

# Chem Soc Rev

Chemical Society Reviews

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ISSN 0306-0012

**REVIEW ARTICLE**



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Cite this: *Chem. Soc. Rev.*, 2022, 51, 513

# Revisiting the grammar of Tau aggregation and pathology formation: how new insights from brain pathology are shaping how we study and target Tauopathies

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Converging evidence continues to point towards Tau aggregation and pathology formation as central events in the pathogenesis of Alzheimer's disease and other Tauopathies. Despite significant advances in understanding the morphological and structural properties of Tau fibrils, many fundamental questions remain about what causes Tau to aggregate in the first place. The exact roles of cofactors, Tau post-translational modifications, and Tau interactome in regulating Tau aggregation, pathology formation, and toxicity remain unknown. Recent studies have put the spotlight on the wide gap between the complexity of Tau structures, aggregation, and pathology formation in the brain and the simplicity of experimental approaches used for modeling these processes in research laboratories. Embracing and deconstructing this complexity is an essential first step to understanding the role of Tau in health and disease. To help deconstruct this complexity and understand its implication for the development of effective Tau targeting diagnostics and therapies, we firstly review how our understanding of Tau aggregation and pathology formation has evolved over the past few decades. Secondly, we present an analysis of new findings and insights from recent studies illustrating the biochemical, structural, and functional heterogeneity of Tau aggregates. Thirdly, we discuss the importance of adopting new experimental approaches that embrace the complexity of Tau aggregation and pathology as an important first step towards developing mechanism- and structure-based therapies that account for the pathological and clinical heterogeneity of Alzheimer's disease and Tauopathies. We believe that this is essential to develop effective diagnostics and therapies to treat these devastating diseases.

Received 22nd April 2021

DOI: 10.1039/d1cs00127b

rsc.li/chem-soc-rev

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## Introduction

The microtubule-binding protein Tau is an intrinsically disordered protein, which is expressed as six isoforms in the adult human central nervous system (Fig. 1A). In cells, Tau is most prominently associated with dynamic regulation and stabilization of cytoskeletal and mitotic microtubules (MTs) through direct binding to tubulin dimers,<sup>1</sup> however other non-canonical functions of Tau have been proposed<sup>2</sup> (Fig. 1B). In neurons, Tau is important for regulating axon outgrowth and maintenance of axonal cytoskeletal integrity and transport.<sup>3</sup> However, factors such as mutations,<sup>4</sup> post-translational modifications (PTMs),<sup>5</sup> interaction with other molecules<sup>6,7</sup> (Fig. 1C), and changes to the biochemical composition of surrounding milieu (*e.g.* pH, drug compounds)<sup>8</sup> may result in weaker interaction between Tau and its natural partners or its dissociation from MTs (reviewed in *ref.* 9). This is thought to lead to Tau accumulation, which creates conditions that favor its aggregation and formation of the  $\beta$ -sheet rich fibrillar aggregates found in the brains of individuals with Alzheimer's disease (AD) and other neurodegenerative diseases (NDDs). Hyperphosphorylated and fibrillar Tau is found in the form of paired helical filaments (PHFs) and straight filaments (SFs) in cytoplasmic neurofibrillary tangles (NFTs) and dystrophic neurites, which represents the main hallmarks of Alzheimer's disease (AD), in addition to amyloid plaques. Tau aggregates, both fibrils, and oligomers are also found in the brain of individuals that suffered from other neurodegenerative diseases (NDs), which include Pick's disease (PiD) and progressive supranuclear palsy (PSP), collectively known as Tauopathies.<sup>10–17</sup> Several structures of brain-derived Tau fibrils have been recently solved using cryo-electron microscopy (cryo-EM), and the list currently includes Tau fibril folds structures from AD, Pick's disease (PiD), chronic traumatic encephalopathy (CTE), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), argyrophilic grain disease (AGD), primary age-related tauopathy (PART), familial British dementia (FBD), familial Danish dementia (FDD), globular glial tauopathy (GGT), and author-coined limbic-predominant neuronal inclusion body 4R tauopathy' (LNT)<sup>18</sup> (for recent reviews see *ref.* 19–21).

Increasing evidence points to Tau aggregation (oligomerization and fibril formation) and PTMs as central events in the pathogenesis of AD and Tauopathies, which made Tau an appealing target for drug discovery and development.<sup>26–28</sup> However, the molecular and cellular factors that trigger Tau misfolding and aggregation and drive the spreading of Tau pathology in the brain remain unknown. Furthermore, the functional significance of Tau aggregation is still unclear. Whether it is a pathogenic gain-of-function (GOF) linked to the formation of cytotoxic Tau species or represents a neuroprotective process that sequesters soluble toxic forms of Tau, or a balance of both, remains to be elucidated. These processes are also accompanied by the loss of physiological function(s) (LOF) due to the depletion of soluble and functional Tau proteins. It is likely that both GOF and LOF mechanisms contribute to the development and progression of Tauopathies. However, the relative contributions of each to the various stages of disease development remain unknown.

Crucially, which forms of Tau are the primary initiators of these processes and how PTMs influence the course of Tau aggregation and pathology formation and spreading remain subjects of intense investigation and debate. In this review article, we will (1) review how our understanding of Tau aggregation and pathology formation has evolved over the past few decades; (2) present analysis of new findings and insights from recent studies illustrating the biochemical, structural and functional heterogeneity of Tau aggregates; and (3) discuss the importance of adopting new experimental approaches that embrace the complexity of Tau aggregation and pathology as an important first step towards developing mechanism- and structure-based therapies that account for the pathological and clinical heterogeneity of Alzheimer's disease and Tauopathies. We believe that this is essential to develop effective diagnostics and therapies to treat these devastating diseases.

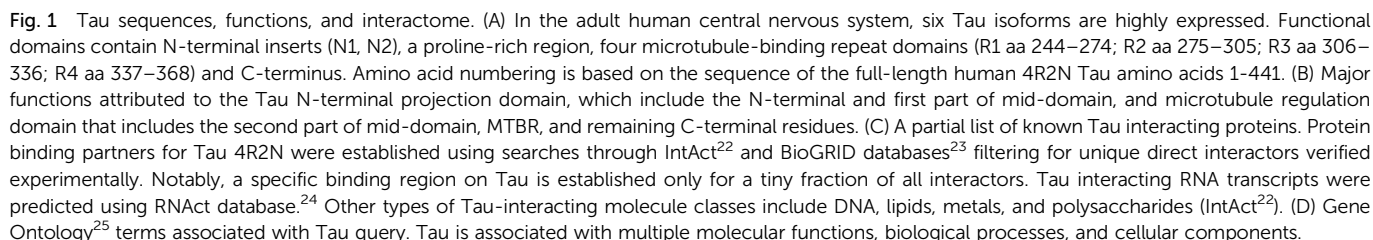
### Tau protein expression

Six major Tau isoforms are found in the central nervous system that differs based on the inclusion and exclusion of the exons 2, 3, and 10 of the *MAPT* gene, resulting in the Tau protein isoforms containing 0, 1, or 2 N-terminal segments, and differential exclusion of the second MT-binding repeat<sup>29</sup> (Fig. 1A). On the level of protein expression, Tau is most abundant in the frontal and occipital cortices, followed by white matter, whereas its levels are significantly reduced in putamen and cerebellum in concordance with gene expression data.<sup>30</sup> The protein levels or distribution of 3R-Tau and 4R-Tau isoforms are approximately equal across brain regions, with 1N-Tau isoforms accounting for about 50%, 0N-Tau isoforms for ~40%, and 2N-Tau isoforms for ~10% of total Tau.<sup>30</sup> However, in neurodegenerative diseases, the ratio of Tau protein isoforms containing 3 or 4 MT-binding repeats is perturbed.<sup>31–36</sup>

### Cellular processes

The Tau proteins have been implicated in multiple cellular processes (Fig. 1D). It was long thought that the primary function of Tau was to stabilize MTs. Still, more recently, the focus shifted to its abilities to regulate the MT dynamics, more so than to stabilize them<sup>37</sup> (reviewed in *ref.* 38). Tau is involved in the insulin signaling pathway by inhibiting phosphatase and tensin homolog (PTEN)<sup>39</sup>, nuclear functions such as stability of heterochromatin,<sup>2</sup> and synaptic processes such as long-term depression<sup>40,41</sup> and potentiation<sup>42</sup> (reviewed in *ref.* 43). Depletion of functional Tau leads to impairments of the processes related to the subset of highly cycling and dividing brain cells, such as hippocampally-positioned neuronal precursors, involved in neurogenesis<sup>44–47</sup> and glial precursors to neuronal cells,<sup>48,49</sup> as well as incomplete neuronal cell-cycle re-entry.<sup>50</sup> This contributed to the neurodegeneration that underlies impairment in memory, increased anxiety, and reduced learning ability.<sup>51,52</sup> Recent studies have also shown that Tau proteins are secreted and propagated from one cell to another. Although this process has been proposed to play central roles in the spreading of Tau aggregates, whether extracellular







Tau also plays a physiological role in the brain remains unknown.

What happens upon complete depletion of Tau in neurons? Interestingly, complete mouse Tau knockout mice that do not express any Tau isoforms showed variable pathological phenotypes at an advanced age, manifesting in cognitive and motor deficits, which varied according to the genetic background of mice.<sup>53</sup> Another commonly used mouse line is hTau.<sup>54</sup> It is hemizygous for overexpression of all six human Tau isoforms on mouse Tau knockout background, and the litters result in the expected half of the mice to express human Tau only, and the other half to be complete mouse and human Tau knockout, providing the isogenic experimental controls. Tau proteins are ubiquitously expressed throughout tissues, and function in processes related to cell division and dynamic cytoskeleton reorganization. Consistent with this, hTau mice showed peripheral deficits in functions related to the highly cell-cycling, dividing, and metabolically-active secreting pancreatic cells in the Tau knockouts, and overexpression of human Tau in islet cells did not rescue the deficits in glucose homeostasis.<sup>55,56</sup> Also, impairment in memory, increased anxiety, and reduced learning ability were observed in these mice, which could not be rescued by the expression of human Tau isoforms.<sup>56</sup> Incomplete cell-cycle re-entry (CCR) of post-mitotic neurons was also observed in hTau mice in ref. 50, suggesting that loss of physiological Tau could contribute to neurodegeneration *via* mechanisms independent of Tau aggregation (LOF) and formation of NFTs. Consistent with this hypothesis, aberrant CCR was implicated in the processes of neuronal loss in AD patients,<sup>51,52</sup> and in mouse models,<sup>57,58</sup> and was found to be independent of the A $\beta$  plaque or NFT formation. Further, the ectopic cell CCR was found to require soluble A $\beta$  oligomers-mediated activation of Tau kinases Fyn, PKA, and CCKII, which phosphorylated Tau at positions pY18, pS409, and pS416, respectively.<sup>59</sup> Tau knockout neurons did not enter the CCR despite the activation of kinases. However, the expression of Tau restored the CCR, in contrast to the phosphorylation-resistant Tau mutants which failed to restore it.<sup>59</sup>

In summary, depletion of Tau may lead to impairments of the processes related to the subset of highly cycling and dividing brain cells, such as hippocampally-positioned neuronal precursors, involved in neurogenesis<sup>44,45</sup> and glial precursors to neuronal cells.<sup>48,49</sup>

One way to gain insight into the functions of Tau in health and disease is through understanding its interactome network. Tau is involved in multiple interactions with a wide range of molecules, with different domains implicated in regulating these interactions (Fig. 1B). These include proteins, nucleic acids, lipids, and polysaccharides, many of which have been shown to regulate many aspects of Tau functions under physiological and/or pathological conditions<sup>60–62</sup> (IntAct database,<sup>22</sup> BIOGRID database,<sup>23</sup> RNAct database<sup>24</sup>) (Fig. 1C). Tau interacts with more than 200 proteins (Fig. 1C, proteins). This versatility and broadness of Tau interactions with proteins are highly complex and it differs between healthy and pathological human conditions.<sup>63</sup> Experimental approaches such as

neuroproteomics,<sup>25</sup> laser capture microdissection coupled with mass spectrometry,<sup>64,65</sup> and interactome studies have been instrumental for the interrogation of Tau interactions with other molecules,<sup>63,66,67</sup> and in organoid<sup>68</sup> and mouse models.<sup>69</sup> Tau was found to interact with deoxyribonucleic acid (DNA)<sup>70</sup> and ribonucleic acid (RNA),<sup>71</sup> strongly suggesting that it has important regulatory roles in the nucleus,<sup>2,72</sup> such as wide-scale chromatin reorganization, detected in AD brain,<sup>73</sup> as well as through post-transcriptional regulation of multiple gene transcripts (Fig. 1C, RNA transcripts).

Tau interactions with other molecules are often heavily influenced and modulated by the extensive Tau PTMs.<sup>74</sup> One of the most prominent PTM-regulated Tau interactions is with tubulin, the subunits composing MTs. Hyperphosphorylation in the MT-binding domain (MTBR) decreases Tau affinity for MTs and leads to an increased rate of its dissociation. This may lead to a higher rate of Tau self-association conducive to aggregation.<sup>75</sup> Further functional validations of the Tau interacting partners are necessary to understand the significance and extent of the molecular functions of Tau beyond its canonical association with the tubulin.

### Tau contribution to neurodegenerative disorders

The presence and spreading of Tau-positive detection of pathology in the brain of normally-aging individuals is well-known, and is not in itself considered to be a functional Tau pathology, and may not be associated with cognitive impairment, however, could indicate the incipient pathology.<sup>76–79</sup> In older adults, Tau accumulates predominantly in medial temporal lobes and is concurrent with low cognitive deficits that accompany the normal aging processes.<sup>80,81</sup> Regional brain vulnerability to Tau pathology is well-known in humans and is thought to vary depending on the different Tauopathies, for example in AD the pathology tends to follow the “subcortical to cortical” spreading pattern affecting the cortex at the later stages, whereas in PSP and CBD pathology is confined to the subcortical regions, with later stages affecting basal ganglia and cerebellum.<sup>82–84</sup> Entorhinal, temporal isocortices, and hippocampal formations are highly susceptible to Tau aggregate formation and neurodegeneration.<sup>82,85–96</sup> Brain circuits between these areas are anatomically connected and are implicated in memory formation, consolidation, and cognition. The aggregation of Tau observed in these neocortical regions temporally follows the deposition of amyloid- $\beta$ , and correlates with the loss of cognitive abilities in Alzheimer's patients.<sup>97,98</sup> Non-neuronal and glial cell populations can also contribute to Tau-mediated pathology formation in mouse models,<sup>99,100</sup> and microglia-expressed triggering receptor expressed on myeloid cells 2 (TREM2) gene variants contribute to the increased AD risk in humans.<sup>101</sup> Tau pathology is thought to proceed through the primary mechanism of direct Tau aggregation in the cell cycle-arrested non-neuronal oligodendrocyte<sup>102</sup> and astrocytic cells,<sup>103</sup> through secondary pathology events such as gliosis and neuroinflammation *via* cytokine excretion,<sup>104,105</sup> or glia-mediated Tau spreading.<sup>106</sup>

Therefore, it is important to delineate the conditions which flip the switch of physiological-to-pathological Tau aggregation in the brain. These may include (1) regional vulnerability of the



brain circuits to Tau aggregation, (2) neuronal and non-neuronal cell type vulnerability, such as increased susceptibility of the excitatory presynaptic neurons,<sup>107</sup> (3) emergence of particularly toxic Tau protein subpopulations due to PTMs or associations with cofactor molecules or other amyloidogenic proteins, such as amyloid- $\beta$ ,<sup>108</sup> (4) local changes in the brain and cerebrospinal fluid (CSF) acidification levels,<sup>109,110</sup> as well as (5) subcellular mislocalization of Tau protein from axonal to somatodendritic compartments<sup>111</sup> that may contribute to Tau displacement from microtubules in cells<sup>112</sup> and subsequent aggregation. It is yet not fully understood which mechanisms contribute substantially to the inception and progression of human Tauopathies.

In terms of the clinical manifestations of Tau pathologies, loss of cognition coupled with the regional brain Tau NFT distribution detected by live positron emission tomography (PET) imaging may indicate the specific Tau-associated disease. These tests, combined with the post-mortem histochemical stainings and the biochemical properties of the isolated Tau, form the basis of the classification of the Tauopathy disorders, and their stages of progression.<sup>113</sup> The Tauopathies with the known biochemical Tau profile are classified into predominantly 4R-Tauopathies containing the 4R Tau isoforms in the insoluble protein brain fractions, such as PSP, CBD, and AGD, as well as gliopathies ARTAG and GGT. Predominantly-3R Tauopathies include the neuronopathy and gliopathy PiD and neuronopathy the behavioural variant of frontotemporal dementia (bvFTD).<sup>113</sup> The Tauopathies defined by the presence of all six Tau isoforms include AD, CTE, PART, tangle-only dementia (TOD), dementia with Lewy bodies (DLB), and primary progressive aphasia (PPA) (Table 1). Also, co-occurrence of Tau pathology may be observed in the late stages of other proteinopathies, such as Huntington's disease,<sup>114</sup> synucleinopathies,<sup>115</sup> and amyotrophic lateral sclerosis (ALS).<sup>116</sup>

Following the findings that cognitive decline correlated with Tau pathology progression in patients<sup>117</sup> to a better extent than amyloid  $\beta$  pathology, the levels of Tau or phosphorylated Tau in

CSF,<sup>118</sup> as well as Tau PET imaging<sup>119</sup> in the brain have been studied as early biomarkers. This is especially prescient due to fact that the sporadic and secondary Tauopathies, such as AD, are not associated with specific mutations in the Tau-coding *MAPT* gene and therefore elude early screening. This is in contrast to familial Tauopathies (Table 1), such as autosomal dominant frontotemporal dementia and parkinsonism (FTDP) linked to chromosome 17q21,<sup>120</sup> or Tauopathies with a clear underlying genetic component, such as Pick's.<sup>121</sup> Therefore, early detection of incipient pathological Tau processes as diagnostics biomarkers, as well as therapeutic targets, has recently garnered much attention.

### Spreading of Tau pathology

At the brain level, in AD the Tau pathology spreading follows the sequence of pathology progression (Fig. 2A), starting from entorhinal and transentorhinal cortices at stage I, progressing to hippocampal in stage II and transentorhinal cortical regions in stage III, followed by the cerebral and visual cortices in stage IV and later stages V and VI.<sup>82,122</sup> Evidence suggests that Tau pathology in AD is transmitted along the anatomically connected brain regions (reviewed in ref. 123). Computationally-aided integration of imaging and biochemical data would allow for reconstructing the AD connectome, which, combined with computer modeling approaches, have the potential to provide higher resolution and granularity to our understanding of stages of tauopathy progression in the human brain.<sup>124</sup>

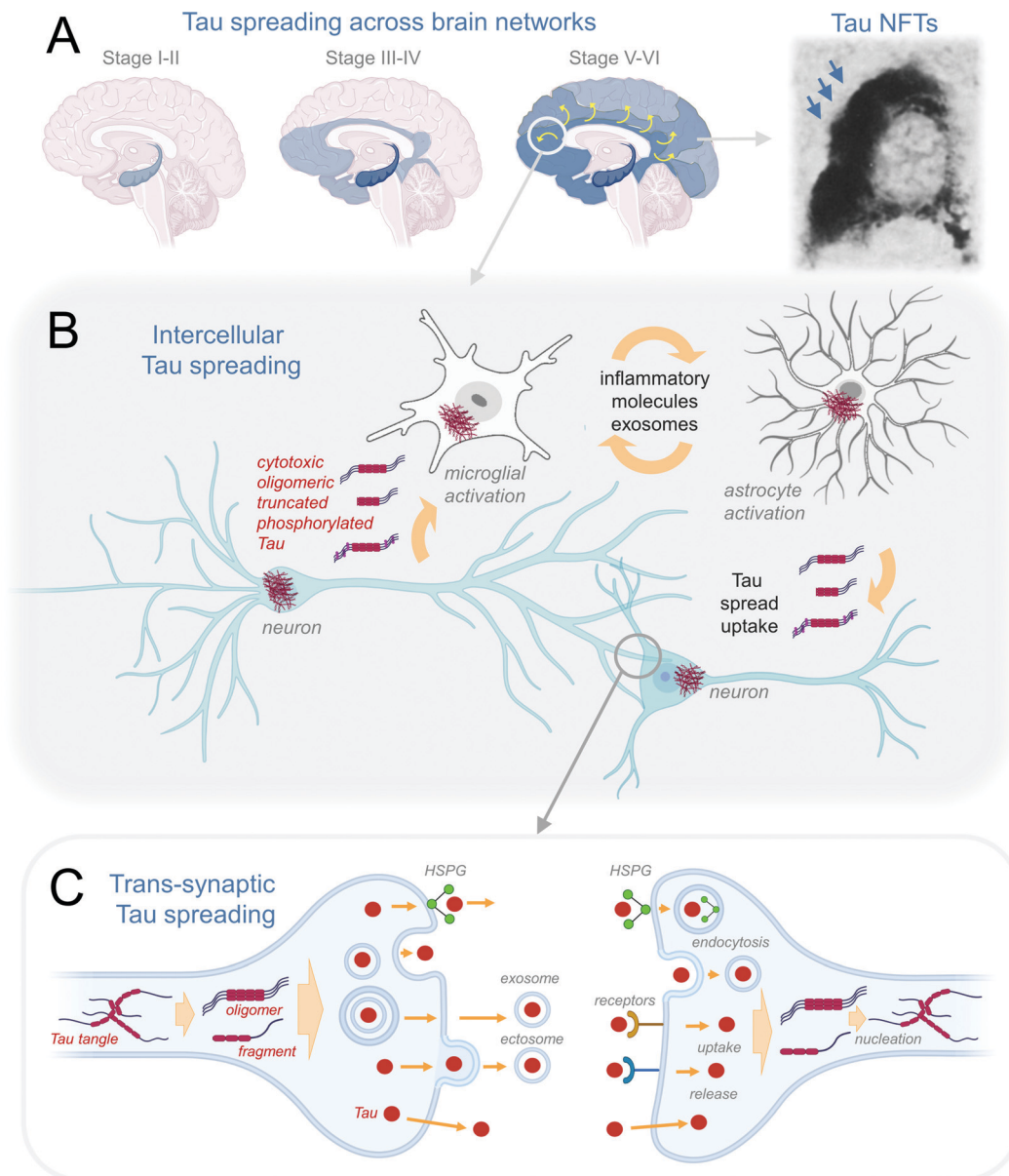
On a cellular level, Tau pathology is thought to spread from one cell to the next through Tau proteoform release from the donor cell *via* multiple potential pathways, such as the direct transport across membrane, exocytosis, endosome-, exosome-, and heparan sulfate proteoglycan (HSPG)-mediated release (Fig. 2B). A further transmission of Tau across synaptic cleft is thought to be followed by Tau uptake by recipient cell *via* direct transport, endocytosis, or receptor-, or HSPG-mediated uptake. In the recipient neurons Tau is thought to nucleate and initiate recruitment and aggregation of

