Review

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The Cellular Environment Guides Self-Assembly and Structural Conformations of Microtubule-Associated Protein Tau (MAPT)

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Abstract: In neurodegenerative tauopathies, such as Alzheimer's disease (AD), microtubule-associated protein tau (MAPT/tau) transitions from a soluble form to insoluble, filamentous lesions inside affected neurons. During this process, tau adopts a range of physical configurations: from misfolded monomers to higher-order oligomers and fibrils. Tau aggregation is also associated with changes in post-translational modifications (PTMs), such as ubiquitination, oxidation, glycation, hyper-phosphorylation and acetylation, which collectively produce an impressive range of possible tau proteoforms. Many of these tau proteoforms are highly cationic and unlikely to self-assemble without neutralization of their charges. Indeed, tau fibrils from patients contain anionic biomacromolecules and bound proteins, suggesting that cytosolic components contribute to fibrilligenesis. Here, we review what is known about how the cytosol impacts tau's aggregation pathways. We also speculate that the composition of each brain region (*e.g.*, redox state, tau proteoforms, levels of permissive polyanions, *etc.*) might play an active role in shaping the structure of the resulting tau fibrils. Although much remains to be discovered, a greater understanding of the role of the cytosol on tau self-assembly might lead to identification of new therapeutic targets.

Introduction

The aberrant aggregation of the microtubule associated protein tau (MAPT; tau) into insoluble fibrils is linked to a group of age-related, neurodegenerative dementias called tauopathies.[1] These disorders are grouped into primary tauopathies, such as Pick's disease (PiD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and frontotemporal dementia with Parkinsonism (FTDP-17) that are characterized by tau pathology in the absence of other protein lesions. In the secondary tauopathies, tau pathology is often observed alongside inclusions of other proteins; for example, Alzheimer's disease (AD) is characterized by both tau deposits and extracellular plaques composed of amyloid-β (Aβ). Thus, there has been great interest in understanding how tau assembles into fibrils, with the therapeutic goal of finding ways to prevent or reverse this process.[2] While early work in the field focused on studying how tau self-associates *in vitro*, advances in both analytical methods (*i.e.*, mass spectrometry) and genetics (*i.e.*, CRISPR) have driven a growing appreciation for how the cytosol, including proteins, metabolites and other biomacromolecules, shapes the aggregation process.

Tau aggregation is a fascinating biophysical transition, in part because tau is normally exceedingly soluble. Tau belongs to the class of intrinsically disordered proteins (IDPs), which are largely void of stable secondary and tertiary folds.[3] Instead, purified tau exists as an ensemble of rapidly interconverting and highly soluble conformers.[4] The *MAPT* gene is composed of four regions: an N-terminal domain, a proline rich domain (PRR), a variable number of microtubule binding repeats (MTBRs) and a C-terminal domain (Figure 1A).[5] Each domain encodes for a protein sequence that has distinct physiochemical properties. For example, the N- and C-terminal domains are disordered and negatively charged, while the PRR and MTBRs are positively charged and contain transient secondary structures.[6] Two hexapeptide motifs, VQIINK and VQIVYK, are located in MTBRs (Figure 1A), and these are required for tau's assembly into β-sheet-rich fibrils.[3,7] Alternative mRNA splicing produces six tau isoforms that vary in the number of segments in the N-terminal domain (0N, 1N or 2N) and the inclusion or exclusion of VQIINK and the second microtubule binding repeat (3R or 4R) (Figure 1B). Because tau's domains have distinct physicochemical properties, each splice isoform (*i.e.*, 0N4R, 2N4R, *etc.*) is predicted to have its own propensity to aggregate. For instance, 3R tau variants tend to be generally less aggregation prone compared to 4R tau,[8] likely due to the exclusion of the VQIINK motif. Yet, splicing is not the only factor that alters the physicochemical properties of tau proteoforms. Tau is subject to a wide number of post-translational

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modifications (PTMs), including proteolysis, phosphorylation, acetylation and more.[9] Each of these PTMs also impacts the structure, charge and solubility of tau isoforms; for example, some of the products of tau proteolysis are significantly more aggregation prone, while others are not.[5,10] In this way, tau has a remarkable number of potential proteoforms; as each combination of splice isoform and PTM pattern creates, in theory, biomacromolecule with individualized chemical features. Mass spectrometry studies are beginning to confirm that there are a large number of proteoforms present in tauopathy brains,[11,12] highlighting the importance of better understanding their unique properties.

Recent evidence suggests that tau assembles into a diverse range of structural states, including misfolded monomers,[13] soluble oligomers and a wide range of insoluble, higher order fibrils.[14–16] Thus, in addition to understanding the physicochemical features of individual tau proteoforms, we also need to understand how their self-assembly culminates in a distinct set of structures. One of the most exciting developments in recent years has been the realization that the fibril cores from patients with a single tauopathy (*i.e.*, AD, PSP, *etc.*) adopt similar folds.[17,18] Because tau pathology tends to appear in distinctive brain regions in these diseases, one wonders whether the composition of the cytosol in the affected cells might shape the conformational preferences of the tau fibrils? In other words, does the disease state create cellular conditions that are permissive to only a subset of tau aggregation pathways?

In this review article, we first briefly introduce the types of tau structures that have been observed, with a focus on insights gained from nuclear magnetic resonance (NMR) and cryo-electron microscopy (cryo-EM). Then, we review how tau's splicing, mutations and PTMs combine to create a remarkable range of potential proteoforms, each having their own electrostatic properties and aggregation propensities. Then, we speculate that constituents of the cellular milieu, including the concentration(s) of certain proteins, polyanions, metals and metabolites, might collectively create an environment that is either permissive or detrimental to the formation of specific tau fibril conformations. While many questions remain, we hypothesize that a deeper understanding of how the cellular environment shapes tau self-assembly might hold the key to the creation of next-generation therapeutic strategies.

Tau's Conformational Landscape is Diverse. The self-assembly of IDPs, such as tau, does not follow a simple folding trajectory to produce an individual conformation.[19] Rather, this pathway seems to be "rough", featuring several minima, many of which might have competing, low energy structures (Figure 2). One result of this complex landscape is that tau samples a diverse ensemble of monomers, which then seem capable of self-assembling into a wide range of oligomers, fibrils and other higher order structures (Figure 3). The diversity of tau structures on this landscape has been extensively reviewed,[18,20] so, here, we only briefly summarize the major structural categories, with the goal of motivating a discussion, in later sections, into how the cytosol could shape this process.

Microtubule Bound. In healthy neurons, tau is largely restricted to axons, where it associates with microtubules (MTs) to regulate their assembly and organization.[21] Although there remains some controversy over the details, recent work has shown that tau binds tubulin heterodimers[22] and that multiple tau molecules come together into "envelopes" on intact MTs.[23] This interaction requires tau's MTBRs, while residues outside of this region are thought to remain flexible in the bound state and behave akin to a polyelectrolyte brush.[24] During cytoskeletal dynamics, tau is thought to cycle off MTs,[25] as supported by fluorescence recovery after photobleaching (FRAP) experiments.[26] On balance, the bulk of tau (>90%) in neurons is bound to axonal MTs under basal conditions and; thus, the "healthy" conformation of tau can likely be considered to be the microtubule-bound state.

