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TOC1: A valuable tool in assessing disease progression in the rTg4510 mouse model of tauopathy

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Abstract

All tauopathies result in various forms of cognitive decline and neuronal loss. Although in some diseases, tau mutations appear to cause neurodegeneration, the toxic “form” of tau remains elusive. Tau is the major protein found within neurofibrillary tangles (NFTs) and therefore it seemed rational to assume that aggregation of tau monomers into NFTs was causal to the disease process. However, the appearance of oligomers rather than NFTs coincides much better with the voluminous neuronal loss in many of these diseases. In this study, we utilized the bigenic mouse line (rTg4510) which conditionally expresses P301L human tau. A novel tau antibody, termed Tau Oligomer Complex 1 (TOC1) was employed to probe mouse brains and assess disease progression. TOC1 selectively recognizes dimers/oligomers and appears to constitute an early stage marker of tau pathology. Its peak reactivity is coincident with other well-known early stage pathological markers such as MC1 and the early-stage phospho-marker CP13. TOC1’s reactivity depends on the conformation of the tau species since it does not react with monomer under native conditions, although it does react with monomers under SDS-denaturation. This indicates a conformational change must occur within the tau aggregate to expose its epitope. Tau oligomers preferentially form under oxidizing conditions and within this mouse model, we observe tau oligomers forming at an increased rate and persisting much longer, most likely due to the aggressive P301L mutation. With the help of other novel antibodies, the use of this antibody will aid in providing a better understanding of tau toxicity within Alzheimer’s disease and other tauopathies.

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Keywords

Neurodegenerative diseases; Tauopathies; Oxidation; Reduction; Oligomer; Filament

Introduction

“Tauopathies” are classified as a particular subset of neurodegenerative diseases characterized by a pathological aggregation of hyperphosphorylated tau protein within the human brain. The most well characterized tauopathy is Alzheimer’s Disease (AD), while others include Frontotemporal Dementia with Parkinsonism linked to tau on chromosome 17 (FTDP-17-tau; caused by exonic or intronic missense mutations in the tau gene), Picks disease (PiD), Corticobasal Degeneration (CBD), Progressive Supranuclear Palsy (PSP) and Argrophilic grain disease (AGD) (Arriagada et al., 1992; Ferrer et al., 2008; Gomez-Isla et al., 1997; Iqbal et al., 2005; Lee, 2001). Although the exact mechanism has yet to be elucidated; post-translational modifications, truncations and conformational changes all play a role within this pathological process (Garcia-Sierra et al., 2003).

Tau is a microtubule-associated protein (MAP) with limited secondary structure. It is highly soluble and is a major component of the neuronal cytoskeleton (Weingarten et al., 1975). It exists as six alternatively spliced isoforms in the CNS (Gamblin et al., 2003a; Goedert et al., 1989). One of tau’s normal functions within the neuron may involve stabilization of the extensive microtubule network that is necessary for intracellular transport in axons and all other subcellular compartments within the nervous system. The efficiency with which tau can bind to and stabilize these microtubules appears to be influenced by the presence of three or four repetitive regions (3R vs. 4R) within the C-terminal one third of the molecule (Alonso et al., 1994). The six tau isoforms are differentiated by the number of repeats and by the presence or absence of one or two amino terminal inserts of unknown function.

Until recently, it was widely accepted that, since NFT progression coincides extremely well with neuronal cell loss within the same brain regions, the NFTs themselves must be producing the toxic effect (Arriagada et al., 1992; Braak and Braak, 1991; Gomez-Isla et al., 1997). However, there is now much data to dispute this theory and to support the hypothesis that non-fibrillar aggregates may play a more toxic role than once believed. For instance, there is now evidence to suggest that oligomers correlate much better with synapse loss and behavioral deficits than NFTs (Berger et al., 2007; Spires et al., 2006). This line of evidence also correlates very well with what is observed in the P301S mouse which demonstrates hippocampal synapse loss and microgliosis preceding the formation of NFTs (Yoshiyama et al., 2007). In a cell culture tauopathy model, a pro-aggregation tau mutant construct displays toxicity before the presence of any β -sheet structures, which are indicative of NFTs (Khlistunova et al., 2006). Similarly, studies on a triple transgenic mouse model of AD indicated that memory deficits are found before NFTs form (Oddo et al., 2003). A mouse model which expresses no mouse tau due to a gene disruption and has been manipulated to over-express all six isoforms of wild-type human tau demonstrates neuronal cell loss in the absence of any observable tangles (Andorfer et al., 2005). In another study using the rTg4510 bigenic mouse model, fibrillar tangles were shown to be dissociated from

neurotoxicity during early pathogenesis; suppression of the mutant transgene (P301L) decreased neuronal death and reversed memory impairment even though NFTs continued to form (Santacruz et al., 2005). Taken together, these data indicate that tau changes prior to NFT formation may be responsible for the neuronal dysfunction and death observed within AD; however, only recently was another form of tau aggregate implicated in these processes.

