Reversal of tau-dependent cognitive decay by blocking adenosine A1 receptors in mouse models of tauopathy

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Abstract

Background: Accumulation of tau is a characteristic hallmark of several neurodegenerative diseases and is associated with neuronal hypoactivity and presynaptic dysfunction. Oral administration of the adenosine A₁ receptor antagonist rolofylline (KW-3902) has been shown previously to reverse spatial memory deficits and to normalize the basic synaptic transmission in a mouse line expressing full-length proaggregant tau (Tau^{ΔK}).

Methods: Using a combination of behavioral assays, imaging by PET with several PET-tracers, and analysis of brain tissue, we studied the curative reversal of tau pathology by blocking adenosine A₁ receptors in transgenic mice expressing different types of tau and tau mutants.

Results: We show by positron emission tomography using the tracer [¹⁸F]CPFPX (a selective A₁ receptor ligand) that intravenous injection of rolofylline effectively blocks A₁ receptors in the brain. Moreover, when administered to Tau^{ΔK} mice, rolofylline can reverse tau pathology and synaptic decay. The beneficial effects are not restricted to the full-length Tau^{ΔK} mouse line as they were also observed in a line with more aggressive tau pathology, expressing the amyloidogenic repeat domain of tau (TauRD^{ΔK}) with higher aggregation propensity. Both transgenic models develop a progressive tau pathology with missorting, phosphorylation, local accumulation of tau, loss of synapses and cognitive decline. TauRD^{ΔK} causes pronounced neurofibrillary tangle assembly concomitant with neuronal death, whereas Tau^{ΔK} accumulates only to tau pretangles without overt neuronal loss. A third model tested, the rTg4510 line has a high expression of mutant Tau^{P301L} and hence a very aggressive phenotype starting at ~3 months of age. This line failed to reverse pathology upon rolofylline treatment, consistent with higher accumulation of tau-specific PET tracers and inflammation.

Conclusions: Blocking adenosine A₁ receptors by rolofylline can reverse pathology if the pathological potential of tau remains below a threshold value that depends on concentration and aggregation propensity. Thus, reversal is possible even in presence of potent pro-aggregant mutants near physiological tau levels but is overwhelmed by high levels of mutant tau, accompanied by intense neuroinflammation.

Background

Alzheimer disease (AD) is a neurodegenerative disorder characterized by progressive accumulation of protein aggregates composed of Amyloid-beta (Aβ) and the microtubule-associated tau protein in brain structures including those relevant for learning and memory (Selkoe et al., 2012). Despite much progress made in recent years with regard to understanding pathophysiology and development of novel therapeutic approaches, a number of promising drug candidates failed in clinical trials (Anderson et al., 2017; Di Santo et al., 2013; Karran et al., 2011; Wischik et al., 2014).

Tau protein is well known for stabilizing microtubules in neurons. However, 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 phosphorylation, acetylation, proteolytic processing, etc.), concomitant with neurofibrillary tangle formation (Takashima, 2013) which are suspected to induce neurodegeneration. Different aggregation-prone tau mutants have been generated to support this hypothesis (Gotz and Gotz, 2019). Some examples are the mutation P301L (Ramsden et al., 2005; Santacruz et al., 2005) and Δ K280 (Sydow et al., 2011a; Van der Jeugd et al., 2012).

Disturbances in the cerebral metabolic rate of glucose (CMRglu) were an early observation in AD (de Leon et al., 1983) and a decrease in glucose consumption is correlated with decreased cognitive performance (Mosconi et al., 2007; Mosconi et al., 2004; Small et al., 2000). Although reductions in CMRglu in AD patients could be explained first for the increase synaptic dysfunction and by the large-scale neuronal loss at later stages, several authors have examined the early onset of these defects in people at risk for AD (Reiman et al., 2001). It appears that hypometabolism occurs in individuals at risk decades before clinical symptoms of dementia are evident, and is unlikely to be due to cell loss (Costantini et al., 2008). On the other hand, such association remains unclear in animal models where inconsistent metabolic activities have been reported, depending on the type of pathology, age, and treatment of the animals (Bouter and Bouter, 2019). In the case of transgenic mice developed in our laboratory, expressing tau with the aggregation-prone mutation Δ K280 at moderate levels caused hypoactivity of neurons, reduction in neuronal ATP levels and mitochondrial density, loss of dendritic spines, and impaired synaptic functioning (Decker et al., 2015; Van der Jeugd et al., 2012), consistent with decreased glucose uptake.

Adenosine is a neuromodulator and has a depressant effect on neuronal activity when bound to the ubiquitously expressed adenosine A₁ receptor, a G_i/G_o-protein coupled receptor (Dunwiddie and Masino, 2001). This makes adenosine a potential therapeutic target for increasing neuronal activity. Indeed, adenosine A₁ receptor antagonist (rolofylline) treatment restored neuronal activity and rescued previously impaired presynaptic plasticity in vitro in organotypic hippocampal slice cultures expressing mutant tau and in vivo in a tau transgenic model (Dennissen et al., 2016). In order to test the validity of this approach we now treated different transgenic mouse models with rolofylline and observed their response by behavioral assays and tau-dependent biochemical and histological assays.

Methods

Animals and rolofylline treatment.

All animal experiments were carried out in accordance with the guidelines of the German Welfare Act and approved by the local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen). Animals were housed in groups of 2–5 animals under standard conditions (23 °C, 40–50 % humidity, *ad libitum* access to food and water) with a 12 hours light/dark cycle (with light on from 6 a.m. to 6 p.m.).

The transgenic mouse models included the following: (a) rTg(tauP301L)4510 (Ramsden et al., 2005; Santacruz et al., 2005), (b) transgenic mice expressing full-length pro-aggregant human tau (2N4R Tau-ΔK280, termed Tau^{ΔK}) (Eckermann et al., 2007) or (c) its repeat domain (TauRD-ΔK280, TauRD^{ΔK}) (Mocanu et al., 2008). Briefly, rTg4510 animals were produced by crossing the activator mouse line CamKII-tTA, with the responder tetO.MAPT*P301L mouse line. Mice having both CaMKII-tTA and tau transgene expressed human mutant P301L tau (0N4R). Transgenic mice expressing pro-aggregant human full-length tau (2N4R, Tau441, with deletion mutant ΔK280, 441-1 = 440 amino acids, here termed Tau^{ΔK}) or pro-aggregant tau repeat domain (construct K18, 4R, residues 244-372, termed TauRD^{ΔK}) were generated as described (Eckermann et al., 2007; Mocanu et al., 2008). Briefly, responder mice carrying either the Tau-transgene together with a luciferase reporter were crossbred with the CaMKIIα-tTA transactivator mice (Mayford et al., 1996) to obtain double-transgenic mice with constitutive expression of luciferase and pro-aggregant Tau^{ΔK} or TauRD^{ΔK}. All bigenic offspring were heterozygous and had an identical C57BL/6 genetic background. Non-transgenic littermates were used as controls. The transgene expression of bigenic mice started roughly around birth (0mo) concomitant with the onset of CaMKIIα activity. The expression was measured in vivo by bioluminescence imaging of luciferase activity (Van der Jeugd et al., 2012).

For oral application of rolofylline, mice were treated with custom-made food pellets (Ssniff Spezialdiäten GmbH) containing 1.5 mg adenosine A₁ receptor antagonist rolofylline (KW-3902) per kg. The dosage of rolofylline was 0.2 mg·kg⁻¹·d⁻¹. Nine to thirteen control littermates and transgenic mice were treated with rolofylline at different ages for 3 weeks, and then behavior analysis was started. Animals were continuously treated until the end of the behavioral study.

