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NEURONAL DEGENERATION, SYNAPTIC DEFECTS, AND BEHAVIORAL ABNORMALITIES IN TAU$_{45-230}$ TRANSGENIC MICE

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Abstract—The complement of mechanisms underlying tau pathology in neurodegenerative disorders has yet to be elucidated. Among these mechanisms, abnormal tau phosphorylation has received the most attention because neurofibrillary tangles present in Alzheimer's disease (AD) and related disorders known as tauopathies are composed of hyperphosphorylated forms of this microtubule-associated protein. More recently, we showed that calpain-mediated cleavage leading to the generation of the 17 kDa tau$_{45-230}$ fragment is a conserved mechanism in these diseases. To obtain insights into the role of this fragment in neurodegeneration, we generated transgenic mice that express tau$_{45-230}$ and characterized their phenotype. Our results showed a significant increase in cell death in the hippocampal pyramidal cell layer of transgenic tau$_{45-230}$ mice when compared to wild-type controls. In addition, significant synapse loss was detected as early as six months after birth in transgenic hippocampal neurons. These synaptic changes were accompanied by alterations in the expression of the N-methyl-D-aspartate glutamate (NMDA) receptor subunits. Furthermore, functional abnormalities were detected in the transgenic mice using Morris Water Maze and fear conditioning tests. These results suggest that the accumulation of tau$_{45-230}$ is responsible, at least in part, for neuronal degeneration and some behavioral changes in AD and other tauopathies. Collectively, these data provide the first direct evidence of the toxic effects of a tau fragment biologically produced in the context of these diseases in vertebrate neurons that develop *in situ*.

Key words: calpain, tau cleavage, cell death, synapse loss, NMDA receptors, neurite degeneration.

INTRODUCTION

The microtubule-associated protein (MAP) tau plays an important role during neuronal development by stabilizing the microtubule network in growing axons (Drubin and Kirschner, 1986; Ferreira et al., 1989; Dreschel et al., 1992; Bramblett et al., 1993). Therefore, conditions that altered the levels of expression of this MAP during development led to abnormal axonal elongation (Caceres and Kosik, 1990; Knops et al., 1991; Dawson et al., 2001). Tau has also been implicated in axonal degeneration and cell death in the context of Alzheimer's disease (AD) and related disorders known as tauopathies (Kosik et al., 1986; Wood et al., 1986; Kondo et al., 1988; Rapoport et al., 2002; Yancopoulou and Spillantini, 2003; Parihar and Hemnani, 2004; Roberson et al., 2007). The mechanisms by which tau mediates neuronal degeneration are not completely understood; however, a growing body of evidence indicates that abnormal posttranslational modifications of this MAP underlie tau pathology. Numerous studies have focused on the role of tau phosphorylation in AD because neurofibrillary tangles, pathological hallmarks of this disease, are formed mainly by hyperphosphorylated tau isoforms (Kosik et al., 1986; Wood et al., 1986; Kondo et al., 1988; Takashima et al., 1993; Ferreira et al., 1997; Alvarez et al., 1999; Ekinci et al., 1999; Parihar and Hemnani, 2004). More recently, it has been suggested that cleavage could also underlie tau toxicity. Thus, we have shown that calpain-mediated tau cleavage is a conserved mechanism in multiple tauopathies (Ferreira and Bigio, 2011). This cleavage induces the generation of the 17 kDa tau$_{45-230}$ fragment (Park and Ferreira, 2005; Park et al., 2007; Reinecke et al., 2011). The toxic effects of this tau fragment were first detected in hippocampal neurons transfected with a tau$_{45-230}$-green fluorescent protein (GFP) construct (Park and Ferreira, 2005). Similar effects were demonstrated in a Drosophila model of tauopathy (Reinecke et al., 2011).

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

To address this discrepancy and obtain insights into the toxic effects of the 17-kDa tau$_{45-230}$ fragment in mammalian central neurons that develop *in situ*, we generated and characterized transgenic mice that

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Abbreviations: Aβ, β amyloid; AD, Alzheimer's disease; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; GFP, green fluorescent protein; IgG, immunoglobulin G; ITI, inter-trial-interval; MAP, microtubule-associated protein; MEM, minimum essential medium; MWM, Morris Water Maze; NMDA, N-methyl-D-aspartate glutamate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction.

