The Tau/A152T mutation, a risk factor for frontotemporal-spectrum disorders, leads to NR2B receptor-mediated excitotoxicity

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Abstract

We report on a novel transgenic mouse model expressing human full-length Tau with the Tau mutation A152T (hTauAT), a risk factor for FTD-spectrum disorders including PSP and CBD. Brain neurons reveal pathological Tau conformation, hyperphosphorylation, mis-sorting, aggregation, neuronal degeneration, and progressive loss, most prominently in area CA3 of the hippocampus. The mossy fiber pathway shows enhanced basal synaptic transmission without changes in short- or long-term plasticity. In organotypic hippocampal slices, extracellular glutamate increases early above control levels, followed by a rise in neurotoxicity. These changes are normalized by inhibiting neurotransmitter release or by blocking voltage-gated sodium channels. CA3 neurons show elevated intracellular calcium during rest and after activity induction which is sensitive to NR2B antagonizing drugs, demonstrating a pivotal role of extrasynaptic NMDA receptors. Slices show pronounced epileptiform activity and axonal sprouting of mossy fibers. Excitotoxic neuronal death is ameliorated by ceftriaxone, which stimulates astrocytic glutamate uptake via the transporter EAAT2/GLT1. In summary, hTauAT causes excitotoxicity mediated by NR2B-containing NMDA receptors due to enhanced extracellular glutamate.

Keywords CA3; excitotoxicity; frontotemporal-spectrum disorders; NR2B; Tau

Subject Categories Molecular Biology of Disease; Neuroscience

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Introduction

Familial tauopathies including FTDP-17 are often caused by mutations in the Tau gene (MAPT), which particularly occur in Tau’s microtubule-binding repeat region [1]. MAPT mutations can lead to a partial loss of function due to a reduction in Tau’s microtubule-binding capability or to a gain of toxic function by increasing the tendency of Tau to aggregate into filaments [2,3]. By contrast, the rare Tau p.A152T substitution is located outside the microtubule-binding domain in the N-terminal half of Tau. It has emerged as a risk factor for both frontotemporal dementias (FTDs) and Alzheimer’s disease (AD), including progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) [4–7]. The mutation decreases Tau’s binding to microtubules and increases the formation of Tau oligomers in vitro [7]. However, the consequences for neuronal function and the exact course of pathogenesis still remain elusive. We therefore generated a transgenic mouse line expressing the Tau-A152T mutation and characterized its pathology.

In healthy conditions, Tau is localized mainly in axons, stabilizes microtubules, and supports axonal transport processes [8,9]. Phosphorylation and detachment of Tau from microtubules following subsequent mis-sorting into cell bodies and dendrites may provoke axonal transport alterations [10,11], which in turn may disturb physiological conditions at postsynapses [12,13]. Presynaptic physiological disturbances in Tau models have been reported as well [14,15]. Phosphorylated Tau within dendritic spines has been proposed to impair synaptic transmission by decreasing the densities of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors [12] and causes calcium dysregulation [16]. Furthermore, dendritic Tau is thought to mediate Aβ-induced excitotoxicity and memory deficits in APP transgenic mice [13,17]. Tau plays a role in neuronal excitability and is required for Aβ and other excitotoxins to execute their degenerative effect, such as aberrant network activity and cognitive decline [13,18]. In primary neurons prepared from mice expressing mutant human APP tau allows Aβ oligomers to inhibit
Axonal transport through activation of GSK3β [19]. Genetic ablation of Tau decreases spontaneous seizures in APP transgenic mice and even in a non-AD epilepsy model [20,21].

Tauopathies are characterized by pathological aggregation of Tau and massive cell loss in particular brain regions. In the case of PSP, this occurs in the basal ganglia and brain stem [22] but also in the hippocampus, which may be one factor contributing to cognitive decline. The molecular mechanisms of Tau-mediated neurotoxicity are not well understood and may be diverse. One suggested mechanism in AD is glutamate-mediated excitotoxicity which may lead to the stimulation of extrasynaptic NMDARs (containing NR2B-subunits) and promote Tau-dependent cell death [23]. Recently, impairments in glutamate metabolism were also reported in the hippocampus of transgenic mice overexpressing Tau-P301L [24,25].

When characterizing the new Tau A152T substitution (hTauAT) mouse model by biochemistry and immunohistochemistry, we noticed that the most prominent Tau pathology occurred in the mossy fiber pathway of the CA3 region. We therefore analyzed this region in acute and organotypic slices for structural and functional abnormalities. This revealed a distinct, excitotoxic phenotype which caused an increase in extracellular glutamate, a rise in intracellular calcium, combined with epileptiform activity and mossy fiber sprouting in the hippocampus. This was reminiscent of the situation in PSP patients, where increased seizure susceptibility was reported [26]. The effects were consistent with an overactivation of extrasynaptic NMDA receptors and could be prevented by pharmacological treatments stimulating the uptake of glutamate by astrocytic glutamate transporters (EAAT2/GLT1). These results agree well with observations from another mouse model based on the same mutation [27], which shows age-dependent neuronal loss, cognitive impairments, and spontaneous nonconvulsive epileptiform activity.

Results

Generation and phenotypic characterization of hTauAT mice

We generated a novel transgenic mouse model (hTauAT) expressing human full-length Tau (hTau40, 2N4R) with the PSP-related point mutation p.A152T (Fig 1A). The transgene is located in the R05A26 locus [28] and its expression is controlled by the neuron-specific Thy1.2 promoter. This results in moderate overexpression levels in the hippocampus of heterozygous (+/-) and homozygous (+/+) hTauAT mice at an age of 14 months (Fig 1B). The molecular ratios of mutant hTauAT to endogenous mouse Tau in the hippocampus are -1:1 (heterozygous) and 1:8:1 (homozygous mice) (Fig 1C). The hTauAT mRNA levels in transgenic heterozygous (1.00 ± 0.35 A.U.) and homozygous (2.19 ± 0.27 A.U.) mice (16 month) were in good agreement with the hTau/mTau protein expression ratios in hippocampi from transgenic animals (compare Fig 1C to D). Immunohistological analysis with the human Tau-specific antibody HT7 confirmed the higher hTauAT expression in the homozygous hTauAT mice (Fig 1E). Both types of mice show intense staining of the CA3-mossy fibers (Fig 1E, asterisks) and mis-sorting of the human Tau into the somato-dendritic compartment, a typical early feature of AD (Fig 1E, arrowheads). The hTauAT-expressing mice show hyperphosphorylated Tau (by antibodies pT217, AT180 (pSer235, pSer202), PHF1 (pSer396/pSer404), and AT8 (pSer202/pThr205); Fig 1F–I, respectively) and a pathological Tau conformation (antibody Alz-50, Fig 2A). Transgenic Tau is prominent in the axonal mossy fibers of the CA3 region (Fig 1E and H, asterisks) and is mis-sorted into the cell bodies of hippocampal pyramidal neurons (Fig 1E–I, arrowheads). Using two complementary techniques, Gallyas silver staining (Fig 2B) and sarcosyl extraction (Fig 2C), we could identify aggregated Tau species in 14-month-old mice. Interestingly, both hTauAT and mouse Tau was found in the sarcosyl-insoluble fraction indicating strong co-aggregation (Fig 2C). The ratio of mutant hTauAT to endogenous Tau within the sarcosyl-insoluble fraction is 0.7:1 (+/-) and 1:4:1 (+/+), demonstrating that endogenous mouse Tau is incorporated into Tau aggregates (Fig 2D).

Electron microscopy revealed typical ultrastructural hallmarks of hippocampal CA3 neuropil, including distinct mossy fiber boutons in the stratum lucidum that appeared intact, densely packed with clear synaptic vesicles in control animals (Fig 2E). By contrast, in transgenic age-matched animals, some presynaptic terminals were electron dense and filled with swollen synaptic

Figure 1. Expression of hTauAT causes Tau phosphorylation and mis-sorting in aged mouse hippocampus.

A The diagram shows the domain structure of wild-type human Tau and the location of the substitution A152T (hTauAT). Tau domains are broadly divided into the N-terminal “projection domain” (amino acids M1-Y197) and the C-terminal “assembly domain” (amino acids S198-L441). The C-terminal assembly domain includes three or four pseudo-repeats (~31 residues each; R1–R4), which together with their proline-rich flanking regions (P1 and P2) constitute the microtubule-binding region. Repeat R2 (exon10) and the two near-N-terminal inserts (N1 and N2, exons 2 and 3) may be absent due to alternative splicing.

B Blots showing expression of hTauAT (hTau, Mr ~67 kDa) and/or endogenous mouse Tau (mTau, Mr ~45–55 kDa) in the hippocampus of 10-month-old heterozygous (+/-) and homozygous (+/+) hTauAT mice in comparison to age-matched control (Ctrl) detected by pan-Tau antibody K9A. Actin at 42 kDa serves as a loading control.

