Tau Isoform Composition Influences Rate and Extent of Filament Formation

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Background: Human brain Tau isoforms differ by the presence or absence of inserts derived from alternative splicing of MAPT transcripts.

Results: Tau inserts modulate Tau aggregation propensity through differing kinetic mechanisms that synergize or compete depending on sequence context.

Conclusion: MAPT splicing patterns associated with tauopathies correlate with aggregation propensity.

Significance: Tau aggregation propensity may contribute to disease pathogenesis.

The risk of developing tauopathic neurodegenerative disease depends in part on the levels and composition of six naturally occurring Tau isoforms in human brain. These proteins, which form filamentous aggregates in disease, vary only by the presence or absence of three inserts encoded by alternatively spliced exons 2, 3, and 10 of the Tau gene (MAPT). To determine the contribution of alternatively spliced segments to Tau aggregation propensity, the aggregation kinetics of six unmodified, recombinant human Tau isoforms were examined in vitro using electron microscopy assay methods. Aggregation propensity was then compared at the level of elementary rate constants for nucleation and extension phases. We found that all three alternatively spliced segments modulated Tau aggregation but through differing kinetic mechanisms that could synergize or compete depending on sequence context. Overall, segments encoded by exons 2 and 10 promoted aggregation, whereas the segment encoded by exon 3 depressed it with its efficacy dependent on the presence or absence of a fourth microtubule binding repeat. In general, aggregation propensity correlated with genetic risk reported for multiple tauopathies, implicating aggregation as one candidate mechanism rationalizing the correlation between Tau expression patterns and disease.


Tau Isoforms Differ in Aggregation Propensity

(14) and modulates spacing between microtubules (15), whereas the C-terminal (microtubule-binding) domain mediates both microtubule binding affinity (16) and Tau self-association (17). Because the 4R isoforms most strongly associated with familial FTLD affect the C-terminal domain, modulation of microtubule biology and aggregation propensity are leading rationales for association of these isoforms with disease. Nonetheless, current models cannot rationalize the potential role of N-terminal insertions, which do not affect microtubule binding affinity (18) or microtubule dynamics (19). In contrast, both N- and C-terminal inserts that arise from alternative splicing have been reported to modulate Tau aggregation propensity in the presence of heparin and anionic surfactant aggregation inducers (20–24). Under these conditions, which leverage a heterogeneous nucleation mechanism (25), isoform aggregation propensity is differentially influenced by Tau:inducer ratios (22–24). As a result, the reported contribution of segments encoded by alternatively spliced exons to aggregation propensity has been inconsistent. Moreover, the mechanisms through which alternatively spliced sequences act to modulate aggregation propensity are unknown.

Recently we showed that the small-molecule anionic inducer Thiazine red can substitute for macromolecular and surfactant inducers in driving aggregation of full-length 2N4R Tau into filaments with twisted ribbon morphology at submicromolar concentrations (26). The interaction approximates a homogeneous nucleation scheme characterized by initial formation of an unstable dimeric nucleus, followed by filament elongation through monomer addition (27). Under these conditions, the inherent aggregation propensity of full-length, un-modified Tau isoforms may be quantified and mechanistically localized under near physiological Tau protein concentrations, pH, ionic strength, and reducing conditions (28, 29).

Here, we use this approach to characterize the aggregation propensity of all six unmodified human brain Tau isoforms. The results show that all three alternatively spliced segments modulate Tau aggregation propensity and that they can do so at both the nucleation and elongation steps of the pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant His-tagged human Tau isoforms were prepared as described previously (23, 30). Aggregation inducer Thiazine red (Chemical Abstract Service registry no. 2150-33-6) was obtained from TCI America (Portland, OR). Formvar/carbon-coated copper grids (300 mesh), glutaraldehyde, and uranyl acetate were obtained from Electron Microscopy Sciences (Fort Washington, PA).