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express this fragment in hippocampal neurons. Our results showed enhanced cell death of pyramidal neurons and synaptic loss in the hippocampus of transgenic tau_{45-230} mice. In addition, these changes were accompanied by behavioral abnormalities. Collectively, these data indicate that indeed tau_{45-230} has toxic effects that could contribute to the progressive degeneration of central neurons in AD and related disorders.

EXPERIMENTAL PROCEDURES

Generation of tau_{45-230}-GFP transgenic mice

Transgenic mice were generated by injecting the pronucleus of a single-cell fertilized C57BL/6J mouse embryo with the tau_{45-230}-GFP transgene under the control of the Thy 1.2 promoter. This transgene was derived from a peGFP-N1 plasmid (Invitrogen, Grand Island, NY, USA) containing the human cDNA coding sequence for the tau_{45-230} fragment cloned into the multiple cloning site as previously described (Park and Ferreira, 2005). The 7.8-kb transgene containing all regulatory elements and the transgene coding regions (tau_{45-230}-GFP) was isolated from the promoter vector by enzymatic digestion and extracted from agarose gels using the Qiaquick Gel Extraction Kit (Qiagen, Germantown, MD, USA). Transgenic founder mice were identified by genomic polymerase chain reaction (PCR) screening for GFP and confirmed by Southern blot analysis using genomic DNA isolated from tail biopsies as previously described (Feng et al., 2000). Founders from two lines were crossed with C57BL/6J mice and the subsequent offspring were backcrossed for at least five generations to produce congenic tau_{45-230}-GFP lines. Both female and male mice from line 1 were used for the experiments described below with the exception of behavioral studies that were performed using only male mice to avoid hormone-dependent changes in behavior. Experiments assessing the effects of tau_{45-230} on neuronal death and synapse loss were also performed using line 3 mice. Since no differences in the phenotype were detected when results obtained using line 1 mice were compared to those obtained using line 3 mice, we have only included data obtained with line 1 mice. The Northwestern University Animal Care and Use Committee approved this experimental protocol in accordance with USPHS regulations and applicable federal and local laws. All efforts were made to minimize the number of animals used and their suffering.

Reverse transcription-polymerase chain reaction (RT-PCR)

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

Histology, immunostaining, and stereological analysis

Wild-type and homozygous transgenic tau_{45-230} mice (3–12-month-old) were injected with an overdose of ketamine/xylazine (120/10 mg/kg) and transcardially perfused with ice-cold saline solution followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brains were removed, post-fixed in the same fixative overnight, and then cryopreserved in increasing concentrations of sucrose (10–30%) in PBS. Brains were sectioned in the sagittal plane on a freezing-stage microtome at 25 μm and serial sections were collected in 0.1 M phosphate buffer. Free-floating sections were blocked for 1 h in 10% normal goat serum in PBS and then incubated overnight at 4 °C with the following primary antibodies: anti-Class III β-tubulin (clone TuJ1, 1: 1000; R&D Systems, Minneapolis, MN, USA); anti-tubulin (clone DM1A, 1:5000; Sigma); anti-GFP (1:500; Millipore, Temecula, CA, USA); and anti-synaptophysin (1:250; Santa Cruz Biotechnology, Dallas, TX, USA). After 3 × 15 min washes in PBS, the sections were blocked for 1 h in 10% normal goat serum in PBS and then incubated with secondary antibodies for 2 h at room temperature. For fluorescence microscopy experiments, sections were incubated with AlexaFluor anti-mouse or anti-rabbit immunoglobulin G (IgG) antibodies (1:200; Molecular Probes, Eugene, OR, USA). For light microscopy experiments, sections were exposed to biotin-conjugated rabbit anti-mouse IgG (Sigma), washed in PBS (3 × 15 min each wash), and then incubated with peroxidase-conjugated Extravidin (Sigma) (1 h at room temperature for each incubation). Finally, sections were reacted with a substrate solution containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.075% hydrogen peroxide (v/v) in 50 mM Tris, pH 7.6. Sections were then rinsed in deionized water to stop the developing reaction, dehydrated with graded ethanol solutions, cleared in xylene, and finally mounted on gelatin-coated glass slides with Permount (Thermo Fisher Scientific, Pittsburgh, PA, USA). Images of the