C Quantification of B. Ratio of hTau/mTau indicating a higher hTauAT expression in homozgygous mice (~1.8) compared to heterozygous mice (~1.0). Each bar represents an average of n = 4 animals; error bars represent SEM.

D Quantification of mRNA levels in hippocampal of heterozygous and homozygous hTauAT, 16-month-old mice (n = 4 animals per group). Error bars indicate mean ± SEM.

E Distribution of hTauAT visualized by the human Tau-specific antibody HT7 in the hippocampal CA3-region of hetero- and homozygous hTauAT mice at the age of 14 months. Notice the mis-sorted hTauAT in cell bodies and dendrites (arrowheads) of pyramidal neurons of the CA3 region and immunoreactivity of the axons (mossy fibers, asterisks). By contrast, the control shows no immunoreactivity.

F Elevated hTauAT expression causes Tau pathology inside area CA3 of the hippocampus. Note the increase in Tau phosphorylation probed with the antibody against pT217, a site upstream of the repeat domain. Arrowheads point to areas with phosphorylated and mislocalized Tau in cell somata of pyramidal neurons in area CA3.

G Tauopathy detected by antibody AT180 specific for phosphorylation sites pT231 + pSer235 (arrowheads denote somato-dendritic mislocalized tau).

H Antibody PHF1 (phosphorylation sites pSer396 + pSer404) illustrates pathological phosphorylation of Tau due to hTauAT expression especially in stratum lucidum (asterisks) of area CA3 and pyramidal cell bodies (arrows).

I Tau phosphorylation detected by antibody AT8 (pSer202 + pThr205) in stratum pyramidale of area CA3 (arrowhead). Note that immunoreactivity increases with expression level.
vesicles (Fig 2F, arrow). Interestingly, relatively high numbers of synapses, specifically with perforated postsynaptic densities, appeared intact in the stratum lucidum of transgenic animals with no obvious signs of degeneration (Fig 2F and J, asterisks). This finding is in apparent contrast to other animal models including a tau-aggregation model associated with the ΔK280 mutation and the classical murine models overexpressing APP/PS1 mutations [29,30]. Neuropathology in these models was generally associated with a profound and early reduction in the numbers of excitatory hippocampal synapses that was not apparent in our study even at late disease stage (12–13 months). Signs of neuronal degeneration were obvious in pyramidal neurons; degenerating profiles were identified by a shrunken cell membrane plus a darkened cytoplasm, as well as darkened nucleoplasm and crenated nucleolemma within cell soma (Fig 2G, arrow). Furthermore, various degrees of structural abnormalities were detected in dendrites and axons (Fig 2G–J). Electron opaque, degenerating neuritic profiles were identified by an increase in mitochondrial density, presence of whorling membrane fragments, and numerous lysosome-like vacuoles (Fig 2J, arrow).

Figure 1.
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Jochen Martin Decker et al

The Tau/A52T mutation leads to excitotoxicity in the mouse brain

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Figure 2. Expression of hTauAT leads to pathological conformation and aggregation of Tau in the hippocampus of aged mice.

A Conformational change of Tau detected by ALZ-50 antibody immunoreactivity in stratum lucidum (asterisks) of area CA3 and somata in stratum pyramidale (arrowheads).

B Tau aggregation confirmed by Gallyas silver staining of NFTs bearing neurons in hetero- and homozygous hTauAT mice at 14 months of age compared to non-reactive control littermate mice. In homozygous hTauAT mice, the extent of neurofibrillary tangles (NFTs) visualized by Gallyas silver staining is enhanced compared to heterozygous hTauAT mice (white arrowheads). The control shows no silver-reactive Tau aggregates.

C Western blot analysis using the pan-Tau antibody K9JA indicates sarcosyl-insoluble Tau species of human hTauAT (upper band) and mouse Tau (mTau, lower band). Note the enhanced Tau aggregation in homozygous hTauAT mice compared to heterozygous hTauAT mice and control mice.

D Quantification of C. The ratio of hTauAT/mTau indicates a stronger aggregation in the homozygous hTauAT mice (~1.4) compared to heterozygous hTauAT mice (~0.7) (n = 4 animals, error bars represent SEM).

E Electron micrograph of CA3 region of the hippocampus illustrating typical synapse with a perforated postsynaptic density (asterisks) in a control animal at 12 months. ds, dendritic spine; pt, presynaptic terminal; m, mitochondrion.

F Example of a degenerating synaptic bouton (arrow) containing swollen synaptic vesicles in proximity to a large terminal with a perforated postsynaptic density (asterisks) in a transgenic animal at 13 months.

G Electron-dense neuronal cytoplasm with dark nucleoplasm and various vacuoles (arrow) detected in CA3 pyramidal neurons (arrow) at 13 months in transgenic animals in proximity to normally appearing neurons (asterisks), degenerating apical dendrite (d).

H Neuropil of a transgenic animal (13 months) with a degenerating neuritic profile (arrow).

I Degenerating dendrite (arrow) in a transgenic animal with darkened cytoplasm and abnormally distributed mitochondria (arrow).

J Degenerating dendrite containing electron-dense whorling membrane fragments (arrow) in proximity to normally appearing synapses (synapse with a perforated postsynaptic density, asterisks).

Expression of hTauAT elevates intracellular Ca\textsuperscript{2+} concentrations in CA3 neurons at resting state and after membrane depolarization through NR2B-containing NMDA receptors

It was reported earlier that mis-sorted, phosphorylated Tau within dendritic spines of primary neurons impairs synaptic transmission by altering the densities of postsynaptic receptors such as AMPARs and NMDARs [12] and causes abnormal changes in intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) [17]. We therefore investigated [Ca\textsuperscript{2+}]\textsubscript{i} by Fura-2AM imaging in organotypic hippocampal slices. Experiments were performed in area CA3 of slice cultures at DIV 15 (Fig 4A and B), when the NR2B to NR2A ratio in synapses is comparable to acute slices [31–33]. We observed a pronounced increase in resting [Ca\textsuperscript{2+}]\textsubscript{i} in hTauAT slices (~129 nM) compared to control littermate slices (~82 nM, Fig 4A–D). Next, slices were incubated and imaged for [Ca\textsuperscript{2+}]\textsubscript{i}, in the presence of drugs to identify the source of the

Tau is hyperphosphorylated and mis-sorted due to hTauAT expression in hippocampal slice cultures

To further clarify whether hTauAT is mis-sorted into dendrites and dendritic spines, we used immunofluorescence in organotypic hippocampal slices. Organotypic hippocampal slices were derived from young heterozygous transgenic hTauAT mice and maintained in culture for several weeks. Western blotting with pan-Tau antibody K9JA revealed a ~onefold overexpression in one adult hTauAT mice (Fig 1C). To detect the subcellular location of hTauAT, we used immunohistochemistry with human Tau-specific antibody TauY9 or pan-Tau antibody K9JA. In slices from non-transgenic littermates, we could not detect any signal with antibody TauY9 (Fig 3C) but found an axonal distribution of Tau with the pan-Tau antibody (Fig EV1A). In contrast, in hTauAT slices the human Tau appeared both in axons (Fig 3C, asterisk) and dendrites (Fig 3C, right panel). Whereas the dendritic marker MAP2 was restricted to the dendritic shaft, Tau was also apparent in dendritic spines when hTauAT was expressed (Fig 3E, asterisk). The somato-dendritically mis-sorted Tau was reported earlier that mis-sorted, phosphorylated Tau within dendritic spines of primary neurons impairs synaptic transmission by altering the densities of postsynaptic receptors such as AMPARs and NMDARs [12] and causes abnormal changes in intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) [17]. We therefore investigated [Ca\textsuperscript{2+}]\textsubscript{i} by Fura-2AM imaging in organotypic hippocampal slices. Experiments were performed in area CA3 of slice cultures at DIV 15 (Fig 4A and B), when the NR2B to NR2A ratio in synapses is comparable to acute slices [31–33]. We observed a pronounced increase in resting [Ca\textsuperscript{2+}]\textsubscript{i} in hTauAT slices (~129 nM) compared to control littermate slices (~82 nM, Fig 4A–D). Next, slices were incubated and imaged for [Ca\textsuperscript{2+}]\textsubscript{i}, in the presence of drugs to identify the source of the

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Figure 3. hTauAT expression causes Tau phosphorylation and mis-sorting in organotypic hippocampal slice cultures.

A. Western blot analysis with pan-Tau 99A antibody to estimate expression levels of hTauAT and endogenous Tau (mTau). Slice homogenates from non-transgenic littermate (lane 1, Ctrl), heterozygous hTauAT (lane 2 (+/-)), or homozygous hTauAT slices (lane 3 (+/+)) were analyzed after three weeks in culture. Actin served as a loading control.

