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NEURONAL DEGENERATION, SYNAPTIC DEFECTS, AND BEHAVIORAL ABNORMALITIES IN TAU₄₅₋₂₃₀ TRANSGENIC MICE

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Abstract

The complement of mechanisms underlying tau pathology in neurodegenerative disorders has yet to be elucidated. Among these mechanisms, abnormal tau phosphorylation has received the most attention because neurofibrillary tangles present in Alzheimer's disease (AD) and related disorders known as tauopathies are composed of hyperphosphorylated forms of this microtubule-associated protein. More recently, we showed that calpain-mediated cleavage leading to the generation of the 17 kDa tau₄₅₋₂₃₀ fragment is a conserved mechanism in these diseases. To obtain insights into the role of this fragment in neurodegeneration, we generated transgenic mice that express tau₄₅₋₂₃₀ and characterized their phenotype. Our results showed a significant increase in cell death in the hippocampal pyramidal cell layer of transgenic tau₄₅₋₂₃₀ mice when compared to wild type controls. In addition, significant synapse loss was detected as early as six months after birth in transgenic hippocampal neurons. These synaptic changes were accompanied by alterations in the expression of the N-methyl-D-aspartate glutamate (NMDA) receptor subunits. Furthermore, functional abnormalities were detected in the transgenic mice using Morris Water Maze and fear conditioning tests. These results suggest that the accumulation of tau₄₅₋₂₃₀ is responsible, at least in part, for neuronal degeneration and some behavioral changes in AD and other tauopathies. Collectively, these data provide the first direct evidence of the toxic effects of a tau fragment biologically produced in the context of these diseases in vertebrate neurons that develop *in situ*.

Keywords

Calpain; tau cleavage; cell death; synapse loss; NMDA receptors; neurite degeneration

The microtubule-associated protein (MAP) tau plays an important role during neuronal development by stabilizing the microtubule network in growing axons (Drubin and Kirschner, 1986; Ferreira et al., 1989; Dreschel et al., 1992; Bramblett et al., 1993).

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Therefore, conditions that altered the levels of expression of this MAP during development led to abnormal axonal elongation (Caceres and Kosik, 1990, Knops et al., 1991, Dawson et al., 2001). Tau has also been implicated in axonal degeneration and cell death in the context of Alzheimer's disease (AD) and related disorders known as tauopathies (Kosik et al., 1986, Wood et al., 1986, Kondo et al., 1988, Rapoport et al., 2002, Yancopoulou and Spillantini, 2003, Parihar and Hemnanni, 2004, Roberson et al., 2007). The mechanisms by which tau mediates neuronal degeneration are not completely understood; however, a growing body of evidence indicates that abnormal posttranslational modifications of this MAP underlie tau pathology. Numerous studies have focused on the role of tau phosphorylation in AD because neurofibrillary tangles, pathological hallmarks of this disease, are formed mainly by hyperphosphorylated tau isoforms (Kosik et al., 1986, Wood et al., 1986, Kondo et al., 1988, Takashima et al., 1993; Ferreira et al., 1997, Alvarez et al., 1999, Ekinici et al., 1999; Parihar and Hemnanni, 2004). More recently, it has been suggested that cleavage could also underlie tau toxicity. Thus, we have shown that calpain-mediated tau cleavage is a conserved mechanism in multiple tauopathies (Ferreira and Bigio, 2011). This cleavage induces the generation of the 17 kDa tau₄₅₋₂₃₀ fragment (Park et al., 2005; Park et al., 2007, Reinecke et al., 2011). The toxic effects this tau fragment were first detected in hippocampal neurons transfected with a tau₄₅₋₂₃₀-GFP construct (Park et al., 2005). Similar effects were demonstrated in a *Drosophila* model of tauopathy (Reinecke et al., 2011).

Conflicting results regarding the identity and toxicity of this tau fragment have been recently published (Garg et al., 2011). Although these authors described the formation of a tau fragment of similar apparent molecular weight, it contained a different N-terminus as a result of the cleavage by a different calpain isoform. This fragment failed to induce neurodegeneration in cultured neurons (Garg et al., 2011).

To address this discrepancy and obtain insights into the toxic effects of the 17 kDa tau₄₅₋₂₃₀ fragment in mammalian central neurons that develop *in situ*, we generated and characterized transgenic mice that express this fragment in hippocampal neurons. Our results showed enhanced cell death of pyramidal neurons and synaptic loss in the hippocampus of transgenic tau₄₅₋₂₃₀ mice. In addition, these changes were accompanied by behavioral abnormalities. Collectively, these data indicate that indeed tau₄₅₋₂₃₀ has toxic effects that could contribute to the progressive degeneration of central neurons in AD and related disorders.

EXPERIMENTAL PROCEDURES

Generation of Tau₄₅₋₂₃₀-GFP Transgenic Mice

Transgenic mice were generated by injecting the pronucleus of a single-cell fertilized C57BL/6J mouse embryo with the tau₄₅₋₂₃₀-GFP transgene under the control of the Thy 1.2 promoter. This transgene was derived from a pGFP-N1 plasmid (Invitrogen, Grand Island, NY) containing the human cDNA coding sequence for the tau₄₅₋₂₃₀ fragment cloned into the multiple cloning site as previously described (Park and Ferreira, 2005). The 7.8 kb transgene containing all regulatory elements and the transgene coding regions (tau₄₅₋₂₃₀-GFP) was isolated from the promoter vector by enzymatic digestion and extracted from agarose gels using the Qiaquick Gel Extraction Kit (Qiagen, Germantown, MD). Transgenic founder

mice were identified by genomic polymerase chain reaction (PCR) screening for GFP and confirmed by Southern blot analysis using genomic DNA isolated from tail biopsies as previously described (Feng et al., 2000). Founders from two lines were crossed with C57BL/6J mice and the subsequent offspring were backcrossed for at least 5 generations to produce congenic tau₄₅₋₂₃₀-GFP lines. Both female and male mice from line 1 were used for the experiments described below with the exception of behavioral studies that were performed using only male mice to avoid hormone-dependent changes in behavior. Experiments assessing the effects of tau₄₅₋₂₃₀ on neuronal death and synapse loss were also performed using line 3 mice. Since no differences in the phenotype were detected when results obtained using line 1 mice were compared to those obtained using line 3 mice, we have only included data obtained with line 1 mice.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Tau₄₅₋₂₃₀-GFP expression levels were evaluated by RT-PCR. For these experiments, hippocampi from three 2 month-old wild type and homozygous transgenic tau₄₅₋₂₃₀ mice were rapidly collected as previously described (Kelly et al., 2005). RNA was extracted using the TRIzol Reagent (Sigma Chemical, St Louis, MO) per manufacturer's protocol. Briefly, hippocampi were homogenized with RNase-free Teflon pestles and glass tissue homogenizers. Total RNA was extracted with TRIzol and chloroform (0.2 ml), precipitated with 0.5 ml isopropanol, and then reverse transcribed using random hexamers. The RT-PCR reaction was carried out using the Superscript III One-Step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen, Grand Island, NY) and the following primers: 5'-TGGTGCAGATGAACTTCAGG-3' and 5'-CAAGAAGGTGGCAGTGGTC-3' for transgene analysis, 5'-AATGGAAGACCATGCTGGAG-3' and 5'-ATTCAACCCCCTCGAATTTT-3' for full-length tau control, and 5'-GCACCACACCTTCTACAATGAG-3' and 5'-ACAGAGTACTTGCGCTCAGGAG-3' for β -actin control. Forty cycles (15 sec at 94°C, 30 sec at 55°C, 45 sec at 68°C, and a final extension for 5 min at 68°C) were performed in a Thermal cycler (Applied Biosystems, Invitrogen). The final PCR products were ran in 1% agarose gels and the resulting bands were quantified by densitometric analysis using a ChemiDoc XRS system (Bio Rad Life Sciences, Hercules, CA). The relative amount of tau₄₅₋₂₃₀ RNA in the tissue was determined by the ratio of transgene to full-length tau and to β -actin.

