Microtubule associated proteins and plasticity in the developing and diseased brain

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CHAPTER 4

Improved long-term potentiation and memory in young Tau-P301L transgenic mice prior to onset of hyperphosphorylation and tauopathy.

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Abstract

The microtubule binding protein tau is implicated in neurodegenerative tauopathies including frontotemporal dementia with Parkinsonism (FTDP-17) caused by diverse mutations in the tau gene. Hyperphosphorylation of tau is considered crucial in the age-related formation of neurofibrillary tangles (NFT) correlating well with neurotoxicity and cognitive defects. Transgenic mice expressing FTD mutant tau-P301L recapitulate the human pathology with progressive neuronal impairment and accumulation of NFT. Here, we studied tau-P301L mice for parameters of learning and memory at young age, before hyper-phosphorylation and tauopathy were apparent. Unexpectedly, in young tau-P301L mice increased long-term potentiation in the dentate gyrus (LTP) was observed in parallel with improved cognitive performance in object recognition tests. Neither tau phosphorylation, neurogenesis nor other morphological parameters that were analyzed could account for these cognitive changes. The data demonstrate that learning and memory processes in the hippocampus of young tau-P301L mice are not impaired and actually improved in the absence of marked phosphorylation of human tau. We conclude that protein tau plays an important beneficial role in normal neuronal processes of hippocampal memory, and conversely, that not tau mutations per se, but the ensuing hyperphosphorylation must be critical for cognitive decline in tauopathies.

Introduction

Protein tau is a microtubule-associated protein (MAP) involved in the assembly and stabilization of microtubuli (MT). The affinity of tau for MT is actively regulated by phosphorylation and by changes in the ratio of its isoforms, containing either 3 or 4 MT binding domains (tau-3R, tau-4R) (Buee et al., 2000). In an increasing number of neurodegenerative disorders, including Alzheimer's disease (AD), intracellular accumulation of hyperphosphorylated tau as neurofibrillary tangles (NFT) parallel memory disturbances (Tolnay et al., 1999). In a diverse group of inherited tauopathies, known as frontotemporal dementia with Parkinsonism (FTDP-17), mutations in the tau gene located on chromosome 17 are the underlying genetic causes. AD and FTDP-17 patients share the diagnostic accumulations of hyperphosphorylated tau as NFT (Goedert and Jakes, 2005).

FTDP-17 mouse models show that increased phosphorylation of tau results in severe tauopathy, often with premature death (for recent review see [Lee et al., 2005]). Furthermore, FTDP-17 modeling transgenic mice have memory impairment (Tatebayashi et al., 2002; Arendosh et al., 2004; Pennanen et al., 2004) and severe hyperphosphorylation of tau at later ages (Chen, 2005; Sun et al., 2005; Terwel et al., 2005).

In most studies, memory was tested when phosphorylation of tau was evident. In the present study, we questioned whether mutant tau per se alters memory and learning, i.e. in the absence of increased phosphorylation and before NFT formation. We analyzed transgenic mice expressing tau-
P301L in the human tau-2N/4R isoform, in an otherwise normal genetic background (Terwel et al., 2005). Hippocampal learning and memory was studied at young age, when phosphorylation of tau is normal or even somewhat lower than in wild-type mice (Terwel et al., 2005). Moreover, the behavioral tests were performed prior to the onset of any motor defects, as tested by rotarod performance that showed motor impairment in older tau transgenic mice (Lee et al., 2005; Terwel et al., 2005). Long term potentiation (LTP) in both the CA1 and dentate gyrus (DG) was measured in parallel with a hippocampus-dependent memory task that imposes a low level of stress, i.e. the object recognition task (ORT). The CA1 is early and severely affected in AD, while the DG is severely affected in FTDP-17 patients (van Swieten et al., 1999; Kobayashi et al., 2003; Bronner et al., 2005). Adult neurogenesis was studied as a possible factor contributing to tau related changes in hippocampal memory function (Gould et al., 1999; van Praag et al., 1999; Shors et al., 2002; Jin et al., 2004; Shors, 2004), particularly since disruption or over expression of human protein tau affects the cell cycle, neuronal maturation and axonal elongation (Takei et al., 2000; Dawson et al., 2001; Zhao et al., 2003; Andorfer et al., 2005). A second factor possibly contributing to hippocampal memory function, i.e. the morphology CA1 pyramidal and DG granular cells, was also studied in detail. The surprising outcome of improved learning and memory in young tau-P301L mice was however not correlated with increased neurogenesis nor with changes in hippocampal morphology.

Materials and methods

Transgenic mice

Unless indicated otherwise, 8 to 10 week old male tau-P301L transgenic mice in the FVB/N genetic background (Terwel et al., 2005) were compared to age- and sex-matched FVB/N non-transgenic mice. In the transgenic mice, the longest human tau isoform bearing the P301L mutation (tau-4R/2N-P301L) is expressed under control of the mouse thy1 gene promoter, resulting specifically in neuronal expression only, which begins in the second week post-natally. Several independent founder lines were obtained and phenotyped initially, demonstrating similar genotypic and phenotypic characteristics. We selected one strain for further experiments, based on a very similar expression of the tau-P301L transgene as the previously characterized tau-4R strain (Spittaels et al., 1999; Spittaels et al., 2000) and both strains were characterized in extensive detail (Spittaels et al., 1999; Terwel et al., 2005). Homozygous tau-P301L mice obtained by inbreeding are normally fertile and transmit the transgene in a stable manner to their offspring.