**Table 1** Tauopathies with known Tau isoform composition

3R + 4R	4R
Alzheimer's disease (AD)	Age-related Tau astroglialopathy (ARTAG)
Amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS)	Argyrophilic grain disease (AGD)
Anti-IgLON5-related Tauopathy	Corticobasal degeneration (CBD)
Chronic traumatic encephalopathy (CTE)	Guadeloupean parkinsonism
Diffuse neurofibrillary tangles with calcification	Globular glial Tauopathy (GGT)
Down's syndrome	Hippocampal Tauopathy
Familial British dementia (FBD)	Huntington's disease
Familial Danish dementia (FDD)	Progressive supranuclear palsy (PSP)
Gerstmann-Sträussler-Scheinker disease (GSS)	Familial frontotemporal dementia and parkinsonism (FTDP, mutations P301S, intronic mutations, coding region mutations in exon 10)
Niemann-Pick disease, type C	
Nodding syndrome	
Non-Guamanian motor neuron disease with neurofibrillary tangles	3R
Postencephalitic parkinsonism	Pick's disease (PiD)
Primary age-related tauopathy (PART)	Familial frontotemporal dementia and parkinsonism (FTDP, mutations G272V and Q336R)
Progressive ataxia and palatal tremor	
SLC9A6-related parkinsonism	
Tangle-only dementia (TOD)	
Familial frontotemporal dementia and parkinsonism (FTDP, mutations V337M and R406W)	







**Fig. 2** Mechanisms of Tau spreading. (A) Schematic representation of Braak stages of Tau pathology across brain networks as detected by immunohistochemistry of NFTs (reproduced from ref. 125 with permission from the Elsevier, copyright 1991). (B) Intercellular Tau pathology spreading between neuronal and neuronal, and neuronal and glial cells. (C) Trans-synaptic spreading of different pathogenic Tau proteoforms is thought to occur through their transport across membrane release, exocytosis, endosome-, exosome-, HSPG-mediated release from the affected donor cell. The uptake of putative pathological Tau species is thought to be mediated by HSPG-, receptor-coupled mechanisms, endocytosis, and direct transport across the plasma membrane. The recipient cell may develop further Tau pathology through nucleation and seeding of the endogenous Tau.

endogenous Tau (Fig. 2C), first affecting the distal, followed by proximal dendrites, then cell soma, eventually reaching the axonal compartment.<sup>126</sup> It has been suggested that the spread of toxic Tau species underlying the pathologies may be mediated by one or a combination of (a) a direct prion-like spreading mechanism of misfolded Tau across the cellular membrane with the recruitment of normal Tau in the receiving cell; (b) exosome-mediated and (c) trans-synaptic spreading of toxic Tau species (reviewed in ref. 127 and 128) (Fig. 2A). Thus far, however, the evidence for the prion-like Tau propagation is only circumstantial in humans, with most

work to elaborate this hypothesis done in cellular and animal models (see recent reviews<sup>26,129,130</sup>). Which forms of Tau are secreted and propagated in the human brain remains unclear, although studies have identified several truncated<sup>131,132</sup> and post-translationally modified forms of the protein<sup>133,134</sup> in CSF and blood of Tauopathy patients.

#### Composition of neurofibrillary tangles

Besides the Tau proteins in fibrillized conformation, NFTs are composed of multiple other molecules, including proteins,

carbohydrates, nucleic acids, and lipids. Formation and diversity of molecular composition of NFTs are established by (1) the molecules that drive the primary formation of NFTs, such as mutant proteins, or proteins in pathological conformations, such as fibrillar Tau, (2) recruitment of other molecules and cellular components through direct binding or diffusion, and (3) accumulation of cellular waste products following cellular impairment and degeneration.

Although the exact molecular composition of pathological Tau aggregates remains unknown, several studies have highlighted their complexity and diverse composition. Cytoskeletal proteins such as MAP1B,<sup>135</sup> MAP2,<sup>136</sup> neurofilament, and vimentin<sup>137</sup> were detected by immunohistochemistry in AD-derived NFTs early on. Further, in AD, extracellular NFTs contained amyloid- $\beta$ , amyloid-P, extracellular signal-related kinase-2, glial fibrillary acidic protein (GFAP), and ubiquitin, whereas intracellular NFTs contained apolipoprotein E (Apo E), basic fibroblast growth factor (bFGF), GFAP, heparan sulfate proteoglycan (HSPG), complement membrane attack complex C5b-9, neurofilament, synaptophysin, and ubiquitin. Other NFT-associated molecules included casein kinase II (CKII), glycogen synthase kinase-3 (GSK3), phospholipase C- $\delta$ , malondialdehyde, heme oxygenase-1, vitronectin, gangliosides C, anti-thrombin III, and lactotransferrin. Pick's bodies were shown to contain Apo E, advanced glycation end-products (AGEs), bFGF, chromogranin B, complements C1, C1q, C4, C2, C3, C5, C6 and C8, clathrin, membrane complement inhibitor CD59, clusterin, GFAP, synaptophysin, and ubiquitin (reviewed in ref. 138). NFTs in PSP contained enriched GFAP and ubiquitin, whereas CBD inclusions contained GFAP and marker for killer lymphocytes Leu-7 (reviewed in ref. 139). The proteomic profiling of NFTs using laser capture microdissection coupled to mass spectrometry revealed peptides derived from 542 proteins.<sup>140</sup> However, the list of these 542 proteins is not openly available. 41 polypeptides were identified in AD NFTs by mass spectrometry and included GAPDH and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) that colocalized with NFTs by immunostaining.<sup>141</sup> Another study identified 72 proteins within the NFTs, with biochemically-verified glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a major constituent.<sup>142</sup> S-glutathionylated GAPDH form was detected in the blood of Alzheimer's patients and is a pro-apoptotic molecule that was not present in blood under healthy conditions.<sup>143</sup>

Given such complexity of NFTs and the implications of their biochemical diversity for targeting and imaging pathological Tau, it is crucial to revisit Tau pathology with more systematic approaches to dissect the molecular compositions of intracellular, extracellular, and ghost tangle NFTs. A better understanding of the proteomic compositions of individual NFTs could provide insight into biochemical changes associated with the formation and maturation of Tau pathology. The knowledge gained from these studies, combined with the structural insight gained from cryo-EM could guide future efforts to develop diagnostic and therapeutic strategies that target specific Tau pathologies or capture their diversity in the brain. Furthermore, a better understanding of the molecular components of

pathological Tau aggregates could also provide novel insight into natural cofactors responsible for triggering Tau pathology formation in different Tauopathies.

### Tau aggregation

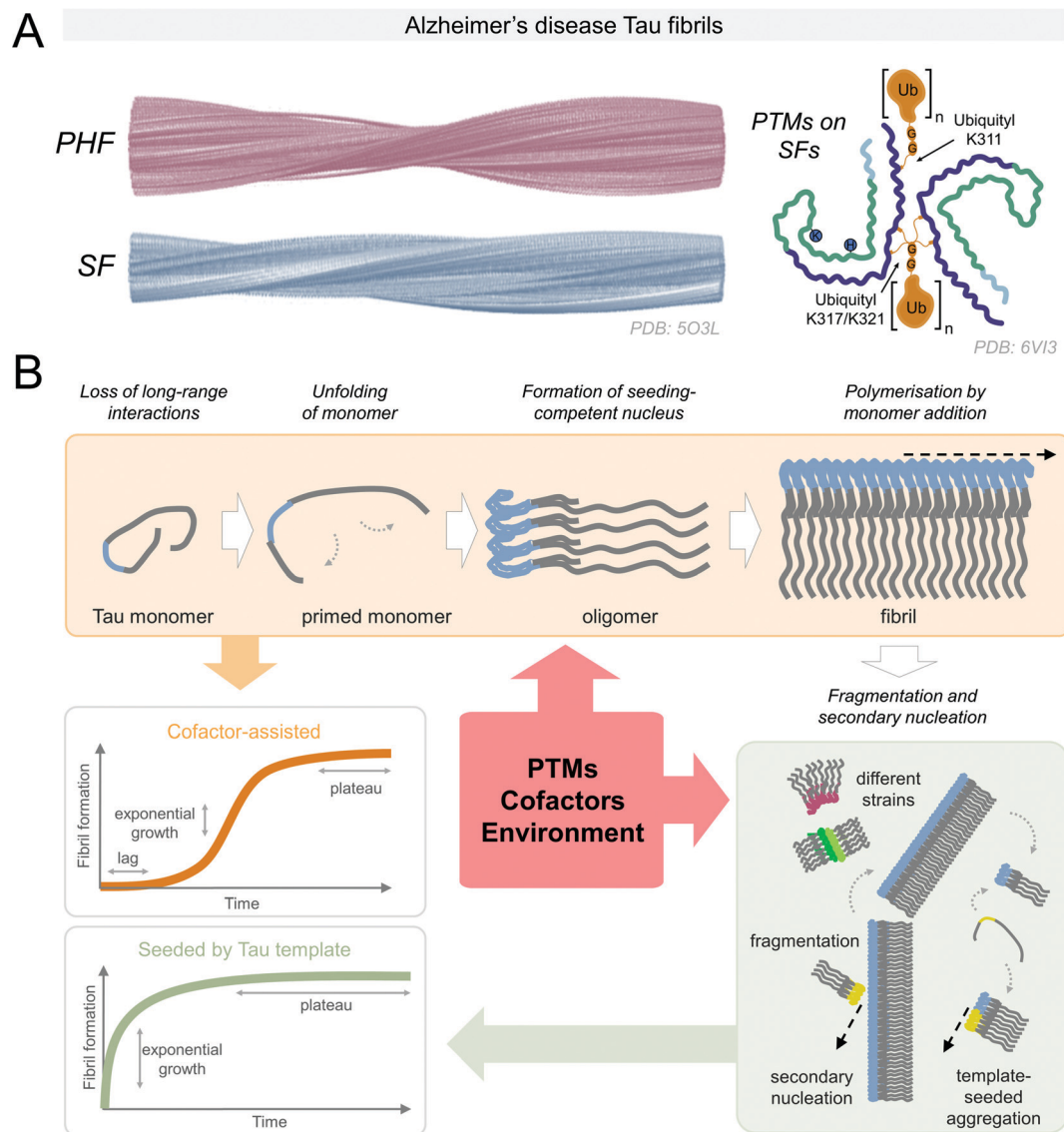
Under pathological conditions, Tau is observed in the aggregated state in the surviving cells of patients with Tauopathies (Fig. 3A). Therefore, there is considerable interest to understand the conditions and molecular processes contributing to Tau protein aggregation. In solution, all Tau isoforms are soluble and exist as an ensemble of disordered conformations, but are capable of acquiring secondary structure upon association with MTs, dimeric tubulin,<sup>144</sup> or lipid membranes.<sup>145</sup> The solubility of Tau proteins could be attributed to the high positive charge density surrounding the aggregation-prone microtubule-binding region, as well as long-range N-terminal interactions with the mid-domain and C-terminal domain. To aggregate and form  $\beta$ -sheet-rich fibrillar structures – Tau proteins must undergo conformational changes or interaction with other molecules that alter the charge distribution along the Tau molecule, leading to the formation of aggregation-competent intermediates (Fig. 3B). This can be mediated and influenced by negatively-charged cofactors, such as heparin,<sup>146</sup> changes in the environmental solvent conditions like the presence of osmolyte urea or trimethylamine N-oxide;<sup>147</sup> fluctuations of the proton density gradient pH;<sup>148</sup> the introduction of specific patterns of post-translational modifications,<sup>149,150</sup> (reviewed in ref. 151), or other yet to be identified factors.

### Biophysical concepts applied to Tau aggregation

Tau is thought to aggregate through a nucleation-dependent mechanism, surpassing the kinetic barrier by forming a seeding where monomeric Tau self-assembles to form an aggregation-competent nucleus (lag phase) that undergoes rapid elongation through templated addition of monomers to form fibrils<sup>154</sup> (see Fig. 3B). The priming of soluble semi-stable monomers existing in a paperclip conformation<sup>155</sup> into partially misfolded conformation is necessary for the formation of the seeding-competent Tau nucleus. The lag phase can be overcome upon the addition of the preformed fibrillar Tau seed or cofactor molecules.  $\Delta$ K280, P301L, V337M, and R406W Tau mutants, found in FTDP-17 patients, were found to have higher aggregation propensity into paired helical filaments than wild type Tau<sup>156</sup>. Both Tau mutations detected in familial (P364S), and sporadic (G366R) FTDP-17 reduced MT polymerization activity of Tau, however, interestingly, only the protein with mutation detected in the sporadic case had higher aggregation propensity *in vitro*.<sup>157</sup> Specific mutations associated with FTDP-17 on the sites P301 and S320 were highly dependent on the residue substitutions in their propensity to be seeded by exogenous preformed fibrils, or facilitate aggregation in cells.<sup>158</sup> Only the threonine substitution of proline 301 of 4R0N Tau resulted in an increase in the detergent-insoluble Tau upon overexpression in HEK293T cells, followed by K18 fibril seeding, whereas overexpressed mutants G303V, G304S, or S305N Tau remained in soluble fractions. Double mutants P301S/S320F and P301L/S320F, however,







**Fig. 3** (A) Alzheimer's disease derived-Tau fibrils in the paired helical filament (PHF) and straight filament (SF) structures (reproduced with modifications from ref. 152 with permission from the Macmillan Publishers Limited, part of Springer Nature, copyright 2017). SFs may be post-translationally modified (reproduced with modifications from ref. 153 with permission from the Elsevier, copyright 2020). (B) Tau aggregation is thought to start by the loss of the long-range contacts on the soluble Tau monomers in semi-stable paperclip conformation. The formation of a seeding competent nucleus is necessary for the fibrillization to proceed. Secondary nucleation events and fragmentation of fibrils contribute to further Tau fibrillization that may result in Tau aggregates in various structural conformations. PTMs, cofactors, and environmental conditions can influence Tau fibrillization. Kinetics of cofactor-assisted Tau fibrillization generally follows the S-shaped curve kinetics, seeded Tau fibrillization follows exponential curve kinetics with the loss of the lag phase.

exhibited high propensity to aggregate even without the addition of the preformed seed. These mutations likely work through destabilizing the semi-stable paperclip conformation of the Tau monomer,<sup>155</sup> leading to the formation of highly aggregation-prone intermediates, however, the exact mechanisms are unknown.

Understanding of biologically relevant Tau protein aggregation is increasingly informed by the concepts commonly used in polymer science fields. Solubility and assembly properties of monomers under supersaturated conditions define the assembly characteristics, such as labile conditions favoring stochastic nuclei formation, or metastable phase, where nuclei turn into elongated structures is favored.<sup>159,160</sup> Conversion of Tau

monomers must proceed through misfolded aggregation-competent conformations to form the aggregation-competent nuclei upon transient association. Formation of the first aggregation-competent oligomers is considered the rate-limiting step in the aggregation process. This process could be induced or promoted by mutations, cellular stress factors, *e.g.*, oxidation stress, PTMs, or interaction with aggregation promoting cofactors or changes in the cellular environment leading to disruption of the long-range intramolecular interactions that stabilize the Tau monomers. On the other hand, in "noisy" biological systems, stable Tau nuclei may also assemble into amorphous OFF-pathway aggregates, predominantly due to transient contacts of



Tau molecules of heterogeneous conformations. Structural template-guided Tau fibril polymerization can be thermodynamically unfavorable in these cases, and these types of aggregates would not result in the formation of the fibrils even with cofactor molecules acting as catalysts to overcome the energetic nucleation barrier.<sup>161</sup> This was observed for some meta-stable cofactor heparin–Tau complexes that did not polymerize, however they could be induced to form ON-pathway fibrillar structures in the presence of nucleation-competent Tau seed.<sup>162</sup> Once the surfaces of the Tau conformational structural template are established, the assembly proceeds predominantly by end-addition of Tau monomers. This further illustrates the versatility and dynamical nature of structures that Tau is capable of adopting.

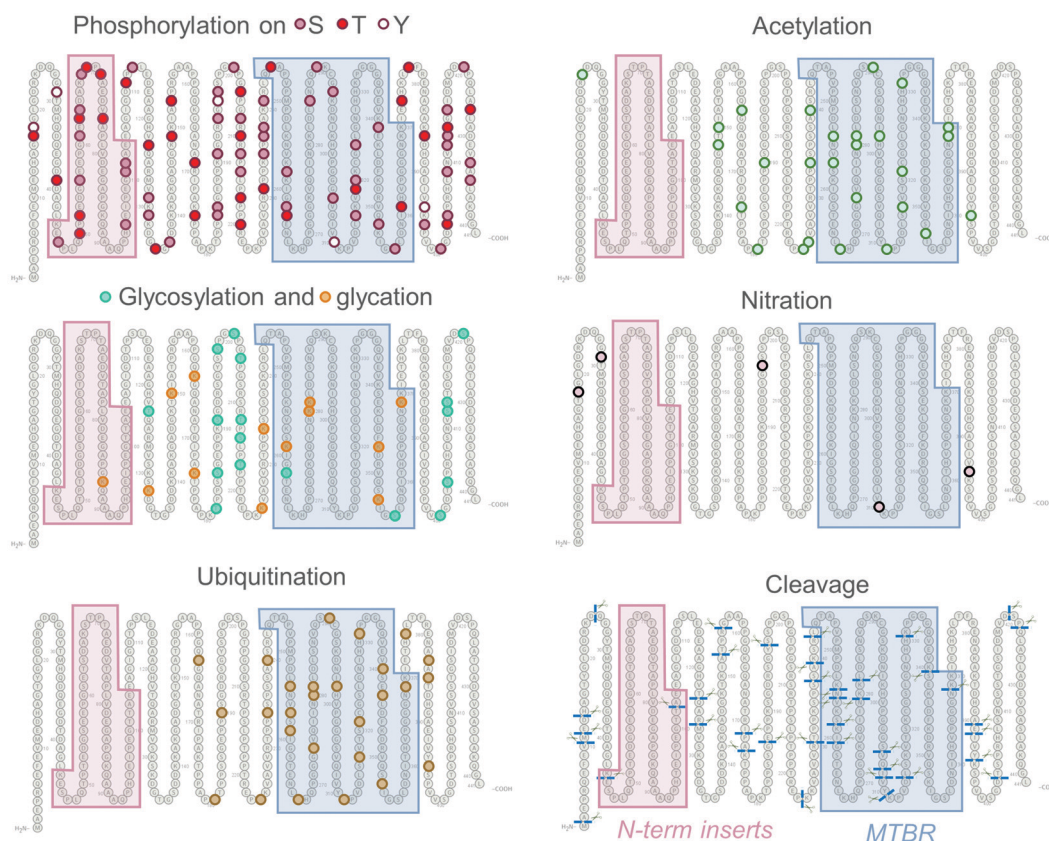
### Determinants of Tau aggregation

**PTMs of Tau.** Tau protein aggregation can be extensively modulated by the patterns of PTMs.<sup>163</sup> Tau can be phosphorylated, nitrated, acetylated, ubiquitinated, proteolytically cleaved, SUMOylated, deaminated, oxidated, glycosylated, and glycated, methylated, and demethylated on numerous residues.<sup>151,164</sup> Importantly, the patterns, co-occurrences, and interactions between PTMs may play key roles in Tau functions and the processes of aggregation.<sup>74,165–167</sup>

In a recent study, Wesseling *et al.*<sup>133</sup> provided the first unbiased qualitative and quantitative profiling of the diversity of the Tau proteoforms and how it changes during AD progression. Although this study revealed that the cumulative number of the detected PTMs was much less than predicted, it still further highlighted the complexity of the Tau PTM patterns in pathological samples with the identification of 55 phosphorylation, 17 ubiquitination, 19 acetylation, and 4 methylation sites. However, several other types of Tau PTMs, which have been detected in the brains of Tauopathy patients, were not investigated in this study, including nitration, oxidation, O-GlcNacetylation, and glycation.

The high abundance of PTMs in pathological Tau aggregates, relative to soluble Tau, led to hypotheses implicating a central role of PTMs in triggering Tau aggregation and pathology formation. The patterns of Tau PTMs evolve and change during disease progression,<sup>133</sup> however, the significance and relations to underlying pathology are not yet understood. It has become clear that the PTM occurrence patterns, cross-interactions, and spatial distribution contribute to the combinatorial Tau PTM code, functions of which are yet to be fully deciphered.<sup>168</sup>

Phosphorylation is one of the most common Tau PTMs and may occur on several of the 85 tyrosine, serine, and threonine residues throughout the longest Tau isoform sequence (Fig. 4, Phosphorylation). Physiologically, Tau phosphorylation patterns modulate Tau-MT interactions, generally



**Fig. 4** PTM patterns on Tau. Phosphorylation sites are distributed along the Tau molecule, whereas acetylation and ubiquitination sites are clustered around mid-domain and MTBR. Two cleavage sites, a single glycation site, and no acetylation, nitration, or ubiquitination sites are present in the N-terminal repeats.

