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Current Opinion in Chemical Biology

Rethinking protein aggregation and drug discovery in neurodegenerative diseases: Why we need to embrace complexity?

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Abstract

More than a century has passed since pathological protein aggregates were first identified in the brains of patients with neurodegenerative diseases (NDDs). Yet, we still do not have effective therapies to treat or slow the progression of these devastating diseases or diagnostics for early detection and monitoring disease progression. Herein, I reflect on recent findings that are challenging traditional views about the composition, ultrastructural properties, and diversity of protein pathologies in the brain, their mechanisms of formation and how we investigate and model pathological aggregation processes in the laboratory today. This article is an invitation to embrace the complexity of proteinopathies as an essential step to understanding the molecular mechanisms underpinning NDDs and to advance translational research and drug discovery in NDDs.

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Current Opinion in Chemical Biology 2021, **64**:67–75

This review comes from a themed issue on **Synthetic Biomolecules**

Edited by **E. James Petersson** and **James Nowick**

For a complete overview see the Issue and the Editorial

https://doi.org/10.1016/j.cbpa.2021.05.006

Keywords

Neurodegenerative diseases, Neurodegeneration, Pathological aggregates, Post-translational modifications, Amyloid, Fibrils, Cryo-EM, Amyloid-beta, Alpha-synuclein, Tau, TAR DNA-binding protein 43, TDP-43.

Several neurodegenerative diseases (NDDs) have been linked to the failure of proteins to fold correctly or maintain their native conformations, both of which render proteins more prone to aggregate in different brain regions. These processes occur both inside and outside cells and lead to the accumulation of aggregated proteins in the form of intracellular inclusions or extracellular deposits that are the defining hallmarks of NDDs, including Alzheimer's, Parkinson's, Huntington's disease, amyotrophic lateral sclerosis, and prion diseases. Despite differences in the affected brain regions and cell types, these pathological aggregates share one common feature: they are enriched in β-sheet-rich filamentous aggregates, termed amyloid fibrils, which form as a result of the misfolding and fibrillization of a single protein. Pathogenic mutations in the genes that code for the amyloid-forming proteins associated with each disease or genetic variations in the proteins that regulate their production, levels, or clearance led to increased aggregation and/or amyloid formation in vitro and pathology formation in preclinical models. These observations supported the hypothesis that the process of amyloid formation plays central and causative roles in the pathogenesis of these disorders [1].

Over the years, our views on pathological aggregates and their mechanisms of formation have been shaped by the prominent appearance of fibrillar assemblies in electron microscopy images of amyloid plaques, neurofibrillary tangles (NFTs), and Lewy bodies (LBs) (Figure 1). Despite early evidence highlighting the complex biochemical composition and morphological diversity of pathological aggregates in the brain, the ability to reproduce amyloid formation in cell-free systems from a single recombinant protein has led to a fibril-centric approach to investigating and targeting pathology formation in NDDs. This approach has dominated the field over the past decades.

In spite of decades of research on the mechanisms of amyloid formation and the structural properties and mechanisms of toxicity of the various types of aggregates that form along the amyloid pathway, many fundamental questions about the role of this process in the pathogenesis of NDDs remain unanswered. Furthermore, despite the discovery of hundreds of molecules and antibodies that inhibit amyloid formation in cell-free systems and preclinical models of NDDs, only a handful of compounds and antibodies have advanced to clinical trials, and non has proven to be effective in slowing the progression of NDDs in the clinic. These failures have led many to question the amyloid hypothesis and whether protein aggregation indeed plays a

causative role in NDDs. In this article, I argue that the failure to appreciate the complexity of the processes underlying protein aggregation and pathology formation in NDDs and reluctance to integrate this knowledge into preclinical models and platforms for drug discovery are major contributors to the high rate of antiaggregation drug failures in clinical trials. I will also highlight how recent advances in protein chemistry, biophysics, advanced imaging, and proteomics could be applied to decipher and embrace the complexity of these processes and advance translational research and drug discovery in NDDs.

What is new and why it matters?