There is an abundance of evidence to suggest that non-fibrillar, oligomeric structures have negative effects within the cell. Prion, A β and α -synuclein oligomers have all been implicated in neurodegeneration for some time (Conway et al., 2000; Novitskaya et al., 2006; Walsh et al., 2002). However, recently tau oligomers have gained considerable attention. Homogeneous populations of A β 42 oligomers have been added to tau monomers in a process known as “seeding”. The resulting tau oligomers were then added to additional tau monomers and this was repeated until all A β 42 oligomers were diluted out. Using an MTS assay for toxicity on SH-SY5Y cells, tau oligomers were shown to be by far the most toxic tau species when compared to tau monomers or filaments (Lasagna-Reeves et al., 2010). Together these data suggest that a pre-fibrillar oligomeric tau structure may also be involved in toxicity and pathogenesis (Maeda et al., 2007).

Previous work from the Binder Laboratory demonstrated the successful generation of a monoclonal antibody that selectively recognizes tau dimers and higher-order oligomers, designated Tau Oligomeric Complex 1 (TOC1) (Patterson et al., 2011). The present study was initiated using this novel antibody, in conjunction with other well-defined antibodies to investigate the progression of tau conformational changes during neurodegeneration. The reactivity of TOC1 is greatly elevated in AD brains over control brains. TOC1 is considered an early stage marker since it co-localizes extremely well with pS422, an early stage pathogenic marker of tau alteration, in the entorhinal cortex in prodromal AD cases (Guillozet-Bongaarts et al., 2006; Patterson et al., 2011). Although TOC1 colocalizes with pS422, it does not display any co-localization with thiazine red (TR)(Patterson et al., 2011), which labels β -sheet structures and is indicative of NFTs, nor does it colocalize with MN423, a late-stage NFT truncation marker (Guillozet-Bongaarts et al., 2005). Therefore, it appears likely that TOC1 is labeling pre-fibrillar structures (dimers and oligomers) during early stage filament formation in AD pathogenesis (Patterson et al., 2011; Ward et al., 2013)

Our findings demonstrate that TOC1 can recognize tau oligomers under native conditions within the soluble fraction. Utilizing biochemical assays we demonstrate that the peak of TOC1 immunoreactivity coincides with that of both phospho- and conformationally-specific antibodies, indicating that early changes in aggregation such as dimerization and oligomerization accompany these other well-defined events. Furthermore, we also determine that tau oligomers preferentially form under oxidative conditions, suggesting that changes in the redox state of the brain may initiate or sustain this process producing toxic tau species without necessitating NFT formation.

Materials and Methods

Mice

The double-transgenic rTg4510 mice, a parental mutant tau responder line, and tTA activator line were generated and maintained as previously described by SantaCruz et al (Santacruz et al., 2005). Briefly, to make the tau responder line expressing the 4R0N isoform of human P301L mutant tau, cDNA was placed downstream of a tetracycline-operon-responder (TRE) construct and this tTA activator system was placed downstream of the Ca²⁺-calmodulin kinase II alpha promoter (CaMKII α). The tau responder mice were maintained in the FVB/NCr strain (Charles River Laboratories, Wilmington, MA) and the tTA activator mice were maintained in the 129S6 strain (Taconic, Germantown, NY). Hemizygous mice from each parental line were crossed to produce F1 offspring to contain rTg4510 double-transgenic mice and respective controls on a 50:50 FVB/129 background. All procedures involving mice were performed in accordance and with approval of the University of Florida Institutional Animal Care and Use Committees.

Tissue extraction and fractionation

Mice were euthanized by cervical dislocation in order to preserve the metabolic environment of the brain and to prevent chemically-induced artifacts that could alter the biochemical profiles of tau. Mouse brains were removed and bisected down the midline. The cerebral cortex and hippocampus (ctx+hip) of the right hemisphere of each animal were quickly frozen on dry ice and stored at -80°C until use. Tissues were then homogenized in 10 volumes of Tris-buffered saline [TBS: 50 mM Tris/HCl (pH 7.4), 274 mM NaCl, 5 mM KCl, 1% protease inhibitor mixture (Sigma, St. Louis, MO), 1% phosphatase inhibitor cocktail I & II (Sigma), and 1 mM phenylmethylsulfonyl fluoride (PMSF)] (Berger et al., 2007; Sahara et al., 2012; Santacruz et al., 2005). The homogenates were centrifuged at 27,000 $\times$ g for 20 min at 4°C to obtain supernatant (S1) and pellet fractions. Pellets were homogenized in 5 volumes of high salt/sucrose buffer [0.8 M NaCl, 10% sucrose, 10 mM Tris/HCl, (pH 7.4), 1 mM EGTA, and 1mM PMSF] and centrifuged as above. The S1 fraction was further separated by centrifugation at 150,000 $\times$ g for 20 min to obtain a supernatant (S1c) and a pellet (S1p). S1p fraction was re-suspended in TBS buffer to 1/5 volumes of original S1. The protein concentration of S1 fraction was measured by the BCA assay (Pierce).

Recombinant Tau Protein Expression and Purification

The tau protein is numbered according to the largest human isoform found in the central nervous system - hTau40. This isoform consists of 441 amino acids, four MTBRs and both alternatively spliced N-terminal exons. Full length hT40 or full length P301L tau constructs were cloned into the vector pT7c, expressed in *Escherichia coli* and purified on a TALON metal affinity resin (Clontech) using batch binding, followed by size exclusion chromatography (SEC) as previously described (Carmel, 1994). Protein concentration was determined via the BCA assay (Pierce).

