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Review Article

Biochemical approaches to assess the impact of post-translational modifications on pathogenic tau conformations using recombinant protein

Mohammed M. Alhadidy[1,2] and ⬤Nicholas M. Kanaan[1,2]

[1]Department of Translational Neuroscience, College of Human Medicine, Michigan State University, Grand Rapids, MI, U.S.A.; [2]Neuroscience Program, Michigan State University, East Lansing, MI, U.S.A.

Correspondence: Nicholas M. Kanaan (nkanaan@msu.edu)

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Tau protein is associated with many neurodegenerative disorders known as tauopathies. Aggregates of tau are thought of as a main contributor to neurodegeneration in these diseases. Increasingly, evidence points to earlier, soluble conformations of abnormally modified monomers and multimeric tau as toxic forms of tau. The biological processes driving tau from physiological species to pathogenic conformations remain poorly understood, but certain avenues are currently under investigation including the functional consequences of various pathological tau changes (e.g. mutations, post-translational modifications (PTMs), and protein–protein interactions). PTMs can regulate several aspects of tau biology such as proteasomal and autophagic clearance, solubility, and aggregation. Moreover, PTMs can contribute to the transition of tau from normal to pathogenic conformations. However, our understating of how PTMs specifically regulate the transition of tau into pathogenic conformations is partly impeded by the relative lack of structured frameworks to assess and quantify these conformations. In this review, we describe a set of approaches that includes several *in vitro* assays to determine the contribution of PTMs to tau's transition into known pathogenic conformations. The approaches begin with different methods to create recombinant tau proteins carrying specific PTMs followed by validation of the PTMs status. Then, we describe a set of biochemical and biophysical assays that assess the contribution of a given PTM to different tau conformations, including aggregation, oligomerization, exposure of the phosphatase-activating domain, and seeding. Together, these approaches can facilitate the advancement of our understanding of the relationships between PTMs and tau conformations.

Introduction

Tauopathies are a heterogenous group of neurodegenerative disorders that present clinically with a broad spectrum of cognitive, behavioral, and motor symptoms, and are associated with degeneration in brain regions related to the clinical impairments [1,2]. These disorders are characterized by the abnormal aggregation of filamentous tau into several forms of pathognomonic neuronal and/or glial inclusions [3,4]. For example, AD is characterized by neuronal neurofibrillary tangles (NFTs), neuropil threads and neuritic plaques, while tufted astrocytes and oligodendrocyte coiled bodies are common in progressive supranuclear palsy (PSP), astrocytic plaques and globose tangles are found in corticobasal degeneration (CBD), neuronal Pick bodies are characteristic of Pick's disease (PiD), and both neuronal and glial tau inclusions are found in chronic traumatic encephalopathy (CTE). In addition to their diversity at the neuropathological level, diversity at the structural level of the filamentous forms of tau has long been appreciated [5–8].

Traditional electron microscopy techniques highlighted differences in paired helical filaments (PHFs) from different tauopathies, namely different diameters and twist periodicities [9–18]. Building on these earlier studies, cryogenic electron microscopy (cryo-EM) was used to resolve structural differences among insoluble tau aggregates in several tauopathies with atomic resolution [19–24]. Collectively, cryo-EM studies indicate that tau assumes conformations within the filament cores unique to each of the tauopathies and this specificity may be helpful in post-mortem disease classification [23,25]. Thus, the physiology and pathophysiology of tau protein is critically important because of its central role as a pathological hallmark in this diverse group of neurodegenerative disorders [26].

Tau is encoded by the microtubule-associated protein tau (*MAPT*) gene located on chromosome 17q21 [27,28]. The MAPT gene gives rise to six primary tau isoforms in the central nervous system through alternative splicing (Figure 1A) [29]. The resulting six tau isoforms contain either three or four of the microtubule binding repeats (MTBRs), called 3R and 4R isoforms, respectively (see Figure 1 for a detailed description of tau domains) [29]. At the protein level within the inclusions, some tauopathies feature primarily 4R tau isoforms (e.g. CBD and PSP), 3R tau isoforms (e.g. PiD) or both 3R and 4R tau proteins (e.g. AD and CTE) [29,30]. Due to the involvement of tau in many neurodegenerative diseases, studying tau functions has drawn significant attention since its discovery in 1975 [31].

Historically, tau is known as a microtubule-associated protein that is involved in modulating microtubule dynamics [31–35]. However, several lines of recent evidence suggest a more diverse functional repertoire for tau that extends to several cellular compartments and pathways [36,37]. As is the case with the other intrinsically disordered proteins, pinpointing specific molecular functions can be complex and this remains an active area of investigation in the field [38]. Moreover, our understanding of the mechanisms mediating tau toxicity and aggregation in neurodegenerative disorders has undergone substantial progress [39,40].

The toxicity of tau protein in neurodegenerative disorders was traditionally linked to tau filamentous aggregates such as NFTs [41]. This is based on myriad studies showing aggregated tau pathology correlates strongly with cognitive decline in patients [42–44]. Moreover, studies in mice demonstrated that behavioral deficits are linked to the overexpression of pro-aggregation versus anti-aggregation tau mutants [45]. However, estimates suggest that tangle-bearing neurons are likely to survive for decades in the human brain [46]. Indeed, tangle-like inclusions are not necessary nor sufficient for neuronal dysfunction in mice [41]. For example, neuronal dysfunction could be reversed by turning off expression of a mutant form of human tau despite the continued presence of tangle-like pathology in the rTg4510 transgenic mouse model [47]. Moreover, tangle-bearing neurons in the rTg4510 mouse model remain functionally intact as indicated by two-photon calcium imaging [48]. Together, these findings highlight that tangles are not as detrimental as initially thought and suggest other pathological species of tau (i.e. pre-tangle forms) may be more critical to tau-mediated toxicity.

Under physiological conditions, tau lacks a stable three-dimensional structure [49] and assumes an ensemble of conformations *in vitro*, such as the paperclip conformation [50,51]. In disease conditions, tau undergoes conformational changes that instigate a cascade of self-assembly that may (on pathway) or may not (off pathway) lead to the eventual formation of filamentous aggregates [52–54]. Given that tau tangles are not as toxic as once thought, earlier conformations of tau that are antecedent to fibrillar aggregation have drawn increasing attention.