**Fibrillization Assays**—Tau preparations were incubated (37 °C) without agitation in assembly buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM dithiothreitol) in the presence of 100 μM Thiazine red inducer for up to 24 h (unless specified otherwise). Aliquots were withdrawn as a function of time up to 5 h post-incubation and then assayed for filament length. The resultant disaggregation time series was fit to an exponential decay function to obtain $k_{app}$, the pseudo-first order rate constant describing the time-dependent decrease in filament length, and $L_{op}$ the total filament length at time zero. The rate constant $k_{crit}$ was estimated from $k_{app}$ and the number of filaments at time zero as described previously (33, 34). The association rate constant for elongation, $k_{app}$, was then obtained from the relationship (27).

$$K_{crit} = k_{app}/k_{app}$$

Aggregation lag times were obtained by Gompertz regression of time series as described in Ref. 35.

**Statistical Tests**—Estimated kinetic parameters were assumed to resemble normally distributed random variables ($X_i$) with mean $\mu_i$ and known standard deviation $\sigma_i$. As a global test of the null hypothesis $H_0$ (i.e. that all compared $\mu_i$ values were the same), the statistic $T$ based on the maximum likelihood ratio test principle (32) was calculated,

$$T = \sum_{i=1}^{k} w_i (X_i - \hat{\mu})^2$$

where $k$ is the number of kinetic parameters being compared, $T$ is the 1 – $\alpha$ point of the Chi-square distribution having $k - 1$ degrees of freedom, $w_i = 1/\sigma_i^2$, and $\hat{\mu}$, the common mean under the null hypothesis, is the weighted sum of $X_i$.

$$\hat{\mu} = \frac{\sum_{i=1}^{k} w_i X_i}{\sum_{i=1}^{k} w_i}$$

If $H_0$ was true, then the probability (p) of obtaining more extreme values of $T$ than actually observed is $\alpha$. 

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Additional details and equations are provided in the text, including calculations for the rate constant $k_{crit}$ and the mean $\hat{\mu}$.
For pairwise comparisons, the probability (p) of obtaining the observed results, assuming the null hypothesis, was assessed by z-test,

\[ z = \frac{x_1 - x_2}{\sqrt{(S_{x1})^2 + (S_{x2})^2}} \]  

(Eq. 6)

where \( x_1 \pm S_{x1} \) and \( x_2 \pm S_{x2} \) are the pair of estimates \( \pm \) SEE being compared, \( z \) is the \( 1 - \alpha \) point of the standard normal distribution, and \( p \) is \( 2\alpha \). All statistical analyses were carried out using JMP (version 9.0, SAS Institute, Cary, NC).

RESULTS

Human Tau Isoforms Differ in Aggregation Propensity—All six Tau isoforms aggregated in the presence of Thiazine red inducer, forming filaments with twisted ribbon morphology (supplemental Fig. 1). To quantify aggregation propensity of each isoform, the minimal concentration required to support filament formation in the presence of Thiazine red inducer was estimated using an electron microscopy assay for total filament length (31). In nucleation-dependent interactions, the minimal concentration is termed the critical concentration (\( K_{\text{crit}} \)) and approximates the dissociation equilibrium constant for elongation (\( K_d \)) (27). Results showed that \( K_{\text{crit}} \) values varied over more than an order of magnitude (Fig. 1B; Table 1). When subjected to a global statistical test (Equations 4 and 5), the null hypothesis was rejected at \( p < 0.0001 \). Furthermore, the Fieller 95% confidence intervals for any pair of isoforms did not overlap. These data indicate that naturally occurring human Tau isoforms differed in their ability to support fibrillization in the presence of Thiazine red and that our analytical methods were adequate to detect and quantify the differences.

To determine whether the observed variation in aggregation propensity resulted from differential sensitivity to Thiazine red inducer, the concentration-effect relationship for Thiazine red-mediated fibrillization was quantified for each isoform at constant Tau supersaturation (i.e. the net difference between bulk concentration and \( K_{\text{crit}} \) was held constant, so that the amount of aggregation at plateau was approximately the same for all six isoforms). Results showed that all isoforms resembled one another with respect to both the potency and efficacy of Thiazine red (supplemental Fig. 2). Together, these data indicate that naturally occurring human Tau isoforms differed in their ability to support fibrillization in the presence of Thiazine red and that our analytical methods were adequate to detect and quantify the differences.