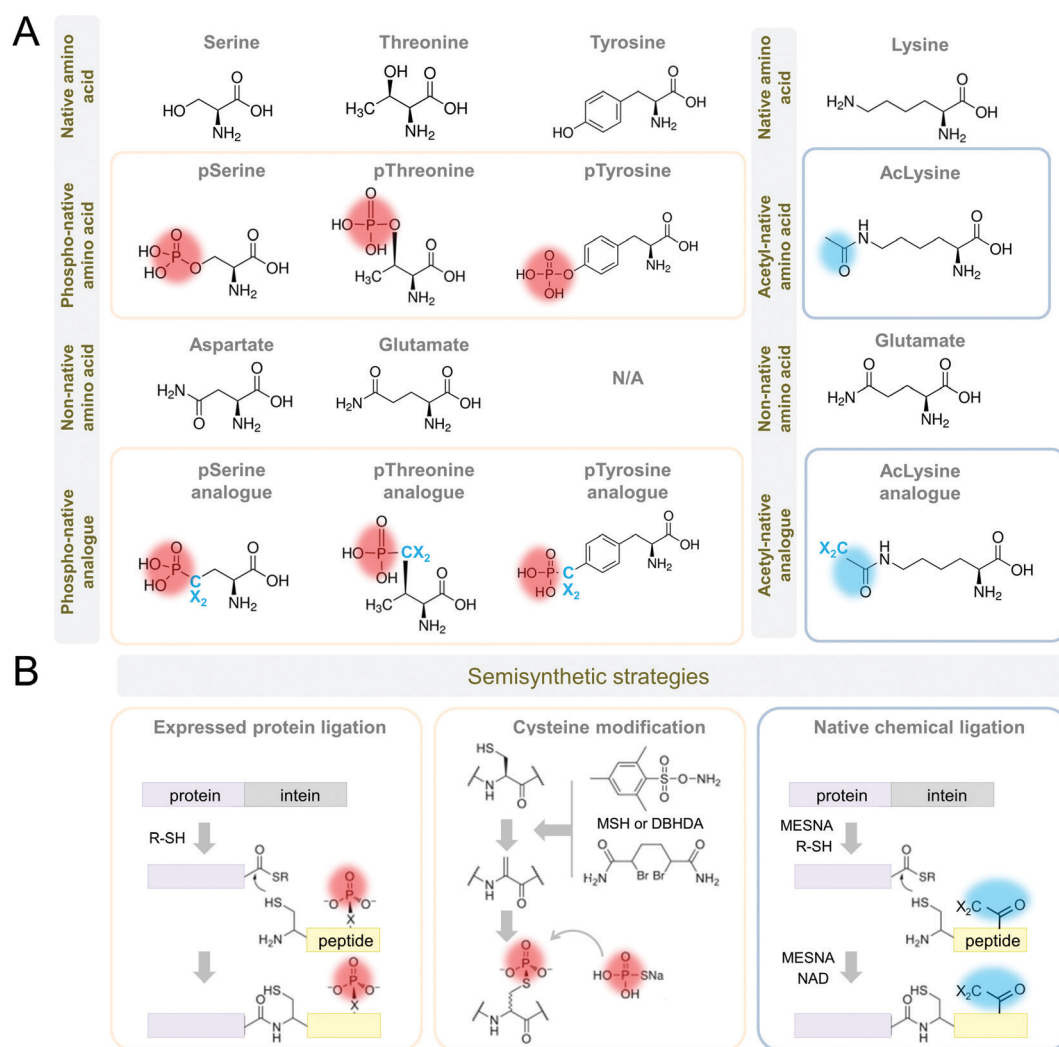




decreasing the Tau binding affinity for tubulin subunits.<sup>169–172</sup> However, specific sites, such as pT50 were found to promote tubulin assembly *in vitro* and in cells.<sup>173</sup> Phosphorylation patterns differ between Tau derived from the brain and biological fluids of healthy individuals and neurodegenerative disease patients<sup>133,167,174,175</sup> Early studies detected higher phosphorylation levels of Tau in AD patients' brains by immunohistochemistry,<sup>176,177</sup> as well as by nanoelectrospray mass spectrometry.<sup>178</sup> Immunolabelling by AT8 antibody detects Tau phosphorylated at mid-domain residues pS199/pS202/pT205/pS208,<sup>179</sup> which are present in all Tau isoforms (see Fig. 1) and is extensively used to assess and quantify the Tau pathology in humans and animal models. Phosphorylation at these sites is also used for histological Braak staging of Tau pathology,<sup>82</sup> and the diagnosis of AD in living patients. Which phosphorylation sites drive Tau aggregation and the role of

different phosphorylation sites or patterns in regulating different aspects of Tau aggregation, pathology formation, interactions with other contributing factors, and spreading in the brain remain subjects of active research and debate.<sup>180</sup> Several strategies have been used to investigate the role of phosphorylation in regulating Tau functions in health and disease. However, many of the commonly used approaches have limitations that preclude deciphering the Tau phosphorylation and PTM code.

**Phosphorylation mimics.** Pseudophosphorylation by amino acid substitution is the most widely used method to mimic the electrostatic and steric effects of a phosphate group and investigate the role of phosphorylation in regulating Tau structure and aggregation properties *in vitro* (Fig. 5A). Despite the popularity of this approach, however, it has several limitations (reviewed in ref. 181). Pseudophosphorylation requires introducing non-native amino acids into protein sequence, which could impact the Tau



**Fig. 5** Strategies for *in vitro* mimics of phosphorylation and acetylation of Tau protein. (A) Amino acid substitutions for phosphorylated serine and threonine include aspartic and glutamic acid, as well as phospho-analog residues, including phospho-tyrosine. Amino acid substitutions for acetylated lysine include glutamic acid, as well as acetyl-analog residues, including acetyl-tyrosine. (B) Semisynthetic strategies include protein ligation and cysteine modification (reproduced with modifications from ref. 181 with permission from the Elsevier Ltd, copyright 2015). Abbreviations: DBHDA: 2,5-dibromohexane diacetamide, MESNA: 2-mercaptoethanesulfonate, MSH: *O*-mesitylenesulfonylhydroxylamine, NAD: nicotinamide adenine dinucleotide, R-SH: thioester. Phospho-group is designated in red, acetyl group is designated in blue.



structure beyond the effects of the introduced charge. Also, aspartic or glutamic acids do not encompass all true physical steric and charge attributes of the phosphate group. Previous studies from our lab and others have shown that phosphomimetics do not reproduce all aspects of phosphorylation<sup>182,183</sup> or the cross-talk between phosphorylation and other PTMs.<sup>184,185</sup> In addition, phosphorylation is a dynamic PTM, and phosphomimetic approaches are generally irreversible.

One alternative and commonly used approach to phosphorylate Tau involves the use of kinases that have been shown to phosphorylate Tau *in vitro* or in cells. However, the enzymatic phosphorylation suffers from a lack of specificity or differential affinity of kinases for specific residues. Almost all the kinases that phosphorylate Tau do so at multiple residues. This can lead to variable rates of phospho-group addition and result in samples containing a mixture of differentially phosphorylated proteins. Furthermore, our knowledge of Tau kinases is still limited, and not all desired residues or their combinations may be efficiently phosphorylated *in vitro*. Previously, the lack of knowledge about the enzymes involved in regulating phosphorylation at specific sites precluded studies from assessing whether phosphomimetics accurately reproduce the effect of phosphorylation. However, recent advances in protein synthesis of Tau and other proteins now make this possible, therefore providing means for assessing the suitability of using phosphomimetics to investigate phosphorylation *in vivo*.

Alternative approaches for precise and clean *in vitro* Tau phosphorylation include combinations of enzymatic, synthetic, and semisynthetic strategies (Fig. 5B)<sup>186,187</sup> that were successfully adapted to Tau.<sup>150,188–193</sup> These strategies provide homogeneous site-specifically modified proteins, including phosphorylations, nitrations, and acetylations, in large quantities for experimental application *in vitro*, *in vivo*, and for drug screening and development. In addition to paving the way for deciphering the Tau PTM code with great precision, these approaches will allow reproducing the complexity of Tau species as they exist *in vivo*. This is essential for developing accurate assays to quantify and monitor changes in total Tau or specific modified Tau species.

**The complicated relationship of Tau phosphorylation and aggregation.** The predominant focus of investigations of Tau isoforms' phosphorylations has been on the modulation of their aggregation propensity, which depends heavily on the spatial distribution of phospho-groups along the Tau molecule. Early studies showed that enzymatic phosphorylated (non-specific phosphorylation) Tau readily formed AD-like fibrils *in vitro*.<sup>194,195</sup> Hyperphosphorylated Tau derived from AD brain cytosolic fraction fibrillized more efficiently *in vitro* compared to non-phosphorylated protein.<sup>196</sup> *In vitro*, full-length Tau readily formed fibrils upon kinase-mediated hyperphosphorylation of mid-domain residues pS202, pT205, pS208, but not S262.<sup>197</sup> In another study,<sup>172</sup> phosphorylation of mid-domain region residues pT181, pS199, pS202, pT205, pT212, pS214, pT217, pT231, or pS262 only moderately increased Tau aggregation propensity. However, phosphorylation reduced tubulin binding and MT-assembly in a phosphosite-specific manner.

The C-terminal domain appears to play a crucial role in modulating Tau aggregation propensity. Mimicking of phosphorylation at residues S396 and S404 by mutating serines to glutamines was found to increase Tau aggregation.<sup>198</sup> A subsequent study by Haase *et al.*<sup>199</sup> using a pseudophosphorylation mutation strategy of selected serine or threonine residues to aspartic or glutamic acids found that N-terminally positioned mutations inhibited, and C-terminally positioned mutations promoted Tau aggregation, particularly the pseudophosphorylation of residue 422. The enzymatic phosphorylation of C-terminal residues pS396, pS404, pS409 or pS422 alone, or combined with the mid-domain residues greatly enhanced Tau aggregation *in vitro*.<sup>172</sup> In addition, FTDP-17 Tau mutations G272V, P301L, V337M, and R406W were found to increase the rate of Tau fibrillization *in vitro* when the C-terminal residues S396, S400, T403, and S404 were phosphorylated, compared to non-mutant protein, that also fibrillized when phosphorylated at these sites.<sup>171</sup> Taken together, additions of negative-charge bulky phospho-group before aggregation onset to N-terminal domain or MTBR tend to decrease Tau aggregation; phosphorylations of C-terminal residues tend to increase Tau aggregation; whereas mid-domain phosphorylations tend to be highly context-dependent concerning the modulation of Tau aggregation.

Conversely, phosphorylation at S262 was previously associated with the inhibition of full-length<sup>200</sup> or truncated Tau aggregation.<sup>150</sup> Also, in a Tau overexpression cell system, the phosphorylation pattern corresponding to AT8 antibody epitope did not result in the formation of high molecular weight Tau species, or observable PHF-like fibrils, even at high amounts of phosphorylated Tau reaching up to 8% of total cellular proteins.<sup>201</sup> Similarly, using eukaryotic Sf9 cellular Tau overexpression system coupled to native mass spectrometry of phosphorylated Tau in these cells, Drepper *et al.* found that even the highly-phosphorylated Tau fractions did not show self-assembly into the fibrils.<sup>202</sup> In addition, recent work from our group showed that phosphorylation of all five tyrosine residues, three N-terminal residues Y18, Y29, and Y197, or a single tyrosine residue Y310 in the MTBR greatly reduced Tau aggregation.<sup>149</sup>

These observations suggest that the pattern of phosphorylation is a key determinant of Tau structure and aggregation. However, a more systematic investigation of the role of Tau phosphorylation using strategies that enable homogeneous and site-specific phosphorylation or hyperphosphorylation is essential for elucidating the role of phosphorylation in the pathogenesis of Tauopathies.

**Glycosylation.** The non-canonical form of glycosylation is O-linked-N-acetylglucosaminylation (O-GlcNAcylation), which is added to protein residues by enzyme O-GlcNAc transferase (OGT), and removed by O-GlcNAcase (OGA). Early studies reported high levels of Tau glycosylation of Tau PHFs, but not normal Tau, in AD patient brains.<sup>203,204</sup> The levels of O-GlcNAcylation and OGT were lower in AD patients,<sup>205–207</sup> which may be linked to the impairment of metabolic processes related to glycans. The knockout of OGT was further shown to increase Tau phosphorylation, aggregation and induce neuro-degeneration in mice.<sup>207</sup>



Interestingly, deglycosylation of PHFs *in vitro* promoted their conversion into straight filaments (SFs),<sup>204</sup> illustrating the profound influence of PTMs on the structure, dynamics, and morphology of the Tau fibrils. Tau can be O- and N-glycosylated on asparagine, serine, and threonine residues,<sup>208</sup> thus competing with phosphorylation of the same serine and threonine sites (Fig. 4, Glycosylation).<sup>209</sup> Several sites modified by OGT were identified *in vitro* and included T123, S400, S409, S412, S413.<sup>210,211</sup>

An inverse correlation between Tau phosphorylation and O-GlcNAcylation levels<sup>212,213</sup> was found in AD patients' brains, implicating glucose metabolism impairment as a contributing factor to AD progression and pathology.<sup>214</sup> O-GlcNAcylation of Tau peptides at S400 was found to inhibit phosphorylation at the neighboring S396 and S404 sites.<sup>215</sup> In cells, different subsets of the overexpressed Tau molecules were found to carry O-GlcNAcylation and phosphorylation, suggesting a function-related balance between these PTMs.<sup>216</sup> In a HEK293 Tau-BiFC cellular system, inhibition of OGT resulted in increased Tau phosphorylation at S199 and S396.<sup>217</sup>

On the other hand, NMR-based studies showed that phosphorylation was not blocked by O-GlcNAcylation.<sup>211</sup> Furthermore, more recent work using antemortem AD patient CSF samples found increased levels of total N-glycans, which correlated with an increase of total and phosphorylated Tau levels, especially in patients with subjective cognitive impairment, an early stage in AD pathology development.<sup>218</sup> This suggests that any offset of Tau phosphorylation by Tau glycosylation may have a negligible effect on the total levels of phosphorylated Tau detected in CSF.

It has been proposed that O-linked Tau glycosylation reduces Tau aggregation through the prevention of its phosphorylation on the same residues,<sup>212,219–221</sup> pointing to the importance of the temporal sequence of Tau modifications. The deglycosylation of already hyperphosphorylated Tau derived from the cytosolic fraction of AD patients' brains, failed to induce its fibrillization.<sup>196</sup> Tau phosphorylation on the PHF-1 antibody epitope, which includes residues pS396/pS404, and spans over O-GlcNAc S400 residue was found to enhance Tau aggregation, whereas glycosylation of residues S400, S412, and S413 slowed Tau aggregation rate.<sup>222</sup> N-linked glycosylation may also contribute to the structural integrity of the AD-derived PHFs, as evidenced by the fact that deglycosylation by F/N-glycosidase F resulted in untwisting of the fibrils into thin filaments.<sup>223</sup> In addition, N-glycosylated Tau had a lower aggregation propensity and showed shorter and thinner fibrils than deglycosylated Tau.<sup>224</sup> Despite this, the impact of increased Tau O-GlcNAcylation may only be prominent in the physiological systems, and it may involve or require complex interactions with other Tau PTMs and/or cofactors. HEK293 Tau-BiFC cell-based studies showed that Tau aggregation was decreased by inhibition of OGA, and increased by inhibition of OGT (Lim, Haque *et al.* 2015), suggesting that this particular Tau PTM must be studied within the complex cellular context.

**Glycation.** Glycation is a non-enzymatic spontaneous reaction between free amino groups of molecules, such as proteins, with free reducing sugars leading to the formation of Amadori

products, precursors to AGEs, and reactive oxygen species.<sup>225</sup> As opposed to functional glycosylation PTMs, glycation is irreversible, and glycated proteins tend to become defective and lose their functionality.<sup>226</sup> AGEs were found colocalized with Tau PHFs in NFTs in AD brain,<sup>227–229</sup> and are associated with AD pathology.<sup>228,230</sup> AGEs may impact neuronal cell survival through increases in Tau phosphorylation and its dissociation from MTs.<sup>231</sup> Glycation has not been reported to induce fibrillization of Tau *in vitro*,<sup>232</sup> but may contribute to stabilization and accrual of the bundles of Tau fibrils.<sup>233,234</sup> Another work found that glycation preceded and promoted phosphorylation of all six Tau isoforms, indirectly leading to enhanced aggregation,<sup>234</sup> suggesting that Tau glycation may be an early PTM signifying incipient pathology.

**Acetylation.** Multiple lysine residues in the Tau sequence can be acetylated and are predominantly located around the proline-rich and MTB regions (reviewed in ref. 235) (Fig. 4 Acetylation). Tau acetylation at K280 (AcK280) was immunohistochemically detected in brain tissue of AD, CBD, and PSP patients, mirroring the Tau hyperphosphorylation staining pattern.<sup>236</sup> In the recent study by Wesseling *et al.*, high acetylation frequency was detected at the residues K535, K369, K370, and K375 located in the MTBR repeat R4, the region outside the fibrillar core fold.<sup>133</sup>

Acetylation at K280 has been reported to be associated with the early stages of AD. It is thought to occur in CTE patients' brains before Tau hyperphosphorylation, suggesting that Tau acetylation might be an early marker of incipient Tau pathology.<sup>237</sup> Acetylation at K174 was also detected at early stages in AD patients by mass spectroscopy,<sup>238</sup> whereas AcK274 and AcK281 were found at the later stages of the AD progression.<sup>239</sup> *In vivo* rodent experiments showed that AcK174, AcK274, and AcK281 disrupted synaptic homeostasis, suggesting that Tau acetylation on specific residues leads to hippocampus-associated memory loss at the early stages of dementia.<sup>240</sup> Contrary, Choi *et al.*<sup>241</sup> used AD patient-derived organoids and the rodent model of AD to show that the inhibitor of histone deacetylase 6, which was found to deacetylate Tau and modulate its clearance,<sup>242</sup> reduced the synaptic defects and pathology associated with Tau. In addition, acetylation of potential and canonical degron motifs, including the Tau sequence KFERQ, may be required for enhanced protein recognition and degradation by autophagy systems.<sup>243</sup> This suggests that at least some residues and patterns of Tau acetylation may be protective. Acetylation may have cross-talk with other PTMs, such as phosphorylation. When acetylated on KXGS motifs that modulate Tau binding to MTs, the phosphorylation of these sites on Tau was reduced, and Tau MT-binding capacity was preserved, thus preventing Tau detachment and downstream aggregation.<sup>244</sup> Acetylation modifies lysine residues, which can also be ubiquitinated, methylated, glycated and SUMOylated. This introduces the competition between modifications, which in case of decreased ubiquitination due to acetylation resulted in a decrease of Tau turnover.<sup>245</sup>

*In vitro* investigations of Tau acetylation at lysine residues predominantly focused on its connection to Tau aggregation.





Furthermore, previous work predominantly utilized pseudoacetylation by lysine-to-glutamic acid substitution, which may not fully represent the effects of acetylation, or *in vitro* enzymatic acetylation, which may result in a non-specific and differentially-modified mixture of proteins.<sup>188,246</sup> Acetylation-mimic of residues K280 and K281 was found to promote Tau fibrillization<sup>247,248</sup> (see Fig. 5A). On the other hand, singular acetylation or mimic of K321, or in combination with other lysines, including K280 and K281, was found to decrease Tau fibrillization.<sup>244,249,250</sup> Using precise site-specific acetylation of K280 through protein semisynthesis (Fig. 5B) to obtain homogeneously-acetylated protein,<sup>188</sup> our group showed that AcK280 greatly enhanced the rate of Tau fibrillization and promoted the formation of short fibrils.

Overall, Tau acetylation may be a promising PTM marker of incipient Tau pathology. However, the underlying mechanisms linking it to Tau aggregation and pathology formation and spreading must be investigated further.<sup>244,245</sup>

**Ubiquitination.** One of the most elusive Tau PTMs is ubiquitination despite being one of the earliest identified Tau PTMs linked to pathological Tau aggregates in the brain. Under normal conditions, Tau was shown to be ubiquitinated and proteolytically degraded by ubiquitin-proteasome system.<sup>251</sup> However, under pathological conditions ubiquitinated Tau was detected in PHFs following the occurrence of PTMs such as glycosylation and phosphorylation.<sup>125,252–254</sup> Tau with high levels of ubiquitination was detected in AD patients' CSF.<sup>223,255</sup> In AD patients, Tau was singly<sup>256</sup> or multiply ubiquitinated.<sup>257</sup> Differential patterns of Tau ubiquitination were detected in AD patient samples according to the stage of disease progression,<sup>133</sup> and electron densities on AD brain-derived Tau fibrils detected by cryo-EM and cross-referenced by mass spectrometry were attributed to ubiquitin groups<sup>153</sup> (Fig. 3A).