The formation of tau oligomers is considered an early event in the progressive accumulation of pathological tau in AD [55–57]. Oligomers are sufficient to cause synaptic and mitochondrial dysfunction in neurons of wild-type mice [58]. Remarkably, oligomeric tau impairs long-term potentiation in tau knock out mice [59]. Moreover, treating primary mouse neurons with oligomeric tau induces electrophysiological dysfunction, such as inhibition of long-term potentiation [60,61]. In agreement, neutralizing oligomeric tau with immunotherapy rescues tauopathy-induced memory deficits in both hTau and tau P301L mice [62,63].

Tau undergoes additional conformational changes early during the progressive accumulation of pathology in human disease that also are associated with mechanisms of toxicity. For example, exposure of a biologically active motif in the extreme N-terminus of tau, called the phosphatase-activating domain (PAD), activates a signaling pathway that disrupts axonal transport [37,64–66]. In addition, recent work shows that monomeric tau can exist in a conformation capable of seeding aggregation in cell and animal models [67,68]. Another conformational change of tau involves its N-terminus coming near the MTBR, an event that happens early in AD [69,70]. A soluble fraction of this conformation can readily assemble into PHFs, suggesting that it facilitates tau aggregation [69,71].
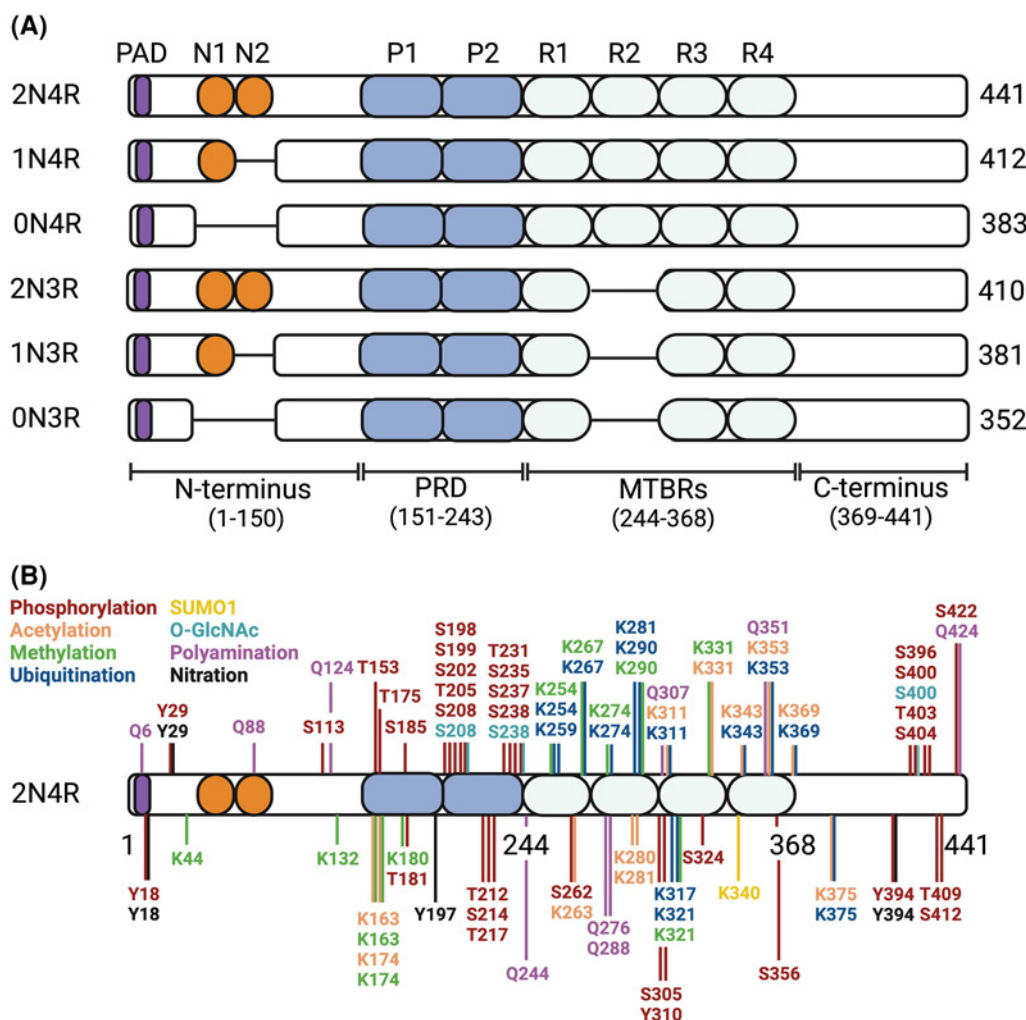
Figure 1. Domains and post-translational modifications (PTMs) of tau protein.

(**A**) Tau proteins contain multiple domains, including the acidic N-terminus (aa 1–150) containing the phosphatase-activating domain (PAD, aa 2–18) and the alternatively spliced N-terminal inserts (N1, aa 45–73 and N2, aa 74–102); the highly flexible proline-rich domain (PRD, aa 151–243) comprised of two subdomains (P1, 151–198; P2, 199–243); the microtubule-binding region (aa 244–368) that harbors four or three microtubule-binding repeats (MTBRs); and the C-terminal domain (aa 369–441). Alternative splicing of exons 2, 3, and 10 in the MAPT gene results in six different isoforms. Inclusion or exclusion of exon 10 yields three forms of four MTBR containing isoforms (4R) or three forms of three MTBR containing isoforms (3R), respectively. Each 3R and 4R isoform protein has either the N1 and N2 (2N), only the N1 (1N) or neither the N1 nor N2 (0N) N-terminal inserts. The amino acid length of each isoform is indicated to the right of the protein and the amino acids comprising each domain is indicated in parentheses below the domain name. (**B**) Several approaches (e.g. mass spectrometry and antibody-based assays) have been used to identify many tau PTMs in tauopathies, including Alzheimer's disease, corticobasal degeneration, progressive supranuclear palsy, and Pick's disease. This panel summarizes many of the known phosphorylation, acetylation, methylation, ubiquitination, SUMOylation, polyamination, glycosylation, and nitration sites in tau that were identified using human tissues (longest tau isoform, 2N4R, used as the numbering reference). Several PTM sites involving lysine, serine or threonine residues overlap or have high proximity, which may lead to competition and/or complex interplay between different PTMs at specific sites.