B. Quantification of hTauAT expression levels (n = average of 3–6 slice homogenates per group, error bars indicate mean ± SEM). The ratio between hTauAT and endogenous mouse Tau is shown.

C. Immunohistochemistry with antibodies against human Tau (TauY9 antibodies; middle panel) and dendritic marker MAP2 (left panel) in slice cultures at DIV 10. Stratum radiatum of area CA3 is shown. Although MAP2 staining is apparent, no signal is detected by the TauY9 antibody in control slices. In contrast, in hTauAT slices human Tau is detected in both dendrites and axons (see asterisk in merged image).

D. Immunohistochemistry against phosphorylated Tau at the PHF1 epitope in area CA3 of a control (upper lane) or hTauAT slice (bottom lane). PHF1 staining is slightly visible in some axons in control slices. In contrast, PHF1-phosphorylated Tau is apparent in somata, dendrites and axons of neurons in hTauAT slice cultures.

E. Higher magnification image of apical dendrites in area CA3 in a hTauAT slice after immunohistochemistry against MAP2 (red) and Tau (99A antibody, green). Tau staining is seen in dendritic spines (asterisks) in contrast to MAP2, which is restricted to the dendritic shaft.

[Ca^{2+}]_{i} increase. We applied the L-type voltage-gated calcium channel (L-VGCC) blocker nifedipine (NIF), the AMPAR antagonist CNQX, the NMDAR antagonists APV and ifenprodil (IFEN, inhibitor of NMDARs composed of NR1 and NR2B subunits [34]), memantine (MEM: preferentially blocking extrasynaptic NMDA receptor currents; [35]), the sodium channel blocker tetrodotoxin (TTX), and the presynaptic vesicle release inhibitor tetanus neurotoxin (TeNT), or imaged in the absence of extracellular Ca^{2+} (see Table E1 for a summary of all drugs and concentrations used). In control littermate slices, the resting [Ca^{2+}]_{i} was not significantly affected by these drugs (Fig 4D). However, as expected, the removal of extracellular Ca^{2+} leads to a pronounced decrease in [Ca^{2+}]_{i} (~50%, ~40 nM, P < 0.05, Fig 4D). In agreement, Ca^{2+}-free saline caused the strongest [Ca^{2+}]_{i} reduction in hTauAT slices (~55%, ~55 nM, P < 0.0001, Fig 4D) but additionally the blockade of NMDAR with APV leads to a reduction in resting [Ca^{2+}]_{i} (~83.5 nM, P < 0.05, Fig 4D).

The inhibition of extrasynaptic NMDARs (either by IFEN or by low-dose MEM) or the blockage of L-VGCC (by NIF) was sufficient to reduce resting [Ca^{2+}]_{i} in hTauAT-expressing slices to control levels (IFEN: ~70 nM, P < 0.05; MEM: ~83 nM, P < 0.01; NIF: ~83 nM, P < 0.05, Fig 4D). [Ca^{2+}]_{i} levels were not significantly altered in presence of either TTX, TeNT, or CNQX in both control and hTauAT slices under resting conditions (Fig 4D).

Next, changes in calcium influx after membrane depolarization were monitored by applying high potassium chloride (KCl). After activity induction, we observed a steep rise in [Ca^{2+}]_{i} levels in hTauAT slices (~575 nM, Fig 4E), whereas control slices achieved only maximum values of ~300 nM (Fig 4E). KCl-evoked Ca^{2+} influx was blocked by nifedipine (NIF: ~47%, P < 0.05, Fig 4E) and less prominently by APV (~36%, Fig 4E) in control littermate slices.

In contrast, treatment of hTauAT slices with APV caused a stronger inhibition of Ca^{2+} influx than application of NIF (~56%, P < 0.001; APV: ~69%, P < 0.001, Fig 4E).

Comparison of the effects of MEM and IFEN on KCl-evoked Ca^{2+} influx in control (MEM: ~1%; IFEN: ~13%, Fig 4E) and in hTauAT slices (MEM: ~44%, P < 0.05; IFEN: ~48%, P < 0.01, Fig 4E) demonstrated a prominent contribution of NR2B-mediated Ca^{2+} influx to the overall [Ca^{2+}]_{i} increase in hTauAT slices.

hTauAT expression is neurotoxic by increasing extracellular glutamate, thereby activating the CREB shutoff pathway

Adult excitatory neurons of the hippocampus express NMDARs with mainly two subunit compositions, NR1/NR2A and NR1/NR2B [36,37]. Synaptic NMDARs are predominantly of the types NR1/NR2A or NR1/NR2A/NR2B (ifenprodil insensitive), whereas somatic or extrasynaptic NMDARs are predominantly of the type NR1/NR2B (ifenprodil sensitive) [36]. The pathway downstream of NMDAR activation is dependent both on the subunit composition and on the location of the receptors [38,39]. For example, Ca^{2+} influx through synaptic NMDARs initiates changes in synaptic efficacy and promotes pro-survival events, whereas Ca^{2+} influx through extrasynaptic NMDARs is coupled to cell-death pathways [38,40,41]. The pharmacology of calcium imaging experiments (IFEN, MEM) pointed to a strong contribution of NR2B-containing extrasynaptic
The Tau/A152T mutation leads to excitotoxicity in the mouse brain

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**Figure 4. hTauAT causes elevation of Ca** sup+ levels in CA3 hippocampal neurons at resting state and after membrane depolarization through extrasynaptic NMDA receptors.**

A Slices (DIV 15) from control mice were loaded with the Ca** sup+**-sensitive dye Fura-2AM. Ratiometric images are presented at baseline and after application of high potassium. Note the increase in intracellular Ca** sup+** ([Ca** sup+**]) after KCl application.

B Slices (DIV 15) from hTauAT mice were loaded with the Ca** sup+**-sensitive dye Fura-2AM. Ratiometric images at baseline and after application of high potassium chloride in area CA3 are depicted. Note the increase in [Ca** sup+**], (false color legend) under both conditions in hTauAT slices compared to controls.

C Electrophysiological example traces (control, black; hTauAT, red) of excitatory postsynaptic field potentials (EPSP) depict examples of depolarizations that are induced by a single high potassium chloride application in stratum pyramidale (s.p.) of area CA3 in slices in which calcium imaging experiments were conducted. The example depolarization evokes averaged calcium influxes into neurons depicted in the calcium imaging graph. The graph shows the mean of [Ca** sup+**], changes in response to high KCl in stratum radiatum and stratum pyramidale of area CA3 in Fura-2AM-loaded hippocampal slices. After depolarization, [Ca** sup+**] i rises up to ~600 nM in hTauAT slices (red trace, n = 6 slices, prepared from at least three animals), compared to ~300 nM in control slices (black trace, n = 6 slices, prepared from at least three animals). Note that even under resting conditions, [Ca** sup+**], is elevated to ~120 nM due to hTauAT expression.

**D Quantification of the [Ca** sup+**],** from ratiometric images as depicted in (A) and (B) after background subtraction and selection of regions of interest (10 circles of fixed diameter) in stratum radiatum border to stratum pyramidale of area CA3. Under resting conditions, [Ca** sup+**], was elevated in transgenic slice cultures (~120 nM, n = 11) compared to controls (~80 nM, n = 8). Slices were incubated with one of the following channel blockers: nifedipine (NIF, VGCC; Ctrl n = 8, A152T: n = 7), APV (NMDAR; Ctrl n = 7, A152T: n = 8), ifenprodil (IFEN, NMDAR; Ctrl n = 8, A152T: n = 7), memantine (MEM, NMDAR; Ctrl/A152T n = 6), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, AMPAR; Ctrl n = 8, A152T: n = 7), tetrodotoxin (TTX, VGNaC; Ctrl n = 4, A152T: n = 5), tetanus neurotoxin (TeNT, neurotransmitter release; A152T n = 6) or in Ca** sup+**-free buffer (Ctrl n = 10; A152T: n = 7). For each experiment, slice preparations from at least three different mice were used.

One-way ANOVA followed by Tukey’s post hoc test: Ctrl: F(7/51) = 3.533, P = 0.0036; A152T: F(8/50) = 5.230, P < 0.0001. For an overview of drug concentrations see Table EV1.

**E Quantification of the maximum peak increase in intracellular calcium after high KCl stimulation.** One-way ANOVA followed by Tukey’s post hoc test 

Ctrl: F(7/45) = 4.080, P = 0.0015; A152T: F(8/50) = 7.580, P < 0.0001.

Data information: Error bars represent SEM; one-way ANOVA: *P < 0.05, **P < 0.01, ***P < 0.001.

NMDARs under both conditions, resting and activity-induced Ca** sup+** influx in hTauAT slices, not seen in control littermate slices.

