Adenosine A_1 receptor antagonist rolofylline alleviates axonopathy caused by human Tau ΔK280

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Accumulation of Tau is a characteristic hallmark of several neurodegenerative diseases but the mode of toxic action of Tau is poorly understood. Here, we show that the Tau protein is toxic due to its aggregation propensity, whereas phosphorylation and/or missorting is not sufficient to cause neuronal dysfunction. Aggregate-prone Tau accumulates, when expressed in vitro at near-endogenous levels, in axons as spindle-shaped grains. These axonal grains contain Tau that is folded in a pathological (MC-1) conformation. Proaggregant Tau induces a reduction of neuronal ATP, concomitant with loss of dendritic spines. Counterintuitively, axonal grains of Tau are not targeted for degradation and do not induce a molecular stress response. Proaggregant Tau causes neuronal and astrocytic hypoactivity and presynaptic dysfunction instead. Here, we show that the adenosine A_1 receptor antagonist rolofylline (KW-3902) is alleviating the presynaptic dysfunction and restores neuronal activity as well as dendritic spine levels in vitro. Oral administration of rolofylline for 2-wk to 14-mo-old proaggregant Tau transgenic mice restores the spatial memory deficits and normalizes the basic synaptic transmission. These findings make rolofylline an interesting candidate to combat the hypometabolism and neuronal dysfunction associated with Tau-induced neurodegenerative diseases.

tauopathies | rolofylline | hypoactivity | axons | treatment

The Tau protein is well known for stabilizing microtubules in neurons, although in a subset of neurodegenerative disorders called tauopathies [e.g., Alzheimer disease (AD), frontotemporal lobar degeneration (FTLD), Pick disease, etc.] Tau becomes modified (e.g., by hyperphosphorylation, acetylation, proteolytic processing, etc.), leading to neurofibrillary tangles (1). Alternatively, Tau can assemble as spindle-shaped grains, as in argyrophilic grain disease (AGD). Mutations within the repeat domain of the Tau protein can increase its β-sheet propensity (e.g., mutations P301L, ΔK280, and others), leading to missorting and aggregation of Tau (2, 3). In humans, such mutations can cause typical FTLD pathology with corresponding neurofibrillary tangles (4). In transgenic mice expressing human Tau with the ΔK280 mutation, the Tau protein is missorted into the somatodendritic compartment, (hyper)phosphorylated, and folded into a pathological conformation (MC-1 epitope) (5). These mice are still functionally impaired from ∼12 mo onward despite the absence of neurofibrillary tangles (6). Here, we use this transgenic human Tau model (ΔK280, proaggregant) in parallel with its antiaggregant counterpart (ΔK280-PP line) where Tau cannot aggregate because of Ile-to-Pro mutations that serve as β-sheet breakers (7). Both types of Tau bind similarly to microtubules but differ in their aggregation potential (8). Noxious stimuli [e.g., hypoxia or amyloid-β (Aβ)] increase adenosine levels 30–100 times in the brain (9). Adenosine is a neuromodulator and has a depressant effect on neuronal activity when bound to the ubiquitously expressed adenosine A_1 receptor, a G_i/G_0-protein coupled receptor (10, 11). Hypometabolism (i.e., diminished neuronal activity) is strongly associated with neurodegeneration (12–14). Moreover, pathological Tau can disrupt ongoing network activity even at early asymptomatic stages (15). The relationship between hypometabolism seen in human tauopathies, Tau aggregation, and its effect on neuronal activity is not well established. Here, we show that moderate levels of aggregation-prone Tau protein induces hypoactivity of neurons with a reduction in neuronal ATP levels, loss of dendritic spines, and impaired synaptic functioning. The neuronal activity and impaired presynaptic compartment can be restored by application of the adenosine A_1 receptor antagonist rolofylline in vitro and in vivo, suggesting that restoration of the diminished neuronal activity may be a yet-unexplored treatment strategy to combat cognitive impairment in tauopathies.

Results

High Aggregation Propensity Is Not Necessary for Tau Missorting but Causes Tau to Accumulate in Axons as Grains. In organotypic hippocampal slices, both proaggregant and antiaggregant Tau is missorted to the somatodendritic compartment as has been shown before (Fig. 1 *A* and *B*, asterisks) (5). This missorted Tau in transgenic slices consists of, at least partially, transgenic human Tau (Fig. S1). Only proaggregant Tau transgenic slices reveal Tau-positive beaded structures in the neuropil oriented mostly perpendicular to the apical dendrites of the CA1 pyramidal cells (Fig. 1 *C* and *D*, arrowheads) resembling grains in human AGD (16). This suggests that proaggregant Tau accumulates in the axons as grains. Transgenic proaggregant Tau is expressed at 83% of wild-type Tau levels, whereas antiaggregant Tau is expressed at 50% of wild-type Tau levels in 30 d in vitro (DIV30) organotypic slices (Fig. 1*E*), which is approximately threefold less as has been found in the in vivo Tau transgenic mouse brain (6). The quantitative difference between proaggregant and antiaggregant Tau could be partially

Significance

Tau-driven neurotoxicity occurs in multiple neurodegenerative diseases that have a severe impact on families and the society at large. However, its mode of toxicity is poorly understood, and therefore no effective drug treatments have been discovered. Here, we show that aggregate-prone Tau accumulates in axons where it causes presynaptic dysfunction and matching neuronal hypoactivity. The adenosine A_1 receptor antagonist rolofylline, a drug developed for patients with acute heart failure and renal dysfunction, normalizes neuronal functioning in vitro and restores cognition in Tau-transgenic mice. We hypothesize that rolofylline could be used as a treatment by increasing bona fide neuronal activity, which is diminished in tauopathy patients. In turn, this should delay the onset or the progression of these neurodegenerative diseases.

