Microtubule associated proteins and plasticity in the developing and diseased brain

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CHAPTER 3

Disturbance of the tau-3R to 4R switch affects early neurogenesis and maturation of the dentate gyrus resulting in an enlarged hippocampus that is paralleled by improved memory function.

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Tau isoform expression affects hippocampal development

Abstract
In the hippocampal dentate gyrus (DG), most neurons are formed in the first two postnatal weeks, whereafter neurogenesis steeply declines. Precisely around this period, also a switch in expression from the cytoskeletal protein tau-3R to the -4R isoform occurs. Given the role of tau in structural plasticity, this switch could be implicated in hippocampal maturation. To define the differential roles of the tau-3R and -4R isoforms in neuronal maturation, we first studied proliferation, differentiation and neuritic outgrowth in primary hippocampal cultures in a tau knockout background. Absence of tau increased cell-birth, whereas outgrowth of neurites and neuronal differentiation were decreased. Introduction of the tau-4R isoform reverted these effects, whereas introduction of tau-3R only modestly increased neurite outgrowth. To examine the relevance of these findings in vivo, we subsequently studied humanized tau knock-out/knock-in mice, in which only human tau-4R/2N is expressed on a mouse tau null background from postnatal day 12 onwards, and at low expression levels, preventing axonopathy. These KOKI mice showed a transient increase in hippocampal proliferation in both CA and DG subregions from the second until the fourth week of age, a less complex DG neuronal morphology at 2 months of age and increased doublecortin expression, indicative of enhanced numbers of young neurons. The net outcome, as confirmed also at later ages, was a lasting and significant increase in both hippocampal neuron number and volume in tau-KOKI mice. This structural alteration was paralleled by an improved cognitive function, but not by changes in long term potentiation. We conclude that the 3R to 4R tau isoform switch is critically involved in hippocampal maturation.

Introduction
The microtubule associated protein (MAP) tau is involved in morphogenesis, axonal outgrowth and vesicle transport (Caceres and Kosik, 1990; Ebnet et al., 1998; Gonzalez-Billault et al., 2002; Mandelkow et al., 2003), most likely by regulating microtubule (MT) dynamics. Upon tau binding e.g., MT stability increases. The affinity of protein tau for MT can be altered by phosphorylation and alternative splicing (Andorfer and Davies, 2000; Buee et al., 2000; Goedert and Jakes, 2005). The latter gives rise to 6 tau variants with either 3 or 4 MT binding repeats, with the 4 repeat (tau-4R) variants having a higher MT affinity than 3R tau (Goedert et al., 1996; Buee et al., 2000; Lee and 5534, 2001).

The shortest tau-3R/0N isoform predominates in early stages of development in both humans and rodents. While adult human brain contains approximately equal levels of tau-3R and tau-4R isoforms, a practically complete switch from 3R to tau-4R expression occurs in rodent brain in the second postnatal week that is maintained throughout adult life (Goedert et al., 1989; Kosik et al., 1989; Larcher et al., 1992; Takuma et al., 2003). It has been speculated that the tau-3R to -4R switch is related to structural plasticity changes during specific developmental processes like axonal outgrowth, pathfinding and synaptogenesis. These processes all require a more dynamic
cytoskeleton than in adulthood (Bunker et al., 2004). Indeed, specific MAPs are involved in neuronal migration and mitotic spindle formation (Andersen, 2000; Boekhoorn et al., 2006; Shu et al., 2006), while also differential effects of tau-3R and tau-4R on microtubule dynamics have been reported (Levy et al., 2005). In this respect, the hippocampal dentate gyrus (DG) is an interesting area as the majority of DG neurons is formed in the first two postnatal weeks. After this period, a steep decline in neurogenesis occurs, that coincides with the decline in 3R and onset of tau-4R expression (Altman and Bayer, 1975; Schlessinger et al., 1975; Bayer and 1, 1980). We therefore hypothesized that the tau-3R to -4R isoform switch is of particular relevance for the DG and DG-specific processes like neurogenesis and neuronal maturation.

Intronic mutations in the tau gene result in a perturbed isoform ratio and cause fronto-temporal dementia (FTD) (Hutton et al., 1998; Spillantini et al., 1998; D’Souza et al., 1999; Hasegawa et al., 1999). In general, but not always, these mutations cause an up-regulation of tau-4R expression (Hogg et al., 2003). Although difficult to separate completely from the additional effects of tau hyperphosphorylation, this suggests that altered tau-4R/3R isoform ratios are directly implicated in cognition and pathology. To address this, several tau transgenic mouse lines have been generated, including lines with altered tau-isoform ratios without mutations (Ishihara et al., 1999; Spittaels et al., 1999; Gotz et al., 2004; Terwel et al., 2005)). Although these reports confirm important roles of the different tau isoforms, most focus on changes during aging, whereas its role during early postnatal development has received little attention.

To address the morphological and functional relevance of the different tau isoforms and particularly 4R during hippocampal maturation, we here first studied tau-3R and tau-4R specific effects on proliferation, differentiation and axonal outgrowth in primary hippocampal cultures from a murine tau null background. Next, we examined the functional role of particularly the 4R isoform in more detail in vivo, making use of a humanized tau knock-out/knock-in (tau-KOKI) mouse strain, in which all endogenous murine tau is deleted and only the longest isoform of human tau, tau-4R/2N, is expressed from the second post-natal week onwards (Terwel et al., 2005). Notably, this time-window largely coincides with the switch from tau-3R to tau-4R and with the post-natal maturation of the rodent hippocampus (Goedert et al., 1989; Stanfield and Cowan, 1979; Takuma et al., 2003). Given its relevance for structural plasticity, our main focus was on proliferation and morphological changes. As both the DG and tau are also important for cognition, we subsequently addressed LTP and memory performance as well.

**Experimental procedures**

**Generation of transgenic tau-KOKI mice**

The tau knock-out-knock-in strain of human tau-4R (tau-KOKI) was engineered to inactivate the endogenous murine tau gene and to replace it
with a single copy of the thy1- human tau-4R/2N recombinant DNA construct. The construct ligated into the mouse thy-1 gene expression cassette was identical to the one used in generating the tau-4R transgenic mice (Spittaels et al., 1999). The excised insert was ligated in the BamHI site of the pBluescript vector, followed by ligation into a unique SmaI site of a 1.9 kb Not I fragment encoding the hygromycin B phosphotransferase gene driven by the phosphoglycerate kinase (PGK) promoter, (Uman s et al., 1999). The total insert of this construct was excised to remove vector sequences and ligated in the unique NcoI site in exon1 of the mouse tau gene, partially sub-cloned from the 129 mouse strain (Harada et al., 1994). The targeting vector was linearized by NotI restriction, purified and electroporated into embryonic stem (ES) cells and cultured in selective media containing hygromycin, by standard procedures (Umans et al., 1999; Dewachter et al., 2002).

Single colonies were expanded for genotyping by southern blotting with different probes against external and internal fragments of the murine and human tau gene and the marker gene. ES cell colonies with the desired homologous recombination were sub-cultured and genotyped again before microinjection into 4 day old blastocysts isolated from pregnant C57/Bl6 female mice. Re-implantation into pseudo-pregnant CD1 females by uterine transfer resulted in coatcolor chimeric mice proving germline transmission of the ES cells. Offspring from the chimeras were crossed with FVB/N mice and their offspring genotyped by southern blotting of tail DNA with the same probes described above. Heterozygous transgenic mice were mated to establish the tau-KOKI strain. Breeding was continued by backcrossing into FVB/N for 8 generations to obtain homozygous humanized tau-KOKI mice in the FVB/N background. Homozygous and heterozygous tau-KOKI mice were continuously differentiated by the two independent PCR reactions for the recombined and for the wild-type mouse tau gene.

Western blotting
Snap frozen brain tissue was homogenized in 2 ml of 0.1 M MES Buffer (pH 6.4), 0.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 20 mM NaF, 0.2 mM Na3VO4, 1 μM okadaic acid, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml soybean trypsin inhibitor, 1% sodium deoxycholate, 1% Triton-X-100 and 0.1% SDS. After centrifugation, aliquots of the supernatant brain extract were denatured in SDS and reduced with 2-mercaptoethanol by heating for 10 min at 95°C.

