It takes more than tau to tangle: using proteomics to determine how phosphorylated tau mediates toxicity in neurodegenerative diseases

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Two of the most common causes of dementia are Alzheimer's disease (AD) and frontotemporal dementia (FTD). AD is an irreversible, progressive neurodegenerative disorder that is clinically characterized by severe memory loss and behavioral impairment that eventually interferes with everyday function. AD is neuropathologically defined by the presence of extracellular β-amyloid plaques and intracellular accumulation of neurofibrillary tangles (NFTs) that primarily contain aggregated, hyperphosphorylated tau (pTau). Intriguingly, pTau is also the central protein in multiple subtypes of FTD (e.g. corticobasal degeneration, progressive supranuclear palsy, Pick's disease). FTD is an umbrella term for a group of neurological conditions that primarily affect the temporal and frontal regions of the brain. Mutations in the tau gene (MAPT) can cause familial FTD, providing further evidence of the integral role of tau in FTD. Physiologically, tau regulates microtubule structure and dynamics, as well as axonal transport through interaction with tubulin. Tau is also involved in neuronal development and synaptogenesis. In AD and FTD, tau becomes hyperphosphorylated and undergoes major conformational changes, causing it to aggregate into the characteristic neuropathological lesions that define AD and FTD. Despite the known involvement of tau in these diseases, exactly how tau mediates toxicity is still unclear.

Examination of protein-protein interactions between tau and neighboring proteins is an excellent way to determine how tau causes toxicity in the brain in neurodegenerative diseases. Previous studies targeting human AD tissue have shown that interactions between pTau and specific neuronal proteins are critical for pTau to convey its toxic effects. These studies identified important disease-associated pTau interactors such as ubiquitin, 14-3-3 protein, sequestosome-1 and several kinases/phosphatases. Many of these proteins have been shown to be mechanistically involved in AD pathogenesis, highlighting the key role of these interactions in AD. However, our knowledge of the proteins that pTau interacts with in AD and FTD is still incomplete. State-of-the-art, unbiased proteomics approaches are an ideal way to efficiently identify all proteins that interact with pTau directly in human brain tissue (the "pTau interactome").

Our team recently developed localized proteomics approaches that specifically identify proteins present in neuropathological lesions and proteins that directly interact with key neurodegenerative disease associated proteins such as pTau (Drummond et al., 2017, 2020). In our most recent study, we examined the pTau interactome in human AD brain tissue using two complementary proteomic approaches (Drummond et al., 2020). First, we used localized proteomics to identify the proteins present inside NFTs. For this, we used laser capture microdissection to micro-dissect NFTs directly from formalin-fixed, paraffin-embedded human brain sections that were collected at autopsy and label-free quantitative LC-MS to quantify all proteins present. Using this approach, we generated the most comprehensive proteome of NFTs to date, identifying > 500 proteins in NFTs. Besides tau, we found that NFTs contained many proteins previously detected in NFTs including ubiquitin, neurofilament proteins, apolipoprotein E and cyclin dependent kinase 5. We used several enrichment analysis methods to analyze NFTs associated proteins at the network level and found that NFTs were highly enriched in proteins associated with mitochondrial dysfunction, EIF2 signaling, phagosome maturation and 14-3-3 signaling, indicating that these processes may be relevant to understand NFT formation. Proteins involved in the protein ubiquitination pathway and the unfolded protein response were also particularly enriched. Specific protein families were also particularly enriched in NFTs including RNA binding proteins and heat-shock protein 70 and 90 families, suggesting that these may play a critical role in the formation of these lesions. To determine which of these neuronal proteins were present in NFTs because of their direct interaction with pTau, we used a second proteomics approach: affinity purification-mass spectrometry (Drummond et al., 2020). For this, we extracted complexes of pTau and interacting proteins from frozen human brain tissue using co-immunoprecipitation with a pTau specific antibody (PHF1) and quantitative mass spectrometry to identify all pTau interacting proteins. The pTau extracted using this approach was highly phosphorylated (23 different phosphorylated residues were identified) and contained all 6 human tau isoforms (0N3R, 1N3R, 2N3R, 0N4R, 1N4R and 2N4R). This approach also allowed us to identify 125 proteins that significantly interacted with pTau in human AD brain tissue. We were pleased to identify proteins known to be interacting with pTau, such as ubiquitin, sequestosome-1, and numerous kinases/phosphatases (CAM2A, CDK5, PPP2RA1). Seventy-five of these pTau interactors were also present in NFTs, highlighting their likely pathological importance in AD. We observed a striking interaction between pTau and proteins from the two main protein degradation systems in the cell, the ubiquitin-proteasome system and the phagosome-lysosome system. Specifically, pTau was found to interact with many subunits of the 19S proteasome lid (PSMC1, PSMC2, PSMC3, PSMC4, PSMC5, PSMD2, PSMD3, PSMD8, PSMD11, and PSMD13) and also three subunits of the v-ATPase proton pump (ATP6V0D1, ATP6V1B2, ATP6V1H), which is responsible for acidifying lysosomes during the proteolysis process. Our study, in line with previous findings, suggested that pTau may impair general proteasome function and autophagy, hence promoting the accumulation of toxic aggregated proteins in the brain.

