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# Cleavage of Tau by calpain in Alzheimer's disease: the quest for the toxic 17 kD fragment

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**Abstract**

The amyloid cascade hypothesis of Alzheimer's disease (AD) posits that the generation of β-amyloid (Aβ) triggers Tau neurofibrillary pathology. Recently a "17 kD" calpain-induced Tau fragment, comprising residues 45–230 (molecular weight [MW], 18.7 kD), was proposed to mediate Aβ-induced toxicity. Here, we demonstrate that the "17 kD" fragment is actually much smaller, containing residues 125–230 (molecular weight, 10.7 kD). Inducing Tau phosphorylation by okadaic acid or mimicking phosphorylation by Glu mutations at the epitopes of Alzheimer-diagnostic antibodies AT100/AT8/PHF1 could not prevent the generation of this fragment. The fragment can be induced not only by Aβ oligomers, but also by other cell stressors, e.g., thapsigargin (a $Ca^{2+}$-ATPase inhibitor) or glutamate (an excitatory neurotransmitter). However, overexpression of neither $Tau_{45–230}$ nor $Tau_{125–230}$ fragment is toxic to Chinese hamster ovary (CHO) cells, neuroblastoma cells (N2a) or primary hippocampal neurons. Finally, the calpain-induced fragment can be observed both in Alzheimer's disease brains and in control normal human brains. We conclude that the 17 kD Tau fragment is not a mediator of Aβ-induced toxicity, leaving open the possibility that upstream calpain activation might cause both Tau fragmentation and toxicity.


*Keywords:* Tau; Amyloid Aβ; Calpain; Fragmentation; Toxicity; Neurodegeneration

## 1. Introduction

Alzheimer's disease is characterized by 2 main pathologic types of protein aggregation, intracellular neurofibrillary tangles (NFTs) made up of Tau protein and extracellular senile plaques (SPs) formed by β-amyloid (Aβ) (Ballatore et al., 2007; Haass and Selkoe, 2007). The amyloid cascade hypothesis posits that Aβ triggers Tau pathology (Hardy and Selkoe, 2002), but the details of this relationship are still poorly understood. Cell and mouse models have suggested that exposure of neurons to Aβ is toxic and elicits abnormal changes in Tau (Canu and Calissano, 2003; King et al., 2006; Nicholson and Ferreira, 2009; Park and Ferreira, 2005). Conversely, Tau is thought to be necessary for the toxic effects of Aβ (King et al., 2006; Roberson et al., 2007). In some experimental settings, the changes in Tau were ascribed to a toxic Tau fragment of ~17 kD generated by calpain cleavage and located in the N-terminal half of Tau (Canu et al., 1998; Park and Ferreira, 2005), but other N-terminal parts of Tau were reported to be toxic as well (King et al., 2006). In these cases, the toxicity could be triggered by Aβ, but there was no apparent relationship to the aggregation of Tau. By contrast, studies on other cleavage reactions had shown that truncation of Tau in the C-terminal domain by caspase-3 (behind D421) or by lysosomal proteases (around residue 360) could generate Tau fragments with a high tendency for aggregation (Gamblin et al., 2003; Khlistunova et al., 2006; Rissman et al., 2004; Wang et al., 2007, 2009).

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While most of the caspase-induced cleavage sites of Tau have been determined precisely, the calpain-induced cleavage sites of Tau have not been well defined, partly due to the lower specificity of this protease. The claim that the "17 kD" fragment comprises residues 45–230 was derived from sequence-based predictions of potential calpain cleavage sites in Tau (Park and Ferreira, 2005). These predictions were based on the P2-P1 rule, which states that the preferred residues (for calpains 1 and 2) are Leu or Val at position P2, and Arg or Lys at P1, just before the scissile bond (Hirao and Takahashi, 1984; Sasaki et al., 1984). However, this rule has recently been shown to be questionable, and in fact there is no well defined consensus sequence for cleavage by calpains (Cuerrier et al., 2005; Tompa et al., 2004). This means that there is a need for re-evaluating the nature and mode of action of the "17 kD" fragment. The issue is important because this fragment was considered the culprit for A$\beta$-induced toxicity in certain cell and mouse models (Park and Ferreira, 2005; Roberson et al., 2007).