For cell lysates, cells were scraped in a small volume of cold PBS, pelleted and lysed in PBS containing 1% Triton X-100, 0.9% NaCl, 0.05 mM orthovanadate and the complete protease inhibitor mixture (Roche, Vilvoorde, Belgium). The protein content was assayed by bichinchoninic protein assay (Pierce, Polylab, Antwerp, Belgium). From each sample, the equivalent of 20 μg protein was diluted in SDS-PAGE sample buffer and denatured and reduced at 95°C for 5 min.

Proteins were separated by SDS-PAGE on 8% or 4-20% Tris-glycine gels (Novex, Gent, Belgium) after which they were transferred to nitrocellulose membranes that were subsequently blocked with fat-free milk, probed
sequentially with primary and secondary antibodies and developed (ECL, Amersham, UK).

**Cell culture**

Embryonic hippocampal neuronal cultures were prepared from E17.5 foetuses from tau-KOKI and WT FVB/N mice according to standard procedures (Banker and Cowan, 1977; Dawson et al., 2001). Following gross dissection and tissue dissociation by trypsin and trituration, the cells were seeded on glass coverslips (Menzel-Glaser) coated with poly-L-lysine (Sigma, St Louis, MA, USA) at 1 mg/ml in 0.1 M borate buffer, pH 8.5, at a density of 100,000 cells/13 mm² coverslip. Cells were cultured on an astroglial feeder layer in Neurobasal/B27 medium supplemented with 2 mM L-glutamine and 0.1% v/v penicillin-streptomycin (Invitrogen, Gent, Belgium). Some cultures were pulsed with 10 μM S-phase marker BrdU (Sigma, St Louis, MA, USA) and left for 6 h, to allow immunocytochemical detection of proliferating cells.

**DNA constructs and transfection**

Recombinant DNA constructs for tau-3R and tau-4R described in (Vandebroek et al., 2005) were subcloned into the pcDNA3 vector (Invitrogen, Gent, Belgium) and transfected into hippocampal cells by electroporation. Immediately after dissociation of the hippocampal tissue, 106 cells were mixed with 10 μg DNA in 0.5 ml Hanks balanced salt solution (Invitrogen, Gent, Belgium) and electroporated (0.2 kV, 960 μF). Transfected cells were plated on glass coverslips coated first with poly-L-lysine as above, and additionally with 10 μg/ml laminin (Invitrogen, Gent, Belgium). The transfection rate was quantified by immunocytochemical staining for protein tau, as described below, and ranged between 15-25%.

**Immunocytochemical staining**

Cells were fixed with 4% paraformaldehyde in 0.1M phosphate buffered saline (pH 7.4) (PBS) for 10 min. For BrdU staining, the cells were treated with 2N HCl for 10 min as well. Non-specific binding was blocked with 10% fetal calf serum in 0.1% Triton-X100 PBS (blocking buffer). Cells were stained with primary antibody at 4°C overnight. Dilutions as follows: HT-7 (1:2,500), JRF5 (1:2,500), BrdU (1:100), nestin (1:1,000), NeuN (1:1,000), SMI-312 (1:10,000), βIII-tubulin (1:1000), doublecortin (1:5,000) and MAP-2 (1:1,000). After washing with 0.1% Triton-X100 in PBS, the cells were incubated with biotinylated anti-goat, anti-mouse, anti-rabbit or anti-rat IgG (1:500, Vector) in blocking buffer, then with avidin-biotinylated peroxidase complex (Vectastain ABC Elite, Vector labs, Burlington, CA, USA). The peroxidase reaction was developed using 3,3’-diaminobenzidine (DAB), 0.3% H2O2 in Tris-HCl, pH 7.6. Alternative substrates for DAB, used as indicated in the results section, were Vector SG or VectaRed peroxidase substrate. For double labeling, the cells were treated with 3% H2O2 in PBS for 10 min after the first primary antibody staining was developed, then re-stained with the second primary antibody. Programmed cell death of hippocampal cultures was quantified by TUNEL staining (Promega, Leiden, the Netherlands) according
the manufacturers protocol. DNase digestion treatment was used as positive control. After completed staining, cells were counterstained with hematoxylin, dehydrated in graded ethanol and xylene solutions and mounted in DePex. All experiments were repeated on three or more independent cell preparations and cultures. At least 1500 cells per group were scored for each experiment, using a Axioplan 2 (Zeiss) microscope at 40x. Group comparisons were made with the Kruskal-Wallis method and subsequent post-hoc tests were performed using the Mann-Whitney test. Data are presented as average values ± standard error of the mean (mean ± SEM).

**Neurite outgrowth quantification**

Neurite outgrowth was assayed by culturing the cells in a filter-based, compartmentalized culture system, according to the manufacturers instruction (Chemicon, Heule, Belgium). Hippocampal neurons were seeded on laminin coated membrane inserts in 24 well-plates at a density of 100,000 cells per well and cultured in Neurobasal/B27 medium with glutamine and penicillin/streptomycin for 4 or 10 days, as described above. The membrane inserts were removed and the neurites present at the opposite side of the membrane were quantified and in some experiments visualized by immunostaining. Group comparisons were made with the Kruskal-Wallis method and subsequent post-hoc tests were performed using the Mann-Whitney test. Data presented as average values ± standard error of the mean (mean ± SEM).

**Antibodies**

Antibody HT-7 (Innogenetics, Gent, Belgium) specifically recognizes human tau, whereas antibody JN-RF.5 (generous gift from M. Mercken) reacts only with murine tau. Antibody RD3 (USBiological) specifically detects tau-3R (de Silva et al., 2003). Antibody R2 and RD4 (UsBiological) are specific for the second repeat domain and thereby for tau-4R isoforms (de Silva et al., 2003; Takuma et al., 2003). Antibody tau-5 (BD, Aalst, Belgium) detects all tau species. The Brd U antibody (Abcam, Cambridge, UK or Roche Diagnostics, The Netherlands) was used to detect newly generated cells of 1 month old. Antibodies against Nestin (Abcam, Cambridge, UK) and NeuN (Chemicon, Heule, Belgium) detect non-proliferating precursor cells and differentiated neurons, respectively. The antibody to doublecortin (C-18, Santa Cruz, CA, USA) detects young, migratory neurons. SMI-312 is a mixture of monoclonal antibodies (Sternberger, Lutherville, MD, USA) that stains medium and high molecular weight neurofilaments specifically in axons. Antibodies against βIII-tubulin (Promega, Leiden, Belgium), and MAP-2 (Sigma, St Louis, MI, USA) were used as indicated in the results section.

**Immunohistochemistry**

Unless otherwise noted, mice were anaesthetized (Nembutal) and transcardially perfused with ice-cold saline. Brains were immersion-fixed in 4%
paraformaldehyde for 24 h and used for vibratome sectioning (30 μm) or embedded in paraffin and sectioned serially (10 μm).

For BrdU immunohistochemistry, P30 mice were injected intraperitoneally with 5 mg/ml BrdU dissolved in 0.007 M NaOH/0.9% NaCl at a dose of 50 mg/kg, for seven consecutive days. The mice were sacrificed four weeks post-injection and BrdU-labeled nuclei were visualized as described earlier (Heine et al., 2004). After blocking endogenous peroxidase with 1% H₂O₂ in 0.05 M Tris buffered saline pH 7.6 (TBS) for 20 min and denaturing DNA in 50% hot formic acid and 2N HCl, free-floating sections were stained with the primary antibody mouse α-BrdU (1: 3000, Roche, Switzerland) diluted in phosphate buffer containing 0.1% bovine serum albumin (BSA), 0.3% Triton X-100 and 1% goat serum for 1 h at room temperature and then overnight at 4°C. Sections were then incubated with biotinylated sheep a-mouse IgG (1: 200) for 2 h, followed by 2 h with avidin-biotin complex (ABC) (1:1000) (Vector Laboratories, Amsterdam, The Netherlands). Chromogen was developed with diaminobenzidine (0.50 mg/ml in 50 mM Tris.HCl, 0.01% H₂O₂) for 30 min. The sections were washed, mounted, counterstained with hematoxylin, dehydrated in graded alcohols and xylene and covered with Entellan (Merck, Brussels, Belgium).

