SUPPLEMENTARY MATERIAL

Potentiating tangle formation reduces acute toxicity of soluble Tau species in the rat.

BRAIN

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I. SUPPLEMENTARY MATERIAL & METHODS

1. Animal experiments

Adult male Wistar rats (2 months old, 250 g; JANVIER, St Berthevin, France, n=124) were used for AAV transduction. Rats were housed in a temperature-controlled room maintained on a 12 hour light/dark cycle. Food and water were available ad libitum. All animal studies were conducted according to the French regulation (EU Directive 86/609 – French Act Rural Code R 214-87 to 131). The animal facility was approved by veterinarian inspectors (authorization n°B 92-032-02) and complies with Standards for Humane Care and Use of Laboratory Animals of the Office of Laboratory Animal Welfare (OLAW – n°#A5826-01). All procedures received approval from the local ethical committee (Comité d’Ethique en Expérimentation Animale CEA) and the French Ministry of Research (2015063015326177_v1 (APAFIS#985)).

Four groups of rats were included in all analyses. Two human 1N4R Tau constructs were cloned: WT (hTAUWT) and pro-aggregation Tau (hTAUProAggr). A control group termed “utGFP” was designed to express a GFP transgene where all ATG initiating codons were mutated. This resulted in the production of a GFP mRNA that would then not be translated into a protein. This construct was used in our study to overcome the described toxicity of GFP protein expression in some AAV-mediated models (Klein et al., 2006). An additional control group was injected with saline (“PBS”).

2. AAV production

Viral particles were produced by transient co-transfection of HEK-293T cells with an adenovirus helper plasmid (pXX6-80), an AAV packaging plasmid carrying the rep2 and cap8 genes, and the AAV2 transfer vector containing the above-mentioned expression cassettes. Seventy-two hours following transfection, virions were purified and concentrated from cell lysate and supernatant by ultracentrifugation on a iodixaniol density gradient followed by dialysis against PBSMK (0.5 mM MgCl₂ and 1.25 mM KCl in PBS). Concentration of the vector stocks was estimated by quantitative PCR according to the method described by (Aurnhammer et al., 2012) and expressed as viral genomes per ml of concentrated stocks (vg/ml).

3. HEK cells transfections with vectors plasmids

For in vitro validation of Tau constructs, HEK-293T cells were transfected with the different plasmids. For this purpose, 750,000 cells were plated on 6 wells plaques. 200 µl transfection mix, containing 5 µg of plasmids, 50 µl of CaCl₂ and 100 µl of HBS, was then prepared and left to precipitate for 15-20 min. The precipitate was then deposed onto cells and left to incubate for 4-5 h at 37° C. The culture medium containing the transfection mix was then removed and fresh culture medium was added onto cells. Cells were then left to incubate at
37° C for 36-48 h before lysis in a buffer containing 137 mM NaCl, 20 mM Tris pH 8, 1% NP-40, 10% glycerol, 2 mM EDTA and protease inhibitors (Complete, Roche, Basle, Switzerland). Lysed cells were then centrifugated at 13,000g for 30 min at 4° C and BCA dosage of proteins was performed on the supernanant before processing of the samples for western blot analysis.

4. Evaluation of Tau expression on infected HEK-293T cells by western blot analysis and quantitative RT-PCR

HEK-293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO2/air atmosphere. HEK-293T cells were plated at a density of 200’000 cells per well in 24-wells plate. One day later, cells were infected with AAV particles (M.O.I. of 5x10e6 VG/cell, n=4 wells/condition) expressing utGFP, hTauWT or hTauProAggr. Seventy-two hours after infection, cells were either processed for western-blot analysis or quantitative RT-PCR. For biochemical analysis, cells were sonicated in Tris-NP40 buffer (Tris pH7.6 25 mM, NaCl 150 mM, NP40 1%, Glycerol 10%, EDTA 2 mM) before centrifugation at 13,000 x g for 15 min. Proteins from supernatant were quantified using bicinchoninic acid assay (Pierce BCA protein assay kit, ThermoFisher).