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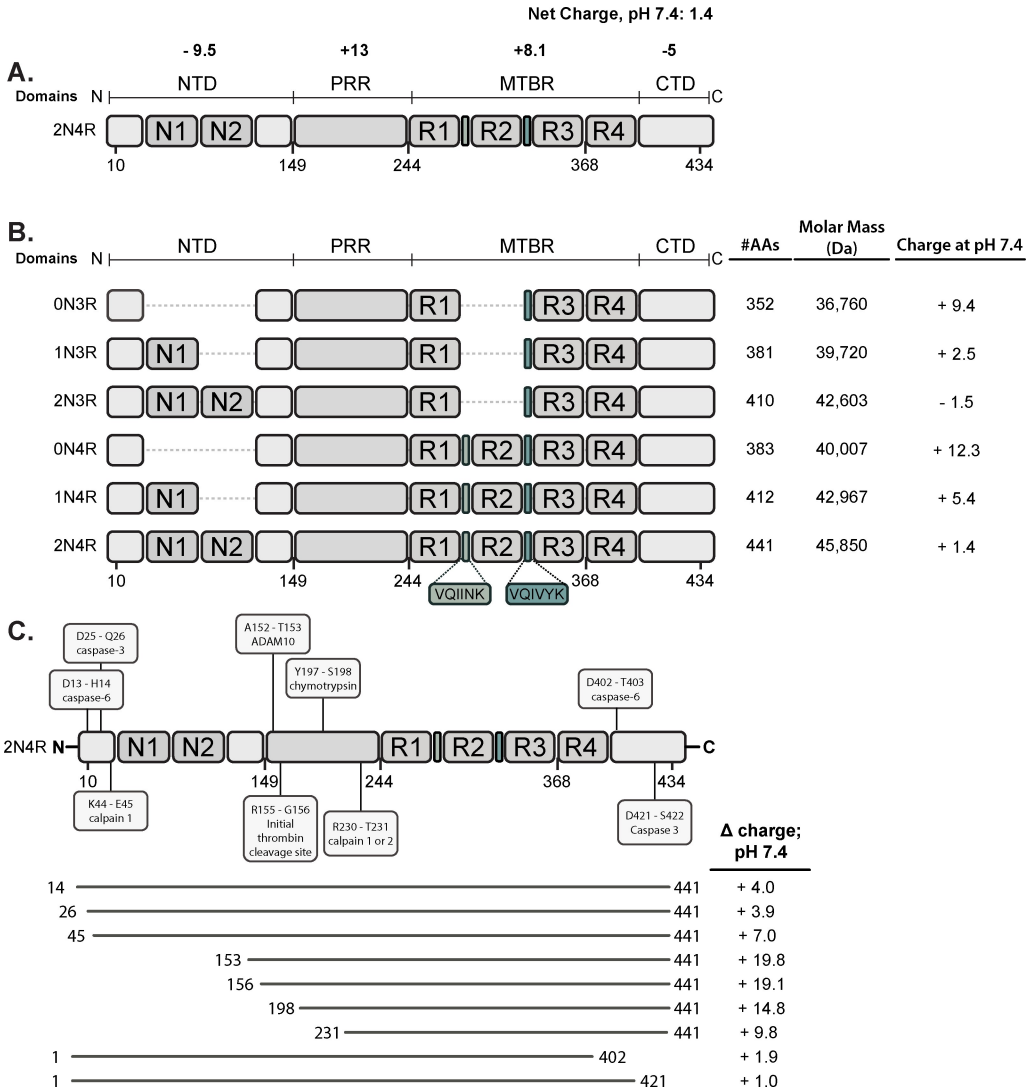

Figure 1. Domain architecture of tau isoforms and location of common proteolytic modifications. Amino acid numbering corresponds to the 441-residue 2N4R tau isoform. (A) Domain architecture of the full-length 2N4R tau, with the overall charge and charge of each domain indicated. Abbreviations: N-terminal domain (NTD); proline-rich region (PRR); microtubule-binding repeats (MTBR); C-terminal domain (CTD). (B) Domain architecture of tau's six splice isoforms, with the size and charge at neutral pH indicated. (C) Common tau proteolytic truncations, with the charge (compared to 2N4R) for each truncation indicated.

Soluble, Monomeric Tau. When tau is transiently displaced from MTs, either in response to MT dynamics, PTMs or physical perturbations, it is placed into a category of states that we refer to as "MT-free". Much of what we know of this structural state comes from NMR and computational studies on tau monomers. In solution, tau monomers sample a dynamic, conformational ensemble,[27] including a restricted capacity to adopt local alpha helices.[28] More broadly, structural studies using a shortened form of tau suggest that the preferred state is a "hairpin-like" fold in which the N- and C-termini reach back onto the MTBRs.[29] Specifically, Förster resonance energy transfer (FRET) experiments have measured relative compaction of the MTBRs in the MT-free state, along with transient, and likely electrostatically driven, interactions of the MTBRs with the N- and C-terminal domains. While these studies have been highly informative and foundational for the field, it is important to consider that, in cells, proteins and other biomolecules will likely bind tau monomers and shape their conformers. In other words, tau is likely never fully "free" of partners, as it is in the NMR tube. For example, the molecular chaperone, Hsc70, is known to bind tau soon after its MT release.[30] Thus, the true range of structures adopted by tau monomers in cells is likely to be significantly restricted by its interactions with partners.

Membrane-less Organelles & Biomolecular Condensation. Tau is known to undergo liquid-liquid phase separation (LLPS),[31] an emerging concept for dynamic regulation of biological processes in cells.[32] Tau's propensity to de-mix in