Since NR2B-containing NMDARs get activated by excessive glutamate [42], we hypothesized the possibility of high levels of extracellular glutamate in hTauAT slices. To test this, culture medium from hTauAT-expressing (or control littermate) slices was collected at different time points and glutamate concentrations were measured. The extracellular glutamate levels from hTauAT slices were increased by ~45% at DIV 5 and DIV 10 and declined at DIV 20–25 almost to control levels (~28% (DIV 20) and ~4% (DIV 25); Fig 5A). The maximum glutamate concentrations were already observed at early time points (DIV 5 and 10; Fig 5A). Remarkably, the increase in glutamate was already maximal several days before the increase in cytotoxicity as seen by LDH (Fig 5B), which argues in favor of a rise in extracellular glutamate as a trigger for toxicity.

To test whether augmented, NR2B-mediated calcium influx in hTauAT slices leads to downstream cell-death signaling pathway, we...
The Tau/A152T mutation leads to excitotoxicity in the mouse brain

**Figure 5.** hTau<sup>AT</sup> expression enhances extracellular glutamate, cytotoxicity and progressive neuronal loss in the hippocampus.

A Glutamate content in the culture medium ([Glu]) from either hTau<sup>AT</sup> (n = 10 culture inserts containing 6 slices each) or control slices (n = 5–12 culture inserts) analyzed at DIV 5, 10, 20 and 25. Glutamate content from medium cultured with hTau<sup>AT</sup> slices is shown in percent of control at each time point. Increased levels were observed between DIV 5 and DIV 20. At DIV 25, glutamate levels already declined to control levels. One-way ANOVA followed by Tukey’s post hoc test: ***P < 0.001, **P < 0.01, *P < 0.05.

B Lactate dehydrogenase (LDH) release (a measure of cytotoxicity) analyzed in hTau<sup>AT</sup> (n = 17–19 culture inserts containing 6 slices each) or control slices (n = 15–16 culture inserts) at DIV5, 10, 20 and 25. LDH release of hTau<sup>AT</sup> slices is shown in percent of control at each time point. A pronounced increase (~25%) in LDH release was observed due to hTau<sup>AT</sup> expression starting at DIV 10 but not at DIV 5. One-way ANOVA: ***P < 0.001.

C Slice cultures from control (left), hTau<sup>AT</sup> (middle) or hTau<sup>AT</sup> treated with ceftriaxone (CEF) 100 μM (right) stained against neuronal nuclear protein (NeuN) at DIV 30. Representative neuronal cell layers are shown. hTau<sup>AT</sup> slices show neuronal loss in the granular cell layer of the dentate gyrus and the pyramidal cell layer in the region of the hilus in area CA3 (middle panel, red arrows). Treatment with ceftriaxone for 30 days prevents neuronal loss in DG and area CA3.

D NeuN-positive cell bodies were counted in the DG and within the pyramidal cell layer in area CA3 and CA1 at DIV 30 in defined regions of interest (ROI). The number of neurons was reduced in hTau<sup>AT</sup> slices, both within area DG and CA3, whereas the number of neurons in the CA1 was only slightly affected. Neuronal loss was largely prevented in cultures treated with ceftriaxone (CEF) in dentate gyrus and area CA3 (n = 14–19 slices per group and area, prepared from at least 6 animals). One-way ANOVA followed by Tukey’s post hoc test: *P < 0.05.

Data information: Error bars represent mean ± SEM.

evaluated the effect of hTau<sup>AT</sup> on cytotoxicity. This revealed an increase in lactate dehydrogenase (LDH) release, a marker for dying cells, starting at DIV 10 (+23%; Fig 5B) and lasting over the whole cultivation period of 25 days (+27% at DIV 20, +20% at DIV 25; Fig 5B). An exception was the earliest time point of measurement (DIV 5) where toxicity stayed close to control levels (+5%, Fig 5B), demonstrating that toxicity progresses from young to older hTau<sup>AT</sup> slices. The question therefore arose, what is the cause of the rise in Ca<sup>2+</sup> and of cytotoxicity in slices from hTau<sup>AT</sup> mice?

To further differentiate between cytotoxicity and neurotoxicity and to test a possible differential vulnerability of hippocampal subregions, NeuN immunohistochemistry was performed. NeuN-expressing cells were counted at DIV 30 and showed a selective neuronal loss in area CA3 (~30%, P = 0.015) and DG (~23%, P = 0.032, Fig 5C and D) in hTau<sup>AT</sup>-expressing slices, whereas CA1 neurons were only mildly affected (~11%, P = 0.15, Fig 5D). We further analyzed neurons in slices at an earlier (DIV 10; Fig EV2A) and a later time point (DIV 60; Fig EV2B). At DIV 10, no significant cell loss was detected in areas DG, CA1, and CA3. In contrast, at DIV 60 neuronal loss was even more pronounced when compared to values observed at DIV 30. These findings in slice cultures were supported by the observation of neuronal cell loss in the hippocampus of aged hTau<sup>AT</sup> mice. Neuronal cell loss was prominent in the pyramidal layer of area CA3 in 12-month-old heterozygous hTau<sup>AT</sup> mice (Fig EV5A). Next, we analyzed NeuN protein levels in hippocampi of 12- and 18-month-old hTau<sup>AT</sup> mice (Fig EV5B and C). Here, we found a progressive loss of NeuN expression which was reduced by ~10% already in 12-month-old hTau<sup>AT</sup> mice and neuronal loss became more pronounced to ~35% in 18-month-old hTau<sup>AT</sup> mice when compared to control mice (Fig EV5B and C). Hippocampal neurotoxicity was also confirmed in hippocampi from 16-month-old, heterozygous hTau<sup>AT</sup> animals by quantitative real-time PCR. NeuN mRNA was significantly decreased in hippocampi from hTau<sup>AT</sup> mice compared to control littermate hippocampi (n = 4 animals per group, unpaired t-test: P = 0.0323, Fig EV2C). The activity of the pro-apoptotic caspase 3 was not increased in hTau<sup>AT</sup> slices compared to control (P = 0.4143; Fig EV2D). This points to a necrotic caspase-independent pathway rather than apoptotic events leading to neuronal cell death.

Ca<sup>2+</sup> influx through extrasynaptic NMDARs is known to induce dephosphorylation of CREB protein (at Ser133) which in turn causes shut-off of the CREB pathway, leading to neuronal death [38,40,43]. Therefore, we monitored a potential CREB shut-off in hTau<sup>AT</sup> slices by Western blot analysis for total CREB and pS133-CREB (Fig EV2E). There was a marked reduction (~50%; P = 0.0064) in active pCREB levels in the mutant slices (Fig EV2E). Taking these findings together with the elevated levels of extracellular glutamate and elevated [Ca<sup>2+</sup>], we conclude that a pathological increase in Ca<sup>2+</sup> influx through extrasynaptic NMDARs takes place in hTau<sup>AT</sup> slices resulting in...
activation of the pro-death CREB shut-off pathway and subsequent neurotoxicity.

The hTauAT mutation enhances synaptic transmission and susceptibility for epileptiform activity in area CA3 without affecting synaptic plasticity

The shut-off of CREB activity influences not only synaptic plasticity and memory formation but also neuronal survival [44] which is based on the proper function of dendritic spines [45]. We thus analyzed dendritic spine density of 10- to 12-month-old, heterozygous hTauAT mice. Apical dendrites of hTauAT CA3 pyramidal neurons displayed a 50% increased spine density compared to control littermate animals (2.04 ± 0.15 spines/µm and 1.36 ± 0.05 spines/µm, P = 0.039; Fig 6A–C). In organotypic slices at DIV 20, the spine density of CA3 apical dendrites was unchanged in hTauAT slices (control: ~1.6 spines, hTauAT: ~1.4 spines/µm, Fig EV3A–E).

Long-term potentiation (LTP) is a common way to measure learning and memory on a cellular scale because memories are encoded by changes in synaptic strength through LTP and LTD [46]. We used excitatory postsynaptic field potentials (fEPSP) in stratum lucidum of area CA3 to measure basal synaptic transmission, short-term plasticity and long-term plasticity of the mossy fiber pathway,

Figure 6. hTauAT enhances synaptic transmission and dendritic spine density in area CA3 but does not interfere with synaptic plasticity.

A Representative microphotograph of spines on dendrites from control littermate mice at the age of 12 months. Apical dendrites from CA3 pyramidal neurons are visualized by Golgi staining. The contrast of photomicrographs was inverted.

B Dendritic spines from heterozygous hTauAT mice, seen by Golgi staining.

C Quantification of dendritic spine densities in control littermate and hTauAT mice. Note that the spine density in heterozygous hTauAT is increased (control littermates: n = 2 animals; heterozygous hTauAT: n = 4 animals; an average of 5 dendrites of CA3 pyramidal neurons were analyzed per animal; unpaired t-test).

D Input/output curve of field potential recording in the mossy fiber pathway in acute slices of 12- (±1)-month-old mice demonstrating significantly enhanced basal synaptic transmission [multiple repeat ANOVA, F(9, 126) = 2.00, P = 0.044] in area CA3 in mice expressing hTauAT. Scale bars: 500 µV vertical bar and 20 msec horizontal bar.

