Supplementary Material

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Title:
Cognitive defects are reversible in inducible mice expressing pro-aggregant full-length human Tau

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Running title:
Recovery of Tau-induced memory defects

Supplemental Material and Methods

Generation of pro- and anti-aggregant hTau40 transgenic mice

Transgenic mice expressing either pro- or anti-aggregant human full-length Tau (hTau40, 441 amino acids) were generated as described previously [3]. In the pro-aggregant form of hTau40 lysine 280 was deleted (hTau40/ΔK280), anti-aggregant hTau40 carried the ΔK280 deletion and two additional proline point mutations (hTau40/ΔK280/I277P/I308P). A plasmid containing the Tet-operon-responsive element (TetO), the bidirectional cytomegalovirus promoter (P_{tet}bi-1), the firefly luciferase reporter gene and either the pro- or anti-aggregant hTau40 was constructed to generate responder mice. Responder mice were crossbred with the CaMKIIα-tTA transactivator strain [6] to obtain inducible (Tet-OFF system) double-transgenic mice with constitutive expression of luciferase and human Tau mutants. All bigenic offspring were heterozygous and had an identical C57BL/6 genetic background. Non-transgenic littermates were used as controls. The transgene expression in bigenic mice was measured in vivo by bioluminescence imaging of luciferase activity. For switch-OFF experiments pro- and anti-aggregant hTau40 mice received doxycycline-containing food pellets (200mg/kg) for 0.5, 1, 2 or 4 months. In addition doxycycline was applied to a group of control mice to exclude side effects of the antibiotic on behavioral performance and electrophysiological recordings. All animal procedures were approved by the German Animal Welfare Act.

In vivo bioluminescence imaging of luciferase activity

In vivo bioluminescence imaging was performed using an Ivis Spectrum imaging system (Caliper Life Science). Fifteen minutes prior to imaging, mice received an intraperitoneal injection of 150mg/kg D-luciferin (Caliper Life Science) dissolved in sterile PBS. Mice were anesthetized using 2% isoflurane (Abbott) vaporized in a constant O_2 with a flow rate of 2l/min. Anesthesia was maintained during the whole imaging session. Mice were placed into the heated, light-tight imaging chamber of the Ivis Spectrum and images were collected using a highly sensitive charged coupled device (CCD) camera. Images were analyzed using Living Image 4.0 software (Caliper Life Science). The bioluminescence emission was normalized and the surface radiance was displayed in photons per second per centimeter squared per steradian (photons/s/cm^2/sr). For quantification of bioluminescence signals, a region of interest (ROI) was defined to convert surface radiance (photons/s/cm^2/sr) into total flux of the bioluminescent source (photons/s).

Preparation of brain homogenates

To estimate total Tau levels and synaptic marker proteins, fresh or snap-frozen brain tissue was homogenized in 8 volumes of lysis buffer [50mM Tris-HCl, pH 7.4, 10% glycerol, 1% NP-40, 5mM DTT, 1mM EGTA, 20mM NaF, 1mM Na_3VO_4, 150mM NaCl, protease inhibitors (Complete Mini; Roche), 5mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 100U/ml benzonase, 5µM okadaic acid]. The total protein concentration of homogenates was measured by bicinchoninic acid assay (Sigma) and adjusted to 1mg/ml.

Extraction of sarcosyl-insoluble Tau

The sarcosyl-insoluble fraction of Tau was isolated from brain tissue as described [5]. Briefly, brain tissue was homogenized in 3 volumes of cold buffer H (10mM Tris, pH 7.4, 0,8M NaCl, 1mM EGTA, 10% sucrose, 1mM phenylmethylsulfonyl fluoride, protease inhibitors) and centrifuged at 27,000 x g for 20min at 4°C. The supernatant was collected and the residual pellet again resuspended in 3 volumes of buffer H followed by another centrifugation at 27,000 x g for 20min at 4°C. The supernatants from both centrifugation steps were combined and adjusted to 1% N-lauroylsarcosine and 1% β-mercaptoethanol and incubated at 37°C for 60-90min with shaking. After centrifugation at 150,000 x g for 35min at 20°C, the supernatant was collected. The resulting pellet was washed with 100µl 1xTBS to remove any remaining soluble Tau, centrifuged again and finally resuspended in 50mM Tris-HCl, pH 7.4 using 0,5µl of buffer for each milligram of the initial brain tissue. Soluble Tau present in the supernatant and sarcosyl-insoluble Tau in the pellet were analyzed by western blotting.

