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A current view on Tau protein phosphorylation in Alzheimer's disease

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Abstract

The functions of the neuronal microtubule-associated protein Tau in the central nervous system are regulated by manifold posttranslational modifications at more than 50 sites. Tau in healthy neurons carries multiple phosphate groups, mostly in its microtubule assembly domain. Elevated phosphorylation and aggregation of Tau are widely considered pathological hallmarks in Alzheimer's disease (AD) and other tauopathies, triggering the quest for Tau posttranslational modifications in the disease context. However, the phosphorylation patterns of physiological and pathological Tau are surprisingly similar and heterogenous, making it difficult to identify specific modifications as therapeutic targets and biomarkers for AD. We present a concise summary of - and view on - important previous and recent advances in Tau phosphorylation analysis in the context of AD.

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Abbreviations

Ser, serine; Thr, threonine; Tyr, tyrosine; Lys, lysine; Arg, arginine; Leu, Leucine; G, Gly; TBS, tris-buffered saline; GG, glycine–glycine; LRGG, Leu–Arg–Gly–Gly; AD, Alzheimer's disease; CSF, cerebrospinal fluid; MAP, microtubule-associated protein; MAPT, Tau gene; MT, microtubule; MS, mass spectrometry; NFTs, neurofibrillary tangles; PTM, post-translational modification; P_i, phosphate group; PRR, proline-rich region (of Tau protein); RD, repeat domain (of Tau protein); R_g, radius of gyration.

