FTDP-17 tau mutations decrease the susceptibility of tau to calpain I digestion

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Abstract Frontal temporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) is caused by splice site and missense mutations in the tau gene, and characterized by the accumulation of filamentous tau in cerebral neurons and glia. The missense mutations reduce the ability of tau to promote microtubule assembly and increase the ability of tau to form filaments. In this report we demonstrate that mutants V337M and R406W are less susceptible than mutant P301L or corresponding wild type tau to degradation by calpain I. The differences were at least in part due to changes in accessibility of a cleavage site located about 100 amino acids off the carboxy-terminus. The results suggest that the pathogenesis of some forms of FTDP-17 may involve tau accumulation due to decreased proteolytic degradation.

Key words: Frontal temporal dementia; Parkinsonism; Chromosome 17; Tau gene; Calpain I

1. Introduction

Frontal temporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) is characterized clinically by behavioral, cognitive and motor disturbances, and histopathologically by neuronal and glial inclusions which contain insoluble forms of the microtubule associated protein tau [1]. Genetic analyses reveal that the disorder is caused by missense, splice site or deletion mutations in the tau gene [2–6]. Intronic mutations have been located close to the 5′ splice site of exon 10 (+3, +13, +14 and +16) and missense mutations in exons 9–13. A single deletion of residue K280 has been found in exon 10. The exon 10 splice site mutations and the exonic mutations at N279K, L284L and S305N have been shown to affect the expression of different tau isoforms, resulting in an increase of the ratio of tau containing four versus three tandem repeats (3R) in its microtubule binding domain [2–10]. Most of the missense mutations (e.g. P301L, P301S, V337M and R406W) were shown by in vitro assays to reduce the ability of tau to bind microtubules and to promote microtubule assembly [8,11,12]. The extent of reduction varied depending on the site and the nature of the mutation. Mutations also affected the ability of tau to form filamentous structures through self-interactions [13–15]. Among the FTDP-17 tau mutants studied, P301L and P301S displayed the highest potential to form filaments. Studies of microtubule and tau filament assembly clearly demonstrated that FTDP-17 mutations have a dual effect, suggesting that the pathogenesis of FTDP-17, which results in the formation of filamentous tau inclusions, involves at least two mechanisms. Assembly of tau filaments as revealed by in vitro studies is a process that requires the presence of a critical concentration of tau and includes both a nucleation and an elongation phase [16]. The cellular concentration of tau could be elevated by an increase in the synthesis of tau or a decrease in the degradation of tau proteins, or both. The turnover of tau may also be affected by mutations. The latter issue was addressed in the present study using calpain I, which is a calcium activated neutral protease rich in neurons and capable of degrading tau [17]

2. Materials and methods

2.1. Antibodies

Six rabbit polyclonal (E1, E2, WKS44, E9, WKS45, WKS46) and two mouse monoclonal (Tau-1, Tau46) anti-tau antibodies were employed. The epitopes recognized by these antibodies were mapped to amino acid residues 19–33, 44–55, 162–176, 192–199, 226–240, 258–266, 358–370, and 420–436 [18,19], respectively. Tau-1 [20] was generously provided by Dr. L. Binder (Northwestern University) and Tau46 by Dr. V.M.-Y. Lee (University of Pennsylvania). All polyclonal antibodies were generated in our laboratory and used at 1:500 dilution. Tau-1 was hybridoma supernatant and used at 1:5 and 1:10 dilutions. Tau46 was ascites fluid used at 1:1000 dilution.

2.2. Generation of constructs and mutagenesis

The cDNA for four repeat wild type tau (4R, +2 +3, or T40) was initially provided by Dr. M. Goedert (MRC, Cambridge). Utilizing the pRK172 construct, a three repeat sibling cDNA was created as follows. Using the *Bcl*I and *Sfi*I sites, the fragment of DNA containing exon 10 was removed and replaced with the same digestion fragment from a pBS tau clone lacking exon 10. Mutagenesis was performed according to the manufacturer's instructions. The sequences of mutagenesis oligos for generation of mutants [4R: P301L, V337M and R406W; 3R: V306M, R375W] were P301L 5′-AACAC-GTCCTGG-GAGGCG-3′, R406W (or R375W) 5′-GGGACACGTCTCCATGG-CATCTCAGCAAT-3′, V337M (or V306M) 5′-CAGGAGGTGGC-CA-GATGGAAGTAAAA-3′. DNA was isolated from mutant and wild type clones and the tau cDNA subcloned in frame into the expression vector pET30a+ (Novagene) containing either full length 4R, +2 +3 or 3R, +2 +3 tau. Based on the location of the P301L mutation, only the four repeat tau construct contains this mutation. Tau cDNA constructs were sequenced using the Big Dye Terminator Cycle Sequencing kit (Perkin Elmer). Sequencing was performed on an ABI377 automated sequencer with Sequence Navigator software (Perkin Elmer).