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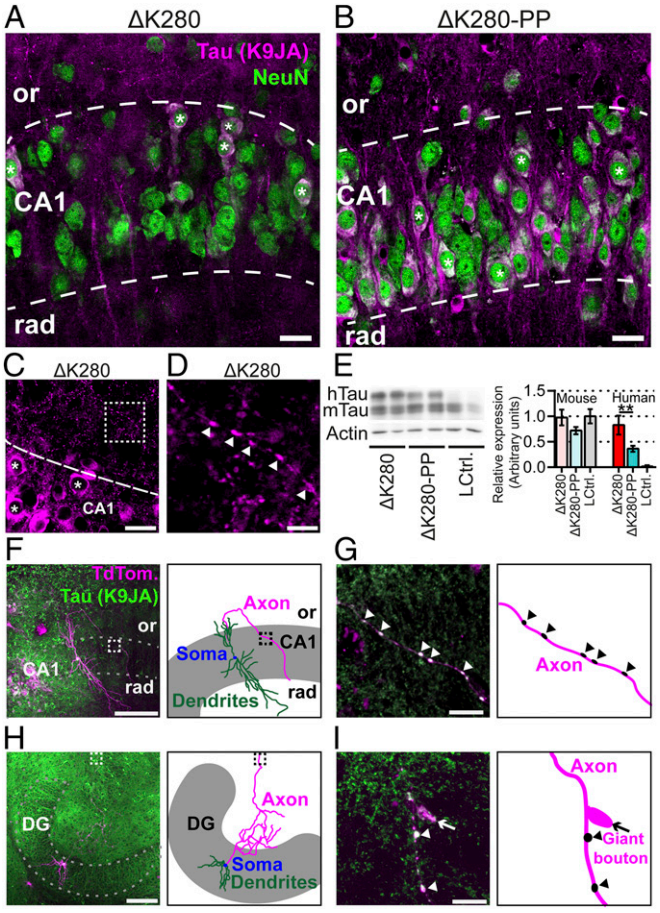

Fig. 1. Aggregate-prone Tau accumulates in the neuropil as axonal grains. (*A* and *B*) CA1 region of proaggregant Tau (ΔK280) and antiaggregant Tau (ΔK280-PP) transgenic organotypic hippocampal slices stained for neurons [NeuN and pan-Tau (K9JA)]. (Scale bar: 20 μm.) Asterisks indicate missorted Tau. (*C* and *D*) Higher magnification of the CA1 and neuropil of proaggregant Tau transgenic slices. [Scale bar: 20 μm (*C*) and 4 μm (*D*).] (*E*) Representative immunoblot of total Tau (antibody K9JA) and quantification. $^{**}P < 0.01$ (one-way ANOVA with Tukey's test). Error bar indicates SEM. (*F–I*) Littermate control slices cotransfected with the red fluorescent protein TandemTomato (TdTom.) and proaggregant Tau [Tau (K9JA)]. (*F* and *H*) A transfected CA1 pyramidal cell (*F*) and a transfected dentate gyrus granule cell (*H*) with schematic representations. (Scale bar: 200 μm.) Dotted boxes in *F* and *H* are magnified in *G* and *I*. (Scale bar: 10 μm.) Or, stratum oriens; Rad, stratum radiatum.

explained by the fact that proaggregant Tau accumulates in the neuropil as well, whereas antiaggregant Tau does not. To confirm the axonal nature of the grains of Tau, we performed a biolistic cotransfection of the red fluorescent protein TandemTomato (for morphology) and proaggregant Tau (Fig. 1 *F–I*). Single neurons are cotransfected, for example, in Fig. 1*F*, which shows a transfected CA1 neuron or a dentate gyrus granule cell in Fig. 1*H* and Fig. S2. The axons of transfected neurons (Fig. 1 *G* and *I*) clearly reveal small inclusions of Tau (~1 μm in size, arrowheads), although presynaptic boutons (e.g., giant mossy fiber boutons) are only marginally stained for Tau (Fig. 1*I* and Fig. S2 *A* and *B*; arrow), indicating that Tau does not accumulate at presynaptic boutons in these slices. Furthermore, we did not see colocalization of the grains of Tau and presynaptic marker synaptophysin (Fig. S3). We also observed that Tau missorts into a subgroup of proximal dendrites, which correlates with a dramatic spine loss in the affected dendrites (Fig. S2 *C* and *F*). By contrast, dendrites that do not contain Tau are richly decorated with spines (>1 spines per μm), indicating that there is only local impairment of dendritic function in case of proaggregant Tau missorting.