Factors driving conformational changes of tau that culminate in its toxicity and aggregation remain largely elusive. A well-studied factor that alters tau conformations and is linked to dementia symptoms is tau mutations causing autosomal dominantly inherited frontotemporal dementias [72]. Some of these mutations may alter splicing increasing the expression of 4R tau isoforms (e.g. P301L) [73], alter microtubule binding, and/or

enhance tau aggregation [74–79]. Another emerging factor that elicits conformational changes of tau is protein–protein interactions. Examples include heat shock protein 90 and bassoon that enhance the multimerization of tau, whereas histone deacetylase 6 decreases tau multimerization [80–83]. Finally, post-translational modification (PTMs) are potential mediators of conformational changes linked to tau pathology [84].

Tau is subject to a broad range of PTMs, including phosphorylation, acetylation, methylation, ubiquitination, SUMOylation, polyamination, glycosylation, and nitration (Figure 1B), among others [84,85]. A significant body of literature demonstrates that PTMs have roles in regulating tau localization, degradation, and aggregation, but the precise impact of specific and combinatorial PTMs on the tau's physiology and pathophysiology requires continued investigation [86]. Therefore, increasing efforts have been put forth to characterize the PTMs of tau through mass spectrometry [87–91]. Perhaps not surprisingly, different tauopathies demonstrate different PTM profiles of tau in both sarkosyl-soluble and insoluble fractions, and now efforts are being made to better understand the interplay between tau PTMs and structural differences in tau aggregates. For example, the structural diversity of tau aggregates between AD and CBD can be explained by difference in the PTM profile of tau [88]. As mentioned earlier, cryo-EM clearly corroborated that heterogeneity of tau aggregates extracted from different tauopathies [19–24]. Taken together, the current advances in our understanding of tau conformations and characterization of PTMs are highly suggestive that PTMs play a role in mediating the diversity of tau conformers in tauopathies.

In fact, several modifications cause the adoption of PAD-exposed conformations and/or cause toxicity through PAD-dependent mechanisms, including specific phosphorylation modifications, multimeric aggregates, and mutant forms of tau associated with inherited tauopathies [64–66,92–95]. Furthermore, mimicking phosphorylation of tau at the S199/S202/T205 and PHF-1 epitopes (S396/S404) makes the paperclip structure more compact [51]. In addition, seeding activity of recombinant tau aggregates is linked to phosphorylation and acetylation specific residues of P301L tau mutant [96].

In conclusion, several lines of evidence demonstrate that PTMs of tau can change tau biology, leading to alterations in the levels of pathogenic conformations that are associated with neurotoxicity [93,97,98]. Subsequently, a better understanding of how specific PTMs affect tau aggregation, conformation and function is of high interest in the field.

Methods to produce recombinant tau protein with PTMs

The ability to produce modified forms of highly purified proteins facilitates the ability to examine the impacts of PTMs on tau conformations. We describe several methods through which tau can be modified with PTMs *in vitro* (Figure 2A):

1. ***In vitro* modification with enzymatic and non-enzymatic reactions**

The use of enzymatic modifications is the cornerstone of studying tau PTMs *in vitro*, especially for phosphorylation and acetylation. Purified recombinant tau protein is incubated with an enzyme (kinase or acetyltransferase) in the presence of an adequate substrate (phosphate or acetyl-CoA). This approach provided opportunities to map specific tau sites that are modified by individual kinases and acetyltransferases [99–107]. Moreover, the enzymatic approach informed a plethora of studies elucidating how phosphorylation and acetylation affects tau's regulation of microtubule dynamics [102,108]. Enzymatic modification was used to study how PTMs alter the conformation of tau and impact its potential to aggregate *in vitro* [102,109–116].

Incubation of recombinant tau with a substrate can directly induce PTMs in the absence of enzymes [117,118]. An example is glycation of tau induced by incubating tau with glucose. Subsequently, tau becomes heavily glycated (>10 sites) within the microtubule-binding region [117]. Glycation differentially regulates aggregation propensity of tau isoforms using heparin as an inducer *in vitro* [109].

These methods provided a substantial base of knowledge about the effect of PTMs on tau; however, they are limited by the inability to introduce PTMs in a site-specific manner. Moreover, the isolated nature of *in vitro* reactions (i.e. containing only purified tau and enzymes) and using substrates at concentrations higher than those observed in cells raises the possibility of modifying sites that are not modified *in situ*.