The roles of ubiquitination in Tau fibril formation are still unclear. Ubiquitination is a complex PTM that remains understudied because of the lack of knowledge of the enzymes that regulate Tau ubiquitination in this domain. Tau is known to be ubiquitinated by E3 ligases such as axotrophin/MARCH7,<sup>258</sup> C-terminus of the Hsc70-interacting protein (CHIP),<sup>259</sup> and TNF receptor-associated factor 6 (TRAF6), which was also found to colocalize with sequestosome/p62 in the Alzheimer's brain-derived Tau aggregates.<sup>260</sup> The polyubiquitination of Alzheimer's brain-derived Tau by CHIP-Hsc70 complex was dependent on its hyperphosphorylated state only in the presence of E2 ligase Hbch5B,<sup>261</sup> indicating the process specificity and interplay between PTMs. Thus far, only one deubiquitinating Tau enzyme OTUB1 has been identified in mice.<sup>262</sup> Further investigations must focus on at which stages of Tau aggregation and at what Tau sites ubiquitination occurs, and how it impacts fibril structure and clearance. This can be achieved using precision protein semisynthesis strategies successfully applied to study impacts of singular ubiquitination, as well as the impact of multiple additions of ubiquityl groups on  $\alpha$ -synuclein aggregation properties.<sup>263,264</sup>

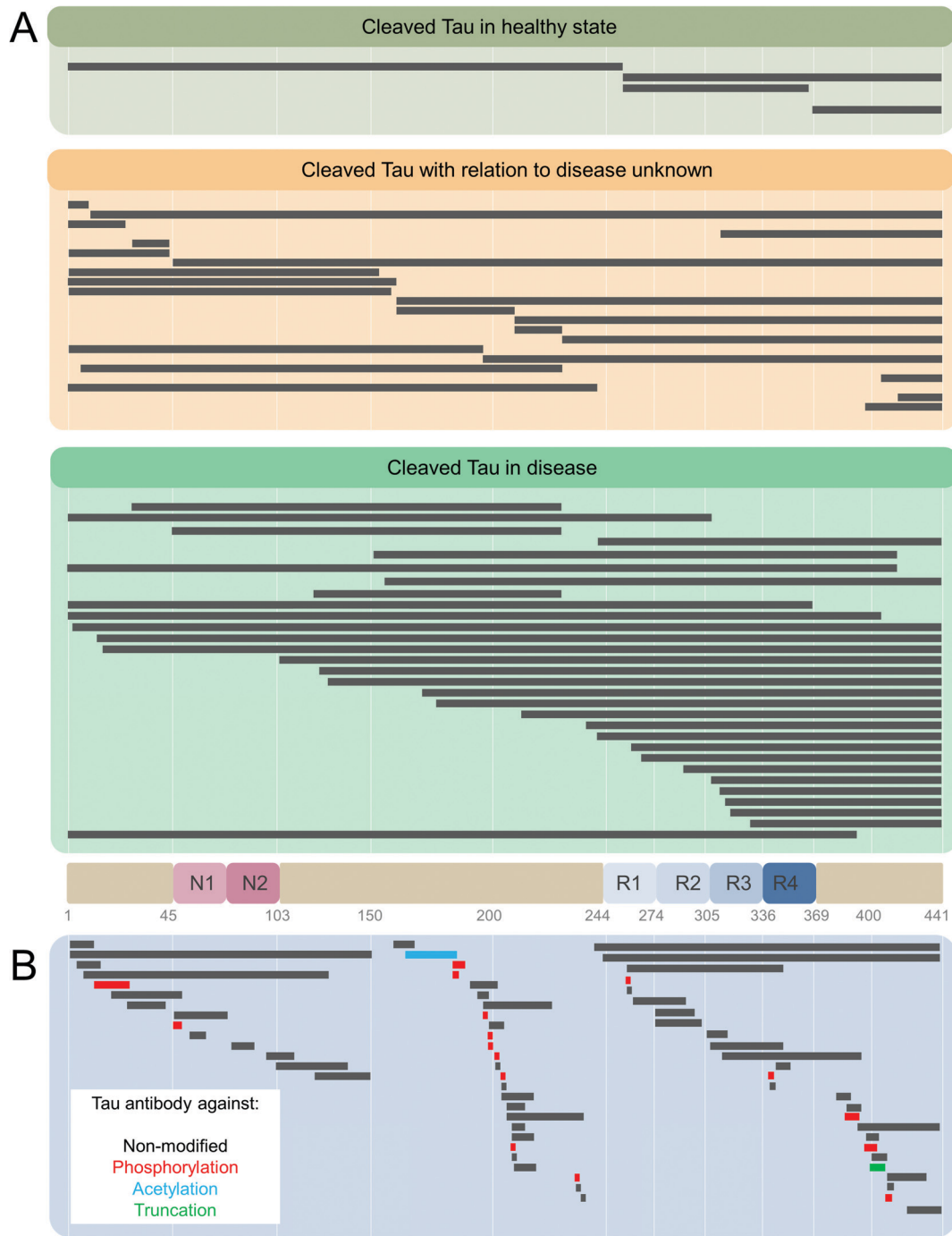
**Tau cleavage.** Another prominent modification of Tau is the proteolytic cleavage of the protein, which occurs at multiple

sites along the protein sequence (Fig. 4, Cleavage, Fig. 6A). Several lines of evidence suggest that the generation of Tau fragments of different lengths plays an important role in driving Tau pathology formation and the pathogenesis of AD and other Tauopathies. Several Tau fragments have been detected in the brain and CSF both in healthy individuals and in neurodegenerative disease patients, including AD,<sup>265–269</sup> PSP,<sup>270</sup> CBD,<sup>271</sup> and in traumatic brain injury (TBI),<sup>272</sup> reviewed in ref. 131 (Fig. 4A). Tau 1–402 was used as an early CSF biomarker for early neurodegeneration processes accompanying AD.<sup>266</sup> A recent study,<sup>273</sup> using brain microdialysis, and biochemical and cellular assays of interstitial brain fluid in mice to detect secreted Tau, demonstrated that Tau was predominantly present in truncated forms at different stages of pathology progression, as well as across brain regions.

Tau fragments resulting from truncations at positions D13,<sup>274</sup> E391, and D421<sup>275,276</sup> were reported to promote Tau aggregation, accumulate in AD brains, and their levels correlated with the progression of disease.<sup>274</sup> 46 kDa fragment was detected using mass spectrometry and primary amino acid sequencing in AD and was determined to be a result of caspase-cleaved Tau at D421.<sup>277</sup> Multiple Tau truncations were also found in PHFs,<sup>278</sup> illustrating the heterogeneity of Tau forms present within the Tau aggregates found in the brain. Truncated Tau fragments were found in NFTs in AD (4R + 3R Tau),<sup>10,279–282</sup> Pick's disease (PiD) (3R Tau),<sup>283</sup> PSP (4R Tau),<sup>284</sup> and other neurodegenerative disorders.<sup>285</sup>

Several studies suggest that proteolytic Tau fragments may contribute directly to Tau neurotoxicity and the initiation of Tau pathology. The Tau 45–230 (~17 kDa) fragment produced by proteolytic cleavage by calpain-1 and thrombin between residues 45 and 230, lead to functional impairments in synapses and axonal transport in cells, as well as behavioral deficits in animals.<sup>286–288</sup> The Tau 26–230 (20–22 kDa) fragment could seed full-length Tau aggregation *in vitro*.<sup>289</sup> In cells, it was enriched in mitochondria and impaired its activity,<sup>290</sup> and induced cell death in differentiated human SH-SY5Y cell line,<sup>291</sup> and primary neurons.<sup>292</sup> A 24 kDa C-terminal Tau fragment produced by calpain 1 cleavage between residues R242 and L243 showed higher aggregation capacity and lower affinity for MTs *in vitro*.<sup>293</sup> In the SH-SY5Y cell line, it showed enhanced propagation and seeding of Tau in the neighboring cells.<sup>294</sup> Another 35 kDa Tau fragment, likely generated by cleavage between residues in the region of 182 and 194, and is present in 4R Tauopathies such as PSP and CBD.<sup>284</sup> Further investigations in the Chinese hamster ovary cell line showed that overexpression of Tau 35 kDa led to its hyperphosphorylation, lower MT-binding affinity, induction of unfolded protein stress response, and aberrant insulin signaling.<sup>295</sup> Tau truncations produced by removing from 12 to 121 C-terminal residues were also found to increase Tau aggregation.<sup>198</sup> Finally, it has been widely known that truncated Tau fragment K18 has a higher propensity to aggregate compared to full-length Tau. These findings suggest that some of the Tau fragments are capable of acting as seeds that could initiate the aggregation of full-length Tau proteins.





**Fig. 6** (A) Enzymatic cleavage-generated Tau truncation products identified in healthy and Taupathy patients.<sup>131</sup> (B) Anti-Tau antibody list from www.alzforum.com database. Alzforum lists a total of 572 Tau antibodies, 45 laboratory-produced, and 527 commercial. 534 are human-reactive, 360 with listed epitopes identified, with unique sites mapped in the figure. Where epitope is unknown, antibodies are raised against full-length,<sup>6</sup> C-terminus,<sup>18</sup> and N-terminus.<sup>14</sup> The break in the Tau sequence coverage is at amino acids 150–159 in the proline-rich domain.

Some Tau truncations resulted in subcellular mislocalization or misdirection of Tau thus impairing its normal functions. The 4R isoform of the Tau fragment 151–391 was displaced from axonal to nuclear compartment in human neuroblastoma cells and primary rat neuronal cultures.<sup>289</sup> Tau fragment 1–314 was found to detach from MTs and promote loss of hippocampal neurons concurrent with induction of mislocalization of

full-length Tau to the somatodendritic compartment and synaptic impairment.<sup>296</sup>

Other<sup>292</sup> Tau fragments 1–255 and 369–441 showed impaired MT-binding and polymerization, whereas fragments 1–368, 256–441, and 256–368, in addition, had higher aggregation propensities as compared to full-length Tau.<sup>131,297</sup> N-terminal fragment 1–13 resulted in degeneration of the axonal structures,



whereas its counterpart Tau fragment 14–441 contributed to the formation of Tau aggregates.<sup>298,299</sup>

Other fragments<sup>290,296,298,299</sup> seem to have no associated toxicity in cellular models, such as Tau 1–25<sup>291</sup> and 125–230,<sup>131</sup> however, the functional or pathogenic roles of the most known enzyme-cleaved and pathology-associated Tau fragments remain understudies. These include Tau fragments 1–242,<sup>293</sup> 45–441,<sup>286</sup> 258–372,<sup>300</sup> 315–441,<sup>296</sup> 403–441,<sup>266</sup> 1–152, 1–197, 3–124, 3–230, 156–209, 156–441, 198–441, 210–230, 210–441, 231–441 and 422–441.<sup>131</sup> Equally unclear are the functional or protective roles, if any, of some Tau fragments. Interestingly, some truncated species may enhance proteolytic and autophagic degradation of Tau and its turnover through specific interactions with co-chaperones and ubiquitin ligases.<sup>301</sup>

Whether Tau cleavages found in pathological aggregates occur before, during, or after the onset of Tau aggregate formation<sup>279,302</sup> needs further investigation. Some studies have reported that Tau hyperphosphorylation preceded Tau cleavage in cell cultures and human AD brain,<sup>283,303,304</sup> whereas others reported that NFT formation followed the Tau cleavage events.<sup>305</sup> Tau cleavage was also reported to precede Tau ubiquitination<sup>256</sup> and glycation<sup>254</sup> events in AD. Early and late NFT formation-specific markers were associated with Tau cleavage by caspase-3, -6, -7, and -9 in the AD brain and correlated with the scores of cognitive decline.<sup>274,306–310</sup> Tau cleavage by calpains<sup>311,312</sup> was detected in AD and FTPD.<sup>313</sup> Despite these findings, multiple truncated Tau species were also detected in normal human hippocampal, cerebellar, entorhinal, prefrontal, and motor cortical brain regions across the age range of 18 to 104 years old,<sup>314</sup> functions and significance of which alone, or in combination with other Tau PTMs, are yet to be determined (see Fig. 6A).

Taken together, these observations suggest that cleavage of Tau plays important roles in Tau aggregation, AD pathogenesis, and possibly in the disease progression and severity.<sup>309,315</sup> This also underscores the critical importance of mapping the Tau cleavage patterns in health and disease. They also suggest that Tau fragments play important roles in the pathogenesis of AD and other Tauopathies. Finally, the abundance of Tau fragments underscores the importance of reassessing their physiologic and pathogenic roles and the extent to which assays used to quantify total Tau can capture these various fragments. These studies should consider all six Tau parent isoforms, which could be cleaved at equivalent sequence positions but produce fragments of varying lengths and sizes.

### Cross-talk between Tau PTMs and impacts on aggregation

Increasing evidence suggests that the Tau PTM code is a combinatorial code that involves the co-occurrence of and cross-interactions between multiple PTMs depending on the type of neuropathology and the stage of its progression. Tau PTMs were found to cluster in specific regions of the protein in the brain samples of AD patients.<sup>133</sup> The cross talks between Tau PTMs were and are still being investigated in the context of Tau phosphorylation, primarily due to the unavailability and poor characterization of antibodies for other PTMs.

For example, Tau cleavage and Tau dephosphorylation co-occurrences have been observed in hippocampal, cortical, and cerebellar granule neurons.<sup>288,316,317</sup> This could link the decreased cleavage of Tau hyperphosphorylated at the specific sites to impaired degradation of Tau. This may be followed by the accumulation and subsequent aggregation of Tau in neuronal and glial cells, leading to degeneration. Short Tau fragments containing MBD sequences such as the PHF6 motif were found to aggregate *in vitro* without the addition of cofactors,<sup>318,319</sup> whereas PTMs in this region, such as pY310, were found to reduce the fibrillization propensity of truncated K18 or full-length Tau.<sup>149</sup> Furthermore, Guillozet-Bongaarts *et al.*<sup>304</sup> reported that dephosphorylation of residue S422 was necessary for caspase-3 mediated cleavage at D421 *in vitro*, emphasizing the importance of cross-talk between Tau PTMs. A recent report<sup>269</sup> using Tau proteins truncated at various positions expressed in HEK-293T cells showed that Tau truncation after the first 50 amino acids reduced its phosphorylation at T205 and T231. Truncation after amino acid 150 enhanced its phosphorylation at T205, S212, S214, T217, T231, and S235 irrespective of the presence of the C-terminus. Truncation after amino acid 231 increased its phosphorylation at S262, S396, and S404.<sup>269</sup>

Cross-talks between other PTMs were also observed, for example between the lysine-targeting PTMs such as acetylation, methylation, glycation, SUMOylation, and ubiquitination (reviewed in ref. 235). Hyperphosphorylation was associated with hypoacetylation – less acetyl PTMs – of the four KXGS motifs present in MTBR, which increased Tau aggregation propensity.<sup>242</sup> AD patients' brain-derived co-phosphorylated and ubiquitinated Tau peptides containing KXGS sequences were enriched compared to control-derived samples.<sup>255</sup> In cellular system,<sup>320</sup> Tau phosphorylation was increased by SUMOylation of K340. This was correlated with the decrease in Tau ubiquitination and degradation, resulting in enhanced levels of insoluble Tau. These findings suggest that Tau aggregation may be modulated by the synergistic effects of the multiple PTMs.

O-GlcNAcylation has also been shown to influence the site-specific phosphorylation pattern of Tau *in vitro* and in a cellular system.<sup>212</sup> The crosstalk between Tau phosphorylation and O-GlcNAcylation was confirmed in cells, where the lower levels of Tau phosphorylation correlated with increased glycosylation and higher nuclear localization of Tau.<sup>216</sup> Interestingly, an NMR study by Bourrée *et al.*<sup>211</sup> found that O-GlcNAcylation did not impact Tau phosphorylation by the rat brain extract or mitogen-activated protein kinase 1 enzyme. However, phosphorylation slightly increased Tau O-GlcNAcylation by the O-linked N-acetylglucosamine transferase enzyme through indirect cross-talk mechanisms which remain to be fully understood.

Spatially, phosphorylation events seem to accrue in the N-terminal and proline-rich Tau region during the Tau NFT aggregate formation and maturation, whereas acetylated and non-ubiquitinated regions seem to dominate in the MTBR overtime.<sup>133</sup> These studies further underscore the complexity





of Tau PTMs' cross-talks in the modulation of Tau isoforms' normal functions, its aggregation propensities, and remodeling of the fibrils.

A complex interplay of multiple Tau PTMs in a tightly regulated spatiotemporal manner is likely to occur during the various stages of Tau aggregation and pathology formation.<sup>167</sup> The phosphorylation patterns of Tau oligomers were heterogeneous among AD patients and correlated with the severity of the disease progression in biophysical, biochemical, cell- and animal-based functional assays.<sup>134</sup> Further investigations of the Tau oligomer PTM landscapes are needed to understand their functional significance *in vivo* (reviewed in ref. 321 and 322).

#### Advantages and limitations of the current approaches to investigate the Tau PTM code

**Mass spectrometry-based techniques and experimental design.** Mass spectrometry-based proteomic approaches are increasingly used to capture the heterogeneity and co-occurrence of Tau PTMs. The high sensitivity and specificity of mass-spectrometry offer many advantages, but deciphering the PTM code of Tau using this technique requires careful experimental design. For example, recent work by Wesseling *et al.*<sup>133</sup> successfully used mass spectrometry to determine the PTM profiles in patient cohorts. However, the frequency and evolution of the PTM profiles within individual patients over time were impossible to establish due to the cross-sectional data collection. Another major gap in our knowledge of the Tau PTM code includes the lack of comprehensive profiling of Tau PTM co-occurrences in healthy young and aged subjects without dementia, which could inform us of their normal functions. As a statistical aggregate, the PTM occurrences and their relative frequencies are increased as the pathology progresses. The fine stratifications of patients are necessary to mitigate high inter-patient variability and enable us to delineate specific Tau PTM patterns in the different regions of the molecule. Furthermore, the current methods of enzymatic digestions and mapping of PTMs do not allow to determine the co-occurrence of PTMs on the same Tau molecule, and therefore their interplays and functional significances. Future studies should be designed to allow the longitudinal profiling of Tau PTM patterns' evolution, determination of PTM patterns on the same Tau molecule, and PTM maps of the different soluble, oligomeric and fibrillar Tau species.<sup>168</sup>

Similarly, most studies attempting to map and identify the naturally occurring Tau cleavage products in biofluids<sup>323</sup> rely on mass spectrometry-based experimental approaches<sup>324</sup> often in combination with immuno-enrichment of Tau species using antibodies, followed by the enzymatic digestion, labeling, and mass spectrometry analyses of fragments. Several antibodies, which cover virtually the whole sequence of the Tau molecule (www.alzforum.org) have been produced (Fig. 6B). However, the most often utilized Tau enrichment antibodies tend to target the proline-rich middle domain,<sup>103–188,194–246,325–327</sup> present in all six Tau isoforms, and peptides from which tend to be overrepresented in mass spectrometry hits.<sup>328,329</sup> Previously, N-terminally truncated Tau fragments found in AD samples by

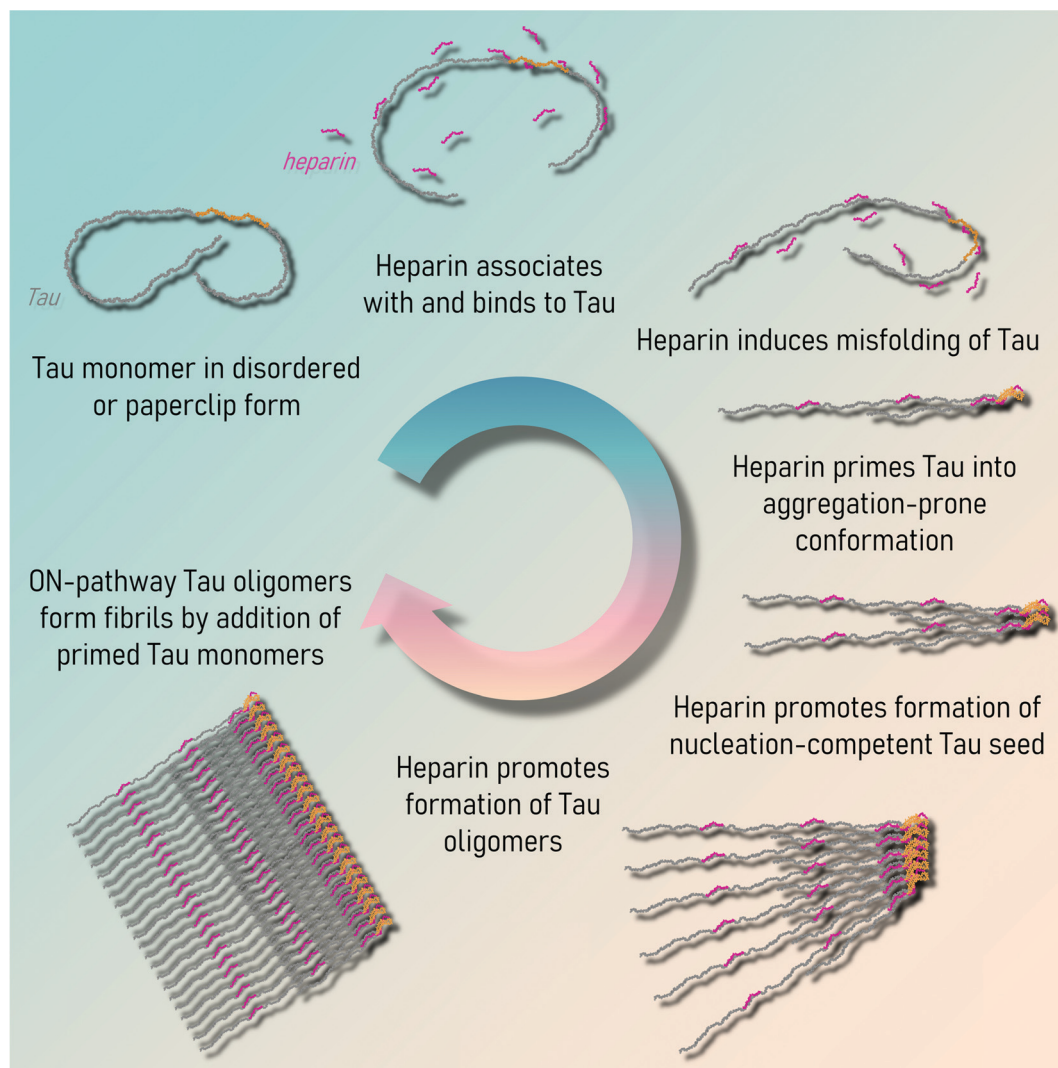
Derisbourg *et al.*<sup>330</sup> were identified by immuno-enrichment using Tau5 antibody against Tau amino acids 218–225. However, the possible range of the Tau species devoid of this region, such as nucleation-competent and aggregation-prone MTBR, or conformations where this region is inaccessible was not captured. Similarly, Portelius *et al.*<sup>331</sup> identified 19 tryptic Tau fragments in AD patients' CSF by nanoflow LC-ESI mass spectrometry using immunoprecipitation with antibodies BT2 (epitope 194–198), HT7,<sup>158–162</sup> AT120 (against phosphothreonine-181), or AT270 (phospho-dependent epitope 176–182), thus limiting the detection to Tau species containing the middle domain, whereas Tau is known to undergo extensive N-terminal<sup>271,284,291,332</sup> and C-terminal<sup>333,334</sup> truncations. Therefore, future studies aimed at the comprehensive mapping of the Tau PTM profile should employ approaches and a combination of antibodies that capture the diversity of Tau proteins and fragments in brain tissues and biological fluids.

#### Co-factor-induced Tau aggregation mechanisms determined *in vitro*

**Heparin.** In general, the unifying characteristic of the Tau aggregation inducers is a polymer with a net negative charge. Heparin and heparan sulfate (HS) remain the most commonly used glycosaminoglycan (GAG) polysaccharides to induce Tau aggregation *in vitro* and have shaped our current understanding of the mechanisms of Tau aggregation and pathology formation (Fig. 7). With the advent of recombinant protein technologies for large-scale Tau production, in 1996 heparin was shown to act as an inducer for the aggregation of unmodified recombinant Tau.<sup>13</sup> It was soon after widely adopted to produce large amounts of Tau fibrils for *in vitro*, in cells, and *in vivo* experiments. Heparin-fibrillized Tau was used as a proxy for the Tau fibrils derived from pathological brain samples for biochemical, biophysical, and kinetic characterization, as well as for screening of Tau fibril-binding molecules. But it became clear that despite some similarities, the heparin-fibrillized Tau differs from pathological Tau fibrils in multiple ways.<sup>335</sup> These findings are not unique to heparin, but other Tau fibrillization cofactors such as RNA and lipids, which also induce the formation of polymorphic Tau fibrils with a physicochemical profile different to patient-derived Tau. Here we focus on heparin given that it is the most widely used and accessible Tau fibrillization cofactor and formed the cornerstone of the experimental investigations of Tau fibrillization for nearly three decades.