Both Proaggregant and Antiaggregant Tau Are Phosphorylated, but Only the Grains of Proaggregant Axonal Tau Appear in a Pathological Conformation. The proaggregant transgenic mice have aberrantly phosphorylated human Tau-ΔK280, although the relation between these posttranslational modifications and Tau toxicity is poorly understood (6). Therefore, to differentiate between toxic and nontoxic modifications of Tau, we compared the phosphorylation status of Tau in proaggregant (ΔK280) and antiaggregant (ΔK280-PP) transgenic organotypic hippocampal slices (Fig. 2 and Table S1). Slices from both types of transgenic mice show the 12E8 epitope (pSer262/pSer356, Fig. 2 *A* and *B*) in the somatodendritic compartment (asterisks). Phosphorylation of these serines is known to induce detachment of Tau from microtubules and to promote missorting of Tau (17). Indeed, the axonal grains of Tau are not 12E8 positive (arrowheads), emphasizing that 12E8 staining is found only in mislocalized Tau. When corrected for the difference in total Tau, 12E8 phosphorylation does not differ between proaggregant and antiaggregant Tau (Fig. 2*C*). The PHF-1 epitope (pSer396+pSer404, Fig. 2 *D–F*) is seen in both types of Tau transgenic slices where it appears in the somatodendritic compartment (asterisks) and in the axonal grains (arrowheads). PHF-1 phosphorylation levels are similar for antiaggregant or proaggregant Tau when corrected for total Tau input (Fig. 2*F*). The antibody AT180 (Tau pThr231) (18) shows (very) weak staining in the cell soma of both types of Tau transgenic slices (Fig. 2

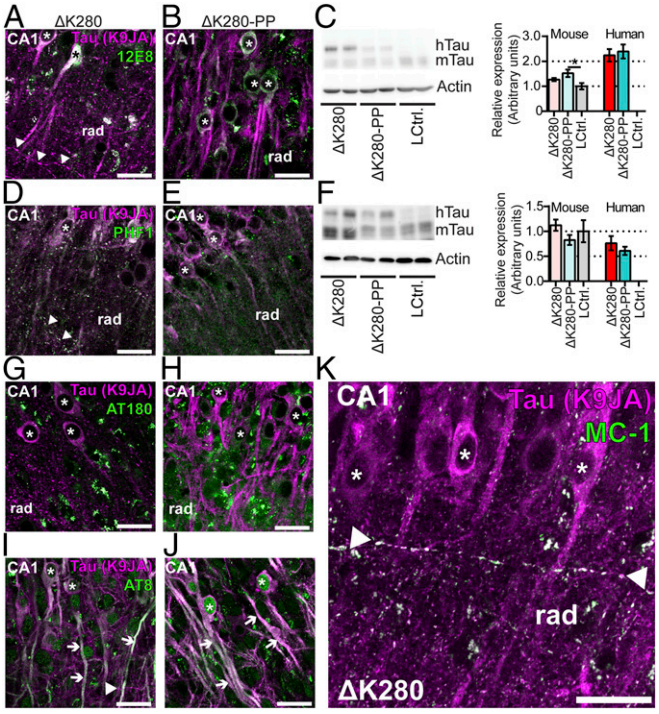

Fig. 2. Proaggregant and antiaggregant Tau are both phosphorylated in organotypic hippocampal slices, but only grains of proaggregant Tau can be stained for pathological (MC-1) Tau. (*A* and *B*) Pan-Tau and 12E8 (pSer262 and pSer356) costaining of proaggregant Tau (ΔK280) and antiaggregant Tau (ΔK280-PP) transgenic organotypic hippocampal slices. (*C*) Quantification of 12E8 phosphorylation by immunoblotting. Data are corrected for total Tau input. Error bar indicates SEM. $^{*}P < 0.05$ (one-way ANOVA with Tukey's test). (*D* and *E*) Costaining of pan-Tau with PHF-1 phosphorylated Tau (Ser396 and Ser404) in organotypic slices of both transgenic lines. (*F*) Quantification of PHF-1 phosphorylation by immunoblotting. Data are corrected for total Tau input. Error bar indicates SEM. (*G* and *H*) Immunostaining of organotypic slices for the AT180 (phospho-Tau pThr231) epitope and pan-Tau. (*I* and *J*) Immunostaining using the pan-Tau antibody (K9JA) and phospho-Tau epitope AT8 (Ser202 and Thr205). (*K*) Costaining for Tau conformation-dependent epitope MC-1 and pan-Tau (K9JA). Rad, stratum radiatum. (All scale bars: 25 μm.)