Behavioral testing

Mice were housed in groups from 3 to 7 animals per cage and placed in the behavioral testing room at least 30 min before the experiments commenced. Two age groups of mice, 5 and 9 weeks old, were subjected to the following tests, performed in chronological order as described below.
Improved memory in young Tau-P301L mice

First, motor ability was tested by rotarod, on a revolving, horizontal rod of 3.2 cm in diameter (Med Associates, Georgia, Vermont). After one (5 week old mice) or two (9 week old mice) training sessions of 5 min at 16 rotations per minute (rpm), the mice were finally tested on the rod, accelerating from 4 to 40 rpm over a 3 min period. The time that mice remained on the rod was recorded automatically.

Exploratory and motor capacities were subsequently analyzed in an open field setting. Each mouse was placed for 5 min on an elevated, white opaque Plexiglas surface of 52x52 cm without bordering-walls, dimly lit from underneath with fluorescent tubes. The travel path was recorded by a video camera linked to a computer and analyzed using dedicated software (Ethovision, Noldus, Wageningen, The Netherlands). The center of the open field was defined as an inner square of 40x40 cm, and the time spent in the center was calculated.

Learning and memory characteristics were analyzed by means of the object recognition test (ORT) (Dewachter et al., 2002). Individual mice were habituated on day 1 for 2 sessions of 5 min in a Plexiglas box (52x52x40 cm), with a white opaque floor and black walls softly illuminated from underneath. On day 2, in the acquisition phase, mice were presented for 10 min with 2 identical objects placed in 2 adjacent quadrants of the box. The time the animal spent exploring an object, with its snout directed towards the object within ± 2 cm, was recorded manually. The travel paths were recorded by computerized video imaging and analyzed using dedicated software, as above. A 10 min retention trial was given after a delay of either 1 or 3.5 hr. During the retention trial the mice were confronted with one familiar and one novel object. We used two sets of identical objects. Combinations of objects, and their positions in the box (left, right) were randomized to avoid preferences not based on novelty. However, after analysis neither wild-type (WT) nor P301L mice showed a preference for the different objects, or for left or right side of the box. After each trial objects were cleaned to eliminate odor cues. The level of discrimination (d2) was calculated by the formula d2 = \((b-a)/(b+a)\), in which \(a\) and \(b\) are the exploration times spent on the old and novel objects, respectively. Furthermore, exploration time during acquisition and retention, and the absolute discrimination index, \(d1 (=b-a)\) were analyzed (data not shown).

Electrophysiology
Slice preparation and recording conditions.

After decapitation between 9:00 and 10:00 a.m., brains were immediately removed and kept at 4 °C in artificial cerebrospinal fluid (ACSF) containing: 120 mM NaCl; 3.5 mM KCl; 5.0 mM MgSO4; 1.25 mM NaH2PO4; 0.2 mM CaCl2; 10 mM D-glucose; 25 mM NaHCO3, gassed with 95% O2 and 5% CO2. The right hemisphere was immersion fixed for histology; the left hemisphere was used for field potential recordings. For Schaffer collateral recordings, 400 μm transversal hippocampal sections were prepared using a tissue chopper; for perforant path recordings, 400 μm horizontal forebrain sections were obtained using a vibroslicer (Leica VT 1000S). After 1h of
incubation at room temperature in oxygenated recording ACSF (120 mM NaCl; 3.5 mM KCl; 1.3 mM MgSO4; 1.25 mM Na2HPO4; 1.25 mM CaCl2; 10 mM D-glucose; 25 mM NaHCO3), slices were transferred to a recording chamber and perfused with oxygenated ACSF at 31.5 °C.

Bipolar, stainless steel, isolated (except for the tip) stimulating electrodes were placed in the Schaffer collaterals or in the perforant path to record field excitatory postsynaptic potentials (fEPSPs) using a glass microelectrode (2-5 MΩ filled with ACSF) placed in the stratum radiatum of CA1 or the middle third of the molecular layer of the DG, respectively. To evoke robust LTP in the DG, γ-aminobutyric acid mediated activity was blocked by adding 10 μM bicuculline methiodide (Sigma Chemicals) to the ACSF (Alfarez et al., 2003).

Stimulation protocol

Before baseline recording commenced the maximal fEPSP amplitude and slope were determined by gradually increasing the stimulus intensity (60 s interstimulus interval) until the response saturated. The relationship between the stimulus intensity and the evoked response was fit to a Boltzmann equation: \( R_i = R_{\text{max}} / (1 + \exp(-i/h)/S)) \), in which \( R_i \) is the response at stimulus intensity \( i \), \( R_{\text{max}} \) is the maximal response, \( h \) is the intensity at which the half-maximal response is observed and slope factor \( S \) is the index that is proportional to the slope of the stimulus-response curve. The half maximal stimulation intensity was used to record baseline responses for at least 20 min. Recordings in which the baseline was not stable or the maximal fEPSP amplitude was <1.5 mV were rejected.