2. **Co-transformation of bacterial cells**

Co-transforming bacterial cells with a tau plasmid and another plasmid that carries the machinery to produce specific PTMs is another established method to study tau PTMs. The co-transformation approach was successfully used to produce O-GlcNAc modified tau protein, leading to the identification of S400 as a potential site
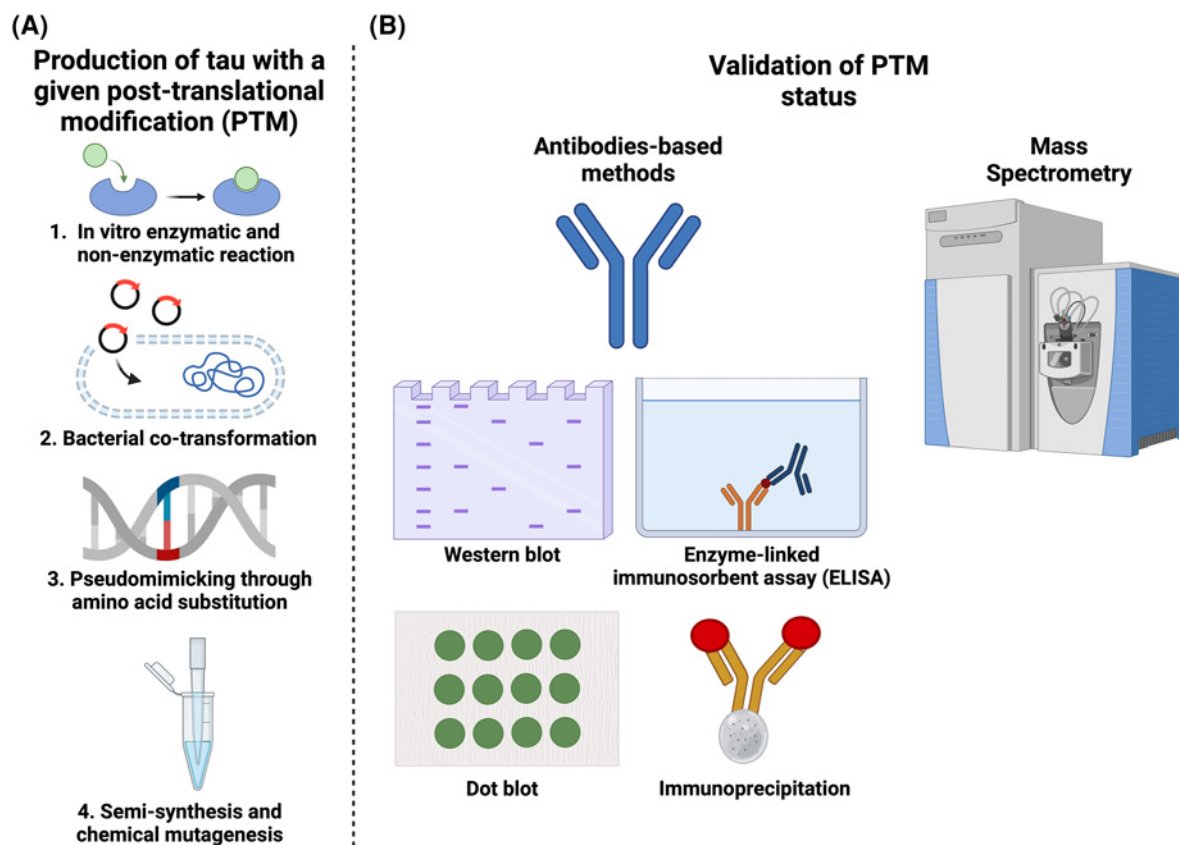
Figure 2. Generation of tau modified with a specific post-translational modification (PTM) *in vitro*.

(**A**) Four different methods can be used to produce tau modified with specific PTMs. First, recombinant tau protein is incubated with a specific enzyme (e.g. kinase) in the presence of adequate substrate (e.g. phosphate) to introduce a given PTM (e.g. phosphorylation). Non-enzymatic reactions involve directly incubating recombinant tau protein with a substrate (e.g. glucose) to introduce the PTM of interest (e.g. glycation). Second, bacteria are co-transformed with a plasmid that encodes tau and other plasmids that carry the machinery required for a specific PTM (e.g. O-GlcNAc transferase enzyme to introduce O-GlcNAcylation). Third, recombinant tau protein is produced with amino acid substitutions that mimic a given PTM (e.g. serine/threonine are substituted with either glutamate or aspartate to mimic phosphorylation). Fourth, semi-synthesis of tau fragments containing site-specific PTMs of interest is followed by ligating the modified fragments together. Alternatively, tau is synthesized with cysteine mutations followed by chemical modifications to introduce the PTM of interest. (**B**) Approaches to validate the PTM of interest on tau proteins. Antibody-based methods require the presence of site-specific antibodies to verify the modification status of tau protein. Mass spectrometry can verify PTMs through targeted methods (e.g. selected reaction monitoring) with or without enrichment of modified peptides. The FLEXITau (Full-Length Expressed stable Isotope-labeled Tau) method is used where isotope-labelled tau is introduced to the sample as a standard to quantify the extent of modification.

of O-GlcNAcylation [119]. O-GlcNAc modification of tau impedes the rate and extent of its aggregation in the presence of heparin. Moreover, O-GlcNAc modification did not induce detectable global conformation changes in tau as assessed using nuclear magnetic resonance (NMR) [119].

A challenge with independently co-transforming bacterial cells with tau and modification machinery is the potential misfolding and trapping of tau into inclusion bodies preventing its modification [120]. To circumvent this problem, a novel approach called protein interaction module-assisted function X (PIMAX) system in which a protein of interest and the enzyme are created in bacteria as fusion proteins with Fos and Jun, respectively. The Fos and Jun interaction successfully brings tau in close proximity to kinases such as GSK-3β and CDK-5. Phosphorylation of tau by this approach enhances the aggregation of tau in the presence of heparin as measured by thioflavin T [120]. In addition, tau phosphorylated by the PIMAX approach can aggregate without an inducer and is toxic to SH-SY5Y cells [121].

These approaches should prove useful for studying tau PTMs catalyzed by other enzymes as well. For example, understudied PTMs of tau, such as SUMOylation, may be effectively studied through this approach [122]. Another advantage of this method is that it induces PTMs inside a biological system, such as bacterial cells. However, this method is still limited by not allowing site-specific PTMs.

3. Mimicking PTMs with alternative amino acids

Modification mimicking approaches involve the substitution of specific amino acids with alternative amino acids that recapitulate the effects of a specific PTM. In the case of phosphorylation, serine and threonine residues are mutated to glutamate or aspartate to mimic the additional negative charge from phosphorylation [93,123,124] or to alanine to prevent phosphorylation [125]. In the case of acetylation, lysine residues are changed to glutamine to mimic the neutralization of charge induced by acetylation or to arginine to mimic a nonacetylated lysine [126]. A recent report utilized mimicked methylation of tau by substituting lysine with phenylalanine to increase the hydrophobicity at a given methylation site [127]. This method offers the advantage of allowing the introduction of one or more site-specific PTMs, but it is limited by the artificial nature of the modifications.