In vitro Aggregation

Aggregation of all tau proteins (4 μ M) was induced with 75 μ M arachidonic acid (AA) at room temperature for at least 6 hours to allow filament formation to attain equilibrium. Assays were performed in a reaction mixture containing 10mM Na-HEPES pH7.6, 100mM NaCl, 5mM DTT (unless indicated as absent), and 0.1mM Na-EGTA. Working solutions of AA were prepared in 100% ethanol immediately prior to use. Efficiency of aggregation was assayed using right angle Laser Light Scattering (LLS) (Gamblin et al., 2000a).

Immunofluorescence

Immunofluorescence was performed on brain tissue from 8-M rTg4510 mice (FFPE) and MCI stage human tissue (free floats) (n = 3). All sections were subjected to antigen retrieval by 10mM sodium citrate pH 6.0 at 95°C for 10mins. Sections were washed in PBS containing 0.5% Triton X-100 and 10% goat serum/2% BSA/PBS-TritonX-100. Primary antibodies were diluted (see table below) and incubated overnight at 4°C. Subsequent to washing in PBS-Triton X-100, the sections were incubated in a mixture of Alexa-Fluor-546 goat anti-mouse IgM μ -chain specific (Invitrogen) 1:500 (for TOC1) and Alexa-Fluor-488 goat anti-mouse IgG (Invitrogen) 1:500 (for Ab39) secondary antibodies for 2h at room temperature. Alternatively, sections were counterstained with 1% Thioflavin-S as indicated. Sections were rinsed in PBS-Triton X-100. Free floating human brain sections from the entorhinal cortex were obtained from the Cognitive Neurology and Alzheimer's Disease Center (CNADC) at Northwestern University and were mounted onto glass slides. Lipofuscin autofluorescence was eliminated by submerging the slides in Sudan Black (2 %). Free floats were then mounted using Vectashield with DAPI mounting medium (Vector) which also served to counterstain the nuclei. Staining was visualized using the Nikon C2 laser scanning confocal microscope.

Immunoblots and Dot blots

Mouse brain homogenate samples overexpressing the human tau mutation P301L were fractionated as outlined above and separated by SDS-PAGE on either 10% or 4-20% linear gradient Tris-Glycine gels. The protein was subsequently transferred onto nitrocellulose membranes as described previously (Reyes et al., 2008). Dot blot samples were also spotted directly onto nitrocellulose membranes at known concentrations across samples. Both Western and dot blot membranes were blocked in 5% non-fat dry milk in TBS-Tween (0.5%) pH 7.4, followed by incubation in primary antibody overnight at 4°C. Membranes were rinsed in TBS-Tween-20 and incubated in peroxidase conjugated horse anti-mouse secondary antibody IgG (H+L) (Vector) 1:5000 for 1 h at room temperature (RT). Signal detection was performed two independent ways. One involved using an enhanced chemiluminescent (ECL) system (Pierce) and developed on X-ray film. Quantification of dot blots shown in Figure 2 was accomplished using ImageJ software (National Institute of Health) and expressed as a ratio of Protein of Interest/Tau5 using GraphPad Prism ver5.0 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). The second method employed an ECL-PLUS kit (PerkinElmer) and visualization by a computer-linked FluorChem E detection system (ProteinSimple, Santa Clara, CA). Quantitative analysis shown in Figure 3 was performed by AlphaView software (ProteinSimple) and

relative signal intensities were normalized to the values obtained from non-Tg S1c sample. As previously described (Sahara et al., 2012; Sahara et al., 2002; Sahara et al., 2007; Sahara et al., 2005), protein loading was adjusted by the volume of original fractions unless specified in figure legend. This volume can be converted to original tissue wet weight.

Alkaline phosphatase treatment

8M old paraffin tissue sections of the cerebral cortex were subjected to antigen retrieval (as above), followed by washing in 50 mM TrisHCl, 1mM EDTA, pH8.0 (5min $\times$ 2). Treated sections were incubated in 250 μ l of 500 unit bovine intestinal alkaline phosphatase (SIGMA-ALDRICH) at 37°C for 16 hours. After treatment, sections were washed with PBS (5min $\times$ 3), blocked and incubated with primary antibodies (TOC1, 1:1000; pS199/pS202 (Invitrogen), 1:1000). Secondary antibodies were Alexa 488 mouse IgM and Alexa 568 rabbit IgG, respectively.

Electron Microscopy/ Immunogold labeling of Recombinant Tau Aggregates

For negatively-stained electron microscopy (EM), aggregated tau was fixed with 2.5% glutaraldehyde and spotted onto 300-mesh formvar/carbon coated copper grids (Electron Microscopy Services). Each grid was rinsed with filtered H₂O and stained with 2% uranyl acetate. Conversely, in the case of negatively-stained TOC1 immunogold, samples were not fixed, as this negatively impacts the TOC1 epitope. Nickel grids were used for immunogold labeling and samples were blocked in 0.2% gelatin, 5% goat serum in 1X TBS. Grids were rinsed in 1X TBS before incubation with TOC1 primary antibody at 1:2500 for one hr at RT. Following rinsing with 1X TBS, 6nm diameter gold-conjugated anti-mouse IgM (μ -chain specific) (Sigma) secondary antibody (1:50) was applied to the samples and allowed to incubate for an hour at RT. Grids were rinsed in 10X TBS to reduce non-specific labeling, followed by H₂O, and finally stained with 2% uranyl acetate. Images were captured using the FEI Tecnai Spirit G2 transmission electron microscope at the Northwestern University Cell Imaging Facility.