Samples were denaturated at +95° C for 5 min and 10µg of each was loaded onto a 10% SDS-PAGE gel and transferred onto a 0.2 µm nitrocellulose membrane. The membrane was incubated in a blocking solution containing 5% bovine serum albumine in Tris buffer saline (TBS), 0.1% Tween-20 for 1 h at room temperature. It was then transferred to the antibody solution (HT7, 1/5000 and actin, 1/10000, diluted in the blocking solution for incubation overnight at +4° C. The signal was then amplified by incubating the membrane 1 h with the appropriate secondary antibody (Goat anti- rabbit-IRDye680RD et Goat anti- mouse-IRDye800CW, 1/1000, LI-COR Biosciences, Bad Homburg, Germany) diluted in the blocking solution. The fluorescent signal was then visualized using Odyssey CLx Imager (Li-cor Biosciences, Bad Homburg, Germany) and analysis performed using Image Studio software (Li-cor Biosciences). For quantitative RT-PCR, cells were lysed in 1 ml trizol. Total RNA (including miRNAs) isolated using miRNeasy mini kit (Qiagen, Hilden, Germany), following manufacturer’s instructions.

5. Stereotaxic injections of AAV vectors

Prior to stereotaxic surgery, anesthesia was first induced by placing the rats in an induction chamber with 4% isoflurane (Iso-Vet, Coumon d’Auvergne, France) until unconscious. Rats were then anesthetized using a mixture of ketamine (Imalgène 1000, Merial, Lyon, France; 75mg/kg) and medetomidine hydrochloride (Domitor, Vetoquinol, Lure, France; 0.5mg/kg), administered intraperitoneally. Lidocaine (Xylovet, Ceva Santé Animale, Libourne, France; 7mg/kg) was administered subcutaneously under the scalp skin 5 minutes before the beginning of surgery. To prevent corneal desiccation, ophthalmic ointment was regularly
placed on the eyes. During the surgery, normal body temperature was monitored and maintained by using temperature-controlled electric heat pads.

AAV vectors were injected bilaterally into the dorsal and ventral HC using a 34-gauge blunt-tip needle linked to a Hamilton syringe (Hamilton, Reno, NV, USA) by a polyethylene catheter at the following stereotaxic coordinates (Paxinos and Watson, 1998): dorsal HC -4.3 mm rostral to bregma, 3 mm lateral to midline and -2.8 mm from the skull surface, ventral HC -5.6 mm rostral to bregma, 5 mm lateral to midline and -7 mm from the skull surface. The tooth bar was set at -3.3 mm. For each injection site, 4 µl containing 2.5 x 10^{10} vg of each vector was delivered using a microdialysis pump (Stoelting, Wood Dale, IL, USA) set at 0.25 µl/min. The injection needle was left in place for an additional 5 min.

At the end of the surgical procedure, the scalp skin was closed using Michel wound clips. Anesthesia was then reversed with a subcutaneous injection of atipamezole (Antisedan, Vetoquinol, Lure, France; 0.5 mg/kg). Rats were rehydrated with a subcutaneous injection of warm saline (10ml/kg) and left for 1h in a ventilated heating box (28°C) until they woke up and fully recovered from anesthesia. Post-surgery analgesia was ensured by the administration of paracetamol (Pracetam, Sogeval, Laval, France; 1.6 mg/ml) in drinking water for 48 hours.