After baseline recordings, paired-pulse responses with inter stimulus intervals of 50, 100, 200 and 500 ms were tested in the DG to make sure the medial perforant path was stimulated. Recordings that showed paired pulse facilitation were discarded. If all of the above criteria were met, LTP was evoked in both the CA1 and the DG using a theta burst protocol consisting of two trains of four pulses at 100 Hz intermitted by 200 ms. This procedure was repeated five times with an interval of 30 s. After theta burst stimulation, LTP was recorded for 60 min.

Brain histology

The histological experiments described below were performed on the same animals, with the other hemisphere used for electrophysiological recordings, except for the histological Golgi-staining, which was performed on a separate set of mice of the same age. After decapitation, the right cerebrum was immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4) for 7 days. The hemisphere was washed in PBS, equilibrated in 30% sucrose overnight, and frozen on dry ice after which 30 μm coronal sections were cut using a sliding microtome. The left cerebrum was used for field potential recordings as described above.

Total volume and cellular density of the DG and other hippocampal sub regions were determined by a stereological approach as described
Improved memory in young Tau-P301L mice

(Heine et al., 2004a). In short, every 10th section was hematoxylin stained and used to determine the total volume of the granular cell layer (GCL), the hilus and the total CA1, calculated according to Cavalieri’s direct estimator. On each section, areas of interest were photographed using a standard magnification, and surface areas were measured on a Macintosh computer, using the public domain program Object-Image (an extended version of NIH Image, developed at the U.S. National Institutes of Health and at the University of Amsterdam; available from http://simon.bio.uva.nl). Total volume was calculated using the formula $V=\sum A \times T$, where $\sum A$ is the sum of area measurements and $T$ is the intersection distance ($300 \mu m$).

The same sections were used to determine the three-dimensional numerical density of neurons ($NV$, neurons per cubic millimeter) in the granule cell layer (GCL), using the optical dissector method. Counting frames were placed randomly over the GCL. Individual neurons were visualized (Zeiss Axiohot microscope, 100x, NA 1.30 oil objective) and counted, according to the dissector approach, if their nuclear profile was present and if they were positioned within the counting frame or intersected by its inclusion edges (i.e. the top and the right edges). Per hippocampus at least 100 dissectors were used to calculate the numerical density ($NV$) of neurons from $NV=\frac{\sum Q}{\sum V\text{DIS}}$, where $\sum Q$ is the sum of the neurons counted in all dissectors and $\sum V\text{DIS}$ is the sum of the dissector volumes. One dissector volume is $7.5 \times 10^4 \mu m^3$, as calculated from $V\text{DIS}=a\text{DIS} \times h$, where $a\text{DIS}$ is the area of the square counting frame, measuring 50 $\mu m$ on a side, and $h$ the dissector height of 30 $\mu m$.

**Golgi staining**

Mice were decapitated and their brains removed and placed in Golgi-Cox solution (1.04 % potassium dichromate, 1.04 % mercury chloride, 0.83 % potassium chromate, dissolved in double distilled water). After rinsing in water for 5 min and dehydration in 70% EtOH (O/N), 96% EtOH (O/N), 100% EtOH (8 h), 1:2 EtOH/ether (O/N), brains were saturated by consecutive overnight incubations in 3%, 6% and 12% celloidine. Celloidine was cleared with chloroform before 200 $\mu m$ coronal sections were cut. Staining was developed by a 5 min rinse in water, 30 min in 16% ammonia, a 2 min rinse in water, 7 min in 1% sodium thiosulphate, a 10 min rinse in water, followed by dehydration for 5 min in 70 % in EtOH, 5 min in 96% EtOH, 5 min in butanol, and 5 min in Histo-clear (Biozym, Landgraaf, The Netherlands). The sections were mounted in Histomount (National diagnostics, Atlanta, Georgia) under glass cover-slips.

In the thick impregnated sections, dentate granule cells were selected from the middle third of the inner pyramidal blade at approximately Bregma -2.54 mm. Pyramidal cells were selected from the same level of the CA1 area opposite to the middle third of the inner pyramidal blade of the DG. If neurons were completely stained and horizontally orientated within the section, 99 Z-stacks of 1 $\mu m$ were recorded with the program Image-Pro Plus, version 5.1.1.38 (Media Cybernetics©, Inc.), using an Axioplan 2 (Zeiss) microscope, equipped with an Evolution QEi FAST (monochrome, 12 bit)
camera (Media Cybernetics©, Inc.), at a 40x magnification (Plan-Apochromat). The drawing tool NeuroDraw (Image-Pro Plus, developed by K. de Vos, J. van Heerikhuize and C.W. Pool, Netherlands Institute for Brain Research, Amsterdam) was used to determine total dendritic length per neuron, number of dendrites per cell, cell area, length per dendrite, number of dendritic ramifications, number of terminal segments, mean terminal segment length and the mean inter segment length (Ramakers et al., 1998).