To validate our findings and place our results in the context of what is already known about proteins associated with tau in AD, we compared our interactome data with previously published studies using a combination of systematic literature searches and data mining of previous proteomics studies. We found that 122/125 proteins (98%) that interacted with pTau had been previously associated with AD, and that 96/125 (77%) had been previously associated with pTau and/or tau, therefore validating that these proteins are of significant interest for future studies examining AD pathogenesis.

A key benefit of proteomics studies is that they are unbiased, which permits the discovery of novel proteins involved in AD pathogenesis (Drummond and Wisniewski, 2017, 2019; Pires et al., 2019). In our study of the pTau interactome, we identified 12 novel proteins that were both present in NFTs and interacted with pTau that had not yet been associated with tau, therefore providing new clues on how they may contribute to AD (Drummond et al., 2020). An additional benefit of proteomics studies is that they provide a more comprehensive overview of AD pathogenesis in comparison to targeted studies that examine individual protein interactions. For example, analysis of protein pathway enrichment highlighted the particularly important role of the ubiquitin-proteasome system and the phagosome-lysosome system in mediating pTau toxicity (Drummond et al., 2020). Localized proteomics approaches also provide an important complement to transcriptomic approaches, as they both provide orthogonal information about disease status in an unbiased, high throughput manner. While transcriptome profiling characterizes disease-specific alterations resulting from multiple genetic, epigenetic and environmental factors, numerous studies have repeatedly observed highly discordant changes at the protein and RNA levels. Therefore, transcriptomic studies do not provide a complete picture of the pathogenic changes in the AD brain. Particularly in diseases such as AD, proteomic analysis better reflects disease-associated protein changes as proteomic studies also account for post-transcriptional and translational modifications on proteins, which are known to have an important pathological role in neurodegenerative diseases.

We are currently evaluating which of these pTau interacting proteins have a mechanistic role in AD. Further characterization of these proteins and their interaction with pTau using well established models to reproduce pTau aggregates *in vitro* are necessary to understand the role of these protein interactions in the context of AD. However, the lack of appropriate *in vitro* models that replicate tau aggregation in AD and other tauopathies is a key challenge in the field. Human tau is a complex protein, comprised of six different isoforms that exhibit a plethora of post-translational modifications that differentially influence disease (Goedert and Spillantini, 2019). The conformation of tau aggregates is different in each tauopathy, likely due to variance in tau isoforms, post-translational modifications and interacting proteins associated with aggregates in each disease (Lippens and Gigant, 2019). The specific features that make pTau toxic in tauopathies is not yet defined. Given this, it is not yet possible to reproduce the complexity of disease-associated pTau using recombinant proteins or simple over-expression of tau. However, recent studies have bypassed these significant limitations by exposing cells *in vitro* to tau aggregates extracted directly

from human brain tissues (Sanders et al., 2014; Woerman et al., 2016). Cells internalize tau aggregates from the media, therefore producing the closest *in vitro* model of tauopathy currently available and an excellent model to study the mechanistic impact of tau aggregates. Most previous studies using this model have studied whether specific tau strains can seed aggregation of monomeric tau in a prion-like manner. However, this model also has great potential for examining the mechanistic consequences of pTau presence and protein-protein interactions in a cell model.

