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# $TAU_{45-230}$ ASSOCIATION WITH THE CYTOSKELETON AND MEMBRANE-BOUND ORGANELLES: FUNCTIONAL IMPLICATIONS IN NEURODEGENERATION

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**Abstract—The dysregulation of posttranslational modifications of the microtubule-associated protein (MAP) tau plays a key role in Alzheimer's disease (AD) and related disorders. Thus, we have previously shown that beta amyloid (Aβ)-induced neurotoxicity was mediated, at least in part, by tau cleavage into the $tau_{45-230}$ fragment. However, the mechanisms underlying the toxicity of $tau_{45-230}$ remain unknown. To get insights into such mechanisms, we first determined the subcellular localization of this tau fragment in hippocampal neurons. $Tau_{45-230}$ was easily detectable in cell bodies and processes extended by these neurons. In addition, cell extraction experiments performed using Triton X-100 and saponin showed that a pool of $tau_{45-230}$ was associated with the cytoskeleton and the cytoskeleton plus membrane-bound organelles, respectively, in cultured hippocampal neurons. Furthermore, they suggested that these associations were independent of the presence of full-length tau. We also assessed whether this tau fragment could alter axonal transport. Our results indicated that $tau_{45-230}$ significantly reduced the number of organelles transported along hippocampal axons. This altered axonal transport did not correlate with changes in the total number of organelles present in these cells or in motor protein levels. Together these results suggested that $tau_{45-230}$ could exert its toxic effects by partially blocking axonal transport along microtubules thus contributing to the early pathology of AD.** 

**Key words: cytoskeleton, mitochondria, lysosomes, axonal transport, degeneration.**

## INTRODUCTION

The microtubule-associated protein (MAP) tau plays an important role not only in neurite elongation but also in a group of neurodegenerative diseases known as tauopathies (reviewed by Huang et al., 2016). The most well studied tauopathy is Alzheimer's disease (AD), a progressive and irreversible neurodegenerative disorder largely affecting the elderly population (Yankner et al., 2008). AD is characterized by the aberrant extracellular deposition of aggregated amyloid beta (Aβ) peptides into senile plaques and the intracellular accumulation of hyperphosphorylated tau forming neurofibrillary tangles (NFT) in affected brain areas (Glenner and Wong, 1984; Hyman et al., 1984; Grundke-Iqbal et al., 1986; Kosik et al., 1986; Davies et al., 1987; Selkoe, 1994). Recent research has focused on the mechanistic link between Aβ deposition and tau pathology in the progression of this disease (Gotz et al., 2011). The widely accepted amyloid cascade hypothesis proposes that Aβ initiates the pathogenic process inducing cell death in central neurons (Hardy and Selkoe, 2002). However, degeneration induced by aggregated Aβ only occurred in neurons that express tau, suggesting this MAP is required for Aβ-induced neurotoxicity (Rapoport et al., 2002; Roberson et al., 2007).

The mechanisms by which tau mediates Aβ toxicity are not completely elucidated. A growing body of evidence suggests that Aβ can induce deleterious posttranslational tau modifications. Tau phosphorylation has been extensively studied in AD due to the composition of NFTs. More recently, a series of studies suggested that proteolytic tau cleavage also played an essential role in neurotoxicity (Canu et al., 1998; Fasulo et al., 2000; Chung et al., 2001; Gamblin et al., 2003; Park and Ferreira, 2005; Amadoro et al., 2006; Reinecke et al., 2011; Lang et al., 2014; Zhang et al., 2014). We have previously shown that exposure of mature cultured hippocampal neurons to aggregated Aβ oligomers increased $Ca^{2+}$influx via the activation of *N*-methyl-D-aspartic acid receptors (Kelly et al., 2005; Kelly and Ferreira, 2006). This enhanced $Ca^{2+}$influx, in turn, induced calpain-mediated tau cleavage leading to the generation of a 17 kDa tau fragment ($tau_{45-230}$) (Park and Ferreira, 2005; Kelly and Ferreira, 2006; Nicholson and Ferreira, 2009; Nicholson et al., 2011). The neurotoxic effects of this fragment have been extensively characterized. Thus, $tau_{45-230}$ induced cell death when

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*Abbreviations:* AD, Alzheimer's disease; Aβ, Beta Amyloid; BSA, Bovine Serum Albumin; E16, Embryonic Day 16; E18, Embryonic Day 18; GFP, Green Fluorescent Protein; kDa, kilo Dalton; MAP, Microtubule Associated Protein; MEM, Minimum Essential Medium; MTSB, Microtubule Stabilizing Buffer; NFT, Neurofibrillary Tangles; PBS, Phosphate-Buffered Saline; SDS, Sodium Dodecyl Sulfate.

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expressed in neuronal and non-neuronal cell types or in an *in vivo Drosophila* model system, (Park and Ferreira, 2005; Reinecke et al., 2011). In contrast, pharmacological inhibition of calpain activity or genetic modification of the putative cleavage sites (Leu43 and Val229) that produced this toxic fragment suppressed the production of the $tau_{45-230}$ and significantly reduced Aβ-induced neurotoxicity (Park and Ferreira, 2005; Amadoro et al., 2006; Sinjoanu et al., 2008; Reinecke et al., 2011). More recently, we have characterized the phenotype of mice expressing $tau_{45-230}$ (Lang et al., 2014). Enhanced neuronal loss, decreased number of synaptic contacts and behavioral defects were easily detected in transgenic $tau_{45-230}$ mice as compared to wild type controls (Lang et al., 2014). Collectively, these data provided strong evidence for an important role of $tau_{45-230}$ in the progression of Aβ-mediated neurodegeneration. However, the mechanism(s) underlying the neurotoxic effects of this tau fragment remained unknown.