G and *H*, asterisks) contrasting the high degree of Tau phosphorylated at Ser202/Thr205 [asterisks (somata) and long arrows (apical dendrites)] (AT8 antibody, Fig. 2 *I* and *J*). We also studied pathologically folded Tau using the MC-1 antibody (Fig. 2*K*). MC-1–positive Tau accumulates in the axonal grains of proaggregant Tau as described above (arrowheads), whereas antiaggregant slices remain unstained. By contrast, Tau missorted in the somatodendritic compartment in either proaggregant or antiaggregant Tau transgenic slices is negative for the MC-1 epitope (asterisks), consistent with the absence of grains in dendrites. We also attempted to costain Tau (K9JA) with dendritic marker MAP2. Although Tau frequently colocalized with MAP2 in dendrites (missorting), the grains never colocalized with MAP2, emphasizing the axonal nature of the grains (Fig. S4). Surprisingly, the axonal grains of Tau appear to resist protein degradation because they are negative for markers of degradation (vimentin, ubiquitin, Lamp1, Sqstm1/P62, Hsc70, and Tia-1) (Fig. S5). Taken together, most phosphoepitopes are present in both antiaggregant and proaggregant Tau transgenic slices. Grains of pathologically folded Tau accumulate only in the axons of proaggregant Tau transgenic neurons, which suggests that these grains play a critical role in Tau-induced neuronal dysfunction. However, these grains are not targeted for degradation, nor do they appear to induce an unfolded protein response within the axon.

Proaggregant Tau Causes Spine Loss, Reduces Axonal Mitochondria, and Lowers Cytoplasmic ATP. We labeled neurons of the organotypic slices diolistically with DiI to investigate the effect of the proaggregant and antiaggregant Tau on spine density and morphology. Proaggregant Tau transgenic slices showed a significant reduction of spines compared with littermate control slices, whereas spine density of antiaggregant Tau transgenic slices was similar to controls (Fig. 3 *A* and *B*). Dendritic spines are usually classified into different categories based on their shape, which represents different functional properties (19). We could not detect a difference in classes of spines between slices of both transgenic lines and littermate controls (Fig. 3*C*). We determined mitochondrial movements in live organotypic slices because aggregation-prone Tau is known to impair mitochondrial transport (Fig. 3 *D* and *E*). Mitochondria transport is similar in both kinds of Tau transgenic slices (Fig. 3*E*) with only a moderately lower mitochondrial density in proaggregant Tau transgenic slices compared with antiaggregant slices (Fig. 3*F* and Table S2). We also investigated the effect of proaggregant and antiaggregant Tau on energy status (ATP level). Transgenic slices were biolistically transfected with the FRET-based ATP sensor (ATeam) (Fig. 3 *G* and *H*) (20). ATP is reduced in the proaggregant transgenic slices, matching the lower mitochondrial density, compared with littermate controls or antiaggregant Tau transgenic slices (Fig. 3*H*). This suggests that the energy status of the neurons is compromised by proaggregant but not by antiaggregant Tau.

The Proaggregant Tau-Induced Phenotype Can Be Rescued with the Adenosine A_1 Receptor Antagonist Rolofylline. The markers described so far did not reveal any clear mode of action of toxic proaggregant Tau despite the functional impairment reported previously in transgenic mice (6). We therefore designed a quantitative PCR-based miniscreen of key genes known to be (up-)regulated at the mRNA level as a result of specific stressors serving as markers for insults (Table S3). Surprisingly, expression of neuronal activity marker *cFos*, astrocytic activity marker *Gfap*, and oxidative stress marker *Hmox1* were reduced in the proaggregant Tau transgenic slices, whereas antiaggregant Tau transgenic slices were not different from littermate controls (Fig. 4*A*). Signs of molecular stress (e.g., protein misfolding, osmotic stress, oxidative stress, etc.) reflected by an increase in stress markers (*Hspa1a*, *Osp94*, *Hmox1*, etc.), however, could not be found in the proaggregant Tau transgenic slices. This confirms the lack of a classical cytotoxicity by this species of aggregate-prone Tau (e.g., chaperones, aggresomes, stress granules, etc.), as has been reported above (Fig. S5).