Immunohistochemistry

Immunohistochemistry for phosphorylation-independent human tau with Mab HT7 (Innogenetics, Gent, Belgium, 1:10,000) and for the specific phospho-epitope Ser(P)\(^{396}\)/Ser(P)\(^{404}\)(AD2, A. Delacourte, 1:1000), Thr(P)\(^{231}\) (AT180, Innogenetics, Gent, Belgium, 1:250) and Ser(P)\(^{202}\)/Thr(P)\(^{205}\) (AT8, Innogenetics, Gent, Belgium, 1:1000) was as described before (Terwel et al., 2005). In short, mice were anesthetized with pentobarbital (120 mg/kg, intraperitoneal), fixed by transcardiac perfusion with 4% paraformaldehyde in phosphate buffered saline (PBS, 2 ml/min for 10 min). The brain was removed, post-fixed overnight in 4% paraformaldehyde, and stored in PBS, 0.1% sodium azide at 4 °C. Sagittal sections (40 μm) were cut, rinsed in PBS, and treated with 1.5% H\(_2\)O\(_2\) in 50% methanol to inactivate endogenous peroxidases. Nonspecific binding of antibodies was blocked by treatment with 10% fetal calf serum, 3% bovine serum albumin in PBS (blocking buffer). The sections were incubated at room temperature overnight with primary antibodies in blocking buffer and 0.1% Triton X-100. The sections were then incubated for 1 h with goat anti-mouse or anti-rabbit IgG-horseradish peroxidase, diluted 1:500 in blocking buffer and 0.1% Triton X-100. Next, the sections were washed with PBS and 50 mM Tris.HCl (pH 7.6) for 5 min and developed with 3,3’-diaminobenzidine (DAB), 0.3% H\(_2\)O\(_2\) in 50 mM Tris.HCl (pH 7.6) for 3–5 min.

Western blotting

Western blotting was performed as described (Terwel et al., 2005) on extracts of isolated hippocampi of 3 individual mice per genotype and age-group. The total amount of tau was detected using Mab Tau-5 (Pharmingen, 1:1000) and selected phospho-epitopes were detected with Mabs AT8 (1:200), AD180 (1:200) and AD2 (1:5000). Tau-4R isoforms were specifically detected with the rabbit polyclonal antibody directed against the second microtubule binding domain (Takuma et al., 2003).

Analysis of neurogenesis

Since hippocampal LTP and learning and memory have been related to parallel changes in neurogenesis, we analyzed 3 different neurogenesis markers, (i) doublecortin, as a cumulative marker for young, migratory neurons (3 days to 3 weeks of age) (Couillard-Despres et al., 2005); (ii) the birth date marker BrdU, and (iii) Ki-67, an endogenous DNA binding protein present only in proliferating cells.
**BrdU immunohistochemistry**

To study the effects of mutated tau on adult generated cell survival, 1 month old mice were injected in the morning with BrdU (5 mg/ml dissolved in 0.007 M NaOH/0.9% NaCl, i.p.) at a dose of 50 mg/kg, repeated for seven consecutive days. The mice were sacrificed 4 weeks after the first injection. After blocking endogenous peroxidase with 1% H₂O₂ for 20 minutes and denaturing DNA in hot formamide and 2 N HCl, free-floating sections were incubated in the primary antibody mouse α-BrdU (Roche Diagnostics, The Netherlands, 1:3000), diluted in phosphate buffer/0.1% bovine serum albumin/0.3% Triton X-100/1% goat serum albumin/0.3% Triton X-100/1% goat serum for 1 h at room temperature and then overnight at 4 °C. The rest of the procedure was identical to that described elsewhere (Heine et al., 2004b).

**Doublecortin immunohistochemistry**

Sections were rinsed in 50 mM Tris buffered saline (TBS, pH 7.6) for 3 times 5 min. After blocking endogenous peroxidase activity by 2% H₂O₂, sections were incubated for 1 hr at room temperature and overnight at 4 °C with the primary goat anti-doublecortin antiserum (C-18, Santa Cruz) (diluted 1:600 in 0.25% gelatin/0.5% Triton X-100 in TBS. The secondary antibody (donkey anti goat Ig, 1:500) was applied for 2 hr at room temperature. Subsequently the reaction was amplified for 2 hrs with the Elite Vectastain avidin-biotin complex (ABC kit; Vector Laboratories, Burlingame). The signal was further amplified with biotinylated tyramide (1:500) and 0.01% peroxide for 30 min followed by 45 min incubation with ABC. DAB was used to visualize antibody labeling.

**Ki-67 immunohistochemistry**

The Ki-67 antigen is a 345 to 395 kDa non-histone protein complex present in proliferating cells during G₁, S, G₂ and M, but not the G₀ phase of the cell cycle (Gerdes et al., 1984; Endl and Gerdes, 2000; Duchrow et al., 2001). Ki-67 is widely used in tumor biology and considered a good proliferation marker. Moreover, numbers of cells visualized with BrdU labeling after a short survival time are highly comparable to the numbers obtained with Ki-67 staining (Kee et al., 2002).

Microwave antigen retrieval was applied before antibody incubation. Sections were mounted on Menzel Superfrost Plus glass sections. After air-drying, sections were placed in 400 ml 0.01 M citrate buffer (pH 6.0) and heated in a household microwave device starting at 800 W and gradually decreasing to 100 W once boiling commenced. Sections were allowed to cool down to RT. The following procedure was identical to that used for doublecortin immunohistochemistry, however, instead of 0.05 M TBS, 0.1 M TBS was used. The primary antibody solution was 1:1500 (NCL-Ki67p Novocastra, New Castle, UK). The secondary antibody solution was 1:200 (biotinylated anti-rabbit, Amersham Life Sciences).
Chapter 4

Statistical analysis

Except for the Golgi data, which were analyzed using a nonparametric Mann Whitney U test, differences between strains were tested using a two-way ANOVA.

