice were placed on a delicate task wiper (Kimwipe, Kimberly-Clark, Irving, TX) and positioned such that the head was placed directly under a hollow guide tube. Mouse's tail was grasped. A 54 -gram metal bolt was used to deliver an impact to the dorsal aspect of the skull, resulting in a rotational acceleration of the head through the Kimwipe. Mice underwent single severe injury (ssTBI, 60-inch height), single mild injury (mTBI, 28-inch height), or repetitive mild injuries (rmTBI, 7 injuries in 9 days). Sham-injured mice underwent anesthesia but not concussive injury. All mice were recovered in room air. Anesthesia exposure for each mouse was strictly controlled to 45 seconds. Blast-induced TBI mouse model was performed as described⁵. Briefly, anesthetized adult wild-type C57BL/6 male mice were exposed to a single blast or sham blast, removed from the apparatus, monitored until recovery of gross locomotor function, and then transferred to their home cage. Maximum burst pressure compatible with 100% survival and no gross motor abnormalities was ascertained empirically. All these and following animal experiments were approved by the Boston

Children's Hospital, Beth Israel Deaconess Medical Center and/or Boston University and IACUC and complied with the NIH Guide for the Care and Use of Laboratory Animals.

Antibody treatment of mice

Mice undergoing TBI were randomized to treatment with anti-*cis* p-tau monoclonal mouse antibody or mouse IgG2b. Mice received 1 dose of *cis* antibody or IgG2b intraperitoneal pretreatment (i.p. 200 µg/per mouse) 3 days prior to the injury (which was omitted in some experiments), post-injury treatment with single intracerebroventricular (ICV) treatment (20 µg in 5 microliters) 15 minutes after injury, then post treatment 200 µg i.p. every 4 days for 3 times and analyzed brains 14 days later for IB or fEPSP recording, followed by 200 µg i.p. weekly for another 1.5 month (with total 2 months of treatment) before the elevated plus maze or the Morris Water Maze in a double blindness manner. After the above treatment, some mice received further antibody treatment, by 200 µg i.p. biweekly for another 4 months before assaying *cis* p-tau spread, tau aggregation and tauopathy and brain atrophy at 6 months after ssTBI, as described^{29, 32, 42}. For all behavioral tests, experimenters were blinded to injury and treatment status, using color-coding stored in a password protected computer.

Electrophysiology

Mice were anesthetized with isoflurane (NDC 10019-360-40, Baxter Healthcare Corporation Deerfield, IL, USA) and decapitated. The brains were quickly removed and placed for sectioning in ice-cold treatment artificial cerebrospinal fluid (tACSF) containing (in mM) NaCl 124, KCl 3, NaH₂PO₄ 1.25, NaHCO₃ 26, CaCl₂ 2, MgSO₄ 2, and glucose 10 (pH 7.4, and bubbled with 95% O₂ and 5% CO₂ gas mixture). Cortical slices (thickness 350 µm) were cut with a Vibratome 1000P (Leica VT1000P, Leica Microsystems Inc., Buffalo Grove, IL, USA) and transferred to a chamber with oxygenated tACSF for 90 min at 30°C before recording.

Field excitatory postsynaptic potentials (fEPSP) were recorded using a multi-electrode array recording system (MED64 system) with MED-P5155 probe (AutoMate Scientific, Inc., Berkeley, CA, USA) in this study. After incubation, one cortical slice was positioned in the center of the MED64 probe (to fully cover the 8 × 8 electrode array) with oxygenated recording ACSF (rACSF) containing (in mM) NaCl 124, KCl 3, NaH₂PO₄ 1.25, NaHCO₃ 26, CaCl₂ 2, MgSO₄ 1, and glucose 10 (pH 7.4) at 30°C. A fine nylon mesh and a mesh anchor were placed on top of the slice to immobilize the slice during recording. The probe with immobilized slice was connected to two MED64 amplifiers [MED64 Head Amplifier (MED-A64HE1) and Main Amplifier (MED-A64MD1), AutoMate Scientific, Inc., Berkeley, CA, USA]. The slice was continuously perfused with oxygenated, fresh rACSF at the rate of 2 ml/min using a peristaltic pump (Minipuls 3, Gilson Inc., Middleton, WI).

Data was collected using Mobius software (Mobius 0.4.2). Field potentials were induced by single pulses (0.2 ms) delivered at 0.05 Hz through one planar microelectrode in layer V of cortical slice. We used stimulus intensity sufficient to induce a 50% of the maximal fEPSP slope in all experiments. The fEPSP was recorded from the channels in layer II/III. A stable fEPSP slope for at least 20 minutes was recorded as baseline. The induction protocol of LTP

that we used is 5Hz theta burst (each burst consists of 4 pulses at 100hz). The data were filtered at 10 kHz and digitized at a 20 kHz sampling rate. Data were analyzed off line by the MED64 Mobius software. For quantifying the level of LTP, the mean of fEPSP slope (10-40%) within the last 10-min's recording was normalized and expressed as a fold change of the averaged baseline (first 10 min of the baseline). Three successive responses were averaged. Statistics were performed using the number of slices as 'n' value, and one to two slices per animal. *p* values was calculated using one-way ANOVA with Bonferroni posthoc test.

Morris water maze

A Morris water maze (MWM) paradigm was used to evaluate spatial learning and memory as described^{25, 57}. Briefly, a white pool (83 cm diameter, 60 cm deep) was filled with water to 29 cm depth. Water temperature was maintained at approximately 24°C. Several highly visible intra- and extra-maze cues were located in and around the pool. The target platform (a round, clear, plastic platform 10 cm in diameter) was positioned 1 cm below the surface of the water. During hidden and visible platform trials, mice were randomized to one of four starting quadrants. Mice were placed in the tank facing the wall and given 90 seconds to find the platform, mount the platform, and remain on it for 5 seconds. Mice were then placed under a heat lamp to dry before their next run. Time until the mouse mounted the platform (escape latency) was measured and recorded. Mice that failed to mount the platform within the allotted time (90 seconds) were guided to the platform by the experimenter and allowed 10 seconds to become acquainted with its location. Each mouse was subjected to a maximum of two trials per day, each consisting of four runs, with a 45-minute break between trials. For visible platform trials, a red reflector was used to mark the top of the target platform. For probe trials, mice were placed in the tank with the platform removed and given 60 seconds to explore the tank. Noldus Ethovision 9 software tracked swim speed, total distance moved, and time spent in the target quadrant where the platform was previously located. When mice underwent repeat MWM testing, 2 to 3 months or 6 months after their final injury, the platform was moved to a different quadrant than that used previously.

Elevated plus maze

The elevated plus maze was used to assess anxiety/risk-taking behavior two months after injury and carried out as described⁵⁸. Briefly, the elevated plus-maze consists of two open and two closed arms (30×5cm) extended out opposite from each other from a central platform (decision zone) to create a plus shape. The entire apparatus is raised 85 cm above the floor (Lafayette Instruments). Mice are placed on the center platform of the maze, facing a closed arm, and allowed to explore the apparatus for 5 minutes. The maze is cleaned between subjects with a weak ethanol solution and dried. A computer-assisted video-tracking system (Noldus Ethovision) recorded the total time spent in the open center (decision zone), and the two closed or "safe" arms and the two open or "aversive" arms. The percent time spent in the open arms is used as a surrogate measure of anxiety/risk-taking behavior; mice with lower levels of anxiety/risk-taking behavior spend less time in the open arms.

