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Review

Combating deleterious phase transitions in neurodegenerative disease

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ABSTRACT

Protein aggregation is a hallmark of neurodegenerative diseases. However, the mechanism that induces pathogenic aggregation is not well understood. Recently, it has emerged that several of the pathological proteins found in an aggregated or mislocalized state in neurodegenerative diseases are also able to undergo liquid-liquid phase separation (LLPS) under physiological conditions. Although these phase transitions are likely important for various physiological functions, neurodegenerative disease-related mutations and conditions can alter the LLPS behavior of these proteins, which can elicit toxicity. Therefore, therapeutics that antagonize aberrant LLPS may be able to mitigate toxicity and aggregation that is ubiquitous in neurodegenerative disease. Here, we discuss the mechanisms by which aberrant protein phase transitions may contribute to neurodegenerative disease. We also outline potential therapeutic strategies to counter deleterious phases.

State without borders: Membrane-less organelles and liquid–liquid phase transitions edited by Vladimir N Uversky.

1. Introduction

Cells are organized into compartments known as organelles. Conventionally, organelles were thought to be restricted to eukaryotes and were defined by a delimiting membrane that allows physiochemical separation from the rest of the cellular environment. However, it is now clear that organelles can also be formed without a membrane boundary. These organelles are termed membrane-less organelles (MLOs) and are found in eukaryotes and prokaryotes [1,2]. MLOs form via spontaneous liquid-liquid phase separation (LLPS) of key biomolecules allowing them to achieve physiochemical separation from the surrounding environment. Some examples of eukaryotic MLOs include stress granules [3], nucleoli [4], P bodies [5], Cajal bodies [6], and paraspeckles [7].

In polymer physics, phase separation is a well-known phenomenon [8]. This phenomenon has also long been observed in structural biology during the process of protein crystallization [9], but is now appreciated anew in biology. In 2009, P granules were found to have liquid-like properties and form by phase separation [10]. In 2011, the nucleolus was also found to display liquid-like properties and forms via LLPS [11]. Several other instances of LLPS in biological systems have been defined, including both physiological and pathological examples. Here, we will describe pathological protein LLPS that is linked to neurodegenerative disease-related proteins. Further, we will outline potential therapeutic strategies to counter deleterious phases in neurodegenerative disease.

2. Common features of proteins that undergo LLPS

Proteins that undergo LLPS share common features. The most important feature that enables a protein to undergo LLPS is multivalency [12–15]. Indeed, the concentration of protein needed to undergo LLPS is directly related to its valency, with a lower concentration needed for proteins with a higher valency [16]. Interestingly, several key neurotoxic proteins that are emblematic of neurodegenerative disease are also highly multivalent, and will readily undergo LLPS [17]. It has been suggested that LLPS may be a common feature of many proteins [18].

One way for a protein to realize its multivalency is to interact with

Abbreviations: AAV, adeno-associated virus; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; ASO, anti-sense oligonucleotide; Cry2, Cryptochrome 2; FTD, frontotemporal dementia; FUS, fused in sarcoma; hnRNPA1, heterogeneous nuclear ribonucleoprotein A1; HD, Huntington's disease; Kapβ, Karyopherin-β; LLPS, liquid-liquid phase separation; LCD, low-complexity domain; MLO, membrane-less organelle; Tau, microtubule-associated protein tau; NIR, nuclear-import receptor; NLS, nuclear-localization signal; PolyQ, poly-glutamine; PD, Parkinson's disease; PTM, post-translational modification; PrLD, prion-like domain; RAN, repeat associated non-AUG; RBP, RNA-binding protein; SCA3, spinocerebellar ataxia type 3; TDP-43, TAR-DNA binding protein 43 kDa; TIA1, T-cell restricted intracellular antigen-1; U1-70K, U1 small nuclear ribonucleoprotein 70 kDa; UBQLN2, ubiquilin 2; WT, wild type.

