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Biochimica et Biophysica Acta 1739 (2005) 167–178

BIOCHIMICA ET BIOPHYSICA ACTA BBA

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Review

Pathways of tau fibrillization

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Received 26 April 2004; accepted 2 June 2004
Available online 23 July 2004

Abstract

New methods for analyzing tau fibrillization have yielded insights into the biochemical transitions involved in the process. Here we review the parallels between the sequential progression of tau fibrillization observed macroscopically in Alzheimer's disease (AD) lesions and the pathway of tau aggregation observed in vitro with purified tau preparations. In addition, pharmacological agents for further dissection of fibrillization mechanism and lesion formation are discussed.

Keywords: Alzheimer's disease; Neurofibrillary tangle; Tau; Folding intermediate; Aggregation; Kinetics

1. Introduction

Neurofibrillary pathology is one of the diagnostic criteria of Alzheimer's disease (AD) and other tauopathic neurodegenerative diseases. It consists of neurofibrillary tangles (NFTs) in neuronal cell bodies, neuropil threads within neuronal processes of the neuropil, and dystrophic neurites associated with neuritic plaques [1]. Each of these lesions contains filamentous aggregates composed of the microtubule-associated protein (MAP) tau. Filamentous tau can contain all six central nervous system splice isoforms [2] and is hyperphosphorylated relative to nonfilamentous tau [3].

Tau filaments adopt multiple morphologies. In AD, filaments consist primarily of paired helical filaments (PHFs) that appear as twisted structures alternating between 10- and 20-nm widths with a half-periodicity of 80 nm [4]. On the basis of morphology [5], seeding experiments in vitro, and mass-per unit length measurements [6], PHF structure is consistent with the presence of two hemifilaments wound around each other. Filaments with straight morphology also exist in AD brain, and examples have been characterized as consisting of one or two hemifilaments [6]. Other tauopathies, such as those arising from corticobasal degeneration, can yield additional morphologies [7]. The regular appearance of each of these filament forms stems from a repeating cross β-sheet structure [8] mediated by ~93-residue sequences of amino acids located in the microtubule-binding repeat region [9]. Although the repeats involved in aggregate formation vary among tau isoforms, self-association minimally involves residues spanning three repeats as demonstrated by proteolysis experiments [9]. Filament populations isolated from late stage AD tissue typically average 300–600 nm in length and adopt an exponential length distribution [10].

The tau fibrillization pathway is thought to consist, therefore, of at least two key steps. First, the microtubule binding function of tau must be neutralized so that tau protein can accumulate in an assembly competent form and intracellular location. Second, tau molecules must self-associate through their microtubule binding repeat regions to form the β-sheet enriched filaments observed in tissue. Hyperphosphorylation accompanies one or both of these steps. Although late stage disease is characterized by large increases in total tau levels [11], the earliest changes in tau

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doi:10.1016/j.bbadis.2004.06.016

biology potentially occur years earlier [12] under presumably near normal intracellular conditions of pH, ionic strength, redox potential, and physiological levels of tau (ranging between 1 and 10 μM) [10,11,13].

Longitudinal studies suggest that tau fibrillization is a surrogate marker for disease, correlating with both cognitive decline and neurodegeneration [14]. The lesions develop according to a common spatiotemporal pattern in AD, which reflects the sequence, type and severity of cognitive decline and neuronal loss [15,16]. Tau aggregation may also contribute to neurodegeneration, as suggested by the synaptic deficits that accompany NFT formation [17], by the discovery of tau's involvement in certain genetic forms of frontotemporal dementia [18], and by the toxicity associated with the overexpression of tau in animal models [19–22]. Thus, clarification of the tau aggregation pathway has important implications for AD diagnosis and treatment. Recent advances in clarifying the tau aggregation pathway from experiments conducted on authentic tissue specimens and in vitro using defined preparations of tau protein are described below.

2. Fibrillization pathway in vivo

Among the fibrillar lesions, neuropil threads precede the appearance of NFTs that in turn precede the appearance of neuritic plaques [14]. Macroscopic characterization of tau lesion maturation has been described in detail for NFTs. The first detectable step by immunohistochemistry using antibodies to different phosphorylated and non-phosphorylated tau epitopes involves the aggregation of tau protein into non-fibrillar deposits [23]. These "pretangles" display a punctate staining pattern in the cytoplasm and are not reactive with β-sheet-sensitive dyes such as thioflavin S (ThS) or thiazine red [24]. The amorphous aggregates within pretangles may appear either free in cytoplasm or associated with membranous organelles [25]. Aggregate formation is accompanied by the appearance of tau phosphoepitopes, suggesting this posttranslational modification may play an early role in the process. Indeed, phosphorylation of tau can lower tau–tubulin affinity thereby raising free intracellular tau concentrations [26,27], suggesting a mechanism underlying the first step in the fibrillization pathway. Tau phosphorylation may also modulate the rate and extent of fibrillization (see below).