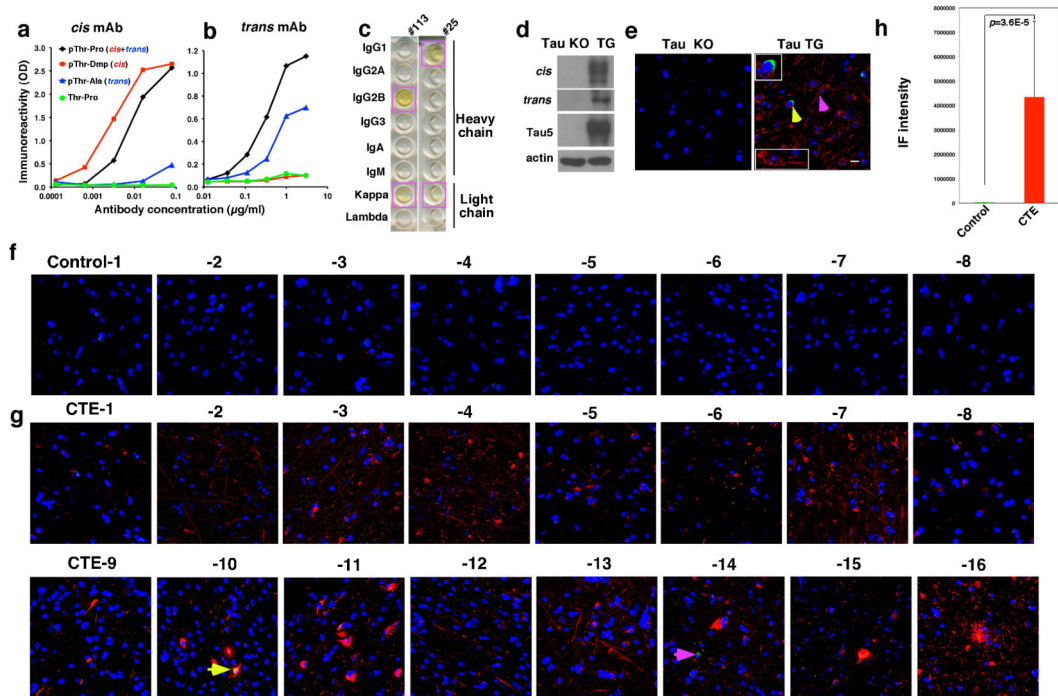
Immunohistochemistry

Mice were intra-cardiacally perfused with 4% paraformaldehyde at various time points after injury and brains were collected for histopathological outcomes. Serial 20µm coronal frozen sections from sham and injured brains were cut on a cryostat (Leica) from the anterior frontal lobes through the posterior extent of the dorsal hippocampus. Every 10th section was collected and mounted on slides.

Statistical analysis

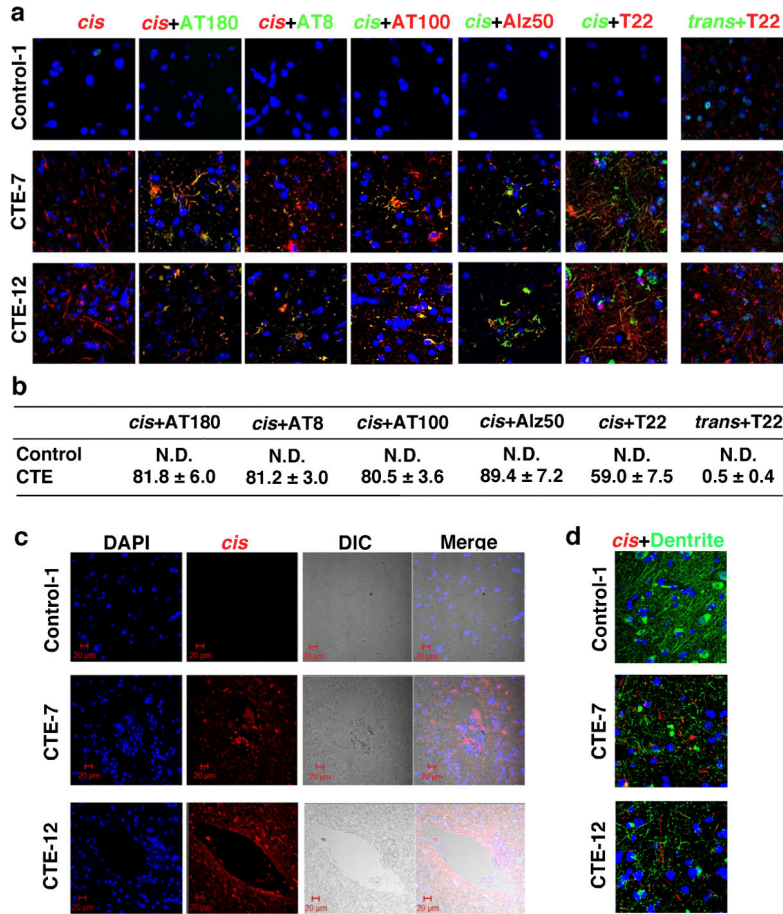
Experiments were routinely repeated at least three times, and the repeat number was increased according to effect size or sample variation. We estimated the sample size considering the variation and mean of the samples. No animals or samples were excluded from any analysis. Animals were randomly assigned groups for *in vivo* studies and for mAb treatment experiments in mice, group allocation and outcome assessment were also done in a double blinded manner. For all behavioral tests, experimenters were blinded to injury and treatment status, using color coding stored in a password protected computer. All data are presented as the means ± s.d. except behavioral tests where data are presented as the means ± s.e.m, followed by determining significant differences using the two-tailed Student's *t* test for quantitative variables or ANOVA test for continuous or three or more independent variables or one-way ANOVA with Bonferroni posthoc test, and significant *p*-values <0.05 are shown.

Extended Data



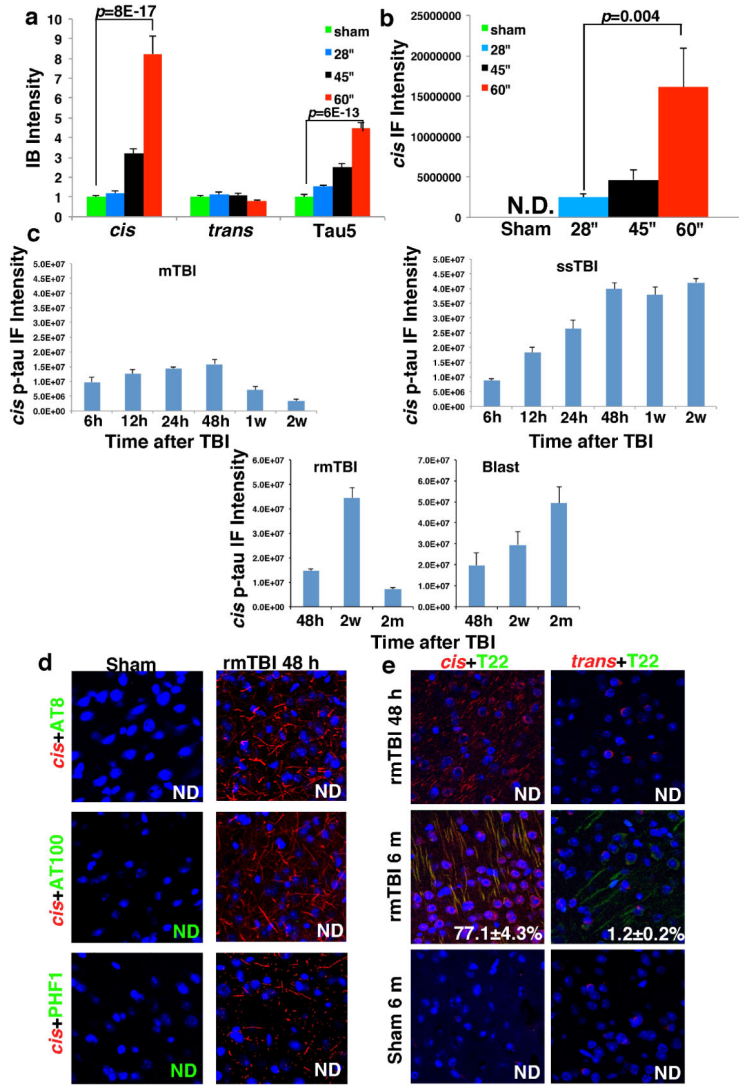
Extended Data Figure 1. Characterization of *cis* and *trans* p-tau mAbs and robust *cis* p-tau in human CTE brains

a, b, Characterization of the specificity of *cis* and *trans* p-tau mAbs by ELISA. *cis* (**a**) and *trans* (**b**) antibodies at various concentrations were incubated with *cis* (pT231-Dmp), *trans* (pT231-Ala), *cis+trans* (pT231-Pro) or T231-Pro tau peptides, followed by detecting the binding by ELISA. Representative examples of ELISA are shown from 3 independent experiments. pT231-Pro, CKKVAVVVRpT(Pro)PKSPSSAK; pT231-Pip, CKKVAVVVRpT(homoproline)PKSPSSAK; pT231-Ala, KVAVVVRpT(Alanine)PKSPS; pT231-Dmp (KVAVVVRpT(5,5-dimethyl-L-proline)PKSPS). **c**, Determination of the isotypes of *cis* and *trans* p-tau mAbs. Isotypes of *cis* and *trans* mAb heavy and light chains were determined by ELISA assay using a commercially available assay kit. **d, e**, Characterization of the specificity of *cis* and *trans* p-tau mAbs by IB and IF. Brain lysates (**d**) or sections (**e**) prepared from tau-deficient (KO) or wild-type tau-overexpressing (TG) mice were subjected to IB or IF with *cis* and/or *trans* antibody. The *cis* and *trans* signals were readily detected in TG, but not at all in KO mouse brains, with *cis* in the soma and neurites (pink arrow), but *trans* only in the soma (yellow arrow) (insets). Similar results were observed in at least three different animals. *cis*, red; *trans*, green; DNA, blue. **f-h**. Robust *cis* p-tau in human CTE brains. 16 CTE brain tissues and 8 healthy controls were subjected to IF, with one representative image from each case being shown) (**f, g**). Yellow arrow points to a neuron expressing both *cis* (red) and *trans* (green) p-tau, while pink one to a neuron expressing only *trans* in the soma. Fluorescence immunostaining intensity of *cis* p-tau was quantified using Volocity 6.3 from Perkin Elmer (**h**). The results are expressed as means \pm s.d. and *p* values determined using the Student's t test.