Histology, Immunostaining, and Stereological Analysis

Wild type and homozygous transgenic tau₄₅₋₂₃₀ mice (3 to 12 month-old) were injected with an overdose of ketamine/xylazine (120/10 mg/kg) and transcardially perfused with ice-cold saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS). The brains were removed, post-fixed in the same fixative overnight, and then cryopreserved in increasing concentrations of sucrose (10–30%) in PBS. Brains were sectioned in the sagittal plane on a freezing-stage microtome at 25 μ m and serial sections were collected in 0.1 M phosphate buffer. Free-floating sections were blocked for 1 h in 10% normal goat serum in PBS and then incubated overnight at 4°C with the following primary antibodies: anti-Class III β -tubulin (clone TuJ1, 1: 1,000, R&D Systems, Minneapolis, MN); anti-tubulin (clone DM1A, 1:5,000, Sigma); anti-GFP (1:500, Millipore, Temecula CA); and anti-synaptophysin (1:250, Santa Cruz Biotechnology, Dallas TX). After 3 $\times$ 15 min washes

in PBS, the sections were blocked for 1 h in 10% normal goat serum in PBS and then incubated with secondary antibodies for 2 hrs at room temperature. For fluorescence microscopy experiments, sections were incubated with AlexaFluor anti-mouse or anti-rabbit IgG antibodies (1:200; Molecular Probes, Eugene, OR). For light microscopy experiments, sections were exposed to biotin-conjugated rabbit anti-mouse IgG (Sigma), washed in PBS (3 × 15 min each wash), and then incubated with peroxidase-conjugated Extravidin (Sigma) (1 h at room temperature for each incubation). Finally, sections were reacted with a substrate solution containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.075% hydrogen peroxide (v/v) in 50 mM Tris, pH 7.6. Sections were then rinsed in deionized water to stop the developing reaction, dehydrated with graded ethanol solutions, cleared in xylene, and finally mounted on gelatin-coated glass slides with Permount (Thermo Fisher Scientific, Pittsburgh, PA). Images of the hippocampal region were captured at high resolution (4000 × 4000 pixels) using a Photometrics Cool Snap HQ2 camera coupled to a fluorescent inverted microscope (Nikon Diaphot, Melville, NY). Images were analyzed using MetaMorph Image Analysis software (Universal Imaging Corporation, Fryer Company Inc.).

The area occupied by the pyramidal cell layer was determined in six to eight sequential sections, at equal intervals, prepared from wild type and transgenic tau₄₅₋₂₃₀ mice using MetaMorph Image Analysis software. To assess neuronal loss and apoptotic cell death using the *In Situ* Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN), sections prepared as described above were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 2 min and TMR fluorescein-labeled nucleotide was incorporated at 3'-OH DNA ends using the enzyme Terminal deoxynucleotidyl transferase (TdT). The sections were counterstained using the Class III β -tubulin antibody as described above. The total number of neurons and the number of TUNEL (+) neurons were manually counted in the pyramidal cell layer of at least six sections per animal, age group (3–12 month-old), and genotype. Five mice per experimental condition were used for this study. The results were expressed as the number of total and TUNEL (+) cells in the pyramidal cell layer of the hippocampal region/field in images of 4000 × 4000 pixels.

Electrophoresis and Immunoblotting

Hippocampi obtained from wild type and homozygous transgenic tau₄₅₋₂₃₀ mice (3 to 12 month-old) were homogenized in 2X Laemmli buffer and boiled for 10 min. Whole cell extracts were also prepared from 1 to 21 days in culture hippocampal neurons prepared from wild type and homozygous transgenic tau₄₅₋₂₃₀ mice. Lysates were loaded and run on sodium dodecyl sulfate (SDS)-poly-acrylamide gels as previously described (Laemmli, 1970). The proteins were transferred onto Immobilon membranes (Millipore, Billerica, MA) and immunoblotted (Towbin et al, 1979). Immunodetection was performed using anti- α -tubulin (clone DM1A; 1:200,000; Sigma), anti-synaptophysin (p38 1:1,000; Santa Cruz Biotechnology), anti-NR1 and NR2A (1:50; Santa Cruz Biotechnology), anti-NR2B (1:50; BD Biosciences, San Jose, CA), anti-Class III β -tubulin (clone TuJ1, 1:1,000; R&B Systems), anti-GFP (1:1,000; Millipore), and anti-integrin β_1 (clone M-106, 1:100 Santa Cruz Biotechnology) antibodies. Secondary antibodies conjugated to horseradish peroxidase (1:1,000; Promega, Madison, WI) were used followed by enhanced chemiluminescence for

the detection of proteins (Yakunin and Hallenbeck, 1998). The ChemiDoc XRS system and Quantity One Software (Bio-Rad) were used to image and analyze immunoreactive bands.

Preparation of Membrane-Enriched Protein Fractions

Membrane-enriched protein fractions were obtained as previously described (Dunah et al. 2000, Simón et al. 2009). Briefly, frozen hippocampi dissected from 9 month-old wild type and transgenic tau₄₅₋₂₃₀ mice were homogenized in ice-cold Tris-ethylenediaminetetraacetic acid (EDTA) buffer (10 mM Tris-HCl and 5 mM EDTA, pH 7.4) containing 320 mM sucrose, a cocktail of protease inhibitors (Roche, Nutley, NJ), and phosphatase inhibitors (0.1 mM Na₃VO₄ and 1 mM NaF). The homogenates were centrifuged at 700 × g for 10 min, the supernatant was then removed and centrifuged at 37,000 × g at 4°C for 40 min, and the pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.4) containing the protease and phosphatase inhibitors. For Western blot analysis, the samples were diluted 1:10 in 10% sodium deoxycholate in 500 mM Tris-HCl buffer, pH 9.0, and incubated at 36°C for 30 min. Samples were then diluted 1:10 with 500 mM Tris-HCl, pH 9, and 1% Triton X-100. After centrifuging at 37,000 × g at 4°C for 10 min, equal volume of 2X Laemmli Buffer was added to the supernatant. The samples were then boiled for 10 min and stored at -20°C. The protein concentration was determined by the method of Lowry et al. (1951) as modified by Bensadoun and Weinstein (1976). Electrophoresis and quantitative Western blot analysis were performed as described above.