For doublecortin staining, sections were incubated for 1 h at room temperature and overnight at 4°C with the primary antibody 1:1500 in 0.25 % gelatin, 0.5% triton-X-100, 0.1 M TBS (defined hereafter as Supermix). The biotinylated anti-goat IgG secondary antibody (1:200 in Supermix) was applied for 2 h at room temperature. Reaction with avidin-biotin complex, as above, was enhanced by incubation with biotinylated tyramide, 1:500, and 0.01% H₂O₂ for 30 min, followed by another round of amplification with avidin-biotin and DAB chromogen development.

Tau-3R detection was done with the same protocol, using 0.05 M TBS, 1:100 dilution of the primary antibody (RD3, USBiological) and a 15 min microwave pretreatment at pH6 (0.01 M citrate buffered). Tau-4R detection was done using 0.05 M TBS 1:10 dilution of the primary antibody (RD4, USBiological) and a 15 min microwave pretreatment at pH9 (0.01 M Tris HCl). DAB staining was intensified using 0.04% nickelammonium sulphate.

Terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated UTP nick-end labelling (TUNEL), which identifies cells undergoing programmed cell death, was performed on 10 μm thick paraffin sections. Sections were first re-hydrated and nick-end labeled with TdT and biotin-16-dUTP as previously described (Lucassen et al., 2004). Sections were washed, mounted, counterstained with hematoxylin, dehydrated in graded alcohols and xylene and covered with Entellan. At least 4 individual mice were used for each age group and at least 3-4 separate sections from each for qualitative and quantitative analysis. All statistical analyses were carried out using a 2-way ANOVA.

**Stereological analysis**

Quantification of the number of neurons was performed by first estimating the volume of the region by means of the point-counting method.
and Cavalieri’s principle. Neurons were counted by registering cells within 3-dimensional optical disectors systematically and randomly spaced throughout the region. (West, 1993). Post-processing section thickness was measured at each dissector location. Only cells with typical neuronal morphology, including a clearly delineated nucleolus, were counted within the optical dissector frame using a 100x oil-immersed objective.

A series of the developing hippocampus was collected at ages p7, p15, p28, p60 and p180. For every age, at least 15 systematically sampled sections per animal were analyzed. For each genotype, 3 mice per age were analyzed. For the DG, the molecular, granular and hilar regions were combined, for the CA, the pyramidal cell layer was analyzed. To address whether the early alterations were lasting, cytoarchitectural changes were also assessed at the age of 6 months in an independent and differently processed cohort (n=7). Total numbers of cells in specified brain regions were quantified in 30 μm sagittal cryostat sections stained with cresyl-violet. A series of systematically sampled sections throughout the hippocampus was prepared of which every 10th section was analyzed, totalling 10-15 independent sections per mouse brain.

To address the contribution and possible parallels with a tau knockout condition, Ki-67 and DCX immunostaining and cytoarchitectural analysis was also performed on “regular” tau deficient mice of the same age. These mice were generated as described before in detail (Dawson et al., 2001) and immunohistochemical procedures were identical to those for the WT and KOKI mice, as described above.

**Golgi impregnation staining**

In order to study structural properties of individual neurons, Golgi impregnation was performed. To that end, mice were decapitated, their brains removed and placed in Golgi-Cox solution (1.04 % potassium dichromate, 1.04 % mercury chloride, 0.83 % potassium chromate in double distilled H2O). After rinsing in H2O for 5 min and dehydration in 70 % EtOH (overnight), 96 % EtOH (overnight), 100 % EtOH (8 h), 1:2 EtOH/ether overnight, brains were saturated by consecutive overnight incubations in 3 %, 6 % and 12 % celloidine. The celloidine was cleared with chloroform before 200 μm coronal sections were cut. Staining was developed by a 5 min wash in H2O, 30 min in 16 % ammonia, a 2 min wash in H2O, 7 min incubation in 1 % sodium thiosulphate, a 10 min rinse in H2O, followed by dehydration for 5 min in 70 % EtOH, 5 min in 96% EtOH, 5 min in butanol, and 5 min in Histoclear (Biozym, Landgraaf, The Netherlands). Sections were mounted in Histomount (National diagnostics, Atlanta, Georgia).

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 supra pyramidal blade of the DG. If neurons were completely stained and horizontally orientated within the section, 99 Z-stacks of 1 μm 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). Statistical analysis was carried out using the Mann-Whitney test.

**Electrophysiology**

After decapitation, brains were quickly 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 and 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 with a tissue chopper; for perforant path recordings, 400 μm horizontal forebrain sections were obtained using a vibroslicer (Leica VT 1000S). After 1 h of incubation at RT in oxygenated recording ACSF (120 mM NaCl, 3.5 mM KCl, 1.3 mM MgSO4, 1.25 mM NaH2PO4, 1.25 mM CaCl2, 10 mM D-glucose and 25 mM NaHCO3), slices were transferred to a recording chamber and perfused with oxygenated ACSF at 31.5°C.

Bipolar, stimulating electrodes isolated with stainless steel were placed in the Schaffer collaterals or in the perforant path to record field excitatory postsynaptic potentials (fEPSPs) using a glass microelectrode (2-5 MO filled with ACSF) placed in the stratum radiatum of the 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, St Louis, MI, USA) to the ACSF.

Before baseline recording commenced, the maximal fEPSP amplitude and slope were determined by gradually increasing the stimulus intensity (interstimulus interval was 60s) until the response was saturated. The relationship between the stimulus intensity and the evoked response was fit to a Bolzmann equation: R(i)=Rmax/(1+exp((i-ih)/S)), in which R(i) is the response at stimulus intensity (i). Rmax is the maximal response, ih is the intensity at which the half-maximal response is observed and slope factor S is the index that describes the slope of the stimulus-response curve. ih 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 less than 1.5 mV were rejected.

After baseline recordings, paired-pulses with interstimulus 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 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. All statistical analyses were carried out using a 2-way ANOVA.

**Behavioral testing**

Mice were housed in groups of 3-7 animals in the behavioral testing room at least 30 min before the experiments commenced. Two cohorts of 5 and 9 weeks of age old WT FVB/N and tau-KOKI mice were subjected to the following behavioral tests.

First, general motor ability was assessed by rotarod testing with a revolving, horizontal rod of diameter 3.2 cm (Med Associates, Georgia, Vermont). After one (5 week old mice) or two (9 week old mice) training sessions of 5 min at 16 revolutions per minute (rpm), the mice were placed on the rod and the speed was increased from 4 to 40 rpm over 3 min. The time that the mice stayed on the rod was recorded electronically.

Exploratory and motor activities were determined in an open field setting. Each mouse was placed for 5 min on an elevated, white opaque plastic surface of 52x52 cm without bordering-walls. The travel-path was recorded on 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 and in the periphery was calculated.

Hippocampus dependent learning and memory were analyzed using the object recognition test (ORT) as previously described (Dewachter et al., 2002; Boekhoorn et al., 2006). Individual mice were habituated on day 1 for two sessions of 5 min in a plastic box (52x52x40 cm), with a white opaque floor and black walls softly illuminated from below. On day 2, in the acquisition phase mice were familiarized for 10 min with two identical objects placed in two adjacent quadrants of the box. The time that the animal spent exploring an object with its snout directed towards the object within nostril reach, was recorded manually. Travel paths were recorded and analyzed using dedicated software. In the actual memory-retention trial of 10 min duration, performed after a delay of either 1 or 3.5 h, the mice were confronted with one familiar and one novel object. The novel object and its position on either side of the familiar object was randomized to avoid preferences not based on novelty. The level of discrimination (d2) was calculated by the equation $d2 = (b-a)/(b+a)$, in which a and b are the exploration times spent on the old and novel objects, respectively. All statistical analyses were carried out using a 2-way ANOVA.

**Results**

**Generation of tau transgenic mice deficient in murine tau and expressing human tau-4R/2N.**

The tau knock-out/knock-in (tau-KOKI) mouse strain was designed to inactivate the endogenous murine tau gene and replace it by a single copy of the thy1-tau-4R recombinant DNA construct (Spittaels et al., 1999; Terwel
et al., 2005). This construct was incorporated into a genomic fragment of 4 kB, previously used to knock-out the mouse tau gene activity completely (Harada et al., 1994). This construct was targeted to exon1 of the mouse tau gene, inactivating it (knock-out) and inserting a single copy of the thy1-tau-4R construct (knock-in). This single copy number generated low expression levels of the human tau-4R transgene (Fig.1A) preventing the axonopathy inflicted by tau-4R overexpression (Spittaels et al., 1999; Terwel et al., 2005).