Results

TOC1 recognizes all forms of denatured tau

TOC1 is a monoclonal oligomeric-selective antibody (Patterson et al., 2011) that was utilized in this study to investigate whether oligomeric inclusions exist within the rTg4510 P301L conditional mouse model of tauopathy (Lewis et al., 2000; Santacruz et al., 2005). An extraction protocol was developed (see Methods) (Figure 1A) and we subsequently focused on the S1 fraction which included solely soluble tau. The S1 fraction contains a high percentage of normal tau (50-60kDa) and the recently reported TBS-extractable 64kDa tau, presumably a hyperphosphorylated version of the tau monomer (Sahara et al., 2012) (Figure 1B & 1C). Under normal reducing conditions, all of the antibodies assayed (including TOC1) react with rTg4510 protein lysates on Western blots in an age dependent manner. Initially, only the tau monomer appears reactive at early ages; this reactivity lessens as the animals age and appears to be replaced, in part, by the 64kDa hyperphosphorylated tau at approximately 6 months of age, peaking at 8 months (Figure 1C). CP13, a mouse monoclonal antibody whose epitope encompasses phosphoserine-202, also reacts with the

64kDa tau species indicating that this tau species is phosphorylated at this site. We demonstrate that the 64kDa species is composed of full length tau, as indicated by its reaction to N-terminal Tau12, C-terminal Tau46, and Tau5 (central portion of tau) (Figure 1C).

The peak of TOC1 expression is coincident with CP13 and MC1 and appears earlier than Ab39

The S1 soluble fractions derived from brains of 2-M, 4-M, 6-M, 8-M and 14-M rTg4510 mice were probed with various antibodies to assess disease progression. To better visualize conformational and phosphorylation changes, the reactivities of TOC1, MC1, CP13 and Ab39 were ratioed to an antibody that reacts with all tau species (Tau5). Dot blot analyses illustrate a distinct peak in the presence of TOC1 (Figure 2A), MC1 (Figure 2B), and CP13 (Figure 2C) reactivities at 6-M of age and a sharp decline and subsequent plateau as the mice continue to age. Ab39, an NFT marker (Dickson et al., 1992; Lewis et al., 2000; Yen et al., 1987), displayed no reactivity in this soluble fraction until 8-M of age, with its peak reactivity at 14-M. This reactivity is much lower than any of the other antibodies in the soluble tau fraction likely indicating a slow transition from more soluble oligomers to tau in a more filamentous state (Figure 2D).

TOC1 detects a dimer size protein only under non-reducing and denaturing conditions

The S1 soluble fraction of tau was fractionated further by additional centrifugation into its supernatant (S1c) and pellet (S1p) (Figure 1A) and each fraction was then separated by SDS-PAGE. Under non-reducing conditions we previously observed that the pellet fraction contained both the TBS-extractable 64kDa tau *and* a 130kDa potential dimer when probed with the tau antibody Tau46 (24). In the present study, we chose to investigate whether this putative tau dimer is recognized by our oligomer selective antibody, TOC1. Using a Bis-Tris gel system and MOPS running buffer for SDS-PAGE, we separated S1c and S1p fractions from 4-M and 8-M old mice. Under reducing and denaturing conditions, the S1c lysates contains noticeable tau monomer in the transgenic lanes only, while only 64kDa hyperphosphorylated tau is observed in the S1p lysates, when probed with TOC1. However, under non-reducing and denaturing conditions, a TOC1-positive 130kDa band in the S1p fraction is observed only in the 8-M rTg4510 mouse (Figure 3A).

To ascertain TOC1's reactivity with native monomers and dimer/oligomers, both 8-M S1c and S1p fraction were analyzed for tau immunoreactivity by dot blot analysis. As expected, TOC1 displays little reactivity against tau monomers (Figure 3B); however, dot blots of S1p show high TOC1 reactivity in the 8-M old transgenic mice (Figure 3B) coincident with the appearance of dimers/oligomers under non-reducing and denaturing conditions in Western blots (Figure 3A). Moreover, rTg4510 mice at 8-M contain ~ 3 times more dimers/oligomers compared to their non-transgenic littermate controls (Figure 3C). The 8-M transgenic lysate contains readily detectable amounts of MC1 and CP13 (Figure 3B and 3C) which further supports our contention (Figure 2) that tau must change its conformation and potentially its phosphorylation profile as it dimerizes and begins to form higher oligomeric species.