Histology and Golgi staining

Immunohistochemistry was performed on 5 μm paraffin sections as described (Sydow et al., 2011b; Van der Jeugd et al., 2012). The following antibodies were used for light microscopy: MC1 (conformational epitope, aa 5-15 + 312-322, 1:10, gift of Dr. P. Davies, Albert Einstein College, NY), AT8 (pSer202/pThr205, 1:500, Thermo Scientific), Alz-50 and PHF-1 (pS396/pS404) (1:50, were a kind gift from Dr. P. Davies), NeuN (1:1000, Millipore), Iba1 (1:1000, Wako) and GFAP (1:2000, Sigma). Secondary antibodies as well as the avidin-biotinylated peroxidase complex were provided by the Vectastain Universal Elite ABC kit (Vector laboratories) and DAB (Dako) was used to visualize the antibody labeling. Gallyas silver impregnation was performed as described (Sydow et al., 2011b; Van der Jeugd et al., 2012).

For Golgi-Cox impregnation of neurons (Glaser and Van der Loos, 1981), the FD rapid GolgiStain TM kit (FD NeuroTechnologies) was used according to the manufacturer's protocol. 120 μm floating sections of transgenic and WT mice at ~17.5 months of age were Golgi-impregnated and hippocampal pyramidal CA1- neurons were used for quantification of dendritic spines as described (Ricobaraza et al., 2012). For each mouse ($n = 3-4$ per group), 3 different brain slices were taken and spines of ~10 neurons per slice were counted. For each neuron, 1-2 secondary dendrites of 20-30 μm lengths were quantified using ImageJ software (NIH). Stainings were imaged by a LSM510 Meta confocal microscope (Zeiss).

Biochemical analysis of brain tissue

Sarkosyl-extraction, total protein preparation and western blots were performed as described previously (Sydow et al., 2011b; Van der Jeugd et al., 2012). Depending on the 1st antibody, 2-20 µg of total protein or 3 µl of sarcosyl extraction lysates from brain tissue were loaded for the detection with pan-Tau antibody K9JA (1:20000, Dako A-0024), phospho-Tau antibodies 12E8 (pSer262/pSer356, 1:500, Elan) and PSD-95 (1:1000, ThermoFischer). Blots were normalized by the concentration of β-actin (1:20000, Sigma) or b-III-tubulin (1:5000, RD Systems), visualized with ECL Plus detection system (GE Healthcare) and quantified by a computer-assisted densitometer (Gel-Pro Analyzer, version 4; Media Cybernetics, Bethesda, MD, USA).

Behavioral tests

Nesting test. Mice were single-housed with one nestlet per cage. The nestlet was removed after 2, 6 and 12 hours, weighted, and the percentage of nesting was assessed at each time point (% nesting = 100 - ((final weight)/(initial weight)) × 100). Nest-building scores were defined as previously described (Deacon, 2012).

Burrowing test. Burrowing test was performed as described elsewhere (Deacon, 2012). The final food pellets weight burrowed overnight was measured next morning and the percentage of burrowing determined (% burrowing = 100 - ((final weight)/(initial weight)) × 100).

Fear conditioning. Mice were tested in the fear conditioning apparatus as described elsewhere (Curzon et al., 2009). Briefly, during the training, mice were introduced into the chamber and allowed to explore it during 2 min. A tone (sound cue) was then presented at a level of 80 dB for 30 s. A mild foot shock (0.5 mA) was administered during the last 2 s of the tone presentation and co-terminated with the tone. After the shock presentation, mice were kept for 30 s in the chamber to allow memory consolidation. Contextual and cued fear conditionings were tested 24 h after the training. For contextual conditioning test, mice were placed into the chamber and the percentage of freezing per minute was analyzed during 4 min. After 4 min, the cue conditioning test was performed by presenting the tone for 30 s. Freezing in the presence and after the tone was analyzed over 2 min and compared to analyze cue-induced memory. Results were expressed as the mean percentage of freezing in the presence and absence of tone.

Morris water maze (MWM). Spatial memory abilities were examined in the standard hidden-platform acquisition and retention version of the MWM (Morris, 1984).

A 2 days pre-training protocol was conducted to familiarize the mice with swimming and climbing to a hidden platform (22°C water temperature, 4 trials/day, maximum trial duration 60 sec, 20 min inter-trial interval). The pre-training platform (10 cm diameter) was placed 1 cm below the water surface.

In the learning and probe trial phase, a 150 cm circular pool was filled with water opacified with nontoxic white paint (Biofa Primasol 3011, Germany) and kept at 22°C, as previously described (D'Hooge et al., 2005). In the middle of the target quadrant, a 15 cm round platform was hidden (1 cm beneath the water

surface). The MWM room was equipped with visual cues to facilitate orientation. The pool was divided into four quadrants: target (T), right adjacent (R), opposite (O), and left adjacent (L). Each mouse performed 4 swimming trials per day (maximum duration 60 sec, 15-20 min inter-trial interval) for 5 consecutive days. Mice started the test from 6 symmetrical positions in a pseudo-randomized order across trials. When mice failed to find the submerged platform within 60 sec, they were guided to the platform, remaining there for 10 sec before returning to their cage. The escape latency was determined. On acquisition days 3, 4, 5, and 3 days after the end of the acquisition phase (day 8), a probe trial was conducted by removing the platform and recording the search pattern of the mice for 60 sec. The following 3–4 learning trials were carried out with the platform placed in the initial position on the target quadrant to avoid extinction. During acquisition and probe trials the EthoVision XT video tracking system was used (Noldus Information Technology).

Open Field and novel object recognition test (NORT). Mice were tested in a square open field (45 cm long) (Panlab) located in a room with dim lighting. NORT was performed as previously described (Anglada-Huguet et al., 2014). Briefly, mice were habituated to the open field in the absence of the objects for 10 min/d over 2 d. During the training period, mice were placed in the open field with two identical objects for 10 min. The retention test was performed 24 h posttraining (long-term memory) by placing the mice back to the open field for 5-min session, and by randomly exchanging the familiar object for a novel one.

Y-maze. The Y-maze task was used to analyze hippocampus-dependent memory in rolofylline treated mice. The dimensions of the used Y-maze were 30 × 6 × 15 cm (length by width by height) (Panlab). In the training session, one arm was closed (novel arm), and mice were placed in the stem arm of the Y (home arm) and allowed to explore this arm and the other available arm (familiar arm) for 10 min. The mice were placed back in their home cage after exploration. To assess long-term memory, 4 h post-exploration, the closed arm was opened, and mice were placed in the stem arm of the Y-maze and allowed to freely explore all three arms for 5 min. Arm preference was determined by calculating the following: time spent in each individual arm*100/total time spent in both arms (both familiar and novel arm).