6. Gallyas silver impregnation

Sections were washed 3 times in sterile 0.1 M PBS, mounted on slides and left for drying overnight prior to Gallyas staining. Sections were then permeabilized by incubation into toluene followed by decreasing ethanol concentrations (100%, 90% and 70%). Slides were then transferred into a 0.25% potassium permanganate solution (Sigma) for 15 min, incubated 2 min in 2% oxalic acid (Sigma) followed by a 60 min incubation into a solution containing 4 g/l lanthanum nitrate (VWR, Fontenay-sous-Bois, France), 20 g/l sodium acetate (Sigma), and 3% H_{2}O_{2}. Slides were then rinsed 3 times in distilled water before incubation for 2 min in a solution containing 0.035% AgNO_{3} (Sigma), 0.04 g/ml of NaOH and 0.1 g/ml KI (Sigma). Reaction was stopped by rinsing sections in 0.5% acetic acid and development performed by incubation for 20 min in a solution containing 2 g/l NH_{4}NO_{3} (Sigma), 2 g/l AgNO_{3} (Sigma), 10 g/l tungstosilicic acid (Sigma), 0.28% formaldehyde and 50 g/l Na_{2}CO_{3} (Fisher Scientific, Illkirch, France). Sections were then washed again in 0.5% acetic acid, incubated 20 min in 1% gold chloride (Sigma) before rinsing in distilled water. Fixation of the staining was then performed by washing sections 3 times in 1% sodium thiosulfate (Sigma). Sections were then dehydrated in increasing ethanol concentrations and xylene before mounting using Eukitt (Dutscher, Brumath, France).

7. Image analysis

Images of HT7/P2A and AT100/P2A colabellings were acquired using a confocal microscope (SPM, Leica, Wetzlar, Germany) using the 40 x objective and a zoom of two. Z-stack images of AT100 immunostaining were acquired at 20 x (10 µm, 1 µm steps) using an axioscan (Zeiss, Oberkochen, Germany). 40 x z-stacks (1 µm steps) images of NeuN staining were
acquired on a spinning-disk confocal microscope (BX51WI, Olympus, Tokyo, Japan). Point-counting/Cavaliere estimation of volume was then performed on those images at each stereological counting site. Briefly, a grid (100 µm side) was sur-imposed onto the section and each object hitting an intersection was counted. Estimation of volume was then performed using the following formula:

\[ V_{CA1/2\,pyramidal\,cells} = A_p \times m \times t \times \sum P_i \]

Where \( V \) is the estimated volume, \( A_p \) is the area of the grid, \( m \) the section sampling, \( t \) the measured thickness and \( P \) the points counted on grid.

In addition, estimation of whole hippocampal volume was performed on 5x mosaic images of NeuN staining acquired with a spinning-disk confocal microscope (BX51WI, Olympus). Manual segmentation of the hippocampus was performed on those images and area of the structure determined using Stereo Investigator software (MBF Bioscience, Williston, VT, USA). Volume of the structure was then estimated using the following formula:

\[ V = \sum (A \times n \times T) \]

Where \( V \) is the estimated volume, \( A \) is the hippocampal area segmented on each section, \( n \) corresponds to the section sampling and \( T \) the section thickness.

8. Real-time quantitative PCR (RT-qPCR)

One HC per rat was dissected as described for biochemical analysis (section 8). The structure was then lysed in 1 ml of trizol using Precellys 24 homogenizer (Bertin Technologies) and total RNA (including miRNAs) isolated using miRNeasy mini kit (Qiagen, Hilden, Germany), following manufacturer’s instructions. RNAs (0.125 µg) were then reverse-transcribed into cDNA using SuperScript VILO cDNA Synthesis Kit (Invitrogen, Waltham, MA, USA). Cycling parameters were +25° C for 10 min, +42° C for 1 h and +85° C for 5 min. RT-qPCR was then performed using iTaq Universal SYBR Green Supermix (Biorad, Hercules, CA, USA) and primers (Biorad or Eurofin Genomics, Les Ulis, France) specific to different targets on 1 ng of cDNA, using 3 nM of primers: human Tau forward: TGGGGGACAGGAAAGA, human Tau reverse: CCTCAGATCCGTCCCTCAGTG; hypoxanthine phosphoribosyltransferase 1 (Hprt1) forward: GGACCTCTCGAAGTGTTGGATAC, Hprt1 reverse: CCCTGAAGTGCTCATTATAGTCAAG, Peptidylprolyl isomerase A (Ppia) forward: ATGGCAAATGCTGGACCAAA, Ppia reverse: GCCTTCTTTCACCTTCCCCAAA; human GAPDH forward AAGGTGAAGGTCGGAGTCAA, human GAPDH reverse GGCAACAATATCCACTTACCAGA. Reactions were run in triplicates in 384-well PCR plates and the cDNA of four non-injected Wistar rats served as an inter-plate reference for in vivo experiments. Reactions were run in duplicates for in vitro experiments. PCR cycling parameters were as follows: +95° C for 3 min, 40 cycles at +95° C for 10 s, and +60° C for 30 s. Dissociation temperature mounted 0.5° C/min from +60° C to +95° C. Cycle threshold (Ct)
values were generated using Bio-Rad CFX manager software (regression mode). Results for each sample were then expressed using the following formula:

\[
\Delta \Delta C_t = \frac{RQ_{\text{interest}}}{\sqrt{RQ_{\text{reference}_1} \times RQ_{\text{reference}_2}}}
\]

where:

\[
RQ = (2^E)^{C_{\text{min}} - C_{\text{sample}}}
\]

and E represents the efficiency of each primer and \( C_{\text{min}} \) the minimum Ct value of all samples. Ppia and Hprt1 were used as the reference genes pair for quantification.

9. Biochemical analysis

Sample preparation

One or three months after injection, six rats per group were anesthetized with 4% isoflurane before receiving a lethal dose of pentobarbital (60 mg/ml). Brains were then extracted and briefly rinsed in 0.1 M PBS before dissection of the HC. One hemisphere served for biochemical analysis, the other one for RT-Q PCR study.

Sarkosyl extraction

One hundred mg of tissue was homogenized and sonicated in 600 μl Tris buffer 10 mM pH 7.4 containing 10% sucrose and protease inhibitors (Complete, Roche, France). 0.23% Triton were added to one aliquot of each sample. The sample was then sonicated before centrifugation at 5,000 x g for 10 min at +4° C. The supernatant was collected for ultracentrifugation at 100,000 x g for 1 h at +4° C. The pellet was resuspended in 800 μl of 10 mM Tris buffer pH 7.6, 0.32 M sucrose, 1% sarkosyl, sonicated and went through a second ultracentrifugation at 100,000 x g for 1 h at +4° C. The third supernatant containing the sarkosyl soluble fraction was diluted in 3X LDS and the pellet containing the sarkosyl insoluble fraction resuspended in 100 μl of 2X LDS.

Western blot

Samples were denatured at +100° C for 10 min and loaded onto a 4-12% Bis-Tris NuPAGE Novex gel (Invitrogen). This was followed by transfer onto a 0.45 μm nitrocellulose membrane using the Novex system from Life technology (XCell II blot module). The membrane was incubated 1 h at room temperature in a blocking solution containing 5% milk-TNT (Tris 15 mM, NaCl 140 mM, Tween 20 0.05 %) for home-made rabbit polyclonal M19G which recognizes the amino terminal region of Tau (Sautière et al., 1994-Sergeant et al., 2001) and anti-phospho Tau rabbit polyclonal anti-pSer396 (Invitrogen, 44-752G, batch FR230433); or without saturation for mouse monoclonal AT100 immunoblotting. Membrane was then transferred to the antibody solution diluted in 5% milk-TNT (M19G and anti-pSer396; 1/ 10,000) or in TNT (AT100; 1/ 1,000) for incubation overnight at +4° C.
signal was then amplified by incubating the membrane 1 h with the appropriate secondary antibody diluted in TNT (anti-rabbit 1/5000; anti-mouse 1/50 000). The chemiluminescent signal was then visualized using the ECL western blotting detection reagents (GE Healthcare). Anti-pSer396 blots were reblotted directly on AT100 blots without stripping of the membrane. Percentage of sarkosyl-insoluble material was calculated as follows: ([IS] * 100) / (IS + S), where IS and S are respectively the quantity of sarkosyl-insoluble and sarkosyl-soluble material.
## II. SUPPLEMENTARY TABLES