2.3. Expression and purification of recombinant tau

Recombinant tau proteins were expressed in *Escherichia coli* and purified from bacterial lysates as described previously [13,21]. Tau separated by CM Sepharose chromatography were subjected to HPLC chromatography (C18 column, 1×25 cm, Vydac), and eluted with a gradient of 20–50% acetonitrile containing 0.1% trifluoroacetic acid in 60 min at a flow rate of 2 ml/min. The eluates were monitored at UV 260 μm. The purity of tau sample was examined by gel electrophoresis, followed by Coomassie blue staining and immunoblotting. Protein content was determined by the bicinchoninic acid method (Pierce).

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2.4. Digestion of tau with calpain I

Tau proteins (100 μg/ml) were incubated with porcine erythrocyte calpain I (EC 3.4.22.17, MW 112 000) at an enzyme:substrate ratio (based on molarity) of 1:100 (Calbiochem, La Jolla, CA). The reaction mixture contained 20 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol, 150 mM NaCl and 100 μM $CaCl_2$. Aliquots of the samples were removed at various time intervals and mixed immediately with a 6× sample buffer containing 12% SDS and 12% β-mercaptoethanol to terminate the digestion. The samples were boiled and used for gel electrophoresis and immunoblotting.

2.5. Electrophoresis and blotting

Aliquots of the calpain I digested samples, each containing 1 μg of tau, were loaded on 12.5% or 15% SDS-polyacrylamide gels and gel electrophoresis was performed as described. Proteins separated on gel were stained with Coomassie brilliant blue or silver stain (Bio-Rad, Hercules, CA). For immunoblotting, the protein loading was 0.3 μg. The separated proteins were electrotransferred to nitrocellulose paper, and the papers were incubated with 5% non-fat milk in Tris buffered saline to block non-specific binding, followed by incubation with anti-tau antibodies. The bound immunoglobulin was detected by enhanced chemiluminescent system (Amersham, Buckinghamshire). The amount of undegraded tau was quantitated by the MCID system (Imaging Research Inc., Ontario).

3. Results and discussions

3.1. Kinetics of tau degradation

3.1.1. Four repeat tau. Wild type and three mutated forms (P301L, V337M, R406W) of tau containing four tandem repeats in the microtubule binding domain and amino-terminal inserts encoded by exons 2 and 3 were used. These proteins [4R, +2 +3] were purified from lysates of bacteria expressing the tau cDNAs. The proteins were incubated for up to 32 min at 30°C with calpain I, a calcium dependent protease. Small aliquots of the mixtures were removed after 0, 1, 2, 4, 8, 16 and 32 min of incubation and mixed immediately with a 6× sample buffer containing SDS to terminate the digestion.