Site-specific PTMs using mimetic approaches enriched our understanding of the roles PTMs play in regulating tau biology in health and disease. Pseudophosphorylation at either T175 or the S199/S202/T205 alters tau conformation leading to increased exposure of tau's PAD [51,93,98], which is a conformational change linked to tau-induced axonal transport impairment [64,93]. Moreover, pseudophosphorylation at S202 and T205 enhances the aggregation of tau in the presence of arachidonic acid [128], and modification of S199 or T205 alone is sufficient to impair axonal transport [129]. In addition, pseudophosphorylation at S199/S202/T205 impedes the cleavage of tau by caspase 3 at D421 [130]. Similarly, pseudophosphorylation of tau at S422 inhibits caspase-3-mediated cleavage at D421 [131] and is linked to causing transport impairment [97]. Another example is pseudoacetylation of tau within the KXGS motifs, which reduces its ability to form filaments as assessed by thioflavin S [132]. Conversely, pseudoacetylation at either K274 or K280/K281 of tau increases its propensity to aggregate [106,133].

PTM mimetics, such as pseudophosphorylation, can faithfully recapitulate the effects of their physiological counterparts. For example, pseudophosphorylation at regulatory phosphorylation sites often reproduce the effects of bona fide phosphorylation with GSK3β and cyclin-dependent kinase 5 [101,115,128,134–139]. Despite the advantages of targeting specific residues, PTM mimetics have important caveats. It must be acknowledged that they are not the physiological PTM and may produce artifactual changes warranting validation studies with the physiological modifications [140,141]. Moreover, not all PTMs of tau have amino acid substitution that mimic their effects (e.g. O-GlcNAcylation). Finally, this approach requires the investigator to choose specific amino acids to modify, leaving other sites potentially modified *in situ* unchanged.

4. Semi-synthesis and chemical mutagenesis

The semi-synthesis method is a more recent approach that allows the introduction of site-specific PTMs on tau [142]. The full-length tau protein is synthesized in a series of fragments that can be chemically modified with the PTM of interest. Then, the modified fragments are brought together by fragment ligation reactions. The semi-synthesis strategy was first used to introduce acetylation at K280. Interestingly, tau acetylated at K280 aggregated with the same rate as the acetylation mimetic K280Q in the presence of heparin [142]. However, electron microscopy showed that acetylated K280 tau produced oligomeric and short fibrillar aggregates while the K280Q mimetic produced short filaments with no oligomers observed [142]. This result, along with the others observed with pseudophosphorylation, emphasizes the importance of expanding the approaches for introducing site-specific PTMs to fully understand their impact on tau behavior.

Semi-synthesis was also used to study the effects of tau phosphorylation at specific residues. For example, phosphorylation of tau at S356 inhibited tau aggregation with heparin *in vitro* and reduced seeding in tau biosensor cells that measures seeding activity [143]. Not only was this method used to introduce phosphorylation, but also to introduce uncommonly studied PTMs of tau such as carboxymethyllysine [144] and ubiquitination [145]. Diubiquitination of tau at K353 inhibits aggregation, whereas carboxymethyllysine modification left aggregation unaltered.

A newer strategy to introduce site-specific PTMs in tau is known as chemical mutagenesis [146]. This method uses cysteine mutants of tau as the means to introduce site-specific PTMs in a two-step reaction. The first reaction involves the formation of dehydroalanine using bis-alkylation elimination strategy with methyl

2,5-dibromopentanoate followed by a second reaction where the PTM of interest is introduced in a site-specific manner through thia-Michael addition. Phosphorylation of tau is introduced through sodium thiophosphate and dimethylation through captamine. However, this process requires substituting the two native cysteine of tau (C291 and C322) with serine to circumvent inadvertently modifying these sites. Even though modifying both cysteines to serine did not appear to change the microtubule-polymerizing activity of tau [147], it is not clear whether this modification may alter other aspects of tau pathobiology (e.g. aggregation and conformational changes).

Semi-synthesis and chemical mutagenesis allow for the introduction of site-specific PTMs, circumventing one of the main obstacles faced in other methods. Moreover, they represent a bona fide PTM (semi-synthesis) or versions of PTMs that more closely resemble the *in situ* modifications (chemical mutagenesis) when compared with mimetic mutants. However, it is not yet clear whether these approaches can be expanded to include all possible PTMs.

Validation of PTM status

After producing tau carrying a specific PTM, the modification status must be verified using methods such as those described below (Figure 2B):

1. Antibody-based approaches

This approach utilizes antibodies specific for a given PTM to determine the modification status of a target protein. Over the years, a plethora of antibodies that detect PTMs were generated, with a heavy focus on phosphorylated tau. Some of these antibodies are tau-specific and/or site-specific, such as AT8, pS422, PHF1, Tau AcK280, and Tau S400 O-GlcNAc, among many others. Antibodies can verify the modification status of tau in multiple biochemical assays such as western blot [102,119,148], enzyme-linked immuosorbent assays (ELISAs) [149], dot blots [149] and immunoprecipitation [150]. However, it is difficult to determine the stoichiometry of a given modification site using antibody-dependent approaches, and the efficacy of confirming PTMs with antibodies is inherently linked to the validation and degree of specificity of the antibody. A good example of the later concern was demonstrated in a report that tested more than 30 commercially available antibodies for tau [151]. Some antibodies are specific, while others can be easily affected with modifications at neighboring sites. Validation and assessing the degree of specificity paved the way to then use the validated tau antibodies to identify PTM profiles of tau in different stages of AD [152].

2. Mass spectrometry

Mass spectrometry is a technique used to identify and quantify specific PTMs on tau. This approach was used to study tau PTMs in cognitively unimpaired and tauopathy patients [153]. In addition, tryptic digestion and mass spectrometry was used to determine phosphorylation [101], acetylation [102], methylation [154], and glycation sites on tau *in vitro* [109]. Moreover, tryptic digestion was used to determine the double glycine remnants of ubiquitination on lysine sites of tau [155]. Indeed, the double glycine remnants on tau were used to identify ubiquitination sites at K254, K311, and K353 within the MTBR of tau purified from AD brains [156].