Positron Emission Tomography (PET)

In order to ascertain that systemically administered rolofylline reached the brain and engaged with the drug target, PET measurements were performed with six control mice at the age of seven months. Each animal received one measurement with 10 MBq [¹⁸F]CPFPX alone (a selective A1 receptor ligand), and one measurement with 10 MBq [¹⁸F]CPFPX + 10 mg/kg rolofylline, on two separate days. Animals were anesthetized (initial dosage: 5 % isoflurane in O₂/air 3:7, then reduction to 2 %), and a catheter for tracer injection was inserted into the lateral tail vein. Mice were placed on an animal holder (medres® GmbH, Cologne, Germany) and fixed with a tooth bar in a respiratory mask. Body temperature was maintained at 37 °C using a feedback-controlled warming system (medres®). Eyes were protected from drying with Bepanthen eye and nose ointment (Bayer). A PET scan in list mode was conducted using a Focus 220 micro PET scanner (CTI-Siemens, Erlangen, Germany) with a resolution at the center of field of view of 1.4

mm. Data acquisition started with intravenous tracer injection (volume: 125 μ l) and lasted for 30 min. This was followed by a 10 minute transmission scan using a ^{57}Co point source for attenuation correction. After the scan was finished, the catheter was removed and the mouse was returned to its home cage. To visualize tau accumulation and inflammation, the tracers [^{18}F]PI-2620 and [^{18}F]DPA-714 were administered to rTg4510 mice (n=8 rTg4510 and 8 non-transgenic littermates) and TauRD $^{\Delta\text{K}}$ mice (n=9 TauRD $^{\Delta\text{K}}$ and 7 non-transgenic littermates) as described above with scan durations of 1 h ([^{18}F]PI-2620) and 30 min ([^{18}F]DPA-714).

After full 3D rebinning, summed images (0-30 min for [^{18}F]CPFPX and [^{18}F]DPA-714, 30-60 min for [^{18}F]PI-2620) were reconstructed using the iterative OSEM3D/MAP procedure resulting in voxel sizes of $0.47 \times 0.47 \times 0.80$ mm. For all further processing of the images including statistics, the software VINCI 4.72 for MacOS X (Max Planck Institute for Metabolism Research, Cologne, Germany) was used. Images were first co-registered and then intensity-normalized to background. For this, a spherical volume of interest (VOI) with a volume of 4 mm^3 was placed inside the neck musculature immediately caudal to the cerebellum ([^{18}F]CPFPX) or into the midbrain ([^{18}F]PI-2620 and [^{18}F]DPA-714). Each image was divided by the mean value of the background VOI, resulting in the "background standardised uptake value ratio" (SUVR_{bg}).

[^{18}F]CPFPX images with and without rolofylline were compared voxel-wise using the paired t-test. This was followed by a threshold-free cluster enhancement (TFCE) procedure with subsequent permutation testing (Smith and Nichols, 2009), resulting in a statistical map corrected for multiple testing, thresholded at $p < 0.05$. Because TFCE values are used for final thresholding only, color bars of TFCE maps were labeled with the original t-values, marked t_{TFCE} . In addition, VOIs were drawn for different brain areas and VOI mean values were extracted. They were used for paired t-tests in Graphpad Prism 6.0 for MacOS X. Significance level was always $p < 0.05$.

Results

1. Adenosine A1 receptor binding of [^{18}F]CPFPX is reduced by rolofylline treatment

Wild type animals were intravenously injected with 10 mg/kg rolofylline together with [^{18}F]CPFPX, a tracer that binds specifically to adenosine A₁ receptors with high affinity (Bauer et al., 2003a). Mean [^{18}F]CPFPX uptake of the whole brain was significantly lower with rolofylline (Fig. 1), indicating that rolofylline effectively blocks adenosine A₁ receptors. Voxel-wise comparison revealed that blocking effects were not homogeneous throughout the brain, but were highest in the midbrain (a region where A1 receptor density is low) and cerebellum (where A1 receptor density is high) (Bauer et al., 2003a; Bauer et al., 2003b). VOI analysis demonstrated that rolofylline decreases [^{18}F]CPFPX uptake significantly in midbrain and cerebellum by ~ 25 and 20% respectively ($p < 0.05$). A tendency of decreased [^{18}F]CPFPX uptake ($0.10 > p > 0.05$) was found in the basal forebrain, frontal cortex, hippocampus, and thalamus (Fig. 1E).

2. Decreased tau accumulation and synaptopathology after blockade of adenosine A₁ receptors.

Using a mouse model of tauopathy expressing human full-length tau with $\Delta K280$ mutation (Tau ^{ΔK}), we had shown previously that the adenosine A₁ receptor antagonist rolofylline (KW-3902) is able to restore spatial memory deficits and normalized the basic synaptic transmission of 14-month-old Tau ^{ΔK} transgenic mice (Dennissen et al., 2016).

When checking pathology in the Tau ^{ΔK} mice after rolofylline treatment, a reduction in pre-aggregated tau species detected by Gallyas silver staining and a decrease of conformationally-changed tau (Alz-50) were detected in the hippocampus of the mice (Fig. 2A). Additionally, levels of human transgenic phosphorylated tau (12E8; epitope pSer262/pSer356) detected by Western Blot were reduced after rolofylline treatment (Fig. 2B, C). No differences in hippocampal volume were observed in any of the groups in accordance to our previous results (Van der Jeugd et al., 2012) (Supplementary Fig. S1). Although no neuronal death was observed in this mouse line, the presence of mutant tau results in a depletion of synaptic proteins in the hippocampus, which was partially reversed after rolofylline treatment (Fig. 2D-F). We observed a decrease in the PSD-95 protein levels (Fig. 2D) accompanied by decrease in the number of spines analyzed by Golgi staining (Fig. 2E, F) in the Tau ^{ΔK} mice, whereas no differences were observed between LCtrls and rolofylline-treated Tau ^{ΔK} mice.

3. Rolofylline decreases cognitive deficits in mice expressing Tau-repeat domain (TauRD ^{ΔK})

The phenotype of tau pathology in the full-length Tau ^{ΔK} described above was relatively mild, correlating with a low expression of exogenous mutant Tau ^{ΔK} (~2-fold overexpression of hTau40 over mTau) (Van der Jeugd et al., 2012), a moderate propensity for forming β -structured tau amyloid fibrils, and a late onset of cognitive decline (~ 12 months). In related studies with a tau aggregation inhibitor, methylene blue (MB), onset of cognitive decline could be delayed by several months by preventive treatment with MB. However, therapeutic treatment after onset did not reverse the disease progression, showing that rolofylline was clearly a superior drug compared to MB (Hochgrafe et al., 2015).

Given this background, we wanted to test the potential of rolofylline in mice with a more aggressive course of tau pathology. We chose the inducible line TauRD ^{ΔK} , expressing the repeat domain of human tau with the FTDP mutation $\Delta K280$. This domain contains the sequences responsible for the aggregation of tau (PHF1, PHF1*, (von Bergen et al., 2000)) and has much higher propensity for β -structure. At an expression level of ~ 70% or less of endogenous tau (Mocanu et al., 2008), the age of decline was earlier (~ 10 months), the mice had a more severe tangle burden, synapse loss, neuronal loss, and loss of brain volume (Hochgrafe et al., 2013; Joseph et al., 2017; Sydow et al., 2011b). We therefore wanted to investigate whether the beneficial effect of rolofylline was specific to the full-length Tau ^{ΔK} mice or could be observed also in the more severe case of TauRD ^{ΔK} mice (Hochgrafe et al., 2013).

Fifteen-months old TauRD ^{ΔK} and litter-mate control mice were treated with rolofylline for 2 weeks at 5 months after cognitive decline started (Sydow et al., 2011). Cognitive performance was assessed by

different behavioral paradigms after rolofylline treatment (Fig. 3A). The body weight, reflecting the welfare of the animals, was increased to control levels after blocking adenosine A1 receptor in TauRD^{ΔK} mice (Fig. 3B). However, rolofylline did not reverse the ~ 10% decrease in brain weight observed in the transgenic animals (Fig. 3F). In the burrowing test, which is correlated with hippocampal function (Deacon, 2012), rolofylline reversed the loss in burrowing behavior back to ~ 93% of controls (Fig. 3C) and was significantly higher than TauRD^{ΔK} untreated group. Additionally, for fear conditioning testing, the effects of systemic rolofylline administration on different stages of contextual and cue-based (sound) fear learning were investigated 24 h after the training session. Control groups showed a cue-induced freezing response and rolofylline-treated TauRD^{ΔK} group a clear tendency to increase freezing in the presence of the sound, whereas hardly any increase was seen in the untreated TauRD^{ΔK} mice (Fig. 3D). This result shows that the impaired learning association between the sound and the foot shock can be partially reversed by rolofylline treatment. Although TauRD^{ΔK} mice showed increased contextual freezing during the testing day compared to LCtrl, these mice also presented significantly higher freezing behavior during the training session before the shock (4.73% freezing in LCtrls VS 24.59% in TauRD^{ΔK} mice; $F(3,35) = 7.189$, $p = 0.0007$, Tukey's post-hoc $p = 0.0082$).