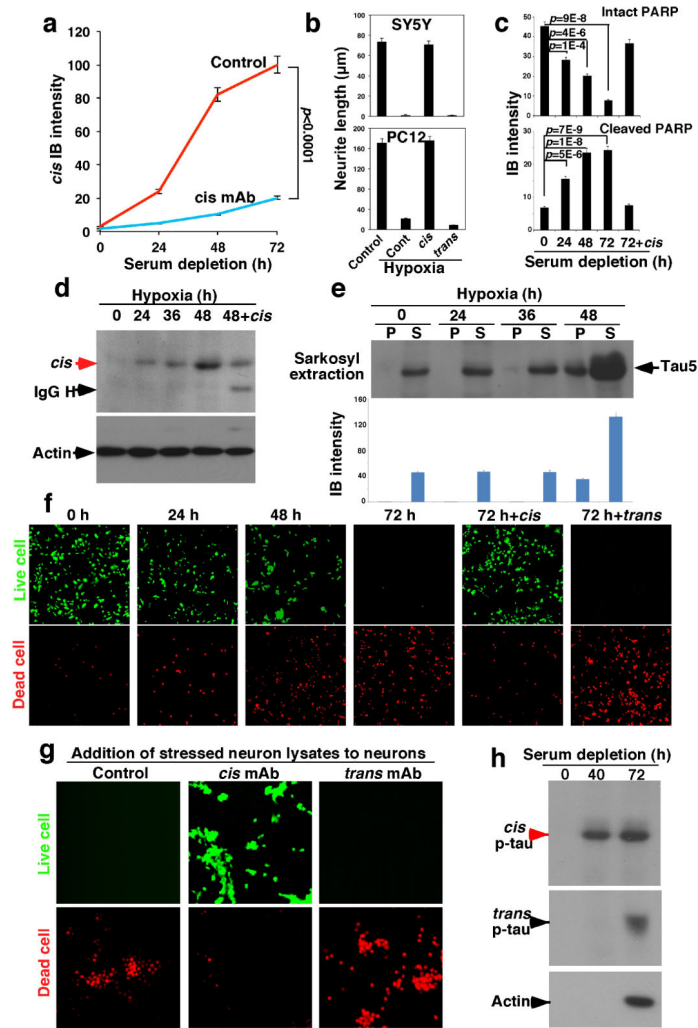


Extended Data Figure 2. Colocalization of *cis* p-tau with other tau epitopes and its concentration near blood vessels in CTE brains

a, b, Colocalization of *cis* p-tau with other tau epitopes in CTE brains. CTE brain tissues and healthy controls were stained with *cis* mAb and AT180, AT8, AT100, Alz50 or T22 antibodies, or *trans* mAb and T22 antibodies, with two examples being shown (**a**), and then quantified their colocalization using Coloc 2, with the results being expressed in a percentage (mean ± s.d.) (**b**). N.D., not detectable. **c,** CTE brain tissues and healthy controls were stained with *cis* mAb, with two examples being shown. *cis* is more prominent near blood vessels, which corresponds to the typical perivascular distribution of p-tau in CTE. **d,** CTE brain tissues and healthy controls were stained with *cis* mAb (red) and the dendritic marker MAP2 (green), along with DNA dye (blue). Colors in the text correspond to their fluorescence labels. n=4.



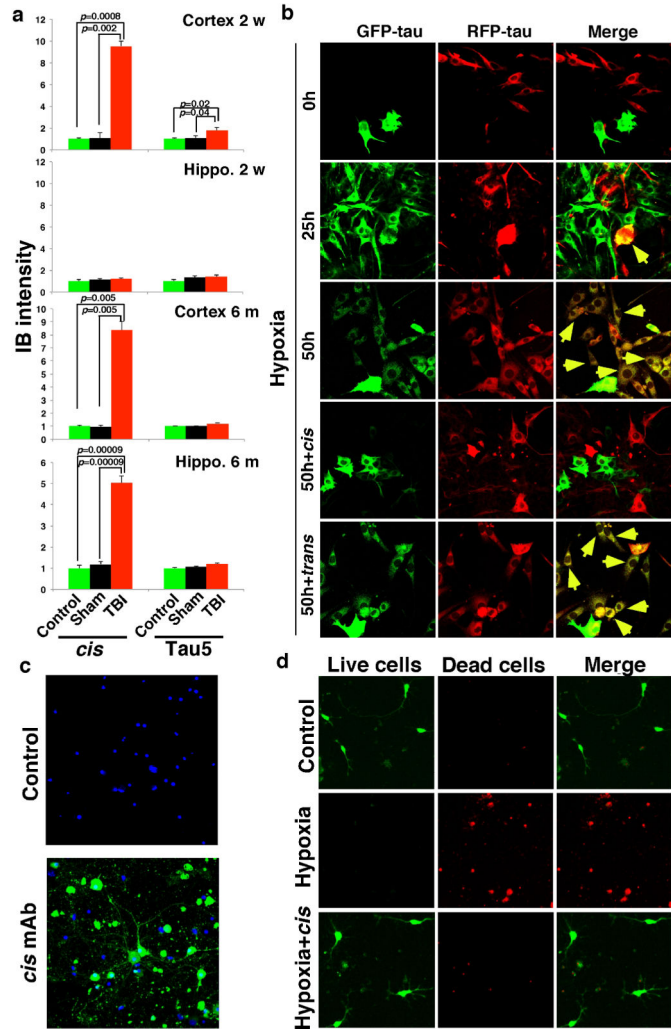
Extended Data Figure 3. TBI induces *cis* p-tau in a severity- and time-dependent manner long before other known tauopathy epitopes
a-c, Severity- and time-dependent induction of *cis* p-tau after TBI. Quantification results of Fig. 2a-f. **d**, Robust *cis* p-tau signals were detected in neurons 48 hr after rmTBI without any other tangle-related tau epitopes. 48 h after rmTBI, brain sections were stained with *cis* mAb (red) and AT8, AT100 or PHF1 (green). **e**, Robust *cis* p-tau signals were detected in neurons 48 hr after rmTBI without tau oligomerization, which appears and colocalizes with *cis* p-tau at 6 month after TBI. 48 h or 6 m after rmTBI or sham treatment, brain sections were immunostained with T22 (green) and *cis* or *trans* mAb (red). The results in 48 hr sham mice were similar to those at 6 m (data not shown). The colocalization of red and green signals was quantified using Coloc-2, with the results being shown in percentages. ND, not detectable. n=3-4. The results are expressed as means ± s.d. and *p* values determined using the Student's *t* test.



Extended Data Figure 4. Stressed neurons robustly produces *cis* p-tau, *cis* p-tau is released from stressed neurons and neurotoxic, but are effectively blocked by *cis*, but *trans*, mAb

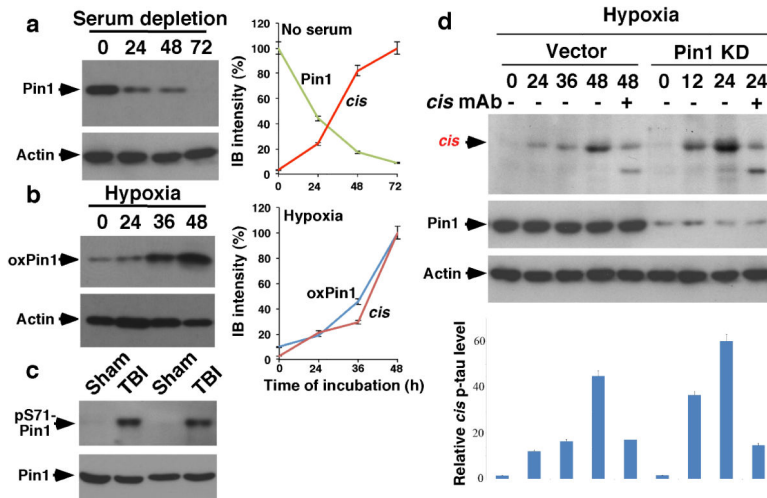
a-c, Quantification results of Fig. 4a, b and f, respectively. The results are expressed as means \pm s.d. and p values determined using the two-way ANOVA test (**a**) and Student's t test (**c**). **d**, Hypoxia induces *cis* p-tau, which is blocked by *cis* mAb. SY5Y neurons expressing a control vector were cultured in the hypoxia chamber in the absence or presence of *cis* or *trans* mAb for the times indicated, followed by IB for *cis* p-tau. **e**, Hypoxia induces *cis* p-tau before tau aggregation. SY5Y neurons were subjected to hypoxia for the times indicated, followed by sarkosyl extraction before IB with Tau5 mAb and quantification. **f**, Hypoxia induces cell death, which are blocked by *cis*, but *trans*, mAb. SY5Y neurons were cultured in the hypoxia chamber in the absence or presence of *cis* or *trans* mAb for the times indicated, followed by live and dead cell assay using the LIVE/DEAD® Viability/Cytotoxicity Kit. **g**, Stressed neuron lysates are neurotoxic, which is neutralized by *cis*, but not *trans*, mAb. Cell lysates were prepared from stressed SY5Y neurons and then added to growing SY5Y neurons directly (Control) or after immunodepletion with *cis* or *trans* mAb to remove *cis* or *trans* p-tau, respectively for 3 days, followed by live and dead cell assay. **h**, *cis* p-tau is released from stressed neurons. SY5Y neurons were cultured in the absence of

serum for the times indicated and culture media were collected and centrifuged, followed by analyzing the supernatants for *cis* and *trans* p-tau with actin as a indicator of cell lysis.