Non-reducing conditions appear to favor oligomer formation

Since our mouse studies indicate that oxidative conditions favor formation of oligomers, we sought to determine whether this was true in our recombinant tau aggregation assay *in vitro*. To test this, recombinant hT40 and P301L tau protein were generated and aggregated in the presence and absence of 5mM DTT. The resulting aggregates were then visualized via electron microscopy. As expected, hT40 assembles into long straight filaments (white arrow) with some smaller aggregates also present (Figure 4A). However, in the absence of DTT, we observe structures that are more globular in nature (black arrowheads) and we no longer detect any long filaments (Figure 4B). Interestingly, the same phenomenon is observed with recombinant P301L tau; although, in the presence of DTT, the filaments are much shorter and less abundant than those formed from hT40 (white arrow) (Figure 4C). In the absence of DTT, P301L also forms an abundance of globular structures (black arrowheads) and fewer filaments (Figure 4D).

Identifiable oligomeric structures enriched from the rTg4510 mouse are TOC1-positive

Recombinant hT40, P301L, and 8-M rTg4510 protein lysates were used for TOC1 immunogold labeling. Few filaments are labeled in any instance (white arrowheads); in fact, TOC1 only seems to label clusters of oligomers with the recombinant protein (red arrows) (Figure 5A and C). Negative stains of the S1p fraction from the rTg4510 transgenic mouse appear to confirm the *in vitro* data (Figure 5) in that both short filaments (white arrow) and the presence of oligomers (Figure 5B) are observable. TOC1 immunogold labeling of this fraction again revealed the same result as that seen with recombinant protein, in that very few gold particles are observed decorating the filaments. However, a few are detected at the filament ends, (white arrow) consistent with our previous findings for this antibody (Patterson et al., 2011). As expected, we also see TOC1 labeling tau oligomers (black arrowhead) (Fig. 5D).

Does oligomeric tau (TOC1+) precede formation of filamentous tau (Ab39+) in the rTg4510 mouse model?

As tau pathology forms in the medial temporal lobe during the course of AD, a stepwise evolution of aggregation types appear to exist. Abnormal phosphorylation of tau either codifies or induces conformational changes that appear to coincide with the formation of tau dimers/oligomers evidenced by the colocalization of specific markers with TOC1 (Patterson et al., 2011). Tau then forms filaments that coalesce into NFTs, the hallmark fibrillar lesion of AD. These bind Thioflavin S (a beta sheet-specific dye) and a set of tau antibodies specific for changes that occur during NFT formation such as TauC3, MN423, and Ab39 (Gamblin et al., 2003b; Lewis et al., 2000; Novak, 1994). In mild cognitive impairment (very early AD), there is no colocalization between TOC1-positive oligomers and Ab39-positive structures in human MCI tissue (Figure 6A). Similar results were observed for TOC1 and Thioflavin-S (Figure 6B). Within the rTg4510 mouse model of tauopathy, however, we observe that TOC1 colocalizes with Ab39 (Figure 6C). A double stain with TOC1 and the NFT marker Thioflavin-S reveals the same phenomenon although less so than the TOC1/Ab39 stains (Figure 6D). This may be indicative of a faster transition from oligomers to polymers in the mouse due to the voracious aggregation of P301L and/or the

fact that the mouse expresses the P301L mutation at a level approximately thirteen times that of endogenous mouse tau (Santacruz et al., 2005) (see Discussion). Clearly, these results indicate that tau pathogenesis in the rTg4510 mouse model appears to occur at an accelerated rate when compared to wild type human tau in prodromal AD (see Discussion).

Discussion

In recent years, there has been a great body of evidence to suggest that NFTs are not the primary toxic tau aggregate within the brain in Alzheimer's disease and other tauopathies (Berger et al., 2007; Ramsden et al., 2005; Santacruz et al., 2005). For example, NFTs are suggested to persist in neurons for decades (Guillozet-Bongaarts et al., 2006; Morsch et al., 1999) indicating a dissociation between NFT formation and memory impairment (Santacruz et al., 2005). Other *in vivo* investigations have only served to strengthen the argument that oligomers appear to be more neurotoxic than other tau aggregates. When tau monomers, oligomers and filaments were injected into the CA1 region of the right hemispheres of wild-type mice, *only* the oligomers caused memory deficits (Lasagna-Reeves et al., 2012a), and cellular damage only occurred in the hemisphere where the oligomers were initially injected and accumulated in the highest concentrations. This may suggest that oligomers need to reach a critical level before they achieve toxicity (Khlistunova et al., 2006; Lasagna-Reeves et al., 2012b). A recent human study focusing on the nucleus basalis of the cholinergic basal forebrain (CBF) demonstrated through the use of antibodies (pS⁴²² and TauC₃) that cognitive defects are apparent before the onset of NFT deposition (Vana et al., 2011). Furthermore, pre-fibrillar inclusions are linked to memory deficits that emerge prior to the formation of NFTs in the rTg4510 FTDP-17 mouse model of tauopathy, the same model used in this investigation (Berger et al., 2007).

Recently, we succeeded in isolating a hyperphosphorylated pool of tau (64kDa) in the TBS-extractable fraction from rTg4510 mice (Sahara et al., 2012). We hypothesized that the majority of this hyperphosphorylated tau species within the soluble fraction may be a precursor to tau oligomers due to their size on an SDS-PAGE gel. Using the novel TOC1 dimer/oligomer-selective antibody, we previously demonstrated that dimerization/oligomerization is an early event in the aggregation process (Patterson et al., 2011). Utilizing this antibody, it was demonstrated that tau oligomers are greatly increased in people affected with AD compared to non-cognitively impaired controls (Patterson et al., 2011). Preliminary studies suggested that TOC1 had a discontinuous epitope which was destroyed by SDS denaturation and subsequent Western blotting (Patterson et al., 2011). However, additional characterization indicates that TOC1 appears to recognize a discrete epitope between amino acids 209-224, that is masked in the non-pathological native protein, but can in fact be revealed when denatured by Western blotting (Ward et al., 2013).