E Long-term potentiation in the mossy fiber pathway demonstrates no differences in long-term plasticity between control (black) and hTauAT-expressing mice (red).

F Input/output curve of field potential recording in stratum pyramidale (s.p.) of area CA3 in organotypic hippocampal slices at DIV 30. Somatic field potentials are increased in hTauAT compared to control littermates. Scale bars: 800 µV vertical bar and 20 msec horizontal bar.

Data information: Error bars represent SEM; t-test: *P < 0.05.
since this pathway was found to be particularly affected as judged by tauopathy markers (e.g., PHF1, Alz-50, Figs 1 and 2). Recording input/output curves of the mossy fiber pathway from 12±1)-month-old hTauAT (+/−) mice revealed an increase in basal synaptic transmission compared to non-germinal siblings (Fig 6D). At 10 μA stimulation intensity, fEPSP responses still stayed at the same level in control littersmates and hTauAT animals (40.4 ± 9.1 mV/s, n = 7 and 44.8 ± 8 mV/s, n = 9; Fig 6D), but when comparing all other stimulation intensity steps the responses of hTauAT mice were clearly increased. The maximum of the i/o curve of hTauAT animals was reached at 50 μA stimulation intensity (248.49 ± 33.1 mV/s, n = 9; Fig 6D), whereas control littersmates reached the maximum fEPSP only at 60 μA stimulation intensity (171.4 ± 30.9 mV/s, n = 7; Fig 6D). However, short-term plasticity (paired-pulse facilitation) remained unaffected in hTauAT mice (paired-pulse ratio control littersmates: 1.44 ± 0.08, n = 31 and hTauAT: 1.48 ± 0.05, n = 33; Fig EV3F). The same was true for long-term plasticity evoked by theta burst stimulation in the mossy fiber pathway. There were no differences in the level of potentiation of the last 10 min of LTP recording (control littersmates: 152 ± 9.7% of baseline, n = 6 and hTauAT: 141.2 ± 12.2% of baseline, last 10 min of recording, n = 7; Fig 6E).

Similar to the situation in acute hippocampal slices, recording evoked fEPSPs in stratum pyramidale of area CA3 in DIV 26–30 old hTauAT organotypic slices revealed elevated amplitudes of somatic fEPSPs in stratum pyramidale (Fig 6F). At an input stimulus intensity of 10 μA, the average output fEPSP amplitude in control littersmate slices was 102.5 ± 33.6 μV (n = 5) and 158.58 ± 34.79 μV (n = 15) in hTauAT slices; however, overall amplitudes were increased in hTauAT slices compared to control littersmates (multiple repeat ANOVA, F(7,89) = 2.1874, P = 0.042; Fig 6F). In younger slices at DIV 10, this effect was not observed (no significant difference between control (n = 5) and hTauAT slices (n = 12); multiple repeat ANOVA, F(7,77) = 1.1472, P = 0.34, Fig EV3G), which suggest a progression of this phenotype with age.

Given that basal synaptic transmission in the mossy fiber tract of hTauAT mice was increased, we hypothesized that this might be accompanied by elevated susceptibility for epileptiform activity. To investigate this, we recorded field potentials (fEPSPs) in stratum pyramidale of area CA3 in hTauAT-expressing slices. Picrotoxin (50 μM) was used to induce epileptiform discharges by blocking GABAergic transmission in control slices and hTauAT-expressing slices at two time points (DIV 10 and DIV 30). After washing in picrotoxin diluted in ACSF, recurrent epileptiform discharges occurred approximately after 3 min (Fig 7A and B). At DIV 10, when extracellular glutamate concentrations are enhanced in hTauAT slices (Fig 5B), we observed an increase in burst frequency (bursts per minute) compared to control littersmate slices (control: 1.07 ± 0.21 burst per minute, n = 8 slices and hTauAT: 1.82 ± 0.169 bursts per minute, n = 8, two-sample t-test: P = 0.001; Fig 7C). In addition, the number of firings per burst was enhanced by ~40% (control: 5.80 ± 0.73 firings per burst, n = 7 slices and hTauAT: 8.32 ± 0.41 firings per burst, n = 5; two-sample t-test: P = 0.004; Fig 7D). Recording at a later time point (DIV 30), when excessive extracellular glutamate levels drop down to control levels (Fig 5A), demonstrated that enhanced epileptiform activity becomes even more pronounced. At this time point, hTauAT-expressing slices display on average 2.56 ± 0.26 bursts per minute (n = 10, Fig 7E) after picrotoxin wash-in, which was ~100% higher than recorded in control littersmate slices (1.24 ± 0.37 bursts per minute, n = 5, two-sample t-test: P = 0.006; Fig 7E).

Applying the β-lactam antibiotic ceftriaxone (CEF), which is known to upregulate the astrocytic excitatory amino acid transporter EAAT2/GLT1 expression and its biochemical activity [47], prevented the burst frequency increase in hTauAT slices, presumably by normalizing extracellular glutamate levels (1.74 ± 0.35 bursts per minute, n = 5, Fig 7E). Similar to the burst frequency, firings per burst were strongly enhanced (~80%) at DIV 30 in slices expressing hTauAT (control: 4.47 ± 0.42 firings per burst, n = 5 and hTauAT: 8.74 ± 0.45, n = 10, Fig 7F, t-test: P = 0.0000001). Finally, a pathological change in slices expressing hTauAT recorded at DIV 30 was prevented by ceftriaxone (4.71 ± 0.32 firings per burst, n = 6, Fig 7F).

Staining by pan-Tau antibody (K9JA) indicated that hTauAT slices used for epileptiform activity recordings displayed increased mossy fiber sprouting (Fig 7G and H). In adult heterozygous A152T mice, we confirmed network hyperactivity in the hippocampus by a significant increase in c-Fos mRNA expression in comparison to control littersmates (n = 4 animals per group, P = 0.001, Fig EV4A). c-Fos is upregulated in the hippocampus under a variety of activity inducing conditions, for example, seizure induction [48]. At the same time, Homer1a was downregulated (Fig EV4B) in such animals pointing to a disturbed Homer scaffolding with metabotropic glutamate receptors, which is causative for an epileptic phenotype in a mouse model with fragile X syndrome [49,50]. However, we did not observe any changes in transcription levels of NR2B and NR2A receptors (Fig EV4C and D).

**Enhanced presynaptic glutamate release is responsible for excitotoxicity due to hTauAT expression**

The observation that extracellular glutamate concentrations are elevated even before cytotoxicity becomes enhanced (Fig 5A and B) was indicative of a mechanism of glutamate-mediated excitotoxicity in hTauAT slices. To prove that excess glutamate in the slice medium was due to enhanced neuronal activity (causing elevated neurotransmitter release), we next incubated the slices with either tetrodotoxin (TTX: 1 μM) to inhibit action potentials driven by voltage-gated sodium channels or tetanus neurotoxin (TeNT: 50 nM) to inhibit glutamate release from presynaptic terminals. In both conditions, glutamate levels in hTauAT slices dropped down to control levels (TeNT: ~57%; TTX: ~35%; Fig 8A). Both inhibitors had smaller effects in control slices (TeNT: ~35%; TTX: ~25%; Fig 8A), indicating that elevated glutamate levels are due to hTauAT expression.

**EAAT2/GLT1 plays important roles in restricting extracellular glutamate concentrations in order to limit the activation of glutamate receptors, thereby protecting neurons from excitotoxicity [51]. To test this, EAAT2/GLT1 was blocked by treating slices with benzyloxyaspartic acid (TBOA, 50 μM). This caused a strong increase in glutamate in control slices (+66%; P < 0.01), but only a small rise in hTauAT slices (+24%, Fig 8B) accompanied by an increase in cytotoxicity (LDH; control: +50%; P < 0.001; hTauAT: +28%, P < 0.001, Fig 8C).

To investigate mechanisms causing the rise in extracellular glutamate due to hTauAT expression, we treated organotypic cultures
Figure 7. Cultured slices expressing hTauAT show a progressive increase in epileptiform activity and mossy fiber sprouting which can be prevented by CEF application.

A Example of electrophysiological fEPSP recording in stratum pyramidale of area CA3 in a control slice (DIV 30) during wash-in of 50 μM picrotoxin. This leads to blockage of GABAergic transmission followed by epileptiform discharges (burst) occurring approximately after 3 min. The zoom-in reveals that such an epileptiform discharge (burst) consists of repetitive firings.

B Similar example trace as in (A) recorded in a hTauAT-expressing slice (red) demonstrating an increase in burst frequency as well as in firings per burst due to hTauAT expression.

C Quantification of bursts per minute in DIV 10 slices from control littermates and hTauAT slices demonstrating a ~twofold increase in hTauAT slices (n = 8 slices per group).