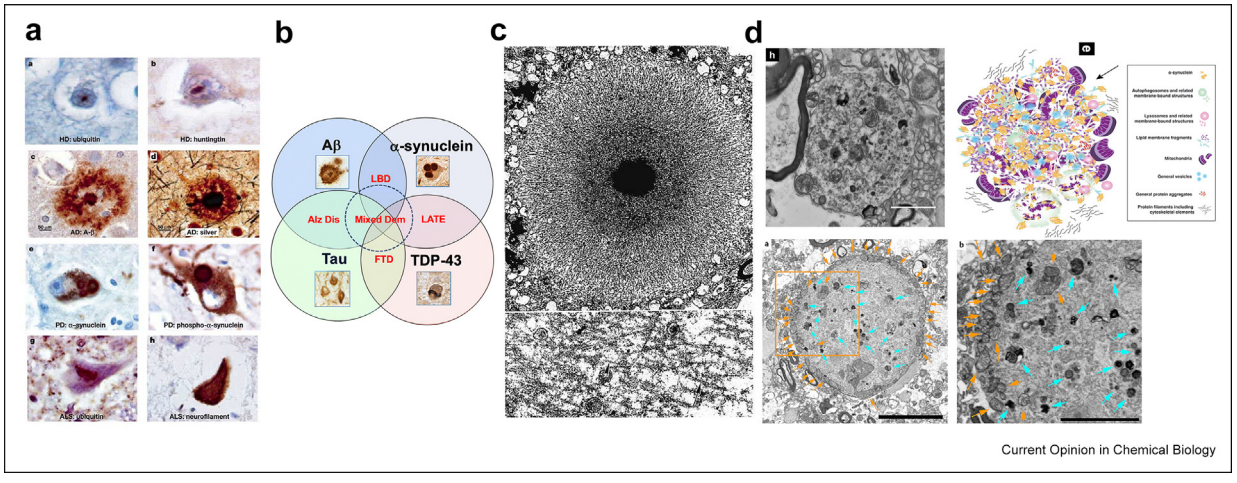
Pathological aggregates, beyond amyloid formation

Different approaches have been used to dissect the proteome of pathological aggregates, revealing the presence of 100s of proteins in amyloid plaques (~500) [2,3], NFTs (~900) and LBs (~300–500) [4,5], representing different cellular pathways and cellular structures. Nonproteinaceous components, such as metals, lipids, lipid rafts, nucleic acids, cholesterol, and carbohydrates, are also found at different levels in these pathological aggregates (Figure 2) [6]. However, the types and distribution of these macromolecules has not been systematically assessed. Furthermore, except for a very few molecules, the role of these nonproteinaceous components in pathology developments and aggregation-mediated toxicity has not been thoroughly investigated (Figure 2). This knowledge gap and the sheer large number of candidate proteins, metals, lipids, and other macromolecules, have discouraged efforts to systematically deconstruct and reconstruct how pathological aggregates form beyond fibril formation in vitro and in preclinical models.

Recent correlative light electron microscopy (CLEM) studies of LBs from Parkinson's disease (PD) brains revealed that they are not, as previously thought, predominantly composed of alpha-synuclein (αSyn) fibrils but are also enriched in lipids, membranous organelles, and hundreds of other proteins (Figure 1d) [7]. These observations were recently confirmed by CLEM studies in seeding neuronal models that reproduce many of the key steps in LB formation [8]. This model suggests that fibril formation occurs before the appearance of LB-like inclusions, formation of which involves post-translational modifications (PTMs) of αSyn aggregates and the active participation and/or recruitment of a complex set of proteins, lipids, and membranous organelles into LBs. Many of our observations related to αSyn amyloid formation in this model were recently validated by cryo-electron tomography studies of seeded mediated aggregation of αSyn fused to Green fluorescent protein (GFP) (αSyn-GFP) in primary neurons [9]. It is noteworthy that this model recapitulates the formation of αSyn fibrils, but not LB formation, which forms upon fibril-mediated seeded aggregation of endogenous untagged αSyn [8]. These observations and others from our group [10] highlight the limitations of investigating the mechanisms of inclusion formation using overexpression systems and amyloid proteins fused to large fluorescent proteins.