GAGs are a large class of long anionic unbranched polysaccharides consisting of repeating disaccharide units.<sup>336</sup> Based on the compositional differences, GAGs comprise five classes of molecules, such as hyaluronic acid, predominantly associated with skin tissues, synovial fluid and articular cartilage;<sup>337</sup> chondroitin sulfate, which is a major component of perineuronal nets, changes in sulfation patterns of which were linked to cognitive decline in AD,<sup>338</sup> as well as dermatan sulfate, keratan sulfate, and HS, all of which play important structural and signaling roles in neural and other tissues.<sup>336,339–341</sup> Heparan sulfate proteoglycans (HSPGs) have





**Fig. 7** Hypothetical mechanism of heparin-induced Tau fibril formation. Tau exists in solution as a disordered monomer that could undertake the paperclip conformation. Negatively-charged heparin can bind to positively-charged regions on Tau and prime it into aggregation-prone conformation. Tau forms the nucleation-competent seed, ON-pathway oligomers, and fibrils upon the addition of primed monomers. Heparin molecules may tightly associate with Tau fibrils. Tau MTBR is designated with coral, N- and C-termini in grey, heparin molecule in magenta.

been implicated in Tau pathology formation and spreading at the stages of its release from the affected cell, as well as in facilitating the internalization of pathogenic Tau forms by the neighboring cell (reviewed in ref. 342). Despite the popularity and ease of use of polysaccharides as cofactors for *in vitro* Tau fibrillization, the formed fibrils differ from pathological in several ways. The heparin-induced fibrils are highly heterogeneous, predominantly single protofibril, and composed out of the single Tau isoform, which contrasts with the highly conserved disease-specific Tau fibril fold. In addition, the heparin-induced fibrils are generated from recombinant unmodified proteins and thus lack the PTMs found on the patient brain-derived Tau fibrils.<sup>21,335</sup> Other concerns for the downstream applications *in vitro*, in cells and *in vivo* arise when using added heparin as a cofactor to fibrillize Tau. These may include interference with the Tau fibril-targeting compounds,<sup>343</sup> disruption of the cellular uptake of Tau fibrils,<sup>344</sup> and causing

vasodilation or immune response in animals,<sup>345,346</sup> all of which may contribute to the modification of Tau seeding propensity non-contingent on the inherent properties of the Tau seed itself.

### Mechanisms of heparin-induced Tau aggregation

#### Kinetics of Tau aggregation and heparin-to-Tau stoichiometry.

For Tau aggregation to proceed efficiently and on reasonable time scales,<sup>347</sup> the aggregation-inducing cofactors must have a high affinity for Tau. Heparin displays properties of fast Tau protein binding and forms stable heparin-Tau complexes. Kinetic biolayer interferometry assays, using biotinylated monomeric full-length Tau immobilized on the biosensor and incubated with variable concentrations of heparin or synthetic heparinoid (SN7-13) solutions, demonstrated the high heparin affinity to Tau with ON rate constant of around  $9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  and the lower OFF rate constant of  $6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ .<sup>348</sup> This is consistent



with a previous report suggesting that heparin–Tau binding in the submicromolar range.<sup>349</sup>

At physiological pH and in the presence of a reducing agent, such as dithiothreitol (DTT), the 4R Tau MTBR formed a stable complex with ~7 kDa heparin at a ratio of one-to-one<sup>350,351</sup> or two Tau MTBR molecules per one 12 kDa heparin molecule.<sup>351</sup> These interactions were slower in the absence of reducing agents, which suggests that the formation of intramolecular cysteine bridge could stabilize Tau MTBR monomer and/or disrupt its interactions with heparin, thus preventing efficient 4R Tau MTBR fibrillization even in the presence of cofactors.<sup>352–354</sup> Interestingly, the lag phase was unaltered when the Tau MTBR to heparin molar ratio was either one-to-one or one-to-two. However, it decreased sharply at higher molar ratios of heparin. Conversely, the exponential growth rate increased with a decreasing ratio of heparin to Tau MTBR.<sup>351</sup> Quantitation of Tau MTBR and heparin-binding kinetics, based on calculations of the stoichiometry of the molecules and association and dissociation rate constants, suggested that the interaction between Tau MTBR and heparin occurs at the nucleation step. The exponential growth phase, which proceeds by the sequential addition of Tau MTBR monomers to aggregation competent nuclei or fibril seeds, was reduced at lower heparin to Tau MTBR molar ratios and inhibited at higher ratios. This demarcates the narrow optimal range of heparin-to-Tau molecule ratios necessary for the efficient Tau fibrillization reactions. Furthermore, these studies show that the heparin association rate with Tau is higher than the dissociation rate. On average, more heparin molecules are likely to be associated with Tau than are released back into the solution.

The kinetics of heparin-induced Tau fibrillization was also dependent upon the length of heparin polysaccharide chains, with longer chains providing higher Tau-heparin binding and nucleation efficiency,<sup>355</sup> likely due to a higher number of the binding sites along the Tau molecule. However, due to the high complexity of the glycochemistry of the heparin, the quality of the commercial heparin products depends on the purification or synthesis methods, and the origin or the raw material. This might lead to high batch-to-batch variability and large heterogeneity in terms of the heparin molecular sizes.<sup>344</sup>

**Electrostatic charge contributions to heparin–Tau interactions.** The heparin–protein interactions are primarily mediated by the iduronic acid epimerization of heparin, polysaccharide chain conformations, and negative charges of sulfate groups<sup>356,357</sup> (Fig. 8A). Polysaccharide sulfation patterns are key determinants of the specificity and the affinity of heparin–protein interactions.<sup>358,359</sup> In general, medium (MMW; ~5–12 kDa) to high (HMW; >12 kDa) molecular weight heparins induce Tau fibrillization. Heparin–Tau interactions have been determined to rely largely on the electrostatic, rather than covalent, van der Waals, or other forces. Heparin provides charge compensation for the lysine- and histidine-rich stretches<sup>360</sup> that form upon the in-register, parallel stacking of the Tau repeat regions R2 and R3.<sup>349</sup> The full-length Tau aggregation conditions most often feature a

Tau-to-heparin ratio of four-to-one to achieve charge neutralization,<sup>361</sup> a ratio within the range experimentally determined using kinetics assays (see below). For full-length TauΔ187 under physiological conditions, the charge was estimated to be approximately +13.5. For 11 kDa heparin, the charge was estimated at around –50, which is the equivalent of approximately Tau-to-heparin molar ratio of four-to-one, resulting in a charge balance of +54 to –50.<sup>362</sup>

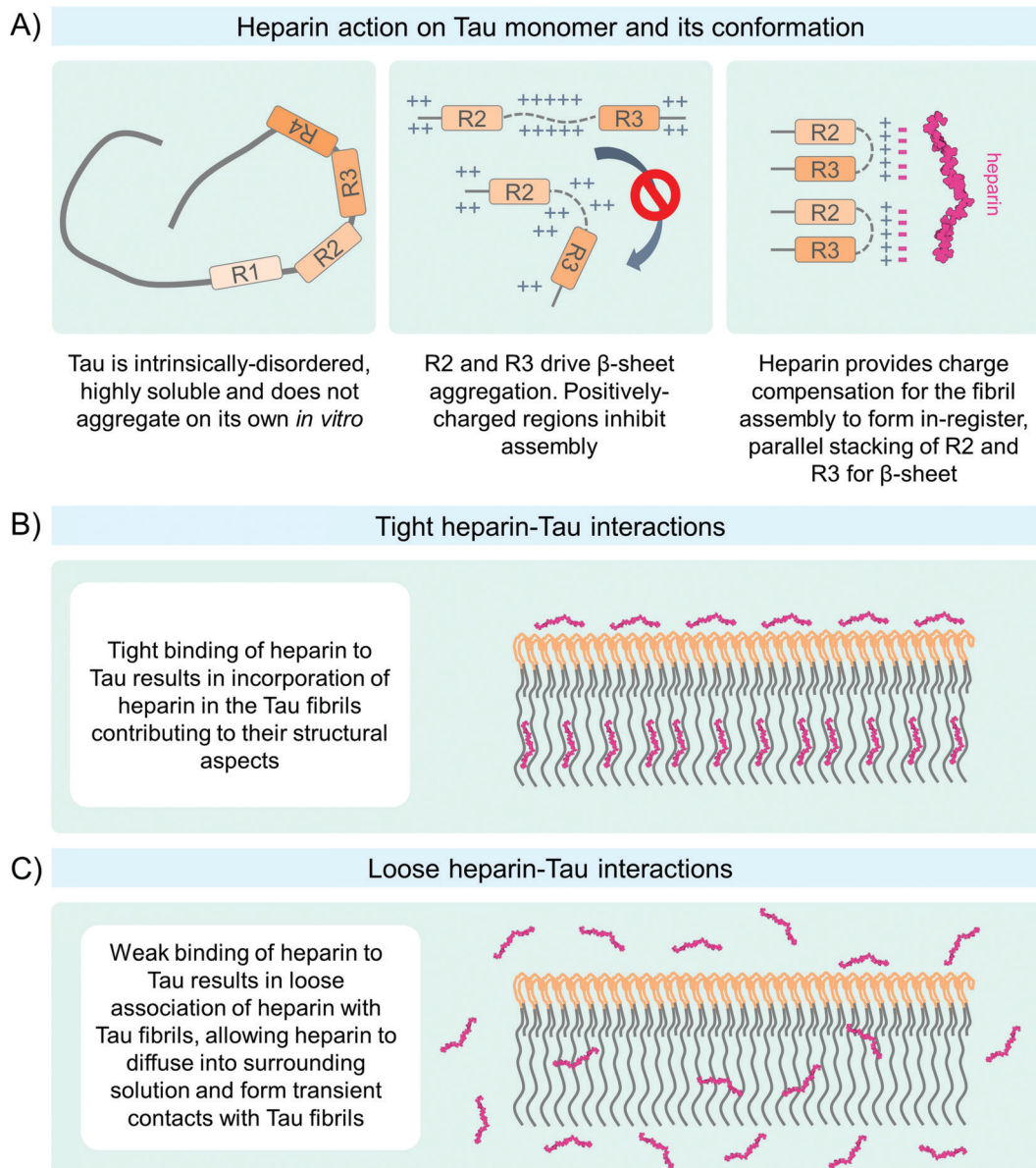
**Heparin-induced conformational changes of Tau.** Heparin interactions with Tau alter the conformational landscape of the protein, resulting in the population of partially-structured Tau intermediates with an increased propensity for self-assembly and fibril formation. Upon heparin binding to Tau monomer, Tau undergoes conformational transitions characterized by the presence of two distinct Tau populations, one possessing the unstructured features of the IDP, another, however, showing compaction of MTBR and restructuring of the N- and C-termini upon heparin binding to Tau.<sup>363,364</sup> This dichotomy in Tau molecular states is opposed to the expected continuum of expanding-contracting Tau conformers under the conditions of the sole effect of charge redistribution along the Tau molecule by heparin. Under these conditions, the Tau conformations are expected to undergo progressive loss of intrinsic disorder and a gradual shift towards a more structured protein. However, this was not the case, as the Tau protein either retained most of its IDP character and long-range intramolecular contacts or collapsed into structured compact MTBR conformation.

*In vitro* chemical cross-linking performed at various time points during Tau aggregation showed that Tau formed stable dimers, tetramers, and higher-order assemblies in the presence of heparin.<sup>364</sup> This suggests that in addition to direct effects on Tau molecular conformation, heparin promotes Tau aggregation and fibril formation through stabilizing or increasing the formation of nucleation-competent Tau oligomers.<sup>365</sup> Heparin was shown to associate with the rigid core region of Tau PHFs composed of the MTBR. However, the N-terminal residues upstream of the N-terminal inserts N1 at amino acid position 45–74 and N2 75–103 were also important for Tau-heparin interactions.<sup>349,355,360,366</sup> Studies by electrochemical impedance spectroscopy and cyclic voltammetry showed that Tau monomers immobilized on the gold electrode surface by the N-termini had a lower capacity to bind heparin compared to the Tau molecules immobilized by cysteine residues 291 and 322 in the middle of the protein, which allowed the N- and C-termini mobility.<sup>367</sup> These studies indicate that heparin binds tightly to several regions of Tau molecules and influences its conformational and aggregation properties.

**Heparin-binding sites on Tau.** Based on the experimental evidence from biophysical studies, several heparin-binding sites on Tau have been proposed. Using variable length Tau fragments, the minimal Tau fragment sequence competent of the heparin-induced assembly into the fibrils was determined to comprise 18 amino acid stretch 317KVTSKCGSLGNIHHKPGGG335, located in the R3 repeat.<sup>368</sup> This contains two basic lysine and two histidine residues, and cysteine residue, conducive to







**Fig. 8** Heparin-induced Tau aggregation mechanisms. (A) Proposed mechanism of charge neutralization by heparin around R2 and R3 regions of Tau molecule conducive to Tau aggregation. (B) Tight interactions between heparin and Tau may lead to the incorporation of heparin into fibrils and contributions to their structure. (C) Loose associations of heparin with Tau may lead to the release of heparin molecules into the surrounding medium.

the formation of inter-peptide sulfur bridges, that may promote the formation of nucleation-competent peptide dimer *in vitro*.<sup>365,369,370</sup> Within this peptide, the triplet 329HHK331 was hypothesized as a likely heparin-binding site.<sup>371</sup> The K331 residue was identified to have a substantial low-molecular-weight (LMW) heparin-binding affinity (10  $\mu$ M range), among other positively charged residues. In the same study, using NMR spectroscopy, the highest affinity heparin-binding sites were mapped to the stretches 139DKKAKG144 in the proline-rich domain; within the PHF6\* (275VQIINK280) in the R2 and the PHF6 (306VQIVYK311) in the R3; and 336QVEVK370 in R3–R4.<sup>349</sup> The individual positively-charged lysine residues along the Tau molecule also showed heparin-binding affinity, with the highest affinity for K257 within the R1; K298 within

the R2; K311 in the PHF6 and K331 within the R3; as well as K343 and K347 within the R4. Notably, the heparin-binding affinity of Tau lysine residues could be modified by the charges of other residues in the vicinity, providing support for the strong impact of electrostatic forces in heparin–Tau interactions. Other studies also indirectly suggested the heparin-binding sites were present within the Tau MTBR.<sup>350</sup>

**Insights into PHF6 region as the backbone of Tau fibrils.** Despite the wide use of Tau peptides harboring the PHF6 sequence as a representative for the pathological Tau fibril formation, stark differences exist in residue configurations within the Tau fibrillar core of *in vitro* and pathological Tau fibrils. Generally, the two stable  $\beta$ -sheet forming polymorphs include the front VIY and back QVK side residues of the PHF6



sequence VQIVYK. Crystal structures of fibrils composed of Tau fragments 305SVQIVY310<sup>372</sup> and 274KVQIINKKL282<sup>373</sup> formed steric zippers<sup>374</sup> with the residues of the same sequence on the opposing molecule. This may suggest that the homotypic association of the aggregation-driving sequences, including the PHF6, may be key to Tau aggregate formation. However, the cryo-EM structures of pathological Tau fibrils show much greater diversity in association side-chain partners of these regions. In AD-derived PHFs<sup>152</sup> VQIVYK sequence formed the heterosteric zipper with the sequence THKLTF, in PiD<sup>375</sup> VQIVYK  $\beta$ -sheet was opposed to GQVEVK, and in CBD folds<sup>153</sup> residues VIY of VQIVYK were opposite to DNIKHV, and on the flip side of the sequence VQIVYK the residues QVK were opposite to GQVEVK. Both CTE Type I and II fibrils<sup>376</sup> were formed by the heterosteric zippers running in the opposite directions through phenol on oxygen atom O $\epsilon$  in VQIVYK and histidine on nitrogen atom N $\delta$  in HKLTFR with the distances of the magnitude permissible for the hydrogen bond formation. In the heparin-fibrillized Tau polymorphs<sup>377</sup> only 3R2N fibrils showed a steric zipper between opposing VQIVYK-VQIVYK sequences, which also differed from crystal structures of these regions.<sup>372</sup> NMR studies of 4R0N fibrils showed the region VQIVYK constituting part of the  $\beta$ -sheet, among other regions yet to be mapped.<sup>378</sup> These high-resolution studies, as well as computational modeling,<sup>379</sup> revealed the importance of  $\pi$ -stacking of the tyrosine rings for the stabilization of all known Tau fibrillar structures. The occurrence of PTMs in the regions that may lead to destabilization or prevent the formation of the bonds instrumental for the formation of stable Tau polymers could decrease the aggregation propensity of Tau.<sup>149</sup>

#### Tau dimerization under reducing or oxidizing conditions.

Induction of Tau aggregation by adding  $\sim 18$  kDa heparin under reducing conditions<sup>380</sup> depended on cysteine residues within MTBR,<sup>381</sup> illustrating the importance of this region for the binding of heparin to Tau. The formation of Tau dimers is thought to be an early event of the ON-pathway of Tau fibrillization. Dimerization of Tau through the covalent formation of cysteine-cysteine intermolecular sulfur bridges was observed under oxidizing conditions with 3R Tau isoforms, which in the presence of cofactors proceeded to form fibrils. However, the 4R isoforms were found to form an intramolecular sulfur bridge that compacted the monomers thus significantly delaying or preventing the formation of fibrils even in the presence of cofactors.<sup>352</sup> Under reducing conditions, however, the Tau dimers were not covalently linked and could form fibrils in the presence of cofactors.<sup>352</sup> The recent cryoelectron microscopy (cryo-EM) of Tau fibril structures support the formation of Tau fibrils under reducing conditions from both 3R and 4R Tau isoforms in patients and *in vitro*,<sup>152,335,375,376,382</sup> as the distances between cysteine residues 291 or 322 observed are  $\sim 2.5$  times larger than the disulfide bond<sup>383</sup> (Fig. 9 and Table 2).

**Is heparin a major structural component of Tau fibrils?** The initial clues that heparin could be involved in the formation of Tau pathological aggregates (Fig. 8B) came from studies on patient brain-derived Tau PHFs. Heparinase enzyme treatment of AD patient brain-derived PHFs resulted in the loss of the

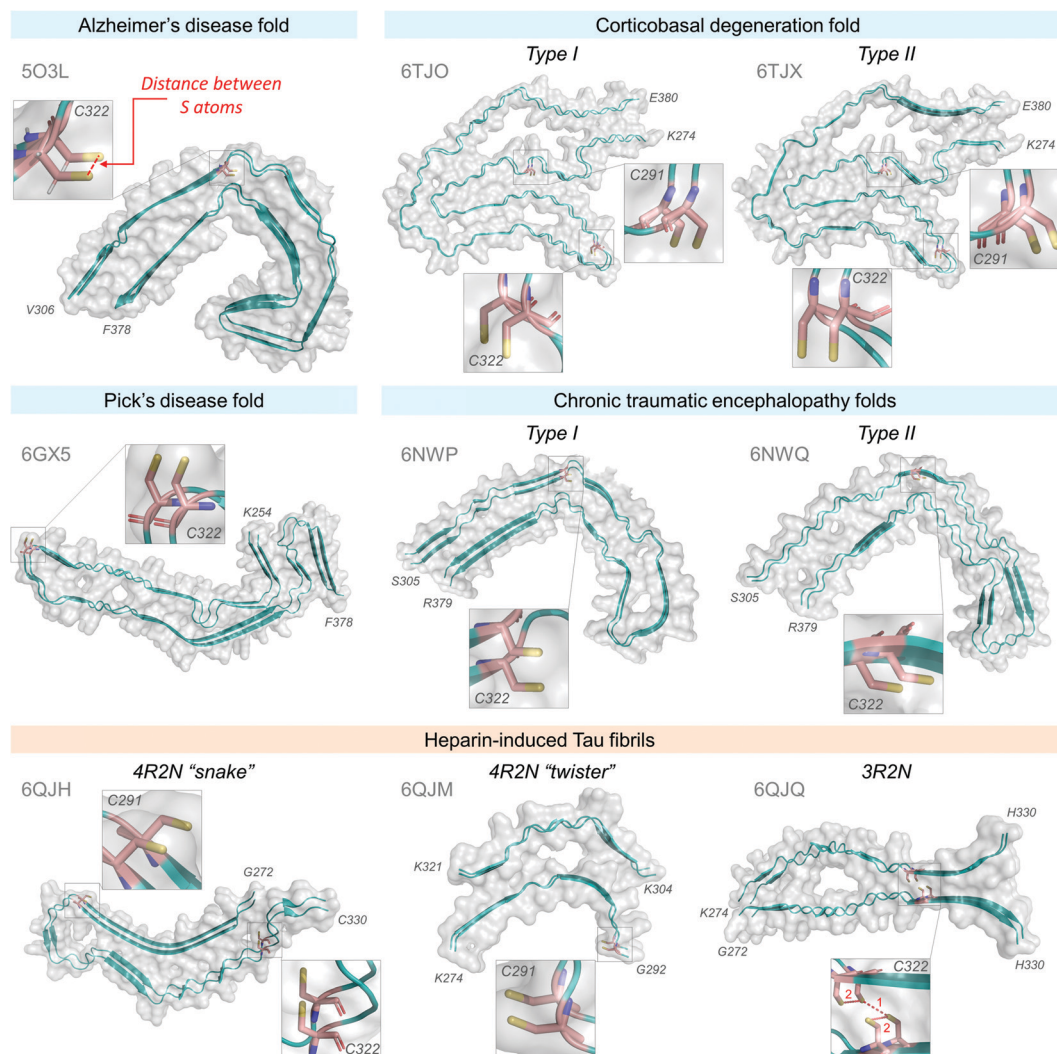
PHFs' helical twist signature<sup>386–388</sup> and a shift of PHFs' twist periodicity from  $69.5 \pm 5$  nm to  $78.2 \pm 7.7$  nm. Interestingly, this treatment decreased PHFs' immunoreactivity to the N-terminal antibody Tau14 (epitope recognition of amino acids 141–176) and phosphorylation-dependent antibody AT8 (pS199/pS202/pT205/pS208<sup>179</sup>), but increased the detection by PHF-1 antibody (pSer396/pSer404).<sup>386</sup> Curiously, the labeling of PHFs and SFs with Tau14 antibody was restricted to a subpopulation of fibrils and was nearly abolished by the heparinase treatment. This is puzzling when considering that part of the Tau14 epitope spans over amino acids 140KKAK143, which were previously identified as heparin-binding sites.<sup>388</sup> The authors surmised that the fibril untwisting could be attributed to increased conformational flexibility in the C-terminus and/or degradation of the heparin bound to sites near MTBR. Also, likely, the diminished accessibility of the heparinase enzyme to this domain on Tau fibrils could have contributed to the retention of the heparin molecules bound at this site, thus reducing the recognition by Tau14.