D Firings per burst are increased ~1.5-fold already at the early time point DIV 10 due to hTauAT expression.

E Analysis of burst frequency in slices at DIV 30 demonstrates the progression of the pathological effect beyond that observed at DIV 10. Burst frequency is enhanced in hTauAT-expressing slices (n = 10 slices). If ceftriaxone (CEF) is applied to hTauAT slices, the pathological burst frequency enhancement is partly prevented (n = 5 slices).

F Firings per burst are strongly enhanced due to hTauAT expression in slices at DIV 30. Ceftriaxone prevents this pathological phenotype if applied during the entire period of cultivation (n = 6 slices).

G Example of a control littermate organotypic slice culture at DIV 24 stained against NeuN (red) and pan-Tau (K9J antibody, green). Axonal projections (green) of granule cells (red) in the dentate gyrus are depicted.

H Hippocampal slice culture expressing hTauAT stained against NeuN (red) and pan-Tau (green) showing mossy fiber sprouting in the hilar region, CA3 and dentate gyrus (white arrows).

Data information: Error bars represent SEM; t-test: *P < 0.05, **P < 0.01, ***P < 0.001.
In the present study, we used aged hTauAT mice and organotypic hippocampal slices from P8 mice to characterize the pathophysiological phenotype of the recently described Tau mutation p.A152T [4,7]. This mutation is a risk factor for FTD-spectrum disorders such as PSP or CBD, and AD. However, its pathogenic effects are poorly understood. In transgenic mice and slice cultures, we found characteristic signs of Tau pathology, that is, phosphorylation, pathological conformation, somato-dendritic mis-sorting, aggregation, neuronal degeneration, and loss due to hTauAT expression, similar to human carriers [5,6]. The slices revealed a remarkable increase in intracellular Ca^{2+} concentration in hippocampal CA3 neurons, both at resting state and after membrane depolarization. These alterations were apparent before Tau aggregates, suggesting oligomeric forms of Tau as mediators of these changes. Indeed, in vitro experiments had shown that p.A152T favors the formation of oligomers [7]. This is remarkable because the mutation lies in the N-terminal domain, far away from the repeat domain that forms the core of aggregation, and

with ceftriaxone (CEF: 100 μM, starting at DIV 1) (Figs 8D–F and 5C and D). Indeed, treatment with CEF prevented all pathological phenotypes in hTauAT-expressing slices mentioned above, that is, (i) extracellular glutamate (Fig 8D), (ii) maximum calcium influx after membrane depolarization (Fig 8F), (iii) neurotoxicity measured by LDH release (Fig 8E), (iv) epileptiform activity (Fig 7E and F), and (v) neuronal loss (by NeuN counting; Fig 5C and D).

**Discussion**

In the present study, we used aged hTauAT mice and organotypic hippocampal slices from P8 mice to characterize the pathophysiological phenotype of the recently described Tau mutation p.A152T [4,7]. This mutation is a risk factor for FTD-spectrum disorders such as PSP or CBD, and AD. However, its pathogenic effects are poorly understood. In transgenic mice and slice cultures, we found characteristic signs of Tau pathology, that is, phosphorylation, pathological conformation, somato-dendritic mis-sorting, aggregation, neuronal degeneration, and loss due to hTauAT expression, similar to human carriers [5,6]. The slices revealed a remarkable increase in intracellular Ca^{2+} concentration in hippocampal CA3 neurons, both at resting state and after membrane depolarization. These alterations were apparent before Tau aggregates, suggesting oligomeric forms of Tau as mediators of these changes. Indeed, in vitro experiments had shown that p.A152T favors the formation of oligomers [7]. This is remarkable because the mutation lies in the N-terminal domain, far away from the repeat domain that forms the core of aggregation, and
therefore, one would not expect the mutation to have an effect on tau aggregation.

To identify the origin of the intracellular Ca\(^{2+}\) increase, we tested different drugs and found that intracellular Ca\(^{2+}\) concentrations were normalized by ifenprodil and memantine (targeting NR2B-containing NMDA receptors) but also by nifedipine (targeting VGCCs). Since we also observed enhanced extracellular glutamate levels in hTau\(^{AT}\) slices, we assume this phenotype to cause network hyperactivity. This was confirmed by the measurements of epileptiform activity. In conclusion, an increased network activity could lead to constant enhancement of extracellular glutamate concentrations, which would in turn lead to increased NR2B activation causing increased intracellular Ca\(^{2+}\) concentrations.

For future investigation, it would be worth investigating whether a Na/Ca exchanger reversal is contributing to the high intracellular calcium concentration comparable to the situation in other excitotoxic phenotypes (e.g., ischemia, stroke). Since we found no indication of disturbances in NR2A or NR2B transcription by real-time PCR measurements (Fig EV4C and D), one can speculate that the enhanced calcium influx through NR2B-containing NMDAR at resting state should be due to receptor/channel dysfunction caused by somato-dendritic mis-sorted Tau. Indeed, we observed enhanced epileptiform activity in hTau\(^{AT}\) slices at DIV 10 and 30 as well as an increase in synaptic transmission. Consistent with this, several reports have suggested the involvement of the NR2B subunit in the mediation of seizures [52], and the reduction in Tau leads to seizure resistance in epilepsy models [21,53]. Moreover, when rats were treated with transient middle cerebral artery occlusion to initiate an excitotoxic insult, NR2B-containing NMDARs were overactivated, resulting in Tau hyperphosphorylation and aggregation [54].

It is notable that different Tau mutations may have opposite consequences on synaptic transmission in mice: Pro-aggregant mutations in Tau’s repeat domain (A280) lead to reduced transmission in the mossy fiber tract [15,55,56], whereas the amino-terminal mutation A152T, described here, enhances transmission in the same pathway. By analogy, there can be opposite effects in terms of calcium levels, which are reduced in neurons expressing Tau\(^{RD/280}\) [15,16] and increased when A152T Tau is expressed. Enhanced calcium levels were also observed in SH-SY5Y cells expressing Tau with a V337M mutation [57]. EEG studies of human hTau\(^{AT}\) carriers are not available so far, but increased seizure susceptibility was described for PSP [26] and Alzheimer’s disease patients [58].

Notably, we did not observe any changes in synaptic plasticity of the CA3 synapses but an increase in spine density of CA3 pyramidal neurons. An increase in spine density is also observed under excitotoxic conditions in animals which have undergone transient global ischemia [59]. This is in strong contrast to mouse models expressing Tau with mutations in the repeat domain which show reduced plasticity and synapse loss [14,55,56].

The CREB shut-off pathway is well established, assuming that enhanced intracellular calcium concentrations through NR2B-containing NMDAR lead to necrotic cell death [38]. In agreement with this pathway, we observed a decreased ratio of phosphorylated CREB to CREB, at the same time no alteration in caspase-3 activity but progressive neuronal cell death due to hTau\(^{AT}\) expression in organotypic slices and in adult animals. This correlates well with the observation of enhanced glutamate content in the slice medium of hTau\(^{AT}\) slices. Elevated levels of glutamate cause excitotoxic insults which finally lead to neurotoxicity [60]. The strongest vulnerability was seen in the DG and CA3, the same regions that were affected in aged hTau\(^{AT}\) mice by enhanced synaptic transmission in acute slices as well as in organotypic slices and by increased epileptiform activity in organotypic slices. Moreover, in cultured slices we observed mossy fiber sprouting demonstrating this pathway to be severely affected during the process of pathogenesis. At the ultrastructural level, degenerative hallmarks were observed in aged transgenic mice by electron microscopy in the somato-dendritic compartment as well as in axons.

It is interesting to compare our results with those of Mucke and colleagues who created regulatable transgenic mice with the same mutation (A152T), but in a different human Tau isoform (1N4R), and under a different promoter, CaMKII-tTA [27]. These mice developed a comparable phenotype including age-dependent neuronal loss and spontaneous nonconvulsive epileptiform activity. Tau pathology markers and neuronal loss were most prominently found in hippocampal areas CA3 and DG; however, synaptic plasticity was not affected in both mouse lines. The similarity of phenotypes argues for a common pathway of toxicity induced by the Tau-A152T mutation. Finally, our observations of neuronal toxicity are broadly consistent with the neurotoxicity reported for human-induced pluripotent stem cells (iPSCs) carrying the Tau-A152T mutation [61].