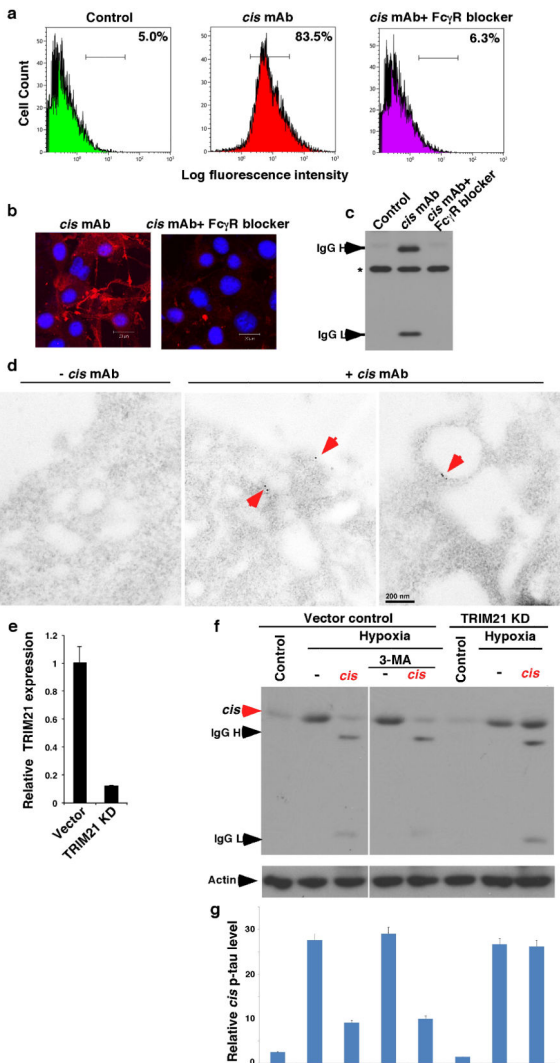
Extended Data Figure 5. *cis* p-tau spreads after rmTBI or neuronal stress, and hypoxia induces cell death in primary neurons, which is blocked by *cis* mAb

a, *cis* p-tau spreads in the brain after rmTBI. Quantification results of Fig. 3c. **b**, *cis* p-tau spreads after neuronal stress. GFP-tau or RFP-tau SY5Y neurons were co-cultured and subjected to hypoxia or control treatment in the presence or absence of *cis* or *trans* mAb for different times, followed by assaying cells expressing both GFP-tau and RFP-tau (arrows) to determine tau spreading among cells. The results are expressed as means \pm s.d. and *p* values determined using the Student's *t* test. **c**, *cis* mAb enters primary neurons. Primary neurons were established from mouse embryos and differentiated in vitro and *cis* mAb was added to culture media, followed by immunostaining with secondary antibodies. **d**, Hypoxia induces cell death in primary neurons, which is effectively blocked by *cis* mAb. Primary neurons were cultured in the hypoxia chamber in the absence or presence of *cis* mAb for 48 h, followed by live (green) and dead (red) cell assay using the LIVE/DEAD® Viability/Cytotoxicity Kit.



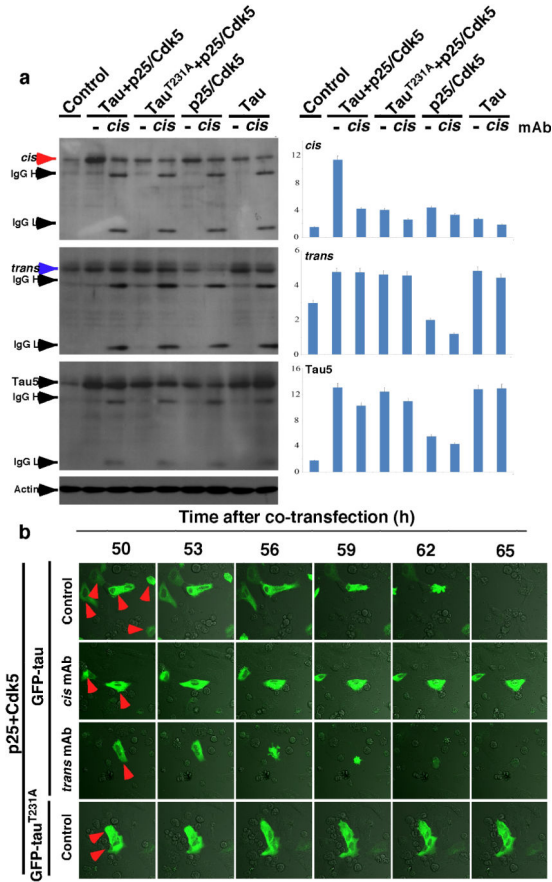
Extended Data Figure 6. Pin1 inhibition by multiple mechanisms contributes to *cis* p-tau induction after neuron stress and TBI

a, Pin1 was downregulated and correlated with *cis* p-tau induction after serum starvation. Cells were subjected to serum starvation for times indicated, followed by IB, with the right panel showing the correlation of Pin1 down regulation with *cis* p-tau induction from Figure 4a. **b**, Pin1 was oxidized and correlated with *cis* p-tau induction after hypoxia. SY5Y cells were subjected to hypoxia for times indicated, followed by IB for C113 oxidized Pin1, with the right panel showing the correlation of Pin1 oxidation with *cis* p-tau induction from Extended Data Figure 6d. **c**, Pin1 is inhibited in TBI mouse brains. Mouse brains 48 hr after ssTBI were subjected to IB and quantification for Pin1 and S71 phosphorylated Pin1. **d**, Pin1 knockdown potentiates the ability of hypoxia to induce *cis* p-tau. Pin1 KD or vector control SY5Y cells were subjected to hypoxia treatment for the times indicated in the presence or absence of *cis* mAb, followed by IB and quantification for *cis* p-tau levels.



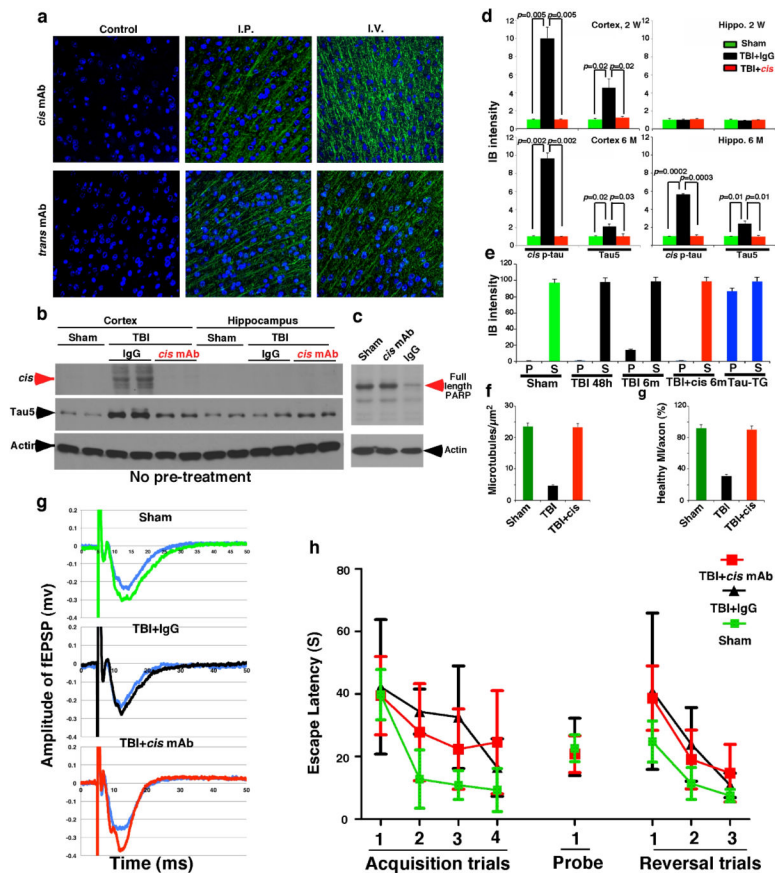
Extended Data Figure 7. Inhibition of Fc γ R binding blocks *cis* mAb from entering neurons and TRIM21 KD fully prevented *cis* antibody from ablating *cis* p-tau in neurons