Results

Tau expression and phosphorylation

Previously, we analyzed the phosphorylation status of protein tau in whole brain and in spinal cord, and its progression with age. We observed that in young tau-P301L mice, mutant tau was less phosphorylated than wild-type tau-4R in brain of age-matched tau-4R mice, which differ only in the mutation (Terwel et al., 2005).

Here we compared specifically the phosphorylation status of tau in the hippocampal formation in both the transgenic strains, and relative to non-transgenic mice, all at age 2 months. First, IHC with mAb HT7 specific for human tau demonstrated expression in all hippocampal sub-areas in tau-P301L mice (Fig 1, color figure). Some differences in localization, i.e. tau-P301L more in somatodendritic compartments than tau-4R, were evident in the CA1, dentate gyrus and perforant pathway (Fig 1). Secondly, IHC with Mabs AT8, AT180 and AD2, directed against specified phospho-epitopes, revealed that in brain of tau-P301L mice, less phosphorylated epitopes were present than in tau-4R mice, and even hardly more than in non-transgenic mice (Fig 1).

Since IHC is not well suited for quantitative estimations, we quantified by western blotting the IHC results, using the same phospho-epitope specific antibodies AT8, AT180 and AD2, as well as the pan-tau antibody Tau-5. Particularly the AT8 epitope was less abundant in hippocampal extracts from tau-P301L mice, but also the other phospho-epitopes were less prominent (Fig 2). The lesser phosphorylation of transgenic tau-P301L was also inferred from its higher electrophoretic mobility in SDS-PAGE (Fig 2) as observed previously (Terwel et al., 2005).

Protein tau isoforms with 3 or 4 microtubule binding domains were differentiated in western blotting with an antiserum that specifically recognizes the second microtubule binding domain present only in tau-4R, and not in tau-3R (Takuma et al., 2003). The data unequivocally demonstrate that the tau isoforms expressed in brain of young adult mice examined here was tau-4R (Fig 2, lower panel), in accordance with the reported complete post-natal switch in expression from tau-3R to tau-4R in mouse brain (Andorfer and Davies, 2000; Takuma et al., 2003).
Improved memory in young Tau-P301L mice

Human protein tau was detected with mAb HT7 in the hippocampal formation of tau-P301L and tau-4R transgenic mice (8 weeks of age) but not in nontransgenic mice (FVB/N). IHC with the phosphorylation-specific antibodies AD2, AT8, and AT180 demonstrated less phosphorylation in tau-P301L mice. Except for IHC with HT7, all sections were counterstained with hematoxylin (original magnification, 5x).

The current observation of lesser phosphorylation of tau-P301L relative to tau-4R are completely conform our previous findings in brain and spinal cord of older tau-P301L transgenic mice (Terwel et al., 2005). Although in young mice, tau-P301L is relatively less phosphorylated at the AT8 and AD2 epitopes, no immunoreactivity at all was detected with Mab AT100, which defines a typical pathological epitope (Terwel et al., 2005). Moreover, the electrophoretic mobility of tau-P301L was not markedly slow, which further underlines the minimal degree of post-translational phosphorylation.
We therefore conclude that in young tau-P301L mice the phosphorylation of protein tau in the hippocampus is, if anything, lower than in age-matched non-transgenic mice.

**Figure 2.** Western blotting for human tau and phosphorylated tau in the hippocampus of young nontransgenic and tau-4R and tau-P301L transgenic mice.

Protein extracts from the hippocampus of nontransgenic mice (FVB) and tau-P301L and tau-4R transgenic mice (n 3, each) were analyzed by Western blotting with mAbTau5 to detect total tau (i.e., mouse tau and transgenic human tau). Western blotting with phosphorylation-dependent antibodies AT8, AT180, and AD2 demonstrated that transgenic tau-P301L was less phosphorylated than wildtype mouse tau and transgenic tau-4R. Only tau-4R isoforms were present as demonstrated with antibody 2R (bottom panel) directed against the second microtubule binding domain in protein tau-4R (Takuma et al., 2003). The different tau-4R isoforms are indicated. Protein loaded was threefold higher for the nontransgenic than for the transgenic mice to compensate for the overexpression relative to endogenous mouse tau for different levels of the epitopes and for differences in titer and avidity of the respective antibodies. Samples loaded for tau-P301L and tau-4R transgenic mice were equivalent to 2.8 g of protein for mAbs Tau-5 and AD2, 9 g for mAb AT180, 18 g for mAb AT8, and 5.6 g for 2R antibody.
Improved memory in young Tau-P301L mice

<table>
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<th>WT CA1 (n = 12)</th>
<th>P301L CA1 (n = 7)</th>
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Table 1. Basal neural transmission in tau-P301L transgenic and nontransgenic mice.
Basal neural transmission is unchanged in tau-P301L transgenic mice, whether recorded in the CA1 or DG. Values are mean SEM. $R_{\text{max}}$, Maximal response; $I_h$, half-maximal stimulation intensity.

Electrophysiology

To characterize typical hippocampal properties, we recorded field potentials and induced LTP in brain sections, both in the DG and in CA1. By fitting the input-output curves to a Boizmann equation, the three major determinants for basal transmission were calculated, i.e. maximal amplitude or slope of the fEPSP, half maximal stimulation intensity and slope factor (Table 1). None of these parameters were significantly different in brain sections of young tau-P301L mice compared to age-matched non-transgenic mice, regardless of whether recording was in the CA1 or DG.