CLEM studies on Huntingtin inclusions in mammalian cells overexpressing mutant exon 1 of the huntingtin

Figure 1

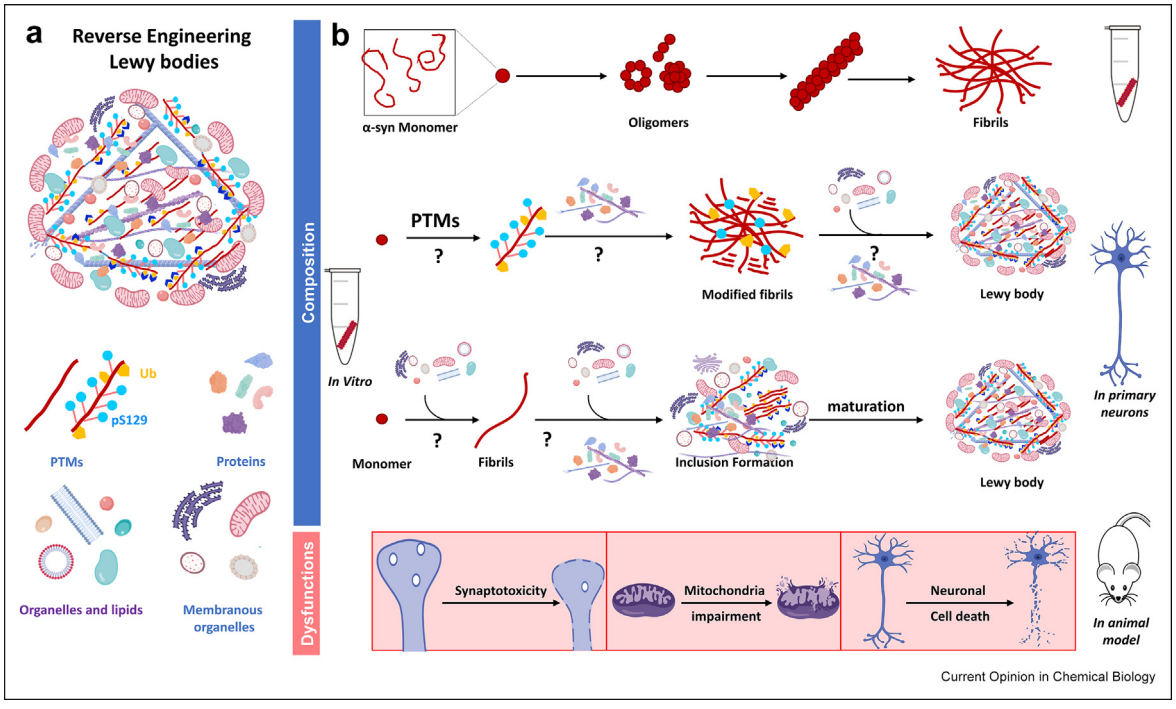


Unmasking the complexity of pathological aggregates in brain proteinopathies. (**a**) Images of protein aggregates found in the brain and that represent the pathological hallmarks of Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), and Amyotrophic lateral sclerosis (ALS) (adapted with permission from Ross C. et al. [57]). (**b**) Schematic depiction illustrates the co-occurrence of multiple Aβ, αSyn, Tau, and TDP-43 pathological aggregates in NDDs (adapted with permission from Kwon S. et al. [56]). (**c**) An electron microscopy image of a classical LB showing the dominant appearance of radiating filamentous structures that are surrounded by membranous organelles (Adapted with permission from a study by Forno [58]). (d) Recent Electron microscopy (EM) images of LBs illustrating the diversity of their ultrastructure properties and the complexity of their composition, including the presence of proteins, vesicles, and membranous organelles (Adapted with permission from a study by Shahmoradian et al. [7]).

protein (mHttex1) also revealed complex architecture and biochemical composition that is dependent on the polyQ length and cell context [10]. Cytoplasmic mHttex1 inclusions showed a core–shell organization with a high degree of organization of the Htt fibrils in the periphery, giving rise to a ring-like structure. Inclusion formation appears to occur in two phases: an initial phase driven by the polyQ-dependent formation of a dense aggregate core (possibly driven by phase separation) and a second phase that involves concomitant fibril growth and interactions with cytoplasmic proteins and organelles. In contrast, the inclusions formed in the nucleus did not exhibit this core–shell morphology. Instead, they showed the predominant accumulation of tightly packed mHttex1 aggregates lacking specific organization, lipids or cellular organelles. Interestingly, interactions with membranous organelles, except for the endoplasmic reticulum, were less prominent in Htt inclusions than in LBs or LB-like inclusions in neuronal models [10]. These observations demonstrate that the mechanism of inclusion formation is context dependent and that aggregation in different cellular compartments may occur via distinct mechanisms. Altogether, the emerging picture from these cellular models and recent ultrastructural properties of LBs in the brain demonstrate that the formation of pathological aggregates and neurodegeneration in NDDs are driven by multiple processes that are cell context dependent and involve a complex interplay between protein aggregation, PTMs, and interactions between various forms of the disease-associated protein and other proteins, lipids, and cellular organelles (Figure 2). They also suggest that different approaches may be required for targeting the different types of inclusions or aggregation in different cellular compartments.