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---, sequence present in GFP but absent in utGFP; G, mutation of initiating codons

**Supplementary Table 1** Nucleotide sequence of the utGFP construct
hTAU<sup>ProAggr</sup> Chimeric construct allowing the co-expression of hTAU<sup>WT</sup> and the pro-aggregation peptide TauRD-ΔK280 linked by a 2A peptide (P2A) sequence

ATGGGACAGACAGCCCCCGTGCCCATGCCAGACCTGAAGAATGTCAAGTCCAAGATCGGCTCCACTGAGAACCTGAAGCACCAGCCGGGAGGCGGGAAGGTGCAGATAATTAATAAG---CTGGATCTTAGCAACGTCCAGTCCAAGTGTGGCTCAAAGGATAATATCAAACACGTCCCGGGAGGCGGCAGTGTGCAAATAGTCTACAAACCAGTTGACCTGAGCAAGGTGACCTCCAAGTGTGGCTCATTAGGCAACATCCATCATAAACCAGGAGGTGGCCAGGTGGAAGTAAAATCTGAGAAGCTTGACTTCAAGGACAGAGTCCAGTCGAAGATTGGGTCCCTGGACAATATCACCCACGTCCCTGGCGGAGGAAATAAAAAGATTGAA

GGATCCGGGCCAAGCTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCT

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AGGCACCTCCCGGCAAGCGGCGCTCCGACCCCGCTCCCTCCACCCACAAAAACGCCGCGGCTTGGGCAGAGTCAGACAGCTGCAACATGTCTCCTCCACCGGCAGCATCGACATGGTAGACTCGCCCCAGCTCGCCACGCTAGCTGACGAGGTGTCTGCCTCCCTGGC

CAAGCAGGGTTTGTGAAATG, TauRD-ΔK280; GGA, linker; GCC, P2A sequence; ATG, hTAU<sup>WT</sup>; CAG, sequence common to TauRD-ΔK280 and hTAU<sup>WT</sup>; AAG and ---, deletion of lysine 280

Supplementary Table 2 Nucleotide sequence of the hTAU<sup>ProAggr</sup> construct
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**Supplementary Table 3** Summary table of antibodies used in this study and the concentrations used for different applications. Abbreviation: NGS, normal goat serum, p, phospho-, S, serine, T, threonine, n.a., non available.
III. SUPPLEMENTARY FIGURES

Supplementary Fig.1 *In vitro and in vivo* validation of the hTAU\textsuperscript{ProAggr} construct. 