Preparation and Immunostaining of Primary Hippocampal Cultures

Embryonic day 16 (E16) pregnant wild type and transgenic tau₄₅₋₂₃₀ mice were euthanized by CO₂ overdose. Embryos were removed and their hippocampi were dissected and freed of meninges. The cells were dissociated using trypsin (0.25%) for 15 min at 37°C followed by trituration with a fire-polished Pasteur pipette. The cell suspension was plated in minimum essential medium (MEM) with 10% horse serum (MEM10) on poly-L-lysine-coated dishes at 800,000 cells per 60-mm dish. After 4 h, the medium was changed to glia-conditioned MEM containing 0.1% ovalbumin, 0.1 mM sodium pyruvate, and N2 supplements (N2 medium, Bottenstein and Sato, 1979). For immunocytochemical analysis, neurons were plated (150,000 cells per 60-mm dish) onto poly-L-lysine-coated coverslips in MEM10. After 4 h, the coverslips were transferred to dishes containing an astroglial monolayer and maintained in N2 medium. Hippocampal neurons cultured on coverslips were fixed in 4% paraformaldehyde in PBS containing 0.12 mM sucrose for 15 min and permeabilized in 0.3% Triton X-100 in PBS for 4 min. Coverslips were then incubated in 10% bovine serum albumin (BSA) in PBS at room temperature for 1 h before overnight incubation with the primary antibody. The following antibodies were used: anti-tubulin (clone DM1A; 1:1,000; Sigma), and anti-tau₄₅₋₂₃₀ (1:100). Anti-mouse and anti-rabbit AlexaFluor secondary antibodies (1:200; Molecular Probes) were used for protein detection. To assess the specificity of the tau₄₅₋₂₃₀ immunoreactivity, sister cultures were stained omitting the corresponding primary antibody.

The number of synapses per neuron was determined by labeling 21 days in culture hippocampal neurons with anti-synaptophysin antibody (p38; 1:500, Santa Cruz Biotechnology) as described above. The number of synapses per cell was calculated by

counting the synaptophysin immunoreactive puncta using MetaMorph Image Analysis software and dividing these values by the total number of neurons in the field. For synaptic marker localization studies, 21 days in culture hippocampal neurons were fixed with methanol for 15 min at -20°C and incubated with BSA for 1 h at room temperature prior to their incubation with anti-synaptophysin (p38 1:500, Santa Cruz Biotechnology), anti-NR1 (1:50, Santa Cruz Biotechnology), and anti-NR2B (1:50; Biosciences). Biotin-conjugated anti-goat (1:50; Invitrogen, Carlsbad, CA) or anti-mouse (1:50; Chemicon, Temecula, CA) secondary antibodies followed by fluorescein-Avidin (1:50; Vector Labs, Burlingame, CA) incubation were used to detect NMDA receptor subunits. Images were taken using a Photometrics Cool Snap HQ2 camera coupled to a fluorescent microscope (Nikon Diaphot). Images were analyzed using MetaMorph Image Analysis software (Universal Imaging Corporation, Fryer Company Inc.). For these experiments, neurons obtained from three independent culture preparations per genotype were analyzed.

Production of Tau₄₅₋₂₃₀ Antibodies

Polyclonal antibodies against tau₄₅₋₂₃₀ were produced by BioSources International (Hopkinton, MA). The following KLH-conjugated peptides were used for immunization of rabbits: KESPLQTC (amino acids 45–50) and CKKVAVVR (amino acids 224–230). The Spanning peptides TDAGLKESPLQTC (amino acids 39–50) and CKKVAVVRTPPKS (amino acids 224–235) were used to negatively absorb the serum to remove antibodies that recognize the uncleaved forms. Polyclonal sera were affinity purified and concentrated to at least 1 mg/ml. The specificity of the antibody was assessed by means of Western blot analysis and immunocytochemistry. For Western blot analysis, whole cell extracts were obtained from 21 days in culture hippocampal neurons incubated in the absence or presence of 20 μM preaggregated β -amyloid as previously described (Park and Ferreira, 2005). To aggregate the peptide, synthetic A β_{1-40} (American Peptide, Sunnyvale, CA) was dissolved in N2 medium to a concentration of 0.5 mg/ml and incubated for 3 days at 37°C (Park and Ferreira, 2005). For immunocytochemistry experiments, hippocampal neurons incubated in the presence or absence of aggregated A β were double stained with tubulin and the tau₄₅₋₂₃₀ antibodies as described above.

Morris Water Maze Test

Spatial memory was assessed by the Morris water maze assay as previously described (Watase et al., 2002). Five male homozygous transgenic tau₄₅₋₂₃₀ and wild type mice were tested at seven months of age. Two paradigms (visible and hidden platform tests) were used for conditioning (training) mice to locate an escape platform in a circular pool (148 cm in diameter) of water at 24.5°C . During the first week, each mouse was given eight trials of visible platform per day for four consecutive days in two blocks of four sequential trials for each mouse with at least a minute inter-trial-interval (ITI) between trials. The visible platform was located in the same place for the duration of the first block of trials. In the second block, the platform was moved to another quadrant to ensure that the mice were not pursuing a preferred location or possible cue. During the second week, each mouse was given eight trials (two blocks of four trials) of hidden platform per day for four consecutive days. The hidden platform was submerged (0.5 cm under water line) in water mixed with cloudy, white, non-toxic liquid paint to ensure the platform was invisible to the mouse. For

these trials, the time taken to locate the platform (escape latency), the percent of time the mouse spent in the target quadrant, the swimming speed, and the distance traveled were determined. On the last day, each animal was given a “probe” trial one hour after trial 32. For this test, the platform was removed, and each animal was allowed 1 min to search for a platform in the pool. The amount of time that each animal spent in each quadrant was recorded (quadrant search time) and the number of times a subject animal crossed the exact target location of the platform during training was determined and compared with crossings of the equivalent location in each of the other quadrants (platform crossing). The previously described parameters were recorded using the WaterMaze™ Software (Actimetrics, Inc. Wilmette, IL 60091).

Fear Conditioning Test

Five male homozygous transgenic tau₄₅₋₂₃₀ and wild type mice (seven month old) were tested for their performance in a conditioned fear paradigm as previously described (Watase et al., 2002). Briefly, Coulborn shockers were calibrated to 0.7 mA intensity, 2 sec duration, sound pulse-hiss at 75–76 dB, frequency of 5 Hz, 5 millise (rise time), and 50% duty cycle using a RadioShack digital sound meter prior to acclimation (1 h in the original home cages) and assay of mice in the isolation testing chambers. Each fear apparatus was clean-wiped with 70% ethanol, set up in the respective enclosure cabinet, and calibrated for sound, shock, and camera recording focus within a yellow light environment where Venetian blinds were down on each side. The research technician was dressed in yellow smock, facemask, and blue gloves. The Training Conditioning Assay had a 726 sec total time duration, 180 sec baseline, followed by three CS (hiss-tone)-US (unconditioned stimulus) onsets with ITI between each CS-US onset per trial. Each squad (4 mice per 4 chambers) was tested in a round-robin style. Freezing behavior (%) was recorded as an indicator toward fear. For the original context assay, mice acclimated for one hour and then were placed in respective chambers for 180 sec. Mice were tested with original gowning and testing conditions as Day 1 but without any stimulus (sound/shock) or movement and freezing behavior was recorded using the Actimetrics FreezeFrame Software. A one-hour ITI was allowed prior to the next set of testing while changing chambers to a novel context. This novel environment included a large (~3' × ~2') transparent cage within a grey open field box and red lights within enclosure chamber. The research technician was dressed with an orange shirt, yellow gloves, and red-taped facemask. For this trial, the Venetian blinds were up on one side. Each mouse followed round-robin style testing with four mice per squad and one mouse per test chamber. The testing was completed with the following assay design: 180 sec baseline, 60 sec Hiss-Tone cue (75–76 dB, 5 Hz), and 180 sec post Hiss-Tone to end the 391 sec duration assay. Freezing behavior was recorded using the Actimetrics FreezeFrame Software.