TOC1 was one of the major tools used in the current investigation to assess the initial aggregation of tau protein at the onset of disease. It is a selective aggregation marker, meaning that under dot blot analysis of native protein, it largely recognizes dimers or higher order oligomers but not monomers and filaments (Patterson et al., 2011). By contrast, upon SDS denaturation, TOC1 recognizes all forms of tau tested, including tau monomers. Therefore, while TOC1 can detect denatured monomers, in certain folded states the epitope

is obscured, while it is available for antibody binding in conformations induced via dimer/oligomer formation. Dot blot analysis indicates that unmasking of the TOC1 epitope in non-denatured tau is coincident with the appearance of both MC1 and CP13 epitopes, which are conformation-specific (Jicha et al., 1997; Spire et al., 2006) and phosphorylation-specific antibodies (Spire et al., 2006) respectively. Hence, the conformational change and this phosphorylation event would appear to be heralding the dimerization/oligomerization of the tau molecule. Another antibody, Ab39, is a late-stage NFT marker (Dickson et al., 1992; Lewis et al., 2000; Yen et al., 1987), which is not present in soluble tau fractions until 8-M within these transgenic mice. The contemporaneous peaks in appearance between TOC1, MC1 and CP13 further support our previous studies indicating that TOC1 is an early stage marker, and also suggest a structural context for the formation of these other early markers of tau pathology (Jicha et al., 1997; Spire et al., 2006). Alkaline-phosphatase treatment, which serves to dephosphorylate the potential phosphoepitopes present, demonstrates that TOC1 staining is relatively unchanged between treated sections and untreated controls (Figure 7). This suggests that TOC1 is truly recognizing a conformational change of tau as opposed to a phosphorylational or post-translational modification within the protein.

The S1c and S1p fractions were analyzed for tau immunoreactivity by Western blot and dot blot to assess reactivity under denaturing and native conditions, respectively. As expected from our earlier data, TOC1 reacts with all types of tau species under denaturing conditions. However, under non-reducing conditions a high molecular-weight protein (~130kDa) is also present. Additionally, it was observed that TOC1 does not recognize tau monomers under native conditions (Patterson et al., 2011). MC1 displays similar reactivity to TOC1 in an age-dependent manner. This suggests that a conformational change occurs as tau monomers transition to dimers/oligomers allowing the TOC1 epitope to become exposed. Interestingly, we observe CP13 activity within *both* the S1c and S1p fractions whereas we only see appreciable reactivity in the S1p fraction with both TOC1 and MC1 antibodies. Since monomeric tau remains in the S1c fraction, this may suggest that phosphorylation of the monomer at the CP13 site is a pre-requisite to oligomer formation or in the very least an early event that occurs prior to any conformational change. However, more investigation is needed to confirm this hypothesis.

For many years there has been much speculation regarding the deleterious effects of oxidation within the brain as humans age. The presence of a high-molecular weight tau protein band (ca. 130kDa) was noted on Western blots only under oxidizing conditions; therefore, we next investigated whether the reduction/oxidation environment of the protein impacts the formation of oligomers. Both hTau40 and P301L tau predominantly form filaments when the proteins are aggregated under reducing (5 mM DTT) conditions, although in the case of the mutant protein, the filaments are much shorter. However, when the proteins were not reduced (-DTT), we observed an abundance of oligomers present, along with only a few short filaments in both aggregated hT40 and P301L tau proteins. We explored this further using TOC1 immunogold labeling. As expected, TOC1 does not decorate the filaments (Patterson et al., 2011), but only binds to small clusters of oligomers, in *both* hT40 and P301L recombinant proteins. Many studies have implicated oxidative conditions as most favorable for tau aggregation (Schweers et al., 1995; Yao et al., 2003). It

is believed that one or two cysteines, present in the 3R and 4R isoforms respectively, are necessary for both oligomer and filament formation (Schweers et al., 1995). However, Gamblin et al. demonstrated that the double cysteine mutant “cys-less” can readily form filaments both in the absence and presence of DTT, indicating that the oxidation of cysteines is not essential for filament formation (Gamblin et al., 2000b). Collectively, these data would seem to suggest that the increase in oligomer formation under oxidative conditions is kinetically favored over filament formation.

We subsequently carried out the same experiments using the S1p fraction from the rTg4510 mice. The negative stain of an 8M old mouse frontal cortex clearly displays short filaments and many oligomers. TOC1 immunogold labeling is more difficult on the mouse extract as these proteins are much better visualized using fixative, but fixative obscures the TOC1 epitope. However, we were still able to observe the same phenomenon as that seen with recombinant protein. TOC1 does not fully decorate short filaments; occasionally it labels the filament ends, but consistently labels small clusters of oligomers.