Spatial learning was assessed in the Morris water maze task (MWM) that requires mice to learn to find a hidden platform in a circular pool, guided by spatial cues. During MWM acquisition, TauRD^{ΔK} mice showed a slower rate of learning, which was partially reversed by rolofylline treatment (Fig. 3E). As a control, mice were tested for their general motor and exploration behavior in the Open Field test. There was no significant difference between groups (Supplementary Fig.S2A), so we can exclude that the difference in learning rate was attributable to a difference in neuromotor disability.

Finally, rolofylline treatment improved the expression of the postsynaptic protein PSD-95 in TauRD^{ΔK} mice (Fig. 3G-H), similar to what we observed in the Tau^{ΔK} mice treated with rolofylline, and similar to the reversal after switching off the expression of the transgene in regulatable mice (Van der Jeugd et al., 2012).

All of these results indicate that even though cognitive abilities are lost in the Tau-transgenic TauRD^{ΔK} mice, the loss can be reversed by rolofylline treatment. This holds not only for the relatively mild phenotype of the full-length Tau^{ΔK} expression, but also for the expression of the more aggressive repeat domain TauRD^{ΔK}. We note that in both cases, the pathology can be reversed by "genetic" treatment (i.e. by stopping the inducible expression of mutant tau by addition of doxycyclin); however none of the Tau-directed drugs tested so far (e.g. aggregation inhibitors (Pickhardt et al., 2019; Schafer et al., 2013)) have achieved a reversal, with the exception of rolofylline (Dennissen et al., 2016).

4. Rolofylline does not improve behavior in rTg4510 mice strongly overexpressing mutant Tau^{P301L}

In the mouse lines described above, tau pathology sets in at late time points, roughly corresponding to stages of AD. There are, however, other tau transgenic lines with stronger phenotypes designed to allow a

faster analysis of tau pathology. This includes lines 3xTg-AD and 5xFAD by LaFerla and colleagues which combine several AD-related mutations (Oakley et al., 2006; Oddo et al., 2003a), and the line rTg4510 which expresses human 0N4R-Tau with the P301L mutation at a high level (~ 13x endogenous Tau) and in an inducible fashion, created by Ashe & coworkers (Ramsden et al., 2005; Santacruz et al., 2005). This line has become a popular model, as it phenocopies the tau pathology and the pronounced neurodegeneration of human tauopathies with an early onset (~ 3 months).

To select the time window for a therapeutic-rolofylline treatment in this transgenic mouse model, we studied histopathological features during the expression of Tau^{P301L} at different ages. We observed high expression of Tau^{P301L} with development of sarkosyl-insoluble tau already at 3 months of age

(Supplementary Fig. S3. A- B), whereas Gallyas silver staining of neurons appeared later (Supplementary Fig. S3C).

In AD and other tauopathies, tau appears to undergo hyperphosphorylation and conformational changes, accompanied by the translocation of pathological tau from the axonal to the somatodendritic compartment of neurons (Buee et al., 2000; Mandelkow and Mandelkow, 2012). These changes are also observed in rTg4510 mice as early as 3 months of age, *viz.* hyperphosphorylation [AT8 antibody, epitope pS202/pT205) and PHF-1 antibody (epitope pS396/pS404)] and pathological conformation of tau (MC1 antibody) appearing in the hippocampus and the cortex (Supplementary Fig.S 3D-F). The number of cortical neurons bearing hyperphosphorylated tau (e.g. AT8- and PHF1-Tau) and tau conformational changes (MC1) increase progressively with age (Supplementary Fig. S3D-F). At 6 months of P301L expression, mice show histopathological features of AD, including neuronal loss in the CA1 layer of the hippocampus (by NeuN staining, Supplementary Fig. S4A). Finally, Iba-1 and GFAP staining against activated microglia and astrocytes, respectively, indicate inflammatory processes in the hippocampus of rTg4510 mice already at 3 months of age (Supplementary Fig. S4B, C).

Rolofylline treatment was tested on these mice for its beneficial effect by similar procedures as for the mouse lines described above. Based on the histopathological and biochemical results (Supplementary Fig. S3, Fig. S4), we selected 5 months of age for the therapeutic treatment because at this age the rTg4510 mice present extensive tau pathology and also cognitive deficits. In this therapeutic treatment protocol rolofylline application started at 5 months of age for 2 weeks in rTg4510 mice and control littermates and cognitive assessment with continuous rolofylline treatment was performed using several behavioral tests (Fig. 4A).

First, we analyzed the body weight without treatment as an assessment of general wellbeing of the animals. The rTg4510 transgenic mice had reduced body weight compared to their littermates (-12%) as early as 2.5 months of age although it didn't gain statistical significance until 4.5 months of age (Fig. 4B), There was no improvement by rolofylline treatment (Fig. 4C). In fact, surprisingly, rolofylline increased body weight in control animals, an effect not seen with the rTg4510 tested. rTg4510 mice also showed reduced brain weight, which could not be reversed by rolofylline (Fig. 4D). In the nesting and

burrowing tests, rTg4510 mice performed significantly worse than control animals with no reversal after blocking A₁ receptors (Fig. 4E, F). Indeed, rolofylline seemed to inhibit nesting behavior in the transgenic animals (Fig. 4E). In the Open Field test, no differences were observed between non-treated control and transgenic animals, whereas rolofylline induced pronounced hyperactivity in rTg4510 (Fig. 4G). In the Y-maze test, control mice spent more time in the novel arm, in contrast to rTg4510 mice which did not show any arm preference (Fig. 4H), showing no beneficial effect of rolofylline in restoring spatial memory in these animals.

Consistent with the effects observed in behavior, rolofylline treatment in rTg4510 mice at 5 months of age could not prevent or improve molecular markers of tau pathology. rTg4510-treated mice show similar degree of tau hyperphosphorylation (PHF-1 (pS396/pS404); Fig. 5A), tau pathological conformation (MC1; Fig. 5B) and tau aggregation (Gallyas silver positive cells; Fig. 5C).

Since no recovery was observed in rTg4510 mice after therapeutic treatment, we tested the potential of rolofylline in preventing cognitive decline. For the preventive intervention, rolofylline was initiated at 2.5 months of age for 2 weeks and continued throughout the behavioral experiments (Fig. 6A). Rolofylline treatment had no effect on behavioral assays, similar to what we observed in case of the brain weight which was still reduced in rTg4510 mice compared to control mice after treatment (Fig. 6B).