Statistical Analysis

All experiments performed in this study were conducted with at least five mice per time point and genotype in addition to three independent cultures per experimental condition. The compiled data were analyzed using either a Student's *t* test (when comparing only 2 experimental conditions) or two-way ANOVA followed by Fisher's LSD *post hoc* test (when comparing more than 2 experimental conditions). The values within the graphs

represent the mean $\pm$ standard error of the mean (S.E.M.). The statistical significance is indicated within the graphs.

RESULTS

Generation of Transgenic Tau₄₅₋₂₃₀ Mice

We have previously shown that the expression of the 17 kDa tau₄₅₋₂₃₀ fragment in otherwise healthy cultured hippocampal neurons led to degeneration and cell death (Park and Ferreira, 2005). Similar results were obtained in a tauopathy model system in *Drosophila* (Reinecke et al., 2011). To test whether the expression of this fragment could also induce neurodegeneration in vertebrate hippocampal neurons that develop *in situ*, we generated strains of transgenic mice carrying tau₄₅₋₂₃₀-GFP cDNA under the transcription control of the Thy 1.2 promoter on a C57BL/6J background (Figure 1A). This promoter has been extensively used in the generation of transgenic models of AD because it drives the expression of different transgenes mainly to areas affected by the AD process in human brains (i.e. hippocampal region and to a lesser extent neocortex) (Spires and Hyman, 2005). Genotyping by PCR and southern blotting were used in order to identify the transgene positive founder mice. Two transgenic mouse strains exhibiting similar expression levels of the transgene were generated and expanded (Figure 1B). Transgenic tau₄₅₋₂₃₀ mice had both normal posture and corporal weight and were indistinguishable from wild type mice throughout the entire period analyzed (0–12 months after birth). To evaluate the brain expression of this fragment in transgenic tau₄₅₋₂₃₀-GFP mice, we performed immunocytochemistry, RT-PCR, and Western blot analysis. Two month-old homozygous tau₄₅₋₂₃₀-GFP and wild type mice were fixed and their brains were sectioned. A weak GFP signal was detected in unlabeled sections obtained from the transgenic mice. To enhance this signal, sections prepared from tau₄₅₋₂₃₀-GFP mice and wild type controls were labeled with an anti-GFP antibody. As expected, no GFP immunofluorescence was detected in the hippocampus or throughout different brain regions of wild type brain sections (Figure 1C). On the other hand, the pyramidal cell layer of the hippocampus was GFP-positive in transgenic tau₄₅₋₂₃₀ mice (Figure 1D). High-magnification images showed intense GFP immunoreactivity in the cytoplasmic area of the cell body of these neurons. In contrast, no staining was detected in their nuclei (Figure 1F). Faint GFP immunostaining was also detected in the processes elongated by pyramidal neurons (Figure 1E). GFP immunofluorescence was mainly limited to this brain region although GFP-positive neurons were seldom detected in the cortex, cerebellum, or midbrain (Figure 1G). Next, we determined the expression of the tau₄₅₋₂₃₀ fragment and full-length tau in the hippocampus of transgenic mice in both lines (line 1 and line 3) by RT-PCR. Tau₄₅₋₂₃₀ mRNA was easily detectable in both transgenic lines but was absent from the hippocampus of wild type controls (Figure 1B). As expected, full-length tau was detected in wild type mice as well as in transgenic tau₄₅₋₂₃₀ line 1 and line 3 mice (Figure 1B). No statistically significant differences in the expression of tau₄₅₋₂₃₀ or full-length tau were detected when these lines were compared (Figure 1B). We also attempted to quantify tau₄₅₋₂₃₀-GFP expression by means of Western blot analysis using GFP and tau₄₅₋₂₃₀ antibodies. Tau₄₅₋₂₃₀-GFP levels were below the detection limit of this method in whole hippocampal extracts, most likely due to its restricted expression to the pyramidal cell layer (Figure 1D).

Signs of Degeneration Were Detected in the Hippocampus of Transgenic Tau₄₅₋₂₃₀ Mice

Numerous studies have described brain atrophy, enhanced neuronal death, and synapse loss in brain areas affected by the AD process. To assess whether the expression of tau₄₅₋₂₃₀ induces any of these signs associated with neuronal degeneration, we first determined the wet weight of the hippocampus of transgenic mice. No differences were detected in the hippocampal weight when transgenic tau₄₅₋₂₃₀ mice (3 to 12 month-old) were compared to wild type ones (data not shown). We then determined both the area occupied by the pyramidal cell layer and neuronal loss in this layer of the hippocampus. For these experiments, sequential sections were immunostained with Class III β -tubulin, a neuron specific isoform, and the area (pixels) occupied by the pyramidal cell layer of the hippocampal region was determined in images of 4000 $\times$ 4000 pixels obtained at 20 X. No significant differences in the area occupied by pyramidal neurons were detected when transgenic tau₄₅₋₂₃₀ mice were compared to wild type controls (data not shown). To evaluate cell death, we quantified the number of pyramidal neurons present in this layer and assessed the presence of neurons undergoing apoptosis. For these experiments, sequential sections obtained from 3 to 12 month-old mice were labeled with the Class III β -tubulin antibody and reacted with the *In Situ* Cell Death kit reagents as described in the Experimental Procedures section. Quantitative analysis showed a significant decrease in the number of neurons in the pyramidal cell layer as early as 6 months after birth when transgenic tau₄₅₋₂₃₀ mice were compared to wild type controls. Neuronal loss was also significantly higher in 9 and 12 month old transgenic tau₄₅₋₂₃₀ mice when compared to wild type controls (Figure 2D). In wild type mice, few of the pyramidal neurons were TUNEL (+) throughout the entire period analyzed (Figure 2A). In contrast, TUNEL (+) nuclei were readily detectable in sections obtained from transgenic tau₄₅₋₂₃₀ mice (Figure 2B). Quantitative analysis showed that the number of TUNEL (+) neurons in the pyramidal cell layer of the hippocampus of transgenic mice was a significantly higher when compared to wild type controls. This enhanced number of TUNEL (+) was evident as early as three months after birth and remained significantly higher than in the wild type controls throughout the period analyzed (Figure 2C).

We then evaluated whether the enhanced cell death observed in transgenic tau₄₅₋₂₃₀ mice was accompanied by synapse loss. For these experiments, we stained sections from wild type and transgenic tau₄₅₋₂₃₀ mice ranging from 3 to 12 month-old with synaptophysin. This integral synaptic vesicle membrane protein localizes in virtually all nerve terminals where it is associated with small synaptic vesicles (Navone et al., 1986; King and Arendash, 2002). In addition, a pool of synaptophysin is concentrated in the Golgi complex of neuronal cell bodies representing either newly synthesized or recycled synaptophysin from nerve terminals (Navone et al., 1986). Numerous studies have shown that experimental conditions that induce synaptic loss are associated with a significant decrease in synaptophysin levels (Fletcher et al., 1991; Fykse et al., 1993; Qui et al., 1995; King and Arendash, 2002). Immunostaining of hippocampal sections showed faint synaptophysin immunoreactivity in the pyramidal cell layer of transgenic mice (Figure 3B & D). This pattern of synaptophysin immunoreactivity was more evident in the CA3 area when sections obtained from transgenic tau₄₅₋₂₃₀ mice were compared to those from wild type controls (Figure 3A & B). To quantify these changes in synaptophysin levels, Western blot analysis was performed using

hippocampal homogenates prepared from 3 to 12 month-old mice. Quantification of immunoreactive bands showed a significant decrease in synaptophysin levels as early as 6 months after birth when transgenic mice were compared to wild type controls. Synaptophysin levels remained low throughout the entire period studied in tau₄₅₋₂₃₀ expressing mice (Figure 3E & F).