The second step involves the adoption of β-sheet structure by tau. The earliest secondary structure detectable with fluorescent dyes corresponds to tau aggregates associated with membranous structures [24,25], suggesting that the folding of tau protein into β-sheet containing species may be facilitated by interaction with intracellular membranes and organelles.

The third step involves the nucleation of tau filaments and formation of mature NFTs. Filaments gradually replace amorphous deposits to dominate the tau immunostaining of cells [24]. Whereas some lesions develop predominantly straight filaments [28], late stage disease is dominated by PHF morphology, which may represent a minimal energy conformation [29]. In biopsy specimens, individual PHFs appear in endwise association with membranes, consistent with surface-mediated nucleation and polar extension from stable tau–membrane complexes [30].

Final steps include the loss of cell viability and formation of "ghost tangles", which appear as extracellular fibrillar aggregates that retain the ability to bind the small molecule fluorophores ThS [31], Congo red [32], and thiazine red [33]. In the transition, NFTs undergo proteolytic modifications [34] and become highly insoluble [35], consistent with the presence of covalent cross-linking and ubiquitination in late stage disease.

Together these data are consistent with an assembly pathway involving amorphous aggregation followed by facilitated fibrillization, with a role for phosphorylation from the earliest stages.

3. Tau fibrillization in vitro

3.1. Methodology

Modelling of the tau assembly pathway in vitro has employed highly purified recombinant preparations of individual tau isoforms and mutants. In addition, synthetic tau peptides have been used for mechanistic studies [36,37]. Non-histidine-tagged recombinant tau protein is typically purified using heat denaturation and cation exchange chromatography [26,38–40], sometimes followed by further purification by gel filtration [41]. The acid solubility of tau also has been employed in tau preparations [38,42,43]. Because it has been claimed that harsh conditions can modify tau aggregation behavior [44], tau has also been prepared without boiling and acid treatment as a poly-His fusion protein using immobilized metal affinity chromatography followed by gel filtration [45]. The poly-His tag introduces one additional positive charge into tau but otherwise does not appear to affect its assembly characteristics [46]. The claim that poly-His tags can promote tau dimerization based solely on Stokes radius measurements [47] appears to be premature owing to the relative contributions of size, shape, and hydration to this parameter.

Tau fibrillization reactions have been quantified using electron microscopy [48], dye-based fluorescence spectroscopy [5,49], static light scattering [50], sedimentation [44,51–53], and intrinsic tau fluorescence [53,54] assays. Each method has specific strengths and weaknesses.

The electron microscopy assay has been characterized in the greatest detail [48]. It yields accurate estimations of filament length, length distributions, and morphology when performed at constant bulk tau concentration. The utility of the assay stems from the nature of tau filaments, which typically appear as well-separated linear objects after

adsorption onto EM grids. This is not true of filaments formed by many other aggregating proteins, such as α-synuclein [55]. Care must be exercised in comparing reactions at different bulk protein concentrations, however, owing to the ability of soluble tau to compete with filaments for adsorption to the grid surface. Solutions to this problem have been proposed, including the use of macroscopic inducer particles such as anionic microspheres as internal standards for grid adsorption. Using this approach, one can not only quantify tau fibrillization at different bulk protein concentrations, but also report values in units of molar concentration. Although definitive, the assay is time-consuming when applied to the study of a large number of samples.

Aggregation assays can be processed more rapidly using solution methods. Because ThS is characterized by an increase in fluorescence upon binding to β-sheet structures typical for amyloids [56], ThS-based assays are among the most popular tools for following tau fibrillization in real time [49]. Nonetheless, interpretation of reaction kinetics is not straightforward because dyes such as ThS react with partially folded intermediates as well as mature tau filaments [10]. The relative fluorescence yield when bound to each of these targets is unknown, as is the minimal unit required to bind ThS. Thus, ThS fluorescence is useful as a rapid but semiquantitative tool for estimating tau aggregation reactions.