When analyzing cognitive decline, in the nesting test the rTg4510 mice performed significantly worse when comparing to control littermates regardless the rolofylline treatment (Fig. 6C). Just at 12h after the introduction of the nestlet, rTg4510-treated mice were performing not significantly different than controls, although there was a clear trend to show reduced nesting behavior ($p = 0.0569$). Likewise, no improvements were observed in the MWM. Transgenic animals presented higher latency to scape in the learning phase, showing that they cannot remember where the hidden platform was located. The same results were observed in the rolofylline-treated transgenic animals (Fig. 6D). When checking the long term memory 72 hours after the training phase, no differences were observed between treated and non-treated transgenic animals (Fig. 6E). Finally, in the open field test, contrary to the assays described so far, no differences were present between groups when analyzing locomotor activity (Supplementary Fig. 2B), even though rolofylline-treated rTg4510 animals showed increased anxiety, as shown by the lower exploration of the center of the arena (Fig. 6F).

In summary, in case of rTg4510 mice neither therapeutic nor preventive rolofylline treatment could reduce the cognitive decline, prevent or reverse markers of tauopathy, in pronounced contrast to what we observed for the Tau^{ΔK} and TauRD^{ΔK} lines.

Discussion

In the present paper, we assess the potential beneficial effect of the adenosine A₁ receptor antagonist rolofylline in transgenic mouse models of tauopathy. For this study, we used 3 different transgenic mouse lines: a transgenic mice expressing full-length pro-aggregant human tau (2N4R Tau-ΔK280, termed

Tau^{ΔK}) (Eckermann et al., 2007) or its repeat domain (TauRD^{ΔK}) (Mocanu et al., 2008) and the rTg(tauP301L)4510 (rTg4510) (Ramsden et al., 2005; Santacruz et al., 2005). We have shown that Tau^{ΔK} and TauRD^{ΔK} mice with moderate levels of aggregation-prone tau protein present hypoactivity of neurons with a reduction in neuronal ATP levels and mitochondrial density, loss of dendritic spines, and impaired synaptic functioning (Decker et al., 2015; Dennissen et al., 2016; Messing et al., 2013; Van der Jeugd et al., 2012). Rolofylline, a drug developed for patients with acute heart failure and renal dysfunction, normalizes presynaptic functioning, neuronal activity as well as the reduction in the number of spines in the in vitro model of Tau^{ΔK} organotypic hippocampal slices. Moreover, when administrated orally to Tau^{ΔK} and TauRD^{ΔK} transgenic mice, rolofylline restores cognitive functioning, which is known to be subdued as a result of adenosine signaling (Dennisen et al., 2016) (Fig. 3). On the contrary, rTg4510 mice do not improve after rolofylline treatment (Fig. 4–6).

Adenosine, when bound to the A₁ receptor, has an inhibitory function on many organs including the brain where it reduces neurotransmitter release (Dunwiddie and Masino, 2001). Postsynaptic activation of adenosine A₁ receptors causes decreased cellular excitability (Thompson et al., 1992). In the hippocampus, adenosine is predominantly inhibitory due to high expression levels of the high-affinity A₁R (Ochiishi et al., 1999) and in vivo evidence showed that prolonged A₁R activation may lead to increased hippocampal neuronal death (Stockwell et al., 2017). Moreover, a continued intravenous infusion of a high dose rolofylline for the treatment of heart failure was shown, in some cases, to induce epilepsy (Massie et al., 2010), suggesting that a systemic administration of rolofylline can cross the blood brain barrier (BBB). Using a selective A₁ receptor PET ligand [¹⁸F]CPFPX, we showed for the first time that rolofylline can cross the BBB and successfully engage the target (adenosine receptors) when administered systemically, since intravenously injected rolofylline was able to reduce the binding of [¹⁸F]CPFPX to adenosine A₁ receptors in the brain.

Using the mouse model of tauopathy Tau^{ΔK}, we have previously shown the beneficial effect of the adenosine A₁ receptor antagonist rolofylline in Tau^{ΔK} mice and in organotypic hippocampal slice cultures (Dennisen et al., 2016). In these mice, the tau protein shows the typical hallmarks of a pathological state - it is missorted into the somatodendritic compartment, phosphorylated at key sites (e.g. AT8-epitope), and folded into a pathological conformation (MC-1 epitope) (Eckermann et al., 2007); presenting cognitive impairment from ~12 mo onward despite the near-absence of mature neurofibrillary tangles (Van der Jeugd et al., 2012). Rolofylline treatment clearly reduced the Gallyas-silver positive cells in the hippocampus of old mice with Tau^{ΔK} expression, reduced the level of misfolded tau (Alz50 staining) as well as the levels of Alzheimer-like phosphorylation (12E8; epitope pSer262/pSer356), both indicators of subsequent formation of insoluble NFTs (Hardy, 2003).

Remarkably, the reversal of cognitive decline upon rolofylline treatment was observed not only in fulllength Tau^{ΔK} mice (Dennisen et al., 2016), but also in TauRD^{ΔK} mice expressing only the mutant repeat domain which has a much higher aggregation propensity and earlier onset of cognitive decline.

The overall recovery of body weight in this mouse line was also a marker for improvement of general welfare. Tau-related pathological changes predominantly affect the hippocampal formation in these mice (Sydow et al., 2011b). The hippocampus plays an important role in spatial and contextual learning processes and memory consolidation after initial acquisition (Dudai, 2004). To examine hippocampus-dependent learning and memory, MWM test, burrowing test and cue and context fear conditioning paradigm were used (Curzon et al., 2009; Deacon, 2012; Morris, 1984). Different types of hippocampal lesions can decrease burrowing rate (Deacon, 2012), which was shown to be altered in TauRD^{ΔK} mice which also exhibited longer latency to reach the hidden platform in the MWM. These acquisition deficits were not due to impairment in neuromotor performance as no impairment in the open field test was observed. In both tests, rolofylline treatment reversed the loss of cognitive performance. With a more subtle effect, the same was true for cue-based (sound) fear learning, where rolofylline-treated transgenic animals showed a clear tendency to increase freezing behavior in the presence of the cue. All these reversal effects, achieved by initial 15 days of rolofylline treatment (and continuous treatment during behavior assessment), were analogous to those previously reported for the Tau^{ΔK} mouse line (Dennissen et al., 2016), in spite of the higher pathogenicity of TauRD^{ΔK}, underscoring the curative potential of the adenosine A₁ receptor antagonist.

By contrast, a different picture emerged for the rTg4510 mice which express much higher levels of aggregation-prone tau (~ 13x endogenous Tau) (Ramsden et al., 2005; Santacruz et al., 2005) than the Tau^{ΔK} and TauRD^{ΔK} mice (~ 2x and 0.7x endogenous Tau, respectively) (Mocanu et al., 2008; Van der Jeugd et al., 2012). This line undergoes pronounced tau pathology and cognitive decline at early stages (~ 3 months) (Ramsden et al., 2005) (Supplementary Figs. 3 and 4; Fig. 6) and therefore reaches the irreversible “point of no return” much earlier than the Tau^{ΔK} and TauRD^{ΔK} lines where tau protein aggregates more slowly and at lower expression levels. Indeed, neither a therapeutic treatment with rolofylline (of 5 months old mice, after cognitive decline) nor a preventive treatment (at 2.5 months, before decline) could rescue the cognitive decline in this high expression tau line (Fig. 4–6). It appears that the very high Tau^{P301L} aggregation propensity in this line overwhelms the protective actions of the drug. Different behavioral paradigms were used to check cognitive performance in rTg4510 mice after rolofylline treatment, but no reversal effects were observed in any of the tests, except a slight improvement in the 12h nesting test. This also holds for tau pathology markers such as PHF1, MC1 or Gallyas silver staining and correlates with the failure of reversal of cognitive performance in this mouse line (Fig. 5). Notably, the presence of tau tangles in the hippocampus and olfactory bulb of rTg4510 mice, judged by PET imaging with the tracer [¹⁸F]Pi2620 and confirmed by immunohistochemistry and biochemistry is much higher than in mice accumulating Tau^{ΔK} or TauRD^{ΔK} (Supplementary Figs. 3 and 5). Indeed, the olfactory dysfunction is among the earliest features of AD (Murphy, 2019) and the accumulation of aggregated tau protein in this brain structure correlates with the progression and the severity of the disease in other brain regions (Attems et al., 2014), underscoring the potential utility of olfactory tissue in the early diagnosis of AD. Moreover, a significantly higher level of inflammation was observed in the cortex and hippocampus of rTg4510 mice by PET imaging using the tracer [¹⁸F]DPA-714,