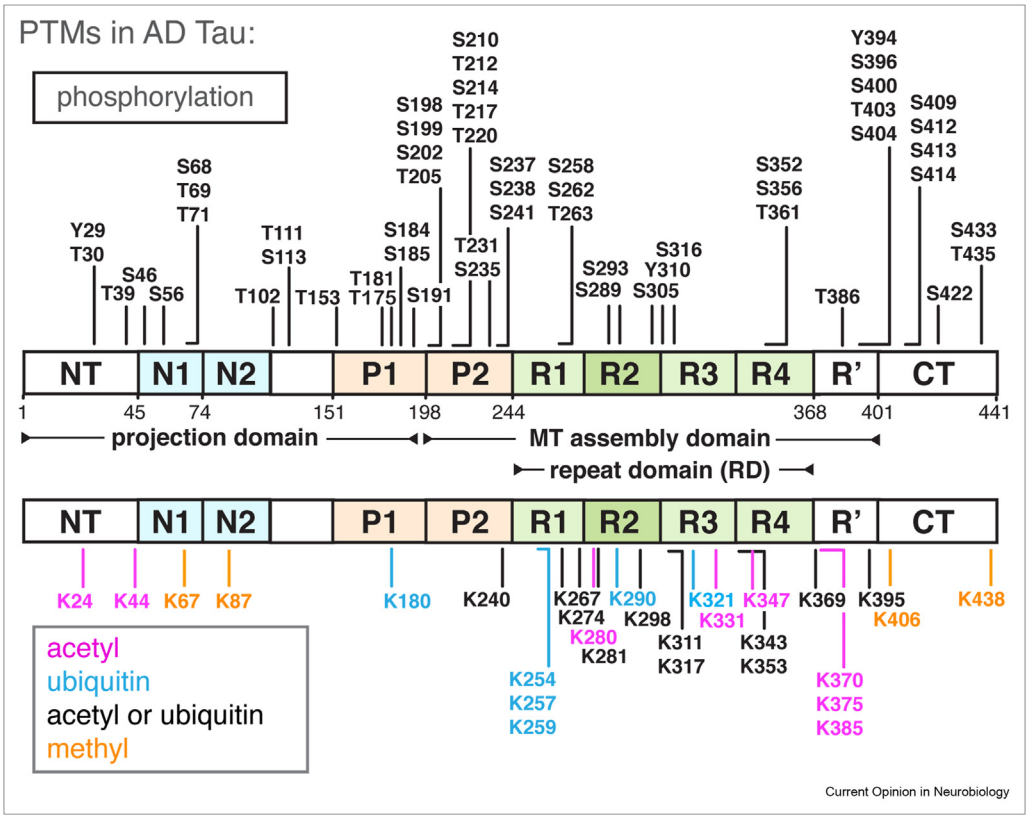
Introduction

The microtubule-associated protein Tau contributes to the stability of axonal microtubules (MTs) in the brain and, in this role, is involved in the regulation of axon outgrowth and axonal transport. The binding of Tau to MTs is regulated by post-translational modifications (PTMs), mostly phosphorylation [14], which also controls various other less characterized functions of Tau [51]. Tau was initially discovered by *Kirschner* and colleagues, in a search for MT-associated proteins (MAPs) that enhance MT self-assembly [61]. From a biomedical perspective, the interest in Tau rose sharply when Tau was identified as a principal component of neurofibrillary tangles (NFTs), a pathological hallmark found in the brains of Alzheimer's disease (AD) [27]. By implication, the progressive pathological accumulation of phosphorylated and aggregated Tau has since been used for the staging of AD and other tauopathies [10,33]. However, the causal relationship between phosphorylation and aggregation is still a matter of debate. Interestingly, fetal Tau and Tau from AD brain appear to have a similar high Tau phosphorylation levels, despite the absence of aggregation of fetal Tau during neuronal development [12,38,63].

Tau is a highly soluble intrinsically disordered protein that occurs in six main isoforms in the human central nervous system [25]. The isoforms differ in their content of three alternatively spliced exons, producing Tau isoforms with 0, 1, or 2 inserts in the N-terminal projection domain (N1 and N2 isoforms), and 3 or 4 pseudo-repeats (3R and 4R isoforms) in the Tau repeat domain (RD), which covers most of the MT-assembly domain[1] and is responsible for Tau aggregation (Figure 1). Along the amino acid (aa) sequence of Tau, ~85 putative phosphorylation sites (p-sites) have been predicted (Ser, Thr, Tyr), of which >50 are found to be modified in Tau ([21,42]; Tau phosphorylation site table: https://www.kcl.ac.uk/people/diane-hanger). The distribution of p-sites in the Tau sequence is asymmetric, such that most of the nonmodified sites are located in the acidic N-terminal half (up to residue

[1] The RD (Figure 1) is often described in the literature as 'microtubule-binding domain' (MTBD). This term is misleading, as the RD alone has only a weak affinity for microtubules. Efficient binding and assembly of microtubules is achieved by the RD plus the flanking domains, herein denoted as 'MT assembly domain' (Figure 1).

Figure 1



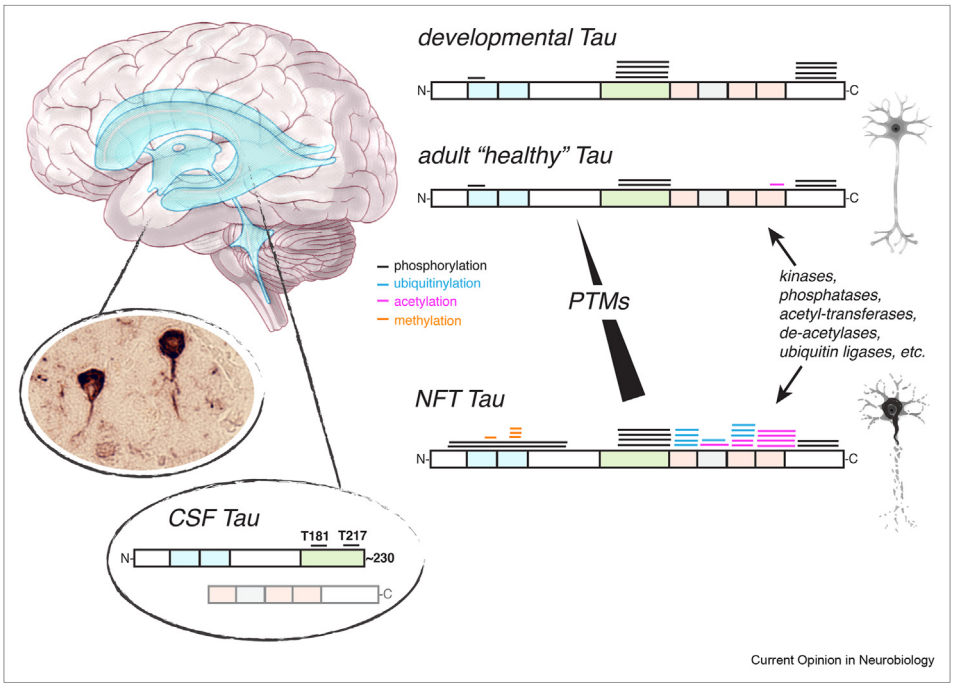
PTMs occurring in Tau isolated from *postmortem* AD brains. PTMs found in Tau from AD brains are mapped on the sequence of the longest Tau isoform (2N4R, UniprotKB P10636-8; modification sites taken from Ref. [62]). Phosphorylation (residues indicated above the domain structure in gray) is the major PTM of Tau and is distributed heterogeneously in the Tau sequence, with most abundant p-sites clustering in P1 and P2 of the proline-rich domain (PRD) between residues T181 and S235, and in regions C-terminally of the RD (repeats R1–R4) in between residues S396 and S404 (R′ and CT). The N-terminal projection domain includes the inserts N1 and N2 and is frequently phosphorylated as well. Acetylation (pink) and ubiquitinylation (blue) occur mostly in the MT-assembly domain, and often alternatively at the same residues (black). Methylation (orange) is rare and found only in the projection domain and the C-terminal domain (CT). The position of PTMs other than phosphorylation is indicated below the Tau domain structure.

