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Intracerebral injection of preformed synthetic tau fibrils initiates widespread tauopathy and neuronal loss in the brains of tau transgenic mice

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ABSTRACT

Neurofibrillary tangles composed of hyperphosphorylated fibrillized tau are found in numerous tauopathies including Alzheimer's disease. Increasing evidence suggests that tau pathology can be transmitted from cell-to-cell; however the mechanisms involved in the initiation of tau fibrillization and spreading of disease linked to progression of tau pathology are poorly understood. We show here that intracerebral injections of preformed synthetic tau fibrils into the hippocampus or frontal cortex of young tau transgenic mice expressing mutant human P301L tau induces tau hyperphosphorylation and aggregation around the site of injection, as well as a time-dependent propagation of tau pathology to interconnected brain areas distant from the injection site. Furthermore, we show that the tau pathology as a consequence of injection of tau preformed fibrils into the hippocampus induces selective loss of CA1 neurons. Together, our data confirm previous studies on the seeded induction and the spreading of tau pathology in a different tau transgenic mouse model and reveals neuronal loss associated with seeded tau pathology in tau transgenic mouse brain. These results further validate the utility of the tau seeding model in studying disease transmission, and provide a more complete *in vivo* tauopathy model with associated neurodegeneration which can be used to investigate the mechanisms involved in tau aggregation and spreading, as well as aid in the search for disease modifying treatments for Alzheimer's disease and related tauopathies.

Introduction

An important hallmark of Alzheimer's disease (AD) is the accumulation of insoluble hyperphosphorylated and fibrillized tau in neurofibrillary tangles (NFTs) (reviewed by Ballatore et al., 2007). Since there is a clear correlation between NFT density and cognitive decline, misfolded and aggregated tau is likely a key pathological agent in AD (Arriagada et al., 1992; Gómez-Isla et al., 1997). The hypothesis that tau plays a critical role in AD pathogenesis was further strengthened by the discovery that inherited forms of frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) resulted from mutations within the tau gene (Goedert, 2005a; Goedert and Jakes, 2005b). This provided evidence that alterations of tau itself can directly lead to neurodegenerative disease and suggested that tau aggregation in the AD brain could, indeed, be involved in disease onset and progression.

The events leading to tau aggregate formation are not completely understood and were long thought to be entirely cell-autonomous, with protein misfolding occurring independently in many cells. Recent studies have shown, however, that prion-like cell-to-cell spreading mechanisms might be important in the propagation of tau pathology. In AD, tau inclusions occur in a particular brain area and progress in a well-defined predictable manner (Braak and Braak, 1991; Braak et al., 2011) suggesting a prion-like spreading of aggregated tau or tau fragments with altered conformation. Recent studies in cultured cells indicate that exogenously supplied tau preformed fibrils (PFFs) are

Abbreviations: AD, Alzheimer's disease; FTDP-17, frontotemporal dementia with Parkinsonism linked to chromosome 17; NFT, neurofibrillary tangle; PFFs, preformed fibrils; Tg, transgenic.

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internalized by cells, likely *via* endocytosis, and seed the recruitment of endogenous tau to adopt misfolded conformations (Frost et al., 2009; Guo and Lee, 2011; Wu et al., 2013; Holmes et al., 2013). Intracellular tau aggregates can be externalized by degenerating axons or somatodendritic compartments, released *via* secretion or through other unknown mechanisms (Saman et al., 2012; Simón et al., 2012; Chai et al., 2012) and can then be taken up by surrounding or interconnected neurons and template the conversion of soluble tau into insoluble aggregates, giving a plausible explanation for the propagation of tau pathology in the AD brain.

Also, *in vivo* studies have demonstrated that the intracerebral injection of both brain extracts from diseased tau transgenic (Tg) mice or synthetic tau PFFs induce spreading of tau pathology in the brain of tau Tg mice (Clavaguera et al., 2009; Iba et al., 2013). These data, together with the observation that tau pathology can spread along anatomically connected networks *via* trans-synaptic transport in the mouse brain (Liu et al., 2012a; de Calignon et al., 2012), provide further evidence for a prion-like spreading mechanism of tau pathology.

In this study, we injected tau PFFs in the hippocampus or frontal cortex of tau Tg mice expressing human mutant P301L tau found in FTDP-17 pedigrees and we observed induction of tau fibrillization and spreading of tau pathology to interconnected brain regions. Notably, since tau pathology as a consequence of injection of tau PFFs induced selective neuronal loss in the hippocampus, our data provide an *in vivo* model of tau pathological spread with associated neurodegeneration.

Material and methods

Tau Tg mice

Tg tauP301L mice, expressing the longest human tau isoform with the P301L mutation (tau-4R/2 N-P301L) under control of the *thy1* gene promoter were generated as described previously (Terwel et al., 2005). The animals were used for surgery at the age of 3 m, which is 6 m before they develop tau pathology driven solely by the tau transgene. Transgenic tau-4R/2N mice, expressing the longest wildtype human tau isoform (Spittaels et al., 1999) and non-Tg mice were used as controls. All experiments were performed in compliance with protocols approved by the local ethical committee.