The heparinase-treated PHFs also showed altered biochemical properties, such as higher sodium-dodecyl-sulfate (SDS) solubility and reduced PHF-1 immunoreactive high molecular weight species by Western blotting. Although several *in vitro* studies have attempted to address the question of whether heparin is integrated into Tau fibrils,<sup>349,351,389–392</sup> at this time no consensus has been reached. It is likely that heparin associates strongly with specific sites on Tau fibrils. Several aspects of the biochemical properties of heparin (*e.g.* molecular weight and sulfation patterns) and Tau molecules (*e.g.* isoform, modifications, mutations, and aggregation) have been shown to influence the interactions between these two molecules. Finally, the binding and the heparin-Tau association-dissociation equilibrium might maintain a stochastic component, *i.e.* not readily attributable to any predictive variable. All these observations motivated researchers to look increasingly closer at heparin-Tau interactions to disentangle the complex relationships between heparin and Tau.

**Evidence for the heparin integration into Tau fibrils.** Sibille *et al.* were the first to experimentally test whether heparin was integrated into the Tau fibrils using NMR spectroscopy, and found that HMW heparin interacted with the positively-charged regions flanking the MTBR of Tau monomers.<sup>349</sup> After Tau fibrillization reaction completion, followed by the depletion of residual heparin, no relaxation of the heparin-Tau interacting residues or detectable heparin free signal were seen, indicating that all heparin was tightly associated with Tau. In the case of a weak association of heparin with Tau, the expectations were to observe the gradual release of heparin into the solution, accompanied by the NMR shift of heparin-binding amino acids in Tau, which was not the case. This suggested that heparin was stably integrated into the surface of Tau fibrils in parallel with the direction of fibril growth, rather than being intercalated perpendicularly within  $\beta$ -sheets, in which case the  $\beta$ -sheet packing of amino acids would have been perturbed, or resulted in longer distances between packed molecules.

Electron paramagnetic resonance (EPR) studies showed that RNA induced the formation of Tau fibrils containing in-register





**Fig. 9** Cryo-EM structures of Tau fibrils and cysteine residue positions. Tau fibril structures available at Protein Data Bank,<sup>384</sup> accession codes are in grey, structures were rendered using PyMOL software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC<sup>385</sup>). Two molecule strands are shown for each structure. Atomic distances between sulfur atoms on adjacent cysteine residues (as depicted in Alzheimer's disease fold, red dashed line) were measured using the PyMOL<sup>385</sup> Measure tool and are summarized in Table 1. All distances between cysteine residues are significantly larger than the distance required for the formation of the disulfide bond at 2.3 Å.<sup>383</sup>

stacking of in-parallel  $\beta$ -sheet-containing monomers, conformationally resembling heparin-induced Tau fibrils. However, RNA affinity for 3R MTBR fibrils was higher than heparin, which suggests other structural differences between Tau fibrils induced by heparin or RNA. Subsequent studies by Fichou *et al.*<sup>390</sup> (see below) showed that RNA was stably associated with the Tau fibrils, but could be displaced into the soluble fraction by the addition of heparin. No change in fibril morphology was detected after heparin addition and RNA displacement. The majority of fluorescein-conjugated heparin (around 80%) was directly shown to be associated with 3R or 4R MTBR fibrils, with a low amount remaining in the soluble fraction. These results show that the binding of these negatively-charged cofactors to the surfaces of Tau fibrils is mediated by electrostatic forces that are more pronounced in the case of heparin, most likely due to high negative charge density per heparin molecule.<sup>393</sup> The main limitation of this study was the use of

truncated Tau proteins with non-native amino acid residues, which could limit the translation of these results to the full-length Tau isoforms. However, increasing evidence suggests that Tau fragments are present in appreciable amounts under physiological and pathogenic conditions.<sup>265–268,270–272,329</sup>

So far, the most compelling evidence for heparin as a constituent of Tau fibrils came from the direct detection of spin-labeled heparin within Tau fibrils. Using continuous-wave EPR and biochemical approaches, Fichou *et al.*<sup>390</sup> showed that heparin ( $\sim 15$  kDa) and RNA ( $\sim 900$  base pairs) were integral parts of the *in vitro* formed Tau fibrils. Upon removal or heparin, using enzymatic digestion by heparinase which removed around 20% of bound heparin, a fraction of the Tau fibrils depolymerized into the Tau monomers and dimers. Similarly, treatment with RNase led to the removal of around 60–70% of bound RNA. The detection of spin-labeled heparin in the thioflavin-positive heparin/RNA-containing Tau





Table 2 Atomic distances between cysteine residues in Tau fibrils

Disulfide bond distance <sup>383</sup>		~2.3 Å	
		S-S distances (Å) between residues	
	PDB	C291-C291	C322-C322
Pathological Tau fibril fold			
Alzheimer's disease	5O3L	—	4.79
Corticobasal degeneration			
Type I	6TJO	4.80	4.86
Type II	6TJX	4.81	4.82
Chronic traumatic encephalopathy			
Type I	6NWP	—	4.72
Type II	6NWQ	—	4.90
Pick's disease	6GX5	—	4.85
Heparin-induced Tau fibrils			
4R2N “snake”	6QJH	4.80	4.76
4R2N “twister”	6QJM	4.72	—
		<b>1. C322-C322</b>	<b>2. C322-C322</b>
3R2N	6QJQ	3.70	5.13

fibril-containing fraction post-heparinase/RNAs treatment showed that some of the heparin/RNA bound were structurally integrated into Tau fibrils and were not easily accessible by enzymes. Nevertheless, in both cases, heparin and RNA cofactors were instrumental for maintaining the thioflavin-positive and continuous-wave electron paramagnetic resonance of paramagnetic spin spectra signatory of  $\beta$ -sheet arrangement within Tau fibrils. These observations suggest that these two cofactors contribute to the structural integrity of Tau fibrils. Interestingly, thus far no non-proteinaceous electron densities in cryo-EM imaging of Tau fibrils were attributed to heparin, possibly due to sample preparation procedures and reversible and dynamic nature of heparin–Tau contact. It remains unclear to what extent the level of heparin/RNA integration into the fibrils alters their morphology or stability. Also, whether the interactions happen before or after Tau fibril formation *in vivo* must be clarified.

**Evidence against the heparin integration into Tau fibrils.** A few studies have argued against the significant heparin integration into the Tau fibrils (Fig. 8C). Based on the mass-per-length analysis of scanning transmission electron micrographs, von Bergen *et al.*<sup>391</sup> failed to detect the expected increase of ~11% mass in fibrils formed by the Tau MTBR variant (lysine 280 deletion) in the presence of LMW heparin (6 kDa). Also, following the centrifugation of the Tau aggregation solution, fluorescently-labeled MMW heparin (16 kDa) was predominantly detected in the soluble fraction, with a low but detectable amount in the polymerized Tau protein pellet fraction containing the fibrils. Based on these observations, it was concluded that heparin was not incorporated into the fibril core,<sup>391</sup> which is in line with the earlier findings<sup>349</sup> that heparin is likely to associate with the fibril surface residues, some of which may be lacking in fibrils composed of just Tau MTBR. However, this study had several limitations, including the use of (1) a mutant form of a Tau fragment (MTBR) that lacks one of the important lysines ( $K^{280}$ ) for heparin interaction; (2) LMW heparin (6 kDa) that was likely to possess reduced Tau-binding affinity compared to the HMW heparin, and (3) large fluorescent tag on the heparin molecules which could affect their biochemical properties and binding to Tau. These issues might limit the

extrapolation of the results from this study to the non-mutant Tau MTBR or full-length Tau and heparin interactions.

Using kinetic and steady-state analyses, Carlson *et al.*<sup>392</sup> suggested that heparin-to-Tau molar ratios were more important factors in Tau polymerization than the concentration of Tau molecules. These authors argued for an allosteric impact of heparin on Tau molecules resulting in a low thermodynamic barrier to nucleation.<sup>392</sup> Based on the classical model of allosteric regulators,<sup>394</sup> the authors concluded that heparin was not integrated into the Tau fibrils. However, the data in the study did not directly demonstrate that on the structural level. Similarly, Ramachandran and Udgaonkar<sup>351</sup> estimated the heparin-to-Tau molecule stoichiometry at the end of fibril formation to be one-to-twenty. Based on the theoretical calculations using heparin–Tau dissociation constant and Tau and heparin molecules' concentrations values, they argued that heparin was only a minor constituent of the formed fibrils. Analyses of the heparin-induced full-length Tau or MTBR fibrils at three different heparin concentrations by AFM and FTIR spectroscopy showed no significant size or structural differences of fibrils between these three conditions.<sup>351</sup> One obvious limitation of this experimental design is the lack of independent controls, such as alternative inducer-generated fibrils or reassessment of the fibril biophysical properties after enzymatic heparin removal treatment.

In summary, kinetic, structural, and biochemical studies strongly suggest the possibility of tight heparin association with or integration into Tau fibrils under certain conditions *in vitro*. However, further investigations using complementary experimental techniques and quantitative approaches are warranted to determine the precise mechanisms of heparin–Tau fibril interactions (Fig. 7B and C) and whether these interactions occur at the monomer or fibril levels in the brain.

The tight binding of heparin or other cofactors to Tau fibrils has significant implications for using heparin-induced fibrils to investigate Tau fibril toxicity mechanisms of seeding activity in cells or *in vivo* as the Tau-non-contingent responses, due to the presence or release of these molecules may occur. In the case of heparin, this includes vasodilation of the blood vessels<sup>346</sup> and immune<sup>345</sup> or aberrant cell stress responses.<sup>395–400</sup> Also, the presence of heparin or other cofactors might directly impact the post-translational modifications of Tau fibrils,<sup>401</sup> alter their surface properties, secondary nucleation events, and thus their seeding activity and spreading through extracellular matrix interactions.<sup>344,348</sup> Furthermore, the interference of the polysaccharide with the Tau-targeting molecules<sup>343</sup> cautions the use of the current systems in the development of Tau pathology targeting drugs and tracers.

**Do Tau and heparin interact in patients' brains *in vivo*?** Although heparin moieties were detected within intracellular NFTs,<sup>402–408</sup> it is still puzzling how the extracellular heparin and HSPGs might directly impact the fibrillization of predominantly intracellular Tau *in vivo*, which by definition requires direct exposure to these molecules. Physiological and pathological forms of Tau were demonstrated to be released into the extracellular fluid (ECF) in the free form, as well as in ectosomes or exosomes, and internalized by the neighboring cells *via* bulk dynamin-mediated



endocytosis or HSPG-dependent micropinocytosis<sup>409</sup> (reviewed in ref. 410 and 411). This forms the basis of the hypotheses on the pathological Tau propagation in the brain along the neuroanatomically connected regions (reviewed in ref. 412 and 413). However, the initiation events that spur the primary Tau conversion from physiological to pathological forms remain unknown.

Tau molecules in physiological conformation present in the ECF might have ample opportunities to encounter heparin, and other cofactors, especially in the events of heparin-mediated neuroinflammatory responses. Thus, one may speculate that during these events, heparin exerts conformational changes on Tau molecules, priming them into fibrillization-competent forms and/or tightly binding to Tau. The subsequent cellular intake of these primed Tau molecules or Tau-heparin complexes might cascade into the pathological Tau oligomerization, fibrillization, and formation of NFTs, concomitantly sequestering the functional pool of Tau molecules and inciting cellular stress responses. Further investigations are necessary to determine whether the ECF heparin mediates the Tau conformational changes from physiological to pathological forms *in vivo*. Addressing this question might be achieved using techniques such as microdialysis of ECF in the brain of model animals,<sup>414–417</sup> or recovery of the material from the brain electrode implants of neurodegenerative disease patients undergoing the procedures such as deep brain stimulation. The Tau proteoforms and heparin molecules then can be determined by highly sensitive biophysical techniques, such as mass spectrometry<sup>418</sup> at different stages of Tau pathology formation.

#### Indirect heparin-mediated induction of Tau aggregation

*Heparin activates Tau kinases.* Interestingly, apart from the direct interaction of heparin with Tau to induce the formation of fibrils, heparin might also indirectly influence Tau's biochemical and biophysical properties through stimulation of Tau hyperphosphorylation by several Tau kinases. This is thought to occur due to heparin-induced conformational changes that ultimately lead to increased exposure of phosphorylation sites on Tau.<sup>364</sup>

Heparin was shown to greatly enhance Tau phosphorylation by p34/cdc28 serine-threonine kinase<sup>419</sup> and by protein kinase A at least on serine 156.<sup>420</sup> However, the physiological relevance of modifications by these kinases is unknown. On the other hand, heparin enhanced Tau phosphorylation by protein kinase FA/glycogen synthase kinase-3 $\alpha$  (GSK-3 $\alpha$ ) *in vitro*, which phosphorylated residues found in the AD pathology, such as serines 235, 262, 404, 324 and 356, and threonines 212 and 231.<sup>401,421</sup> Similarly, heparin was shown to enhance Tau phosphorylation by stress-activated serine-threonine kinases (SAPK1 $\gamma$ , SAPK2a, SAPK2b, SAPK3, SAPK4) on the serines (S202, S396, S404, S422) and threonines (T181 and T205, T231)<sup>422</sup> *in vitro*. Interestingly, Hasegawa *et al.*<sup>423</sup> showed that the heparin concentration required to induce Tau phosphorylation by MAP kinase, NCLK, and GSK3 $\beta$  on multiple serine residues, and notably on serine 262, was lower than the heparin concentration required to induce Tau fibrillization. This suggests possible precedence of heparin-induced Tau phosphorylation to fibrillization, however subsequent studies showed that cofactor-induced Tau

fibrillization kinetics were complex and dependent on other factors in addition to heparin–Tau concentration stoichiometry.<sup>351,392</sup> Serine 262 is considered abnormally phosphorylated in AD.<sup>424,425</sup> However, our group<sup>150</sup> and others<sup>197,200</sup> showed that phosphorylation of serine 262 in combination with other residues inhibited, rather than promoted, heparin-induced Tau aggregation *in vitro*. It is still unclear whether and how the predominantly extracellular heparin or plasma membrane-associated molecules of HSPGs can directly impact Tau kinase-mediated downstream fibrillization of Tau in the intracellular milieu *in vivo*.

Taken together, cofactors such as heparin and others, known and unknown, seem to play a much larger role in the Tau fibril formation, their biochemical characteristics, and their impact on neuropathology formation and propagation than is generally thought and should be investigated further.

#### Other Tau aggregation inducers and mechanisms

*RNA.* Tau was first discovered to directly bind to RNA molecules in 1984 by Schröder *et al.*,<sup>426</sup> later corroborated by others.<sup>389,427</sup> RNA was also found in association with hyperphosphorylated Tau in NFTs, Pick bodies, and neuritic plaques,<sup>428,429</sup> providing credence to the physiological relevance of investigating RNA-induced Tau fibrillization. In 1996, Kampers *et al.*<sup>430</sup> showed that RNA induced Tau assembly into fibrils through the formation of the intermolecular cysteine 322 disulfide bridges, which led to the formation of Tau dimer intermediate before Tau fibrillization. Subsequently, RNA has been successfully used to induce and study Tau fibrillization mechanisms *in vitro*.<sup>431</sup> Nevertheless, the formation of disulfide-mediated dimer formation is not necessary for Tau fibrillization in the brain<sup>352</sup> as exemplified by their absence in the cryo-EM-solved fibrils from sporadic AD, Pick's, CBD, and CTE patients (see Fig. 9). The presence of RNA catalyzed the conversion of soluble Tau into insoluble forms, likely through the charge balance to allow the tighter packing of the molecules.<sup>432</sup> Recently, the role of RNA–Tau interactions has received increased attention due to converging data implicating liquid–liquid phase separation as a key mechanism in the initiation of Tau aggregation and fibril formation.<sup>427</sup>

Another recently proposed model for the aggregation of Tau involves liquid–liquid phase separation (LLPS) (reviewed in ref. 433 and 434). In biology, LLPS defines biophysical processes of condensation of biomolecules driven by transient interactions.<sup>435</sup> These condensates are involved in the formations of membrane-less organelles in cells, such as nucleoli, stress granules, and liquid droplets. Recent findings suggest that these condensates may be implicated in the protein aggregation processes in neurodegenerative diseases spurred interest among researchers to study LLPS processes further<sup>436</sup> which have been reviewed in ref. 435. RNA and heparin were found to induce LLPS of Tau into reversible condensed droplets that increased local Tau concentration, where Tau retained mobile conformations.<sup>427</sup> In cells, the transfer RNAs were the predominant RNA species that were associated with Tau droplets.<sup>427</sup> Wegmann *et al.*<sup>437</sup> showed that Tau condensation into droplets coincided with the formation of Tau fibrils in the presence of heparin or RNA cofactors *in vitro*, suggesting the possibility of the coacervate heparin–Tau complex



formation as an intermediate to Tau fibrillization.<sup>438</sup> However, whether these two processes were independent was unclear.

Recent studies using turbidity assays with varying salt concentrations showed that in the presence of heparin, the formation of thioflavin-positive Tau aggregates occurred under a much wider range of sodium chloride concentrations, in contrast to complex coacervation, which occurred only in a small subset of the conditions.<sup>439</sup> These results indicated that canonical heparin-induced Tau fibrillization was preferential to electrostatically driven heparin-Tau coacervate complex formation. The two processes – Tau LLPS formation and fibrillization – were likely to occur under overlapping conditions, but were independent processes. On the contrary, another study showed that LLPS-promoting conditions increased the fibrillization kinetics of Tau.<sup>440</sup> This was exacerbated by the presence of the disease-associated mutations  $\Delta$ K280, P301L, and G272V, which, in their own right, did not influence the phase transition propensity of Tau compared to non-mutant protein. Under physiological buffer conditions and Tau concentration of 2  $\mu$ M,<sup>290,441,442</sup> resembling that of the protein levels in the neurons, Tau required molecular crowding agents such as polyethylene glycol to phase separate. However, the P301L mutation or AT8-positive phosphorylation at pS202/pT205/pS208 increased the formation of droplets.<sup>443</sup>

These studies indicate that total Tau concentration and molecular crowding conditions, isoform composition and ratios, and the presence of pathological mutations can all impact Tau's propensity to phase separate. Nevertheless, whether the LLPS and ordered amyloid assembly of Tau fibrils are co-occurring or co-dependent processes has not yet been definitively demonstrated. Furthermore, the LLPS should be modeled under physiological conditions to provide useful insights into Tau biology. Although the Tau LLPS is an exciting new line of research into mechanisms leading to Tau fibrillization, thus far the lack of robust tools to study these delicate and dynamic biophysical processes in cells and *in vivo* restricted studies to explore the therapeutic potential of modulating these processes and investigating their role in the pathogenesis of AD and other neurodegenerative diseases (reviewed in ref. 444).

**Lipids and membranes.** Early studies showed the presence of lipids and membranes in association with the neuropathological lesions in neurodegenerative disease patients,<sup>445–447</sup> and directly bound to Tau.<sup>448,449</sup> Therefore, Tau-lipid interactions and anionic lipid-mediated aggregation of Tau were subsequently extensively studied (reviewed in ref. 450). Lipid membranes promoted Tau self-assembly at the membrane surface.<sup>145,451</sup> MTBR was identified as a lipid-binding domain,<sup>452,453</sup> with interactions specifically mediated by the PHF6 region.<sup>454</sup> Lipid membranes were found to induce a shift in full-length Tau molecular conformation towards  $\beta$ -sheet-containing structures, that formed stable oligomeric complexes.<sup>455</sup>

Tau interactions with membranes could be modulated by Tau PTMs. Phosphorylation had differential effects on Tau interactions with lipid membranes in various systems. Phosphorylation of Y310 in the MTB region<sup>149</sup> reduced its affinity for lipids *in vitro*. In neuronal cells casein kinase 1

and GSK3 $\beta$  inhibition led to increased Tau association with the membrane, whereas inhibiting protein phosphatase 2A, or mimicking phosphorylation in the N-terminal and proline regions, led to a decrease of Tau affinity for membranes.<sup>456</sup> Similarly, mimicking phosphorylation of 3R0N fetal Tau isoform on multiple residues significantly decreased its membrane association.<sup>457</sup> Membrane fraction-containing extracts of cell lines<sup>458,459</sup> and neuronal cultures<sup>456</sup> contained predominantly dephosphorylated non-aggregated Tau. On the other hand, oligomeric Tau phosphorylated by GSK3 $\beta$  *in vitro* had a higher affinity for di-palmitoylphosphatidylcholine (DPPC) or 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) unilamellar vesicles, whereas phosphorylation of monomeric 4R1N did not affect its lipid-binding affinity.<sup>460</sup> During apoptotic cell death phosphorylated aggregated Tau was enriched in the plasma membrane-containing PC12 cell line extracts.<sup>461</sup> Tau phosphorylation by Fyn kinase resulted in its recruitment and enrichment at the membrane in neuronal cells,<sup>462</sup> and oligomeric phosphorylated Tau was enriched at plasma membrane of human 4R0N-overexpressing murine neuronal N2a cell line.<sup>463</sup> As evident from the contradicting reports, the role of Tau phosphorylation and PTMs on its lipid interactions is dynamic and multifaceted, and must be investigated further in light of its impact on transmembrane Tau spreading (see Fig. 3A).<sup>128,464</sup>

From a Tau aggregation tool development perspective, unsaturated long-chain fatty acids, such as arachidonic acid (5,8,11,14-eicosatetraenoic acid (20:4)) and 9-palmitoleic acid (16:1), were found to efficiently promote full-length Tau assembly into long fibrils *in vitro*.<sup>465</sup> Arachidonic acid-induced Tau fibrils showed twists at the  $88 \pm 15$  nm intervals, were thioflavin-positive and immunoreactive to PHF-specific Alz50 antibody.<sup>466</sup> Nevertheless, the lipids were not extensively adopted for *in vitro* Tau fibrillization assays, likely due to the difficulty of the post-fibrillization purification and lipid removal, as well as the heterogeneity of the fibrillar assemblies.