~200), whereas the basic C-terminal half contains most of the observed p-sites (i.e. the RD and its adjacent flanking domains; Figure 1). The addition of a single phosphate group (P_i) adds a net charge of −1 to the protein, and the clustering of p-sites in Tau induces large local charge effects that can alter molecular interactions (e.g. MT binding) and Tau conformation [17,50]. Notably, the addition of acetyl or methyl groups to a lysine residue also changes the Tau net charge by removing positive charges. These modifications can compensate much of the basic character of the protein.

Because aggregated and soluble Tau from AD brains shows reactivity to antibodies detecting phosphorylated Tau [4], and to an antibody reactive for nonphosphorylated Tau after alkaline phosphatase digestion [27], it was concluded that Tau 'hyperphosphorylation' is associated with Tau aggregation and toxicity. Until today, the detection of specific phosphorylated sites in Tau—together with silver or thioflavine staining of beta-sheet containing Tau aggregates—is the main method to describe 'pathological alterations' in Tau (Figure 2; [9,11,27]).

However, a caveat for PTM studies on human tissue might be the rapid change (minutes) of enzymatic activities after death, compared to the extended *postmortem* period (hours), which holds in particular for phosphorylation of Tau: in a simple model, ATP-dependent enzymes, such as kinases, become inactive fast, whereas energy-independent enzymes, such as phosphatases or proteases, may remain active. However, in fact, a much more complex reality has been shown for the activity of Tau's main kinases and phosphatases, which shows a differential decrease in enzymatic activity upon lowering of the body temperature and in postmortem brains [24,45]. A similarly complex scenario may exist for other Tau modifying enzymes and PTMs.

Figure 2



Changes in Tau PTMs during AD. Tau extracted from healthy adult brains (middle bar, right) is moderately phosphorylated in the 'phosphorylation hot spots' between epitopes S198–S217 in the PPR (green region) and S396–S404 in the C-terminal part, with some phosphorylation in the insert N1. In contrast, detergent-insoluble *NFT Tau* isolated from AD brain (bottom right), is phosphorylated in the same protein regions but with much higher probability, and extra p-site occupancy occurs in the N-terminal end. In addition, *NFT Tau* is extensively ubiquitinylated and acetylated in the RD (orange region), and to some extent in the N-terminal inserts (blue region). As Tau modifying enzymes can be active on healthy Tau as well as on aggregated *NFT Tau*—also *postmortem*—it remains unclear to which degree Tau PTMs contribute to its aggregation. During neuronal development (top), Tau is phosphorylated to a similar degree as *NFT Tau*, suggesting that high Tau phosphorylation may have a functional role in cells and is not *per se* associated with Tau aggregation. *CSF Tau* from AD patients, which is used as a biomarker for AD progression, contains mostly N-terminal Tau fragments covering aa residues 1 to ~230. Small fractions of C-terminal Tau fragments have been identified as well in the CSF. If and how CSF Tau PTMs (phosphorylation and truncation) occur before Tau release from neurons or during its 'journey' into the CSF need to be further evaluated. Image shows NFT neurons filled with phosphorylated Tau labeled with an antibody against epitope pS396/pS404 (PHF-1) in human AD temporal cortex.