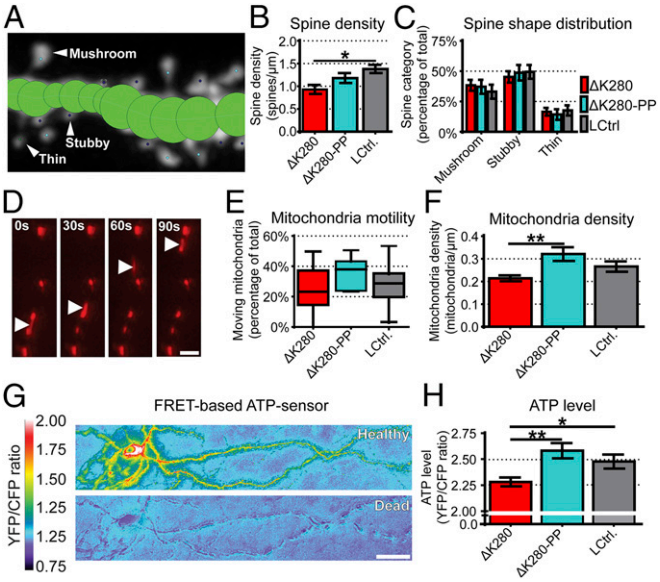

Fig. 3. Proaggregant Tau transgenic slices have fewer spines, less axonal mitochondria, and reduced ATP levels compared with antiaggregant Tau transgenic or control littermates. (*A*) Example image of semiautomated spine counting. (*B*) Graph representing total number of spines per micrometer for proaggregant (ΔK280), antiaggregant (ΔK280-PP), and control littermate (LCtrl.) slices. $*P < 0.05$. (*C*) Graph showing the distribution of different spine categories obtained using NeuronStudio. Data are expressed as a percentage of total number of spines analyzed. (*D*) Images showing an example of a moving mitochondrion within an axon at different time points. (Scale bar: 10 μm.) (*E* and *F*) Graph representing the percentage of moving mitochondria in the different groups analyzed (*E*) and the density of mitochondria per micrometer of axon. $**P < 0.01$ (*F*). (*G*) Representative image of a neuron expressing the ATP sensor in a healthy state (*Upper*, YFP/CFP > 2) and after death (*Lower*, YFP/CFP ~ 1). (Scale bar: 50 μm.) (*H*) ATP levels displayed as the background-corrected ratio between YFP and CFP. ($**P < 0.01$ and $*P < 0.05$, compared with Tau-ΔK280 slices.) All error bars indicate SEM. Significant differences determined by using one-way ANOVA with Tukey's test.

Given that grains of Tau accumulate within axons (Fig. 1) and that axonal mitochondria density is reduced (Fig. 3), we tested next whether axonal (presynaptic) functioning is indeed impaired in organotypic hippocampal slices. We therefore measured the paired-pulse ratio (PPR) by applying a paired-pulse stimulus of the Schaffer collaterals (Fig. 4 *B* and *C*). We observed a typical paired-pulse facilitation (PPF) response in littermate controls and antiaggregant Tau transgenic slices, whereas in proaggregant Tau transgenic slices, the same stimulus paradigm resulted in a paired-pulse depression (Fig. 4*B*). This indicates that proaggregant Tau induces presynaptic impairment, whereas presynapses of antiaggregant Tau slices are unaffected. Adenosine downmodulates neuronal activity (cFos levels), impairs the presynapse, and attenuates long-term potentiation (LTP) via the A_1 receptor (21). Because this resembles our presynaptic phenotype (Fig. 4*B*) and the outcome of the miniscreen, we attempted to counterbalance the observed phenotype by using the adenosine A_1 receptor antagonist rolofylline. An adenosine A_{2A} receptor antagonist (ZM-241385), an adenosine A_1 receptor agonist (N^6-cyclopentyladenosine), and a Tau aggregation inhibitor (BSc3094) were used as controls (Table S4) (22–24). Rolofylline increases neuronal activity (*Fos* mRNA) both in proaggregant Tau transgenic slices and controls, although in case of the proaggregant slices neuronal activity is almost doubled, yielding levels similar to those of treated littermate control slices (Fig. 4*D*). In line with these observations, the presynaptic impairment in proaggregant Tau transgenic slices can be reversed by rolofylline or BSc3094 without causing adverse effects in controls (Fig. 4*F* and Fig. S6).

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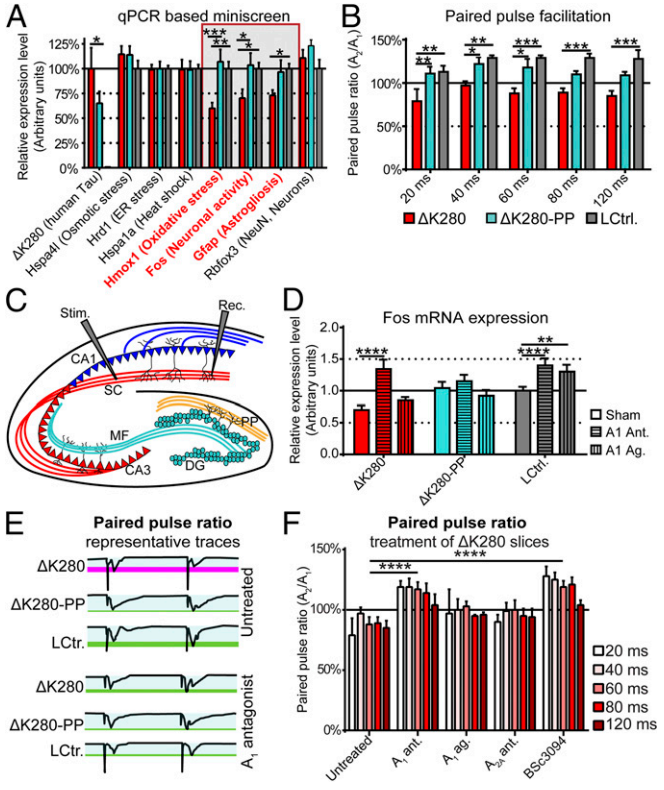