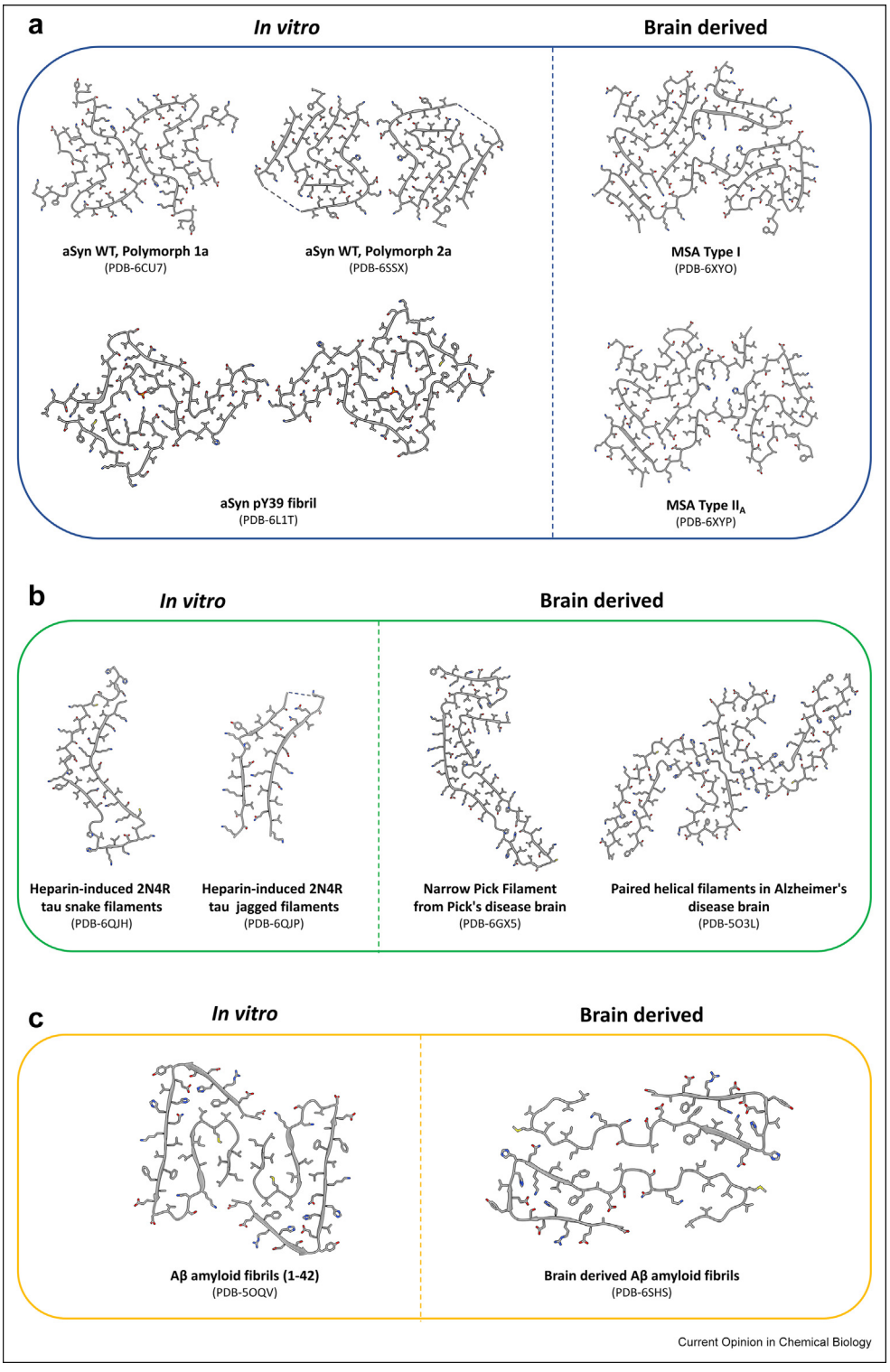
Interestingly, in both PD and HD cellular models discussed above, the presence of amyloid fibrils in the cytosol, was not sufficient to cause neurodegeneration or cell death, which occurred only during the transition from fibrils to inclusions. In contrast the accumulation of nuclear mHtt inclusions was associated with increased toxicity in primary neurons. These findings suggest that in some NDDs the process of inclusion formation rather than the formation of fibrils is one of the primary drivers of neuron loss in NDDs. One implication of these findings is that investigating the relationship between protein aggregation and neurodegeneration requires the