Studies conducted in brain samples obtained from AD subjects have shown that decreased synaptophysin levels were associated with changes in the NMDA receptor subunits levels (Geddes et al., 1986, Mishizen-Eberz et al., 2004). Therefore, we determined next whether synaptic loss was accompanied by changes in the levels of NR1, the obligatory subunit, and either NR2A or NR2B subunits of the NMDA receptors in transgenic tau₄₅₋₂₃₀ mice. First, we determined the levels of these subunits in hippocampal extracts prepared from tau₄₅₋₂₃₀ transgenic and wild type mice 3, 6, 9, and 12 months after birth. Immunoreactive bands were easily detectable for all three subunits in extracts prepared from the hippocampal region of both wild type and transgenic tau₄₅₋₂₃₀ mice (Figure 4A). Densitometry of immunoreactive bands, performed using tubulin content as a standardization value, showed no statistically significant differences in the levels of NR1 throughout the period analyzed when mice from each genotype were compared. In contrast, a significant increase in NR2A and NR2B subunits was detected in 6 and 9 month-old transgenic tau₄₅₋₂₃₀ mice when compared to wild type controls (Figure 4A). In addition, the main NR2B immunoreactive band ran slightly slower indicating higher molecular weight in samples obtained from transgenic tau₄₅₋₂₃₀ mice. To further analyze these changes in NMDA receptors, we prepared membrane fractions from 9 month-old transgenic tau₄₅₋₂₃₀ and wild type controls and the levels of the subunits were determined by quantitative Western blot. To quantify the content of these subunits, equal amounts of total protein were loaded in each lane. As an additional loading control, we reacted the Immobilon membranes with an antibody against β_1 integrin a membrane protein highly expressed in hippocampal neurons (Anderson and Ferreira, 2004; Pinkstaff et al., 1999). Densitometry of immunoreactive bands showed no differences in the levels of β_1 integrin when membrane fractions obtained from transgenic tau₄₅₋₂₃₀ mice were compared to wild type controls (126% $\pm$ 13% vs. 100 %, n=3). On the other hand, a significant increase of all three subunits was detected in the membrane of tau₄₅₋₂₃₀-expressing hippocampal neurons as compared to wild type controls (Figure 4B).

In older mice transgenic mice (12 month-old), the whole cell content of NR2 subunits returned to levels comparable to (NR2A) or significantly lower than (NR2B) those of wild type controls (Figure 4A).

The Expression of Tau₄₅₋₂₃₀ Induced Progressive Degeneration in Cultured Hippocampal Neurons

We determined the extent to which the abnormalities described above in hippocampal pyramidal neurons of transgenic mice expressing the tau₄₅₋₂₃₀ fragment could be reproduced when these neurons were placed in culture. For these experiments, dissociated neurons were analyzed for up to three weeks in culture. The expression of tau₄₅₋₂₃₀ in these cells was assessed using a rabbit specific antibody against this tau fragment c-terminus recently generated and characterized in our laboratory. While commercially available antibodies

directed to epitopes located between tau amino acids 45 and 230 recognized both full-length tau and tau₄₅₋₂₃₀, our polyclonal antibody only recognized tau₄₅₋₂₃₀ when whole cell extracts obtained from hippocampal neurons cultured in the absence (control) or in presence of aggregated β amyloid (A β) were analyzed (Figure 5). We also used this antibody to stain sister cultures prepared on coverslips. As shown in Figure 5, no immunoreactivity for this fragment was detected in control hippocampal neurons (Figure 5D). In contrast, punctate tau₄₅₋₂₃₀ immunoreactivity was detected in the cell body and neurites of degenerating hippocampal neurons treated with A β (Figure 5F). To detect the presence of this fragment in cultured neurons obtained from transgenic tau₄₅₋₂₃₀ mice, coverslips were double-labeled with a monoclonal tubulin antibody and the tau₄₅₋₂₃₀ polyclonal antibody. As expected, tubulin immunoreactivity was detected in all cell compartments of hippocampal neurons throughout the whole period analyzed (Figure 6A, C, E, G, & I). On the other hand, changes in the distribution of tau₄₅₋₂₃₀ were detected as neurons matured in culture. Thus, one day after plating, homogenous tau₄₅₋₂₃₀ immunoreactivity was concentrated in the cell body of hippocampal neurons (Figure 6B). This immunoreactivity was not present when the corresponding primary antibody was omitted (Figure 6D). By seven days in culture, localized tau₄₅₋₂₃₀ immunoreactive spots were detected along the processes extended by hippocampal neurons (Figure 6F). Tau₄₅₋₂₃₀ immunoreactivity increased thereafter and a diffuse signal was detected throughout the whole extension of the neuritic tree. Initial signs of degeneration, including the formation of tortuous processes, were detected in these cells (Figure 6H). By three weeks in culture, most of the processes extended by hippocampal neurons were retracted, contained varicosities, and/or appeared fractioned (Figure 6J). It is worth noting that most of wild type neurons did not show any sign of degeneration at this stage (Figure 7A & B, see also Rapoport et al., 2002).

To assess changes in synaptic contacts, 21 days in culture hippocampal neurons prepared from wild type and transgenic tau₄₅₋₂₃₀ mice were double-labeled with synaptophysin and tubulin or NMDA receptor subunit antibodies. Synaptophysin immunoreactive puncta were easily detectable around cell bodies and along the neurite processes extended by wild type hippocampal neurons (Figure 7A). Synaptophysin immunoreactive spots were also detected in transgenic tau₄₅₋₂₃₀ mice (Figure 7C & D). However, quantitative analysis showed that the number of synaptophysin immunoreactive spots was significantly lower as compared to wild type controls (61 ± 4 vs. 154 ± 10 synapses per cell, respectively; $p < 0.01$, $n=150$ neurons/experimental condition). Most of these immunoreactive spots colocalized with NR2B immunoreactive spots in both wild type and transgenic tau₄₅₋₂₃₀ hippocampal neurons. To further quantify these differences, Western blot analysis was performed using synaptophysin, NR1, NR2A, and NR2B antibodies (Figure 7E). Densitometry of immunoreactive bands showed a significant decrease in the levels of synaptophysin, NR1, and NR2B in transgenic tau₄₅₋₂₃₀ hippocampal neurons as compared to wild type controls (Figure 7F). NR2A levels were below the detection level in hippocampal neurons obtained from both transgenic and wild type mice.

Behavioral Deficits Were Detected in Transgenic Tau₄₅₋₂₃₀ Mice

Finally, we used the Water Morris Maze (MWM) and fear conditioning tests to evaluate whether the hippocampal expression of tau₄₅₋₂₃₀ affected spatial learning and memory.

During the cued platform version of MWM, seven month-old transgenic tau₄₅₋₂₃₀ and wild type mice learned to find a visible platform using a visual strategy. Transgenic tau₄₅₋₂₃₀ mice performed similarly to wild type controls but they swam slightly faster, indicating that they have no visual or swimming deficiency (Figure 8A). To test whether transgenic tau₄₅₋₂₃₀ mice have spatial memory deficits, the mice were evaluated in the hidden-platform version of the MWM. The performance of transgenic tau₄₅₋₂₃₀ mice did not differ from wild type controls (Figure 8B). Probe trials, in which the platform was removed and mice were given a minute to explore the pool, indicated that transgenic tau₄₅₋₂₃₀ mice swam significantly longer distances although their swim speed was significantly faster than age-matched wild type controls (Figure 8C).