a-d, Inhibition of Fc γ R binding potently blocks *cis* mAb from entering neurons. *cis* mAb was added to neurons in the absence or presence of a human Fc γ R-binding inhibitor, followed by detecting the binding of *cis* mAb to the cell surface by FACS (**a**), entry of *cis* mAb into cells by IF (**b**), IB (**c**) and electron microscopy after immunogold labeling (**d**). The FcR binding inhibitor fully blocked *cis* mAb from binding to the cell surface and entering neurons. Electron microscopy showed that *cis* mAb binds to the cell surface and endocytic vesicles (red arrows). **e, f**, TRIM21 knockdown fully prevented *cis* antibody from ablating *cis* p-tau in neurons. TRIM21 was stably knocked down in SY5Y neuronal cells using a validated TRIM21 shRNA lentiviral vector and confirmed by real-time RT-PCR analysis of TRIM21 mRNA expression (**e**). TRIM21 knockdown or vector control SY5Y cells were subjected to hypoxia treatment in the presence or absence of *cis* mAb and/or 3-Methyladenine, an autophagy inhibitor, followed by IB, followed by quantifying *cis* p-tau levels normalized actin levels (lower panel) (**f**).



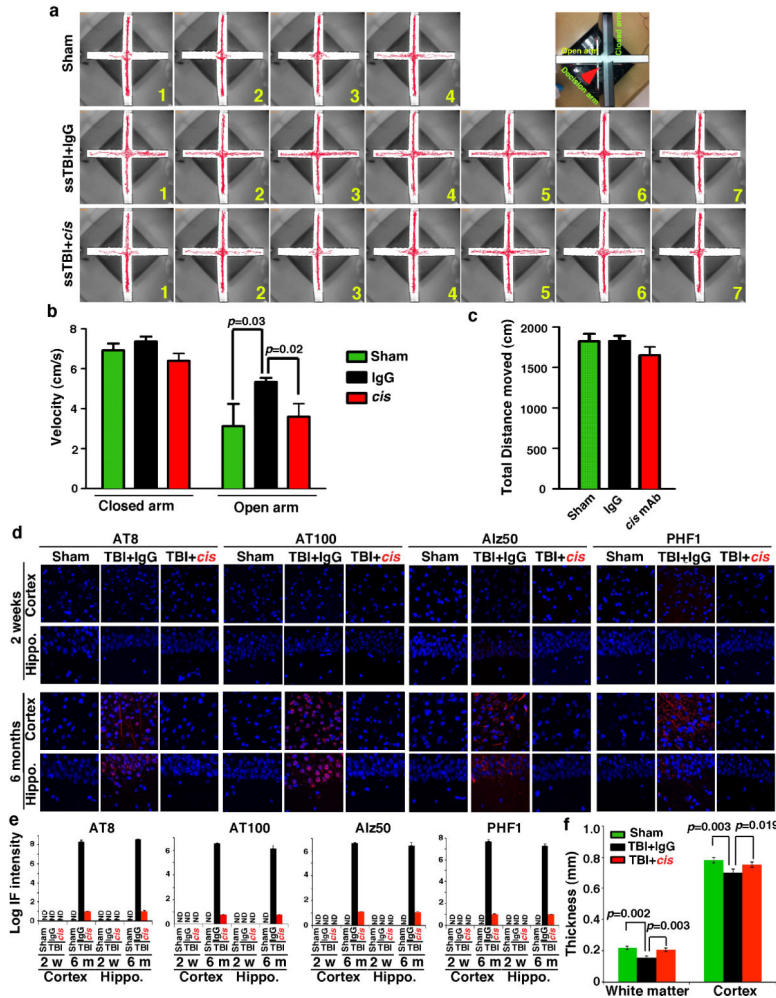
Extended Data Figure 8. *cis* pT231-tau is both necessary and sufficient for p-tau to induce neuronal cell death *in vitro*

a, SY5Y cells were co-transfected with non-tagged indicated constructs in the absence and presence of *cis* mAb followed by IB with quantification on the right panel. **b**, SY5Y cells were co-transfected with GFP-tau, or GFP-tau^{T231A} and p25/Cdk5 in the absence and presence of *cis* or *trans* mAb followed by live-cell confocal video (see videos 5, 6). Red arrows point to GFP-tau or -mutant expressing cells.



Extended Data Figure 9. *cis* mAb effectively blocks *cis* p-tau induction and spread, tau aggregation, and restores neuronal ultrastructures, apoptosis and defective LTP after TBI
a, Peripherally administrated *cis* and *trans* mAbs enter neurons in brains. 250 μ g of biotinylated *cis* or *trans* mAb was injected i.p. or i.v. into B6 mice, followed by detecting the biotinylated *cis* mAb in brains 3 days later. **b, c**, *cis* mAb effectively blocks *cis* pT231-tau induction and apoptosis. ssTBI mice were randomly and blindly treated with *cis* mAb or IgG isotype control, i.c.v. 20 μ g per mouse 15 min after injury, and then i.p. 200 μ g every 4 days for 3 times, followed by subjecting brains to IB for *cis* p-tau (**b**) and PARP cleavage (**c**), with sham as controls. **d-f**, *cis* mAb effectively blocks *cis* pT231-tau induction and spread, tau aggregation and restores neuronal ultrastructures. ssTBI mice in (**c**) received additional i.p. 200 μ g per mouse 3 day prior to injury. **d**, Quantification of IB in Fig. 5a. **e**, Quantification of IB in Fig. 5b. **f**, Quantification of EM images in Fig. 5c. n=3. The results are expressed as means \pm s.d. and *p* values determined using the Student's *t* test. **g**, *cis* mAb treatment of ssTBI mice rescues defective long-term potentiation in the cortex. fEPSPs were recorded in the layer II/III by stimulating the vertical pathway (the layer V to II/III) in the cortex. Robust LTP was induced by 5 Hz theta-burst in the cortical slices of sham mice (n=15 slices, 9 mice), but was deficient in the cortex of IgG-treated TBI mice (n=9 slices, 5 mice). However, LTP magnitude was restored to the control level in *cis* mAb-treated TBI animals (n=9 slices, 5 mice). The representative recordings were presented. **h**, No significant effects of *cis* pT231 tau mAb treatment on Morris Water Maze performance. 8 weeks after ssTBI, mice underwent Morris Water Maze (MWM) testing consisting of 4 acquisition trials

(hidden platform) daily for 4 days (4 runs/trial), a probe trial, followed by a 3 reversal trials (hidden platform) daily for 3 days. Compared to sham mice, injured mice demonstrated increased latency to find the hidden platform in acquisition and reversal trials ($p < 0.001$). There was no difference in injured *cis* mAb mice compared to injured IgG treated mice in acquisition trials ($p = 0.5$) or reversal trial ($p = 0.9$). For probe trials, injured mice performed similarly to sham mice ($p = 0.7$) and injured *cis* mAb treated mice performed similarly to injured IgG treated mice ($p = 0.2$). $n = 4-7$. The results are expressed as means \pm s.e.m. and p values determined using the ANOVA test.