Figure 2



Reverse engineering pathological aggregates in NDDs. (**a**) Schematic depiction illustrating the complex composition of Lewy bodies as discerned from CLEM, proteomics, and biochemical studies. (**b**) Schematic depictions illustrating how a combination of cell-free and neuronal models that recapitulate key features of the human pathology could be used to decipher the mechanisms by which PTMs and interactions with various cellular molecules and membranous organelles influence protein misfolding and aggregation along the pathway to fibrillization and pathology formation. These studies could pave the way for testing new hypotheses, assessing the therapeutic potential of new targets and pathways and drug candidates in multiple animal models that reproduce different aspects of neuronal dysfunction and degeneration in NDDs. CLEM, correlative light electron microscopy; NDDs, neurodegenerative diseases.

Figure 3



Brain-derived fibrils and in vitro-generated fibrils are not the same. Cryo-EM structures of amyloid fibrils from alpha-synuclein (αSyn) (**a**), tau (**b**), and amyloid-beta (**c**) prepared in vitro and isolated from human brain tissues. In vitro–generated αSyn fibrils (a, left side) were prepared from; recombinant full-length protein under different fibrillation conditions, hence polymorph 1a [20] and polymorph 2a [21] and αSyn monomers with phosphorylation at tyrosine 39 [47]. αSyn human-derived fibrils (a, right side) were isolated from patients with multiple system atrophy (MSA) [59]. In vitro-generated Tau fibrils (b, left side) prepared from recombinant full-length protein with four (2N4R) microtubule-binding repeats in the presence of heparin [19]. The tau

human-derived fibrils (b, right side) were isolated from patients with Pick's disease [14] or Alzheimer's [13]. In vitro generated Aβ fibril (c, left side) produced from recombinant [$^{15}N/^{13}C$]-Aβ(1–42) labeled peptide [60]. Human-derived Abeta fibril (c, right side) isolated from patients with Alzheimer's disease [18].

use of model systems that capture the entire process of pathology formation from protein misfolding and aggregation to inclusion formation and maturation and not only fibril formation.

Brain-derived fibrils and in vitro-generated fibrils are not the same

Recent advances in Cryogenic electron microscopy (cryo-EM) and data processing have enabled, for the first time, detailed and unprecedented insight into the structure of amyloid fibrils derived from proteins linked to several NDDs (Aβ, αSyn, Tau, TDP-43-derived peptides). In the case of Aβ, Tau and αSyn (Figure 3) [11], we have access to 3D structures of amyloid fibrils produced in vitro and fibrils isolated from brain-derived pathological aggregates [12–18]. The cryo-EM structures revealed several unexpected features: (1) the structure of brain-derived fibrils showed different core-sequence arrangements and structural characteristics from fibrils of the same protein generated in vitro (Figure 3) [11,19]; (2) PTMs contribute to the structural diversity of amyloid fibrils in the brain [17]; (3) brain-derived fibrils isolated from different patients with the same disease (e.g. AD or multiple system atrophy) showed similar structures, whereas fibrils produced in vitro showed a high degree of polymorphism [20,21]. This suggests that amyloid formation in the brain occurs under highly controlled conditions and via distinct mechanisms that may be difficult to reproduce in cell-free systems using only recombinant or synthetic forms of the disease-associated amyloidogenic proteins.