compared to the TauRD^{ΔK} mice that showed neuroinflammation at later time points (Mocanu et al., 2008) (Supplementary Fig. 5). Although preventive rolofylline treatment was started as early as 2.5 months of age in the rTg4510 mice, this line presents impairment in the MWM as early as 1.3 months of age (Ramsden et al., 2005), indicating that the initial trigger for cognitive decline occurs at a very early age so it becomes irreversible by rolofylline treatment. Even though, we cannot rule out that an earlier intervention with rolofylline could be also beneficial in this transgenic mouse line. Tau^{ΔK} and TauRD^{ΔK} lines showed a milder and more progressive tau pathology when compared to the rTg4510 mouse line. Tau^{ΔK} and TauRD^{ΔK} lines have a low rate of neuronal death which occurs only at late time points when the synaptopathology and cognitive decline are present, and they have a normal life span. In contrast, rTg4510 mice present cognitive deficits, loss of body weight, and tau pathology very early. Therefore, the beneficial effect of rolofylline seen in Tau^{ΔK} and TauRD^{ΔK} lines can be overwhelmed when the expression of mutant tau becomes excessive, as in the rTg4510 line.

With regard to synaptic effects, expression of pro-aggregant Tau^{ΔK} and TauRD^{ΔK} caused a profound synaptic decay and loss of dendritic spines (Sydow et al., 2011b; Van der Jeugd et al., 2012). Synaptic degeneration precedes neuronal loss and best correlates with tau pathology and cognitive performance in dementing illnesses (Spires-Jones and Hyman, 2014; Tracy et al., 2022). Several AD mouse models present early synaptic failure (Billings et al., 2005; Oddo et al., 2003b; Tampellini et al., 2009) and reduction or regression of dendritic spines (Kremer et al., 2011; Moolman et al., 2004; Ricobaraza et al., 2012). Treatment with rolofylline restores the dendritic spine level in organotypic hippocampal slices from Tau^{ΔK} mice and normalizes basal synaptic transmission (Dennissen et al., 2016). Indeed, rolofylline treatment increases the expression of the synaptic protein PSD-95 in the hippocampus of Tau^{ΔK} and TauRD^{ΔK} together with a recovery of the number of spines, as analyzed by Golgi staining in the Tau^{ΔK} mice. Changes in the levels of PSD-95 are likely to induce alterations in synaptic signaling and plasticity, which in turn leads to learning impairments (Xu, 2011). Therefore, amelioration of PSD-95 protein deficits could be related, in part, to the rescue of memory deficits in rolofylline-treated Tau^{ΔK} and TauRD^{ΔK}.

Refining treatment paradigms with respect to treatment initiation, concentration and duration together with an early detection of the pathology are key steps in the reversal or delay of disease progression. Therefore, selection of the right time point and window of intervention is crucial for each treatment to reduce tau pathology. A clear example is the methylene blue (MB) treatment, a drug that inhibits tau aggregation. MB therapeutic treatment (after the onset of cognitive impairment) failed to avert or recover learning and memory deficits in Tau^{ΔK} and TauRD^{ΔK}. In contrast, preventive MB application starting before onset of functional deficits preserved cognition in Tau^{ΔK}. Moreover, preventive MB application starting at young age showed a greater impact on the cognitive performance, reflecting the importance of the treatment design in order to assess the drug efficacy (Hochgrafe et al., 2015).

The adenosine A₁ receptor is a G_i/G_o-protein coupled receptor that inhibits adenylate cyclase and reduces cAMP levels (Dunwiddie and Masino, 2001). Rolofylline treatment could produce an increase in the levels of cAMP inducing the activation protein kinase A (Abel and Nguyen, 2008), which in turn promotes the

activation of the ubiquitin-proteasome system (Chiang et al., 2009; Zhang et al., 2007) that plays a central role in the clearance of unfolded proteins (Glickman and Ciechanover, 2002). Although these pathways need to be further investigated, they could be playing a role in the reduction of aberrant phosphorylated and misfolded tau observed in the Tau^{ΔK} mice. Consistent with this, several studies revealed that drugs promoting increased cAMP levels have beneficial effect in different neurodegenerative diseases, e.g. AD, Huntington's and Parkinson's disease which are associated with tauopathy (Anglada-Huguet et al., 2016; Congdon and Sigurdsson, 2018; Delhay and Bardoni, 2021). This makes drugs targeting adenosine A₁ receptors promising for the treatment/prevention of neurodegeneration, independently of drugs targeting other adenosine receptors such as A_{2A} receptors (also alleviating tau pathology, (Laurent et al., 2016)).

Conclusions

There are currently no drugs to reverse AD in humans, and only few that slow down disease progression with limited efficacy. By contrast, on the level of experimental mouse models of AD-like tauopathy it is possible not only to slow down but also to halt or reverse symptoms such as cognitive decline. Examples are the switch-off of mutant tau expression (in case of regulatable gene expression (Eckermann et al., 2007; Santacruz et al., 2005), tau aggregation inhibitors (e.g. methylene blue or derivatives that halt progression, (Hochgrafe et al., 2015; Wischik et al., 2014), or compounds that affect tau only indirectly (e.g. adenosine A₁ or A_{2A} receptor antagonists, (Dennissen et al., 2016; Laurent et al., 2016). In the latter cases the signalling pathways affecting tau are not well understood; however, known tau-interactors point to presynaptic or mitochondrial components (McInnes et al., 2018; Tracy et al., 2022). As shown here for the case of A₁ antagonists, the curative potential holds for different proaggregant mutations of Tau and for different variants of tau, as long as the level of tau remains below a critical limit.

Limitations

This report has some limitations. Although rolofylline induced activation of adenosine A₁ receptor rescues the memory impairment, the exact mechanism and its relation to Tau pathology need further investigation.

Abbreviations

Tau 2N4R largest tau isoform in human CNS, 441 residues

[¹⁸F]CPFPX (18)F-8-cyclopentyl-3-(3-fluoropropyl)-1-propylxanthine

rolofylline KW-3902, 1,3-dipropyl-8-(3-noradamantyl)xanthine, 8-(Hexahydro-2,5-methanopentalen-3a(1H)-yl)-3,7-dihydro-1,3-dipropyl-1H-purine-2,6-dione

VOI Volume of interest

Tau^{ΔK} full length tau (2N4R) with mutation ΔK280

TauRD^{ΔK} Tau repeat domain (4R) with mutation ΔK280

AD Alzheimer's disease

FTLD Frontotemporal lobar degeneration

CMRglu Cerebral metabolic rate of glucose

CaMKIIα Ca²⁺/Calmodulin-dependent protein kinase type II

PHF paired-helical- filament

GFAP glial fibrillary acidic protein

Iba 1 ionized calcium-binding adapter molecule 1

DAB 3,3'-diaminobenzidine

NeuN neuronal nuclear protein

WT wild-type

MB methylene blue

MWM Morris water maze

NORT novel object recognition test

PET positron emission tomography

PSD-95 post-synaptic density protein-95

CA1 hippocampal area Cornu Ammonis 1

LCtrl littermate controls

Declarations

Ethics approval and consent to participate

All animal experiments were carried out in accordance with the guidelines of the German Welfare Act and approved by the local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen).