**Interaction with other amyloid-forming proteins.** Most neurodegenerative disorders are characterized by overlapping proteinopathy profiles.<sup>467</sup> In addition to Tau-containing NFTs, amyloid- $\beta$  plaques are another pathological characteristic of AD.<sup>468</sup> It has been stipulated, primarily based on the results from preclinical AD models, that amyloid- $\beta$  was associated, and may directly contribute to the initiation of Tau fibrillization.<sup>469–472</sup> Amyloid- $\beta$  was found to exacerbate the AD-derived Tau-seeded pathology in the mouse brain, resulting in the extensive formation of NFTs and neuropil threads, and the pathology spread throughout the brain regions, possibly through the secondary nucleation mechanism.<sup>473</sup> Amyloid- $\beta$  and Tau interconnections were recently reviewed by Ciccone *et al.*<sup>474</sup>

Furthermore, the co-occurrence of Tau and  $\alpha$ -synuclein in neuropathological inclusions has been observed in other neurodegenerative diseases since the early 1990s, including AD with Lewy bodies<sup>475</sup> and dementia with Lewy bodies (DLB).<sup>476–478</sup> Only recently, however, the direct relationship between these proteins and Tau fibrillization has been investigated. Heterotypic induction of Tau fibrillization has been





demonstrated upon co-incubation with pre-aggregated amyloid- $\beta$  fragments<sup>469</sup> or monomeric  $\alpha$ -synuclein, which bound Tau through its C-terminus.<sup>479</sup> The latter case also resulted in the formation of homotypic  $\alpha$ -synuclein fibrils under a low concentration of  $\alpha$ -synuclein monomers.  $\alpha$ -synuclein oligomers were found to nucleate oligomeric Tau formation, but not  $\beta$ -sheet forming fibrils.<sup>480</sup> Tau K18 was found to form co-oligomeric complexes with  $\alpha$ -synuclein,<sup>481</sup> indicating that this region is important in Tau interactions with  $\alpha$ -synuclein.  $\alpha$ -Synuclein was also found to modulate the spread of Tau pathology in the mouse brain.<sup>482</sup> Fibrils composed from both Tau and  $\alpha$ -synuclein monomers showed differential pathology propagation when injected into the mouse brain, where Tauopathy was induced at higher levels than synucleinopathy.<sup>483</sup>

Both Tau and  $\alpha$ -synuclein have been implicated in pathology spreading through anatomically connected brain regions in a protein strain-specific manner, resulting in distinct disease phenotypes in humans (reviewed in ref. 484 and 485). Presence of high levels of Tau in the AD-subtype of Parkinson's disease correlated with age, early cognitive impairment scores, grey matter reduction in the frontal cortex, and APOE  $\epsilon$ 4 allele status.<sup>486</sup> Increasingly, evidence suggests that heterotypic induction of Tau aggregation by other amyloidogenic proteins contributes to the co-occurrence of different proteinopathies found across neurodegenerative disorders.<sup>27</sup> These findings underscore the critical importance of conducting systematic studies to determine the key sequence, structural and cellular determinants of Tau interactions with other amyloid proteins and how they influence each other's aggregation, toxicity, and pathology spreading. The fact that the pathological aggregates of Tau,  $\alpha$ -synuclein, and other amyloid proteins such as TDP-43 are heavily modified emphasizes the importance of conducting these studies in models that reproduce the biochemical complexity of disease-relevant pathological aggregates of these proteins.

### How can the new biochemical and structural insights into physiological and pathogenic Tau inform drug discovery?

**The Tau PTM patterns as drug targets.** How the complex patterns of PTMs influence Tau aggregation and pathology formation remains unclear. However, it is becoming abundantly clear that the diversity and the distribution of Tau PTMs suggest that they play key roles in regulating Tau aggregation and pathology formation, and therefore require closer investigations. A large body of careful investigations over several decades has shown that Tau aggregation propensity depends heavily on the specific residue PTM positions, overall patterns along the Tau molecule, and their impact on the local protein structure (reviewed in ref. 163) (see Fig. 3). However, most of these studies were based on investigating Tau PTMs one at a time. They did not account for the complex interplay between different PTMs, which is likely to be tightly regulated during pathology formation and maturation. Also, the patterns of PTMs may differ between different Tau proteoforms. In a recent study, Dujardin *et al.*<sup>118</sup> conducted a systematic study to investigate the relationship between the phosphorylation of different Tau species

(soluble, oligomers, and seed-competent Tau) from 32 patients with AD and their seeding activity in cellular and animal models of Tauopathies. Their findings revealed striking patient-to-patient variation in the hyperphosphorylation profile of these different forms of Tau. The authors suggested that variations in PTMs may underlie the clinical heterogeneity of AD.

Increasing evidence points to the importance of cross-talk between the different PTMs in the modulation of Tau aggregation and seeding propensities, remodeling of the fibrils, and potentially disease progression and clinical heterogeneity of Tauopathies.<sup>487</sup> These observations underscore the importance of employing more precise tools and experimental approaches to (1) refine our knowledge of the distribution maps of the Tau PTMs in healthy individuals at different stages of aging and disease progression; (2) identify the pathology-driving or preventing PTM patterns; and (3) map co-occurring PTMs, as well as their Tau isoform origin. This will pave the way for elucidating the role of cross-talk between PTMs in regulating Tau functions, aggregation, pathology formation, and spreading during disease progression. The knowledge gained from these studies will also help guide more disease-relevant reductionist approaches by defining the precise chemical identity of the Tau proteoforms that should be used to model Tau aggregation and toxicity *in vitro*. Finally, the complexity of the Tau PTMs should be taken into consideration when developing therapeutic antibodies targeting specific domains of Tau, or antibody-based tools used to quantify Tau species and pathological aggregates. In the section below, we provide an overview of previous studies to assess the therapeutic potential of targeting Tau PTMs to treat AD and other tauopathies. Although there are many studies on targeting Tau kinases in preclinical models, we will focus the discussion below on kinases and drugs that have been evaluated in clinical trials.

**Phosphorylation.** As mentioned above, Tau is known to be phosphorylated by at least 37 protein kinases (reviewed in ref. 75), which have variable specificity for Tau residues,<sup>488</sup> and is subject to dephosphorylation by at least four phosphatases.<sup>489</sup> Because early studies implicated hyperphosphorylation as a pathogenic event that drives the initiation of Tau aggregation, several kinases were shown to phosphorylate specific residues on Tau and pursued as potential targets for the treatment of AD and other Tauopathies. In contrast, phosphatase enzymes are nonspecific and generally act upon many more client proteins other than Tau and have not been extensively investigated for applications in Tauopathy interventions and treatment. Most Tau kinases are known to phosphorylate multiple client proteins and have already been investigated for their involvement in other diseases. In many cases, the availability of inhibitors of these kinases, including some that were in the advanced development stages, facilitated their repurposing as potential therapeutics for AD and Tauopathies.<sup>490</sup>

One promising kinase candidate is glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) (reviewed in ref. 491 and 492), which phosphorylates Tau on at least 26 residues, and its levels are increased as the disease progresses.<sup>493,494</sup> Overactivation of GSK3 $\beta$  resulted in Tau hyperphosphorylation and led to neuronal death.<sup>495,496</sup> Lithium is a potent inhibitor of the GSK3 $\beta$ , inhibition of which



was shown to lead to lower levels of hyperphosphorylated Tau in mice.<sup>497,498</sup> Lithium has also been shown to reduce GSK3 $\beta$ -mediated phosphorylation of Tau and to increase Tau binding to the microtubules and their assembly in neurons,<sup>499–501</sup> in mice,<sup>502</sup> and prevented NFT pathology formation.<sup>503</sup>

Lithium salts are drugs commonly used for symptom management in bipolar and other psychiatric disorders.<sup>504</sup> Lithium has been studied as a modifying treatment for AD, mild cognitive impairment, and dementia.<sup>505,506</sup> However, its efficacy is still unclear. One important consideration is the toxicity, poor tolerance, and side effects of lithium, which led to discontinuation of the recent clinical trial (ClinicalTrials.gov Identifier: NCT00703677) evaluating lithium for the treatment of tauopathies CBD and PSP. Epidemiological observations of lower incidence of dementia in populations with access to tap water containing higher long-term levels of lithium,<sup>507</sup> although later contested,<sup>508</sup> still spurred interest in the use of microdose lithium as a preventative measure to stave off dementia, as well as disease progression modifying treatment in Alzheimer's disease (ClinicalTrials.gov Identifier: NCT03185208, LATTICE trial, currently recruiting). Microdosing of lithium (NP03) at levels 400-fold lower than standard formulations is being considered for clinical trials in humans based on the preclinical studies.<sup>509</sup> The selective GSK3 $\beta$  inhibitor tideglusib (NP031112)<sup>510</sup> was also evaluated for the treatment of AD (ClinicalTrials.gov Identifiers: NCT00948259 and NCT01350362) and PSP (ClinicalTrials.gov Identifier: NCT01049399).<sup>511</sup> However, it failed to show a clinically relevant modification of disease progression despite reducing brain atrophy in PSP,<sup>512</sup> no levels of phosphorylated Tau were reported.

Another Tau-phosphorylating enzyme of interest is Src-family tyrosine kinase Fyn. It specifically phosphorylates the residue Y18, which was found in the Alzheimer's brain NFTs.<sup>513</sup> Despite promising preclinical data<sup>514,515</sup> and good drug penetration and tolerability profile (ClinicalTrials.gov Identifier: NCT01864655),<sup>516</sup> Fyn inhibitor saracatinib (AZD0530) showed no clinical efficacy in Alzheimer's patients with no significant changes in the CSF total or phosphorylated Tau (ClinicalTrials.gov identifier: NCT02167256).<sup>517</sup>

Other Tau kinases have also been considered potential targets for pharmacological agents with expected lowering of Tau phosphorylation and aggregation. These include cyclin-dependent kinase 5,<sup>518</sup> c-Abl tyrosine kinase (c-Abl),<sup>519</sup> lemur tyrosine kinase 2 (LTK),<sup>520</sup> dual specificity tyrosine-phosphorylation-regulated kinase 1A (Dyrk1A),<sup>521</sup> and thousand-and-one amino acid kinases (TAOKs).<sup>522</sup> However, thus far few have proceeded beyond preclinical investigations, or their clinical efficiency is not yet known, for example for the c-Abl inhibitor Nilotinib (ClinicalTrials.gov Identifier: NCT02947893). Tau kinases that (a) phosphorylate Tau *in vitro* or (b) promote its phosphorylation by other kinases, and (c) are implicated in the neuropathological profile of patients with Tauopathies are the potential targets for further investigations.<sup>523</sup> These include casein kinase 1 (CK1),<sup>524</sup> c-Jun amino-terminal kinase (JNK),<sup>525</sup> extracellular signal-regulated kinases 1 and 2 (Erk1 and Erk2),<sup>526</sup> adenosine-monophosphate activated

protein kinase (AMPK),<sup>527</sup> cyclic AMP (cAMP)-dependent protein kinase (PKA),<sup>528</sup> protein kinase N1,<sup>529</sup> tau-tubulin kinases 1 and 2 (TTBK1 and TTBK2),<sup>493,530</sup> Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) and microtubule-affinity regulating kinases (MARKs).<sup>531</sup> Also, targeting of the specific regulatory subunits of Tau phosphatases has been proposed as a viable strategy to selectively reduce pathology-associated phosphorylation of Tau. Tau phosphatases that are implicated in Tauopathies include protein phosphatases 2A and 2B (PP2A and PP2B),<sup>532,533</sup> protein phosphatase 5 (PP5),<sup>534</sup> and calcyclin binding protein and Siah-1 interacting protein (CacyBP/SIP).<sup>535</sup> Recently, we proposed that focusing on inhibiting Tau phosphorylation events that lead to its disassociation from MTs may offer an alternative and more effective strategy to prevent Tau aggregation by stabilizing its native conformations.<sup>150</sup>

**O-GlcNAcylation.** Preclinical studies have shown that increasing Tau O-GlcNAcylation by inhibiting O-GlcNAcase (OGA) can slow aggregation and prevent neurodegeneration in rTg4510,<sup>536,537</sup> Tau-P301L,<sup>538</sup> and JNPL3<sup>326</sup> mouse models. Thiamet G is a potent inhibitor of OGA, and it was demonstrated to reduce Tau phosphorylation at T181, T212, S214, S262, S356, S404, and S409 after acute lateral ventricle injection in mouse brain.<sup>539</sup> Based on this, an OGA inhibitor compound MK-8719, the Thiamet G derivative,<sup>540,541</sup> and ASN120290<sup>542</sup> were developed, and both were granted the orphan drug status for the treatment of PSP, however, their clinical efficacies beyond Phase I trials remain unknown.<sup>543</sup>

**Acetylation.** Salsalate, the derivative of salicylate, primarily a CBP/p300 inhibitor,<sup>544</sup> was investigated in the preclinical studies in the PS19 mouse line overexpressing P301S-Tau. Salsalate reduced Tau acetylation at K174 by inhibiting acetyltransferase p300, and subsequently decreased neuronal loss in the hippocampus, thus reserving memory.<sup>238</sup> Subsequently, salsalate was investigated in Phase I clinical trial for PSP (ClinicalTrials.gov Identifier: NCT02422485). However, it was discontinued due to a lack of efficacy. Further investigations in early Alzheimer's patients are ongoing (ClinicalTrials.gov Identifier: NCT03277573).

**Ubiquitination.** Currently, no therapies are directed at lowering Tau ubiquitination levels or enhancing pathological Tau clearance. Possible strategies might include increasing Tau acetylation at the lysine residues, thus preventing their ubiquitination and Tau aggregation into PHFs that lead to the formation of NFTs. Furthermore, as the impairments of lysosomal and proteasomal protein degradation systems may result in a build-up of ubiquitinated Tau species,<sup>545,546</sup> targeting these pathways to enhance protein clearance might have favorable effects in clearing some of Tau pathology. It is important to stress, that there are no studies on the effect of site-specific mono- or poly-ubiquitination of Tau on its aggregation. Therefore, whether ubiquitination prevents or enhances Tau aggregations remains unclear and might depend on at what stages during aggregation and pathology formation Tau becomes ubiquitinated.

**Tau aggregation inhibitors.** Targeting Tau monomers: initial efforts aimed at targeting Tau focused on trying to identify



small molecules that stabilize the native state of Tau or prevent its aggregation. The dynamic and flexible structure of Tau combined with the fact multiple domains play a role in the initiation of its self-assembly make targeting Tau monomers very challenging. Indeed, no drugs have been shown to stabilize the native monomeric state of Tau and prevent its aggregation.

Preclinical studies provide insights into the possible targets for inhibition of Tau aggregation and  $\beta$ -sheet-containing fibrillization intermediates or oligomers (reviewed in ref. 547). Small molecules, such as methylene blue,<sup>548</sup> orange G,<sup>549</sup> oleocanthal,<sup>550</sup> “molecular tweezer” CLR01,<sup>551</sup> phthalocyanine tetrasulphonate,<sup>552</sup> curcumin<sup>553</sup> and diazodinitrophenol,<sup>549</sup> thiophene,<sup>554</sup> and polythiophene<sup>555</sup> compounds have also been reported to inhibit Tau aggregation *in vitro*. Thus far, methylene blue derivative and curcumin are the only small molecule Tau aggregation inhibitors that have advanced to clinical trials, and none have been approved for clinical use.<sup>556</sup> The most prominent attempt to target Tau aggregation directly has centered on methylene blue. Phenothiazine compound methylene blue (methylthioninium chloride) was found to prevent Tau fibrillization, but not the formation of oligomeric Tau,<sup>557</sup> likely through oxidation of cysteines.<sup>558,559</sup> Phenothiazine could also mitigate Tau-related neurodegeneration and pathology through autophagy induction,<sup>560</sup> reduction of Tau phosphorylation by mitogen-associated kinase 4, reduction of synaptotoxicity,<sup>561</sup> and upregulation of genes by NF-E2-related factor 2/antioxidant response element in mice.<sup>562</sup> Furthermore, due to its primary function as a redox cyler, the total impact of methylene blue is thought to be the result of stimulating the mitochondrial function, metabolism, and reduction of inflammatory responses. Its oldest application in toxicology includes treatment of methemoglobinemia,<sup>563–565</sup> and against malaria from 1891,<sup>566,567</sup> psychiatric conditions such as depression,<sup>568,569</sup> claustrophobia or fear,<sup>570</sup> and ifosfamide-induced encephalopathy.<sup>571</sup> Despite such widespread use of methylene blue in various conditions, unfortunately, no epidemiological studies capturing incidence rates of dementia or AD have been done in these patient populations. Therefore, despite the possible direct effects of methylene blue on Tau fibrillization, it is likely to have pleiotropic effects on brain pathology. Recently, despite promising preclinical data,<sup>572,573</sup> the derivative of methylene blue in a reduced form, LMTX (hydromethanesulphonate; TRx0237) failed to show clinical efficacy in Phase III clinical trials (NCT01689246 and NCT01689233). However LMTX is being investigated as a monotherapy in early stage AD patients (NCT03446001).

**Targeting aggregated and pathological Tau.** Contrary to the extracellular localization of AD-associated amyloid- $\beta$  aggregates, Tau-containing NFTs are predominantly intracellular. This represented a challenge to large antibody-based Tau-targeting strategies due to poor cell membrane penetrance and antibody uptake. However, the discovery that Tau pathology propagates in the brain by mechanisms of cell-to-cell Tau protein spreading<sup>411,413</sup> sparked a huge interest to develop antibodies and therapeutic agents targeting the propagating Tau proteoforms in the extracellular and transsynaptic space. The ultimate goal is to interfere with the propagation of Tau pathology to the

different brain regions after its inception by direct sequestration and neutralization of the toxic and/or seeding competent Tau species.<sup>574</sup>

Despite decades of research, it is still unclear which Tau proteoforms are driving Tau pathology. The reductionist biophysical concepts have dominated the Tau aggregation field and have been useful tools for informing on the kinetics of *in vitro* to advance our understanding of the basic mechanisms and molecular determinants of Tau aggregation. However, it must be stressed that they can only be applied to *in vitro* systems. The processes of Tau aggregation taking place under pathological conditions in cells likely involve multiple yet unidentified cofactors, complex PTMs, and pathological diversity, which could be driven by parallel or cell-type dependent mechanisms of aggregation.

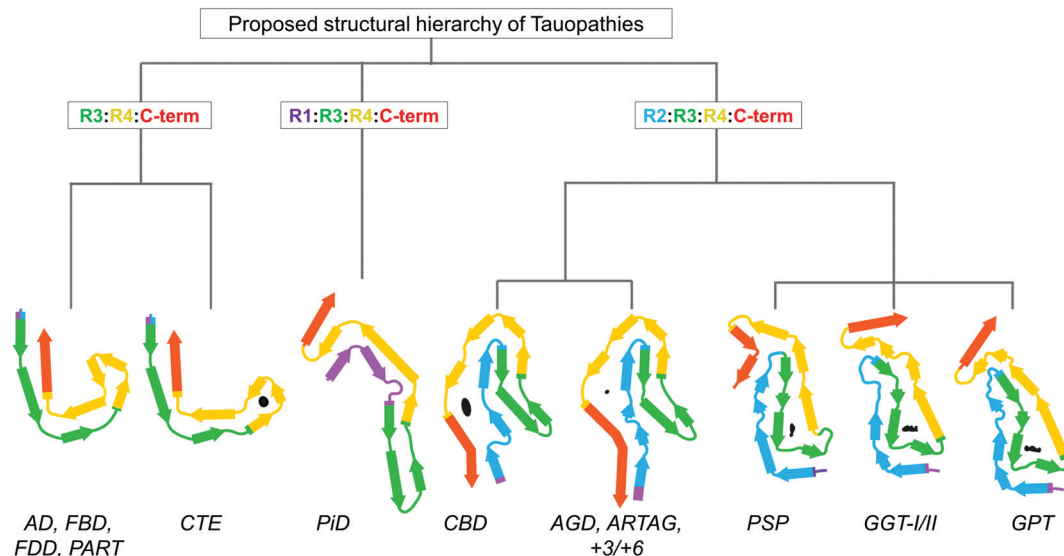
The attempts to systematically identify toxic Tau species, such as oligomers or fibrils, from patient samples are confounded by the difficulties of their purification without disrupting their native structure, in addition to the possibility that there are multiple toxic forms of Tau. Although *in vitro* approaches allowed investigators to produce large quantities of pure Tau fibrils, their biophysical, biochemical, and structural properties substantially differed from the brain-derived material. These factors complicate the rational design of antibodies targeting specific Tau proteoforms due to our limited knowledge of the relevance of these proteoforms to pathological processes, as well as the difficulties to reconstruct the complexity of native Tau proteoforms *in vitro* for the antibody design and screening. Therefore, the exact species of Tau targeted by antibodies are not well defined,<sup>321</sup> and their formation pathways and clinical relevance remain unknown.

The advent of super-resolution imaging techniques, such as cryo-electron microscopy (cryo-EM), allowed for a direct structure visualization of human brain-derived Tau fibrillar cores from postmortem brain tissues of patients with different Tauopathies (Fig. 10).<sup>19,21</sup> These studies have enabled structure based classification of Tauopathies, thus paving the way for a rational design of disease-specific therapeutics and diagnostics.<sup>575</sup> Recently, Cryo-EM was used to directly map the ligand-binding sites within the AD Tau fold.<sup>576</sup> It showed that fibril core residues in multiple diseases primarily comprise the MTBR repeats R3 and R4, which is important in designing the antibodies with epitopes directed outside of these regions. However, this technique is limited to detecting the Tau sequence that constitutes the solid core of fibrils, thus overlooking the less structured Tau domains that might bind ligands, promote Tau aggregation or regulate pathological Tau spreading, for example heavily post-translationally modified proline-rich and C-terminal regions. Furthermore, robust methodological approaches are yet to be developed to isolate the putatively pathological Tau intermediate species and oligomers from the human brain in large amounts and good enough quality for cryo-EM and related techniques.