As the phenotype suggests, enhanced extracellular glutamate concentrations in the hTau\(^{AT}\) slice medium may be caused by an increase in presynaptic release (as revealed by the effect of tetanus toxin, which reduces the increase in extracellular glutamate in hTau\(^{AT}\) slices to control levels). However, electrophysiology demonstrated that paired-pulse ratios are unaffected arguing against presynaptic vesicle release enhancement due to hTau\(^{AT}\) expression, but leaving the possibility that extracellular glutamate increase is due to an increase in total number of presynaptic release sites. Additionally, a dysfunction of the glutamate transporter EAAT2/GLT1 in astrocytes may contribute to the observed increase in extracellular glutamate levels as revealed by pharmacological application of the EAAT2/GLT1 blocker TBOA. Dysregulation of glutamate was also reported in other tau transgenic mouse lines [25,62]. Glutamate is largely taken up by astrocytic glutamate transporters (EAAT2/GLT1), but in many neurodegenerative diseases the expression of the EAAT2/GLT1 is reduced [63,64] and mice expressing mutant tau P301L show lower EAAT2/GLT1 expression levels [25]. Indeed, in our slice system the activation of EAAT2/GLT1 by ceftriaxone [47] was able to preserve normal intracellular calcium levels and extracellular glutamate levels; it prevented neurotoxicity and mitigated epileptiform activity to control levels. However, transcription levels of EAAT2/GLT1 remained unchanged in hTau\(^{AT}\)-expressing mice (Fig EV4E).

In conclusion, the pathological effects of the hTau\(^{AT}\) mutation are explained by an enhanced presynaptic glutamate release which results in an overactivation of NR2B-containing, extrasynaptic NMDA receptors that finally initiate an excitotoxic cascade ending up with CREB shutoff and neuronal death. The pathology can be ameliorated by lowering extracellular glutamate (e.g., by enhancing glutamate re-uptake into astrocytes by ceftriaxone), or by de-sensitizing extrasynaptic NR2B receptors (e.g., memantine).
Materials and Methods

Generation of hTauAT mice

To achieve ubiquitous transgene expression in the brain at moderate levels, the transgene (human full-length Tau carrying the mutation A152T) was inserted into the ROSA locus of C57BL/6NTac embryo stem (ES) cells and injected C57BL/6NTac ES cells into BALB/c blastocysts (TACONIC, Germantown, NY). Injected blastocysts were transferred into the uterine horn of pseudopregnant NMRI females. Chimerism was determined in chimeras by coat color contribution of ES cells to the BALB/c host (black/white). Highly chimeric mice were bred to C57BL/6 females. Germline transmission was identified by the presence of black offspring. The transgene expression is controlled by the neuron-specific Thy1.2 promoter and occurs in the entire brain and spinal cord.

Biochemical analysis of mice

Mice were anesthetized with an O2/CO2 mixture and killed by decapitation. Sarcosyl extraction, total protein preparation, and Western blot analysis were performed as described previously [29]. About 3 µg of total protein or 3 µl of sarcosyl extraction lysates from different tissues (cortex and hippocampus) were loaded for the detection with the pan-Tau antibody K9JA (1:20,000, Dako). Blots were normalized to the concentration of GAPDH (antibody 1:60,000, Sigma). To achieve ubiquitous transgene expression in the brain at moderate levels, the transgene (human full-length Tau carrying the mutation A152T) was inserted into the ROSA locus of C57BL/6NTac embryo stem (ES) cells and injected C57BL/6NTac ES cells into BALB/c blastocysts (TACONIC, Germantown, NY). Injected blastocysts were transferred into the uterine horn of pseudopregnant NMRI females. Chimerism was determined in chimeras by coat color contribution of ES cells to the BALB/c host (black/white). Highly chimeric mice were bred to C57BL/6 females. Germline transmission was identified by the presence of black offspring. The transgene expression is controlled by the neuron-specific Thy1.2 promoter and occurs in the entire brain and spinal cord. The mouse strain was conceived on an identical C57BL/6/N background. The present study presents data from heterozygous and homozygous hTauAT mice. Non-transgenic littermates were used as negative controls. All animals were housed and tested according to the standards of the German Animal Welfare Act.

Genotyping of hTauAT mice

The hTauAT mice (hetero- or homozygous) were identified by PCR of genomic mouse tail DNA using the primer pair 5'-AGCACCTTATACTGATGAC 3' and 5'-TGCTACTCTTTCAGTCTGAACTCC 3', which amplified an expected DNA fragment of 192 bp (human Tau construct). Heterozygous and non-transgenic littermates differ by the appearance of the 299 bp control fragment (ROSA26-locus) amplified by the primer pair 5'-CTCTCCCTGTTGCTTCATGGG 3' and 5'-CATGTCTTTTAATCTACTTCAGTGG 3' to homozygous hTauAT.

Real-time PCR

The RNA extraction was performed by using a standard Trizol/1-bromo-3-chloropropane extraction protocol followed by cDNA generation using the first strand cDNA synthesis kit (Thermo Scientific). RNA was extracted from hippocampus homogenates of 16-month-old heterozygous and homozygous animals and control littermates (for all groups n = 4 animals were analyzed). The Primer3Plus program was used for primer design [65]. Primers were required to be intron spanning and to be yielding an amplicon of 70–120 basepair. The three most stable housekeeping genes were selected (out of a group of five commonly used housekeeping genes) based on the geNorm value [66]. The qPCR analysis was done using the iQ5 optical system real-time PCR machine (BIO-RAD) and the Maxima SYBR Green qPCR Master Mix (Thermo). The raw fluorescence data were exported to Excel (Microsoft) after which mRNA levels were determined based on an assumption free analysis by processing the raw fluorescence data with the LinRegPCR program [67]. A list of primers is found in Table 1.

Immunohistological analysis of mice

Immunohistochemistry was performed on 5-µm paraffin sections as described previously [29]. Brains were placed into histofix (Roth; 4% formalin for 24 h) and dehydrated by sequential incubation in a series of ethanol and chloroform. Sagittal brain sections were incubated with primary antibody prepared in 1% horse serum overnight at 4°C. The following antibodies were used: human Tau-specific antibody HT7 (1:2,000, Thermo Fisher Scientific, Waltham, MA, USA), AT1B (pThr231/pSer235, 1:500, Pierce, Rockford, USA), AT8 (pSer202/pThr205, 1:500, Thermo Scientific), NeuN (1:1,000, Millipore), Alz-50 (1:50), and PHF-1 (1:50, both gifts from Dr. P. Davies, Albert Einstein College, NY, USA). Rabbit polyclonal peptide antibody was generated against pT217 (1:1,000, Biosource, Camarillo, CA, USA). Counterstaining with hematoxylin (Roth, Karlsruhe, Germany) was performed according to the company instructions.

Gallyas silver staining

A total of 5-µm-thick paraffin sections were stained as published [68].

Table 1. Primers.

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Transmission electron microscopy

Heterozygous hTau\(^{AT}\) mice (hTau\(^{AT}\) mice as described above were crossed with black C57Bl/6N background to Glap-luc mice with a white FVB/N background receiving mice with a brown coat color and mixed background 50:50) and controls only carrying the reporter gene but not the mutation (12–13 months old) in groups (n = 4) were terminally anesthetized using ketamine-xylazine and euthanized by perfusion fixation for transmission electron microscopy as described previously [69]. Sections were examined using a Zeiss LEO922 transmission electron microscope with a 2k-CCD camera and subsequently processed using Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA, USA).

Golgi staining and dendritic spine counting

For Golgi-Cox impregnation of neurons [70], the FD rapid GolgiStain TM kit (FD NeuroTechnologies, Columbia, MD, USA) was used according to the manufacturer’s protocol. A total of 80-μm floating sections of transgenic and WT mice at 10 months of age were Golgi-impregnated and hippocampal pyramidal CA3-neurons were used for quantification of dendritic spines as described [71]. The number of animals used for spine quantification was four for heterozygous A152T mice, while two littermate control animals were used for spine counting. An average of 5 dendrites of CA3 pyramidal neurons were analyzed per animal. The sections were imaged using the FV-1000 microscope (Olympus, Shinjuku, Tokyo, Japan) in stacks by application of a 60× objective and a 5× digital zoom. Stacks were loaded in NeuronStudio (CNIC, NY, USA) and inverted after which the dendrite length and spine detection was done in an automated fashion with the trainable spine classifier. Groups were compared and tested for statistically significant difference by applying an unpaired t-test.

Hippocampal organotypic slice cultures

Hippocampal organotypic slice cultures were prepared as described [72], with modifications [16]. Briefly, 8-day-old mice were decapitated, brains were rapidly removed, and hippocampi dissected at 4°C. A McIlwain tissue chopper (Gabler, Bad Schwabach, Germany) was used to prepare 400-μm-thick transverse slices which were transferred to semiporous cell culture inserts (Millipore, Bedford, MA, USA; 0.4 mm). Inserts containing 6 slices were placed in 8 slices were placed in 6-well culture trays containing 1 ml of culture media (50% Minimum Essential Medium (MEM), 25% Hank’s Balanced Salt Solution (HBSS), penicillin/streptomycin (all from PAA, Pasching, Austria), 25% horse serum, 4.5 mg/ml glucose (Sigma-Aldrich, St. Louis, MO, USA), pH 7.4). The culture medium was changed on the first day after preparation and afterward every third day. Slices were kept in culture for up to 60 days.