Transgenic tau₄₅₋₂₃₀ and wild type mice were also tested for conditioned fear as a measure of Pavlovian learning and memory. During the training period, transgenic tau₄₅₋₂₃₀ mice showed enhanced freezing behavior when compared to wild type controls. This behavioral difference was more robust during the first CS-US onset (Figure 9A). During two subsequent days after fear conditioning, we analyzed fear memories for the tone alone when presented in a novel context and in the context in which the fear was acquired. No differences were detected when tone was tested (Figure 9B). On the other hand, a significant increase in freezing observations was detected in transgenic tau₄₅₋₂₃₀ as compared to controls when context was assessed (Figure 9C).

DISCUSSION

This report provides the first direct evidence of a tau fragment (tau₄₅₋₂₃₀) generated in the context of AD and related disorders capable of inducing neuronal death and synaptic loss when expressed in mouse hippocampal neurons that develop *in situ*. In addition, the data presented herein indicate that these hippocampal changes are accompanied by functional abnormalities that could have some bearings on the cognitive and/or behavioral deficits associated with these diseases.

Previous studies have shown that tau plays an essential role in AD (reviewed by Yancopoulou and Spillantini, 2003; see also Rapoport et al., 2002; Roberson et al., 2007). However, the mechanisms by which tau mediates beta amyloid-induced neurodegeneration are not completely known. Most of the research effort in this field has been focused on tau phosphorylation and its effect on the aggregation of this MAP (reviewed by Martin et al., 2011; Takashima et al., 1993; Busciglio et al., 1995; Grynspan et al., 1997; Alvarez et al., 1999; Ferreira et al., 1997; Gong et al., 2000; Rapoport and Ferreira, 2000; Yoshida et al., 2004). Other posttranslational modifications of tau have been recently implicated in tauopathies (reviewed by Martin et al., 2011; see also references within). Among them, tau truncation or cleavage by caspases and calpains have received the most attention (Shea et al., 1996; Canu et al., 1998; Fasulo et al., 2000; Marin et al., 2000; Chung et al., 2001; Rohn et al., 2001; Su et al., 2001; Gamblin et al., 2003; Park and Ferreira, 2005; Park et al., 2007; Sinjuano et al., 2008; Liu et al., 2011). Caspase 3-mediated tau cleavage at the C-terminus (amino acid 421) results in the formation of an aggregation-prone truncated isoform (Harada and Sugimoto, 1999; Gamblin et al., 2003). In contrast, calpain 1 cleavage leads to the formation of a tau fragment in the N-terminal half of the tau molecule. We first identified

the tau₄₅₋₂₃₀ fragment in hippocampal neurons cultured in the presence of oligomeric β amyloid. Our results also showed that this fragment of 17 kDa apparent molecular weight was the result of the proteolytic action of calpain I (Park and Ferreira, 2005). Furthermore, we demonstrated that the expression of tau₄₅₋₂₃₀ in otherwise healthy CHO or cultured hippocampal neurons induced cell death and neuronal degeneration, respectively (Park and Ferreira, 2005). The identity and toxic effects of this fragment became controversial due to data showing that a tau fragment of similar molecular weight failed to induce cell death when expressed in a neuroblastoma cell line and in cultured central neurons (Garg et al., 2011). This apparent discrepancy could be due to the different N-terminal ends of these fragments (amino acid 45 for the fragment described by our research group vs. amino acid 125 for the non-toxic fragment described by Garg et al., 2011). These different N-terminal ends could be the result of the protease involved in tau cleavage. The fragment studied by Garg et al. is the result of calpain 2 cleavage unlike the calpain 1 cleavage described by our research group (Park and Ferreira, 2005; Garg et al., 2011). An alternative explanation for these contradictory results could be related to the levels of expression of the construct and/or a cell-type specific response to the presence of tau fragments. An independent research group has recently addressed some of these discrepancies (Reinecke et al., 2011). This study confirmed and extended our results showing the generation of the 17 kDa tau fragment (aa 45–230) by calpain 1 cleavage and the intrinsic toxic effects of this fragment in a *Drosophila* tauopathy model (Reinecke et al., 2011). These authors showed that mutating the calpain cleavage sites we have identified in the generation of the tau₄₅₋₂₃₀ fragment was sufficient to preclude the formation of this fragment and to abrogate tau toxicity *in vivo*. In contrast, the expression of the tau₄₅₋₂₃₀ fragment induced significant toxicity in the fly retina (Reinecke et al., 2011). The data resulting from the generation and characterization of transgenic mice that express the tau₄₅₋₂₃₀ fragment in hippocampal pyramidal neurons presented above extend these results and provide evidence of the direct toxic effects of this fragment in vertebrate central neurons that develop *in situ*. Our results showed enhanced neuronal loss in the pyramidal cell layer of the hippocampus. This effect of tau₄₅₋₂₃₀ on neuronal survival is consistent with reports showing that overexpression of tau isoforms is associated with neuronal loss in other brain areas (Andorfer et al., 2005). However, neuronal loss in transgenic tau₄₅₋₂₃₀ mice was detectable in much younger mice than in full-length tau-expressing ones (Andorfer et al., 2005). Furthermore, enhanced neuronal cell death was observed in hippocampal neurons expressing low levels of this fragment ruling out the possible toxic effects of any given protein when overexpressed in neurons.

In addition to neuronal loss, our results showed synaptic abnormalities in hippocampal neurons expressing this tau fragment. Thus, a significant decrease in synaptophysin levels was detected in transgenic tau₄₅₋₂₃₀ mice as early as 6 months after birth. Similar results were observed when hippocampal neurons obtained from these mice were placed in culture. It is worth noting that AD is characterized by synapse loss in the hippocampus and cerebral cortex (Masliah et al., 1993; DeKosky et al., 1990 & 1996). In addition, studies have shown that the hippocampal content of synaptophysin significantly decreases during the progression of AD in moderate to severe cases of dementia (Mukaetova-Ladinska et al., 2000; Sze et al., 1997). Moreover, this change in synaptophysin content is preceded by its transient increase in AD subjects (Mukaetova-Ladinska et al., 2000). No such biphasic

response was detected in the hippocampus of transgenic tau₄₅₋₂₃₀ mice or in cultured neurons obtained from these animals. The absence of this type of bimodal response could be due to the accelerated degeneration process in these model systems as compared to a much longer period of time involved in the development of the human disease. Alternatively, the early increase in synaptophysin detected in AD subjects could be reflecting an adaptive synaptic response to de-afferentation in early stages of the disease. This compensatory reaction might be triggered by factor(s) absent under the experimental conditions analyzed in this study.

In contrast to the pattern of synaptophysin changes described above, our results did show a biphasic response when the levels of NMDA receptors subunits were analyzed in hippocampal neurons expressing tau₄₅₋₂₃₀ that develop *in situ*. Thus, a significant increase in both in the cytosolic NR2A and NR2B levels and in all three membrane-bound subunits (NR1, NR2A, and NR2B) levels up to 9 months after birth were detected in mutant neurons as compared to wild type ones. The levels of these subunits declined to similar (NR2A) or significantly lower (NR2B) levels than wild type counterparts by 12 months after birth. A similar response has been detected in other pathological conditions (Gazzaley et al., 1997, Grossman et al., 2000, Clinton et al., 2006). A transient up-regulation of NMDA receptor subunits has been observed in response to spinal cord injury and in response to the transection of afferent inputs in the hippocampus (Gazzaley et al., 1997, Grossman et al., 2000). Increased levels of NR1, NR2A and/or NR2B could represent an attempt to restore synaptic activity after synapse loss. Although the mechanisms underlying the down regulation of these receptor subunits are not known, it is tempting to speculate that as neurons degenerate they are unable to maintain an increased rate of synthesis or transport of these receptors to the membrane. Further studies are needed to elucidate such mechanisms.