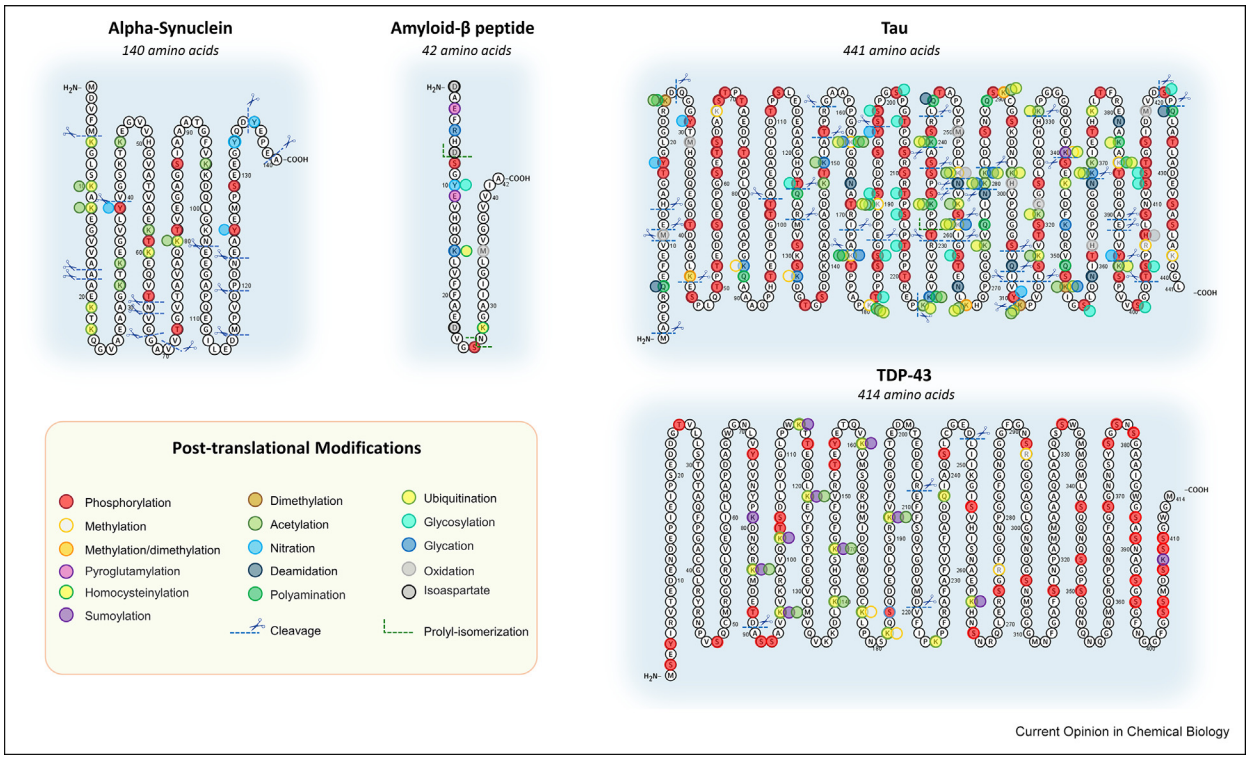
These findings have significant implications for ongoing efforts to develop antibodies and drugs targeting pathological aggregates using recombinant proteins. Pathological aggregates, especially LBs, are present at very low numbers in the brain, which limits the amount that can be isolated and made accessible to scientists. Therefore, there is an urgent need to develop robust methods to amplify brain-derived aggregates or reconstruct the ideal conditions for their formation in vitro or in cells. Studies from several groups have shown that it is possible to use protein misfolding cyclic amplification [22] and real-time-quacking induced conversion [23–25] procedures to amplify brain- or CSF-derived aggregates, thus enabling the generation of sufficient materials for structural characterization using nuclear magnetic resonance (NMR) and EM techniques, including Cryo-EM [26,27]. However, a recent study by Lovestam et al. [28] reported the cryo-EM structures of amplified structures generated using three αSyn filament preparations from three different multiple system atrophy brains differ from those of the brain-derived seeds. Further studies are needed to validate these findings and determine if these observations extend to amyloid filaments associated with other NDDs. Nonetheless, it is imperative that we reassess how we study and model amyloid formation *in vitro* and in preclinical models. These efforts should be guided by a deeper understanding of the biochemical composition and structural properties of pathological aggregates in the human brain. This approach could pave the way for developing more robust methods for reprocuding or amplifying the structural and biochemical complexity of disease-associated pathological aggregates in vitro.