Generation of tau PFFs from recombinant tau

Truncated human tau fragments containing the four microtubule binding repeat domain (K18; residues Q244-E372 of the longest human tau isoform) with a P301L mutation (abbreviated as K18-PL) were produced in *Escherichia coli* (Tebu-bio, Le Perray-en-Yvelines, France). The fragments are flanked by a Myc tag at their C- or N-terminus. To obtain tau PFFs, tau K18-PL fragments (66 μM) were incubated at 37 °C for 5 days in the presence of 266 μM heparin (MP Biomedicals, Illkirch, France). Afterwards, the fibrillization mixture was centrifuged at 100,000 ×*g* for 1 h. The resulting pellet was resuspended in 100 mM ammonium acetate buffer (pH 7.0) and sonicated before stereotaxic injection. Successful tau fibrillization was confirmed using Thioflavin T fluorescent assay (Sigma).

Stereotaxic surgery

Three months old mice of either sex were deeply anesthetized with isoflurane. Unilateral stereotaxic injections (right hemisphere) were performed in the hippocampus (A/P, −2.5 mm from bregma; L, +2.0 mm; D/V, −2.4 mm) or frontal cortex (A/P, +2.0 mm; L, +2.0 mm; D/V, −2.7 mm). According to the experiments performed the injection volume varied between 2 and 5 μl and was applied at a speed of 1 μl min^{-1}. After injection, the needle was kept in place for an additional 5 min before gentle withdrawal.

Biochemistry

Mice were sacrificed by decapitation in accordance with protocols approved by the local ethical committee. Left and right hemispheres were weighed and homogenized in 6 volumes of homogenization buffer (10 mM Tris; 0.8 M NaCl; 10% sucrose; 1 mM EGTA; pH 7.6). Whole hemispheres were utilized to circumvent variability due to dissection errors to the maximal extend. The homogenate was centrifuged at 27,000 ×*g* for 20 min and 1% N-lauroylsarcosine (Sigma) was added to the supernatant. After 90 min, the solutions were again centrifuged at 150,000 ×*g* for 1 h. The supernatants were kept as sarkosyl-soluble fraction, whereas the pellet containing the sarkosyl-insoluble material was resuspended in homogenization buffer.

Levels of total and phosphorylated tau in the sarkosyl-insoluble fractions were quantified using sandwich ELISA. PT4 (total tau, epitope 215–227, made in-house) and AT8 (pSer202/Thr205, Vandermeeren et al., 1993) were used as a capture antibody and Horseradish Peroxidase (HRP)-conjugated human tau 10 (htau10, epitope 29–36) was applied as a secondary antibody. The signal was measured on an EnVision Multilabel reader after applying QuantaBlu fluorogenic peroxidase substrate solution (Thermo Scientific, Rockford, USA).

Histology and immunohistochemistry

Mice were sacrificed by decapitation in accordance with protocols approved by the local ethical committee; brains were dissected, postfixed in a formalin-based fixative for 24–36 h and embedded in paraffin. For fluorescent labeling, perfusion of the animals and subsequent histological processing were performed as previously described (Bottelbergs et al., 2012; Huyghe et al., 2006). Briefly, following deparaffinization, rehydration, heat induced antigen retrieval in citrate buffer (pH 6.0) and blocking of endogenous peroxidase activity by 3% hydrogen peroxide; the samples were incubated with the primary antibodies. The sources and dilution factors of the different antibodies are listed in Supplementary Table 1. Peroxidase labeled secondary anti-mouse (Jackson Immunoresearch, Suffolk, UK or Dako, Glostrup, Denmark) or anti-rabbit (from Sigma or Dako) antibodies were used in all studies. Labeling for bright field microscopy was performed using 3,3-diaminobenzidine DAB (Dako), while fluorescent labeling was achieved using cyanine 2 or 3 TSA kits (Perkin Elmer Life Sciences, Boston, USA). In case of double fluorescent staining, AT8 antibody was directly labeled with an Alexa 488 fluorophore by the use of a protein labeling kit (Invitrogen Life Technologies, Gent, Belgium) and primary antibodies were applied sequentially. After mounting with Vectashield hard set containing DAPI (Vector Laboratories, Burlingame, USA), slices were analyzed with a LSM510 confocal laser scanning microscope (Carl Zeiss) equipped with an axiocam camera. For brightfield microscopy, slides were counterstained with hematoxylin and bluing reagent, dehydrated and permanently mounted. Virtual images of the DAB labeled sections were made using a Zeiss Mirax Virtual Slide scanner.

Thioflavin S (ThioS) staining was performed to visualize the tau aggregates. Following dewaxing, the tissue sections were incubated in ThioS (1%) and subsequently differentiated in 70% ethanol. Counterstaining was performed using nuclear Hoechst stain. Digital images of ThioS were made using Zeiss Axio Image Z2.

Nissl staining was used to demonstrate neuronal integrity. Following dewaxing, the tissue sections were incubated in 0.2% cresyl violet acetate and subsequently dehydrated in graded ethanol and permanently mounted.

Image analysis of the AT8, GFAP, Iba-1 and ThioS stains was performed using either Definiens analysis software package v1.5 (Definiens AG), Panoramic Viewer software package v1.15 (3DHistech) or AxioVision software package v4.6 (Zeiss). Per sample, predefined regions were manually delineated on the digital images. For each region separately, the % of marker area was measured, defined by the area occupied by all marker labeling that is darker than a user-defined