Recombination in ES cells yielded 336 independent hygromycin resistant ES cell colonies, of which six were correctly recombined as genotyped by Southern blotting. One ES cell-line yielded appreciable coat-colour chimeras, from which one male offspring was mated to FVB/N females to produce F1 offspring with the correctly recombinant tau gene.

Heterozygous breeding was performed for eight generations with FVB/N mice to finally breed homozygous tau-KOKI mice in this background that were maintained as an independent strain by inbreeding. Mice were initially genotyped by Southern blotting (Fig. 1B) and later routinely identified by two independent PCR reactions, detecting the thy1-tau-4R construct (positive criterion) and the normal exon1 of the murine tau gene (negative criterion) (Fig. 1C). Complete deficiency of murine tau was demonstrated at the protein level by western blotting of brain tissue from tau-KOKI mice with antibodies against human and murine tau (Fig. 1D). Only human tau-4R (htau-4R) was expressed (Fig. 1D).

The post-natal expression of three different mouse tau-4R isoforms, i.e. tau-4R/0N, 4R/1N and 4R/2N, in brains of WT FVB/N mice is thus replaced solely by the human tau-4R/2N isoform in tau-KOKI mice. It must be noted that the level of expression of the human tau-4R transgene is never higher than that of the corresponding mouse isoform and in this respect the tau-KOKI mice do not "overexpress" human tau (Terwel et al., 2005) but rather reflect a knockdown situation.

Until six months of age, homozygous tau-KOKI mice were phenotypically indistinguishable from WT FVB/N mice with respect to body weight, behaviour in the home-cage, rearing and grooming, fertility, frequency and size of litters. Only late in life do tau-KOKI mice become hampered in their sensory motor functions, most likely related to 4R expression (Terwel et al., 2005).
Tau isoform expression affects hippocampal development

A. Schematic representation of recombinant DNA construct (upper) used to target exon 1 (black box) of the mouse tau gene (middle). Triangles represent loxP sites that are not relevant for the current study. BSSK+ denotes the pBluescript cloning vector, PGK-hyg represents the phosphoglycerate kinase-hygromycin marker gene used for positive selection of the ES cells. The thy1-tau-4R construct was introduced into the unique Ncol restriction site in exon 1 of the tau genomic fragment. Correct homologous recombination of exon 1 of the tau gene (lower panel) as demonstrated by southern blotting of genomic DNA from ES cells or mouse tails, restricted with BamHI (B) and KpnI (K), respectively.

B. Southern blot with the 3 probes of mouse tail DNA isolated from non-transgenic (WT), tau-KOKI heterozygous (KOKI/het) and tau-KOKI homozygous (KOKI/hom) mice restricted with KpnI. The size of the restriction fragments is indicated on the left, whereby the upper band (11.2 kb) was diagnostic for the recombinated tau gene.

C. PCR for the thy1-tau-4R transgene and for exon 1 of the mouse tau gene in non-transgenic (WT), tau-KOKI heterozygous (KOKI/het) and tau-KOKI homozygous (KOKI/hom) mice. Size of amplicons is indicated on the left.

D. Western blots of brain extracts from mice (aged 6 weeks) with antibody tau5 recognizing both murine and human tau; antibody JRF-5 specific for murine tau; antibody HT-7 specific for human tau. Equal amounts of brain protein extract were loaded from non-transgenic (WT), tau-KOKI heterozygous (KOKI/het) and tau-KOKI homozygous (KOKI/hom) mice. Apparent molecular weight as indicated (kDa).

Figure 1. Generation of tau-KOKI mice
Effects of hTau-4R expression on primary hippocampal cultures derived from tau-KOKI mice

Given the complexity of the described model we first set out to study the effects of Tau knock-out and subsequent Tau-4R or -3R introduction in vitro, where they can be separated. To this aim primary hippocampal cultures derived from E17.5 tau-KOKI embryos were used. Htau-4R could not be detected at 4 DIV in tau-KOKI cultures (Fig. 2A-B, color). Between 4 DIV and 10 DIV, htau-4R expression increased and reached stable levels at 10 DIV. Expression of endogenous murine tau was absent at all times in these cultures, whereas western blotting revealed the expected full range of different murine tau isoforms in lysates of WT cultures, with an excess of murine htau-4R at 4 DIV and exclusively htau-4R at 10 DIV (Fig. 2A). These biochemical data were complemented and confirmed by parallel immunocytochemical staining (Fig. 2B).

Proliferation is suppressed and neuronal proloferation promoted in the absence of tau-4R

Hippocampal cultures were labeled and stained for the S-phase marker BrdU and for the neuronal marker NeuN. At 4 DIV, the BrdU labeling index (LI) was significantly increased (p = 0.00005) in tau-KOKI compared to WT cultures, as was the ratio of the precursor marker Nestin (p = 0.005). In contrast, the ratio of NeuN positive (NeuN+) cells was decreased (p = 0.0018) (Fig. 2C-E). These data indicated that in the absence of protein tau, the population of proliferating precursors (BrdU+ cells and Nestin+ cells) significantly expands while differentiation into a mature neuronal phenotype (NeuN+ cells) is significantly reduced. At 10 DIV, htau-4R expression was abundant and indeed the BrdU-LI in tau-KOKI cultures decreased to levels similar to those in WT cultures, while Nestin expression was decreased compared to WT cultures of the same age (p = 0.0018). Moreover, the percentage of NeuN+ cells was increased (p = 0.0209). Thus, tau-4R expression reduces proliferation and cell birth and promotes neuronal differentiation.

TUNEL staining revealed the ratio of cells undergoing programmed cell death in 4 DIV cultures to be less than 10%, with no differences observed between cultures from tau-KOKI and WT mice (data not shown). At 10 DIV, the number of TUNEL positive cells was higher than at 4 DIV, but still not significantly different between the two genotypes, indicating that programmed cell death was contributing neither importantly nor differentially to the observed differences between tau-KOKI and WT primary hippocampal cultures.
Tau isoform expression affects hippocampal development

A

Total tau (tau5)

4 DIV 10 DIV

Human tau (HT-7)

4 DIV 10 DIV

64 kDa

B

Total tau (tau5)

WT Tau-KOKI WT Tau-KOKI

Human tau (HT-7)

WT Tau-KOKI

4 DIV 10 DIV

C

BrdU+ (%)

4 DIV 10 DIV

WT = Tau-KOKI

D

NeuN+ cells (%)

4 DIV 10 DIV

WT = Tau-KOKI

E

nestin+ cells (%)

4 DIV 10 DIV

WT = Tau-KOKI

F

BrdU NeuN nestin

G

Neuroprotein levels (%)

4 DIV 10 DIV

WT = Tau-KOKI

H

WT tau-KOKI

4 DIV 10 DIV
Figure 2. Tau expression affects proliferation, differentiation and axonal outgrowth in primary hippocampal cultures from tau-KOKI mice

A. Western blotting for total tau (antibody tau5) and human tau (antibody HT-7) in cell lysates from non-transgenic (WT) and tau-KOKI primary hippocampal cultures analyzed at 4 DIV and 10 DIV. Asterisks denote unspecific bands reacting with the secondary antisera. Apparent molecular weight as indicated (kDa). Tau is expressed at 10 DIV but not at 4 DIV in tau-KOKI cultures, in WT cultures the predominant tau isoform is of the 4R variant.

B. Immunocytochemical staining for total tau (antibody tau5) and human tau (antibody HT7) shows again that tau is expressed at 10 DIV but not at 4 DIV in tau-KOKI primary cultures.

C. The BrdU labeling index (LI) in hippocampal cell cultures at 4 DIV is increased in tau-KOKI mice but normal at 10 DIV compared to non-transgenic (WT) mice at 4 and 10 DIV.

D. The percentage of NeuN positive cells is decreased in hippocampal cell cultures from tau-KOKI mice at 4 DIV but increased at 10 DIV compared to non-transgenic mice (WT).