A second solution-based assay employs static laser light scattering [50]. This assay is well suited for time course experiments and should be insensitive to bulk protein concentrations. However, initial experience with this method using anionic fibrillization inducers yielded hyperbolic reaction progress curves that correlated poorly with electron microscopy or intrinsic fluorescence assays [47,50] despite resembling the shape of progress curves for other aggregation proteins such as β-amyloid [57]. The latter reactions involve homogeneous nucleation mechanisms that progress spontaneously from small aggregate formation through nucleation and fibrillization, whereas the former do not. Indeed, recombinant tau protein aggregates poorly when incubated alone in solution (see below). Instead, the most efficient tau aggregation assays include a condensing agent such as anionic polymers or micelles that first forms a potentially large light scattering complex with tau before subsequent intermediate formation and nucleation. Thus, light scattering of experimental samples should be corrected for this initial complex formation before they are interpreted. This may be done by running a negative control consisting of aggregation incompetent tau protein in parallel with the experimental sample and subtracting the two resultant progress curves. Mutation I277P/I308P, which does not assemble in the presence of anionic inducers [58–60], is ideal for this purpose. The difference between these progress curves yield a net reaction time course that correlates more closely with other assays (M. Necula and J. Kuret, unpublished data).

Sedimentation assays have been used to separate aggregates from bulk tau [44,51–53]. This assay is only semi-quantitative, however, because it cannot distinguish between filaments, insoluble amorphous aggregates, and insoluble intermediates [61]. With these limitations in mind, the use of these synthetic tau preparations and assay methods to study tau fibrillization in vitro is summarized below.

3.2. Tau assembly pathway

A hypothetical pathway for tau fibrillization is shown in Fig. 1. Unlike most other amyloid forming proteins, recombinant full-length tau proteins purified by the methods summarized above typically do not fibrillize spontaneously when incubated at physiological pH, temperature, ionic strength, and concentration (1–10 μM) [36,38,46,49,62,63]. Exceptions where full-length tau or C-terminal truncation mutants formed aggregates at submicromolar concentrations have been reported, however [44,64]. The inconsistencies in aggregation behavior have been ascribed to differing purification methods [44]. More typically, tau proteins and peptides spontaneously fibrillize above 200 μM [38,43]. It has been suggested that this behavior results from high concentrations being required to overcome charge repulsion [65], but it is also consistent with fibrillization competing with alternative folding pathways (Step 1, Fig. 1). One example of the latter is disulfide bond formation. Because three repeat tau molecules contain only one Cys, they tend to form intermolecular disulfide bonds under oxidizing conditions. In contrast, four repeat tau molecules contain two Cys residues and tend to predominantly form intramolecular disulfide bonds under similar conditions [60]. The altered tau "compact" monomers containing an internal disulfide bridge do not participate in tau filament formation, and constitute a trapped or dead end side reaction in tau fibrillization. Maintenance of reduced sulfhydryls and minimization of this competing pathway may underlie the ability of reducing agents such as dithiothreitol to promote fibrillization of tissue-derived tau preparations [66]. These data support the presence of at least one alternative reaction competing with fibrillization in vitro. The poor efficiency of seeding tau fibrillization with preformed filaments in the absence of anionic inducers suggests there are others unrelated to disulfide bond formation [5,65].

Most kinetic and thermodynamic barriers to fibrillization can be overcome by the presence of anionic condensing agents. Chief among these are anionic polymers (heparin, poly-Glu, nucleic acids, etc.) and anionic surfactants (fatty acids, alkyl sulfate detergents, etc.). The latter agents act in micellar form and promote fibrillization by binding tau on their anionic surfaces [10,51]. The importance of adsorption in the reaction has been shown by inducing tau fibrillization using solid anionic microspheres [51]. The first step in the reaction appears to be the stabilization of an assembly competent intermediate that can be detected by ThS fluorescence (Step 2, Fig. 1). Intermediate formation is

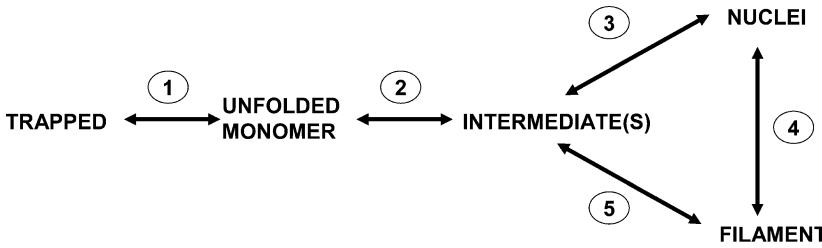

Fig. 1. Hypothetical tau fibrillization pathway in reponse to anionic inducers; (1) the fibrillization pathway is in competitive equilibrium with misconformers unable to support fibrillization. Such "trapped" or assembly-incompetent conformations could result from covalent modifications, such as disulfide bond formation, or from noncovalent intramolecular interactions stemming from partially folded species of tau. (2) Kinetic and thermodynamic barriers to fibrillization can be overcome by anionic inducers, which stabilize one or more species of assembly-competent intermediate. (3) Nuclei are formed from assembly-competent intermediates. (4) Elongation follows nucleation as a thermodynamically favorable step in the reaction so long as assembly competent conformations of tau are present. (5) Final equilibrium is attained between filamentous and all nonfilamentous tau species, including unfolded and partially folded species.