Immunoblot analysis

Proteins were separated by SDS-PAGE (10% polyacrylamide gels) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). After blotting, membranes were blocked in 5% non-fat milk in TBS/0.1%Tween for 1h at room temperature. Membranes were incubated with primary antibodies at 4°C overnight, followed by 1h incubation with secondary antibodies at room temperature. 2-5µg of total protein were loaded for the detection with pan-Tau antibody K9JA (1:20000, Dako), the human Tau specific antibody TauY9 (1:2000, Biosource), phospho-Tau antibodies 12E8 (pSer262/pSer356, 1:500, Elan), PHF-1 (pSer396/pSer404, 1:500, Dr. P. Davies), AT180 (pThr231/pSer235, 1:500, Pierce) and AT8 (pSer202/pThr205, 1:500, Thermo Scientific) and antibodies against synaptic

proteins: synaptophysin (1:20000, Sigma), PSD95 (1:2000, Dianova) and GluR1 (1:1000, Millipore). Blots were normalized by the concentration of actin (1:20000, Sigma), developed using the ECL Plus detection system (GE Healthcare) and analyzed by densitometry (LAS 3000, AIDA software, Raytest).

Histology

Mouse brains were fixed for 24h in 4% formalin and dehydrated by incubation in a concentration series of ethanol and chloroform. The tissue was embedded in a paraffin/paraplast mixture and 5µm coronal brain sections were prepared. Sections were rehydrated in xylene substitute (Fluka) followed by a descending series of ethanol. Endogenous peroxidase activity was blocked using 0.6% H_2O_2. Antigen retrieval was performed in citrate buffer (10mM sodium citrate tribasic, 0.05% Tween 20, pH 6.0) at 95°C for 5min. Section were rinsed in 1x TBS-T (50mM Tris, 0.1% Triton X-100, pH 7.4) and unspecific antibody epitopes were blocked with 10% horse serum in 1x TBS-T at room temperature (RT) for 1h. Primary antibodies were diluted in 1x TBS-T containing 1% horse serum. The following antibodies were used: TauY9 (1:1000, Biosource), 12E8 (pSer262/pSer356, 1:500, Elan), MC-1 (epitope 5-15 + 312-322, 1:10) and PHF-1 (pSer396/pSer404, 1:50, both gifts from Dr. P. Davies, Albert Einstein College, NY), AT180 (pThr231/pSer235, 1:1000, Pierce), NeuN (1:1000, Millipore). Secondary antibodies as well as the avidin-biotinylated peroxidase complex were provided by the Vectastain Universal Elite ABC kit (Vector laboratories) and used as described by the manufacturer. The liquid DAB + substrate chromogen system (Dako) was used to visualize the antibody labeling. The stained sections were dehydrated in a series increasing ethanol concentration and xylole substitute (Fluka) and mounted with HistoKitt^{TM} (Roth).

For immunofluorescent stainings, 400 µm thick horizontal hippocampal slices were fixed overnight in 4% PFA/0.5% glutaraldehyde at 4°C. Slices were cryoprotected in 20% sucrose/10% glycerine for 24h and 25µm cryo-sections were prepared. Sections were rinsed in PBS and blocked in 10% normal goat serum (NGS)/0.1% Triton X-100 in PBS at RT for 30min. Sections were incubated overnight at 4°C with the primary antibody anti-synaptophysin (Sigma), diluted 1:200 in 10% NGS/ PBS. After washing in PBS, the secondary antibody goat-anti mouse-Cy2 (Jackson Immunoresearch) was applied for two hours at RT. Finally, sections were rinsed in PBS and ddH_2O, dried at 50°C for 10min and mounted using Entellan (Merck). Sections were analyzed using a Zeiss laser scanning confocal microscope (LSM 500 META). Photomicrographs were taken with constant laser intensity and the pixel intensity over the region of interest was measured using ImageJ (NIH). Mean pixel intensities of different groups (n = 3 mice/group) were compared by students T-test.