E. The percentage of nestin positive cells in hippocampal cell cultures from tau-KOKI is increased at 4 DIV but decreased at 10 DIV compared to non-transgenic (WT) mice.

F. Representative BrdU, NeuN and nestin staining of primary hippocampal cultures from tau-KOKI mice at 4 DIV.

G. Relative neurite protein levels are decreased in hippocampal cell cultures from tau-KOKI at 4 DIV but normal at DIV 10 compared to non-transgenic (WT) mice.

H. Representative axon-specific staining with antibody SMI-312 showing delayed axonal outgrowth in primary hippocampal cultures from tau-KOKI mice at 4 DIV, which is completely restored at 10 DIV compared to non-transgenic (WT) mice.

In total, more than 1500 cells per group were scored from at least 3 different cell culture preparations. Data presented as normalized average OD values ± SEM. Statistical analysis by Mann-Whitney test, * = p = 0.05 and ** = p = 0.01 relative to control.

**Tau-4R promotes axonal and neuritic outgrowth**

The nature of the delayed neuronal maturation and differentiation in tau-KOKI cultures was further explored by analyzing neurite and axonal outgrowth in vitro. Quantification of total neurite outgrowth of primary hippocampal cultures showed that Tau-KOKI primary cultures had significantly lower neurite outgrowth at 4 DIV compared to WT cultures (p = 0.0495). At 10 DIV, when Tau-4R is robustly expressed in tau-KOKI cultures, this phenotype was almost fully rescued and similar to WT cells (Fig. 2G).

We subsequently immunostained primary cultures with the axon-specific marker SMI-312 (Fig. 2H). The results showed a marked decrease in axonal development at 4 DIV, due to the absence of tau and consistent with the delay in differentiation. Again, this delay of the tau-KOKI cultures was restored at 10 DIV when htau-4R was fully expressed. No marked differences were observed between tau-KOKI and WT primary cultures when stained for the dendritic marker MAP-2, for the early neuronal marker doublecortin or for βIII-tubulin (data not shown).

**The effects of tau-4R are isoform specific**

Next, we investigated whether the changes in proliferation and neuronal differentiation in tau-KOKI primary cells could be restored by introduction of protein tau expression (i) at an earlier time point in neuronal development and/or (ii) in an isoform-specific mode. We transfected tau-KOKI primary cultures with vectors to express either the human tau-3R/2N or tau-4R/2N isoform. Transfection by electroporation was performed prior to plating. At 4 DIV, the cultures contained comparable levels of either the htau-3R or htau-4R isoform (Fig. 3A-B).
Tau isoform expression affects hippocampal development

A. Western blotting for total tau (antibody tau5) in cell lysates from tau-KOKI primary hippocampal cultures. Untransfected (U), vector-only (V), htau-3R transfected (tau-3R) and htau-4R (tau-4R) transfected neurons. Apparent molecular weight as indicated (kDa).

B. Immunocytochemical staining for total tau in vector-only transfected, htau-3R transfected and htau-4R transfected E17.5 hippocampal tau-KOKI cell cultures, showing tau constructs are being expressed.

C. BrdU labeling index (LI) shows proliferation is only reduced in htau-4R transfected E17.5 hippocampal tau-KOKI cell cultures, not in htau-3R transfected cultures compared to cultures expressing the empty vector.

D. The percentage of NeuN positive cells is only increased in htau-4R transfected E17.5 hippocampal tau-KOKI cell cultures, not in htau-3R transfected cultures compared to cultures expressing the empty vector.

E. Relative neurite protein levels shows that both htau-3R and htau-4R transfection increase neuritic outgrowth in E17.5 hippocampal tau-KOKI cell cultures compared to cultures expressing the empty vector, although the effect of htau-4R is more robust.

In total, more than 1,500 cells per group were scored from at least 3 different cell preparations. Data are normalized average OD values ± SEM. Statistical analysis by Kruskal-Wallis test and by Mann-Whitney test as post-hoc test, "*" = p = 0.05 and "**" = p = 0.01 in comparison to vector control values, "#" = p = 0.05 and "##" = p = 0.01 in comparison to htau-3R transfected cell values.
Chapter 3

Tau-KOKI primary cultures transfected with htau-4R, but not those with htau-3R, showed a reduced BrdU LI (p < 0.05), similar to that of WT cultures at 4 DIV (Fig. 3C). Similarly, expression of htau-4R but not of htau-3R, strongly increased the fraction of NeuN+ cells in tau-KOKI cultures compared to that of WT cultures (p < 0.05) (Fig. 3D). In addition, transfection of tau-KOKI primary cultures with either of the tau isoforms significantly increased neurite outgrowth at 4 DIV (p < 0.05) (Fig. 3E), implying that both isoforms have the capacity to promote neurite extension and/or stabilization. Expression of the htau-4R isoform did, however, generate a 3-fold larger increase in neurite protein levels than the htau-3R isoform, indicating that in this respect again the htau-4R isoform dominated over htau-3R.

In addition, subsequent staining with the axon-specific antibody cocktail SMI-312 revealed that only htau-4R promoted axonal outgrowth (data not shown). These experiments suggest that the function of tau-3R during fetal and early post-natal murine maturation is far less prominent than that of tau-4R and its absence may have well been compensated by other MAPs (Harada et al., 1994; Dawson et al., 2001).

Developmental regulation of tau expression in vivo

In order to study the role of different tau isoforms in early development, neurogenesis and hippocampal maturation in vivo, we investigated the tau-KOKI mouse model. This allows investigation of relevant neurons (e.g. cells in the DG) in their natural environment as well as an examination of complex functions in which the DG circuit participates. First, tau expression levels were analyzed in a developmental series. As show in figure 4A, tau-4R/2N expression was initiated around P12 to reach robust levels around P21 in both KOKI and WT mice (Fig. 4A). The timeline of tau-4R expression was validated by western blotting with the tau-4R isoform specific antibody R2 (Takuma et al., 2003) (Fig. 4A). This time-window overlaps with that of the isoform-switch from tau-3R to tau-4R in WT mice (Takuma et al., 2003) and with post-natal maturation of the murine hippocampus (Stanfield and Cowan, 1979).
Tau isoform expression affects hippocampal development

A. Timeline of expression of protein tau in brain of non-transgenic and tau-KOKI mice. Total brain lysates from mice at indicated ages as analyzed by Western blotting with antibody tau5 for human and mouse tau and antibody R2 specific for the second microtubule binding domain in tau-4R. Asterisks denote unspecific bands detected by the secondary antiserum. Apparent molecular weight is indicated (kDa). In non-transgenic mice before birth only 3R isoforms are being expressed. Between p1 and p15 also tau-1N/4R expression is high, reducing after p15. At this age expression of tau-0N/4R and tau 2N/4R increases. In tau-KOKI mice tau 2N/4R expression starts already at p12 (not shown).

B. Tau-3R immunohistochemistry in the developing hippocampus. Tau-3R immunohistochemistry was present in the WT at p7. At p15 it was only detectable in the hippocampal DG. At p28 it was detected in a subpopulation of cells in the SGZ, from p60 onwards tau 3R expression was virtually absent (data not shown). Tau-3 was not expressed in tau-KOKI mice.

Figure 5. Tau-4R expression in WT and KOKI brain

A. In the WT hippocampus, highest tau 4R expression is found in the hilus and CA3 stratum radiatum; staining is prominent in the mossy fibers, consistent with previous observations on tau (Dawson et al.,) and the concept of tau enrichment in axons.

B. Tau-4R expression is additionally enriched in large axonal tracts of e.g. the brainstem (pons), both in WT and in KOKI brain (picture from WT).

C-F Differences in tau-4R expression between WT (left column) and KOKI (right column) at higher power.

C. Within the hippocampal DG subfield, tau-4R expression is markedly reduced in the hilus. Also, an inverse tau-4R expression is evident in the DG molecular layer. Tau-4R expression in the WT is high in the inner molecular layer but lower in the outer molecular layer, tau-4R expression in the KOKI mice appears low in the inner molecular layer and higher in the outer molecular layer.

D. Within the CA3 subfield, the considerable expression levels of tau-4R in the mossy fiber tract (stratum radiatum) in WT are absent in KOKI mice.