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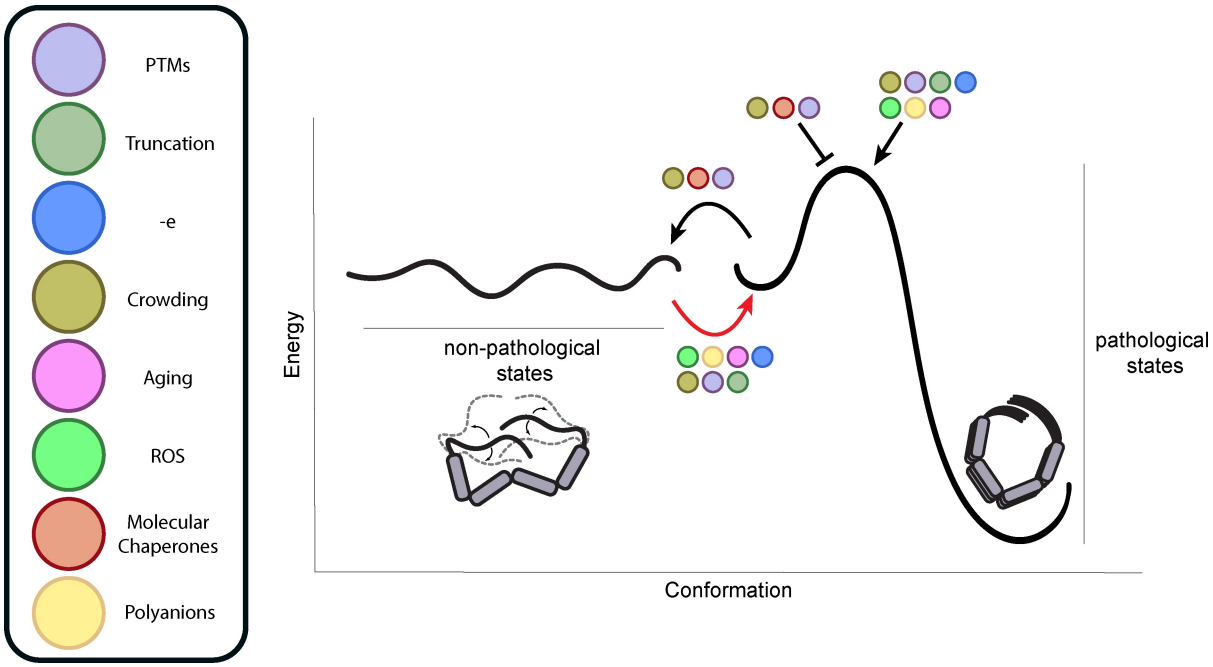

Figure 2. Impact of the cellular environment on tau's free energy landscape. Tau's folding energy landscape is rough and its conformational states (and the energy barriers between them) are influenced by interactions with metabolites, polyanions, protein partners and more components of the cellular environment. Under conditions such as aging, changes in those interactions, such as a decrease in chaperone activity or increase in redox stress, may allow access to conformational states that are on-pathway to oligomer and fibril formation. Here, the ensemble of tau's non-pathological states (e.g., microtubule-bound tau, microtubule-free tau) and its pathological states (e.g., oligomers, protofibrils, fibrils) are pooled together for clarity. See Figure 3 for more details.

solution is driven primarily by electrostatic intermolecular interactions (simple coacervation),[33] but it is also prompted by crowding agents[34] and interactions with negatively charged polymers, such as RNAs (complex coacervation).[35] It is still not clear what triggers tau to undergo LLPS *in vivo;* however, it is clear that tau's dynamics are significantly reduced in coacervates. This change in dynamics, combined with the high local concentration, might favor aggregation under some conditions. Indeed, in aged, tau-rich droplets, aberrant liquid-to-solid transitions also seem to occur, producing fibrils.[34] Insights into this process have been gained by modeling of tau's free-energy landscapes, revealing that the MTBRs can follow multiple paths to aggregation: one of which converges toward static, fibrillar aggregates and another which converges toward amorphous states.[36] Recently, it has been confirmed that tau undergoes LLPS with RNA prior to oligomerization.[37] However, not all LLPS events for tau are pathologic. For example, recent results show that tau forms condensates on the surface of MTs[38] and that these condensates contain protein quality control factors, such as Hsp70, BAG2 and the 20S proteasome, suggesting that LLPS is also an important mechanism for tau clearance.

Oligomers & Protofibrils. As envisioned, the earliest steps in tau's aggregation process involve the shielding of unfavorable electrostatic repulsions, followed by the thermodynamically favorable, hydrophobic collapse of the aggregation motifs from juxtaposed tau monomers. This growing "seed" then serves as a template for the recruitment of additional tau protomers, eventually maturing to oligomers and soluble protofibrils, before progressing to form insoluble fibrils. Here, we use the terms "oligomer" (and "protofibril") to encompass a wide range of soluble structures with varying molecular weights and stoichiometries, with morphologies that resemble either spherical or slightly elongated particles by negative stain EM.[39] Because they are often transient and heterogeneous,[40,41] it has proven challenging to structurally characterize oligomers and protofibrils; thus, direct molecular evidence of templating has been difficult to collect. One clear example comes from cell-based and animal models of tau "spreading" and this mechanism has been used to explain the patterns observed in the maturation of tau pathology in patients.[42] In brief, tau is thought to act in a prion-like manner – the structural information in the initial fold of the oligomeric "seed" is propagated to other protomers.[43] *In vitro*, this process appears as a reduced lag time in the aggregation reaction. In cells, tau oligomers and protofibrils have been seen in many different locations, including associated with microtubules,[41] suggesting that this "seeding" activity could occur in multiple, local environments. Finally, there has been a particular focus on better understanding how tau oligomers and protofibrils form because they are often more neurotoxic than other tau structures.[44]

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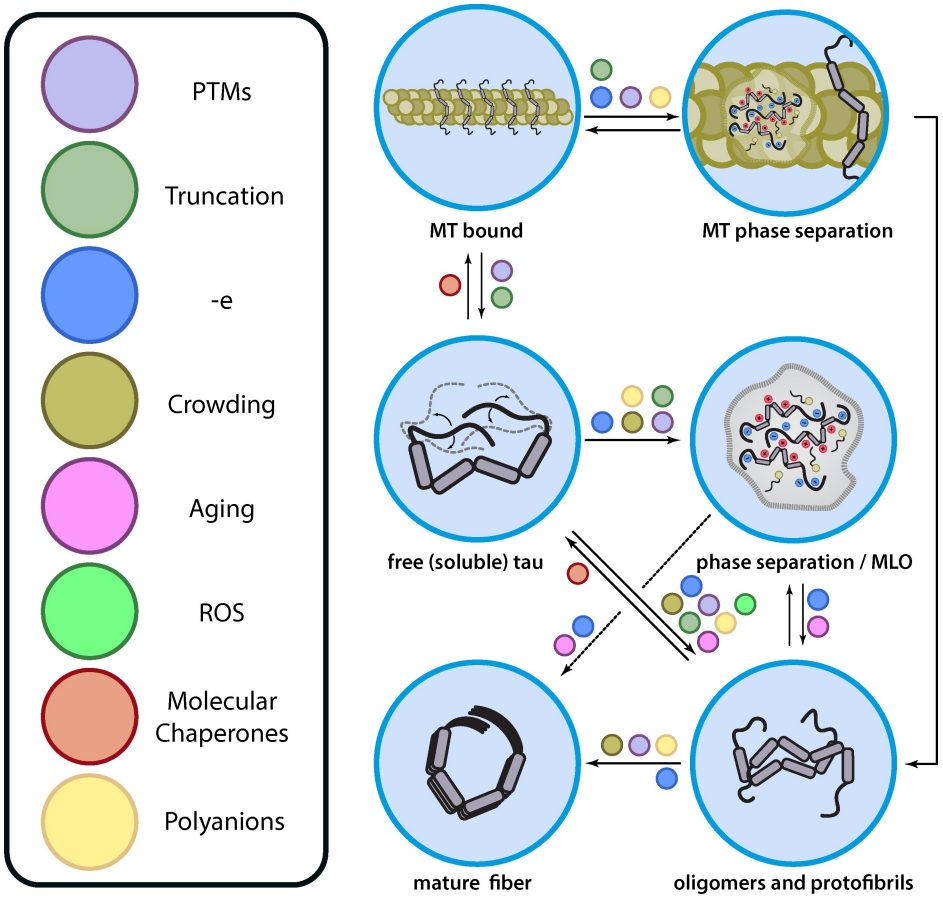