Extended Data Figure 10. *cis* mAb treatment effectively restores risk-taking behavior and prevents tauopathy development and spread as well as brain atrophy after TBI

a-c, *cis* mAb treatment effectively restores risk-taking behavior 2 months after ssTBI. Video-tracking data of each of all mice shows that ssTBI mice treated with *cis* mAb ($n = 7$) spent similar and very little time in the open arm compared to sham mice ($n = 4$), but much less time than TBI mice treated with IgG2b ($n = 7$) (**a**). *cis* mAb-treated ssTBI mice had similar performance to sham in traveling velocity, but IgG2b-treated ssTBI mice traveled a greater velocity in the open arm (**b**). All three groups traveled similar total distance (**c**). Results are expressed as mean \pm S.E.M. and p values determined using the Student's t test.

d-f, *cis* mAb treatment effectively prevents tauopathy development and spread as well as brain atrophy 6 months after ssTBI. ssTBI mice were treated with *cis* mAb or IgG control for 2 weeks or 6 months, with sham mice as controls, followed by IF with various tauopathy epitopes (**d**), with immunostaining fluorescence intensity in the cortex and hippocampus being quantified (**e**), or to NeuN immunostaining for determining the thickness of the cortex and white matter at 6 months after TBI (**f**). n=4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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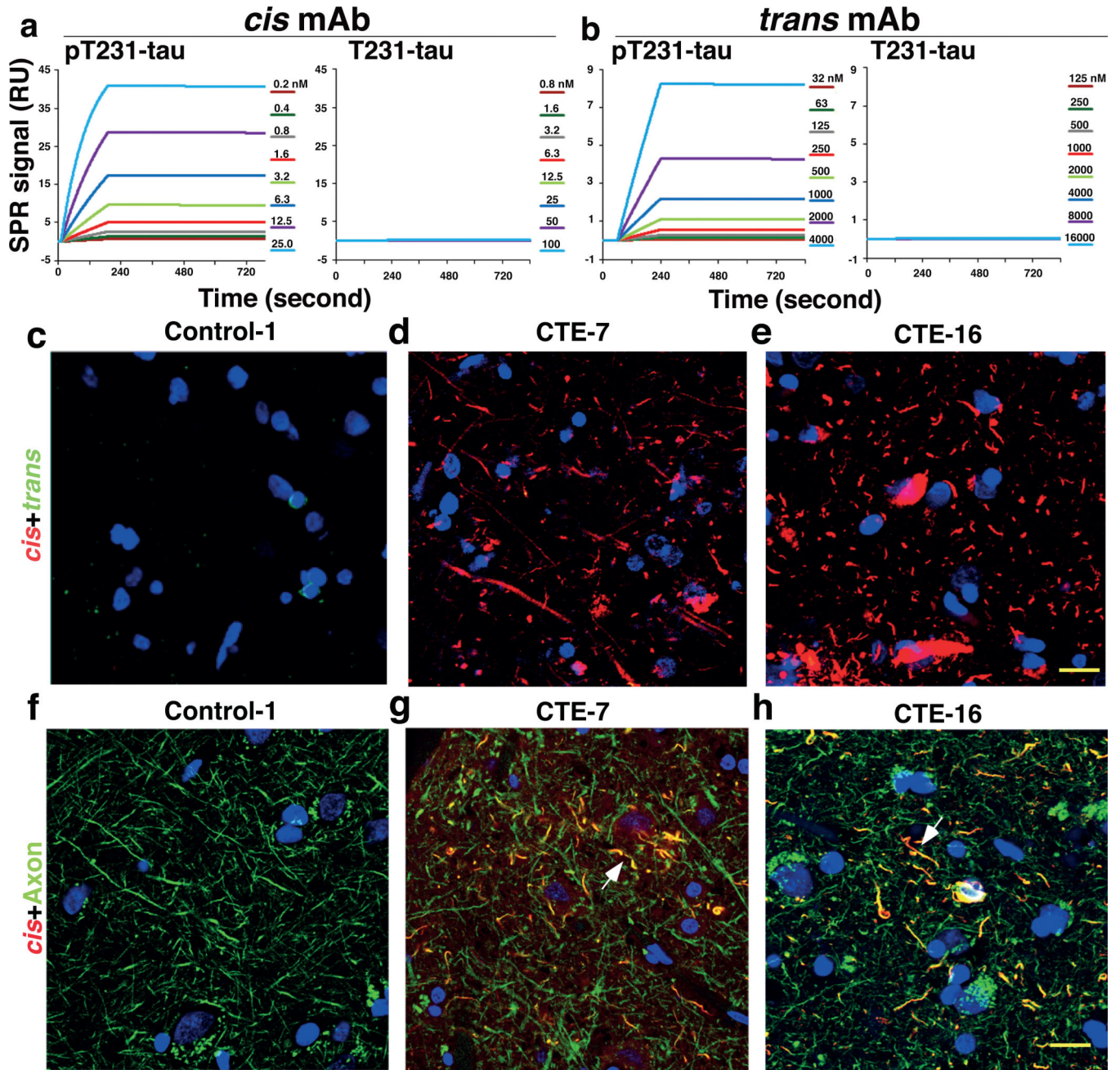


Figure 1. Robust *cis*, but not *trans*, p-tau at diffuse axons in human CTE brains
a, b, *cis* (**a**) or *trans* (**b**) mAb were immobilized on a sensor chip CM5 for surface plasmon resonance and their binding to pT231- or T231-tau peptide at different concentrations were recorded by SRP sensorgrams. **c-h**, The frontal cortex of neuropathologically verified human CTE brains and normal controls were subjected to double IF with *cis* (red) or *trans* (green) mAbs (**c-e**), N=16 for CTE and 8 for controls, or with *cis* pT231 (red) and the axonal marker tau (green), along with DNA dye (blue) (**f-h**), N=4. Two typical *cis* p-tau immunostaining patterns are presented, with all cases being shown in Extended Data Fig 1f. Arrows, colocalization; Bars, 20 μ m.

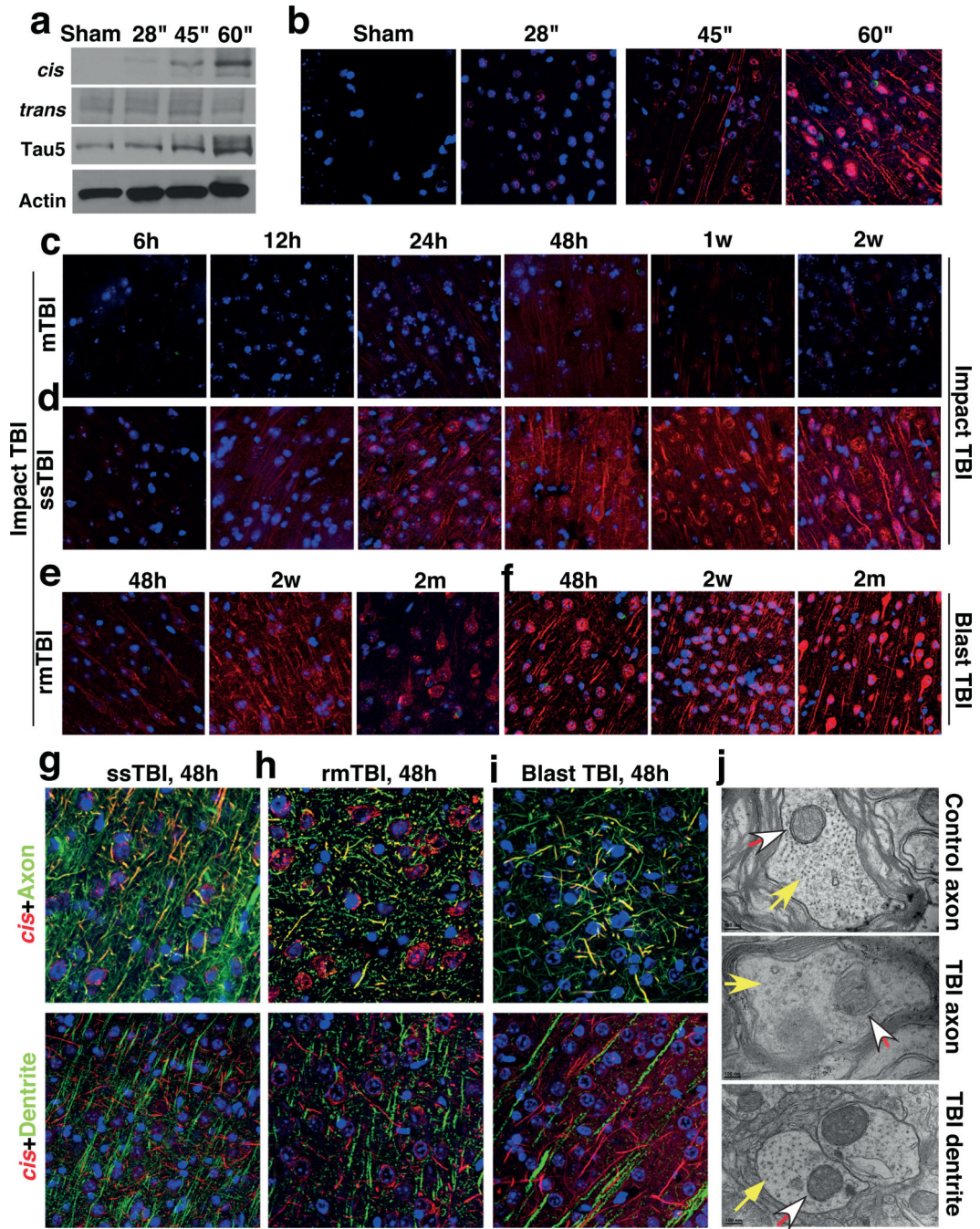


Figure 2. While mTBI has moderate and transient effect, rmTBI, ssTBI or blast TBI leads to robust and persistent *cis* p-tau induction notably in diffuse axons starting at 12-24 hr
a, b, Mice were subjected to single TBI by 54 g weight drop from varying heights, followed by IB (**a**) and IF (**b**) to detect *cis* and *trans* p-tau 48 h later. *cis*, red; *trans*, green; DNA, blue.
c-e, Mice were subjected to single mTBI (**c**), ssTBI (**d**) or rmTBI (**e**), followed by IF to detect *cis* and *trans* p-tau at different times after last injury. N=4. **f,** Mice were subjected to blast-induced TBI, followed by IF to detect *cis* and *trans* p-tau at different times. N=3. **g-i,** ssTBI (**g**), rmTBI (**h**) and blast TBI (**i**) brain sections at 48 hr after last injury were subjected

to double IF with *cis* pT231 (red) and axon marker neurofilament SMI312 (green) or dendrite marker MAP2 (green), along with DNA dye (blue). N=4; Arrows, colocalization; Bars, 20 μ m. **j**, ssTBI or sham mice were subjected to EM analysis 48 h after injury to examine the structure of MTs (filled arrows) and MI (open arrows) at axons and dendrites.