Golgi staining and quantification of spines

For Golgi-Cox impregnations of neurons [4] the FD rapid GolgiStain^{TM}kit (FD NeuroTechnologies) was used according to the manufacturer´s protocol. Briefly, brains were rinsed in distilled water and stored in Solution A/B (1:1) for 2 weeks in the dark at room temperature. The tissue was then transferred into Solution C and kept for 1 week in the dark at 4°C. Brains were rinsed in PBS and 150µm thick sections were prepared using a vibratome (Leica VT1200S). The sections were transferred to gelatine-coated microscope slides (Thermo Scientific), mounted with Solution C and allowed to dry overnight. The sections were washed in distilled water, incubated for 10min in Solution D/E/H_2O (1:1:2), washed again in distilled water, dehydrated in an ascending series of ethanol and xylole substitute (Fluka) and mounted with HistoKitt^{TM} (Roth).

Golgi-impregnated pyramidal neurons of the CA1 and CA3 layer of the hippocampus were used for quantification of dendritic spines as described before [8]. Apical CA1 dendrites originating from the cell soma were classified as primary and dendrites which branched off the primary dendrite were defined as secondary. For quantification of spines z-stacks of secondary, apical dendrites of the CA1 layer, which were at least 100µm apart from the soma, were taken at a high resolution using an Olympus laser scanning microscope FV1000. For each mouse (n = 3 per group) 10-13 neurons and 1-2 dendrites per neuron of 20-30µm lengths were analyzed using ImageJ software (National Institute of Health). Results were analyzed using Graph Pad Prism 5.0 software (Graph Pad). One-way analysis of variances (ANOVA) followed by Bonferroni´s post-hoc was applied when appropriate. The null hypothesis was rejected on the basis of $P < 0.05$. Results are given as spines/µm ± standard error.

Behavioral and memory tasks

Neuromotor tests

Grip strength was tested using a T-shaped bar, connected to a digital dynamometer (UgoBasile). Mice were placed on the apparatus so that they grabbed the bar spontaneously, and gently pulled backwards until they released the bar. Maximal strength (mN) was recorded ten times per animal. Motor coordination and equilibrium were tested on an accelerating rotarod (MED Associates Inc.). Mice were placed on the rod with their body axis perpendicular to the rotation axis. Their head was directed against the direction of the rotation such that the animal had to progress forward to maintain

its balance. Mice were first trained on a constant speed (4rpm, 2min), before starting with four test trials (intertrial interval, 10min). During these trials, mice had to balance on the rotating rod that accelerated from 4 to 40rpm within 5min. The latency until the mice fell from the rotating rod was recorded, up to a maximum of 5min. To measure cage activity, mice were put individually in 27×21cm² transparent cages that were placed between three photo beam sensors. Beam crossings (representing ambulatory cage activity) were registered for each 30min interval, during a 23h recording period, using an interfaced PC counter.

Learning and memory tasks
Morris water maze
Spatial memory abilities were examined in the standard hidden-platform acquisition and retention version of the Morris water maze [7]. A 150cm circular pool was filled with water opacified with non-toxic white paint, and kept at 26°C as previously described [2]. A 15cm round platform was hidden 1cm beneath the surface of the water at a fixed position. Four positions around the edge of the tank were arbitrarily designated 1, 2, 3, and 4; thus dividing the tank into four quadrants: target, adjacent 1, opposite, and adjacent 2. Each mouse was given four swimming trials per day (10min intertrial interval) for five consecutive days. The start position was pseudo-randomized across trials. Mice that failed to find the submerged platform within 2min were guided to the platform, where they remained for 15s before being returned to their cages. The time required to locate the hidden escape platform (escape latency), the distance travelled (path length), and swimming speed (velocity) were determined. Two days after the acquisition phase ended, a probe trial was conducted, during which the platform was removed and the search pattern of the mice was recorded for 100s. During acquisition and probe trials the Ethovision video tracking system was used to record and analyze behavior (Noldus Information Technology). Statistical comparison between groups and control littermates were accomplished by two-way repeated ANOVA (one factor repetition) followed by all pairwise multiple comparison procedures (Fisher LSD method). For analysis of probe trials one way ANOVA was performed.

Step-through passive avoidance task
Single-trial passive avoidance learning was examined in a step-through box with a small illuminated compartment and a larger dark compartment with a grid-floor. The grid-floor was connected with a constant current shocker (MED Associates Inc.). Animals were dark adapted for 30min, and then placed in the small illuminated compartment. After 5s, the sliding door to the dark compartment was opened and the entry latency was recorded. When the mouse entered the dark compartment with four paws on the grid, the door was closed and a slight foot shock (0.3 mA, 1s) was delivered. Retention was tested 24h later according to the same procedure, excepting that the animals did not receive a shock. The entry latency was recorded with a cut-off of 300s. For statistics one way ANOVA followed by all pairwise multiple comparison procedures (Fisher LSD method) was performed.