Figure 3. Tau's cellular interactome helps guide its structural states. In cells, tau interacts with diverse cellular components, including proteins, polyanions, metals, metabolites, etc. It is also subjected to PTMs, proteolysis, modification by ROS, redox potential and crowding agents (left). These various partners and stressors help guide tau into distinct states, including phase separated particles, oligomers and fibrils (right). The conversion of tau to different states has been shown to be impacted by the indicated cellular factor, although this list is likely not inclusive. The major take-home message is that tau's inter-conversion between structural states is guided by direct and indirect interactions with its environment.

Tau Fibrils. Tau fibrils are insoluble, highly ordered, β-sheet rich, amyloid structures composed of repeating tau protomers.[45] Many *in vitro* experiments, supported by simulations, have shown that maturation of tau into a fibril is a multistep process initiated by misfolding events, followed by iterative annealing, oligomerization, and fragmentation, resulting in higher-order structures.[46] Analyses of this energetic landscape also suggest a nucleation step, in which oligomers containing parallel β-strand registry proceed relatively rapidly to amyloid fibrils.[36] However, in many tauopathies, this is not the final step in tau self-assembly. Rather, tau fibrils often further assemble into quaternary structures, such as paired helical filaments (PHFs) and straight filaments (SFs).[47] Both straight (SFs) and twisted (PHF) architectures are found in AD patient brains, as revealed by negative stain EM.[16] More recently, tau fibrils have been partially purified from patient tissue,[16,18] enabling high resolution cryo-EM studies and revealing the repeating structure(s) of the fibril cores. These results have been reviewed recently,[18] so here we only reiterate that the core folds have been reported for AD, PiD, CTE, PSP, CBD, and more (*vide infra*). The key takeaways from these studies are that the folded part of the core typically encompass the MTBRs and amino acids flanking the repeats. However, the major surprise is that a variety of folds are adopted by these sequences, depending on the disease. It is also important to note that, in these cryo-EM studies, proteases are used to trim off the regions outside of the core. The removed region is termed the "fuzzy coat" and partial proteolysis studies have shown that it can also adopt local structures that differ between tau fibrils.[48,49] Thus, the true conformational diversity of fibrils might be under-represented by cryo-EM alone. Taken together, the conclusion of ongoing structural studies is that an array of possible tau fibril structures are possible and some of these structure appear to be associated with specific tauopathies.

An important observation from recent structural studies is that the tau fibril cores from multiple AD patients have a similar set of folds.[18] Why aren't other conformations

observed, such as those seen in PSP? One possibility is that other structures are disfavored in AD, either because the specific environment is not permissive for them or because they are cleared by degradation pathways, such as autophagy.[50] The alternative is that the cellular environment specifically favors (or even catalyzes) only a subset of folding trajectories, perhaps by lowering the activation energy for a specific fold by providing a cofactor, such as a polyanion or metal ion. In this review, we explore the unproven hypothesis that the combination of cellular factors (*i.e.*, polyanions, salts, protein partners, *etc.*) present where the tau seeds emerge might favor a subset of fibril structures.[51] Because different tauopathies arise in partially distinct brain subregions and have different morphological appearances,[47] it is then possible to speculate that different cellular environments might favor alternative structures in AD, PSP, CBD and other tauopathies. In the next sections, we review how splicing, PTMs and mutations create a diverse set of tau proteoforms with their own physicochemical properties and then discuss what is known about how cellular factors (*i.e.*, proteins, biomolecules, ions, *etc.*) interact with these proteoforms. As a theme of this review, we especially focus on how changes to tau impacts its electrostatic charge, interactions with polyanions and aggregation propensity.

Tau Proteoforms: Chemical, Physical and Genetic Perturbations Influence Tau Self-Assembly. Mass spectrometry studies have shown that tau can be found in a variety of possible proteoforms[12] that differ in their chemical and physical features. Here, we briefly review the major splicing, mutagenesis and PTM events that give rise to tau proteoform diversity.

Splicing Isoforms. As mentioned above, splicing produces six different isoforms of tau, comprising between 352 to 441 residues (Figure 1B). Splicing directly impacts tau's aggregation propensity. For example, inclusion of R2 (in the 4R splicing isoforms) adds a second aggregation motif, VQIINK (Figure 1B).[52] Less well appreciated is the fact that splicing also has a major impact on tau's overall charge. For example, while one of the most predominate isoform in adult neurons, 0N4R, has an overall charge of +12.3 at neutral pH, adding N-terminal repeats partially neutralizes this charge (*i.e.*, 1N4R = +5.4, 2N4R = +1.4) (Figure 1B). At the extreme, the 2N3R isoform even has a predicted −1.5 negative charge. The Kuret group has performed careful *in vitro* studies to measure the relative aggregation propensity of purified, tau splice isoforms[8] and the results are consistent with both total charge and inclusion/exclusion of an extra aggregation motif being important. Why is overall charge important? As one potential mechanism, charge can change the requirements for polyanions to neutralize tau's intra- and inter-molecular repulsion, thus altering its propensity for self-association. However, a systematic study of the effects of splicing on sensitivity to polyanions has not yet been reported.

Proteolytic Cleavage. In the human brain and in murine models, tau is subject to extensive proteolysis and a substantial proportion of endogenous tau exists as truncated fragments.[53] The enzymes responsible for these cleavages are known in a few cases, and several caspase-mediated and calpain-mediated truncations are detected in AD brains.[54] One under-appreciated aspect of tau cleavage is that the products have dramatically altered overall charge (Figure 1C). For example, many cleavages occur in the N-terminus, where they remove negatively charged regions and produce tau fragments with higher cationic character. For example, while full length 2N4R has a predicted charge of +1.4 at neutral pH, cleavage at residues 14, 26 or 45 in the N-terminus produces truncations with charges of +4.0, +3.9 and +7.0, respectively (Figure 1C). Unfortunately, a comprehensive and systematic study of the effects of truncation on aggregation propensity has not been reported. That being said, certain truncated forms of tau exhibit faster aggregation than full-length.[55] On first glance, this enhanced aggregation propensity seems to go against the hypothesis of overall charge driving self-assembly, but another important consideration is whether the truncation alters the stability of the "paper clip" structure. Specifically, truncation of the N-terminus might be expected to disrupt the paperclip interactions and promote tau's aggregation. However, other tau truncations are indeed protective. For example, a truncated form of tau generated by intron 12 retention termed TIR-MAPT (Truncated by Intron Retention MAPT) is less prone to aggregation.[56] Thus, a combination of mechanisms seems to be relevant, including the truncation's effects on overall charge and the relative stability of the paper-clip structure.