Consent for publication

Not applicable

Availability of data and materials

All relevant data are within the paper and its supporting information files. Additional datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

The acquisition of funding and project administration was provided by EM, and EMM. MAH, HE, EM and EMM designed the study. MAH and HE performed most of the study. SR, AS, RH and SK involved in immunohistochemistry and biochemistry studies. BN, AD and HE involved in PET tracers experiments. MAH, HE, EM and EMM wrote the manuscript. All the authors read and approved the final manuscript.

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Authors' information

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Figures

Figure 1

PET imaging: Blocking of $[^{18}\text{F}]\text{CPFPX}$ uptake with rolofylline in brains of WT mice. Animals were injected intravenously with 10 mg/kg rolofylline together with 10 MBq $[^{18}\text{F}]\text{CPFPX}$, a tracer that binds specifically to adenosine A1 receptors.

- (A+C)** Average images of [^{18}F]CPFPX SUVR_{bg} in horizontal and sagittal view, projected onto an MRI template in the absence of rolofylline (A) or injected with [^{18}F]CPFPX + 10 mg/kg rolofylline (C).
- (B)** Significant differences on the voxel level between the two measurements, analyzed by paired t-test corrected for multiple testing ($p < 0.05$).
- (D)** Box plots of VOI values. $*p < 0.05$ when comparing to unblocked [^{18}F]CPFPX uptake.
- (E)** Analysis of the [^{18}F]CPFPX SUVR_{bg} in different brain areas. Results are expressed as a mean $\pm$ SD ($n = 6/\text{group}$). Abbreviations: Cer: cerebellum; MB: midbrain, WB: whole brain.

Figure 2

Rolofylline decreased hippocampal tau pathology in Tau^{ΔK} transgenic mice. Treatment with rolofylline was started at ~15 months, ~3 mo after onset of cognitive decline. At age ~17.5 months (after 2.5 months of treatment), brain tissue was obtained for Western blotting and histological analysis. For all the figures, data are expressed as a mean ± SEM.

(A) Gallyas silver staining of CA1 hippocampal region (upper panels) and Alz50 antibody staining (lower panels). Note the reduction in both pathological markers after rolofylline treatment. Scale bar: 20µm.

(B) Protein levels of phosphorylated human tau (antibody 12E8; pSer262/pSer356) in the hippocampus of rolofylline-treated Tau^{ΔK} mice were significantly lower compared to the untreated group (n=6–8 per group; Student's t-test; p=0.0204).

(C) Example of Western blotting with identified bands of human tau (hTau), murine tau (mTau) and loading control β-actin.

(D) Protein levels of PSD-95 in the hippocampus of untreated Tau^{ΔK} mice were significantly lower when compared to LCtrl (one-way ANOVA, F(3,21)=3.23, p=0.0429; Dunnett's post-hoc p=0.0202; n = 6–7/group).

(E) High magnification of CA1 apical dendritic spines from TauRD^{ΔK} and LCtrl mice in the presence or absence of rolofylline treatment. Scale bar: 5µm.

(F) Quantification of **(E)**. Rolofylline treatment recovers the number of spines in transgenic Tau^{ΔK} animals. There was a significant difference between LCtrl and Tau^{ΔK} mice (n=2–4 per group; F(3,8)=5.09, p=0.0293; Tukey's post-hoc p=0.0254), but not between LCtrl and Tau^{ΔK} Rol mice (p=0.9991). The comparison Tau^{ΔK} versus Tau^{ΔK} Rol revealed a statistic tendency (p=0.0843).

Figure 3

Rolofylline decreased cognitive deficits in $\text{TauRD}^{\Delta K}$ mice. Fifteen months-old animals expressing $\text{TauRD}^{\Delta K}$ and their littermate controls were treated with rolofylline for 2.5 months, ~6 mo after onset of cognitive decline. Data are expressed as a percentage mean $\pm$ SEM.

(A) Time line of the behavior paradigms used during the treatment.

(B) Rolofylline treatment prevented the decrease in body weight observed in animals expressing mutant TauRD^{ΔK}. Two-way ANOVA (n=9–13 per group) revealed significant main effects of the factors "time" (F(14,546)=11.79, p<0.0001) and "treatment" (F(3,39)=3.27, p=0.0314), as well as a significant factor interaction (F(42,546)=2.34, p<0.0001). The latter indicates that body weight developed differently within groups. Body weight of LCtrl mice remained constant throughout the observation period. In contrast, body weight of TauRD^{ΔK} mice was significantly different from LCtrl from days 6 onwards (Tukey's post-hoc; *p<0.05) and to rolofylline-treated LCtrls from day 10 onwards (Tukey's post-hoc; #p<0.05). Rolofylline-treated TauRD^{ΔK} Rol mice no statistical difference compared to control animals at any time point analyzed.

(C) After 20 days of rolofylline treatment, TauRD^{ΔK} mice show improved burrowing behavior (F(3,36)=4.87, p=0.0061; n=8–12 per group). There was a significant difference between LCtrl and TauRD^{ΔK} (Tukey's post-hoc p=0.0125; *) and between TauRD^{ΔK} and TauRD^{ΔK} Rol (p=0.0471; \$). LCtrl and TauRD^{ΔK} Rol were not significantly different with respect to burrowing (p=0.8368).

(D) Rolofylline treatment also improved performance in the fear conditioning test. There was a significant difference between contextual (no sound) and cue-induced (sound) freezing for LCtrl and LCtrl Rol (F(1,76)=19.62, p<0.0001, Sidak's post-hoc p<0.05, black *), but not for TauRD^{ΔK} mice (p=0.7525). Rolofylline-treated TauRD^{ΔK} Rol mice showed a statistic tendency (p=0.0905, grey *). TauRD^{ΔK} mice showed more contextual freezing (no sound) than the other groups, which was significant compared to the LCtrl Rol group (F(3,76)=3.99, p=0.0108, Sidak's post-hoc p=0.0138; #).

(E) Rolofylline treatment improved learning speed of TauRD^{ΔK} mice in the Morris water maze test (Two-way ANOVA F(3,39)=3.56, p=0.0227 for factor "treatment", n=9–13 per group). TauRD^{ΔK} mice needed more time to decrease the latency to escape with a significant difference between LCtrl and TauRD^{ΔK} on learning day 3 (Dunnett's post-hoc p=0.0084, *). In contrast, there was no significant difference between LCtrl and TauRD^{ΔK} Rol (p>0.28 on all days tested).

(F) The loss in brain weight in TauRD^{ΔK} animals was not reversed by rolofylline treatment (F(3,36)=7.38, p=0.0006, n=8–12 per group). Both TauRD^{ΔK} and TauRD^{ΔK} Rol mice had significantly lighter brains compared to LCtrl (Dunnett's post-hoc p=0.0006 *** and 0.0022 **, respectively).

(G) Protein levels of PSD-95 in the hippocampus of untreated TauRD^{ΔK} mice were significantly lower when compared to LCtrl (one-way ANOVA, F(3,14)=4, p=0.0285; Tukey's post-hoc p=0.0263, *; n = 4–5/group), meanwhile no differences are observed between LCtrl and rolofylline-treated TauRD^{ΔK} (p=0.8637). There was no difference between transgenic animals, although PSD-95 had a clear trend to increase after rolofylline treatment (p=0.0831).

(H) Representative western blot of PSD-95.