In the present study, we first analyzed the subcellular distribution of $tau_{45-230}$ in cultured hippocampal neurons. We also assessed the effects of this tau fragment on the transport of organelles along the axons extended by these neurons using time-lapse microscopy. The data obtained provided insights into a mechanism by which the $tau_{45-230}$ could induce the formation of dystrophic neurons and cell death in the context of AD and related disorders.

## EXPERIMENTAL PROCEDURES

### Hippocampal culture preparation

Hippocampal neuronal cultures were prepared from embryonic day 18 (E18) Sprague–Dawley rats (Taconic; $n = 30$ E18 pregnant rats) and from E16 C57BL/6J mice (wild type and tau knockout mice, Lang et al., 2014; $n = 21$ E16 pregnant mice) as described previously (Banker and Goslin, 1998; Rapoport et al., 2002). In brief, hippocampi were dissected, stripped of meninges, and trypsinized (0.25%) for 15 min at 37 °C. Neurons were dissociated by pipetting gently through a fire-polished Pasteur pipette and plated (~800,000 cells/60-mm dish or ~240,000/35-mm dish) in minimum essential medium (MEM) containing 10% horse serum (MEM10) on poly-L-lysine-coated dishes. After 4 h, the medium was replaced with glia-conditioned MEM containing N2 supplements, ovoalbumin 0.1%, and 0.1 mM sodium pyruvate (N2 medium, Bottenstein and Sato, 1979). For immunocytochemical analysis, neurons were plated (150,000 cells/60-mm dish) onto poly-L-lysine-coated coverslips in MEM10. After 4 h, the coverslips were transferred to dishes containing an astroglial monolayer and maintained in N2 medium.

### Preparation of astrocyte cultures

Astrocyte cultures were prepared from the cerebral cortex of E16 mice embryos ($n = 5$ E16 pregnant mice) as previously described (Ferreira and Loomis, 1998). Briefly, embryos were removed and their cerebral cortex dissected and freed of meninges. The cells were dissociated by trypsinization (0.25% for 35 min at 37 °C) and then centrifuged in MEM plus 10% horse serum at 1,000 rpm for 10 min. The cells were resuspended in fresh MEM plus 10% horse serum, triturated with a fire-polished pipette, and plated at high density (800,000 cells/60-mm dish) on non-coated culture dishes.

### Plasmid constructs and cell transfection

cDNA encoding for the longest human tau (hTau40) isoform ($tau_{1-441}$) and the $tau_{45-230}$ fragment were generated as described previously (Park and Ferreira, 2005). Both constructs were subcloned into the mammalian expression vector, enhanced green fluorescent protein -N1 (p-eGFP-N1) (Invitrogen) to produce C-terminal GFP-labeled full-length tau (hTau40-GFP) and $tau_{45-230}$ ($tau_{45-230}$-GFP) constructs. These constructs were nucleofected into dissociated hippocampal neurons as previously described (Park and Ferreira, 2005). Briefly, dissociated neurons were resuspended in nucleofection solution containing 3 μg of the respective constructs, transferred to an electroporation cuvette, and nucleofected using the Amaxa Nucleofection system (Lonza, Inc. Allendale, NJ) according to the manufacturer's protocol (program O-03). Non-transfected cells and neurons transfected with an empty p-eGFP-N1 vector were used as controls. For some experiments, astrocytes were nucleofected with the $tau_{45-230}$-GFP construct using the T-20 program (Lonza) as previously described (Paganoni et al., 2004).

### Aβ aggregation and cell treatment

Synthetic $A\beta_{1-40}$ (American Peptide, Sunnyvale, CA) was dissolved in N2 medium to a concentration of 0.5 mg/ml and incubated for 3 days at 37 °C to induce its preaggregation (Park and Ferreira, 2005). Neurons kept in culture for 21 days were incubated in the presence of the preaggregated peptide at final concentrations of 20 μM for 24 h to induce calpain-mediated tau cleavage into the $tau_{45-230}$ fragment (Park and Ferreira, 2005; Nicholson and Ferreira, 2009).

Cultures prepared from 5 E18 pregnant rats per experimental condition were used for these experiments.

### Preparation of whole-cell lysates and detergent-resistant subcellular fractions

To prepare whole-cell lysates, hippocampal and astrocyte cultures were washed once in warmed (37 °C) phosphate-buffered saline (PBS), scraped into Laemmli buffer, and homogenized by boiling in a water bath for 10 min (Laemmli, 1970). Detergent-resistant subcellular fractioning was performed to isolate membrane-bound organelle plus cytoskeleton fractions and pure cytoskeleton fractions as previously described (Paganoni et al., 2004). In brief, hippocampal neurons or astrocytes plated in 60-mm dishes were washed once with warmed PBS, and stabilized by 30-s incubation with warmed microtubule stabilizing buffer (MTSB: 130 mM HEPES, 4 mM $MgCl_2$, 10 mM EGTA, pH 6.9). Cultures were extracted in MTSB containing 0.02% saponin (Sigma) or 0.2%