Post Transitional Modifications. In addition to proteolysis, tau is subject to other post translational modifications (PTMs). Tau has many sites that can be modified by phosphorylation, acetylation, ubiquitination, glycation, glycosylation, SUMOylation, methylation, oxidation, and nitration.[9,57] Moreover, many of these sites are used *in vivo;* for example, in AD brain, tau is phosphorylated at more than 40 phosphorylation sites.[58] Some of these PTMs likely serve physiological roles; for example, acetylation at some lysines has been shown to block ubiquitination and slow tau's turnover.[59] Other PTMs impact aggregation propensity; for example, some phosphorylation events favor aggregation, while other inhibit it.[60] Likewise, phosphorylation at some sites weakens tau's MT binding without impacting its aggregation propensity.[61] Together, these observations, and more, suggest that the effects of an individual PTM are dependent, in part, on its context. Recent reviews have explored these questions[62] and this is a vibrant area of research. Here, we focus on another way of considering the impact of PTMs on tau's aggregation. Specifically, we find it interesting that many PTMs either add negative charge (*i.e.*, phosphorylation) or remove positive charge (*i.e.*, acetylation). For example, as a thought exercise, 0N4R with five Ser/Thr phosphorylations and an acetylation would go from a net charge of +12.3 to approximately +6. We anticipate that these changes in net charge might impact tau's aggregation propensity be altering the extent of charge repulsion between monomers. There is some experimental evidence for this effect; for example, charge neutralization of certain lysines by carbamylation tends to increase aggregation propensity.[63]

However, site specific effects must still contribute, as clearly observed by studies on lysine methylation, which showed that over-methylation inhibits aggregation.[64] Together, these studies suggest that PTMs impact tau aggregation through both local and global effects, while also influencing its interactions with cellular factors. At the same time, in the context of the cell, PTMs are expected to be dynamic and subject to regulation by metabolism and signaling. In this way, the relative contributions of tau proteoforms are likely to be shifted by cellular processes, such as cell stress and inflammation.

Genetic Modifications. A number of *MAPT* missense mutations are linked to tauopathies and many of them favor aggregation.[65] Only a subset of these mutations (*i.e.*, ΔK280) change charge status directly, but many (*i.e.*, P301S) seem to exacerbate aggregation through a mixture of damaging MT binding and favoring intermediates on the aggregation pathways. For example, P301S destabilizes the local structure flanking the aggregation motif ^{306}VQIVYK^{311}, resulting in high aggregation propensity.[66] The A152T mutation, a risk factor for FTD, damages microtubule binding affinity.[67] There are more than 50 missense mutations associated with tauopathy[68] and, for most, the molecular mechanisms by which they contribute to disease are not yet clear. Indeed, the contributions are likely complex and involve a combination of effects on tau's intrinsic dynamics and interactions with the broader cellular environment. For example, some genetic mutations alter binding of molecular chaperones to tau.[69] Some of those mutations are located far from the chaperone-binding site, suggesting that they limit chaperone binding indirectly (*e.g.*, they alter tau's conformational ensemble). In another example, the P301L mutant has been shown to interact with a wider range of polyanions than WT tau *in vitro*,[48] again showing that mutations impact tau's interactions with cellular factors. Together, these observations suggest that genetic mutations restrict tau's conformational ensemble; thus, changing the way that tau proteoforms are "seen" by polyanions and other binding partners.

Interactions of Tau with Polyanionic Biomacromolecules. Several polyanionic biomolecules including heparins, anionic micelles and nucleic acids have been found to be co-colocalized with tau pathology in postmortem brain slices of individuals with tauopathies (*vide infra*). *In vitro*, polyanions have been used for many years to accelerate the kinetics of tau aggregation,[70] and recent evidence suggest they are also needed for ongoing maintenance of the fibril fold.[71] Mechanistically, polyanion binding is believed to partially shield tau's positive charge, facilitating self-association of the aggregation motifs.[72,73] Other reports suggest that some electrostatic interactions can destabilize, rather than stabilize, the hexapeptide motifs, suggesting that both synergistic and antagonistic interactions with polyanions are possible.[74] Most recently, it has been shown that polyanions with distinct chemical structures can also guide tau assembly to yield distinct conformations (Figure 4A).[48] This is an important observation because it suggests an active role for polyanions in shaping tau folding. For example, using heparin or mRNA as the polyanion *in vitro* produces wildly different tau core structures, as revealed by cryo-EM (Figure 4B).[75,76] What chemical features of a polyanion might impact this decision? Side-by-side comparisons of ~40 polyanions found that their valency, but not charge density, is important in promoting tau self-assembly and that the chemical identity of the anion has a major impact on tau fibril structure.[48] Similar conclusions were obtained from a recent comparison of five anions, which compared their effects against multiple, disease-associated tau variants.[77] Here, we provide a brief summary of the major polyanion categories and what is known about their interactions with tau.

Heparin and Heparan Sulfate. Heparin and heparan sulfate (HS) belong to the glycosaminoglycans (GAGs) family of polysaccharides that are often found within proteoglycans. These biomolecules are composed of repeating disaccharides units of (1,4) α-l-iduronic or β-d-glucuronic acid (d-GlcA) and α-d-glucosamine (d-GlcN), with chain lengths ranging from 2 to 40 kDa.[78] Each of these polysaccharides is modified by a varying number of sulfate groups that define their complex microheterogeneity.[79] HS tends to have fewer sulfate modifications and, unlike heparin, is naturally found in the human brain at substantial levels (measured to be micrograms to milligrams per gram).[80] Further, HS is associated with tau lesions in AD brains and other tauopathies,[81] suggesting that it might play a role in fibril formation *in vivo*. *In vitro*, both heparin and HS bind tau and initiate its aggregation.[82] Numerous labs have examined the biophysical and biochemical determinants of these early tau interactions. For example, NMR studies have shown that heparin binds to tau near the MTBRs, inducing an alpha-helical tendency in regions outside of the core.[72,82,83] Many synthetic and natural heparin analogs are available, which has been exploited to study structure-activity relationships. For example, the sulfation pattern and oligosaccharide chain length have both been shown to control nucleation kinetics, aggregation kinetics, and fibril morphology.[72] Immobilized heparins have also been used to show that longer-chain oligosaccharides have superior affinity, compared to shorter chains.[84] However, while these GAGs have been valuable probes for understanding how polyanions bind to tau, cryo-EM studies on recombinant tau treated with HS have shown that the resulting fibrils have a core fold that is distinct from those observed in patient samples.[76] This result could mean that HS is not involved in fibril formation *in vivo*. However, we propose that it is premature to make this conclusion because the exact tau proteoform involved in tauopathies is like not identical to the unmodified, recombinant tau that was used for the reported structural studies. Indeed, a recently reported truncation of tau seems to assemble into a fold that better resembles an AD fold[15], highlighting the interplay between tau proteoform identity and structural outcomes.