LTP in the CA1 area was unaffected (Fig 3A), but LTP in the DG was significantly increased in brain sections from tau-P301L mice, both when analyzed over the complete 1 hr recording period ($p=0.031$, $F=6.28$, $n=5$) and over the last 5 minutes of the session ($p=0.036$, $F=5.84$, $n=5$) (Fig 3B).

Since facilitated LTP in the DG is potentially indicative of improved cognition, the mice were tested for motor and cognitive parameters.
Behavioral testing

Prior to the object recognition test (ORT), the basal motor and behavioral parameters were tested by rotarod and open field tests. Tau-P301L mice fell off the rotating rod significantly earlier than non-transgenic mice (182 +/- 39 sec vs 268 +/- 12 sec; mean +/- SEM, p=0.039, F= 4.86, n=10 and 11 respectively) indicating some motor impairment already at the young age of 9 weeks. Locomotor activity in an open field revealed that tau-P301L mice traveled overall significantly more distance than non-transgenic mice, i.e. 2030 +/- 292 cm vs 1223 +/- 136 cm, p=0.006, F= 9.74, n=11 and 10 respectively). Both genotypes exhibited equal relative times spent in the center of the open field (17.2 +/- 4.9 % vs 417.9 +/- 3.0 %) demonstrating no difference in anxiety or exploratory behaviour.

We then substantiated whether the electrophysiological change, i.e. increased LTP in the DG was paralleled by concurrent alteration of hippocampal memory function, by subjecting the mice to the object-recognition task (ORT). Tau-P301L mice performed similar to non-transgenic mice in ORT with a 1 hr delay. High d2 values indicated that both groups had a good recall of the familiar object (0.49 +/- 0.05 vs 0.45 +/- 0.11, respectively 11 wild-type vs 10 transgenic mice). At the 3.5 h interval, however, the non-
improved memory in young Tau-P301L mice

Transgenic mice showed low d2 values, indicating they had low recall of the familiar object in contrast to tau-P301L mice that performed similar at the 3.5 hr as at the 1 hr interval (Fig 4), demonstrating that they still recognized the familiar object. Since this difference was highly significant (p=0.046, F=4.65), we conclude that young tau-P301L mice display improved memory in this task compared to age-matched non-transgenic mice.

Figure 4. Object recognition in young mice at 9 weeks of age.

Young age-matched nontransgenic and tau-P301L transgenic mice were assessed in the object recognition task with 1 h and 3.5 h delay intervals after acquisition. Both genotypes showed a similar preference for the novel object at 1 h, whereas at 3.5 h, the preference of the tau-P301L transgenic mice for the novel object remained high, as opposed to the decline in nontransgenic (WT) mice (*p = 0.02). This demonstration of improved memory was also observed in younger tau-P301L mice at 5 weeks of age (for details, see Results, Behavioral testing). Error bars represent SEM.

Since the rotarod test pointed to motor problems, we additionally tested even younger tau-P301L mice in ORT, i.e. at the age of 5 weeks. At this age, neither a motor impairment was evident in the rotarod test nor increased travel distance in the open field (results not shown). Nevertheless, also at age 5 weeks the tau-P301L mice performed significantly better in ORT at the 3.5 h interval than age-matched non-transgenic mice (0.42+/−0.07 vs 0.05+/−0.09, p=0.011, F=8.00, n=12 and 10 respectively).

Hippocampal morphology

To address possible morphological correlates of the increased LTP in the DG and the improved cognition, we investigated structural changes in several hippocampal subareas of tau-P301L mice in direct comparison to non-transgenic mice of the same age, same gender and same genetic background. No major differences were observed in the volume of the hippocampal subregions or in cellular density (Fig 5).
Then we analysed the morphology of CA1 and DG at the individual, single cell level by Golgi impregnation. Various parameters of the dendritic tree were measured, i.e. cell area, dendritic length, number of dendritic ramifications, number of terminal segments, mean terminal segment length and mean intersegment length.

Two parameters eventually relevant for the problem at hand, e.g. total dendritic length and total number of dendrites, were not different in either brain region of tau-P301L and non-transgenic mice (results not shown). On the other hand, in CA1 the area of individual soma was significantly smaller in tau-P301L mice (160 +/− 6 μm², n=41) than in non-transgenic mice (180 +/− 6 μm², n=44) (p<0.01). The length of the terminal branch of the apical dendrites was increased from 46 +/− 2 μm in non-transgenic mice (n=44) to 52 +/− 2 μm in tau-P301L mice (n=41)(p=0.037). In the DG, the
Improved memory in young Tau-P301L mice

Inter-segment length was increased from $22.2 +/- 1.7 \mu m$ in non-transgenic mice ($n=91$) to $28.8 +/- 2.2 \mu m$ in tau-P301L mice ($n=98$) ($p=0.018$). All other parameters did not differ between the two genotypes.

**Neurogenesis**

Stereological measurements were performed using three independent markers for different stages of the neurogenesis process: DCX as marker for young migrating neurons, Ki-67 as marker for cycling cells and BrdU as a "cell-age" marker, analysed here 4 weeks after injection of BrdU (Fig 7, color figure). Neither of these markers revealed a major difference in either the number of newborn, of proliferating or of surviving young neurons in tau-P301L mice compared to non-transgenic mice (Table 2).