The pronounced phosphorylation of Tau extracted from AD brain is obvious in SDS-PAGE gels, where protein bands of Tau are substantially shifted toward a higher molecular weight [39]. In contrast, normal adult brains show an apparently low phosphorylation state of Tau. These observations support a hypothetical causal relationship between Tau phosphorylation and pathological Tau aggregation in the brain, and therefore trigger the quest for an AD signature pattern of phosphorylation (and other PTMs), which can be explored for therapeutic targets and biomarkers of the disease. Accordingly, most Tau-targeted therapeutic approaches aim at inhibiting 'pathology-inducing' kinases, target pathological phosphorylation sites or aggregation of Tau, or try to prevent Tau spreading across the brain. The current Tau-centric view of neurotoxicity in AD is based on several observations, for example (i) Alzheimer's original discovery of NFTs, (ii) the progressive appearance of Tau aggregates in parallel with clinical symptoms of AD [10], and (iii) the identification of numerous Tau mutations associated with neurodegenerative tauopathies that show Tau phosphorylation or aggregation similar to AD [52]. However, most AD cases are sporadic and not associated with Tau mutations, suggesting that (unknown) cofactors trigger Tau pathology in the disease. Notably, most Tau mutations in primary tauopathies are located in the Tau RD and flanking regions, protein domains involved in fibrillar aggregate formation and MT binding. Tau mutations generally increase the aggregation propensity of Tau molecules independently of phosphorylation, and there is no obvious relationship with mutations and phosphorylation sites.

Mass spectrometry (MS) of Tau peptides generated by enzymatic digestion helped to identify new modifications and their positions on Tau [30]. In recent years, the use of tandem MS, protein isotope labeling, 'native MS,' and 'smart' sample preparation approaches helped to quantify the phosphorylation and other PTMs of Tau in AD, and to define Tau species in brain lysates and cerebrospinal fluid (CSF) as potential therapeutic targets and biomarkers in AD [8,42].

Aggregated Tau in Alzheimer's brains: defining targets for tau-targeted immunotherapies

The abnormal accumulation of phospho-Tau in disease phenotypic inclusions is a pathological marker in primary and secondary tauopathies [34], and occurs in other neurodegenerative diseases as bystander pathology [52], recently shown also for amyotrophic lateral sclerosis [44].

Studies investigating the PTMs of AD Tau focus on the composition of detergent-insoluble Tau aggregated in NFTs, a pathological hallmark in AD. NFT Tau can be enriched through detergent and acid extraction. Using stable isotope-labeled Tau reference peptides, an enrichment of highly phosphorylated 0N4R and 1N4R Tau isoforms was detected in insoluble Tau from AD brains [49,62].

In Tau from AD brains (frontal and parietal cortex), Steen and colleagues identified modifications on 43−55 different phosphorylation, 19 acetylation, 14−17 ubiquitination, and 4 methylation sites [62]. AD is a progressive neurodegenerative disease, in which Tau pathology starts in distinct brain areas (entorhinal cortex and hippocampus) and from there spreads through the brain in a predictable spatial pattern [10]. The spread of Tau aggregation correlates with cognitive decline, hence disease state, of AD patients. The progressiveness and spatial restriction of Tau pathology in AD generates the possibility to generate 'snapshots' of early-, mid-, and late-stage Tau changes. To correlate Tau PTMs with disease state (healthy > mild cognitively impaired > AD), soluble Tau was separated from detergent-insoluble material, and TBS-soluble Tau was further differentiated in early (larger R_g/molecular weight) and late (smaller R_g/molecular weight) size exclusion fractions. PTM analysis of these Tau preparations across 49 subjects suggested that during the progression of AD, first the normal phosphorylation in the regions flanking the Tau repeats, found in physiological soluble Tau from healthy controls, is further extended in aggregated Tau, then additional phosphorylation sites become visible in the N- and C-terminal ends of Tau, and acetylation and ubiquitination sites become apparent at lysine residues of the RD. Notably, the Tau PTM pattern between AD individuals is heterogenous, suggesting no defined AD-associated phosphorylation pattern but rather a certain propensity for phosphorylation at certain p-sites. In Tau from AD brains, the propensity for phosphorylation at T231, S235, and S262 seems to correlate with clinical progression of AD, as well as correlating with a potential to induce local Tau accumulation in HEK cells [22]. The modification of Tau in aggregates from AD brain can impact the formation and structure of Tau aggregates [2], and therefore also influence their potential to seed Tau aggregation [48].

Acetylation and ubiquitination occur mostly on lysine residues in the Tau RD, whereby the same residues can be modified by either PTM [43,62], suggesting a possible competitive modification by these PTMs. Acetylation is a frequent modification on Lys and Arg, and, *in vitro*, was found to enhance Tau aggregation [15] and inhibit liquid−liquid phase separation of Tau [55]. Interestingly, certain types of Tau phosphorylation can inhibit aggregation [29] and enhance Tau phase separation [59], indicating opposite effects of these modifications on Tau (mis)function.