Hyaluronic Acid. Hyaluronic acid (HA) is a non-sulfated homo-polymeric GAG consisting of alternating residues of β-D-(1,3) glucuronic acid (GlcA) and β-D-(1,4)-N-acetylglucos-

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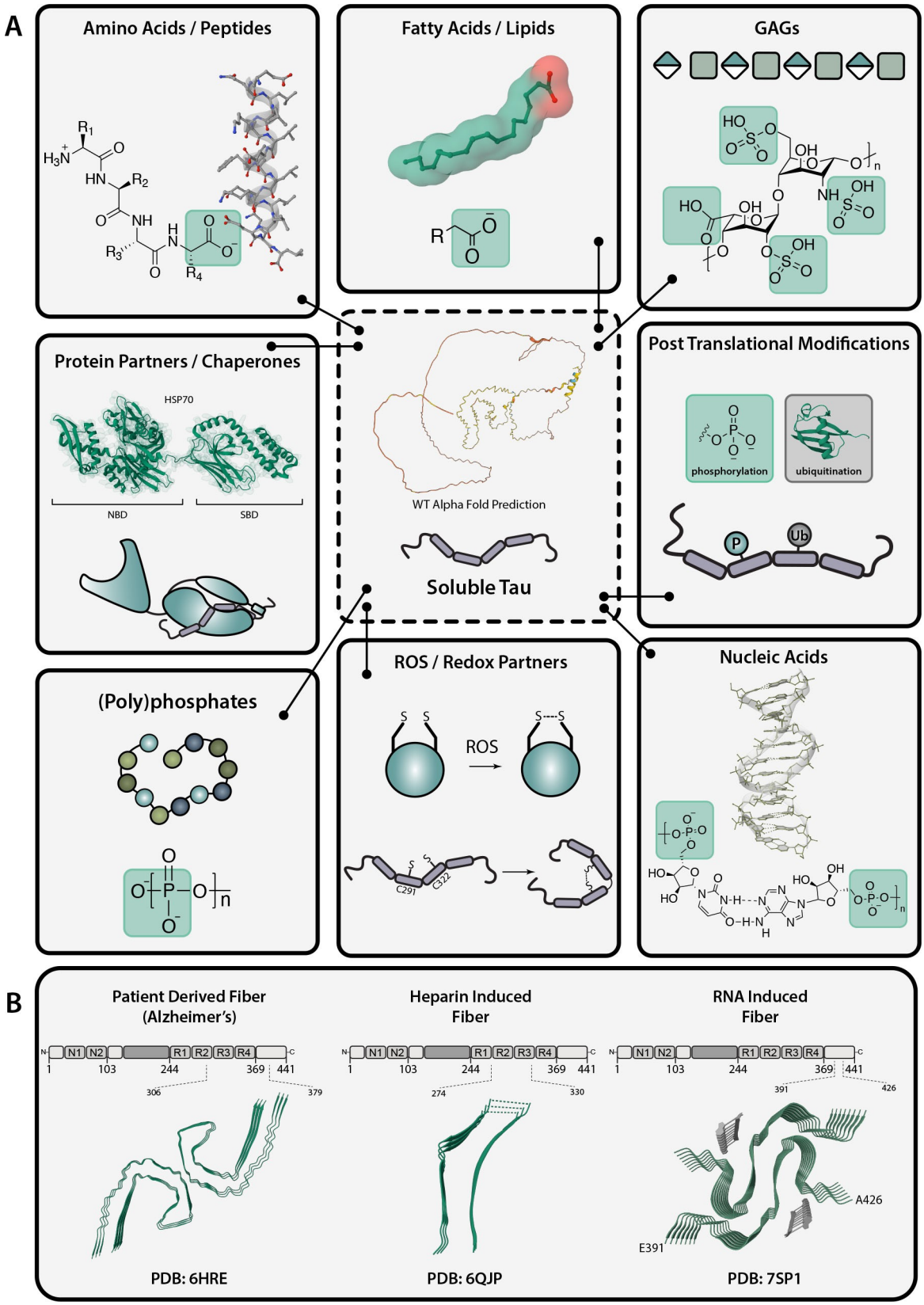

Figure 4. Tau interacts with discrete chemical classes of partners, which guide its fibril structures. (A) Schematic of the types of polyanions and other partners that bind tau and change its structural ensemble and aggregation propensity. In addition, these interactions are tuned by PTMs, such as phosphorylation (and more). (B) Sample cryo-EM structures of tau core folds from AD and those formed in vitro from addition of heparin and RNA, highlighting how the same biomolecule, tau, can adopt wildly different folds depending on the binding partner(s).

amine (GlcNAc). While the precise amount of HA in the human brain is not well characterized, some studies have suggested that it is present in relatively low concentrations (0.1 to 1.0 mg/g of brain tissue).[85] There is no evidence that HA co-localizes with tau pathology and it is not able to promote tau aggregation *in vitro*.[86] Mechanistically, these are interesting findings because HA is a polar, repeating polysaccharide that crudely resembles HS, so one might guess that it would be active in mediating tau's charge neutralization. One possible explanation for this deficiency is that HA's charge density is not sufficient to support tau aggregation, as it lacks the sulfation pattern of heparin or HS. A number of other "inert" polyanion have been reported, including pectin, fusidic acid and kappa carrageenan,[48] supporting the idea that not all polyanions promote tau fibril formation. Indeed, a subset of these molecules, including fusidic acid, even interfere with HS-mediated tau self-assembly.[48] The mechanism of this inhibitory effect is not yet clear, but one can imagine that transient, electrostatic contacts between inert polyanions and tau might sterically limit the productive contacts (*i.e.*, with HS). Thus, one fascinating possibility is that inert polyanions might serve as endogenous inhibitors of tau aggregation in cells and animals, such that an imbalance between the ratio of "active" and "inhibitory" molecules could help dictate the onset of aggregation.

Chondroitin Sulfates. Chondroitin sulfate (CS) is the most abundant GAG in the human brain and it is generally composed of an alternating copolymer of β-glucuronic acid-(1-3)-N-acetyl-β-galactosamine-4-sulfate. Diversity in CS polymer composition emerges due to variable modifications in the sulfation patterning and/or glucuronic acid (GlcA) epimerization into L-iduronic acid (idoA), resulting in a number of defined, CS sub-categories (termed CS–A, CS–B, CS–C, CS–D, CS–E). Various CS forms are observed in tauopathy lesions from AD patients and this interaction has been implicated in regression of neurites.[87] However, CS–A only weakly accelerates aggregation of 0 N4R tau *in vitro*.[48] Thus, the impact of CS in tauopathy is currently considered to be indirect, perhaps through effects on axon outgrowth and neuronal viability[88] rather than on tau itself.