Electrophysiology
CA1 Schaffer collateral recordings
Preparation of hippocampal slices, extracellular electrophysiological recordings and analysis were carried out as previously described [9]. CA1 recordings of pro- and anti-aggregant mice were performed subsequently after finishing behavioral tests at the age of 16 months (16 months ON or 12 months ON + 4 months OFF).

CA3 mossy fiber recordings
Slice preparation
Horizontal hippocampal slices (400µm) were prepared from 12 months old mice (12 months ON or 8 months ON + 4 months OFF) of either sex. Using a vibratome (Leica VT1200S), slices were cut in ice cold, oxygenated artificial cerebrospinal fluid (ACSF). To obtain optimal mossy fiber preservation a cutting-angle of 12° in the fronto-occipital direction was used [1]. Subsequently brain slices were transferred to an interface chamber where they were superfused with carbogenated ACSF (36 ± 0.5°C; flow rate, 1.8ml/min; pH 7.4). ACSF contained 126mM NaCl, 21mM $NaHCO_3$, 3mM KCl, 2mM $CaCl_2$, 1.8mM $MgSO_4$, 1.25mM NaH_2PO_4 and 10mM glucose (saturated with 95% O_2/5% CO_2).

Recordings
After 1.5h recovery time, slices were transferred to a submerged recording chamber at 30°C. Extracellular field potentials were recorded from *stratum lucidum* in area CA3. As stimulation electrode a patch-clamp pipette was used (1.5-2MΩ) to excite *mossy fibers* (mf) in the region of the *hilus*. *Stratum lucidum* mossy fibers were visually identified by infrared (IR)-difference interference contrast (DIC) microscopy. Stimulus- and recording electrodes (2.5MΩ) were both filled with ACSF and separated constantly 200µm from each other. Field excitatory postsynaptic potentials (fEPSP) were filtered at 1kHz, sampled at 10kHz and 10x preamplified (custom made preamplifier) using a HEKA double patch-clamp EPC 10 USB amplifier (HEKA Elektronik Dr. Schulze GmbH).

Stimulation protocols
Constant current pulses were elicited every 20s (0.05Hz) with a pulse width of 0.1ms. Input output (I/O) curves were generated using stimulus intensities ranging from 10-100µA with an increment of 10µA. In the same slice paired pulse facilitation (PPF) was evoked by a 50ms inter stimulus interval (ISI). In a next step frequency facilitation (ff) of the mossy fiber pathway was measured by switching stimulation frequency from 0.1Hz to 1Hz. Only slices which showed robust ff were used further on for mf-LTP recordings. LTP was induced by theta burst stimulation (TBS) at 30-40% of the maximum stimulus intensity as revealed initially by I/O recording. TBS consisted of a series of 10 bursts at 5Hz with 5 pulses per burst at 100Hz, delivered four times with 5s intervals. At the end of each recording 5µM DCGIV (Tocris Biosciences) was applied for at least 5min, if the fEPSP slope was not reduced for at least 50% slices were excluded from mf analysis.

Statistics
Statistical comparison of means in I/O, ff and LTP recordings between Tau-transgenic groups and control littermates were accomplished by repeated measure ANOVA. In LTP measurements the last 10min of recording time were compared. Paired pulse ratios and the last 10min of LTP recording of pro-aggregant on and anti-aggregant on animals were compared by student T-test.