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other proteins in a dynamic network. Indeed, proteins that undergo LLPS also tend to have regions that lack a defined secondary structure, and this intrinsic disorder facilitates the flexibility needed to participate in a high number of multivalent interactions or, in other words, enables interaction with multiple binding partners simultaneously. Thus, it is not surprising that many of the proteins that have been identified with LLPS behavior are intrinsically disordered [19]. It is important to note, however, that multivalency can also be achieved via folded domains [13]. Additionally, proteins with the capacity to oligomerize have a high valency and therefore are also common examples of proteins able to undergo LLPS. In fact, light-sensitive oligomerization domains from the protein cryptochrome-2 (Cry2) have been used to promote phase transitions optogenetically [20–22]. Lastly, nucleic acid binding proteins have the potential to engage nucleic acid and protein partners, and this elevated level of valency makes them robust examples of LLPS-competent proteins [3,23–26]. Additionally, RNA can serve as a scaffold for LLPS to occur for several key RNA-binding proteins (RBPs), including those involved in neurodegenerative diseases such as FUS [27]. Generally, RNAs that serve as scaffolds are longer and have multiple binding sites that can dock several copies of the same RBP on one RNA molecule and therefore can nucleate the formation of higher order structures such as liquid droplets [15,28]. Conversely, short RNAs lack the valency to make these higher-order contacts and therefore can antagonize LLPS [15,21]. RNAs can also maintain proteins in a soluble state [29–31]. Likewise, other multivalent scaffolds that engage RBPs, such as poly(ADP-ribose) can stimulate RBP LLPS as in the case of TDP-43 [32].

3. Pathological protein phase transitions in neurodegenerative diseases

Aberrant phase transitions are increasingly being associated with neurodegenerative disease pathology [3,33–44]. The role of pathological protein LLPS in disease is not well understood, but three potential mechanisms that have been proposed are illustrated in Fig. 1. They include changes in the propensity of the protein to undergo LLPS (Fig. 1A), changes to material properties of phase-separated structures (Fig. 1B), and nucleation of aggregation from sustained confinement in a restricted space that increases the probability for interaction (Fig. 1C) [17].

3.1. Altered propensity to phase separate

Proteins that can undergo LLPS generally do so under specific conditions, as the process is extremely sensitive to changes in both the protein and environment [45]. This sensitivity may increase the possibility for dysregulation of LLPS-driven assemblies. Small perturbations can shift the phase boundary for a protein to undergo LLPS [46], and these shifts could have deleterious consequences for functional neuronal processes that rely on LLPS. There are several examples of these shifts occurring for proteins involved in neurodegenerative disease, and these examples will be discussed below.

3.2. Changes to material properties of phase separated droplets

When proteins undergo LLPS they form liquid droplets with high concentrations of the protein inside. While in a liquid state, these droplets possess certain material properties such as a high level of internal diffusion, the ability to fuse, and flow as a response to shear stress [10,47]. However, with sustained time in a liquid droplet state, the proteins can convert to a more stable gel-like state, eventually converting into a solid [45]. One robust example is in the case of FUS [42]. FUS mutations underlie a subset of familial amyotrophic lateral sclerosis (ALS) cases [48,49]. FUS rapidly undergoes LLPS, but over time converts to a less dynamic gel-like state, and eventually forms solid-like fibrillary aggregates [42,50–52]. Maturation of a liquid droplet into a solid over time is sometimes referred to as “molecular aging,” and could underlie pathological mechanisms that lead to neurodegenerative disease [41,42]. Recent work also suggests that FUS can form soft glassy materials referred to as ‘Maxwell glasses’ with age-dependent material