Fig. 4. Organotypic slices expressing proaggregant Tau show reduced neuronal and astrocytic activity and impaired axonal functioning. This can be alleviated by antagonizing Tau aggregation propensity or stimulation of cell activity with adenosine A_1 receptor antagonist rolofylline. (*A*) The mRNA levels of transgenic Tau and stress-related genes in proaggregant (ΔK280), antiaggregant (ΔK280-PP), and control littermate (LCtrl.) organotypic hippocampal slices. Error bars indicate SEM. $***P < 0.001$, $**P < 0.01$, and $*P < 0.05$ (two-way ANOVA and Dunnett's multiple-comparisons test). (*B* and *C*) Paired-pulse response in ΔK280, ΔK280-PP, and LCtrl. organotypic slices. Error bars indicate SEM. $***P < 0.001$, $**P < 0.01$, and $*P < 0.05$ (two-way ANOVA with Tukey's test). (*C*) Electrodes were placed in the stratum radiatum to excite the Schaffer collaterals. DG, dentate gyrus; MF, mossy fibers; PP, perforant pathway; Rec., recording electrode; SC, Schaffer collaterals; Stim., stimulation electrode. (*D*) The Fos mRNA levels in slices treated with adenosine A_1 receptor antagonist rolofylline and adenosine A_1 receptor agonist N^6-cyclopentyladenosine. Error bars indicate SEM. $****P < 0.0001$, $**P < 0.01$ (two-way ANOVA and Dunnett's multiple-comparisons test). (*E*) Representative traces of the paired-pulse response for proaggregant Tau (ΔK280), antiaggregant Tau (ΔK280-PP) transgenic slices and littermate controls (LCtrl.). (*F*) PPRs in proaggregant Tau transgenic organotypic hippocampal slices after treatment with compounds. Error bars indicate SEM. $****P < 0.0001$ (two-way ANOVA with Tukey's test).

Rolofylline Treatment Restores Dendritic Spine Levels in Proaggregant Tau Transgenic Slices, Rescues Long-Term Memory Deficits, and Normalizes Basal Synaptic Transmission in Proaggregant Tau Transgenic Mice. Because the electrophysiological parameters in the proaggregant Tau transgenic slices were normalized by rolofylline treatment, we investigated whether it would also restore the level of dendritic spines in these slices. Indeed, the reduced level of spines seen in proaggregant Tau transgenic slices are normalized when treated with rolofylline, whereas no significant changes are found in antiaggregant Tau transgenic slices or littermate controls (Fig. 5 *A* and *B*). The axonal density of mitochondria, which is slightly lower in proaggregant compared with antiaggregant Tau transgenic slices, is marginally decreased by rolofylline treatment albeit in a genotype-independent manner (Fig. S7). Having observed that rolofylline restores presynaptic functioning (i.e., PPF, Fig. 4*F*), neuronal activity (induction of Fos, Fig. 4*D*), and dendritic spine levels in proaggregant Tau transgenic organotypic slices (Fig. 5 *A* and *B*), we tested whether we could restore long-term spatial memory in proaggregant Tau transgenic mice as well. We therefore performed the Y-maze test, novel object recognition test (NORT), and the fear conditioning test with 14-mo-old proaggregant Tau transgenic and littermate control mice within 10–20 d of oral rolofylline treatment. In the Y-maze test, (treated) control mice spent more time in the novel arm, whereas untreated proaggregant mice did not show any arm preference (Fig. 5*C*). Rolofylline reestablished novel arm preference in proaggregant mice, suggesting that rolofylline restores spatial memory in these animals. In the NORT, (treated) control mice explored the novel object more compared with the old object, whereas the proaggregant mice did not show any preference for the new or the old object (Fig. 5*D*). Rolofylline treatment improved long-term object recognition memory in proaggregant Tau transgenic mice, as shown by increased novel object preference. For fear conditioning testing, the effects of systemic rolofylline administration on different stages of contextual and clue-based (sound) fear learning were investigated 24 h after the training session (Fig. 5 *E* and *F*). Contextual memory was unaltered as all groups showed similar freezing when reintroduced into the chamber. Control groups and the rolofylline-treated proaggregant group showed a clue-induced freezing response, whereas no effect was seen in the untreated proaggregant mice (Fig. 5*F*). This result suggests an impaired learning association between the sound and the foot shock in proaggregant mice, which can be rescued by rolofylline treatment. Ten weeks of posttreatment (starting at 14 mo), we assessed the electrophysiological properties of the CA1 region of the hippocampus in treated proaggregant Tau transgenic animals and controls (Fig. 5 *G* and *H*). Compared with untreated proaggregant Tau transgenic mice, treated mice (proaggregant Tau transgenics and littermate controls) have significantly larger maximal excitatory postsynaptic potential amplitudes (Fig. 5 *G*–*I*). The slope of the input/output (I/O) curve is significantly reduced in proaggregant Tau transgenic mice compared with controls, indicative of impaired basal synaptic transmission (Fig. 5*I*). Treatment with rolofylline increases the slope of the I/O curve in both proaggregant Tau transgenic slices and littermate controls (Fig. 5*I*). The impairment of the presynapse, as determined by application of a paired-pulse protocol is ambiguous in the acute slices (Fig. S8*A*). However, at the shortest pulse interval (20 ms), PPF is normally strongly suppressed by the feedforward inhibition (25). For the proaggregant Tau transgenic slices, feedforward inhibition seems to be impaired because the PPR is as high as the ratios seen with larger pulse intervals. Synaptic plasticity, measured as the ability to elicit LTP by theta-burst stimulation, is not altered in any condition compared with untreated littermate controls (Fig. S8*B*).