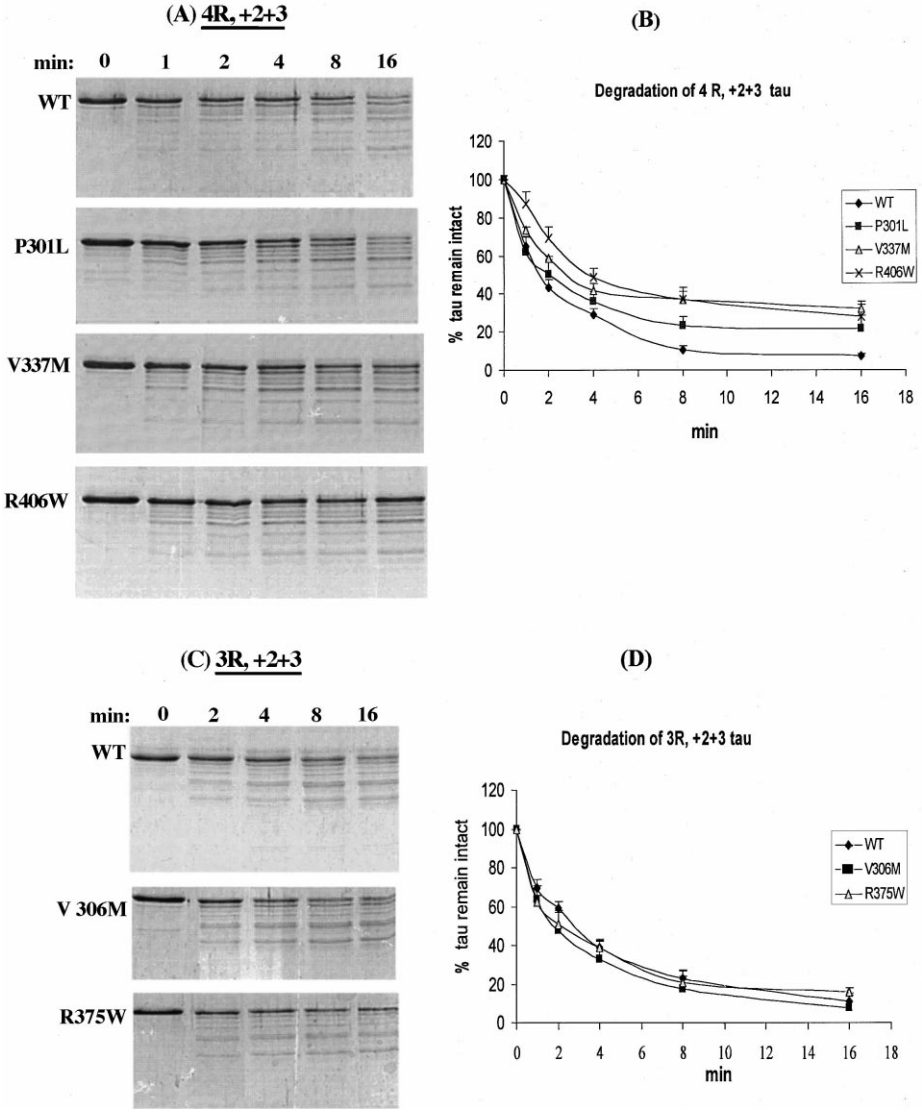

Fig. 1. Degradation of full length four repeat and three repeat tau by calpain I. Undegraded tau and tau incubated with calpain for 1, 2, 4, 8, 16 and 32 min (not shown) were separated by SDS-gel electrophoresis using gels containing 15% polyacrylamide. A and C: Coomassie blue stained gel, demonstrating the decrease of the amount of intact tau with incubation. The decrease was accompanied by the appearance of multiple fragments with molecular weight greater than 35 kDa. Low molecular weight regions of the gel (< 35 kDa) were barely stained by the dye. R406W and V337M mutants were degraded more slowly than P301L mutant or wild type tau. B and D: Kinetics of tau degradation. Error bars represent standard error of the mean.

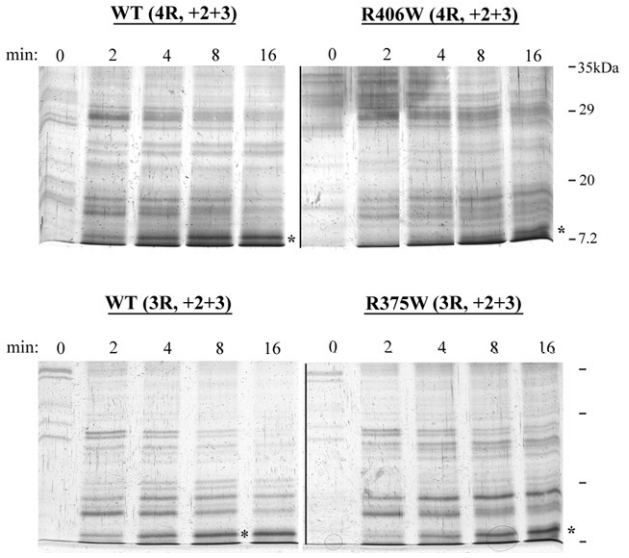

Fig. 2. Silver staining of low molecular weight regions of gels (< 35 kDa) containing calpain I degraded wild type tau, R406W and R375W mutant tau. Degraded R406W mutant differed from degraded four repeat wild type tau in the incubation time required to generate small fragments (marked by *), which migrate between the 7.2 kDa and 20 kDa molecular weight markers. They also differed in the labeling intensity of large sized fragments (between 20 and 29 kDa, or 29 and 35 kDa molecular weight markers). Less difference was detected between the three repeat wild type and R375W mutant tau.