The analysis therefore excludes that changes in basal neurogenesis contributed to the demonstrated functional alterations in the hippocampus of young tau-P301L mice.
Figure 7. Newborn cells and survival in young tau-P301L transgenic mice.

Different markers were examined to define putative changes in cell genesis and/or turnover. A, D, Immunohistochemistry for doublecortin marks young neurons. B, E, BrdU was analyzed 4 weeks after injection as a measure of cell age (see Results for details). C, Immunohistochemistry for the Ki-67 antigen as a marker of proliferating cells. A–C show an overview of the dentate gyrus, and representative individual cells or groups of cells are illustrated in D and E. Sections were counterstained with hematoxylin.
Improved memory in young Tau-P301L mice

<table>
<thead>
<tr>
<th></th>
<th>hilus (WT)</th>
<th>hilus (P301L)</th>
<th>SGZ (WT)</th>
<th>SGZ (P301L)</th>
<th>GCL (WT)</th>
<th>GCL (P301L)</th>
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<tbody>
<tr>
<td>Ki-67</td>
<td>129 ± 21</td>
<td>108 ± 25</td>
<td>665 ± 57</td>
<td>760 ± 70</td>
<td>55 ± 12</td>
<td>94 ± 19</td>
</tr>
<tr>
<td>BrdU</td>
<td>248 ± 30</td>
<td>215 ± 46</td>
<td>2684 ± 134</td>
<td>2573 ± 183</td>
<td>184 ± 23</td>
<td>163 ± 25</td>
</tr>
<tr>
<td>DCX</td>
<td>24 ± 6</td>
<td>43 ± 23</td>
<td>7424 ± 754</td>
<td>7740 ± 840</td>
<td>256 ± 33</td>
<td>313 ± 12</td>
</tr>
</tbody>
</table>

Table 2. Cell birth and survival is unchanged in tau-P301L transgenic mice

The markers used are Ki-67, which labels proliferating cells; BrdU, a marker for cell survival (4 weeks); and DCX, which labels young migrating neurons. The numbers indicate total cells per hemisphere SEM. The number of animals per group is in parentheses (WT vs P301L). SGZ, Subgranular zone.

Discussion
The current study demonstrates for the first time that neuronal expression of a mutant protein tau does not impair hippocampal functions per se. On the contrary, the remarkable and unexpected increase in LTP in the DG of young tau-P301L mice was paralleled by a significant improvement in cognitive performance in the object recognition task. At this young age, no tau-pathology was evident and the phosphorylation of protein tau was normal or, if anything, less than in age-matched non-transgenic mice. Finally, no major morphological or neurogenic abnormalities were detected.

Our previous biochemical and pathological analysis of the tau-P301L mice already established the progressive and age-related nature of the phosphorylation of protein tau, leading to conformational changes and extensive neurofibrillary pathology (Terwel et al., 2005). The progressive morbidity with age is accompanied by progressive motor defects, and increasing hyper-phosphorylation. In the early stages of life, phosphorylation of protein tau is low without any signs of axonal dilations or inclusions (Terwel et al., 2005), this study.

The combined data lend strong support to the hypothesis that not mutant tau itself, but the progressive hyper-phosphorylation with age is critical in FTDP-17 tauopathies, inflicting or at least signaling the onset of memory impairment and neuro-degeneration. Moreover, the current data point to an important physiological function of protein tau in contributing to memory performance in the hippocampus.

Tau transgenic mice including those expressing tau-P301L, have shown cognitive impairment (Arendash et al., 2004; Pennanen et al., 2004; Santacruz et al., 2005). Most of these transgenic mice were tested, however, when tau hyperphosphorylation and pathology was already evident. Most recently, suppression of tau-P301L expression in an inducible transgenic model ameliorated memory functions, without reversing the tauopathy (Santacruz et al., 2005). All these data are in line with our previous and our current study, while the combined results demonstrate that hyper-phosphorylated tau, and not NFT must be the cause of the observed memory defects. This conclusion is based on observations that hyper-phosphorylation of tau invariably precedes the formation of any tau aggregates in all model systems studied (see introduction). In analogy with the evolution of concepts in the "refined" amyloid cascade hypothesis.
proposing soluble oligomers rather than insoluble polymers of amyloid peptides to be the actual culprits in AD (Selkoe, 2005), we underwrite the hypothesis that not NFT are detrimental for neuronal functions (Andorfer et al., 2005) but the soluble isoforms of abnormally phosphorylated tau (Terwel et al., 2005; this study).

Motor impairment has been observed in tau transgenic mice, progressing with age (Lewis et al., 2000; Arendash et al., 2004; Ikeda et al., 2005; Terwel et al., 2005). To exclude possible confounding parameters, we performed rotarod and open field experiments prior to cognitive testing. The higher locomotor activity of tau-P301L mice at age 9 weeks in the open field corroborates the increased explorative activity in other tau transgenic mice (Tanemura et al., 2002; Pennanen et al., 2004). Hippocampal alterations are known to affect explorative activity (Decker et al., 1995; Harley and Martin, 1999; Crusio, 2001). Using a demanding test-protocol in the rotarod paradigm, i.e. involving less training sessions than in a previous study (Terwel et al., 2005), we demonstrate that 5 week old tau-P301L mice do not suffer any motor deficit. Importantly, the cognitive improvement was not influenced to any degree by hyperactivity or motor problems, since tau-P301L mice at 5 weeks of age displayed a similar improvement in memory performance in the object recognition paradigm, without showing any alterations in motor or locomotor faculties. Thereby, the improved cognitive performance in the ORT task, is not related to nor caused by motor disabilities or problems.