Supplemental Figure Legends

Fig. S1 Pathological Tau conformation and phosphorylation at the microtubule binding domain of Tau are most prominent in pro-aggregant ON mice
Pro-aggregant and anti-aggregant ON mice (16 months ON) are compared to pro- and anti-aggregant ON/OFF (12 months ON + 4 months OFF) and controls (16 months). (a) MC1 immunoreactivity (epitope 5-15 + 312-322) indicates a pathological Tau conformation. Pro-ON mice show the strongest MC1 immunoreactivity with missorting of Tau to cell bodies (arrows) of CA1 (a4) and CA3 (a5) pyramidal neurons as well as to somata (arrows) and apical dendrites (arrowheads) of cortical somatosensory neurons (SSCx, a6). In comparison to pro-ON, MC1 immunoreactivity in CA1, CA3 and SSCx of pro-ON/OFF (a7-a9), anti-ON (a10-a12), anti-ON/OFF (a13-a15) or control (a1-a3) mice is clearly diminished. (b) Phosphorylation at the 12E8 epitope inside the KXGS motif indicates detachment of Tau from microtubules. Pro-ON mice show prominent 12E8 immunoreactivity in CA1 (b4), CA3 (b5), SSCx (b6) and *stratum lucidum* (b5, star) with phosphorylated Tau missorted to cell soma (arrows) and apical dendrites (arrowheads). In anti-ON mice only a few cell bodies stain positive for 12E8 (b10-b12, arrows), whereas phosphorylation at the KXGS motifs of Tau is obviously reduced to control levels (b1-b3) in pro-ON/OFF (b7-b9) as well as in anti-ON/OFF mice (b13-b15). Scale bar 50µm.

Fig. S2 Late and dual phosphorylation epitopes of Tau (PHF-1, AT180) analyzed on pro- and anti-aggregant mice
Pro-aggregant and anti-aggregant ON mice (16 months ON) are compared to pro- and anti-aggregant ON/OFF (12 months ON + 4 months OFF) and controls (16 months). (a) A strong immunoreactivity of Tau phosphorylated at residues Ser396 and Ser404 (PHF-1 epitope) is detected in cell somata (arrows) of CA1 (a4) and CA3 (a5) pyramidal neurons and in cortical somatosensory neurons (SSCx, a6) as well as in *stratum lucidum* (a5, star) of pro-ON mice. No PHF-1 positive cell bodies are observed in pro-ON/OFF (a7-a9), anti-ON (a10-a12), anti-ON/OFF (a13-a15) or control mice (a1-a3). (b) Staining of phosphorylated Tau using the AT180 antibody (dual phosphorylation epitope pThr231 + pSer235). Pro-ON mice show a massive mislocalization of phosphorylated Tau to cell somatas (arrows) and apical dendrites (arrowheads) of CA1 (b4), CA3 (b5) and SSCx neurons (b6), whereas the extent of phosphorylated, missorted Tau is markedly decreased in anti-ON mice (b10-b12). Notably, neurons of pro-ON/OFF (b7-b9) appear negative for AT180, similar to control (b1-b3) and anti-ON/OFF mice (b13-b15). Scale bar 50µm.

Fig. S3 Sarcosyl-extraction of cortex tissue to analyze soluble and insoluble Tau of pro- and anti-aggregant hTau40 mice
(a) Sarcosyl-soluble fraction analyzed by the pan-Tau antibody K9JA. The upper band represents soluble hTau40 in pro-ON and anti-ON mice (16 months ON), which is depleted in pro-ON/OFF and anti-ON/OFF mice (12 months ON + 4 months OFF). No hTau40 is detected in controls (16 months). The lower band shows soluble endogenous mouse Tau (mTau), which is present in all genotypes and not affected by doxycycline. β-actin serves as loading control. (b) Sarcosyl-insoluble fraction (antibody K9JA). Insoluble hTau40 and mTau is clearly detected in pro-ON mice. After 4 months OFF, insoluble hTau40 is disappeared, but insoluble mTau still persists in pro-ON/OFF mice. No insoluble Tau is found anti-ON, anti-ON/OFF and control mice.

Fig. S4 Short term plasticity of the Schaffer collateral pathway
Short term plasticity of the Schaffer collateral pathway is studied by paired pulse ratio (PPR) analysis with an inter stimulus interval (ISI) of 50ms. No significant differences in PPR are observed between pro-ON (16 months ON, n = 7), pro-ON/OFF (12 months ON + 4 months OFF, n = 13), anti-ON (16 months ON, n = 7), anti-ON/OFF (12 months ON + 4 months OFF, n = 10) and controls (16 months, n = 10). Bars represent mean ± SEM, two-tailed Student´s t-test.

Fig. S5 Group II mGluR dependency of mossy fiber long term potentiation (mf-LTP)
Example recording of theta burst (TBS) induced LTP in the mossy fiber-CA3 synapse demonstrating the group II mGluR dependency of the potentiation by application of DCG IV. LTP is strongly impaired in pro-ON mice at 12 months of hTau40 expression (a2) relative to controls (a1).

Supplemental References

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