Contribution of Insert Sequences—Visual inspection of Fig. 1B revealed that 4R-containing Tau isoforms aggregated with lower \( K_{\text{crit}} \) than 3R isoforms. Moreover, the rank order of \( K_{\text{crit}} \) within 3R and 4R isoforms consistently was 0N > 2N > 1N, suggesting that the differences resulted from the presence or absence of alternatively spliced segments. To test this prediction, the ratio of \( K_{\text{crit}} \) values observed in the presence and absence of each alternatively spliced segment was calculated for isoform pairs. For example, pairwise comparison of 0N4R with 1N4R, and of 0N3R with 1N3R, gave two estimates of the contribution of the N-terminal segment encoded by exon 2 to \( K_{\text{crit}} \), whereas comparison of 1N4R with 2N4R and of 1N3R with 2N3R provided two estimates of the effects of the exon 3-encoded sequence. Similarly, comparison of 0N4R with 0N3R, 1N4R with 1N3R, and 2N4R and 2N3R provided three esti-
mates the contribution of the alternatively spliced microtubule binding repeat to Tau $K_{\text{crit}}$. Replot of $K_{\text{crit}}$ ratios showed that the presence of the exon 2-encoded insert lowered $K_{\text{crit}}$ ~ 2-fold in both 3R and 4R backgrounds (Fig. 1C). In contrast, exon 3-encoded sequence increased $K_{\text{crit}}$ with stronger effects in 3R than in 4R background (Fig. 1C). The largest effects, however, associated with the exon 10-encoded microtubule repeat, which on average lowered $K_{\text{crit}}$ 6-fold (Fig. 1C). These data indicate that aggregation propensity is increased by exon 2- and exon 10-encoded segments but antagonized by the segment encoded by exon 3 in a manner that is sensitive to the number of microtubule binding repeats.

**Mechanism of Critical Concentration Effects**—$K_{\text{crit}}$ approximates the ratio of dissociation ($k_-$) and association ($k_+$) rate constants for filament elongation (Equation 2). Thus, modulation of $K_{\text{crit}}$ may result from changes in $k_-$ (i.e. filament stability), in $k_+$ (i.e. the efficiency of monomer association with filament ends), or both. To distinguish these possibilities, $k_-$ was estimated for each Tau isoform by diluting preassembled filaments below $K_{\text{crit}}$ and estimating the initial rate of filament shortening in the electron microscopy assay. Loss of filament length followed first order kinetics as predicted for endwise depolymerization from a Poisson-like length distribution (27, 34) (supplemental Fig. 3). On the basis of the relationship between Tau mass and filament length established for wild-type 2N4R Tau (27), the dissociation elongation constant $k_-$ was derived from the disaggregation rate of each isoform. Rate constant $k_+$ was then calculated from estimates of $K_{\text{crit}}$ and $K_{\text{crit}}$ for each isoform through Equation 2. Estimated $k_-$ and $k_+$ values for all isoforms are summarized in Table 1. Pairwise comparisons among isoforms showed that neither exon 2- nor exon 3-encoded segments exerted their effects at the level of dissociation rate constant $k_-$ (Fig. 2). Rather, the aggregation promoting effects of the exon 2 encoded insert resulted from promotion of extension rate through increase in $k_+$, whereas the additional presence of exon 3 partially antagonized this effect (Fig. 2). In contrast, the strong aggregation promoting effects of the fourth microtubule binding repeat were propagatedSEE. A ratio of 1, corresponding to no difference in rate constant in the presence versus absence of the analyzed segment is marked by the dashed line. Exon 2 depressed $K_{\text{crit}}$ by selectively increasing $k_-$, whereas exon 10 both increased $k_+$ and decreased $k_-$. In contrast, exon 3 increased $K_{\text{crit}}$ by decreasing $k_+$, **,** $p < 0.01$ for comparison of average presence versus absence of each alternatively spliced segment.