Discussion

In the present paper, we compared an aggregation-prone species of Tau with its antiaggregant counterpart as well as nontransgenic littermates. We used an organotypic hippocampal slice model to show that both proaggregant and antiaggregant Tau is (hyper) phosphorylated and mislocalized to the somatodendritic compartment in the absence of frank Tau aggregation. Although missorting and/or hyperphosphorylation of Tau may be a prerequisite for Tau pathology to occur, we clearly show here that it is not sufficient to cause neuronal dysfunction. This argues that increased aggregation propensity (the only difference between proaggregant and antiaggregant Tau) is essential for Tau toxicity. Despite of the high (>99.5%) homology of the two Tau species, only proaggregant Tau accumulates as axonal spindle-shaped grains in a pathological "pretangle" conformation, similar to argyrophilic grains in humans. Proaggregant Tau, but not antiaggregant Tau, decreases dendritic spine number and the ATP levels in neurons, which further supports the role of aggregation propensity as the mode of toxic action. Surprisingly, the axonal aggregates of Tau do not colocalize with known aggregation markers. Instead, presynapses are impaired in proaggregant Tau transgenic slices, giving rise to a general reduction of neuronal activity, which has been reported previously for Tau P301L-expressing mice as well

(15). The reduced ATP levels, dendritic spine loss, diminished neuronal activity, and impaired presynaptic functioning are reminiscent of adenosine A_1 receptor signaling. We therefore hypothesized that inhibition of the adenosine A_1 receptor signaling may be able to restore neuronal functioning. Indeed, presynaptic functioning, neuronal activity, as well as the reduction of dendritic spines in the proaggregant Tau transgenic organotypic slices are normalized by application of the highly selective adenosine A_1 receptor antagonist rolofylline. When administrated orally to proaggregant Tau transgenic mice, rolofylline restores cognitive functioning and strengthens basal synaptic transmission, which is known to be subdued as a result of adenosine signaling (26). Adenosine, when bound to the A_1 receptor, has an inhibitory function on many organs including the brain where it reduces neurotransmitter release (11). Adenosine is normally produced both extracellularly and intracellularly where adenosine is formed by degradation of AMP. Because intracellular ATP levels are 50 times higher than AMP levels, small changes in the ATP catabolism lead to dramatic changes in AMP and subsequently adenosine levels. The exact mechanism by which Tau is able to reduce presynaptic functioning and subdue neuronal activity remains to be determined. However, we see a reduction of ATP in proaggregant Tau transgenic neurons, which may be caused by a release of ATP from the neurons or a shift from ATP to AMP intraneuronally, both leading to high extracellular adenosine levels (27). The role for adenosine in the CNS is ambiguous. Adenosine is very important for the circadian rhythm and for neuroprotective effects when bound to the adenosine A_1 receptor (28). Concomitantly, the impaired neurotransmitter release by adenosine A_1 receptor signaling blocks memory formation (29). So adenosine A_1 signaling seems to drive neuronal networks from the (highly) excitable state to the rest and repair state, both of which are important for maintaining synaptic functioning as well as learning and memory. However, prolonged activation of the adenosine A_1 receptor (due to pathological Tau, Aβ, or other chronic stressors) may bring the neurons in a permanent state of hypoexcitability impairing neuronal functioning. In this study, we provide evidence that antagonizing the adenosine A_1 receptor can restore the Tau-induced neuronal dysfunction in a tauopathy mouse model. Rolofylline has never been tested as a treatment for any human neurodegenerative disease. As a diuretic, it failed in a phase III trial for patients suffering from acute heart failure due to unimproved renal function. Adverse effects were, however, limited (30). It has been reported that adenosine receptors are increased in neurons in the degenerating human brain and that administration of an adenosine A_1 receptor agonist induces Aβ production, Tau phosphorylation, and Tau missorting in vitro (31). Down syndrome patients, known to suffer from early-onset AD, have higher levels of adenosine than aged matched controls (32). However, due to the very short half-life of adenosine (<10 s in blood), there have been no studies on adenosine levels in human brain (33). Brain hypometabolism (i.e., neuronal hypoactivity), on the other hand, is a characteristic hallmark during and preceding neurodegeneration (34, 35). Cognitively normal ApoE4 homozygous subjects show reduced glucose metabolism as is seen in AD patients (36). The same reduction of glucose metabolism occurs in preclinical individuals with a genetic predisposition for familial AD, long before the onset of cognitive decline (37). Systemic administration of $GABA_A$ agonists (benzodiazepines), which inhibit neuronal activity, almost double the risk for AD when taken for more than 6 mo (38). On the other hand, the most common psychoactive drug in the world (caffeine), an adenosine receptor antagonist that boosts neuronal activity, protects against AD (39). Stimulation of the perforant path in an Aβ-based mouse model is sufficient to restore memory retrieval (40). Similarly, transcranial