Polypeptides. The impact of polypeptides on tau self-assembly is relatively understudied. Here, we distinguish polypeptides from proteins (which will be discussed below) and, more specifically, only consider repeating homopolymers. For example, poly-L-glutamic acid (PLE) is known to bind tau's MTBRs[83,89] and preferentially decrease the lag time of 0 N4R tau aggregation in a concentration-dependent manner *in vitro*,[48] suggesting that it promotes early events of tau self-association. Polypeptides are relatively straightforward and inexpensive to synthesize, so they could be a good modality on which to carefully explore mechanistic questions related to tau's interactions with polyanions.

Polyphosphates. Polyphosphates (polyPs) are linear homopolymers consisting of as many as thousands of orthophosphate units linked by energy-rich, phospho-anhydride bonds.[90] PolyP exists in the mammalian brain at high levels (low micromolar to low millimolar), depending on the brain region,[91] where it functions as a regulator of neuronal excitability.[92] PolyP has been detected in multiple regions, including the cortex, hippocampus, cerebellum, and striatum, as well as in various cell types such as neurons, astrocytes, and microglia. PolyP has been shown to strongly enhance tau aggregation and it is thought to operate through several mechanisms, including modulation of tau conformation, acceleration of tau aggregation kinetics and stabilization of tau aggregates.[93] Consistent with other polyanions, the valency of the polyphosphate is an important determinant of its potency. For example, low valency triphosphates or monophosphates cannot induce tau aggregation – even when used at concentrations that match the normality of longer polymers.[48] The biosynthetic enzymes that regulate polyP biosynthesis and degradation in mammals are beginning to become clear,[90] so genetic studies to manipulate its levels are becoming feasible. These studies will be particularly exciting because, unlike some of the GAGs, polyP exists in the same subcellular compartments as cytoplasmic tau and it seem likely to be a physiological modulator of early self-assembly steps.

Ribonucleic Acids. Tau lesions from Pick's disease and AD brains are known to be enriched for RNAs.[94,95] These RNAs belong to a number of structural classes, including small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), mRNA and tRNA.[95] Moreover, various RNAs have been shown to bind tau *in vitro*[96] and these interactions also seem able to dictate tau fibril structure. For example, adenine (A), cytidine (C), and uracil (U) heteropolymers bind 2N4R tau and induce unique tau conformers.[97] Mechanistically, RNA is thought to neutralize tau's charge by binding near the MTBRs, favoring tighter packing of tau molecules and promoting cross-β fibril formation.[98] Specifically, polyA and polyG seem to bind residues R406 and H407 in tau, where they support the formation of a fibril core that is composed of residues 391–426, as revealed by cryo-EM.[75] This region is very different than the sequences involved in forming other disease-related fibrils (Figure 4B). For example, the RNA-induced fibers exclude the R2 repeat that is central to many other fibril cores.[48,75] These results provide a striking example of how a polyanion's chemical identity can shift fibril conformation – in this case by stabilizing an alternative fibril core.

Anionic Lipids. Anionic lipids are a broad group of naturally occurring molecules composed of a hydrophilic "head group" and a non-polar, aliphatic tail. In contrast to other polyanions discussed here, lipids are thought to first self-assemble as vesicles or micelles prior to interactions with tau (*e.g.*, they have poor affinity as monomers). On the surface of a vesicle, repeating copies of the anionic head group are thought to recruit tau and favor its self-association. In support of this idea, fatty acids, such as arachidonic acid, linoleic acid, phosphatidyl-L-serine and oleic acid, promote tau aggregation only above their reported critical micellar concentration (CMC).[99] X-ray and neutron scattering experiments also show that lipid membranes impact tau's conformation,[100] suggesting

that they play an active role in promoting self-assembly. Consistent with this idea, recent experiments using NMR have shown that cholesterol is important for recruiting tau, likely by altering the vesicle's curvature, and that the conformation of the fibrils in the presence of lipid vesicles is distinct from that induced by heparin.[101] The interactions of tau with lipid vesicles may be physiologically significant because cholesterol and lipids, including phosphatidylcholine (PC) and sphingolipid, are associated with tau lesions purified from the brains of AD patients.[102]

Protein Binding Partners and Protein Quality Control. Proteins are another broad category of cellular factors that could shape tau's conformational preferences. A number of recent studies have employed proteomics methods to identify tau's interaction partners. For example, affinity purification mass spectrometry,[103,104] as well as APEX- and BioID-based[105,106] studies, have enabled the detection of protein interactions in healthy and pathological states. The key takeaway from those studies is that tau has an extensive interactome, binding to a number of cytoskeletal factors, RNA-binding proteins, molecular chaperones and more. Only a subset of these interaction partners has been studied biochemically to identify the binding site(s); for example, EB3, a protein in the axon initial segment, seems to bind tau in the MTBRs.[107] A full documentation of the interactions of tau with cellular partners is beyond the scope of this review. However, it is interesting to highlight that recent APEX experiments, performed in iPSC-derived neurons, show an especially strong connection to synaptic vesicle transport factors and proteins involved in mitochondrial processes, and the strength of these interactions seems to be enhanced, in some cases, by disease-associated mutations in tau.[106]

To complement these proteomic experiments, CRISPR-based functional genomics experiments have started to reveal genes required for tau uptake, lifetime and aggregation,[108] suggesting key roles for cell surface receptors, vesicle trafficking components, and the autophagy-lysosomal pathway. Again, we will not go into detail on the individual findings. Rather, we suggest that integration of the results of CRISPR screens with the proteomics datasets might eventually be used to better understand the interactome of tau and how it guides tau homeostasis.

To illustrate how such knowledge might be used, we focus here on tau's important interactions with molecular chaperones, such as heat shock protein 70 (Hsp70) and heat shock protein 90 (Hsp90). Briefly, the molecular chaperones are involved in ensuring protein homeostasis (proteostasis), the balance of protein synthesis, folding and turnover.[109] Chaperones are routinely found associated with tau in proteomic studies[104,110] and ~30 of them have been tested for binding to tau in biochemical studies, including NMR and peptide arrays.[69,111–113] Interestingly, those studies have revealed that chaperone binding can be weakened by disease-associated tau PTMs, suggesting that some tau proteoforms might partially avoid quality control by this family. However, Hsc70 (HSPA8), a member of the Hsp70 family, binds tighter to acetylated K280 tau, aberrantly protecting it from degradation in the lysosome and favoring its secretion.[114] Likewise, some chaperones promote tau aggregation.[113] Thus, chaperone binding is not always associated with favorable outcomes. In general, however, interactions of tau with chaperones are thought to promote its solubility and reduce its aggregation.[112] Indeed, many chaperones bind in the aggregation-prone motifs of the MTBRs,[69,115] such that they sterically encumber tau self-association. Other chaperones, such as Hsp22, has also been shown to alter tau's ability to form LLPS structures.[116] Thus, chaperones seem to be active contributors to tau's solubility and aggregation propensity.