**Isoform Structure Influences Nucleation Rate**—In the presence of Thiazine red inducer, Tau aggregation approximates an equilibrium nucleation-elongation interaction (27), where assembly-competent monomer rapidly equilibrates with a thermodynamically unstable species termed the nucleus (36). Once the critical nucleus cluster size is reached, subsequent additions to the nascent filament ends are favorable energetically, and elongation proceeds efficiently. As a result, aggregation rate depends not only on the rate of filament elongation ($k_-$ and $k_+$) but on the efficiency of the nucleation step as well. To assess the effects of primary structure on nucleation rate, the time course of aggregation was quantified for each Tau construct at constant supersaturation. Under these conditions, differences in interaction rates primarily reflect differing rates of nucleation and protein concentrations (37). All resultant progress curves were sigmoidal with lag, exponential growth, and equilibrium phases (Fig. 3A). Lag times, which vary inversely with nucleation rate (38), were obtained ± SEE after fitting each time series to a three-parameter Gompertz growth function as described under “Experimental Procedures.” Resulting values ranged from 0.23–1.36 h, with 4R isoforms having the shortest lags (Table 1). To determine the contribution of each alternatively spliced segment to nucleation rate, the ratio of lag times observed in the presence and absence of exons 2, 3, and 10 encoded segments was calculated and averaged for isofrom pairs. Replot of lag time ratios showed that the presence of a

<table>
<thead>
<tr>
<th>Isoform</th>
<th>$K_{\text{crit}}$</th>
<th>$k_-$</th>
<th>$k_+$</th>
<th>Lag time</th>
</tr>
</thead>
<tbody>
<tr>
<td>0N3R</td>
<td>1.82 ± 0.03</td>
<td>0.051 ± 0.006</td>
<td>27,800 ± 3,300</td>
<td>1.36 ± 0.43</td>
</tr>
<tr>
<td>1N3R</td>
<td>0.92 ± 0.03</td>
<td>0.053 ± 0.002</td>
<td>57,300 ± 2,090</td>
<td>1.18 ± 0.13</td>
</tr>
<tr>
<td>2N3R</td>
<td>1.47 ± 0.05</td>
<td>0.048 ± 0.010</td>
<td>32,300 ± 7,100</td>
<td>0.85 ± 0.20</td>
</tr>
<tr>
<td>0N4R</td>
<td>0.37 ± 0.03</td>
<td>0.021 ± 0.002</td>
<td>55,700 ± 7,600</td>
<td>0.34 ± 0.06</td>
</tr>
<tr>
<td>1N4R</td>
<td>0.16 ± 0.01</td>
<td>0.021 ± 0.001</td>
<td>133,000 ± 8,800</td>
<td>0.29 ± 0.08</td>
</tr>
<tr>
<td>2N4R</td>
<td>0.21 ± 0.01</td>
<td>0.019 ± 0.001</td>
<td>90,000 ± 5,900</td>
<td>0.23 ± 0.02</td>
</tr>
</tbody>
</table>

* Overall constant reflecting events at both filament ends.
fourth microtubule binding repeat decreased lag time >4-fold at \( p < 0.01 \) (Fig. 3B), indicating that nucleation rate was promoted strongly by this segment. N-terminal inserts encoded by exons 2 and 3 also weakly decreased lag time (Table 1), but their effects did not reach statistical significance at \( p < 0.05 \) in pairwise comparisons (Fig. 3B). However, when 0N, 1N, and 2N isoforms were analyzed in separate 3R and 4R backgrounds, linear correlations between lag time and net Tau charge at assay pH were apparent (Fig. 4). In contrast, no correlation was detected (\( r^2 < 0.2 \)) when isoform lag time was plotted against grand average of hydropathy (calculated by the Kyte and Doolittle algorithm; data not shown). These data are consistent with all three alternatively spliced segments accelerating nucleation rate, with the weak effects of the acidic N-terminal inserts potentially being mediated by decreases in isoelectric point and net protein charge (39).