The regulation of this bimodal response might require extracellular signals or the activation of molecular mechanisms that are not present in culture since this transient up-regulation of NMDA receptors was not observed in cultured hippocampal neurons expressing tau₄₅₋₂₃₀. It is worth noting that similar NMDA receptor subunit down regulation has been described in cultured hippocampal neurons that were exposed to A β oligomers (Lacor et al., 2007).

The decrease in synaptic contacts strongly correlated with cognitive impairment in AD patients (DeKrosky et al., 1990, 1996, Masliah et al., 1993; Sze et al., 1997). Therefore, numerous studies have focused on the mechanisms underlying these synaptic changes in the context of AD and related disorders. It has been speculated that they could be the result of neuronal death and neurite degeneration, β -amyloid toxicity, or tau toxicity. Although our results cannot differentiate between direct tau toxicity and indirect effects of neuronal loss on synaptic sites, they do suggest that this tau fragment could induce synapse defects independently of β -amyloid toxicity.

The structural changes described above in tau₄₅₋₂₃₀ hippocampal neurons could have some bearings on the cognitive and/or behavioral deficits observed in the mutant mice. The analysis of the behavior of the transgenic tau₄₅₋₂₃₀ mice in the visible and invisible platform conditions of the MWM indicated that the expression of this fragment neither induced abnormalities in vision or swimming abilities nor did it impair learning ability. On the

contrary, transgenic tau₄₅₋₂₃₀ mice seemed to swim faster than wild type controls. The probe trial portion of this test showed that these mice took more time and swam longer distances to locate the hidden platform than wild type controls. The fact that these mice swam faster than the wild type counterparts might be masking, at least in part, the magnitude of this memory impairment. Alternatively, these mild memory defects could be due to the low levels of expression of tau₄₅₋₂₃₀ in the mutant mice. In addition to these mild defects, classical Pavlovian fear-conditioning tests showed that mutant mice expressed significantly more contextual-dependent fear as compared to wild type controls. A similar enhanced conditioned fear memory was detected in animal models of autism and trait anxiety (Markram et al., 2008; Sartori et al., 2011). This type of response has also been associated with anxiety-like behavior observed in a variety of tauopathies including post-traumatic stress disorder (Peri et al., 2000). Furthermore, anxiety is a common behavioral change present in more than half of early stage AD patients and its prevalence increases as the neurodegenerative process progresses (Mega et al., 1996; Teri et al., 1999). The enhanced fear response of tau₄₅₋₂₃₀ mice could be due to a decreased ability to inhibit fear responses or the acquisition of a stronger fear memory. An in depth study throughout their live span of the mutant mice will be needed to gain insights into the behavioral changes induced by this tau fragment.

The molecular mechanisms by which tau₄₅₋₂₃₀ mediates these effects have yet to be elucidated. However, it is possible that the up-regulation of NMDA receptors could mediate such effects. This seems the case in monoamine oxidase A and B knockout mice which present similar NMDA receptor content and behavioral changes as the mutant mice expressing tau₄₅₋₂₃₀ (Singh et al., 2013). Regardless of the mechanisms, these results suggest that tau₄₅₋₂₃₀ could induce at least some of the behavioral disturbances observed in AD and related disorders.

Collectively, our results provide further insights into the neurotoxic effects of the calpain-mediated tau₄₅₋₂₃₀ fragment in hippocampal neurons that develop *in situ*. Building upon a previous report showing that this tau fragment is readily detectable in the brain of AD subjects, it is tempting to speculate that tau₄₅₋₂₃₀ could be responsible, at least in part, for some of the neurodegenerative signs observed in AD patients. This fragment could exert its effects forming small oligomers; thus, we have previously shown that tau₄₅₋₂₃₀ formed small aggregates in the presence of arachidonic acid (Ferreira and Bigio, 2011). Furthermore, this tau fragment partially inhibited full-length tau aggregation. As a consequence of this inhibitory effect, tau filaments formed in the presence of tau₄₅₋₂₃₀ were smaller, and hence, potentially more toxic than those formed in its absence (Ferreira and Bigio, 2011). Alternatively, tau₄₅₋₂₃₀ could induce cell death acting as a monomer interfering with essential neuronal functions. The identification of such key molecular mechanisms underlying the toxic effects of tau₄₅₋₂₃₀, however, awaits further investigation.

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- Tau₄₅₋₂₃₀ induces cell death and synaptic loss in hippocampal neurons
- Changes in NMDA receptors are present in tau₄₅₋₂₃₀-expressing neurons
- The expression of tau₄₅₋₂₃₀ associates with mild memory loss
- Tau₄₅₋₂₃₀ induces anxiety-like behavior

Figure 1. Generation of transgenic mice expressing tau₄₅₋₂₃₀

(A) Map of the human tau₄₅₋₂₃₀-eGFP targeting vector used to generate the transgenic mice. The location of the primers used for genotyping and RT-PCR is indicated (arrows). (B) RT-PCR analysis of the expression of tau₄₅₋₂₃₀-eGFP in the hippocampus of 2 month-old wild type (WT) and tau₄₅₋₂₃₀ transgenic (TG) lines 1 and 3 mice (200 bp). Actin and full-length tau primers were used as controls (700 and 269 bp products, respectively). The graphs showed the ratios tau₄₅₋₂₃₀/actin (considering the values obtained for line 1 as 100%) and full-length/actin (considering the values obtained in WT controls as 100%). Numbers represent the mean ± S.E.M. No statistically significant differences were detected between transgenic tau₄₅₋₂₃₀ line 1 and line 3 mice. (C–G) Representative frozen sections of the

hippocampus of 3 month-old wild type (C) and tau₄₅₋₂₃₀-GFP (D–G) transgenic mice immunostained using a GFP antibody. No signal was detected in sections prepared from wild type hippocampus (C). In contrast, GFP immunoreactivity was detected in the hippocampal pyramidal cell layer (arrows) of transgenic mice (D). High-power magnification images of the CA3 region of the hippocampus of transgenic tau₄₅₋₂₃₀ mice showed intense GFP immunoreactivity in cell bodies and faint staining (arrow) along the processes extended by pyramidal neurons (E). GFP immunoreactivity was mainly absent in the nucleus of pyramidal neurons (arrow in F). No signal was detected in other brain areas (i.e. midbrain) of the tau₄₅₋₂₃₀ transgenic mice (G). Scale bars: 200 μ m (C & D), 100 μ m (E & G), and 40 μ m (F). bp: base pairs, ND: Not detectable, PCL: pyramidal cell layer.

Figure 2. Increased cell death in the hippocampus of transgenic tau₄₅₋₂₃₀ mice

(A & B) Representative frozen sections of the hippocampus of 12 month-old wild type (A) and transgenic tau₄₅₋₂₃₀ (B) mice were stained using the TUNEL (green) reagent and counterstained with a tubulin antibody (red). Note the increased number of TUNEL (+) neurons (arrows) in the pyramidal cell layer (PCL) of the hippocampus in transgenic tau₄₅₋₂₃₀ mice (B) when compared to wild type controls (A). (C & D) Quantification of the total number of pyramidal neurons and the number of TUNEL (+) neurons in the hippocampus of 3 to 12 month-old wild type and transgenic tau₄₅₋₂₃₀ mice. Results were statistically analyzed using two-way ANOVA followed by Fisher's LSD *post hoc* test. Numbers represent the mean $\pm$ S.E.M. of neurons and TUNEL (+) cells per equal pixel area of pyramidal layer. *Differs from wild type controls *P* $\leq$ 0.05, **Differs from wild type controls *P* $\leq$ 0.01. Scale bars: 100 μ m.