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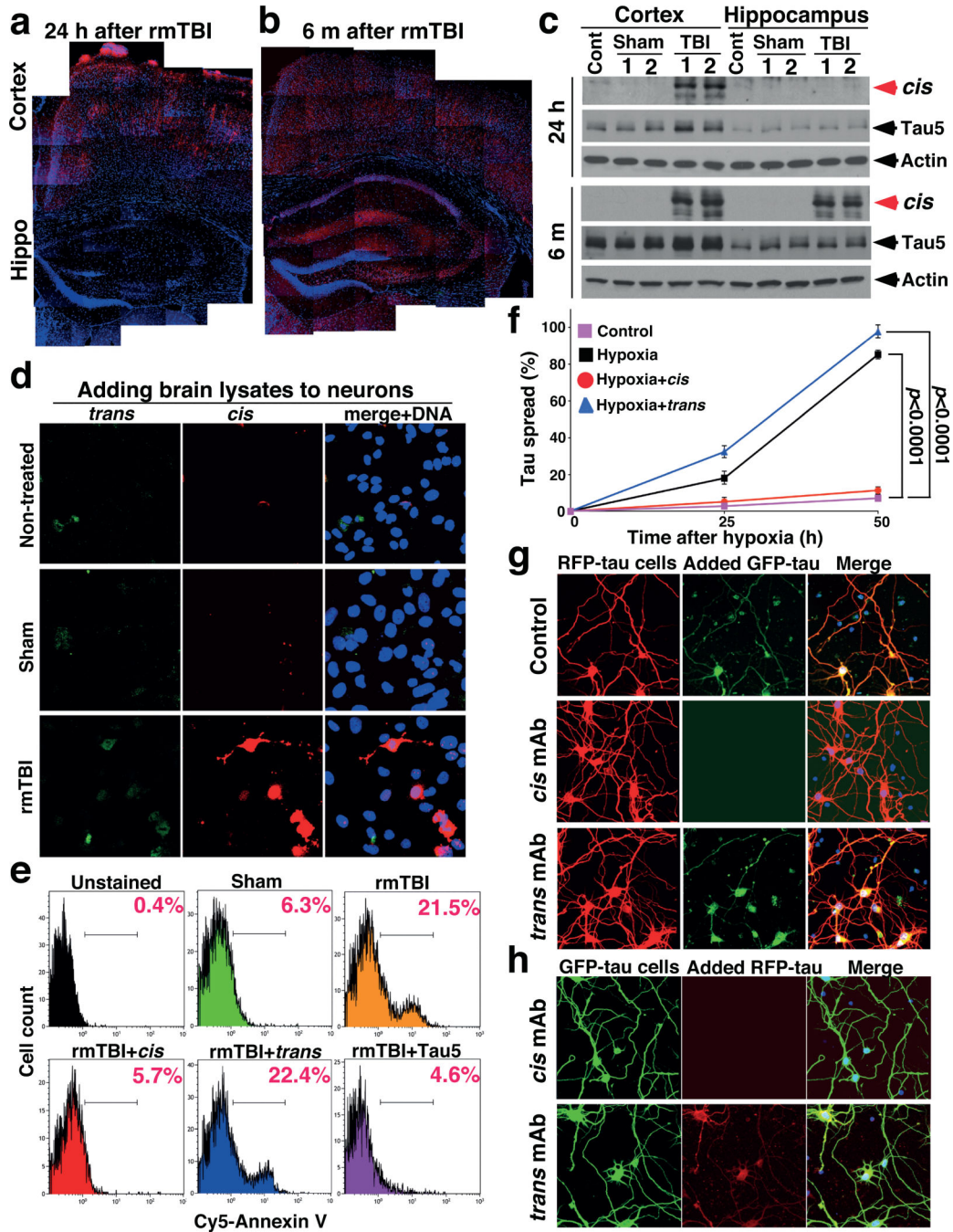


Figure 3. *cis* p-tau spreads in the brain after rmTBI, and spreads and causes neurotoxicity after neuronal stress *in vitro*, which are fully blocked by *cis*, but not *trans*, mAb

a-c, 24 hr or 6 months after rmTBI, mouse brains were subjected to IF (**a, b**) and IB (**c**) to detect *cis* p-tau in different brain regions. N=4. **d, e,** Mouse brain lysates prepared from 6 month after rmTBI or sham controls were added to culture media of SY5Y neurons for 17 h directly or after immunodepletion with *cis* or *trans* mAb, followed by IF with *cis* and *trans* mAbs or Annexin V FACS. N=3. **f,** SY5Y neurons stably expressing GFP-tau or RFP-tau were co-cultured and then treated with hypoxia or control in the presence or absence of *cis*

or *trans* mAb for different times, followed by assaying cells expressing both GFP-tau and RFP-tau (mean \pm S.D.). *p* values, two-way ANOVA test. **g, h**, Primary mouse neurons were transfected with GFP-tau or RFP-tau, and then subjected to hypoxia treatment in the absence or presence of *cis* or *trans* mAb for 36 hours. Resulting filtered soluble media from GFP-expressing neurons were added to RFP-expressing neurons (**g**) or vice versa (**h**), followed by detecting entry of added tau.

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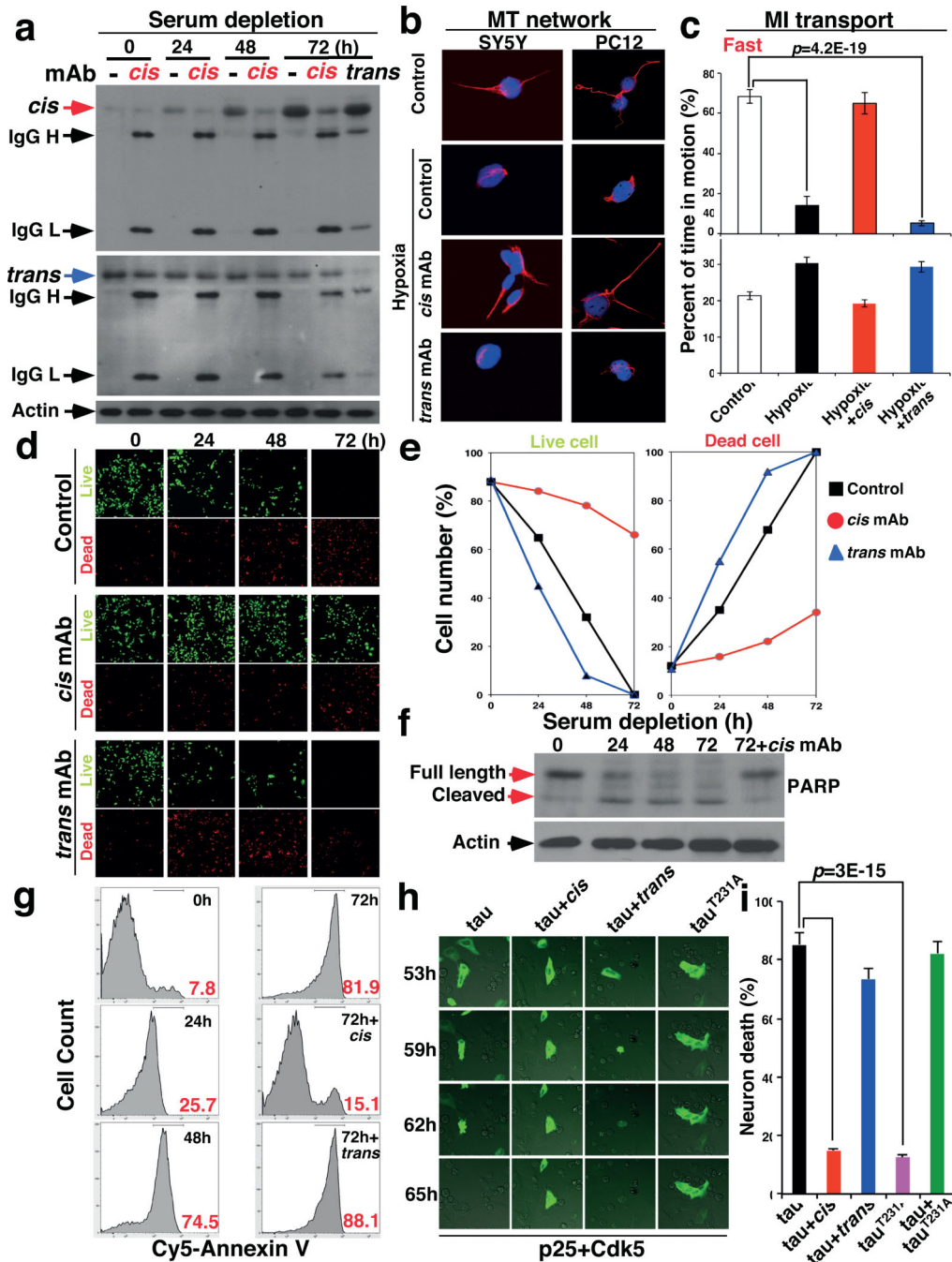