E. Within the CA1 subfield tau-4R expression is low in both WT and tau-KOKI mice.

F. In the basal ganglia, but also in various other areas including the hippocampus and cortex (data not shown) small 4R positive cells are seen with a high perinuclear expression of tau-4R (arrows). These cells were only present in WT mice from p28 onwards but were not observed in tau-KOKI mice. Their morphology resembles that of glial cells although their shape and localization suggests they might also be interneurons.

Picture A is taken from a p15 brain, picture C from p180 WT and KOKI brains. The rest of the pictures are taken from p28 brains. Abbreviations: Hippo: hippocampus; BrSt: Brainstem; OML: outer molecular layer; IML: inner molecular layer; SL-M: stratum lacunosum-moleculare; SR: stratum radiatum; SP: stratum pyramidale; SO: stratum oriens; BasG: basal ganglia.
Chapter 3

WT

KOKI

A Hippo

B BrSt

C DG

D DG

E CA1

F BasG

OML IML GCL Hilus

SL-M SR SP SO

SL-M SP SO

SL-M SP SO
Tau isoform expression affects hippocampal development

A

KI-67

p15

WT
Tau-KOKI

WT
Tau-KOKI

B

TUNEL

Volume DG

Volume pyramidal layer

DT
WT
Tau-KOKI

DT
WT
Tau-KOKI

p7
p15
p28

p7
p15
p28
Figure 6. Developmental dynamics of cell-birth and apoptosis in non-transgenic and tau-KOKI mice

A. Example of Ki-67 immunohistochemical staining for proliferating cells in the dentate gyrus. Quantitative analysis at different developmental stages reveals a significant increase in Ki-67 positive proliferating cells starting at p15 in the hilus, CA1 and CA3 and at p28 in the SGZ and CA1. After p15 apoptosis is a rare event.

B. Analysis of apoptotic cells by tunel staining reveals a significant reduction of apoptosis at p15 in the CA. In the DG the same trend appears. As expected, 3R staining was absent in htau-KOKI mice at any age (data not shown). In contrast, tau 4R IR was apparent at p15 in both WT and KOKI mice and remained visible until later ages (Fig 5). Tau 4R IR was present from p15 onwards in both the KOKI and WT cortex, basal ganglia (Fig 5F) and brainstem (Fig 5B). However, within the hippocampus (Fig 5A) DG (Fig 5C) and CA3 (Fig 5D) hippocampal 4R expression level in the htau-KOKI mice was generally lower than in WT, also at later developmental stages. Tau-4R expression in the DG molecular layer was switched from high expression in the inner molecular layer in WT mice towards high expression in the outer molecular layer in tau-KOKI mice (Fig 5C). In the CA1 tau-4R expression was extremely low in both tau-KOKI and WT mice (Fig 5E). Concluding, unlike the CA1 where tau expression is low in both WT and tau-KOKI the CA3 and DG of htau-KOKI mice resembled the early stages seen in the cell cultures described above, as tau-4R expression is clearly reduced compared to WT while tau-3R expression is largely absent. Remarkable was the high tau-4R somal staining in small cells (possibly interneurons) in WT but never in tau-KOKI mice (Fig 5F). These cells were abundant in the basal ganglia but also observed in the CA3 and DG. Immunoblotting with a panel of phospho-specific antibodies (AT-180; AT-8; AT-270 and AD-2) revealed that except for AT-180 from the second postnatal week, tau phosphorylation is not increased in these mice (data not shown).

Increased proliferation and reduced apoptosis during early postnatal development lead to increased volume and cell number in the tau-KOKI hippocampus.

To assess the effect of tau-4R knock down on proliferation in vivo we quantified Ki-67 expression in tau-KOKI hippocampus. The number of Ki-67+ proliferating cells was significantly increased in the KOKI at p15 in the hilus and the CA area, and at p28 in the SGZ. (Fig. 6A). In addition, apoptosis was
Tau isoform expression affects hippocampal development

Figure 7. Cell birth, survival and hippocampal size in tau-KOKI, tau-KO and non-transgenic mice at 2 months of age

A. Pictures of the immunohistochemical markers doublecortin (DCX), staining young migrating neurons, and BrdU, a marker for survival, used to label 3-4 wks old cells.
B. The amount of Ki-67 positive cells in the DG is not different from WT in tau-KOKI mice. However, the total amount of DCX positive cells is increased in both the SGZ and GCL of tau-KO mice compared to controls.
C. The amount of Ki-67 positive cells in the DG is not different from WT in tau-KO mice, except for the CA where cell-birth is increased in tau-KO mice. However, the total amount of DCX positive cells is increased in both the SGZ and GCL of tau-KO mice compared to controls.
D. The total amount of BrdU positive cells is increased in the SGZ in tau-KOKI mice. The total amount of BrdU positive cells is increased in both the SGZ and GCL of tau-KOKI mice.
E. DG volume is increased in tau-KOKI mice. This is not accompanied by any changes in cellular density; however, the total number of cells has increased. Also the CA pyramidal volume is increased.

HIL: hilus, GCL: granular cell layer, SGZ: subgranular zone. Data presented as average values of total positive cell number per hemisphere ± SEM. Statistical analysis by 2-way ANOVA. Statistically significant differences (p<0.05) indicated by asterisks.

Reduced in tau-KOKI mice at p15 (Fig. 6B). At p60, ki-67 expression in KOKIs was not different from WT (Fig 7B), as was the case at 6m of age (data not shown).

To study whether the increased proliferation and reduced apoptosis would affect hippocampal size or cytoarchitecture in tau-KOKI mice, the DG and CA pyramidal layer volume were studied stereologically during early maturation (Figure 6C). At p7, DG volume was significantly reduced in Tau-KOKI mice. A more detailed analysis showed that this was due to a reduction of size particularly in the hilar region (data not shown), an effect that is likely attributable to the lack of tau-3R expression (a situation that in vitro reduces neuritic growth). At p15 there were no significant differences in both DG and CA volume between the two genotypes; apparently, at this age the earlier reduction in size had been overcome. At p28, DG volume was even significantly enlarged in htau-KOKI mice. Also at two months of age, volumes of both the DG as well as the CA pyramidal cell layer were significantly increased in htau-KOKI mice, in the absence of significant changes in cellular density demonstrating the presence of higher cell numbers (Fig. 7E).

BrdU labeling of adult generated cells after one month survival (Fig 7A,D, color figure) confirmed that indeed increased numbers of newborn cells at p28 did survive in KOKI mice. Additional immunolabeling for DCX, a marker for immature neurons, showed that increased numbers of young cells had adopted a neuronal phenotype (Fig 7A, B). To study whether this was indeed a lasting effect, a separate and larger cohort of animals was investigated at 6 months of age using a stereological/disector approach that again showed both volume and number of neurons in the hippocampus of tau-KOKI mice to be significantly increased by about 20%. The increased volume in CA1 (p<0.005) was paralleled by increased numbers of pyramidal cells in CA1 (p<0.001) and of granular cells in the DG (p<0.003) (data not shown).
The present data suggest that in htau-KOKI mice between p7 and p28 a net increase in cell number occurs, with lasting consequences into adulthood. To possibly relate these transient effects on neurogenesis to tau-knock down, we also studied proliferation and survival in regular tau KO mice (Dawson et al., 2001). At 2 months of age, DCX labeling was increased in tau KO mice in the absence of changes of Ki-67 labeling in the DG. In the CA1 of these mice, however, proliferation was increased (Fig 7C).

These data suggest that tau-4R is involved in promoting differentiation in the early postnatal phase. A decreased or delayed onset of tau-4R expression may hence explain a slower rate or onset of differentiation/maturation. This is supported by the reduced dendritic length in KOKI mice representing a larger proportion of relatively younger cells, as well as by the alterations in the cell-birth and neurogenesis rates during development. Given their timing, i.e. during a period when neurogenesis is maximal, these changes have far reaching and longlasting effects and are likely to contribute to the increased hippocampal volumes and cell numbers at both 2 and 6 months of age.