Moving forward, our unbiased proteomic approach represents an excellent tool to address key unanswered questions about the role of tau in neurodegenerative diseases. For example, to determine which tau species and/or phosphorylation site(s) mediates pathology, particularly at the earliest stages of the disease. Our proteomics approach can be applied at different stages of the disease (e.g. at asymptomatic stages or in cases of mild cognitive impairment) or using different neurofibrillary tangle markers (i.e., Alz50, MC1, CP13, AT8, PHF1) to determine whether distinct pTau interactions are specific to any disease-associated phosphorylation site. For example, previous studies have shown that both the proteasome and the lysosome are impaired at early stages of the disease; a future study examining the pTau interactome in the earliest stages of AD would confirm which proteins are specifically involved mediating tau toxicity early in AD.

Another challenge in the field is whether different subtypes of AD – such as familial and sporadic AD – share the same molecular mechanisms. Although these subtypes eventually converge to a final common clinical and pathological endpoint, there are differences in clinical presentation and distribution of pathology. Going forward, using a technique such as ours to determine if pTau interacts with the same partners in AD subtypes would provide novel insight into whether there are potentially distinct tau-associated mechanisms in sporadic and familial AD. We have previous shown that amyloid plaques have a significantly different proteome in rapidly progressive *vs*. typical AD (Drummond et al., 2017), therefore suggesting that neuropathology associated proteins may also differ between other AD subtypes such as familial and sporadic AD. Determining whether there are distinct mechanisms between familial and sporadic AD is particularly important given that the vast majority of AD experimental models are generated by transgenic expression of familial AD mutations, and therefore model familial AD rather than sporadic AD. Therefore, proteomic studies like ours could inform the field about whether these transgenic models are appropriate models of sporadic AD and could also facilitate the development of more representative models of sporadic AD. Comparison of human and animal model proteomic studies would provide invaluable information about the strengths and weaknesses of animal models, which could in turn improve the translation between preclinical animal studies and clinical trials.

Another key unanswered question is whether there are common or unique pTau-mediated disease mechanisms in different tauopathies. Comparing the pTau protein interactions in different tauopathies is an excellent way to address this question. To date, only a handful of studies have used proteomics to examine protein differences in human brain tissue from progressive supranuclear palsy, corticobasal degeneration or Pick's disease cases, all of which have examined protein expression in bulk tissue homogenate (Johnson et al., 2020; Swarup et al., 2020). Our approach of examining pTau interactors could provide insight into how different tau species in each disease influence pTau pathological interactions and result in distinct molecular mechanisms. We recently identified the first protein to our knowledge that uniquely interacts with pTau in AD and not in other tauopathies – secernin-1 (SCRN1) (Pires et al., 2019). Very little is known about SCRN1 and its function is poorly characterized. Our study was the first to characterize its comparative distribution in neurodegenerative diseases. We found that SCRN1 interacted with pTau in both early and late stages of AD and confirmed its presence in pre-, early and mature neurofibrillary tangles (Pires et al., 2019). In contrast, SCRN1 did not colocalize with pTau aggregates in other tauopathies including corticobasal degeneration, progressive supranuclear palsy and Pick's disease. This supports the hypothesis that pTau interacts with different proteins in different diseases, which in turn could contribute to different clinical and neuropathological presentations in each disease. Going forward, it would be interesting to investigate the pTau interactome in other tauopathies and see whether there are other proteins that, like SCRN1, only interact with pTau in a specific context.

Another exciting avenue for future proteomics studies is the emergence of single-cell proteomics approaches (Labib and Kelley, 2020). Different cell types and regions of the brain are selectively affected in different tauopathies and the role of individual cell types in the development of disease is still unknown. Single cell proteomics studies would allow the development of pathology in tauopathies to be examined in much greater detail and would provide insight about whether a specific cell type has a particularly important role in disease. Although single-cell proteomics approaches would revolutionize the field, particularly approaches that were compatible with formalin-fixed paraffin-embedded post-mortem human brain tissue, these techniques are still in their infancy and more development is still required for cell-type isolation techniques, as well as approaches that quantify low-abundance proteins (Labib and Kelley, 2020).

In conclusion, there is still much to be discovered about how tau contributes to neurodegenerative diseases. Proteomics approaches, particularly localized and targeted proteomics approaches, are an ideal way to examine tau-mediated disease mechanisms directly in human brain tissue. It is our hope that proteomics discovery studies such as these will provide novel insight into disease mechanisms, novel disease-specific biomarkers and novel potential drug targets for AD and FTD.

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