The detection of other PTMs is more challenging using tryptic digestions of proteins, such as SUMOylation and O-GlcNAcylation [157,158]. For SUMOylation, two methods were developed to shorten the SUMO isopeptide tail on lysine residues. This was achieved using either microwave-assisted aspartic digestion [158] or a specialized enzyme called WaLP [159]. Alternatively, this can be achieved by using a combination of Lys-C and Asp-N enzymes [160]. O-GlcNAcylation is difficult to identify using MS as well [157]. Higher-energy collision dissociation (HCD) can cleave the GlcNAc moiety off proteins, eliminating the possibility of detecting site-specific localization on a specific serine and/or threonine residue. Yet, the cleavage of GlcNAc moiety produces diagnostic ions that could be used to verify the modification status of tau. Alternative approaches include using electron transport dissociation, which is a gentler fragmentation method that preserves GlcNAc moieties. A third approach is to replace O-GlcNAc moieties through beta-elimination followed by Michael addition (BEMAD) with dithiothreitol (DTT) [157]. The BEMAD method produces a more stable modification with DTT that is detectable with HCD and provides site-specific information about the location of O-GlcNAc. However, this method requires the dephosphorylation of proteins before the DTT reactions because phosphorylated residues may give false positive results [157].

In the past 5 years, more advanced MS-based strategies were developed to allow quantitative evaluation of PTMs on tau. A prominent example is the Full-Length Expressed stable Isotope-labeled Tau (FLEXITau)

strategy in which isotope-labelled tau is added to the sample followed by tryptic digestion and running the sample on the mass spectrometer using selected reaction monitoring (SRM) [161]. The stoichiometry of PTMs is determined by measuring the reduction in unmodified tau peptides in the biological sample, that contain the light isotope, relative to the isotope-labelled tau (change in heavy to light chain ratios). This method was used successfully to characterize phosphorylation sites on tau expressed in Sf9 cells [157]. Moreover, FLEXITau was later used to determine PTMs on tau extracted from AD with quantitative results showing that PTMs accumulate on tau to varying degrees as disease progresses [87]. Finally, mass spectrometry is being used to measure tau turnover and quantify site-specific tau PTMs (e.g. phosphorylation at T111, T205, S208, T217 and T231) to develop robust biomarker assays for AD in biofluids (e.g. cerebrospinal fluid and plasma) [162–164].

Assays to assess pathogenic tau conformations

Once modified forms of tau are generated and validated, a series of assays are available to determine the impact on pathogenic conformations (Figure 3):
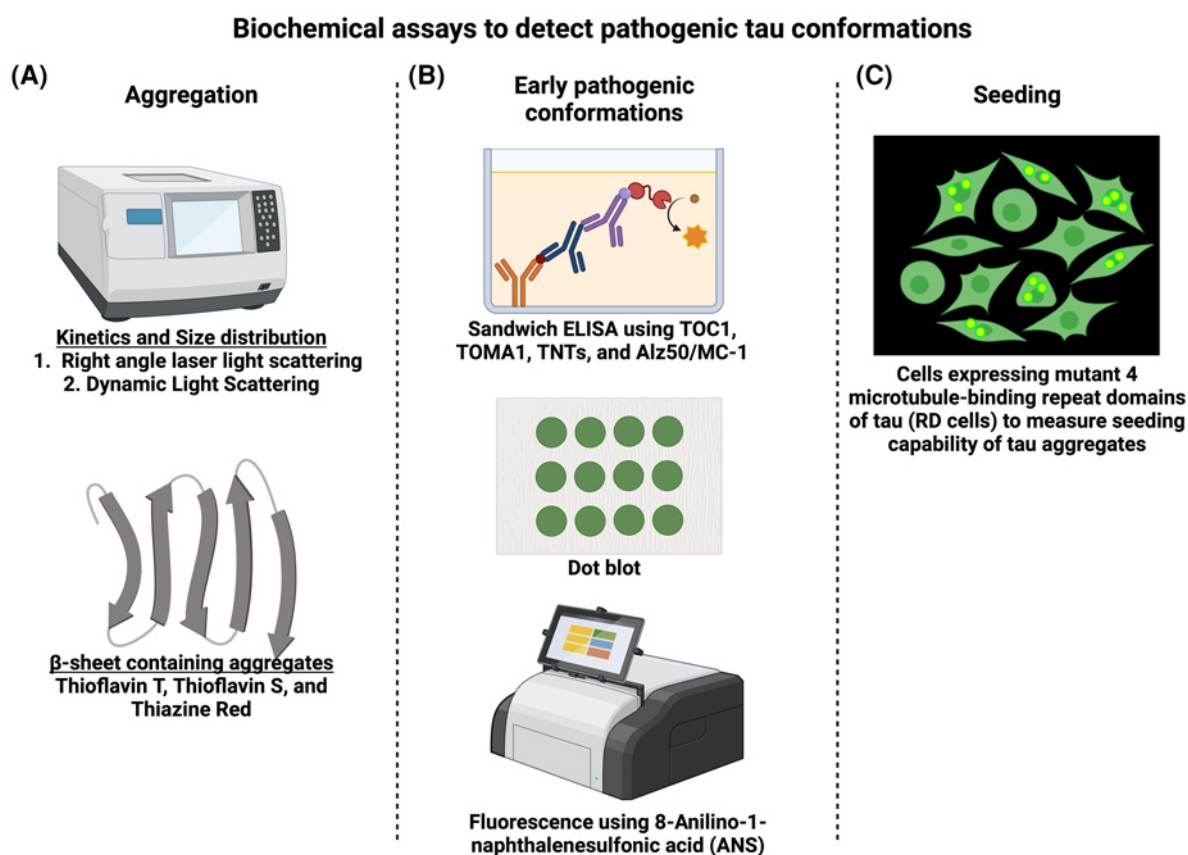

Figure 3. Biochemical methods to assess the impact of post-translational modifications on pathogenic tau conformations.

(**A**) Tau aggregation is induced *in vitro* and monitored by light scattering assays. Right angle laser light scattering allows the determination of aggregation kinetics such as extent of aggregation (i.e. maximum scatter), nucleation rate (i.e. lag time), and elongation rate. Dynamic light scattering helps determine the size distribution of tau populations forming in an aggregation reaction. Alternatively, β-sheet binding dyes, such as thioflavin T, thioflavin S, and thiazine red, can monitor the transition of tau into β-sheet-containing structures (i.e. fibrils). (**B**) Conformational changes that precede aggregation, including exposure of the phosphatase-activating domain and oligomerization, can be monitored with conformation-dependent antibodies (e.g. TNTs, TOC1, TOMA1, Tau22, and Alz50/MC-1) in non-denaturing immunoassays such as ELISAs or dot blots. Oligomerization of tau involves hydrophobic clustering that is monitored by 8-Anilino-1-naphthalenesulfonic acid (ANS) assays. (**C**) RD cells (cells expressing mutant 4 microtubule-binding repeat domains of tau) can be utilized to determine seeding capability.