Reduced dendritic outgrowth in tau-KOKI DG, but not CA1
As mentioned above, 4R tau expression was reduced in the htau-KOKI hippocampal CA3 region and DG but not in the CA1 area (Fig4B). As this condition led in vitro to a reduced neuritic outgrowth, we reasoned that also in vivo neuritic architecture could be altered. Analysis of Golgi-Cox silver impregnated cells revealed a significant decrease in dendritic arborization mainly in the DG of tau-KOKI mice (p = 0.001), and a reduction in the overall dendritic tree (p = 0.001) (Fig. 8A-C). The number and segment lengths of the dendrites were similar in tau-KOKI and WT mice, although the number of filopodia was decreased in tau-KOKI mice (p = 0.014) (Fig. 8D-F). None of these parameters were altered in the CA1 region.
Figure 8. DG Dendritic morphology of non-transgenic and tau-KOKI mice at 2 months of age using Golgi-Cox impregnation and 3D analysis

A. The total dendritic tree is reduced in size in tau-KOKI mice DG (n=45 for both genotypes).
B. However the total dendrite number per cell (branches directly deriving from the soma) is unaffected DG (n=45 for both genotypes).
C. But the average dendritic length (length per branch deriving from the soma) per cell is reduced in tau-KOKI mice (tau-KOKI: n=101, WT: n=91).
D. The average dendritic length of tau-KOKI mice is reduced by a reduction in the number of branchpoints per dendrite (tau-KOKI: n=101, WT: n=91).
E. Not by a change in the inter- or terminal-segment length(tau-KOKI: n=41, WT: n= 40).
F. The average number filopodia per 20 µm counted at 90-110 µm from the soma, but not the number of spines was reduced in tau-KOKI cells (tau-KOKI: n=41, WT: n= 40).

Data presented as average values ± SEM. Statistical analysis of spines and filopodia was by 2-way ANOVA. The rest of the data were analyzed using a Mann-Whitney U test. Statistically significant differences (p<0.05) indicated by asterisks.
No differences in LTP in CA1 and DG

Next, we set out to define the possible functional consequences of an enlarged hippocampal formation, the occurrence of more and relatively younger neurons and the reduced size of the individual dendritic tree in the DG. In order to closely correlate functional properties with morphological alterations we recorded LTP in both the CA1 and DG in the same mice as were used for the neurogenesis analysis described above. The three major determinants of basal transmission -as fitted with a Boltzmann equation-, i.e 1) maximal amplitude or slope, 2) half maximal stimulation intensity and 3) a factor proportional to the slope of the Boltzmann curve, were not different in CA1 nor DG between tau-KOKI and WT mice (Table 1), neither were any differences found in LTP at any time-point during one hour recordings (Fig. 9A).

<table>
<thead>
<tr>
<th></th>
<th>WT CA1</th>
<th>KOKI CA1</th>
<th>WT DG</th>
<th>KOKI DG</th>
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<tbody>
<tr>
<td>Slope</td>
<td></td>
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<td>$R_{\text{max}}$ (mV/ms)</td>
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Table 1: Basal neurotransmission is unchanged in tau-KOKI mice, irrespective whether it was recorded in the CA1 or in the DG.

Values are mean ± SEM. $R_{\text{max}},$ Maximal response; $I_h,$ half maximal stimulation intensity. Averages are of the same animals as LTP was recorded in.

Increased memory performance in tau-KOKI mice

In view of the occurrence of motor deficits particularly in older tau transgenic mice, we first tested various general behavioral and motor parameters before addressing memory function. Assessment of open field performance revealed a reduced level of anxiety in tau-KOKI mice as indicated by the increased time spent in the center of the open field (Fig. 9B). However, locomotor activity of tau-KOKI mice was unaffected in the open field (Fig. 9C) and also in the rotarod test tau-KOKI mice performed identical to WT mice (Fig. 9D).
Figure 9. Functional analysis of tau-KOKI mice and non-transgenic controls at 2 months of age

A. In the absence of changes in basal parameters (data not shown) LTP is unaffected in both the CA1 and DG of tau-KOKI mice compared to non-transgenic (WT) mice. 20 recordings at 1h determined the baseline of which the average was set at 100%, before induction of LTP by theta burst stimulation at t=0. Data presented as average values ± SEM. Statistical analysis by 2-way ANOVA.

B. Tau-KOKI mice spend more time in the center of the field, indicative of reduced anxiety.

C. Tau-KOKI locomotor activity is unaffected as indicated by their total traveled distance in the open field.

D. Tau-KOKI mice had no signs of motor-impairments, indicated by a good performance in the rotarod test.

E. Both Tau-KOKI and non-transgenic (WT) mice had a good performance using the object recognition test with an interval of 1h.

F. Whereas in a object recognition paradigm with a 3.5h interval non-transgenic mice had no recollection of the familiar object anymore, object recognition was still very high in Tau-KOKI mice.

G. Since explorative behavior of Tau-KOKI mice in the training phase of the object recognition test was unaffected changes in the testing phase are more likely the result of changes in memory than to altered explorative behavior due to reduced anxiety.
Tau isoform expression affects hippocampal development

**Figure 9.** Behavior at 5 weeks of age

A. Contrary to the animals recorded at 2 months of age tau-KOKI mice of 5 wks of age do not behave differently from WT mice in the open field test.
B. Also their performance in the rotarod was indistinguishable from WT animals.
C. Yet their object recognition performance with a 3.5h interval between training and testing was significantly improved (p = 0.037).

Memory performance was assessed using the object recognition test (ORT) as this is a hippocampus-dependent task of memory-retention that generates low stress levels, depends on visual as well as tactile stimuli, and is suitable for transgenic mice (Dewachter et al., 2002; Boekhoorn et al., 2006). All mice were first familiarized with one object, and after a delay of either 1 or 3.5 hr, they were confronted with the familiar and with a novel object. The level of discrimination (d2) was defined by the time spent exploring the familiar object relative to the novel one.

Tau-KOKI mice performed similarly to WT mice at the 1 hr delay ORT test, with high d2 values, indicating that all mice remembered the familiar object well (Fig. 9E). At the 3.5 h delay test, the tau-KOKI mice still recognized the familiar object, with d2 values similar to those at 1 hr. In contrast, the WT mice failed to distinguish the familiar from the novel object and spend equal amounts of time exploring both objects after 3.5 hr delay (p = 0.021) (Fig. 9F). This index of superior memory retention of the tau-KOKI mice in this task was observed in two independent experiments, using cohorts of mice of 9 (Fig. 9)
and 5 weeks of age (Fig 10). Moreover, the total exploration time during training (e1) was unaffected in Tau-KOKI mice, showing that the ORT effects were due to alterations in learning and memory rather than to different exploration strategies as a possible result of changes in anxiety (Fig. 9G). In agreement with the latter, in the 5 wks old cohort, improved ORT performance was found in the absence of any change in open field behavior.

Discussion

Most neurons of the hippocampal DG are formed in the first two postnatal weeks, whereafter neurogenesis rapidly declines. Hence we suspected that the parallel isoform switch from tau-3R to -4R around this period was relevant for maturation of this area. This study for the first time shows that the lack of tau-4R is responsible for increased precursor division, and decreased neuritic elongation and neuronal differentiation in vitro. Also in vivo, a relative reduction in expression of tau-4R in the hippocampal DG led to a delay in the decline in cell birth and a delay in dendritic outgrowth. The net result of this was a transient but increased early production rate of DG cells that resulted in an enlarged hippocampus and better memory function in adulthood. These data suggests that the tau-3R to 4R isoform switch is not only of relevance for neuronal maturation, but also for the steep decline of neurogenesis occuring in the DG after the second postnatal week.

The tau-KOKI mouse model was generated to help define the role of tau in a mouse strain that expresses only the human tau-4R isoform specifically in neurons and in a mouse tau-null background. The tau-KOKI mice were, consistent with the idea of redundancy of tau, normally viable and fertile and only developed minor sensori-motor problems at advanced age (18-24 months) (Terwel et al., 2005). However, the tau-KOKI model in fact represents a complete tau-KO before p12, whereas only tau-2N/4R is being expressed after this period at low levels in the hippocampus, and in absence of any hyperphosphorylation (Terwel et al., 2005). To dissociate the effects of a lack of tau-3R from reduced tau-4R expression in the DG, we will first discuss the actions of tau on neuronal maturation in vitro, where tau-3R and -4R effects can be separated.