time-dependent but does not display a lag phase [5,10]. It also does not have a minimal or "critical" concentration requirement, suggesting that the intermediate formation may not require oligomerization. Association with the anionic surface is sufficient to induce the assembly competent conformation. In fact, the efficacy of intermediate formation is directly proportional to surface charge density [10]. On the basis of its reactivity with ThS and metastability, it was proposed that the intermediate represents a partially folded tau molecule containing enriched β-sheet structure. Because heparin and poly glutamic acid also induce large increases in ThS fluorescence without a lag at tau peptide concentrations above 2 μM [49], it is conceivable that anionic polymers also act to stabilize partially folded intermediates.

Because dimer formation affects the rate of formation of ThS fluorescent species in the presence of polyanionic inducers such as RNA and heparin, it has been suggested that dimers may participate in the control of intermediate formation and fibrillization [67,68]. Dimerization is not a requirement, however, and is inefficient in the absence of disulfide formation even at supraphysiological tau concentrations [60]. These data suggest that under normal physiological conditions, which are reducing, dimer formation is likely to make a modest contribution to fibrillization efficiency.

According to our model (Step 2, Fig. 1), partially folded intermediates appear before filament nucleation. Because they contain exposed β-sheet hydrogen bonding edges and solvent exposed hydrophobic patches, which are absent from the fully unfolded or filamentous states, they are prone to self-association [69]. The intermediates either aggregate or change their conformation as they overcome the kinetic energy barrier associated with filament formation (Step 3, Fig. 1). This process, termed nucleation, is characterized by a pronounced lag time that varies with the efficacy of the inducer. For full-length four-repeat tau (4 μM), lag times of ~3 h have been observed with anionic microspheres [10]. The fibrillization reaction then proceeds through an exponential growth phase and finally ceases at equilibrium. Facilitated filament growth differs from typical amyloid forming pathways in being unidirectional with only one actively growing filament end [10].

The size of the tau nucleus has been estimated for dimeric forms of truncated tau (corresponding to the microtubule binding repeats) aggregated in the presence of heparin and other anionic polymers assuming a homogeneous nucleation mechanism and using the ThS-binding assay [65]. This study found that the maximal rate of ThS fluorescence formation rose linearly with increasing protein/inducer concentrations. Using a kinetic model that assumed changes in velocity depended solely on nucleation rate; a nucleus size of four to seven dimers was estimated. This estimate must be considered an upper limit, however, because the analytical treatment ignored the contribution of filament extension (Step 4, Fig. 1) to growth rates, which can be substantial when nucleation is not rate limiting [70]. In fact, filament nuclei correspond to unstable species that need not be oligomeric at all [70]. Further experimentation will be required to refine the quaternary structure of tau intermediates and nuclei.

The full fibrillization progress curve can be modeled mathematically by logistic or other sigmoidal functions that have been used to analyze biological growth phenomena in a variety of contexts ranging from protein crystallization to forestry [10,71]. Each of these sigmoid functions is characterized by a minimum of three parameters: a time constant, which corresponds to the time of maximal growth, a first order rate constant k_{app}, which describes the rate of growth of the entire filament population to equilibrium, and a carrying capacity, or final equilibrium level of fibrillization. Time constants can be used to estimate lag times and thereby gain information on nucleation rates [10,55], whereas maximal growth rates incorporate both nucleation and extension rates [70]. Although these parameters are not directly interpretable as elementary reaction rate constants, they are useful for purposes of comparison in the absence of detailed reaction rate equations. An example of the complexity of a sigmoidal reaction progress curve cast in the form of elementary rate constants for a defined reaction pathway is available for zymogen activation [72].

In the case of anionic surfactant or microsphere inducers, growth equilibrium is reached within 24 h. Up to and including this point, filaments adopt a near exponential distribution of lengths and correspond to a single hemifilament of authentic PHF [66]. The exponential length distributions, which resemble those of authentic brain-derived PHFs, stem from the nucleation dependent kinetics of the fibrillization reaction [10]. However, if incubation is allowed to proceed over a period of days, the fraction of filaments with the mass-per-unit length and morphology of authentic PHF rises dramatically although total filament length does not [6,46]. These data suggest that a second, slower equilibrium exists for PHF formation that does not markedly change length distributions. It has been suggested that this time-dependent evolution of PHF morphology stems from annealing of hemifilaments analogous to the lateral aggregation of β-amyloid fibrils [6,46,57]; however, it may also reflect heterogeneous nucleation with the hemifilament acting as template. Like other aspects of the fibrillization reaction, the rate of conversion to PHF probably depends upon bulk tau concentration and the tau isoform examined [73].