The samples were then subjected to SDS-polyacrylamide gel electrophoresis and Western blotting with a series of antibodies specific to different subregions of the tau molecule. The gels were subjected to Coomassie blue and silver staining, and analyzed by densitometric scanning using the MCID imaging system (Imaging Research Inc.). The immunoblots were visualized with an enzyme-linked chemiluminescent kit (Amersham).

Densitometric analyses of Coomassie blue stained gel revealed that prior to calpain digestion, at least 98% of the tau was intact (Fig. 1A, 0 min). Unlike wild type tau, mutant tau displayed a slower rate of degradation (Fig. 1B). On average, about 8% of the intact wild type tau remained undegraded after 16 min of proteolytic digestion. In comparison, 22–32% of mutant tau remained intact with the same duration of incubation. Most of the degraded fragments (with wild type or mutant tau) were either too small to be retained in the gel system used in present studies, stained poorly with Coomassie blue, or the quantity was below the sensitivity of detection by the dye. About 50% of the intact wild type tau was degraded after 1.3 min of incubation. In comparison it took 1.8, 2.2 and 3.6 min to achieve the same degree of degradation of P301L, V337M and R406W, respectively. The rate of degradation differences observed between the wild type and V337M or between the wild type and R406W were statistically significant at all time points ($P < 0.05$; Student's t-test, $n = 8$). The differences between the wild type and P301L mutant were significant only for those samples with 8 min or longer incubation.

3.1.2. Three repeat tau. Similar studies were also carried out with three repeat wild type tau and tau with mutations at V306M and R375W, which are sites corresponding to V337M and R406W in four repeat tau. In contrast to four repeat tau, there were no significant differences between three repeat mutant tau and wild type tau in the kinetics of degradation (Fig. 1C,D). The results suggest that the presence of the second tandem repeat is required for mutations to affect the susceptibility of tau to calpain I degradation. Comparison of wild type tau containing four repeats with wild type tau containing three repeats did not reveal significant differences between these two isoforms in the rate of degradation. The results are consistent with those reported previously [22].

3.2. Characterization of degraded fragments

3.2.1. Silver staining. Silver stain was used to examine the calpain digests of four repeat wild type and the corresponding R406W mutant tau, focusing on the region of the gel between the 35 kDa molecular weight marker and the bottom of the gel. Unlike fragments with a molecular weight higher than 35 kDa, the low molecular fragments were poorly stained by Coomassie blue (presumably due to low quantities and the relative insensitivity of the dye). However, multiple silver stained bands were observed in this region of gels (< 35 kDa) containing either digests of four repeat wild type or mutant tau (Fig. 2, 4R, +2 +3). Most of the bands were weakly labeled. Some fragments were also detected in undigested controls, presumably derived from degradation that occurs during protein preparation. A number of bands detected in digests of both wild type and mutant tau were stained with different intensities. These were located around the 30 kDa and between the 20 and 7 kDa regions. A prominent band with molecular weight ~10 kDa was detected in digests of wild type tau (marked by *). This band was less apparent in the digests of R406W mutant tau, especially those obtained with shorter incubation periods (compare 2 min versus 16 min samples). A similar low molecular weight (~10 kDa) fragments was also observed after silver staining gels of calpain digested three repeat tau (Fig. 2, 3R, +2 +3, marked by *). In contrast to four repeat tau the intensity of the band and the incubation time required to generate detectable levels of the fragment were comparable in the three repeat wild type and R375W mutant tau.