Tau-3R and -4R affect neuronal differentiation differently

It is well known that MAPs play critical roles during CNS developmental processes like e.g. mitotic spindle stability or migration (Tombes et al., 1991; Riederer et al., 1993; Oudega et al., 1995; Andersen, 2000; Charrasse et al., 2000) (Shu et al., 2006). Even though several studies support an important role for protein tau in axonal extension, neuronal polarity and neuritic outgrowth (Caceres and Kosik, 1990; Liu et al., 1999; Dawson et al., 2001), we here show for the first time that tau-4R is also critically involved in the neuronal progenitor fate.

Tau-3R and -4R were known to have differential effects on microtubule stability (Utton et al., 2001; Levy et al., 2005), but their differential effects are not studied in the DG.
effects on neuronal maturation and neurogenesis had not been addressed before. The current experiments show that both tau-3R and -4R promote neuritic outgrowth whereas only 4R-tau reduces cell-birth and stimulates neuronal differentiation. The actions of tau-4R are hence twofold in that it reduces cell-proliferation and increases differentiation, both promoting neuronal maturation. These data support the assumption that tau-3R is less potent in inducing neuronal maturation than tau-4R.

**Postnatal tau-3R to -4R switch induces DG maturation**

The hippocampal DG is one of the few brain areas that largely matures postnatally, with massive neurogenesis ending at the first two weeks after birth (Altman and Bayer, 1975; Schlessinger et al., 1975; Bayer and 1, 1980; Goedert et al., 1989; Andorfer and Davies, 2000) leaving only low frequencies in adult life. In this study we show that protein tau is an important factor involved in this period as relative reductions in tau-4R expression in the DG of KOKI mice induces the decline in cell proliferation to occur less rapidly. Interestingly, at two months of age the rate of cell birth is again identical to that of WT animals despite the lower expression levels of 4R tau in the htau-KOKI DG. We suggest that once hippocampal maturation is completed the role of tau becomes less important.

Cell birth declined less rapidly in KOKIs at p15 in the hilus and at p28 in the SGZ compared to the WT situation. During embryonic and early postnatal development, neuronal precursors derived from the lateral ventricle wall migrate towards the DG. While on this migratory tract, they maintain the possibility to divide. Once the precursors have reached the DG some of them will differentiate into granule cells whereas others keep their precursor characteristics (Altman and Das, 1967). The former population first migrates from the hilus to the SGZ and then undergoes differentiation while migrating into the GCL. Interestingly, the spatiotemporal order of the changes in proliferation appear to closely follow this migratory pattern; although at p7 no differences are present, the first increase in Ki67+ cells is in the hilus at p15, where precursors enter the DG, followed by an increase in the SGZ at p28, the final destination for most precursors, indicating the transient nature of the structural change induced by the isoform switch.

Quantification of the numbers of BrdU+ cells 4 wks after injection in animals of one month of age demonstrated that the enhanced numbers of newborn cells at approximately p28 do indeed survive. Additional DCX immunostainings indicate many of them maintain an immature neuronal phenotype. CA and DG cell numbers at 6 months of age confirm that the increase in cell number is lasting. Apparently, the strong precursor expansion and extensive neurogenesis during the early postnatal period are very sensitive to disturbances in tau protein or isoform expression, with considerable consequences for the eventual structural make up of the adult hippocampus.

In addition to increased neurogenesis, a significant decrease in apoptosis occurred in the tau-KOKI hippocampus at p15, an effect that has likely also contributed to the increased adult hippocampal size. It is so far
unresolved whether the change in apoptosis is a direct consequence of the tau changes or rather a secondary effect balancing initial alterations in DG turnover, which provides also the major input for the CA. Recent studies have shown that apoptosis and cell-birth in the DG are closely related processes that might be regulated by the same proteins (Heine et al., 2004; Wheatley and McNeish, 2005; Ricard et al., 2006).

Interestingly, apoptosis was not only decreased in the DG but also in the CA area. Also Ki-67 immunoreactivity was increased in this area at p15 indicating that the actions of tau-4R on structural plasticity are not limited to the DG alone. Despite changes in cell-birth and apoptosis, the number of DCX- or BrdU-labeled cells was not altered in the CA of KOKI mice. Possibly, the population of cells undergoing apoptosis in KOKI and WT mice represents a more mature population of cells. We conclude that hippocampal subregion specific factors may determine the eventual maturation of newborn cells into neurons.

The close parallel between the Ki-67 and DCX data obtained from “conventional” tau-KO mice and from the tau-KOKI mice suggests that some of the effects in the KOKI mice may be attributable to low levels of tau. One of the parameters in which the KOKI and tau-KO mice differed, was the CA area in which Ki67+ cell numbers were increased at 2 months of age in the tau-KO mice but not in tau-KOKI mice (Fig 6E). This is probably due to the fact that tau expression in the adult CA1 in tau-KOKI mice is comparable to WT 4R levels.

Golgi analysis showed that dendritic length is significantly reduced in the DG of KOKI mice, which could be the result of proportional increases in the relative number of young granule cells, as such cells generally have a less developed dendritic tree. Another possibility is a direct effect of reduced tau-4R expression in the absence of tau-3R expression. In this respect, it is interesting that also in 4DIV tau-KOKI cultures, neuritic outgrowth was reduced. Since younger neurons are primarily located close to the SGZ, and there was no obvious correlation between the dendritic length of the individual granular cell and its position within the granular cell layer, i.e. either closer to or further from the hilar border (not shown), we conclude that the latter explanation of reduced tau 4R expression is the most likely one.

Moreover, a significantly reduced DG volume was found in KOKI mice at p7. Detailed analysis of these data revealed this was not due to volume changes in the GCL nor were there differences in cell-birth, which implies the following. Firstly, it shows that tau (albeit only proven for tau-3R) can affect neuritic outgrowth in vivo independent of changes in neurogenesis. With time, these volume changes could have been compensated for by increased cell-numbers, which would explain why no or opposite volume differences are seen at p15 and later ages. Secondly, it clearly demonstrates that the effects on proliferation are 4R specific, as also demonstrated in vitro.

More young DG granule cells improves memory performance

To study the functional consequences of an increased hippocampal size and a relatively younger age of the DG granule cell population, we
Tau isoform expression affects hippocampal development

studied memory function by means of an object recognition task. We chose this test as it involves low stress levels, depends on short-range visual as well as tactile stimuli and, as performed here, involves hippocampal activation (Broadbent et al., 2004; Wright et al., 2004; Boekhoorn et al., 2006). Tau-KOKI mice performed better in the ORT than did WT mice, indicative of improved memory function. Although we showed the performance of KOKI mice was not hampered by motor impairments, their open field performance was altered, indicative of some anxiety related changes. Although we cannot completely exclude that this has interfered with ORT performance, it did at least not alter the explorative behavior of the mice towards the objects in the training phase (Fig 8G).

Cognitive performance in tau-KO mice has, to our knowledge, so far only been studied by one group before (Takei et al., 2000) reporting only minor impairments in fear conditioning. Given the fact that both tau-KOKI and tau KO have altered open field behavior, a fear conditioning paradigm may have yielded different results than an ORT paradigm, which involves low stress levels. Another reason why the earlier study is difficult to compare with the presently found improvement in memory performance, is the age of the mice that was much higher in the other study.

In theory, the mean reduction in size of the individual granule cells or the increased granule neuron number could underlie the improved ORT performance of the KOKI mice. Since younger granule cells display enhanced LTP as compared to older ones (Schmidt-Hieber et al., 2004), a population relatively enriched in young cells could provide a possible mechanism by which an improvement in memory function might be explained. However, LTP was unaltered in tau-KOKI mice. Although this does not exclude that LTP at the synaptic level in individual younger cells is enhanced, such changes were apparently not sufficient to contribute to changes in overall DG LTP. We therefore propose that the increased granule cell number, rather than the individual cellular properties, is responsible for the improved memory function in tau-KOKI mice.

In conclusion, we have shown for the first time that tau-4R, both in vivo and in vitro, reduced neuronal precursor division and promotes neuritic elongation and neuronal differentiation, whereas 3R only induces minor increases in neurite elongation. This led us to conclude that the tau-3R to -4R isoform switch is of particular relevance for the early maturation of the hippocampal DG granule cells as well as for the coinciding decline in cell birth.

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