The equilibrium between filamentous and nonfilamentous tau (unfolded monomer and/or partially folded intermediates) is reflected in the concentration dependence of fibrillization at equilibrium (Step 5, Fig. 1). In the presence of anionic surfactant, the minimal or "critical" concentration of tau required to support fibrillization has been estimated as ~2 μM for full-length, four-repeat tau (M. Necula and J. Kuret, unpublished data). These data rationalize the stability and accumulation of tau filaments at physiological tau concentrations found in normal tissue.

3.3. Synthetic filament structure

The products of in vitro fibrillization reactions contain extended β-sheet structure similar to authentic tissue derived PHF, suggesting that the methods described above yield an accurate model of fibrillization [8]. Moreover, experience with tau mutants and synthetic peptides suggests an essential role of the microtubule binding region in fibrillization, consistent with the core sequence identified in authentic PHF. In the presence of anionic inducers, hexapeptide motifs in the second and third microtubule binding repeats, termed PHF6* and PHF6, respectively, appear to be required for fibrillization [58]. In the case of synthetic peptides, the sequence adjacent to PHF6 in the third repeat has been shown to fibrillize spontaneously, suggesting that microtubule binding repeat sequences outside of PHF6/PHF6* can also play a role in aggregation [74]. These findings raise the question of whether multiple sequence motifs fold independently or transiently as part of a kinetic pathway of fibrillization [52]. Multiple pathways of folding may exist and depend on the assembly paradigm. They also suggest that aggregation pathways will vary in the context of full-length tau protein and differ from those more favorable for short synthetic peptides.

4. Role of tau phosphorylation

Whereas normal tau is phosphorylated, PHF-tau contains phosphates with a significantly greater stoichiometry, distributed across ~30 sites [75,76]. These sites are filled in hierarchical fashion as neurofibrillary lesions mature, suggesting that changes in the state of tau phosphorylation may either modulate neurofibrillary pathology formation or reflect changes in the ability of tau to act as a substrate for various phosphotransferases as its aggregated structure evolves [14,77]. Hyperphosphorylation has been suggested to influence tau biology in two principal ways. First, occupancy of specific sites promotes dissociation of tau from microtubules. Phosphorylation sites that mediate this activity include, but are not limited to, Ser199, Ser202, Thr205, Ser214, Ser231, Ser262, Ser356, Ser396, and Ser404 [27,78–81]. Because some of these sites are occupied early in the course of disease [77,82], they may play a key role in shifting the equilibrium toward free tau prior to fibrillization. Phosphorylation-mediated changes in tau/microtubule binding equilibria have been demonstrated for several protein kinases including Cdk5/p25, GSK3β, PKA, and casein kinase-1 [78,83–88]. Tau/microtubule binding equilibria also can be modulated by phosphorylation mimicry [89], suggesting that the introduction of negative charges at specific tau sites is sufficient to affect the microtubule binding function of tau. These studies suggest that control of microtubule binding potentially involves common phosphorylation sites that are differentially regulated through the action of multiple protein kinases.

Hyperphosphorylation of tau has also been suggested to directly influence bulk tau solubility. The incorporation of phosphate into tau facilitates its interaction with non-phospho-tau resulting in the formation of amorphous aggregates [42,90]. Other MAPs such as MAP1 and MAP2 coaggregate in a similar manner [91]. Together these associations may be related to formation of the amorphous pretangle aggregates seen in AD tissue. Although sequestration of MAPs has been proposed to decrease cytoskeletal function in AD, complete loss of tau function does not lead to cytoskeletal collapse in transgenic mice [92,93], and cognitive decline does not correlate with the pretangle stage of pathogenesis [24]. Moreover, the presence of MAPs other than tau in neuritic lesions is not a consistent finding [94].

Phospho-tau isolated from AD tissue (an amorphous aggregate form of phospho-tau) also self-aggregates spontaneously with multiple morphologies at 8 μM total tau concentration once solubilized by urea treatment in vitro on the basis of a nonquantitative electron microscopy assay [95]. However, under the conditions of these experiments, recombinant full-length four-repeat tau also aggregates spontaneously [44]. As discussed above, the latter result is highly unusual, and suggests the presence of endogenous nucleation inducers that are normally absent from most in vitro fibrillization experiments. These have masked poten-

tial effects of phosphorylation on nucleation (Step 3, Fig. 1), and a direct effect of phosphorylation on nucleation rate has yet to be demonstrated [96]. Consistent with this observation, selective phosphorylation mimicry modulates the extent of fibrillization of recombinant tau preparations in the presence of anionic inducers in vitro, but typically does not yield preparations that fibrillize spontaneously [89,97]. Thus, the direct influence of phosphorylation on tau fibrillization may be at the level of filament stability. In quantitative assays, this would appear as a lower minimal concentration of assembly and filament length distributions skewed toward longer lengths.