It is also clear that chaperones promote tau's turnover through the ubiquitin-proteasome system (UPS) and various autophagy-lysosomal pathways. For example, Hsp70 and Hsp90 coordinate with other chaperones[115] and the E3 ubiquitin ligase, CHIP, to promote tau turnover[117] and Hsc70 recruits tau to the chaperone-mediated autophagy (CMA) pathway.[114] Likewise, complexes of BAG1 and Hsp70 drive tau turnover through the UPS,[118] while complexes of BAG2 and Hsp70 couple tau to the ubiquitin-independent proteasome system (UIPS).[38] Because these degradation pathways are initiated by direct binding of Hsp70 or Hsc70 to tau, it seems likely that chaperone-mediated clearance will be more dramatic for some tau proteoforms than for others. In this way, the quality control systems of the cell might "select" for specific tau proteoforms, removing some while sparing others.

Redox-Mediated Alterations to Tau's Cysteine Residues. Tau contains two cysteines, Cys^{322} and Cys^{291}, within its MTBRs, which are prone to oxidation and capable of forming disulfide cross-links.[119] Replacing Cys^{322} with an alanine destabilizes oligomer formation, and promotes toxicity and neuronal dysfunction *in vitro*[120] and in *Drosophila* tauopathy models.[121] Thus, cellular redox state also appears to be an important aspect of tau aggregation and proteostasis. This is a key consideration because redox state could be additionally tuned by neuroinflammation, which elevates reactive oxidative species (ROS).[122]

Tau's Regulation by Other Cellular Factors (Metabolites, Signaling Molecules and More). Other small molecules in the cell have been shown to impact tau proteostasis. Unlike the factors discussed above, molecules in this category tend not to exert their effects on tau by binding it directly; rather, they rely on indirect mechanisms to impact tau aggregation, PTMs and turnover. For example, several second messengers, including cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and diacylglycerol (DAG), have been reported to regulate tau splicing and/or PTMs. The cGMP-dependent protein kinase (PKG), for instance, ensures the proper balance of 3R and 4R tau expression through regulating the alternative splicing of tau exon 10; therefore, pertubed cGMP levels cause an imbalance in 3R/4R-tau expression.[123] However, a select few metabolites also modify tau directly. For example, 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL), a highly reactive molecule found in the locus coeruleus of AD brains, modifies lysine 353 on tau and

promotes its phosphorylation, truncation, and aggregation.[124] Here, we will not attempt to be inclusive of all studies on tau-associated metabolites; rather, the general picture is that tau aggregation in the cytosol occurs within a dynamic environment of organic compounds, which will certainly guide its interactions, reactivity and processing.

The Role(s) of Cell Type. To this point, we have largely focused on cytoplasmic tau and, more specifically, on tau that is expressed in neurons. However, some tau is also found in alternative compartments, such as the mitochondria[106,125] and extracellular space.[126] Moreover, tau is also expressed in glial cells, where it can form aggregates under some conditions.[127] In these alternative cells and locations, we anticipate that parallel processes impact tau and its conformational preferences. For example, HS is known to be important in the cellular uptake of extracellular tau,[128] providing another, parallel role for polyanions. Relatively little is known about tau's homeostasis in these alternative locations and more work is needed.

Discussion

Here, we have summarized tau's many conformational states and discussed how splicing, PTMs, and mutations give rise to a wide variety of potential tau proteoforms (see Figure 1). Then, we presented how the intersection of tau proteoforms with cellular factors, such as polyanions, might shape the formation of distinct tau fibril conformations (see Figures 2, 3 and 4). We envision that a combination of cellular factors, including metabolites, proteins and metals, likely act synergistically or antagonistically to dictate tau's conformation, turnover and aggregation. There have been some fascinating breakthroughs in this space; for example, the demonstration that RNA can produce distinct tau fibril folds.[75] However, there is a massive combinatorial hurdle to overcome if one wanted to systematically ask about the roles of cellular factors on tau's aggregation *in vitro*. Specifically, the theoretical number of tau proteoforms is in the thousands and there are dozens of polyanions that might bind tau and guide its assembly. Moreover, these anions would be expected to work in pairwise and greater combinations *in vivo*. Likewise, it is challenging to even produce individual tau proteoforms with site selective modifications – although major advances have been made using semi-synthesis strategies.[129] Thus, it would be exceedingly challenging to make and test all of the theoretically possible proteoforms against all of the possible cellular components. Rather, we posit that future work must first focus on limiting the search space. For example, if we understood the specific concentrations and identities of individual polyanions in diseased cells, this information would focus the biochemical and structural experiments. One way to achieve this goal is to optimize analytical methods for measuring the metabolites/anions associated with tau inclusions in patient tissues. Which metabolites, metals and polyanions are present? How do they compare in different brain regions? In different cells (*e.g.*, neurons, microglia)? Ideally, a broader base of analytical measurements will focus subsequent research on the most prominent and important cellular factors.

As mentioned above, modern proteomics approaches have transformed our knowledge of which PTMs are found on tau, in both normal and disease states. For instance, we now know which sites are capable of being phosphorylated *in vivo*. Still, it is challenging to translate this information into a comprehensive list of tau proteoforms because, while the number of possible PTMs is known, the intact mass is (typically) not. Therefore, one cannot discern, for example, whether two phosphorylations are present on the same biomolecule or on different ones. Again, advances in analytical methods are expected to transform this field, allowing future focus on the most abundant and disease-relevant tau proteoforms.

Here, we have aimed to present a framework for understanding the intersection of tau proteoforms with cytosolic factors. What can this framework contribute to drug discovery research? We anticipate that a detailed understanding of the cell's impact on tau aggregation might reveal unexpected, new drug targets. For example, if the key polyanion involved in forming disease-associated structures was found, then inhibitors of the biosynthesis of that polyanion might be pursued. Likewise, if a specific tau proteoform was repeatedly found in disease-associated fibrils, then antibodies against that specific structure might be prioritized as therapeutics. We look forward to future advances in this growing field, which promise to create opportunities for patients and their caregivers.

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Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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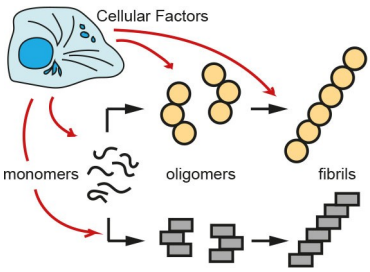
REVIEW



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