Since the data presented herein along with previous studies indicate that oligomers represent an early stage event in tau pathogenesis (Lasagna-Reeves et al., 2012b; Maeda et al., 2007; Meraz-Rios et al., 2010; Patterson et al., 2011; Sahara et al., 2008; Santacruz et al., 2005), we compared IF staining of the frontal cortex of 8M rTg4510 mice and the entorhinal cortex (ERC) of MCI stage human cases. TOC1 was used to label pre-tangles as an early stage marker and either Thioflavin-S (Thio-S) or Ab39 was used to label NFTs as late stage markers (Janocko et al., 2012; Yen et al., 1987). As expected, there is little-to-no colocalization between TOC1-Thio-S and TOC1-Ab39 in the human MCI cases. However, IF staining on rTg4510 tissue instead revealed colocalization between both TOC1-Thio-S and TOC1-Ab39. This result was puzzling, given that TOC1 does not show temporal overlap with these late-stage markers in human AD pathogenesis. One explanation for these conflicting results could be that the P301L tau mutation is so aggressive that tau oligomers form at a more accelerated rate and may persist for longer periods in the mouse model than they do in human disease.. An alternative possibility is that the rTg4510 mouse model, which expresses the mutant transgene at 13 times the level of endogenous murine tau (Santacruz et al., 2005), forces oligomer formation and eventual filament formation contemporaneously. In fact, it was illustrated that the rate of NFT formation is directly related to the amount of transgene expressed (Santacruz et al., 2005). Therefore, it is most likely that the oligomers are forming at an increased rate due to an increased pool of soluble tau becoming available as phosphorylated tau disassociates from the microtubules. This large pool of soluble tau would have a high propensity to aggregate and might subsequently result in NFTs forming at a much earlier stage in the mouse model than would typically occur in the human population. This idea might help to explain the surprising colocalization of TOC1 and the two NFT markers observed in these transgenic mice. However, this by no means indicates that the NFTs are forming from the oligomers directly or from a separate pathway. Such determinations require additional experimentation.

The current investigation has led to a broader understanding of tau oligomers, the elusive new aggregates recently identified within the pathological tau cascade. Here we provide evidence of their abnormal conformation and potential phosphorylation within the rTg4510

conditional mouse model of tauopathy. We demonstrate that these aggregates occur early within the pathological process and that oxidative conditions are most favorable for their formation. Since our TOC1 antibody is strictly selective to tau oligomers but not tau filaments, along with the help of other novel antibodies, the use of this antibody will aid in providing a better understanding of tau toxicity.

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Abbreviations used

AD	Alzheimer's disease
FTPD-17	Frontotemporal Dementia with Parkinsonism linked to chromosome 17
PiD	Picks disease
CBD	Corticobasal Degeneration
PSP	Progressive Supranuclear Palsy
MAP	Microtubule-Associated Protein
NFT	Neurofibrillary tangle
TOC1	Tau Oligomeric Complex 1
MCI	Mild Cognitive Impairment
FFPE	Formalin-Fixed Paraffin Embedded

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Neurobiology of Disease Highlights

- TOC1 recognizes all forms of denatured tau, but only oligomers in their native state.
- Tau oligomers preferentially form under oxidative conditions.
- The peak of TOC1 is coincident with other early stage markers, CP13 and MC1.
- TOC1 detects a dimer size protein only under non-reducing and denaturing conditions
- Identifiable oligomeric structures enriched from the rTg4510 mouse are TOC1-positive

Figure 1. TOC1 recognizes all forms of denatured tau

A. Sample preparation protocol for the detection of tau protein. Tissues were separated into TBS-extractable (S1), high-salt and sarkosyl-soluble (S3), and sarkosyl-insoluble (P3) fractions. S1 fraction was separated further into supernatant (S1c) and precipitate (S1p). Detailed protocol was described in Materials and Methods. **B.** Western blot of brain samples from 4 month-old (4-M) and 8 month-old (8-M) rTg4510 mice probed with human tau specific antibody, E1. S1, S3, and P3 fractions were loaded at a ratio of 1:16:50 (based on tissue weight), which was derived from 0.01 mg tissue, respectively. Upper and lower panel show blot image with short and long exposure, respectively. Arrowheads indicate 64kDa tau. **C.** Western blots of S1 fraction from rTg4510 (tg) and non-tg (nt) mice aged 2-M, 4-M, 6-M 8-M and 14-M using 5 different tau antibodies and GAPDH antibody. The same volume of S1 fraction derived from 0.01 mg (for Tau12, Tau5 and Tau46 Western blots) or 0.1 mg (for TOC1 and CP13 Western blots) wet weight of brain was loaded into the gels. The blot of CP13 was re-probed with GAPDH antibody. Arrowheads indicate 64kDa tau.

Figure 2. The peak of TOC1 expression is coincident with MC1 and CP13

Representative dot blots displaying 500ng of the S1 fraction derived from brains of 2-M, 4-M, 6-M, 8-M and 14-M old rTg4510 mice were probed with various antibodies to assess disease progression. *A*, TOC1 peaks at 6-M with very little expression before this age. It decreases again as the mouse continues to age. *B*, MC1 demonstrates an increase in expression until a peak occurs at 6months of age. *C*, Similarly; CP13 also displays a rapid increase in expression at 6-M of age. *D*, Ab39, which is an antibody directed against NFTs

demonstrates little expression across the lifespan of the mouse, with the peak late in life at 14-M.