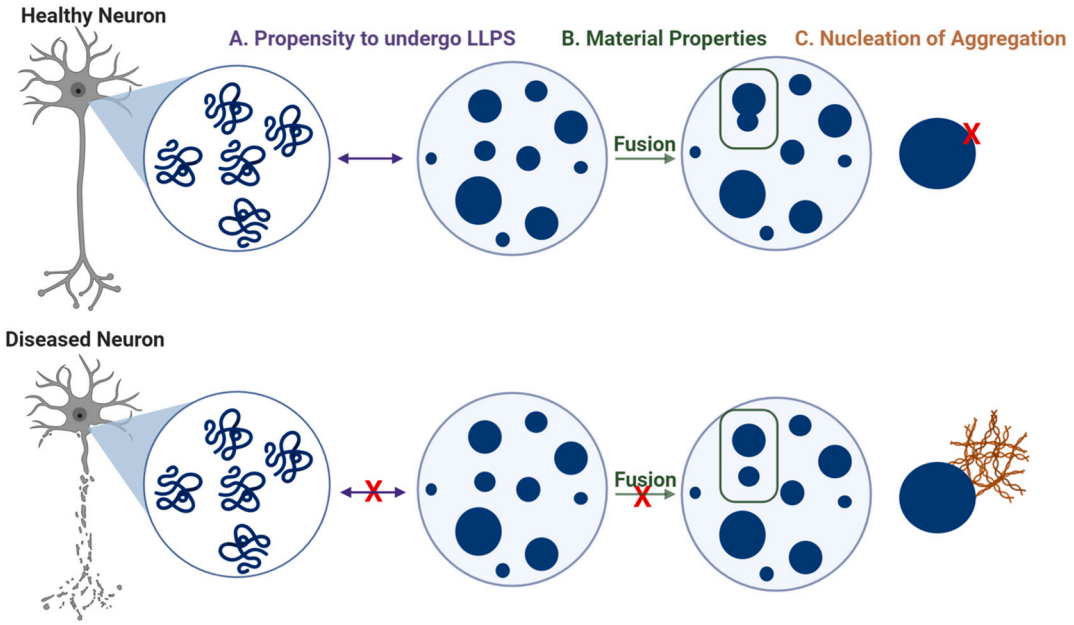

Fig. 1. Potential mechanisms by which aberrant liquid-liquid phase transitions might contribute to neurodegenerative diseases. LLPS is compared between a healthy neuron and a neuron that is deteriorating in neurodegenerative disease. (A) Disease-linked mutations or conditions can affect the ability of the protein to undergo LLPS, and in some cases the ability is enhanced while conversely in others it is diminished. (B) The material properties of a protein that has undergone LLPS may change due to disease-linked mutations or conditions. One such example is the liquid droplet losing ability to fuse. Another example of changes to material properties in a diseased neuron that is not shown is a loss of dynamics. (C) Sustained confinement in liquid droplets can lead to aggregation of the protein contents. This situation often arises for neurodegenerative disease-related proteins that pathologically aggregate. In a healthy neuron this scenario does not occur because LLPS is reversible and proteins in liquid droplets maintain dynamics. Figure made with Biorender.com.

properties [53]. Therapeutics that preserve the liquid-like properties of proteins that have undergone LLPS and prevent progression to gel or solid states would counteract this pathogenic mechanism.

3.3. Nucleation of aggregation

Neurodegenerative diseases share a common theme of protein misfolding leading to the appearance of protein aggregates in disease tissue [54–56]. These aggregates are associated with cytotoxicity and lead to neuronal loss as well as cognitive and motor impairments [54,55]. Elimination of pathological aggregates may serve as a therapeutic approach to treat neurodegenerative disease [57–59]. However, the appearance of aggregates precedes disease symptoms and diagnosis [60]. Therefore, delineating the initial events leading to pathological aggregation could enable the identification of earlier therapeutic targets [60]. Aberrant protein LLPS may be an example of an early aggregation event that could be targeted. If proteins dwell for too long in a liquid-like state they risk conversion to pathological fibrils [3,42]. Therefore, therapeutic agents could be developed to disaggregate or prevent the aggregation of proteins that have undergone LLPS [21,57,61].