The PTM code of amyloids

Disease-associated pathological aggregates of several amyloid proteins, including Tau, αSyn, and TDP-43, have consistently been shown to be post-translationally modified, with phosphorylation, truncation, and ubiquitination being among the most commonly detected PTMs in LBs, NFTs, and TDP-43 inclusions [29–33]. Antibodies against the phosphorylation sites identified in these pathological aggregates, such as pS129 on αSyn, pT181 on Tau, and pS409/pS410 on TDP-43 [34], have become the primary tools for monitoring and quantifying pathological aggregates in preclinical models and in human tissues and biological fluids. The increasing use of unbiased mass spectrometry-based approaches has been instrumental in revealing the complex landscape of PTMs in amyloid proteins (Figure 4). A recent comprehensive analysis of Tau PTMs in post mortem brains from 49 AD and 42 healthy individuals identified 95 PTMs on 88 amino acids in human Tau, including 55, 17, 19, and 4 phosphorylation, ubiquitination, acetylation, and methylation sites in sarcosyl-insoluble Tau [31]. Some degree of variation in the PTM pattern was observed among AD patients and in patients at different stages of AD progression [35]. A similar level of PTM diversity and clustering of PTMs has been observed for other amyloid proteins, including αSyn and TDP-43 (Figure 4).

A great deal of knowledge about the role of PTMs in regulating the function of amyloid proteins in health and disease is based on the use of mutations that do not mimic or only partially mimic the effects of PTMs (e.g., phosphomimetics). Two major shortcomings of these approaches are that they (1) introduce permanent modifications and ignore the reversible and dynamic nature of PTMs; (2) do not allow investigating the role

Figure 4



The missing link: post-translational modifications: schematic depictions illustrating the diversity and distribution of PTMs identified in the amyloid proteins αSyn, Aβ, Tau, and TDP-43.

of cross talk with neighboring or distant PTMs, despite the mounting evidence for the clustering of multiple and different PTMs in different regions of amyloid proteins; and (3) do not allow investigating the role of PTMs at various stages of protein aggregation and of pathology development and spread. Increasing evidence suggests that several phosphorylation events and some ubiquitination events occur after the aggregation of αSyn, Tau and TDP-43 [36,37], reflecting a cellular response aimed at preventing aggregation or targeting protein aggregates for degradation.

Despite the diversity and complex patterns of PTMs associated with amyloid proteins, recent advances in the chemical synthesis and semisynthesis of amyloid proteins, including Aβ, αSyn [38–40], Tau [41–44], and Htt [45], have addressed many of these limitations and should enable new experimental approaches based on embracing the complexity of amyloid proteins and pathological aggregates in the brain. Some of the surprising findings warranted by these methodological advances challenge the conventional thinking about the role of PTMs in the pathogenesis of NDDs. These include demonstration that (1) the most of the PTMs found in LBs, except for C-terminal truncations, either inhibit or do not affect αSyn aggregation; (2) serine/threonine or tyrosine hyperphosphorylation of Tau inhibit rather than promote Tau aggregation [42,46]; and (3) several PTMs on αSyn and Tau were shown to significantly alter the morphology of the final fibril structures [38,47]. These findings suggest that many pathology-associated PTMs may occur after aggregation and that PTMs could be important regulators of fibril strains and potentially fibril seeding activity. Furthermore, these advances have been instrumental in developing novel tools and assays for biomarker discovery and for assessing the potential of PTMs as biomarkers for early diagnosis and the monitoring of disease progression.

The high success in replicating these methodologies in different labs and scaling up the production of these

proteins (10s−100s of mg) shows that the development of similar methods for other amyloid proteins is possible. More efforts and investments in developing these methods are essential to deciphering the PTM code of all amyloid proteins. Complementing these methods with novel chemical probes [48,49] and strategies that enable investigating the role of PTMs with high spatial and temporal resolution in cells is also essential. This will pave the way for a more systematic assessment of PTMs' roles in regulating the biology of these proteins and a deeper understanding of how they influence different stages of pathology formation, pathology spreading, and neurodegeneration in NDDs.