3.2.2. Immunolabelling. To determine the site of calpain I cleavage in other regions of the tau molecule, we used antibodies E2, WKS44, Tau-1, E9 and WKS45 to probe 2 min calpain digests of wild type and mutant tau. These antibodies recognize epitopes located at amino acid residues 44–55, 162–176, 192–199, 226–240 and 258–266, respectively (Fig. 3B). Numerous fragments were detected in the digests. Based on their immunoreactivities, the fragments could be categorized into at least seven groups (Fig. 3A). The approximate size of different groups and the region of tau susceptible for calpain I cleavage is illustrated in Fig. 3B,C. Group 1 represents fragments larger than 35 kDa that displayed immunoreactivities with all tau antibodies tested and are only slightly smaller than intact tau. These fragments are likely missing either the amino- and/or the carboxy-end of tau. They may be missing part of the Tau46 epitopes, since they displayed weaker Tau46 immunoreactivity than that expected according to their immunolabeling with WKS46. Group 2 also represents large fragments but these lack the carboxy-terminus encompassing the Tau46 epitope. Group 3 includes several fragments missing the carboxy-terminus encompassing both the Tau46 and the WKS46 epitopes. Group 4 includes fragments of size approximately 35 kDa. The fragments were mostly weakly labeled by E1, WKS44, E9 and Tau-1 (data not shown), and not labeled

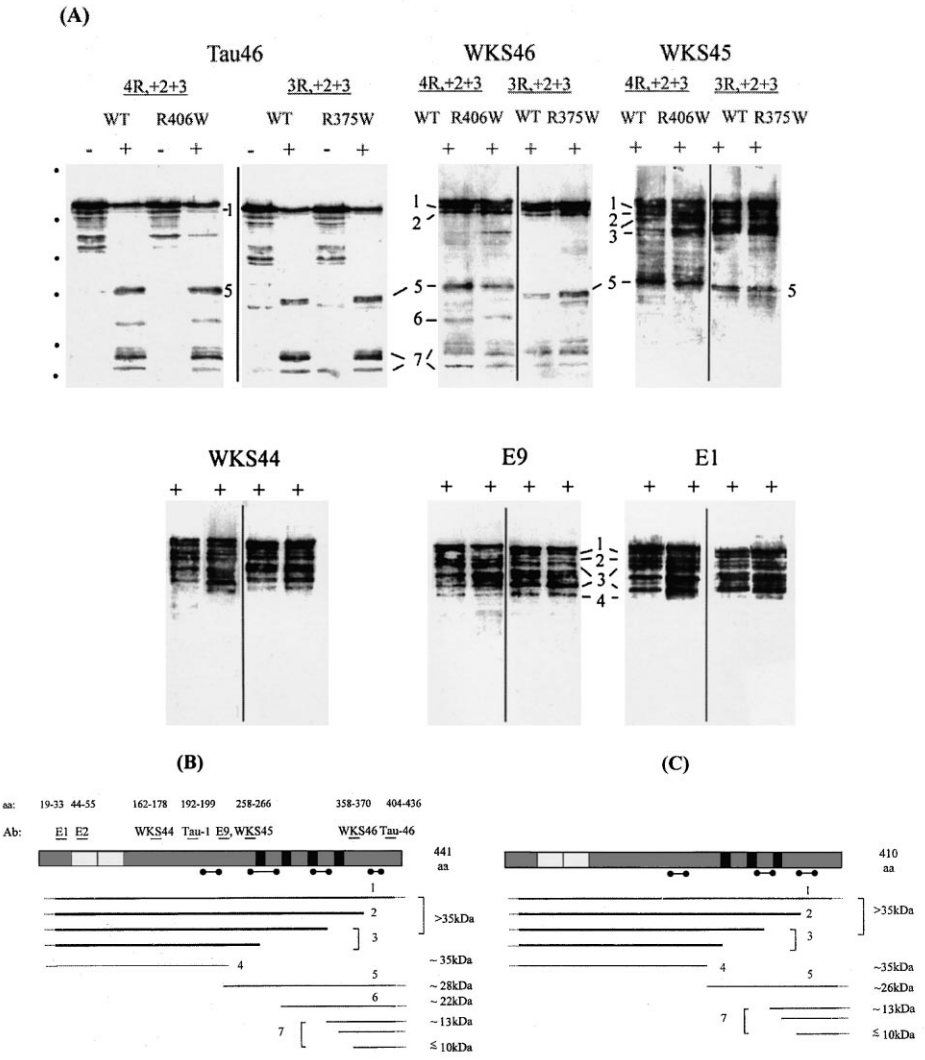

Fig. 3. A: Immunoblotting of calpain degraded tau with antibodies specific to different subregions of the tau molecule. Controls (−) and samples digested for 2 min (+) were compared. Degraded fragments were categorized into at least seven groups (marked 1–7) based on their immunoreactivities. Molecular weight markers were 7.2, 20, 29, 35, 50 and 83 kDa in size. Lower molecular weight fragments (5–7) reacted mainly with antibodies specific to epitopes located at the carboxy-end of tau. B: The location of the epitope recognized by different antibodies. The approximate sizes of different groups of fragments generated from digestion of (B) four repeat and (C) three repeat tau are indicated, and the regions that are likely to contain calpain cleavage sites are marked (●–●). Whether or not the first or the last 30 amino acids (indicated by dotted lines) are cleaved remains uncertain. aa: amino acids, Ab: antibody.