Ubiquitination of Tau occurs mainly in the proline-rich domain (PRD) and RD and is largely increased in AD Tau, where ~28 ubiquitinated sites are modified [1]; a crosstalk between ubiquitination and phosphorylation in KxGS motifs of the RD was suggested [1]. From an MS perspective, digestion of ubiquitinated proteins with trypsin cleaves the ubiquitin chain and leaves residual GG (complete cleavage) or LRGG 'tags' (incomplete cleavage) on modified Lys, independent of the number of attached ubiquitin moieties. As ubiquitination enhances the degradation of (aggregated) Tau by autophagy and the proteasome [40], it becomes difficult to detect this modification unless it is present in high amounts. Controversially, cryo-EM recently suggested a ubiquitin moiety in the core of Tau fibrils from human brains [2], although the size of this PTM, comparable to GFP, is large enough to sterically hinder the formation of beta-sheet stacks in the Tau fibril core [37]. It is therefore likely that only a fraction of Tau molecules in the analyzed aggregates carried this modification.

Other PTMs, such as GlcNAcetylation and methylation, occur at low abundance in the Tau sequence [43]. O-linked glycosylation occurs on Ser or Thr and can compete with phosphorylation [41]. Methylation on Lys residues is present to low but similar degrees in AD and control brains, whereby the methylation changes from primarily di-methyl to mono-methyl in AD and during aging [32]. It was suggested that methylation enhances Tau aggregation [23]. Overall, the observed modifications in AD Tau are surprisingly similar to the ones identified in mouse Tau from WT and A-beta transgenic mice [43].

Up to now, it remains unclear if PTMs in insoluble Tau from AD and control brains occur before or after the formation of insoluble aggregates. Whereas residues in the RD are buried in the core of fibrillary Tau aggregates, and are therefore presumably inaccessible for modification after aggregation, the unstructured N- and C-terminal parts of Tau protrude from the fibril core [60] and therefore in principle remain accessible for the addition or removal of PTMs after fibrillization.

Soluble Tau from Alzheimer's brains: phosphorylation in absence of aggregation

Recent studies showed that many of the p-sites abundant in aggregated Tau from AD brains are, with some changes in the extent of single p-site occupancies, also present in functional soluble Tau from cells and human brain [26,31,42].

The main p-sites used for characterizing pathological AD Tau by p-site–specific Tau antibodies like AT8, AT100, or PHF-1 (e.g. pT181, cluster [pS199, pS202, pS205], pS217, pS235, pS262, pS396, and pS404) are to a lesser extent also modified in Tau from healthy control brains [42,49,62]. Tau phosphorylated at these sites can therefore not *per se* be considered disease-associated or toxic, and using these p-site epitopes to identify AD Tau should thus be complemented with other measures of pathological Tau alterations, such as silver or thioflavin staining of Tau aggregates, PAGE approaches to verify oligomeric Tau species, or immunolabeling of misfolded Tau conformers. Interestingly, the likelihood of phosphorylation in the sequences adjacent to these p-sites (aa243-254 and aa354-369) are modified in 50% of the soluble Tau molecules but vanishes in insoluble AD Tau, indicating a possible role in aggregation inhibition or physiological function of these residues. Identifying the function and origin of modifications in these aa stretches in healthy Tau could host information about changes in Tau leading to aggregation.

Tau is a highly abundant axonal protein, with an estimated average concentration of ~2 μM in the neuronal cytoplasm. When expressed for recombinant protein production in insect cells, cytosolic concentrations of >200 μM phosphorylated Tau can be reached [54], whereby the level of phosphorylation (~8 P_i per Tau molecule; [21,54]) was found to be similar to 'hyperphosphorylated' Tau extracted from Alzheimer's AD brains [42]. Despite the similar level of phosphorylation and occupancy of p-sites, no formation of aggregated Tau species could be detected in Tau expressing insect cells, not even when the phosphorylation was further raised to ~14 P_i per Tau molecule upon PP2A inhibition using okadaic acid [21]. After purification, the phosphorylated Tau formed some oligomers and rarely fibrils.