Hyperphosphorylation of tau in situ using cells overexpressing both tau and selected protein kinases produces relatively few filaments when examined over a period of days [98]. However, these conditions can generate strong ThS fluorescence, indicative of β-sheet formation [98]. The morphology of the fluorescence is typically reticular rather than fibrillar, suggesting formation of species similar to the assembly intermediates introduced above.

Together these data suggest two key roles of tau phosphorylation in early stage disease. The first is to modulate tau/microtubule equilibrium to raise intracellular concentrations of free tau, leading to the formation of amorphous aggregates and assembly competent intermediates. The second is to stabilize filaments once nucleated thereby shifting equilibrium toward the fibrillized state. Quantitative experiments with purified components will be required to confirm this hypothesis and to establish whether phosphorylation directly influences nucleation rate.

5. FTDP mutations

Frontotemporal dementia with Parkinsonism associated with chromosome 17 (FTDP-17) results from mutations in the gene encoding the tau protein (reviewed in Ref. [18]). Though several of these mutations may affect protein aggregation by altering splicing of tau mRNA [99], for the purpose of this review, only those mutations in the tau coding sequence directly affecting the rate of fibrillization will be considered. The assembly characteristics of recombinant tau proteins carrying FTDP-17 mutations suggest that mutation can influence the rate and extent of tau fibrillization [41,50,53,74,100–102]. Indeed, many of the FTDP-17 mutations in the microtubule binding region occur in the vicinity of the PHF6/PHF6* motifs known to mediate polyanion-mediated tau fibrillization. Mutations within this region may act to facilitate intermediate formation through either a direct promotion of β-strand conformation or a reduction in inhibition of β-strand formation through the straightening of β-turns [59].

The influence of β-strand stabilization can be illustrated by FTDP-17 mutation ΔK280, where loss of the Lys residue in position 280 yields the pattern of alternating hydrophilic and hydrophobic residues known to promote β-strand formation in synthetic peptides [103]. Sequestration of hydrophobic residues to one face of the β-strand may also contribute to intermediate stabilization [59,103]. As a result, ΔK280 (in the context truncated tau) can nucleate spontaneously without the need for anionic inducer [59]. Other mutations adjacent to the PHF6/PHF6* motifs, such as P301L and P301S, may also promote β strand formation through the interruption of a potential β turn [59].

Other FTDP-17 mutations lie outside the immediate vicinity of the PHF6/PHF6* motifs and may stabilize β-conformation indirectly through intramolecular interactions. For example, residues near the N-terminus of tau appear to influence the conformation of the microtubule binding repeat region [45], and deletions in the amino terminus of tau have a deleterious effect on the ability of tau to form filaments [102]. Conversely, mutations in the amino terminus have been shown to enhance tau filament formation [102,104]. Substitution of Leu for Arg at position five (R5L mutant) increases the net negative charge of the amino terminus and may facilitate electrostatic interaction with the positively charged microtubule binding region [102]. Further insight into mechanism can be achieved by examining the effect of these and other FTDP-17 mutations on the kinetics of intermediate formation, nucleation, and minimal concentration of assembly.

6. Pharmacology

The complexity of the tau aggregation pathway and the absence of definitive 3-dimensional structure data for any component of it highlight the need for selective probes useful in dissecting mechanism. Pharmacological agents can supply this need and fall into three major groups.

The first group consists of phosphotransferase inhibitors, which have been valuable in assessing the contribution of phosphorylation to tau function [88]. A variety of inhibitors are available for this purpose, and these have been summarized elsewhere [105,106].

The second group consists of agents that modulate microtubule assembly and function. These include compounds that bind directly to tubulin such as the classical microtubule-stabilizing drug taxol. Because neuritic lesion formation is accompanied by disruption of the cytoskeleton, restoration of tau's MT-stabilization function may restore MT function and prevent NFT formation as well. Previous studies have shown that the MT-stabilizing agent taxol protects neurons against β-amyloid, and slows the progression of neurofibrillary pathology [107–109]. Taxol may minimize β-amyloid toxicity through two distinct but interrelated contributions: (1) stabilization of MTs and (2) inhibition of calpain activation by β-amyloid which minimizes proteolysis of p35 to p25, leading to decreased

activation of cdk5/p25 complexes and subsequent tau phosphorylation [110].