Figure 4

Rolofylline does not improve behavior in rTg4510 mice when administered after cognitive impairment (~5 to 7 months of age): Behavioral assessment. Control and rTg4510 animals were treated with food containing 5 mg/kg of rolofylline during 2 months at ~5 month of age. After 3 weeks of treatment several behavior assessments were performed. For all the experiments results are expressed as a mean $\pm$ SEM (n = 9–14/group).

(A) Time line of the behavior paradigms used during the treatment.

(B) Body weight was monitored from 2 to 5.5 months of age in rTg4510 mice without rolofylline treatment (blue curve). It was consistently reduced compared to littermate controls (black curve). 2-way ANOVA revealed a significant main effect of the factor "genotype" ($F(1,16)=11.12$; $p=0.00042$), and Sidak post-hoc comparison showed a significant difference between rTg4510 mice and controls at timepoints 4.5–5.5 months of age ($p<0.05$, *).

(C) Body weight was monitored every 3-4 days during 50 days of rolofylline treatment (from ~5 to 7 months of age). Rolofylline did not reverse the lower body weight observed in the rTg4510 animals. Two-way ANOVA revealed a significant main effect of factor "treatment" ($F(3, 432)=17.83$; $p<0.0001$), "time" ($F(14,588)=18.01$; $p<0.0001$) and a significant interaction of factors "time" and "treatment" ($F(42,588)=5.517$; $p<0.0001$) which suggests a different development of body weight between groups. When comparing the mean body weight between groups Tukey's post-hoc test showed a significant difference between LCtrl and rTg4510 ($p<0.05$, *) and rTg4510 Rol throughout ($p<0.05$, \$\$), while rTg4510 Rol and rTg4510 were only significantly different ($p=0.7898$). Body weight of LCtrl Rol was significantly different from LCtrl ($p<0.05$; #).

(E) Nesting test with rolofylline or without rolofylline treatment for rTg4510 mice and controls, scored after 2, 6, and 12 h after introduction of the nestlet. Transgenic animals show a worse performance (main effect of factor "treatment" in 2-way ANOVA with $F(3, 170)=46.58$; $p<0.0001$; Tukey's post-hoc $p<0.01$ at 2h,*; and , $p<0.001$ at 6 and 12h,**; for comparison with LCtrl). Nesting becomes even more impaired by rolofylline treatment, leading to a significant difference between rTg4510 and rTg4510 Rol after 12 h (Tukey's post-hoc $p=0.0284$, \$). In contrast, treatment has no effect on controls ($p>0.44$ at all timepoints).

(F) Burrowing test: % food pellets burrowed overnight. rTg4510 animals show reduced burrowing behavior which was not improved by rolofylline treatment ($F(3, 43)=11.84$; $p<0.0001$; Tukey's post-hoc test showed significant differences between LCtrl and rTg4510 [$p=0.0254$, *] as well as between LCtrl and rTg4510 Rol [$p=0.0002$, ***], while there was no significant difference between rTg4510 and rTg4510 Rol [$p=0.5054$]. **(G)** Open field test: Graph showing the covered distance (in cm) in the Open Field arena during 10 min for 2 consecutive days. Transgenic mice and littermate controls show similar behavior. Rolofylline treatment has no effect on controls but promotes hyperactive behavior in tau transgenics. Day 1: $F(3, 42)=15.47$; $p<0.0001$; Tukey's post-hoc $p<0.0001$ for rTg4510 Rol versus all other groups (***). Day 2: $F(3, 42)=6.26$; $p=0.0013$; Tukey's post-hoc $p<0.01$ for rTg4510 Rol versus all other groups (**).

(H) Y-maze test: The percentage of novel arm exploration time 4 h after the training session of all groups is shown. In the one-sample T-test control and control rolofylline animals had a increased preference for the new arm ($p < 0.0001$ **** and $p=0.0192$ *, respectively), which was not statistically significant for the transgenic animals ($p=4.227$ non-treated rTg4510 mice and $p=0.2684$ for rolofylline-treated rTg4510 mice).

Figure 5

Rolofylline does not improve pathologically phosphorylated and aggregated tau in rTg4510 mice administered from 5 to 7 months of age: Histology. Histological analysis of conformationally changed and phosphorylated tau in the hippocampus (CA1) of rTg4510 mice after ~2 months of rolofylline treatment. In all cases there was no significant improvement with rolofylline treatment.

(A) Phosphorylated tau PHF1 antibody (dual phosphorylation epitope Ser396+Ser404), (B) pathological tau conformation antibody MC1 (epitope 5-15 + 312-322), (C) Gallyas silver staining of tau aggregates. Scale bar: 50 μ m.

Figure 6

Early (preventive) treatment with rolofylline does not protect against cognitive impairment in rTg4510 mice. Transgenic and control animals were treated with food containing 5 mg/kg of rolofylline at 2.5-3 months of age (at the beginning of the typical onset of tau pathology) for 1.5 months. After 3 weeks of treatment several behavior assessments were performed. For all the experiments, results are shown a mean $\pm$ SEM (n = 7-16/group).

(A) Time line of the behavior paradigms used during the treatment.

(B) Transgenic animals present reduced brain weight, which is not affected by adenosine A1 antagonist treatment ($F(3,33)=34.36$, $p<0.0001$, $n=7-12$ per group). Both rTg4510 and rTg4510 Rol had lighter brains

compared to LCtrl (Dunnett's post-hoc $p < 0.0001$ for both comparisons, ****).

(C) Nesting test with rolofylline or without rolofylline treatment for rTg4510 mice and controls, scored after 2, 6, and 12 h after introduction of the nestlet. Transgenic animals show a worse performance at 2h and 6h (main effect of factor "treatment" in 2-way ANOVA with $F(3, 141) = 44.4$; $p < 0.0001$; Tukey's post-hoc $p < 0.0001$ at 2h and 6h, ****; for comparison with LCtrl). At 12h non-treated transgenics were still performing significantly worse than LCtrl ($p = 0.0019$, **), rolofylline-treated rTg4510 mice had a clear trend ($p = 0.0569$). No significant differences were observed in transgenic animals after rolofylline treatment (Tukey's post-hoc $p > 0.47$ at all points) and treatment has also no effect on controls ($p > 0.43$ at all timepoints).

(D) Morris water maze test shows the time (in seconds) mice take to find the hidden platform during the learning phase. Transgenic animals show an increase of latency to reach the platform during the learning trials with no improvement after rolofylline treatment ($F(3,47) = 37.12$ for factor "treatment", with Dunnett's post-hoc $p < 0.0001$ for the comparisons rTg4510 vs. LCtrl and rTg4510 Rol vs. LCtrl at days 2–5, ****). There was no significant difference between rTg4510 and rTg4510 Rol at any time.

(E) Percentage of time spend in the target quadrant (where the hidden platform was) during the Morris water maze long-term probe trial (LTPT), performed 72 hours after the last training day. In the one-sample T-test all groups explore significantly more the platform quadrant ($p = 0.0001$ for LCtrl, ***; $p = 0.0012$ for LCtrl Rol, **; $p = 0.0138$ for rTg4510, *; and $p = 0.0003$ for rTg4510 Rol, ***). When comparing % of exploration time between groups, One-way ANOVA showed no main effect of treatment ($F(3,47) = 2.24$, $p = 0.0964$).

(F) Open field test: Graph showing the mean percentage of time animals spend in the center of the arena during 10 minutes. Rolofylline-treated rTg4510 mice present increase anxiety shown by the reduced time spend in the center of the arena ($F(3,47) = 5.38$, $p = 0.0029$, Dunnett's post-hoc $p = 0.0023$ for the comparison rTg4510 Rol vs. LCtrl, **).

Supplementary Files

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