The alternative test for cognitive performance, i.e. the water-maze task, proved not suitable for tau-P301L mice as this test depends heavily on swimming ability and performance is negatively influenced by motor defects. Although the FVB mouse strain carries the retinal degeneration gene that can hamper visual tasks, particularly at older age (Pugh et al., 2004). In contrast, the ORT task depends on short-range visual, as well as tactile stimuli, is less stressing than the watermaze task, and requires limited motor skills. Interestingly, a similar improvement in learning performance of tau-P301L mice are observed in a water maze task, in an independent strain of tau-P301L mice with a different genetic background (A. Blokland, Maastricht and H.J. Schröder, Koln; personal communication).

Moreover, hippocampal LTP in CA1 have been demonstrated to correlate well with memory functions measured by ORT (Miller et al., 2002; Wang et al., 2004). The suggestion that ORT would not depend on hippocampal functions (Mumby, 2001) was largely based on tests with short delay protocols in which animals were tested immediately (minutes) after training. Recent studies demonstrate that the delay length determines hippocampal involvement (Hammond et al., 2004) and this is crucial for object recognition at longer delay (hours) like we studied here. We do acknowledge, however, that associated structures like perirhinal and postrhinal cortex may influence ORT performance (Bussey et al., 1999; Kesner
Improved memory in young Tau-P301L mice

et al., 2001) and alterations in these brain regions could contribute to the changes observed in the young tau-P301L mice.

The current data illustrate the clear correlation between improved memory performance and increased LTP in the DG but not CA1 hippocampal areas. To define possible causes of these interesting regional effects, we analysed several cellular and structural correlates. Adult neurogenesis, uniquely occurring in the DG, has been implicated in memory function (Gould et al., 1999; van Praag et al., 1999; Shors et al., 2001; Shors et al., 2002). Despite improvements in cognition and LTP in the DG, no changes in either proliferation or survival of the newborn cells, nor in the extent of neurogenesis were observed in the tau-P301L mice. The tau-P301L mice thereby represent an example of improved cognition not accompanied or paralleled by alterations in neurogenesis, proving that the mutant tau protein affects hippocampal functions through other mechanisms.

Since only relative minor changes were observed in dendritic properties, and in other morphological parameters of hippocampal neurons, and only in DG, we conclude that subtle intracellular effects must underlie the changes in LTP and cognition. One possibility is a differential stabilization of microtubuli by tau that could contribute importantly to vesicular transport (Zhang et al., 2005) and in turn to synaptic plasticity and memory. The trafficking of NMDA and AMPA receptors, critically implicated in LTP and memory formation (Davis et al., 1992; Malinow and Malenka, 2002; Bast et al., 2005) depends heavily on stable MT-mediated transport (Setou et al., 2002; Yuen et al., 2005). Possibly, MT stability is improved in the tau-P301L mice despite a possibly reduced binding of mutant tau (Hong et al., 1998; Perez et al., 2000; Zhang et al., 2004), which could, however, be balanced by the higher concentration of tau. Moreover, the mutant tau-P301L is embedded in the tau-4R isoform, which binds more avidly to MT than the tau-3R isoform.

Whether the alterations are the result of expression of mutant tau or over-expression of tau-4R is unresolved. Although tau-2N/4R mice are available that overexpress the same isoform as the tau-P301L mice, evidently without the mutation, the same experiments cannot be performed in these mice since they suffer a severe axonopathy (Spittaels et al., 1999; Terwel et al., 2005). On the other hand, an interesting analogy is observed in transgenic mice that overexpress p25, since these, also unexpectedly, showed improved hippocampus-dependent memory functions (Angelo et al., 2003). The calpain-truncated cdk5 activator subunit p25 is associated with pathological hyperphosphorylation of tau, and was expected to induce neurodegeneration (for review see (Tsai et al., 2004). Increased tau phosphorylation was not observed in young p25 transgenic mice, but tau expression was increased (Angelo et al., 2003). This is consistent with the hypothesis that increased concentration of protein tau, and not the mutation per se, underlies the improved cognitive performance in young tau-P301L mice.
Although the present transgenic mice replicate many features of the corresponding human tauopathy, including adult onset, progressive neurodegeneration, accumulation of abnormal tau-aggregates and premature death, we are not aware of any reports of similar improvements in memory performance in young adult human subjects bearing tau-P301L or other FTDP-17 mutations. In part, this could also be due to the clinical heterogeneity of FTDP-17 patients (Ingram and Spillantini, 2002). Alternatively, improved hippocampal functioning may be unique to the present type of transgenic model that is essentially different from the human situation in that it expresses only the 2N/4R splice variant of tau.

In summary, we describe an unexpected parallel improvement in hippocampal LTP and memory in young tau-P301L mice, prior to the onset of hyperphosphorylation or aggregation of protein tau. We thereby demonstrate that not the tau-P301L mutation per se is critical for cognitive decline, but conclude that excess phosphorylation of tau, triggered by the mutation is pathogenic. Moreover, the results highlight an important novel role for protein tau in hippocampal memory function.