1. Assessing tau aggregation

The process of tau aggregation involves transitioning into β-sheet-containing structures as assessed by spectroscopic techniques [165]. This conformational transition is predominantly mediated by the two hexapeptide motifs located in tau's MTBR2 and MTBR3 along with the residues flanking the MTBRs [165–168]. *In vitro* tau aggregation is instigated by several inducers, among which heparin and arachidonic acid are the most commonly used [169]. Several reagents allow for the fluorometric quantification of β-sheet-containing aggregates of tau, and other amyloid proteins, including thioflavin T [170], thioflavin S [171], and thiazine red [172]. These fluorometric methods are useful to quantify the β-sheet forming aggregates of tau *in vitro* and are sometimes used to evaluate aggregation kinetics. However, it is worth noting that both thioflavin S and thiazine red also stimulate tau aggregation by lowering the energy barrier to fibrillization [172].

Another way to study the kinetics of tau aggregation is through right angle laser light scattering (LLS) [173]. Right angle LLS allows for the assessment of the nucleation rate, rate of elongation, and extent of aggregation. This approach was used to evaluate the aggregation kinetics of several forms of tau, including full-length, mutant, isoforms, and truncated forms of tau [138,174–176]. Furthermore, this technique was used to assess the impact of PTMs, such as phosphorylation, on aggregation kinetics [97,177].

Dynamic LLS allows for the determination of size distribution of a given population of protein aggregates [178]. To date, tau has not been extensively studied using dynamic LLS experiments. A few studies assessed the extent of oligomerization of tau mutants in the presence of heparin [179] and size of tau condensates during liquid–liquid phase separation [180].

2. Assessing early pathogenic conformations

Several antibodies were developed against the early pathogenic tau conformations mentioned above. For oligomeric tau, three antibodies are used extensively in literature: tau oligomeric complex 1 (TOC1) [56], tau oligomer monoclonal antibody-1 (TOMA-1) [63], and T22 [55]. Other oligomeric or aggregated conformation tau antibodies also exist and will undoubtedly prove useful in studying tau conformation [181,182]. In addition, a set of antibodies called tau N-terminal antibodies (TNTs) are also available that detect conformation-dependent PAD exposure in tau [64,183]. Moreover, the conformational change that precedes tau aggregation where the N-terminus of tau is closely associated with the MTBR is detected with the Alz50 and MC1 antibodies [69,184,185]. These antibodies are useful in detecting pathological conformations and multimers in human and animal model tissues, cell culture models and *in vitro* recombinant protein experiments across many studies in the literature.

The use of conformation-dependent antibodies requires maintenance of the proteins native state in non-denaturing assays, such as sandwich ELISAs or dot blots [56,63,152,183,186]. Using such assays, the impact of PTMs on pathogenic tau conformations is measurable. For example, pseudophosphorylation of tau at S199/S202/T205 and T175 increase exposure of the PAD [93,98]. Conversely, pseudophosphorylation of tau at S422 did not modify the extent of TOC1+tau oligomerization or PAD exposure [98]. Phosphorylation of tau at S199/S202/T205 increases its reactivity with Alz50 and MC1 antibodies in dot blot assays as well [187].

Fluorometric measurements using 8-anilino-1-naphthalene sulfonic acid (ANS) were used to measure the hydrophobic clustering that takes place during tau oligomerization [188]. Interestingly, tau oligomers show higher hydrophobicity when compared with filamentous tau, offering a way to distinguish between the two tau species [189]. A subset of tau oligomers is SDS, heat and reducing condition stable and are visualizable using SDS–PAGE followed by western blot [56]. This may prove useful in studying this subtype of tau oligomers in the presence of a given PTM. For example, phosphorylation at S422 increases these highly stable dimers of tau upon aggregation in the presence of arachidonic acid [97].

3. Assessing conformations associated with seeding activity

Transfer of tau pathology from the entorhinal cortex to other synaptically connected brain regions is proposed as a mechanism by which pathology spreads in AD [190–192]. The Diamond laboratory developed a sensitive assay using tau biosensor cells that measures seeding activity of tau [193]. This and similar assays are relatively widely used to assess seeding activity of tau extracts from both human and animal brains, yielding valuable insights about the prion-like nature of tau, conformers of tau capable of seeding, and tau's interactome [83,194,195]. This work highlighted that tau monomers exist as a dynamic ensemble of conformational states that show varying degrees seeding competence depending upon the accessibility of the regions that mediate

aggregation in microtubule-binding region (i.e. VQIINK/VQIVYK) [68,196,197]. Tau biosensor cells were also used with aggregates prepared from recombinant tau, where the impact of PTMs on seeding behavior was investigated. For example, tau phosphorylated by recombinant extracellular signal-regulated kinase 2 or with a whole rat brain extract can seed aggregation in the absence of tau fibrils [198].