We therefore investigated the calpain-induced cleavage products of Tau by N-terminal sequencing and mass spectrometry and studied their effect in cell models. Contrary to earlier reports (Park and Ferreira, 2005) we find that the "17 kD" fragment comprises residues 125–230 ($Tau_{125–230}$, apparent relative molecular weight [Mr], ~17 kD; molecular weight [MW], 10,680 Da) and therefore is much shorter than residues 45–230 ($Tau_{45–230}$, Mr ~28 kD; MW, 18,702 Da). In both cases, the Mr values are much larger than the true MW because of the anomalous migration of the N-terminal domain of Tau on gels. The cleavages suggest that the specificity of calpains is not governed by amino acid sequence, but rather by conformation of the polypeptide chain (Cuerrier et al., 2005; Tompa et al., 2004). We also found that inducing Tau phosphorylation by okadaic acid (OA) or pseudophosphorylation at AT8*, AT100, and PHF1 epitopes (S199E+S202E+T205E+S396E+S404E+T212E+S214E) could not prevent the generation of $Tau_{125–230}$. A$\beta$ can induce the generation of the $Tau_{125–230}$ in neuronal cells, as shown earlier (Park and Ferreira, 2005), but also other treatments such as glutamate (an excitatory neurotransmitter) or thapsigargin (a $Ca^{2+}$-ATPase inhibitor and activator of calpain), suggesting a more generalized response to $Ca^{2+}$ elevation. However, contrary to other reports, we find that neither $Tau_{45–230}$ nor $Tau_{125–230}$ is toxic to cultured cells. In line with this observation, the Mr ~17 kD fragment is detected not only in Alzheimer's disease (AD) brains, but also in normal human brains. Thus, the Mr ~17 kD fragment of Tau is not the culprit of A$\beta$-induced toxicity but represents a marker of enhanced calpain activity.

## 2. Methods

### 2.1. Cell culture, transfection and treatments

Cortical or hippocampal neurons were obtained from embryonic day 18 (E18) rat embryos and cultured according to Banker and Goslin (1988). Cultures were treated after 4 days with 5 $\mu$g/mL cytosine arabinoside (Sigma, Munich, Germany) to reduce glial growth. Cells were maintained in NeuroBasal medium supplemented with B27 (Invitrogen, Carlsbad, CA, USA) for 3–4 weeks. Chinese hamster ovary (CHO) cells were grown in F-12 medium plus 10% fetal bovine serum (FBS). Tet-on inducible N2a cells were cultured in Eagle's modified essential medium (MEM) with 10% fetal bovine serum, 0.1% nonessential amino acids, and 600 $\mu$g/mL G418. Effectene (Qiagen, Hilden, Germany) was used for transfection according to the manufacturer's manual. To induce Tau phosphorylation, 48 hours after transfection, N2a cells were treated with 0.2 $\mu$M OA for 1 hour before harvest.

### 2.2. Electrophoresis and Western blot analysis

Electrophoresis and Western blot analysis were done as described previously (Wang et al., 2009). The following Tau antibodies were used: 5E2 (reacts with aa 214–233, 1:1000) (Kosik et al., 1988), SA125 (1:400), SA230 (1:400), HT7 (reacts with aa 157–163, 1:10,000) (Mercken et al., 1992), SA4473 (reacts with first insert of Tau, 1:1000), K9JA (Pan-Tau antibody DAKO A10024, 1:20,000), AT8 (pSer202/Thr205, 1:400), PHF1(pSer396/Thr404, gift of P. Davies, Albert Einstein College of Medicine, 1:500), 12E8 (pSer262/Ser356, gift of P. Seubert, Elan Pharma, South San Francisco, 1:1000).

### 2.3. A$\beta$ oligomer preparation and treatment

Soluble A$\beta$ oligomers (A$\beta$-derived diffusible ligands; "ADDLs") were prepared according to Klein and coworkers (Klein, 2002). In brief, $A\beta_{1–42}$ was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma H-8508) at 1 mg/mL, dried with speed vacuum centrifugation, and dissolved in dimethylsulfoxide (DMSO) at 5 mM. The 5 mM stock solution was then diluted into conditional medium to a concentration of 100 $\mu$M and incubated at 4 °C for 24 hours. Following incubation, the samples were centrifuged at 14,000*g* for 10 minutes at 4 °C, and the supernatant was collected as ADDLs preparation. For neuron treatment, ADDLs were added to the culture medium at a final concentration of 1 or 20 $\mu$M. To evaluate whether calpain inhibitors could prevent the generation of the Mr ~17 kD fragment, neurons were pretreated with calpain inhibitor calpeptin (10 $\mu$M) 1 hour before the incubation with ADDLs.

### 2.4. Brain tissue homogenization

Postmortem frozen hippocampal tissues from AD autopsies (aged 82.3 ± 2.3 years) and normal control brains (aged 60.8 ± 5.6 years) were obtained from Drs. J. Matschke and M. Glatzel (Institute of Neuropathology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany). Postmortem frozen temporal cortex tissues from AD brain (aged 78.4 ± 5.4 years) and normal control brains (aged 70.5 ±