A, schematic representation of the hTAU\textsuperscript{ProAggr} construct after cleavage of the P2A sequence. Epitopes recognized by the different antibodies used are indicated. B, western blot analysis on HEK-293T cells transfected with Tau constructs confirms cleavage of the chimeric protein. HT7 antibody detects hTAU\textsuperscript{WT} (but not the pro-aggregation peptide) at the molecular weight of monomeric Tau. The epitope of the anti-P2A antibody, that remains attached to the pro-aggregation peptide (TauRD-ΔK280), is detected only in the hTAU\textsuperscript{ProAggr} construct, at the expected molecular weight. C, confocal images of HT7/P2A co-labelling in hTAU\textsuperscript{ProAggr} expressing animals 1 month post-injection shows hippocampal neurons positive for both HT7 and P2A. Partial subcellular co-localisation is observed with some processes that are stained primarily by HT7 (arrowhead) while others are only P2A positive (arrow). Scale bar: 10µm.
Supplementary Fig. 2 A, the pro-aggregation peptide TauRD-ΔK280 co-aggregates with hTAUWT in the hTAUProAggr group. Confocal images of AT100/P2A co-labelling on an animal of the hTAUProAggr group reveal colocalisation (arrowhead) of aggregated full-length Tau (AT100) and the pro-aggregation peptide TauRD-ΔK280 (P2A) into the same cell. Scale bar: 5µm. B, AT8 and AT100 also colocalise in the hTAUProAggr with the fluorescent aggregation marker Thioflavin S (ThioS, arrowheads). Scale bar: 15 µm.
Supplementary Fig. 3 No significant Tau aggregation can be observed in control groups up to 3 months post-injection. Representative images of AT100 staining detect no aggregation in PBS and utGFP animals at 1 and 3 months post-injection. Scale bars: 150 µm (left) and 50 µm (right)
**Supplementary Fig.4** No Gallyas-positive tangle-like structure can be observed in hTAU\(^{WT}\) and control groups up to 3 months post-injection. Scale bar: 150 µm
Supplementary Fig. 5 Uncropped blots of the western blot analysis presented in Fig. 5. All lanes (1), (4) and (7) in each panel correspond to total homogenates. Lanes (2), (5) and (8) are sarkosyl-soluble fractions and lanes (3), (6) and (9) sarkosyl insoluble fractions. A, B, M19G blots at 1 month (A) and 3 months (B) post-injection. Lanes (1)-(3) utGFP, lanes (4)-(6) hTAUWT and lanes (7)-(9) hTAUProAggr. C, D, AT100 blots at 1 month (C) and 3 months (D).
post-injection. C, lanes (1)-(3) hTAU$^{\text{ProAggr}}$, lanes (4)-(6) hTAU$^{\text{WT}}$, and lanes (7)-(9) utGFP. D, lanes (1)-(3) utGFP, lanes (4)-(6) hTAU$^{\text{WT}}$ and lanes (7)-(9) hTAU$^{\text{ProAggr}}$. E, F, anti-pSer396 blots at 1 month (E) and 3 months (F) post-injection. E, lanes (1)-(3) hTAU$^{\text{ProAggr}}$, lanes (4)-(6) hTAU$^{\text{WT}}$, and lanes (7)-(9) utGFP. F, lanes (1)-(3) utGFP, lanes (4)-(6) hTAU$^{\text{WT}}$ and lanes (7)-(9) hTAU$^{\text{ProAggr}}$. 
Supplemental Fig.6 Biochemical characterisation of Tau shows no difference between groups in the level of total Tau. A, Sarkosyl-soluble (black box) and sarkosyl-insoluble (red box) fractions were quantified and summed to obtain a measure of total Tau (detected with M19G antibody). B, no difference between Tau groups can be observed in the level of total Tau. Mann-Whitney U test $U = 45.0, p = 0.4235$ at 1 month post-injection and independent t-test $t(14) = 0.00, p = 1$. 
Supplemental Fig. 7 Differences in the toxicity of Tau constructs are observed consistently with other HC morphological parameters. A, cavalieri estimation of whole hippocampal volume (One-way ANOVA for each time-point, F(2,24) = 13.91, p < 0.001 and F(2,27) = 4.49, p < 0.05 respectively, Bonferroni post-hoc test) and, B, point-counting Cavalieri estimation of the volume occupied by CA1/2 pyramidal cells (One-way ANOVA for each time-point, F(2,22) = 10.66, p < 0.001 and F(2,21) = 4.71, p < 0.05 for 1 and 3 months respectively, Bonferroni post-hoc test) confirm the toxicity of hTAUWT as well as the neuroprotective effect of hTAUProAggr. Stars above each bar represent the result of post-hoc comparison to controls. *, p < 0.05, ***, p < 0.001. C, the volume occupied by CA1/2 pyramidal cells is negatively correlated to the extent of Tau hyperphosphorylation at 1 and 3 months post-injection (Pearson correlation test, R² = 0.2704, p = 0.067 and R² = 0.4624, p < 0.05 at 1 and 3 months post-injection respectively). D, the total hippocampal volume is inversely correlated to the amount of total Tau in the CSF (Pearson correlation test, R² = 0.6416, p < 0.01).