Conclusions

We provide an overview of the *in vitro* approaches currently available to study pathogenic tau conformations and the impact of PTMs on tau (Figure 4). However, our knowledge of all possible pathogenic tau conformations is far from complete [199]. Biochemical approaches are well-complemented by structural tau studies that utilize biophysics to determine changes in tau conformations [118,200,201]. For example, structural changes leading to changes in tau conformations are measurable using additional biophysical methods such as NMR,
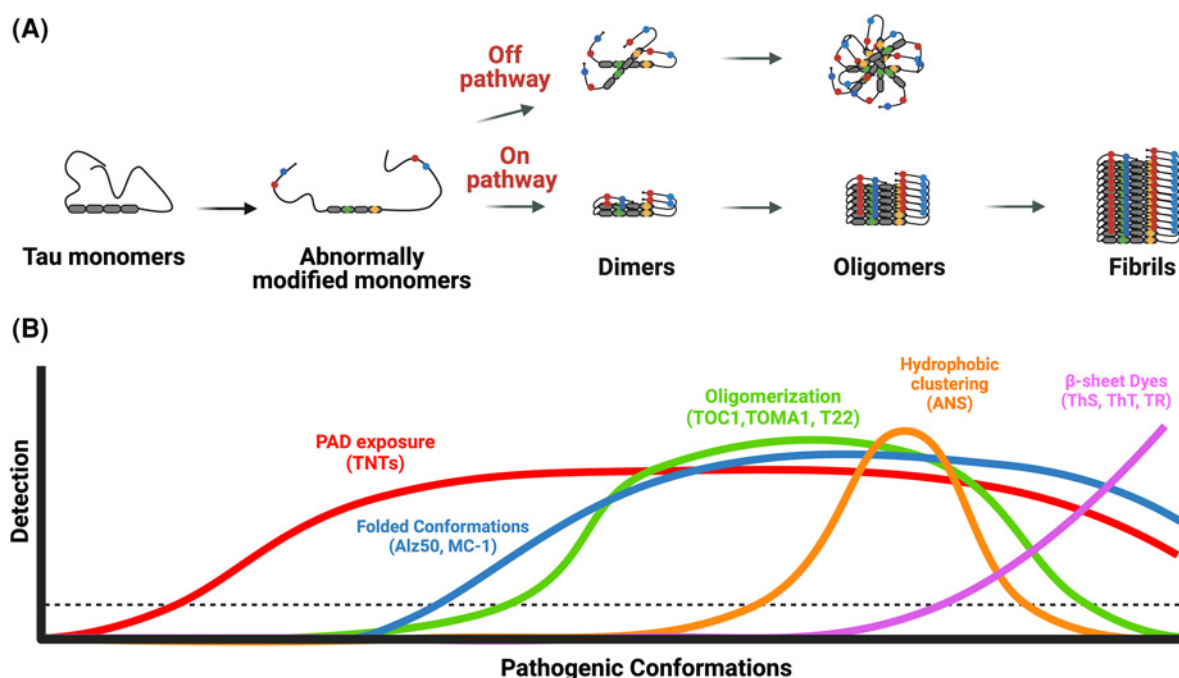

Figure 4. Summary of biochemical approaches to monitor transition of tau into various pathogenic conformations.
(**A**) Soluble tau monomers assume an ensemble of dynamic conformation(s), such as the paperclip structure, under physiological conditions. Abnormal modification with mutations, post-translational modifications, or other factors such as protein–protein interactions are thought to facilitate the adoption of abnormal conformations that facilitate the formation of multimeric species. Tau multimerization is dynamic and a complex ensemble of conformers are possible, some of which are oligomeric forms that are 'off-pathway' for forming fibrils and others are 'on-pathway' and will facilitate the formation of fibrillar structures. (**B**) Specific tools have utility in detecting various forms of tau during the cascade of changes from physiological monomers to abnormal conformations, oligomeric and fibrillar forms of tau. Early abnormal modifications and subsequent conformational changes are identified by some tau antibodies (e.g. exposure of the phosphatase-activating domain (PAD) by tau N-terminal (TNT) antibodies) in biochemical assays. Conformations of tau that precede its aggregation can be monitored by the Alz50 and MC-1 antibodies as well. Many of these early conformational changes are seen in oligomeric species, and additional antibody reagents are available that specifically bind tau oligomers (e.g. tau oligomeric complex 1 (TOC1), tau oligomer monoclonal antibody (TOMA1), and T22). As oligomeric species mature, they become identifiable through 8-Anilino-1-naphthalenesulfonic acid (ANS) assays because of the associated hydrophobic clustering. Finally, fibrillar forms of tau are rich in β-sheet structures and subsequently biochemical assays using fluorometric dyes (e.g. thioflavin S (ThS), thioflavin T (ThT) or thiazine red) that bind these structures identify the formation of filamentous tau aggregates. The seeding capacity of abnormal tau species from monomeric to fibrillar forms are identifiable using the RD biosensor cell assay (cells expressing mutant 4 microtubule-binding repeat domains of tau).

intramolecular Förster resonance energy transfer, differential mobility spectrometry, and ion mobility mass spectrometry (IM-MS) (reviewed in [201]). Moreover, molecular dynamic simulations are useful in detecting conformational ensembles of tau monomers, potentially paving the way to studying tau multimerization [202]. Finally, recent advances in cryo-EM imaging led to unprecedented atomic-level resolution of the fibrillar structures tau forms in human disease and *in vitro* [19–24]. As structural, molecular simulations, advanced imaging, and biochemical approaches converge and continue to advance, the landscape of tau conformations will be deciphered providing clues to the pathobiology of tau in disease.

Perspectives

- Tau is a major contributor to disease pathogenesis in several diseases known as tauopathies. During the process of disease pathogenesis, tau is post-translationally modified and undergoes several conformational changes.

- Current thinking in the field is that the adoption of pathogenic conformations and oligomeric species of tau are key factors in tau-mediated toxicity, and PTMs play an important role in this aspect of tau pathobiology. Several biochemical and biophysical assays exist that facilitate the study of how PTMs affect tau structure.

- The continued use of existing assays and development of new assays to assess the interplay between structural and PTMs will greatly enhance the continued development of our understanding of tau's role in degeneration and disease.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author Contributions

M.M.A. and N.M.K. developed the framework for this manuscript and wrote the manuscript.

Abbreviations

CBD, corticobasal degeneration; cryo-EM, cryogenic electron microscopy; CTE, chronic traumatic encephalopathy; DTT, dithiothreitol; ELISA, enzyme-linked immuosorbent assay; FLEXITau, Full-Length Expressed stable Isotope-labeled Tau; HCD, higher-energy collision dissociation; LLS, laser light scattering; MAPT, microtubule-associated protein tau; MTBR, microtubule binding repeat; NFT, neurofibrillary tangle; NMR, nuclear magnetic resonance; PAD, phosphatase-activating domain; PHF, paired helical filament; PIMAX, protein interaction module-assisted function X; PSP, progressive supranuclear palsy; PTM, post-translational modification; SRM, selected reaction monitoring; TNT, tau N-terminal; TOC1, tau oligomeric complex 1; TOMA-1, tau oligomer monoclonal antibody-1.

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