Ca\(^{2+}\) imaging

Ca\(^{2+}\) imaging experiments of organotypic slices (at 15 DIV) were performed as described previously [16]. Briefly, slices were loaded with Fura-2AM (Invitrogen, Carlsbad, CA, USA) at a concentration of 100 μM at 37°C for 30 min. After loading, slices were washed with 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES)-buffered saline (130 mM NaCl, 5.4 mM KCl; 10 mM HEPES, 25 mM glucose, 1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\); pH 7.4) for another 30 min to allow complete de-esterification of the Fura dye. After washing, cultures were transferred to a submerged imaging chamber of an Examiner A1 microscope (Zeiss, Jena, Germany). Fura-2 fluorescence was imaged at room temperature (RT) in HEPES-buffered saline, using a 10× water immersion objective. The emission of Fura-2-loaded pyramidal neurons was collected at 510 nm after excitation at 340 and 380 nm, respectively, with a Sutter DCIV shutter (Sutter Instrument Co, Navato, CA, USA). Images were taken at a rate of 1 Hz. For baseline recording, intracellular Ca\(^{2+}\) levels were imaged for a 30-s period following superfusion with high potassium (HEPES-buffered saline containing 180 mM KCl) for an additional 30 s. Analysis was performed offline, using 10 regions of interest with constant diameter in area CA3. For each slice, we performed an internal background subtraction, averaged the values in the regions of interest, and normalized with their individual baseline value. Absolute intracellular Ca\(^{2+}\) concentrations were calculated from the ratio of emitted fluorescence (510 nm) after excitation at 340 nm and 380 nm with the standard Gryniewicz formula [73]. The Kd value was calculated by using Invitrogen Fura-2 calibration kit. Initially, Ca\(^{2+}\) imaging was performed in a blinded fashion to compare control littermate slices with slices expressing hTau\(^{AT}\) without drug application.

Microscopy

Images were acquired with an Olympus laser scanning microscope FV1000 (Olympus, Tokyo, Japan), equipped with a confocal laser scanning unit, argon (Ar; 488 nm) and helium/neon (He/Ne 543 nm and 633 nm). For 2 or 3 channel imaging, images were acquired via sequential scanning. Maximum projection images were generated from resulting z-stacks using ImageJ software (NIH, Bethesda, MD, USA).

Dil labeling of slice cultures and spine counting

Hippocampal slice cultures were fixed at DIV 20 in 4% PFA for 2 h, still attached to the semiporous cell culture inserts. A DiI crystal was placed on the CA3 region followed by an incubation of 10 days at room temperature in 4% PFA to allow diffusion. Dendritic spines were imaged by confocal microscopy using tetramethyl rhodamine isothiocyanate (TRITC)-filter settings. ImageJ (NIH, USA) was used to measure spine density from resulting z-stacks.

Cytotoxicity and glutamate assay

Cytotoxicity was assessed by the lactate dehydrogenase (LDH) assay (Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany)). Slice cultures were incubated in serum-free culture medium. The medium was collected at different time points in vitro and processed as prescribed. Finally, the absorbance at 492 nm (indicating dying cells) was assayed in 96-well plates (plate reader Tecan Safire, Manhendorf, Switzerland). The amount of glutamate in the culture medium (serum and glutamate free) was determined with the Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit
(Molecular Probes, US). Medium was collected at different time points in vitro and treated as described. Caspase-3 activity was determined fluorometrically by the EnzChek Caspase-3 Assay Kit with Z-DEVD-AMC substrate (Molecular Probes). Pooled slice cultures (6 slices per sample) were homogenized in 8 M DTT, 1 mM EGTA, 20 mM NaF, 1 mM Na3VO4, 150 mM NaCl, protease inhibitors (Complete Mini; Roche, Indianapolis, IN), 100 U/ml benzonase, 5 µM okadaic acid, 5 mM CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate). Total protein extracts were resolved by SDS–PAGE (10% polyacrylamide gels) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membrane was incubated in 5% non-fat milk in TBS-Tween for 1 h at RT, washed with TBS-Tween and incubated overnight in primary antibody solution at 4°C. The membrane was washed with TBS-Tween and incubated with the secondary antibody (Dako, Hamburg, Germany) coupled to horseradish peroxidase (HRP) for 1 h at RT. The membrane was developed by ECL Western Blotting Detection Kit (GE Healthcare, Chalfont St Giles, UK) and analyzed by densitometry (LAS 3000; AIDA software; Raytest, Straubenhardt, Germany). The following antibodies were used: pan-Tau antibody K9JA (Dako, Hamburg, Germany, 1:10,000), anti-β-actin (Sigma, Germany) (1:10,000), anti-CREB (Cell Signaling) (1:1,000), and anti-p133 CREB (Cell Signaling, Darmstadt, Germany) (1:1,000) and secondary antibodies, HRP-anti-rabbit and HRP-anti-mouse (DakoCytomation, Carpinteria, CA, USA) (1:1,000).

Electrophysiology

Acute, transverse hippocampal slices were prepared from 12±1-month-old male and female mice anesthetized for approximately 15 s by isoflurane inhalation before decapitation. To obtain optimal mossy fiber preservation, a cutting angle of 12° in the fronto-occipital direction was used [74] for vibrating-blade microtome (VS1200T; Leica, Wetzlar, Germany) sectioning. A total of 350-µm-thick slices were transferred into a custom-made interface chamber and superfused with carbogenated ACSF (126 mM NaCl, 26 mM NaHCO3, 3 mM KCl, 2.5 mM CaCl2, 1.3 mM MgSO4, 1.25 mM NaH2PO4 and 10 mM glucose, saturated with 95% O2/5% CO2) at 37 ± 0.5°C; flow rate, 1.8–3 ml/min; pH 7.4. After 1.5-h recovery, slices were transferred to a submerged recording chamber at 30°C. Extracellular field potentials (fEPSP) were recorded from stratum lucidum in area CA3 at a flow rate of ~5 ml/min. The mossy fiber tract was visualized by infrared (IR)/differential interference contrast (DIC) microscopy. As stimulation electrode, a patch-clamp pipette was used (1–2 MΩ) to excite mossy fibers (mf) in the region of the hilus. Recording electrodes (2.5–3 MΩ) were filled with ACSF and separated constantly 200 µm from each other. fEPSPs were 10× preconditioned (custom-made preamplifier) filtered at 1 kHz and sampled at 10 kHz using a HEKA double patch-clamp EPC 10 USB amplifier (HEKA Elektronik Dr. Schulze GmbH, Lambrecht, Germany). Organotypic hippocampal slices were recorded at DIV 10 and 30 with the same ACSF composition, temperature, and electrodes as described above.

Stimulation and recording procedures

Constant current pulses were elicited every 20 s (0.05 Hz) with a pulse width of 0.1 ms. Input/output (I/O) curves were generated using stimulus intensities ranging from 10 to 100 µA with an increment of 10 µA. In the same slice, paired-pulse facilitation (PPF) was
evoked by a 50-ms interstimulus interval (ISI). In a next step, frequency facilitation (FF) of the mossy fiber pathway was measured by switching stimulation frequency from 0.1 to 1 Hz. Only slices which showed robust FF were used further on for ml-LTP recordings. LTP was induced by theta burst stimulation at 30% of the maximum stimulus intensity as revealed initially during the I/O recording. At the end of each recording, 5 μM DCGIV (Tocris Biosciences, Bristol, UK) was applied for at least 5 min to test mossy fiber specificity. In case the fEPSP slope was not reduced to more than 50% of the initial slope steepness, slices were excluded from further mossy fiber analysis.

Statistical analysis

Statistical analysis was performed by using the statistics software Prism5 (GraphPad, La Jolla, CA, USA). Evaluation of data was performed either by Student’s t-test or one-way ANOVA followed by Tukey’s post hoc test or Newman–Keuls multiple comparison test or multiple repeat ANOVA (Statistica, Statsoft (Europe), Hamburg, Germany) as indicated. If not indicated, P-values display Student’s t-test. Data are shown as mean ± SEM. P-values are as follows: *P < 0.05, **P < 0.01, and ***P < 0.001.

Expanded View for this article is available online.

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Author contributions

JMD performed electrophysiological experiments in acute and organotypic slices, calcium imaging in slice cultures as well as analysis, experiment design, writing of the manuscript, and figure artwork. UK contributed immunofluorescence, biochemistry, and calcium imaging in organotypic slices as well as analysis, experiment design, writing of the manuscript, and figure artwork. AS performed biochemical and immunohistochemical analysis of adult transgenic mice. FJAD performed quantitative real-time PCR and dendritic spine analysis in adult mice. ZS carried out EM experiments and analysis. EM and E-MM supervised the study and writing of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

The Tau/A152T mutation leads to excitotoxicity in the mouse brain

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