Figure 3. TOC1 recognizes an oligomeric size structure only under non-reducing and denatured conditions and only in the S1p fraction

S1c and S1p fractions were recovered after $150,000 \times g$ centrifugation of S1 fraction. **A**, Equal volumes of S1c fraction derived from 0.2 mg wet weight of brains and S1p fraction derived from 1 mg wet weight of brains of 4M and 8M rTg4510 (tg) and non-tg (nt) mice were separated by SDS-PAGE under reducing (+bME) and non-reducing (-bME) conditions. This was followed by incubation with the TOC1 antibody. Blots were re-probed and normalized with GAPDH. **B**, Dot blot analysis of 8-M S1c and S1p fractions. Equal volumes of S1c (CP13; volume derived from 0.2 mg of wet tissue weight, TOC1 and MC1; volume derived from 0.02 mg of wet tissue weight) and S1p, (CP13; volume derived from 1 mg of wet tissue weight, TOC1 and MC1; volume derived from 0.1 mg of wet tissue weight) were spotted on nitrocellulose membranes. Only 8-M rTg4510 (Tg+; n=3) and non-tg control (Tg-; n=3) mice were examined. **C**, Graphs show quantification of each antibody signals in different fractions. Relative signal intensities are means $\pm$ SEM normalized to the

value from non-tg S1c sample. Significant differences were found between rTg4510 and non-Tg samples: * $p < 0.05$; *** $p < 0.001$.

Figure 4. Non-reducing conditions appear to favor oligomer formation

Electron microscopy imaging of hT40 or P301L recombinant protein, in the presence or absence of 5mM DTT. *A*, hT40 in the presence of DTT produces long straight filaments (white arrow). *B*, In the absence of DTT there are a lot more globular structures, more indicative of oligomers (black arrowheads). *C*, P301L produces shorter filaments in the presence of DTT (white arrow). *D*, Similar to image *B*, there are an abundance of globular structures present in the P301L recombinant protein in the absence of DTT (black arrowheads). Scale bar 200nm.

Figure 5. Identifiable oligomeric structures enriched from the rTg4510 mouse are TOC1-positive Electron microscopy imaging of recombinant protein and mouse protein lysates. *A and C*, Immunogold labeling of hT40 and P301L protein aggregated in the presence of DTT. TOC1 appears to label few filaments in the hT40 nor the P301L aggregates (white arrowheads), however it does label clusters of oligomers (red arrows). *B*, rTg4510 S1p fraction negatively stained with 2% uranyl acetate, highlights the presence of oligomers and short filaments (white arrow). *D*, TOC1 immunogold labeling of rTg4510 S1p fractions, indicates the gold particle only labeling the end of the filament (white arrow). Oligomers are labeled with TOC1 (black arrowhead). Scale bar 200nm.

Figure 6. Does oligomeric tau (TOC1+) precede formation of filamentous tau (Ab39+) in the rTg4510 mouse model?

A, Human MCI tissue shows no colocalization between TOC1 and Ab39 as expected. *B*, TOC1 and Thioflavin-S display no colocalization within the human tissue. *C*, TOC1 and Ab39 in the cortical neurons of 8M rTg4510 mice. *D*, Colocalization between TOC1 and Thioflavin-S within the rTg4510 mouse model is not as robust as that observed with Ab39.

7.4M rTg4510 Cerebral cortex

Figure 7. TOC1 staining is similar between alkaline-phosphatase treated and non-treated tissue controls

A, Paraffin-section of cerebral cortex treated with alkaline-phosphatase and stained with TOC1. B, Paraffin-section of cerebral cortex stained with TOC1 (–alkaline-phosphatase treatment). C, Paraffin-section of cerebral cortex treated with alkaline-phosphatase and stained with pS199/pS202. D, Paraffin-section of cerebral cortex stained with pS199/pS202 (–alkaline-phosphatase treatment).

Table 1
List of antibodies used

Antibody	Assay	Dilution	From	Reactive to human and/or mouse protein
E1	WB	1:5,000	Provided by Drs. S-H. Yen and D.W. Dickson (Mayo Clinic)	Human tau
Tau5	WB	1:100,000	Generated in the Binder	Human and mouse tau
	DB	1:100,000	Laboratory	
Tau12	WB	1:3,000,000	Generated in the Binder Laboratory	Human tau
Tau46.1	WB	1:5,000	Zymed	Human and mouse tau
CP13	WB	1:200	Provided by Dr. P. Davies	Human and mouse tau
	DB	1:1000		
GAPDH	WB	1:5,000	Biodesign	Human and mouse GAPDH
TOC1	DB	1: 10,000	Generated in the Binder Laboratory	Human tau *
TNT1	DB	1:900,000	Generated in the Binder Laboratory	Human tau *
Ab39	DB	1:1000	Provided by Dr. S-H. Yen	Human tau *
MC1	DB	1:1000	Provided by Dr. P.Davies	Human tau *

* Pathological forms (e.g, abnormal conformation, oligomers, tangles) of human tau have been analyzed.