One disease one amyloid protein or one disease multiple amyloid proteins

Most of us have been educated to associated many NDDs with the aggregation of a single protein, except for Alzheimer's disease, where the pathology is linked to the aggregation of both Aβ (amyloid plaques) and Tau (NFTs). Therefore, it is not surprising that the majority of animal models of NDDs are based on manipulating the expression and aggregation of a single protein. In the case of AD, several models have been developed based on overexpression of several mutant proteins that have been individually genetically linked to AD, although such a situation never occurs in humans. Increasing evidence suggests that pure NDDs associated with a single type of amyloid pathology represent an exception rather than the norm. Several studies have demonstrated the co-occurrence of multiple pathological aggregates formed by different amyloid proteins in the brain of patients with different NDDs [50,51]. LBs are commonly detected in AD brains, and similarly, AD neuropathology (neocortical neuritic plaques and tangles) is frequently seen in the brain of patients with LB diseases [50]. Recently, St-Amour et al. showed that Tau, α-Syn, and TDP-43 proteinopathies are increased in HD [52], confirming previous reports on colocalization of α-Syn and Htt pathology [53]. Although these copathologies can occur independently, the colocalization of multiple amyloid proteins within a particular pathological aggregate is common in many NDDs, suggesting the possibility of cross-seeding as a key mechanism that drives pathological heterogeneity in NDDs. Altogether, these observations underscore the complex interplay between different amyloid proteins in each NDDs and highlight the critical importance of developing in vitro, cellular and animal models and experimental approaches that capture the complexity of protein aggregation and pathological heterogeneity as it occurs in the brain of patients with NDDs.

Conclusions

The emerging picture from the in-depth analyses of pathological aggregates invites us to (1) revisit our early assumptions; (2) rethink how we investigate the mechanisms of protein aggregation and their role in the pathogenesis of NDDs; and (3) embrace the clinical heterogeneity of NDDs and complexity of the proteins and processes associated with each disease. There are several important takeaways from the body of recent investigations tackling the neurodegeneration-associated pathology processes. First, neurodegeneration is most likely driven by multiple processes rather than by a single protein, specific aggregate form, or subset of aggregates. Second, aggregate formation is dependent on both the protein and its surrounding milieu [54], including complex interactions and interplay with other cellular proteins, macromolecules and organelles, composition of which may impact on the disease progression, severity or the clinical heterogeneity of NDDs. Third, the reductionist approach of investigating the amyloid-forming proteins in cell-free systems and one at a time is not sufficient to recapitulate the central disease-causing processes. Fourth, the pathological aggregates in many of the preclinical models do not recapitulate the complexity of the human pathology [55]. Fifth, the identification of drugs or intervention strategies that are successful in targeting pathological aggregates in human patients will require preclinical model systems that represent the human pathology with high fidelity [55]. Finally, the development of diagnostic tools and imaging agents for early diagnosis and the monitoring of disease progression will also require more comprehensive mapping and profiling of pathological aggregates in different NDDs or the development of conditions for reproducing the biochemical and structural diversity in vitro, in cells and in animal models of NDDs.

A more in-depth understanding of the common and distinguishing structural and biochemical features of pathological aggregates in NDDs could pave the way for developing novel disease-specific diagnostics and therapeutic approaches that target pathological aggregation by multiple amyloid proteins. The latter is important because of increasing evidence demonstrating the presence of multiple amyloid pathologies in the brains of patients with NDDs and that these copathologies influence disease progression and symptomology [50,51]. Therefore, future therapies may require strategies that target multiple amyloid proteins [56]. Given that aggregation and amyloid formation by proteins such as Tau and αSyn play important roles in the pathogenesis of several NDDs, it is worth comparing the biochemical composition of αSyn and Tau pathological formations isolated from the brains of patients with different synucleinopathies and tauopathies. The knowledge gained from these studies could inform future efforts to develop disease-specific biomarkers, diagnostics, and therapeutic strategies.

Funding

This work was supported by funding from Ecole Polytechnique Fédérale de Lausanne (EPFL)

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: HAL has received funding from industry to support research on neurodegenerative diseases, including from Merck Serono, UCB, and Abbvie. These companies had no specific role in the conceptualization, preparation, and decision to publish this work. HAL is also the co-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.

Acknowledgements

The authors would like to thank Galina Limorenko for her careful review of the manuscript and valuable feedback and suggestions. The author also thanks Somanath Jangannath for the valuable feedback, and Dr. Anne-Laure Mahul Mellier, Ahmed Sadek, and Galina Limorenko for their help to generate Figure. 2, Figure 3, and Figure 4, respectively.

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