Figure 4. Stressed neurons robustly produce *cis* p-tau leading to cistauosis, which is blocked by *cis* mAb, but enhanced by *trans* mAb

a, SY5Y cells were cultured without serum for different times in the absence and presence of *cis* or *trans* mAb, followed by IB for *cis* and *trans* p-tau. **b**, SY5Y and differentiated PC12 cells were treated with hypoxia in the absence and presence of *cis* or *trans* mAb for 48h, followed by staining for MTs. **c**, Differentiated PC12 cells were treated with hypoxia in the absence and presence of *cis* or *trans* mAb for 48 h, followed by live-cell microscopy to capture fast and slow transport of MI along neurites. **d-g**, SY5Y cells were cultured without

serum for different times in the absence and presence of *cis* or *trans* mAb, followed by live/dead cell assay (**d**, **e**) and apoptosis assays using PARP cleavage (**f**) and Annexin V (**g**). **h**, **i**, SY5Y cells were co-transfected with GFP-tau or -tau^{T231A} and p25/Cdk5, followed by live-cell imaging to observe cell death of GFP-tau expressing cells over 65 h (**h**), with quantification being shown (**i**) (mean ± S.D.). *p* values, ANOVA test.

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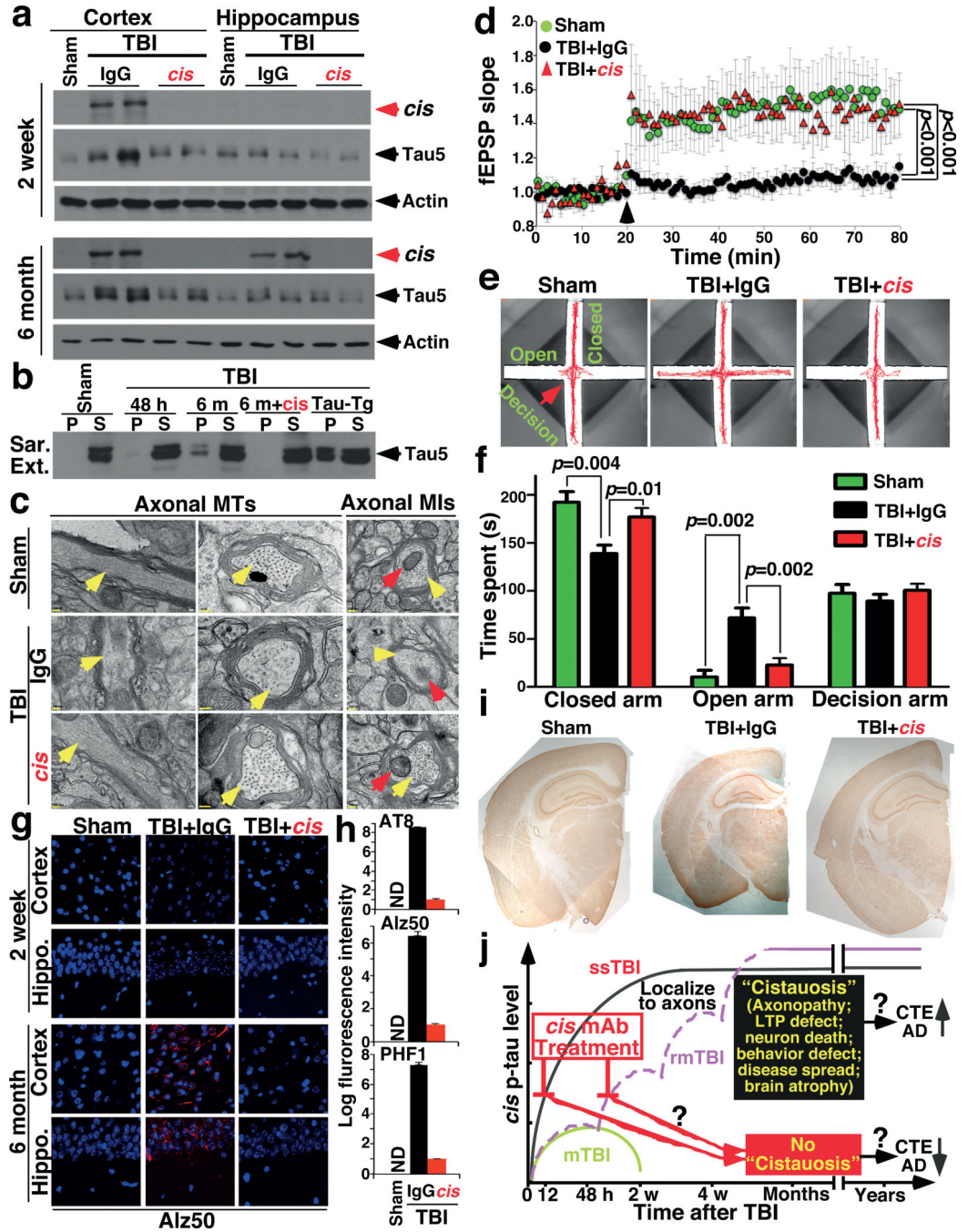


Figure 5. Treating ssTBI mice with *cis* mAb blocks early cistaosis, prevents tauopathy development and spread, and improves histopathological and functional outcomes
a, b, ssTBI mice were treated with *cis* mAb or control IgG for times indicated, along sham mice as controls, followed by IB to detect *cis* p-tau (**a**), and sarcosyl extraction to detect tau aggregation (**b**). N=4. **c, d,** After 2 week treatment, ssTBI mice were subjected to EM for axonal structures of MTs (yellow arrows) and MIs (red arrows) (**c**) or cortical fSPSP recording (**d**) (mean ± S.E.M.). Black arrow, theta-burst application; n=15 slices from 9 sham; n=9 slices from 5 IgG or *cis* mAb mice. *p* values, one-way ANOVA with Bonferroni

posthoc test. **e, f**, after 2 month treatment, ssTBI mice were subjected to the elevated plus maze (**e**) and time spent in three arms was measured (**f**) (mean \pm S.E.M.). n=4 for sham; n=7 for IgG or *cis* mAb. *p* values, Student's t test. **g, h**, After 2 week or 6 month treatment, ssTBI mice were subjected to IF with tauopathy mAbs (**g**), with quantification in the hippocampus being shown (**h**) (means \pm s.d.). n=4. **i**, After 6 month treatment, ssTBI mice were immunostained with NeuN before determining brain thickness. n=4. **j**, Unlike single mTBI, rmTBI or ssTBI causes robust and persistent *cis* p-tau induction within 12-24 hr post-injury, which induces cistauosis, long before commonly known tauopathy and brain atrophy, hallmarks of CTE and AD. *cis* mAb not only blocks early cistauosis, but also prevents long-term neurodegeneration after TBI.

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Table 1Binding affinities of *cis* and *trans* mAbs.

mAb	Peptide	K_a (Ms⁻¹)	K_d (s⁻¹)	K_D (nM)
<i>cis</i>	pT231-tau	40,700	1.10×10 ⁻⁵	0.27
<i>cis</i>	T231-tau	0.17	1.00×10 ⁻⁵	58,820
<i>trans</i>	pT231-tau	250	1.05×10 ⁻⁵	42.0
<i>trans</i>	T231-tau	4.5	1.95×10 ⁻⁵	4,333

The association rate constant (K_a), dissociation rate constant (K_d), and binding constant (K_D) of *cis* and *trans* mAbs toward pT231- or T231-tau peptide were determined by Biacore analysis. Ms⁻¹, millisecond; M, molar; s, second.