Taxol does not penetrate the blood-brain barrier due to the presence of a P-glycoprotein efflux system. However, a blood-brain barrier permeable taxol analog, TX-67, is available and resembles taxol in its ability to protect neurons against β-amyloid-induced cell death. Moreover, neurons obtained from TX-67-treated animals showed decreased basal cdk5 activity and were resistant to the Aβ-induced cdk5 activation in vitro. Importantly, systemic administration of TX-67 at effective doses did not yield severe side effects in experimental animals. These studies suggest that blood-brain barrier permeable taxol analogs may have utility for testing the hypothesis that microtubule instability lies at the heart of the tau defect in AD [110].

The third group includes compounds that directly interact with various conformations of tau protein. These include fluorescent probes such as ThS that bind β-sheet conformation. However, screening random small molecule libraries for antagonism of ThS binding to tau aggregates has elucidated two distinct classes of such ligands. The first class (Class I) has properties similar to ThS itself: these agents bind both intermediates and filaments with high affinity without inhibiting the aggregation pathway at concentrations up to 10 μM. Examples of this class include structures related to thioflavin T (Fig. 2). Compounds of this class selectively bind various amyloids with dissociation constants below 50 nM and may have utility for development of contrast agents suitable for imaging AD pathology [111–113]. To date, much emphasis has been placed on compounds that image β-amyloid bearing plaques. However, the utility of this approach is not clear owing to the lack of correlation between plaques and other markers of AD progression [114]. In contrast, development of Class I ligands with selectivity for tau filaments could have substantial utility for staging AD as is done in postmortem specimens. The challenge is to develop compounds with sufficient selectivity to detect tau-bearing lesions in the presence of a large excess of competing β-amyloid. A hypothetical model for Class I ligand binding has been proposed for the interaction of Congo red with β-amyloid, where dye monomers intercalate with β-sheets [115]. Selectivity of binding among amyloid composed of different proteins has been demonstrated [113] and may stem from differential interactions between dye and protein side chains.

The second class of tau binding ligands (Class II) interact with tau in such a way as to compete with thioflavin S binding and to inhibit fibrillization. Members of this class include the cyanine dyes, which contain two nitrogen centers (Fig. 2A), one of which is positively charged and linked by a conjugated chain of an odd number of carbon atoms to the other nitrogen [116]. The positive charge is delocalized and distributed over the chromophore structure, yielding two symmetric charge resonance forms. One member of the family, 3-(2-hydroxyethyl)-2-[2-[[3-(2-hydroxyethyl)-5-methoxy-2-benzothiazolylidene]methyl]-1-butenyl]-5-methoxybenzothiazolium (Fig. 2B), was shown to inhibit filament growth induced by anionic surfactants and microspheres as estimated by filament length and number, and to drive the disaggregation of mature synthetic filaments [117]. The mechanism of action is not fully established, but may involve trapping of intermediates in an assembly incompetent state. This would be predicted to decrease filament nucleation, which would account for the potent inhibition of filament number. It would also be consistent with the cooperative dose–response relationship for the inhibitor, as nucleation is predicted to vary as a power function of intermediate concentration. Nonetheless, the ability of the inhibitor to destabilize filaments by promoting endwise disaggregation suggests other mechanisms are also involved, including a modulation of the equilibrium at the one filament end that is kinetically active. This mechanism would be manifest as an increase in the minimal concentration of assembly. Further experimentation will be required to clarify these details.

Why do some planar dyes such as thiacarbocyanines inhibit tau fibrillization while others, such as thioflavins S and T, do not? The answer may relate to the supramolecular structure of the dyes. Symmetrical planar dyes such as Congo red (Fig. 2C) are prone to self-aggregation, whereas smaller asymmetric dyes such as thioflavin T (Fig. 2D) are not [118]. Dimerization of cyanine dyes is well established in the literature [119], and the unique activity of these molecules may derive from this property. Indeed, dye aggregation can be initiated at low concentrations by incubation with “templates” in the form of β-sheet-containing proteins or duplex DNA [120,121]. In the latter case, cyanine molecules aggregate to fill the minor groove. Similarly, cyanine dimers could occupy the ThS binding site in such a way as to destabilize filaments. This model is also consistent with cooperative dose–response data for inhibitors [117], in this case arising from the dimerization of ligand rather than the kinetic mechanism of tau aggregation.

Regardless of its mechanism, cyanine dyes will be useful for testing key tenets of the tau hypothesis and potentially clarifying whether fibrillar aggregates are toxic to cells that accumulate large amounts of them. Indeed, their lipophilic nature (despite the presence of a fixed positive charge) allows them to readily enter cells [122] and therefore be accessible to biological models of tauopathy.