Figure 3. Synapse loss in the hippocampus of transgenic tau₄₅₋₂₃₀ mice

(A–D) Synaptophysin staining of the hippocampal region of representative 12 month-old wild type (A & C) and transgenic tau₄₅₋₂₃₀ (B & D) mice. Immunostaining was stronger in hippocampal neurons of wild type mice (arrow in A) when compared to transgenic tau₄₅₋₂₃₀ (arrow in B) mice. (C & D) High-power magnification images of the CA3 regions boxed in A & B. Note the faint synaptophysin staining of the pyramidal cell layer in transgenic tau₄₅₋₂₃₀ mice (arrows). (E and F) Quantitative Western blot analysis of synaptophysin (p38) levels throughout the postnatal development of wild type and transgenic tau₄₅₋₂₃₀ mice. Tubulin was used as loading control. Results were statistically analyzed using two-way ANOVA followed by Fisher's LSD *post hoc* test. Numbers represent the mean $\pm$ S.E.M. (n=5 per age and genotype) **Differs from wild type controls $P < 0.01$. Scale bars: 100 μ M (C & D) and 200 μ M (A & B).

Figure 4. Altered levels of NMDA receptors in the hippocampus of transgenic tau₄₅₋₂₃₀ mice
 (A) Quantitative Western blot analysis of NR1, NR2A, and NR2B glutamate receptor levels in extracts obtained from the hippocampus of wild type (WT) and transgenic tau₄₅₋₂₃₀ (T) mice at 3, 6, 9, and 12 months after birth. Significantly higher levels of NR2A and NR2B receptors were detected in the hippocampus of transgenic tau₄₅₋₂₃₀ mice when compared to wild type controls at 6 and 9 months after birth. (B) Quantitative Western blot analysis of NR1, NR2A, and NR2B glutamate receptor levels in membrane fractions prepared from 9 month-old wild type and transgenic tau₄₅₋₂₃₀ mice. All 3 subunit levels were significantly higher in transgenic tau₄₅₋₂₃₀ mice when compared to wild type controls. Tubulin and β_1

integrin were used as loading control in A and B, respectively. Equal amount of total protein (2 µg/lane) were loaded in B. Results were statistically analyzed using two-way ANOVA followed by Fisher's LSD *post hoc* test. Numbers represent the average $\pm$ SEM. *Differs from controls $P<0.05$; **Differs from controls $P<0.01$ (n=5 per genotype and time point).

Figure 5. Determination of the specificity of the tau 45-230 antibody

(A & B) Western blot analysis of whole cell extracts obtained from control (c) and A β -treated (A β) cultured hippocampal neurons reacted with tau-5 (A) and our specific rabbit anti tau₄₅₋₂₃₀ (c-terminus) antibody (B). While tau-5 detected both full-length tau and the 17 kDa tau₄₅₋₂₃₀ fragment, our antibody specifically recognized only the 17 kDa tau₄₅₋₂₃₀ fragment. (C–F) Control (C & D) and A β -treated (E & F) cultured hippocampal neurons were double stained with tubulin (C & E) and tau₄₅₋₂₃₀ (D & F) antibodies. Note the absence of immunoreactivity for the tau₄₅₋₂₃₀ fragment in control neurons (D). Strong punctate immunoreactivity for this fragment was detected in the cell bodies and neurites (arrow heads) of degenerating neurons (F). Neurons without signs of degeneration were not stained with this antibody (asterisks). Scale bar = 20 μ m.

Figure 6. Progressive degeneration in cultured hippocampal neurons obtained from transgenic tau₄₅₋₂₃₀ mice

(A–J) Hippocampal neurons obtained from transgenic tau₄₅₋₂₃₀ mice were kept in culture for 1 (A–D), 7 (E & F), 14 (G & H), and 21 (I & J) days. Neurons were fixed and immunostained using a tubulin (A, C, E, G, & I) and tau₄₅₋₂₃₀ (B, F, H & J) antibodies. Tau₄₅₋₂₃₀ immunoreactivity increased as the neurons developed in culture. Note the absence of immunoreactivity when the tau₄₅₋₂₃₀ antibody was omitted (D) and the change in the distribution of tau₄₅₋₂₃₀ from a punctate localization (arrows in F) to a more diffuse immunoreactive pattern after the first week in culture (H). Signs of progressive degeneration, including tortuous processes, were readily detected as early as 14 days in culture neurons (arrow in H). Complete degeneration of neuritic processes was evident in 21 days in culture hippocampal neurons (arrow in J). Scale bar: 20 μ m.

Figure 7. Synapse loss in cultured hippocampal obtained from transgenic τ_{45-230} mice (A–D) Hippocampal neurons obtained from wild type (A & B) and transgenic τ_{45-230} (C & D) mice were kept in culture for 21 days, fixed and immunostained using synaptophysin (A, & C) and NR2B (B & D) antibodies. Note the decrease in the number of spots double labeled with synaptophysin and NR2B antibodies around the cell bodies and along the processes extended by transgenic τ_{45-230} hippocampal neurons as compared to wild type controls. Scale bar: 20 μm

Figure 8. Subtle changes in learning and memory in transgenic tau₄₅₋₂₃₀ mice

(A–C) Learning and memory was evaluated in seven month-old wild type and tau₄₅₋₂₃₀ transgenic mice by means of Water Morris Maze test. (A) Cued platform learning curves. Numbers represent the mean $\pm$ S.E.M. of daily trials (8). No significant differences were detected in the latency or distance traveled to find the visible platform when transgenic tau₄₅₋₂₃₀ were compared to wild type controls. In contrast, transgenic tau₄₅₋₂₃₀ mice swam significantly faster than wild type controls. (B) Hidden platform learning curves. Numbers represent the mean $\pm$ S.E.M. of all daily trials. No significant differences were detected in any of the parameters analyzed with the exceptions of the time spend in the quadrant. Transgenic tau₄₅₋₂₃₀ mice remained longer in the quadrant during the first 2 days of training

than wild type controls. (C) Probe trial 24 hours after completion of 3 days of hidden platform training. Note the slight increase in the escape latency and the significant increase in the distance traveled and speed when transgenic mice when compared to wild type controls. The data were analyzed using Student's *t* test. *Differs from controls $P<0.05$; **Differs from controls $P<0.01$, (n=5 mice per genotype).

Figure 9. Enhanced fear conditioning in transgenic tau₄₅₋₂₃₀ mice

(A–C) Fear conditioning testing was performed in seven month-old wild type and tau₄₅₋₂₃₀ transgenic mice. (A) A significant increase in the percentage of freezing observations was detected in the conditioning period when transgenic tau₄₅₋₂₃₀ mice were compared to wild type controls. (B) Transgenic mice also froze slightly more than controls during the exposure to the conditioned stimulus. (C) Note the significant increase in the percentage of freezing observations during exposure to the conditioned novel cue context when transgenic tau₄₅₋₂₃₀ mice were compared to wild type controls. The data were analyzed using Student's *t* test. *Differs from controls $P < 0.05$; **Differs from controls $P < 0.01$, (n=5 mice per genotype).