by WKS45, WKS46 or Tau46. Groups 5, 6, and 7 were carboxy-terminal fragments containing both the WKS46 and Tau46 epitopes. Group 5, in addition, contained the WKS45 epitope. Because fragments belonging to group 6 were detected mainly in four repeat tau, they were likely generated by cleavage of sites within the second repeat. The group 5 fragments were likely generated by cleavage between the E9 and WKS45 epitopes. Group 7 includes low molecular weight fragments generated by calpain I digestion of wild type and R406W tau displayed immunoreactivity with antibodies WKS46 and Tau 46, which recognize epitopes located at the carboxy end (amino acids 358–370 and 420–436 respectively) of tau (Fig. 3A,B). The fragments were not recognized by E1, an antibody that recognizes the amino-end (amino acids 19–33) of tau (Fig. 3A), or other anti-tau antibodies tested. The low molecular weight (~10 kDa) fragment identified by silver stain belongs to group 7. The results suggest that R406W mutation leads to a decrease in the accessibility of the carboxy-end of tau to calpain I digestion. Since the size of the low molecular weight fragment is ~10 kDa, and the smallest carboxy-terminal region of tau encompassing both the Tau46 and WKS46 epitopes contains about 83 amino acids (Fig. 3), the cleavage site affected by the R406W mutation is probably near the WKS46 epitope, which is 21–33 amino acids downstream of V337M and 36–48 amino acids upstream of R406W.

Control samples (marked with −) contained small amounts of endogenously degraded tau, most of which was present as large (>35 kDa) fragments. Some of these control fragments co-migrate with tau fragments obtained after calpain I digestion.

The data obtained from our studies suggest that (1) wild type and mutant tau are digested by calpain I at similar sites, (2) many of the cleavage sites are located in the carboxy-terminal half of tau, and (3) the mutation linked decreases in digestion rate observed in four repeat tau may be due to a

reduced accessibility of calpain I to some of the cleavage sites. How mutations affect protease accessibility remains unclear. It is possible that this is due to changes in tau conformation. In this regard, it is noteworthy that in studies of transfected cultured cells the phosphorylation of four repeat tau was decreased by the R406W mutation, but not by the V337M or P301L mutations [23,24]. Tau proteins with FTDP-17 mutations were also reported in one study to be different from wild type tau in circular dichroic (CD) spectra [25]. However, a subsequent study by others showed that the CD spectra of wild type and mutant tau were comparable [14]. Standard physical analyses may be too insensitive to detect subtle changes of tau structure. For example, standard physical analyses did not reveal any significant changes in the structure of insoluble pathologic tau derived from Alzheimer's disease (AD) and tau from control brains, but immunization of AD pathologic tau has led to the production of antibodies that recognize 'AD specific' conformational tau epitopes [25–27]. Further studies undoubtedly are required to define the effect of FTDP-17 missense mutation on tau conformation.

The results of the present studies of tau degradation together with those of tau filament assembly and microtubule binding demonstrate that FTDP-17 mutations have at least three effects on tau in vitro. They also show that the mutations affect the calpain I degradation of four repeat tau more than three repeat tau. Reduction in the rate of tau degradation could lead to increases in the concentration of cytoplasmic tau. This in turn could contribute to increased tau polymerization into filaments. In discussing the multiple effect of FTDP-17 mutations on tau biology, it is worth noting that while the R406W mutation has the most prominent effects on tau degradation, it has an inhibitory effect on the nucleation process of tau filament polymerization. In contrast, the P301L mutation has little effect on tau degradation, but increases the ability of tau to form fibrils at both nucleation and elongation phases. Further studies are needed to determine the critical tau concentration necessary for the initiation of filament formation in vivo.

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