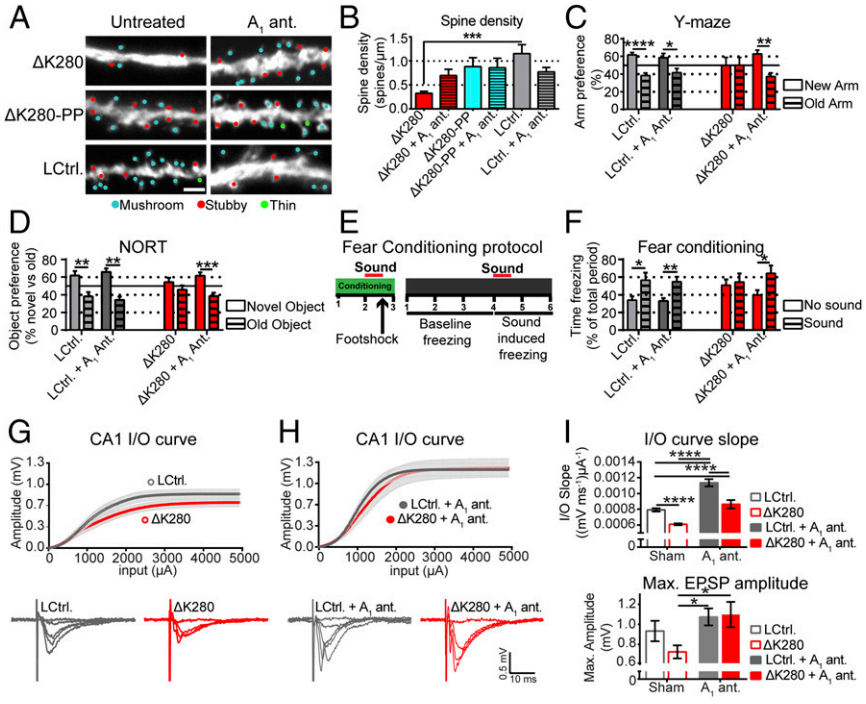

Fig. 5. Treatment with rolofylline restores the dendritic spine level in proaggregant Tau transgenic slices and reverses spatial memory deficits and normalizes basal synaptic transmission in proaggregant Tau transgenic mice. (*A* and *B*) Quantification of dendritic spines of in rolofylline (A_1 ant.) or sham-treated organotypic slices. Error bars indicate SEM. ***$P < 0.001$ (one-way ANOVA with Tukey's test). (Scale bar in *A*: 2 μm.) (*C*–*F*) Outcome of behavior testing after 10–20 d of rolofylline treatment for the Y-maze test (*C*), novel object recognition test (NORT) (*D*), and fear conditioning test (*E* and *F*). Error bars indicate SEM. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$. (*G* and *H*) Basal synaptic transmission (I/O curve) in acute slices from littermate control and proaggregant Tau transgenic. Representative traces of the I/O curves of proaggregants and littermate controls are displayed. Sham treated in *G* and rolofylline (A_1 ant.) treated in *H*. Representative traces of the I/O curves are displayed. (*I*) The slope and maximum amplitude of the I/O curves of CA1 of rolofylline (A_1 ant.) and sham-treated acute slices of proaggregant Tau transgenic and littermate controls. Error bars indicate SEM. ****$P < 0.0001$, *$P < 0.05$ (two-way ANOVA with Tukey's test).

magnetic stimulation in humans increases brain network activity and performance of associative memory, emphasizing the benefit of increased bona fide network activity (41). In conclusion, we show that Tau protein impairs neurons through its ability to aggregate, which in turn leads to reduced neuronal activity, lowered ATP levels, and dendritic spine loss. In both the organotypic slice model as well as in transgenic mice, one can alleviate the process of neuronal dysfunction by administration of the adenosine A_1 receptor antagonist rolofylline, a compound that is proven to be safe in humans. Since neuronal hypoactivity/hypometabolism precedes human neurodegenerative diseases as well, restoration of normal neuronal activity by rolofylline administration may prove to be a successful treatment to counteract the Tau-induced brain dysfunction.

Materials and Methods

See *SI Materials and Methods* for detailed descriptions. All experiments were approved by an animal welfare committee of the agency for Nature, Environment, and Consumer Protection in North Rhine-Westphalia, Germany.

Slices were analyzed at DIV30 to DIV35. The localization of (phosphorylated) Tau was examined by immunofluorescence in organotypic hippocampal slices. Axonal localization of Tau, intraneuronal ATP levels, and mitochondrial motility were studied by using biolistic transfection of organotypic hippocampal slices. The mRNA quantification was performed by using real-time PCR. The synaptic transmission was analyzed by assessing the field excitatory postsynaptic potentials applied in a paired-pulse protocol. Dendritic spine levels in organotypic slices were quantified by biolistic transfection of TandemTomato or diolistic labeling using DiI. Organotypic proaggregant Tau transgenic mice and age-matched controls of 14 mo of age were used to test the effectiveness of rolofylline as a treatment for Tau-induced dysfunction by oral administration. The behavioral performance of mice treated with rolofylline was tested using the Y-maze, novel object recognition task, and fear conditioning testing. The basic synaptic transmission in acute slices was assessed by measuring the I/O responses of field excitatory postsynaptic potentials. All results are presented as mean ± SEM. Statistical comparisons between two groups were tested using Student's *t* test. Comparisons among groups were tested using one-way or two-way ANOVA and Tukey's test or Dunnett's test for post hoc testing. $P < 0.05$ was considered significant.

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