Reversible high phosphorylation of Tau is a normal biological process in hibernating animals [3] and during sleep [28]. Developing neurons naturally have Tau phosphorylation levels similar to 'hyperphosphorylated' Tau from AD brains [12], which becomes reduced upon maturation. Reversible nonpathological phosphorylation of Tau relies on the concerted interplay of Tau kinases (GSK3beta, CdK5, PKA, MARK, Fyn, and others) and phosphatases (mainly PP2A), and a change in activity of either could lead to elevated Tau phosphorylation. High p-Tau levels detected in *postmortem* AD brains may thus in part be due to a reduction in PP2A activity [53] or by inaccessibility of phosphorylated epitopes in Tau aggregates to be dephosphorylated by the enzyme.

Many current therapeutic approaches target specific Tau phosphorylation by modulation of Tau kinase activity or by targeted immunotherapy [16]. Other approaches aim for the activation of Tau phosphatases (under the assumption that AD-like high phosphorylation is responsible for toxicity) to decrease overall Tau phosphorylation, or enhance the degradation of Tau with small molecule drugs. Alternatively, preclinical therapeutic approaches reducing the amount of total Tau in the adult brain on the mRNA level circumvent the necessity of knowing the precise 'toxic Tau' target and have shown beneficial effects on multiple levels of Tau-associated pathology: the accumulation of phosphorylated Tau is inhibited, the pathological accumulation of aggregated Tau is halted, neurotoxicity due to neuronal hyperexcitation is reduced, the number of A-beta induced neuritic dystrophies decreases, and the release of CSF Tau is reduced [19,20,58].

Tau in the CSF: a biomarker for disease states

Soluble Tau is also present in the CSF of healthy and AD individuals [56], whereby the concentration of CSF Tau correlates with the disease state [8]. CSF levels of total and phosphorylated Tau in combination with A-beta function as biomarkers for AD staging and prediction of disease progression [47,57], and are distinct from other neurodegenerative diseases [6,35]. Recently, it was suggested that also Tau in blood samples could function as a biomarker [7].

Early studies immunoprecipitated a ~28 kDa and smaller Tau fragments from the CSF of AD and stroke patients [36]. Using MS, it became clear that these fragments are C-terminally truncated Tau [46], and the more recent use of isotope-labeled references and high-resolution MS techniques showed that mainly N-terminal Tau fragments (aa 1 to ~230) are present in CSF [5,13]. However, also Tau fragments containing (parts of) the C-terminal half of Tau were detected [31].

The amount and phosphorylation of CSF Tau correlate with AD disease state and amyloid load in AD patients [8,31], and are therefore used as biomarkers for disease staging and for the tracking of AD therapy efficacy in clinical trials. The main sites phosphorylated in CSF Tau and characteristic for AD are T181 and T217 in the PRD. Proteolytic truncation and phosphorylation of CSF Tau could occur either intraneuronally to enable the release of the Tau fragments, or occur after the release of Tau into the extracellular space. From studies using metabolic isotope labeling of Tau, Bateman and

colleagues suggest that truncation occurs before the release of Tau in human neurons [49].

Our current detailed understanding of Tau isoform in the CSF is contrasted by the lack of knowledge about the origin and function of these peptides. Actively secreted Tau fragments could, for example, function as signaling molecules for neurons and glia cells in the central nervous system, or—when transported into the blood—even for peripheral cell types.

Conclusion

Most earlier studies, including our own, conceptually considered low-phosphorylated Tau as the normal version of Tau in cells, whereas 'hyper'-phosphorylated Tau was thought to mimic disease-associated Tau. However, the phosphorylation levels of Tau seem insufficient to categorize healthy and disease Tau, and the devil of phosphorylation may lay in the details: competition between PTMs (e.g. lysine residue modification by acetylation or ubiquitination), fine-tuned addition and removal of labile PTMs may differentially regulate the interactions, functions, and aggregation of Tau in neuronal subcompartments. An important question that should and can be addressed by combining 'smart' sample preparation (e.g. by affinity or size exclusion chromatography) and high sensitivity native and tryptic MS techniques is the Tau and protein composition and stoichiometry of toxic and/or seeding-competent soluble tau oligomers, which were shown to occur before NFT formation in the brain [18]. In fact, PTMs other than phosphorylation, like ubiquitination, acetylation, methylation, or GlcNAcetylation, or even other molecular components may encode the toxicity of Tau oligomers and aggregates. Finding the components and PTM signature in these Tau species would enable the identification of therapeutic targets occurring early in the disease cascade, when neuronal loss is still restricted.

Conflict of interest statement

Nothing declared.

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