4R Tau Can Drive Aggregation of 3R Tau—The above experiments show that 4R Tau isoforms aggregate more efficiently and at faster rates than 3R isoforms. Because human brain expresses both 3R and 4R isoforms in certain neurons (11), the potential for isoform co-assembly (40) exists. To test whether 4R Tau can promote aggregation of 3R Tau, the critical concentrations of 2N4R/0N3R mixtures were determined at varying isoform ratios. These two isoforms were chosen for analysis because they differed by nearly an order of magnitude in \( K_{\text{crit}} \) (Table 1). Measured \( K_{\text{crit}} \) values were then compared with theoretical values for a fully dominant interaction (i.e. where all 3R Tau aggregated like 4R Tau) or no interaction (i.e. where \( K_{\text{crit}} \) values reflected the individual, noninteracting 3R and 4R components). Electron microscopy was used as the assay modality so that measured \( K_{\text{crit}} \) values corresponded to filament formation rather than nonspecific trapping of monomers during aggregation. At high (3:1) 4R:3R ratios, \( K_{\text{crit}} \) approximated the dominant interaction scenario, although it differed from the interaction scenario only at \( p = 0.07 \) (Fig. 5). At equimolar and low (1:3) 4R:3R ratios, however, \( K_{\text{crit}} \) shifted to a value intermediate between the fully dominant and non-interacting scenarios (statistical differences when compared with both boundary conditions: \( p < 0.05 \) at 1:1 4R:3R ratio, and \( p < 0.01 \) at 1:3 4R:3R ratio; Fig. 5). These data indicate that the high aggregation propensity of 4R Tau can be partially dominant over 3R Tau, suggesting that the 4R species can recruit less assembly prone Tau isoforms 0N3R (●), 1N3R (□), 2N3R (△), 0N4R (●), 1N4R (□), and 2N4R (△) were incubated (37 °C) at constant supersaturation (i.e. 0.3 μM above \( K_{\text{crit}} \)) in the presence of 100 μM Thiazine red and then assayed for filament formation as a function of time. Each data point represents average filament lengths/field calculated fromtriplicatenelectron microscopy images whereas each normalized curve (solid lines, 4R Tau isoforms; dashed lines, 3R Tau isoforms) represents best fit of the data points to a three parameter Gompertz growth function of time. Each data point represents average filament lengths/field, and 2N4R/2N3R (exon 10) presents best fit of the data points to a three parameter Gompertz growth function (31). Values for lag time were estimated from these plots and summarized in Table 1. Three-repeat isoforms aggregated with longer lag times than four-repeat isoforms. B, replot of data from A, where each bar represents the ratio of lag times determined in the presence relative to the absence of each alternatively spliced segment. ** \( p < 0.01 \) for comparison of average presence versus absence of each alternatively spliced segment.
isoforms into aggregates in the submicromolar free Tau concentration regime.

**DISCUSSION**

These data show that the intrinsic aggregation propensity of naturally occurring, unmodified human Tau isoforms differs owing to the contributions of N- and C-terminal inserts to the nucleation and extension steps of the aggregation pathway (Fig. 6). The Tau isoform lacking all three inserts, ON3R, aggregates with a slow nucleation rate and relatively high $K_{\text{crit}}$. Consistent with this finding, human fetal Tau, which consists of ON3R Tau (41), is not associated with aggregation despite being hyperphosphorylated during development (42). Aggregation propensity of 3R Tau can be increased, however, by the insert encoded by exon 2, which acts to promote filament extension and modestly increase nucleation rate. Rate enhancement correlates with lowering of net protein charge, an established descriptor of aggregation propensity (39). In contrast, the exon 3-encoded segment, which normally is excluded by default (3), partially antagonizes the effects of exon 2-encoded sequences at the level of filament extension. Considered together, however, all three 3R isoforms required higher concentrations and longer nucleation times to support aggregation than their 4R analogs. These results are consistent with the reported resistance of chimpanzees and other primates that express primarily 3R Tau isoforms to neurofibrillary lesion formation (43, 44) and the need for overexpression to drive the aggregation of human 3R Tau in mouse models (45). Interestingly, chimpanzee MAPT haplotype is primarily H2 (46), which supports increased inclusion of the aggregation-resistant segment encoded by exon 3 (47).