**Immuno-based anti-Tau therapies.** Passive and active anti-Tau immunotherapies are being actively investigated in both preclinical and human studies<sup>543,577</sup> (Fig. 11). Extracellular Tau







**Fig. 10** Proposed classification of Tauopathies based on the structural hierarchy of the Tau fold formations based on the similarity. Colors denote different Tau domains (reproduced with modifications from ref. 21 with permission from the author(s), under exclusive licence to Springer Nature Limited, copyright 2021).

is a popular target for passive antibody therapies because it does not require cell penetration, and might prevent the cell-to-cell spread of pathological Tau. Antibody ABBV-8E12 targets the N-terminal Tau region, when administered peritoneally to P301S mice reduced brain atrophy and Tau pathology in hippocampus.<sup>578</sup> In humans, ABBV-8E12 (tilavonemab) was assessed in Phase II clinical trials for PSP (ClinicalTrials.gov Identifier: NCT03391765) but discontinued due to the lack of efficacy, and in an early AD trial, which has completed the Phase II (ClinicalTrials.gov Identifier: NCT03712787). Another anti-N-terminus antibody gosuranemab (BIIB092) was evaluated for PSP and other Tauopathies (ClinicalTrials.gov Identifier: NCT03068468); however, it was discontinued in 2019 due to failure to meet primary and secondary endpoints, and no efficacy was found for AD (ClinicalTrials.gov Identifier: NCT03352557, scheduled to end in 2024). Following encouraging results in Phase I (ClinicalTrials.gov Identifier: NCT02820896), semorinemab (RO7105705), which targets N-terminus of all Tau isoforms in both monomeric and oligomeric forms of Tau and is independent of phosphorylation, has been evaluated in Phase II clinical trial TAURIEL that was terminated in early 2020 due to lack of efficacy (ClinicalTrials.gov Identifier: NCT03828747). Another trial LAURIET assessing semorinemab in patients with prodromal and mild AD (ClinicalTrials.gov Identifier: NCT03289143) was reported in August 2021 meeting only one primary end-point out of two, and not meeting any secondary functional or cognitive endpoints. Several other passive immunotherapies targeting different regions of the Tau protein are currently under development, including the MC1-derived antibody zagotenemab in Phase II for AD (ClinicalTrials.gov Identifier: NCT02754830 and NCT03019536); JNJ-63733657, recognizing phosphorylated T217 in Phase II for AD (ClinicalTrials.gov Identifier: NCT03689153, NCT03375697, and NCT04619420); Lu AF87908, recognizing pS396, in Phase I for AD (ClinicalTrials.gov Identifier: NCT04149860); PNT001,

recognizing *cis*-isomer of pT231, in Phase I for AD and TBI (ClinicalTrials.gov Identifier: NCT04096287 and CT04677829); and bepranemab, binding the residues 235–246, in Phase I for PSP (ClinicalTrials.gov Identifier: NCT03605082, NCT03464227, NCT04185415, and NCT04658199). Another Tau MTBR-targeting antibody E2814 is currently recruiting participants to be investigated in clinical trials commencing in 2021 (ClinicalTrials.gov Identifier: NCT04971733) within the Dominantly Inherited Alzheimer Network Trials Unit (DIAN-TU) program led by the Washington University School of Medicine.<sup>579</sup> Despite the lack of success in the clinic so far, the interest in the research and development of the anti-amyloid Tau-targeting therapies is likely to increase following important, albeit controversial,<sup>580,581</sup> by United States' Food and Drug Administration's approval<sup>582</sup> of amyloid- $\beta$  targeting AD drug BIIB037<sup>583</sup> (aducanumab, ClinicalTrials.gov Identifier: NCT02477800, NCT02484547). It is important to stress that many of these clinical trials have been driven by studies exploring antibodies targeting different regions in Tau and efficacy in preclinical models rather than precise targeting of disease-relevant pathogenic species. Differences in Tau aggregate conformations and biochemical properties between rodent and human brains could explain the failure to translate success in mice to the clinic. The large number of structural data emerging from brain-derived pathological aggregates from Tauopathies is helpful but may not be sufficient to guide disease-specific therapies and diagnostics as they lack information about the large segments of the protein flanking the core of the fibrils, and which could still play important role in the regulation of Tau pathogenic properties. Therefore, a combination of comprehensive structural and biochemical profilings of the Tau species and aggregates in the brain is essential to develop more effective therapies and diagnostics.

Active immunotherapies (vaccinations) for Tau include delivery of Tau sequence fragments to elicit the immune



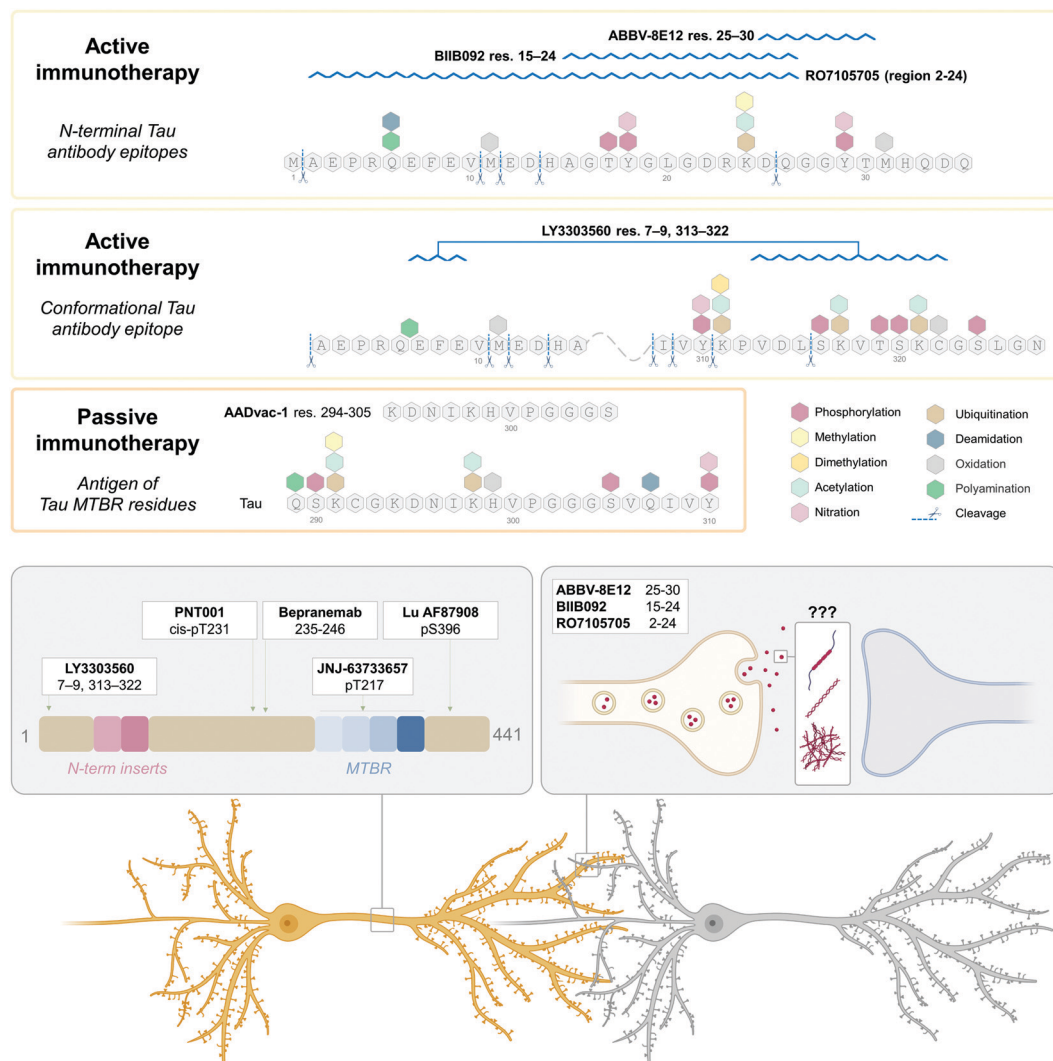


Fig. 11 Active and passive Tau immunotherapies. Known epitopes of antibodies are aligned to the Tau sequence with highlighted PTMs. Putative intracellular and extracellular Tau targeting antibodies with epitope amino acid numbers where known.

response of the recipient.<sup>577</sup> This should arguably prevent the pathology formation, or help clear existing Tau aggregates and inclusion through their recognition by the innate immune system. The furthest therapy along the development pipeline is AADvac-1, which is a Tau peptide consisting of residues 294–305 adjunct to keyhole limpet hemocyanin.<sup>584</sup> It was evaluated in the Phase II trial in AD (ClinicalTrials.gov Identifier: NCT02579252) with promising results that will further its evaluation in the Phase III trial. Another active Tau vaccination is ACI-35 which includes 16 copies of pS396 and pS404 Tau in a liposome carrier. This showed a robust immune response in P301L-Tau and wild-type mice, and produced antibodies that bound the NFTs, and reduced insoluble and soluble Tau levels in the brain homogenates.<sup>585</sup> In human subjects, redesign into ACI-35.030 resulted in a higher immune response compared to the original version. Currently, no investigations in the clinical settings are underway for this therapy.

**Nanobodies.** Another potentially promising approach to target Tau pathology propagation is using nanobodies such

as single-chain variable fragments (scFvs) or intrabodies (iBs) to recognize, bind and intercalate into Tau fibrillar structures.<sup>586</sup> Nanobodies have the advantage of being much smaller in size than conventional multiple-chain antibodies, thus binding to epitopes with low accessibility due to steric hindrance, and can better penetrate across the blood–brain barrier (BBB) and cell membrane. Several preclinical studies investigated the therapeutic potential of nanobodies. The strategy for the development of anti-Tau nanobodies was first published in 2002. A candidate nanobody targeting Tau domain 151–422 (scFv#2) was shown to penetrate the cell cytoplasm and the nucleus, and bind the intracellular Tau.<sup>587</sup> Another nanobody, scFv-RN2N, targeting 2N Tau isoforms, efficiently crossed the BBB, which could be enhanced by scanning ultrasound, in transgenic pR5 human Tau P301L mice.<sup>588</sup> Furthermore, the GSK3 $\beta$ -mediated Tau phosphorylation at mid-domain epitopes recognized by antibodies AT8 (pS202/pT205/pS208) and AT180 (pThr231) was inhibited, whereas C-terminal phosphorylation at S404 was not affected. On a phenotypic level, these mice



showed a reduction of anxiety-like behavior in elevated plus-maze.<sup>588</sup> Studies in rTg4510 and JNPL3 P301L Tau mice showed higher efficacy of iBs than scFvs derived from antibodies PHF1, CP13, and Tau5 when overexpressed in the brain.<sup>589</sup> Nanobodies prevented aggregation upon mutant 4R0N S320F Tau overexpression in HEK293T cells. However, the *in vivo* effects on the motor phenotypes of the mice were only modest, with no assessment of cognitive functions.<sup>589</sup> The latest efforts to develop new nanobodies targeting Tau showed their ability to reduce heparin-induced Tau aggregation *in vitro* and prevent Tau pathology propagation by templating when expressed in the mouse brain (preprint at the time of writing<sup>590</sup>). Further exploration of target engagement of the post-translationally modified Tau epitopes should be explored to determine the feasibility of recognition of the large PTMs, such as ubiquitin groups, by the small nanobodies.

**PROTACs.** Another approach to clearance of toxic Tau species is by using proteolysis targeting chimera proteins (PROTACs).<sup>591</sup> These work through binding of the target molecule such as Tau or Tau aggregates by the ligand connected through the linker to the ligand-binding the ubiquitin ligase molecule.<sup>592</sup> Ubiquitin ligase then ubiquitinates the target molecule, therefore directing the entire complex for the degradation by the proteasome. Recent advances in rational protein engineering and evolution have allowed multiple target proteins to be directed for proteasomal clearance to ameliorate the cellular pathology they may cause. These include B-cell lymphoma 6 (BCL6),<sup>593</sup> focal adhesion kinase (FAK),<sup>594</sup> androgen<sup>595</sup> and estrogen<sup>596</sup> receptors, and Bruton's tyrosine kinase (BTK)<sup>597</sup> for cancer treatments, P300/CBP-associated factor and general control nonderepressible 5 (PCAF/GCN5)<sup>598</sup> and interleukin-1 receptor-associated kinase 4 (IRAK4)<sup>599</sup> for immunotherapies, as well as signal transducer and activator of transcription 3 (STAT3).<sup>600</sup>

The PROTAC approach for Tau clearance has been investigated in preclinical models. TH006 peptide was designed to recruit both Tau and ubiquitin ligase von Hippel-Lindau tumor suppressor protein (Vhl), and reduce Tau levels in 3xTg-AD mice and primary neuronal cultures.<sup>601</sup> PROTAC binding Tau and ubiquitin ligase kelch-like ECH-associated protein-1 (Keap1) efficiently reduced Tau levels in cellular SH-SY5Y human bone marrow cancer model and rodent Neuro-2a and PC-12 cell lines.<sup>602</sup> Small molecule PROTACs that recruited ubiquitin ligase Cereblon were reported to degrade non-modified and phosphorylated Tau in human neuronal models harboring Tau mutations A152T or P301L.<sup>603</sup> Another PROTAC recruiting molecule Cereblon was designed from the Tau PET tracer <sup>18</sup>F-T807.<sup>604</sup> The compound QC-01-175 cleared Tau in FTLN patient-derived neurons, resulting in a higher resilience of cells to stress. Presumably, QC-01-175 selectively bound to and degraded the monomeric aberrant FTD-associated Tau variants, as the clearance of monomeric Tau in the neurons derived from healthy controls was insignificant. Another small molecule, C004019, designed to bind both monomeric Tau and Vhl ubiquitin ligase efficiently targeted it for degradation by the proteasome in cellular and mouse models.<sup>605</sup> C004019 was efficient at improving synaptic and cognitive deficits in hTau and 3XTg-AD mice. The reduction in the high molecular weight

Tau observed was attributed to the shift in the equilibrium between soluble monomeric and oligomeric or aggregated Tau species. The Tau aggregates are not preferentially degraded by the proteasomal pathway.<sup>606,607</sup> A related approach to targeted Tau clearance involves the direct proteolysis of bound Tau, conceptually demonstrated by the artificial synthetic apocyclo-hybrid hydrolase I2-Cu(II),<sup>608</sup> which was designed to recognize the Tau hexapeptide PHF6, VQIVYK, present in MTBR R3 repeat. I2-Cu(II) cleaved the Tau fragments and prevented their aggregation *in vitro*, and reduced the overexpressed Tau-EGFP signal in the N2a cell line.

## Conclusions and implications for targeting Tau

Converging evidence continues to point towards Tau aggregation as one of the central events in the pathogenesis of AD disease and other Tauopathies. The relative contribution of loss of functions *vs.* gain of toxic functions to the pathogenesis of Tauopathies remains unknown. However, it is clear that preventing Tau aggregation in the first place and neutralizing its seeding activity would also contribute to maintaining the normal level of Tau and thus minimize loss of Tau function. Therefore, targeting the process of Tau aggregation, instead of just specific Tau species only, remains a viable therapeutic strategy for treating Tauopathies.

Despite significant advances in understanding the morphological and structural properties of Tau fibrils, many fundamental questions about what causes Tau to aggregate in the first place and the role of cofactors, Tau PTMs, and Tau interactome in regulating Tau aggregation, pathology formation, and toxicity remain unknown. In this review article, we provide an overview of our current understanding of the sequence and molecular determinants of Tau aggregation and the various experimental approaches that have shaped our understanding of its mechanisms of aggregation and role in Tauopathies. Our analyses of the literature revealed a widening gap between the complexity of Tau sequence, structure, and pathology in the brain, and the tools and model systems commonly used to investigate the mechanisms of Tau aggregation and toxicity in research laboratories. We also showed that many of the methods and assays to screen for modifiers of Tau aggregation and toxicity are carried out using unmodified Tau aggregates that do not share the same biochemical signatures or structural properties of pathological Tau. Currently, most experimental approaches do not take into account the diversity of Tau PTMs, the complexity of the Tau PTM patterns in their physiological and pathological forms, or the fact that the Tau PTM patterns change during disease progression. Therefore, it is not surprising that the conventional approaches have not borne tangible results in the clinic. We highlight the necessity to revisit the Tau aggregation processes, associated cofactors, and develop more suitable models that recapitulate the structural and biochemical diversity of pathological Tau. The extensive mechanistic studies and experimental approaches that have been used to investigate and gain insight into the role of heparin in regulating Tau aggregation are useful and can be extended to other newly discovered cofactors.





One important consideration for Tau-targeting antibody-based therapies is defining the precise epitopes or conformational states the antibodies are engineered to recognize. Tau is extensively modified, with cleavage, phosphorylation, ubiquitination, and O-GlcNAcylation potentially interfering and preventing the binding of antibodies raised and tested against non-modified forms of Tau protein produced *in vitro* by conventional approaches. This includes PTMs on the neighboring residues. Also, epitope masking and inaccessibility due to conformational changes in Tau might contribute to less recognition and binding by the antibodies. The design of Tau-specific antibodies should be informed by the data from patient-derived pathological Tau and using reagents that reproduce the key features of the relevant Tau proteoforms. This could be achieved using semisynthetic and synthetic strategies to produce precisely-post-translationally modified Tau, which is then fibrillized using a clean and efficient system with the addition of pathology-relevant cofactor molecules. This will allow to raise and optimize antibodies that have a higher potential to yield efficient Tau pathology modifying molecules that will be successful in clinical settings. One alternative approach is to develop cellular and animal models that recapitulate pathological Tau aggregation at the structural and biochemical levels. Today, it remains unknown whether Tau aggregates in cellular and animal models of Tauopathies possess the same core structure and morphological features of brain-derived Tau aggregates. More detailed biochemical and structural characterization of Tau aggregates in these models is essential to maximize the translation of preclinical success to more effective therapies in the clinic.

Several approaches have been employed to generate antibodies targeting different epitopes and aggregated forms of Tau species, and many are in clinical trials today. Given that many of these antibodies' design was not guided by precise information about the pathogenic Tau species in the brain, it should not surprise anyone that many of these antibodies will not succeed in clinical trials. However, the outcomes of these clinical trials will provide important insight into the potential of sequence and species-specific antibodies for the treatment of AD. They will also help guide future efforts to develop more effective antibodies and therapies. That being said, many steps could be introduced now to minimize failures or ensure that ineffective antibodies are identified early in the process, including (1) employing structure-based and computational approaches that leverage the availability of several cryo-EM structures of brain-derived Tau fibrils,<sup>609</sup> (2) more comprehensive assessment of the biochemical properties (PTMs) of Tau species in the brain and the CSF; (3) evaluating the efficacy of antibodies in multiple animal models of Tau pathology formation and spreading, and (4) rigorous characterization of the specificity of the antibodies towards different pathologic Tau species. This could be achieved using expanded libraries of (a) recombinant Tau species (monomers, oligomers, and fibrils) bearing pathologically relevant PTMs, and (b) well-characterized soluble and insoluble Tau aggregate preparations isolated from brains of different patients with Tauopathies. This approach would determine the extent to which therapeutic antibodies can capture the diversity of pathological Tau species

detected *in vivo*. Although cryo-EM studies of Tau fibrils suggest that the Tau fibrils in the brain are more homogeneous and possess structural features specific for each Tauopathy, it remains unclear if these studies are capturing the diversity of Tau fibrils, or mainly the dominant structures. In contrast, increasing evidence suggests that soluble oligomeric and seeding competent forms of Tau are highly heterogeneous in their size distribution and PTM patterns. Therefore, elucidating the structural properties of recombinant and brain-derived Tau oligomers is essential to any future efforts to improve the precision of therapeutic antibodies and their efficacy.

Although multiple factors may contribute to the failure of clinical trials, the wide range of Tau structures seen in the patient populations may contribute to poor targeting efficiency by the structure-based Tau aggregation inhibitors. This is especially important given recent findings revealing a high level of heterogeneity in the PTM profiles and seeding activity of Tau aggregates from AD patients' brains.<sup>118</sup> Patient stratification based on the disease stage, informed by the robust peripheral biomarkers, may be crucial to determine the appropriate target population and the windows of therapeutic intervention for disease progress-modifying effects. This may involve the early and pre-/asymptomatic stages, where the Tau pathology is developing but has not yet fully progressed and amassed in multiple brain regions. The clearance of the already-formed Tau fibrillar burden and prevention of its spreading is being targeted by immuno-based anti-Tau therapies discussed above.

Like targeted immunotherapies, it is crucial to know precisely which Tau species should be targeted by PROTACs. These target species may include (1) monomeric Tau to reduce the total Tau levels and maintain an equilibrium of Tau species in favor of soluble monomers, (2) monomeric Tau harboring disease-associated mutations, (3) small oligomers that can be efficiently cleared by the proteasome before exerting cytotoxicity or proceeding to form larger aggregates, or (4) seeding competent fibrillar Tau aggregates. Artificially designed hydrolases targeting specific Tau oligomeric and fibrillar species, and directly degrading them, represents a promising strategy to clear the incipient or established Tau pathology.

Although this review focuses on understanding the grammar of Tau aggregation and Tau aggregate-targeting therapies, we would like to re-emphasize that more investments are needed to understand how the loss of normal Tau due to misfolding, aggregation, and seeding contributes to neurodegeneration and clinical symptoms at different stages of disease progression. It is our opinion that the most effective therapies will be combination therapies that, firstly, prevent Tau misfolding and aggregation, and, secondly, neutralize existing bioactive Tau aggregates. The clinical heterogeneity of the AD and other Tauopathies combined with increasing evidence of the possible roles of other Tau-aggregation independent mechanisms in the pathogenesis of Tauopathies suggest that future therapies will involve personalized combination therapies based on molecular mechanisms associated with each disease subtype. Such therapies are more likely to be effective at slowing the progression of the Tauopathies at different stages of disease diagnosis. The sooner



we embrace the concept of combination personalized therapies, the better.

## Conflicts of interest

Hilal Lashuel has received funding from industry to support research on neurodegenerative diseases, including from Merck Serono, UCB, Idorsia and Abbvie. These companies had no specific role in the conceptualization and preparation of and decision to publish this work. H. A. L. is also the Founder and Chief Scientific Officer of ND BioSciences SA, a company that develops diagnostics and treatments for neurodegenerative diseases based on platforms that reproduce the complexity and diversity of proteins implicated in neurodegenerative diseases and their pathologies. Galina Limorenko has no conflicts to declare.

## Acknowledgements

We would like to thank Laura Gasparini, Somanath Jagannath, Rajasekhar Kolla, Senthil Kumar Thangaraj, Pedro Santana Magalhães and Theodora Panagaki for providing valuable critical assessment of the manuscript.

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