7. Summary

The kinetic pathways through which natively unfolded tau protein adopts higher order structure and fibrillizes have important implications for clarifying the pathogenesis

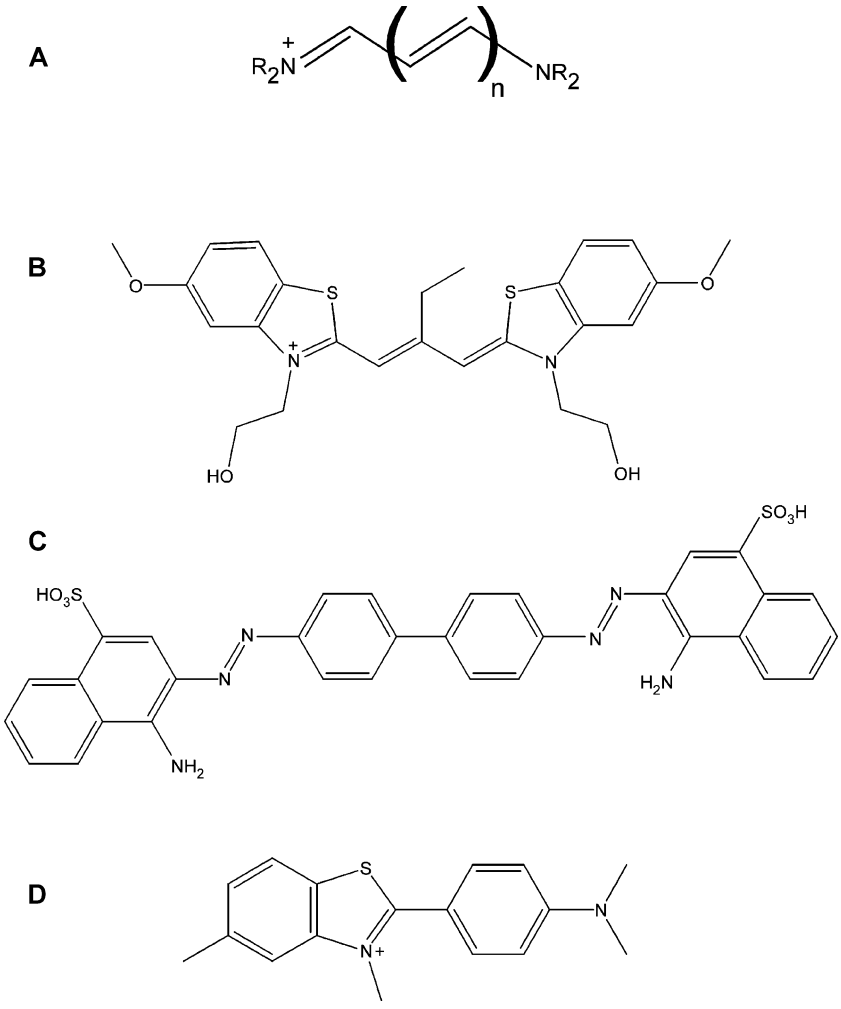

Fig. 2. Amyloid binding compounds. (A) Cyanine dyes contain two nitrogens separated by a conjugated linker (*n*=an integer); (B) 3-(2-hydroxyethyl)-2-[2-[[3-(2-hydroxyethyl)-5-methoxy-2-benzothiazolylidene]methyl]-1-butenyl]-5-methoxybenzothiazolium, a thiacarbocyanine inhibitor of tau fibrillization; (C) Congo red, a planar aromatic dye that binds amyloid; and (D) thioflavin T, a planar thiazine dye that binds tau filaments but does not modulate fibrillization.

of tauopathies such as AD. Experiments performed in situ and in vitro suggest key roles for posttranslational modification such as phosphorylation in creating a soluble pool of tau, and for intracellular membranes and polyanions in facilitating the formation of assembly competent intermediates. These can then accumulate at high local concentration and form fibrillization nuclei. Thus, facilitated assembly can yield tau filaments under conditions where spontaneous, homogeneous nucleation is slow or nonexistent. A challenge for the future is to place the effects of tau phosphorylation and mutation into kinetic context using new analytical methods that have become available. These include selective ligands capable of modulating microtubule function and fibrillization in vitro and potentially in situ. Although full-length tau proteins have little native secondary structure, sequential folding of the molecule in the fibrillization pathway yields species with sufficient structure to bind pharmacological agents with high affinity. Thus, tau fibrillization is a tractable target for drug discovery, and may yield agents useful for premortem diagnosis and treatment of tauopathic neurofibrillary degeneration.

Acknowledgments

This work was supported by a grant from the National Institute of Health (AG14452).

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