Relative to 3R isoforms, the addition of the fourth microtubule binding repeat encoded by exon 10 dramatically lowers critical concentration by stabilizing filaments and by promoting monomer association with filament ends. The nucleation rate is augmented as well through a mechanism that appears to be independent of charge effects. Aggregation propensity of 4R Tau was increased by the N-terminal insert encoded by exon 2, the efficacy of which was indistinguishable in 4R and 3R backgrounds. Although addition of the second N-terminal insert encoded by exon 3 partially antagonized this activity, its efficacy was detectably weaker in the 4R relative to 3R background. The aggregation-resistant segment encoded by exon 3 (47).

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Correlation of Aggregation Propensity with Disease—Animal models have shown that neurodegenerative phenotypes can result from high level overexpression of Tau sequences irrespective of filament formation (49, 50). In sporadic AD, however, total levels of all Tau transcripts do not increase relative to cognitively normal cases (51), and neurodegeneration correlates both spatially and temporally with Tau aggregation (52, 53). The discovery of familial forms of FTLD linked to MAPT (reviewed in Ref. 9) has further extended the correlation between Tau aggregation and disease to the level of Tau isoform composition. Intrinsic mutations that increase exon 10 inclusion are associated with Mendelian inheritance of certain forms of FTLD (54), whereas sub-haplotypes of H1 that increase both exon 10 inclusion and total Tau transcript levels (55) are associated with increased risk for PSP and corticobasal degeneration (6–8). Shift in isoform distribution toward 4R forms has also been observed in mild cognitive impairment and AD (51, 56, 57). On the basis of aggregation propensity investigated herein, exon 10 inclusion will bias Tau isoform distribution toward aggregation-prone species, whereas increases in bulk Tau concentration will accelerate all association steps in the pathway. In particular, the strong increase in aggregation propensity associated with the fourth microtubule binding repeat more than compensates for the depletion of 1N isoforms in AD (56). In contrast, haplotype H2, which is protective against PSP (7, 8), supports increased inclusion of exon 3 as well as decreased inclusion of exon 10 relative to H1 (47, 55). Moreover, levels of 2N forms of Tau are depressed in the Tau aggregates that accumulate in PSP (58–60). These data are consistent with the lower aggregation propensity observed with the insert encoded by exon 3. Overall, the aggregation behavior of naturally occurring Tau isoforms is consistent with the law of mass action in this subset of tauopathies (61).

The aggregation behavior identified herein for unmodified Tau monomers does not account for tauopathies that accumulate aggregates composed primarily of 3R Tau (reviewed in Ref. 1). In myotonic dystrophy, 3R aggregation reflects primarily an abnormal MAPT expression pattern favoring exclusion of exons 2, 3, and 10 (62). In contrast, 3R aggregation in Pick disease occurs in certain cell populations (63), perhaps reflecting selective vulnerability of cells expressing primarily 3R Tau (e.g. neural precursor cells (64)). Post-translational modifications that selectively promote 3R Tau dimerization, including disulfide (21, 65) and dityrosine bond formation (66), may foster 3R Tau aggregation in these cells. These examples indicate that native aggregation propensity is not the only factor underlying neurofibrillary lesion formation in tauopathic neurodegenerative diseases.

In summary, we have provided evidence that naturally occurring Tau isoforms differ in aggregation propensity owing to the contributions of inserts encoded by alternatively spliced exons 2, 3, and 10. The segments differentially modulate rate-limiting steps in the aggregation pathway and can synergize with the effects of missense and pseudophosphorylation mutations. The aggregation propensity of Tau isoforms may influence how Tau misfunction leads to clinically and histopathologically distinct diseases.

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