4. Pathological proteins that undergo LLPS in ALS and frontotemporal dementia (FTD)

ALS is a progressive neurodegenerative disease that is characterized by the degeneration of upper and lower motor neurons [62–64]. FTD is another progressive neurodegenerative disease that is characterized by degeneration of cortical neurons in the frontal and temporal lobes [65,66]. ALS and FTD reside at opposite ends of a clinical spectrum of disorders, and patients can sometimes present with phenotypes of ALS and FTD [65,66]. The pathological development of ALS/FTD is associated with aberrations in several key proteins including FUS, TDP-43, heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), ataxin 2, *C9ORF72*-derived dipeptide repeat proteins poly-GR and poly-PR, and ubiquilin-2 (UBQLN2), which also appear as aggregates in diseased neurons [67–69]. Which of these proteins undergoes aberrant phase transitions depends on the precise form of ALS/FTD [67,70,71]. Most notably, these proteins have all been identified as being components of stress granules, which are stress-induced MLOs containing various RNAs and proteins [67]. Stress granules are highly dynamic. They assemble when the cell is exposed to various stressors but dissipate once the stress subsides, and while they are formed, they readily exchange material with the cytosol [72]. Several links associate aberrant stress-granule assembly to disease pathogenesis, and these connections offer insight into potential therapeutics that may be promising for mitigating disease pathogenesis [22,35,36]. Next, we will focus on each of the proteins listed above, and examine how their LLPS may contribute to the development of ALS/FTD.

4.1. FUS

FUS is a predominantly nuclear RBP that is mislocalized to cytoplasmic inclusions in degenerating neurons in ALS/FTD [67]. FUS associates with stress granules, and the first studies of FUS LLPS demonstrated that it can phase separate into spherical droplets able to fuse and recover after photobleaching, demonstrating their highly dynamic nature [42]. Structurally, FUS has an N-terminal prion-like domain (PrLD) [73–75], which is necessary for its aggregation and LLPS behavior [15,27,51]. A FTD-linked FUS variant with a mutation in its PrLD, G156E, formed non-spherical structures that were unable to recover from photobleaching, indicating a change in material properties compared to wild-type (WT) FUS [42]. Additionally, ALS/FTD-linked mutations alter the mobility of phase-separated FUS in vitro and in vivo, and studies suggest that changing the material properties of FUS may contribute to disease [41]. One promising approach to modulate FUS phase behavior is through phosphorylation of the PrLD. In fact, when FUS is phosphorylated in its PrLD it is less prone to aggregation, shows a decreased toxicity in yeast models, and its phase-separation behavior is altered [76,77]. Indeed, some phosphorylation events permit FUS LLPS but inhibit the pathological transition to gel and solid states [77].

4.2. TDP-43

TDP-43 is another predominantly nuclear RBP that is a major component of cytoplasmic inclusions found in degenerating neurons of ALS/FTD patients [67,78]. TDP-43 proteinopathy and FUS proteinopathy are mutually exclusive in disease [70]. TDP-43 proteinopathy is also more common, being in found in ~97% of ALS cases compared to the ~1% of ALS cases that present with FUS proteinopathy [70]. In disease-linked inclusions, TDP-43 is often found as hyperphosphorylated, ubiquitinated and cleaved C-terminal fragments [78]. TDP-43 has a C-terminal PrLD [73–75], which is phosphorylated and aggregated in motor neurons of ALS patients and the majority of ALS-associated TDP-43 mutations are in this region [67,79,80]. The C-terminal PrLD renders TDP-43 intrinsically aggregation prone [81], and in isolation can phase separate into droplets with rapid dynamics as measured via fluorescence recovery after photobleaching [39]. Likewise, full-length TDP-43 can spontaneously undergo LLPS [15,32,82]. Some ALS-linked TDP-43 mutations accelerate aggregation at the expense of liquid states [21,32,39,81]. Additionally, ALS-associated genes such as UBQLN2 and hnRNPA1 can alter TDP-43 aggregation in cell models [83].

A region within the TDP-43 PrLD spanning residues 320–343 appears critical for LLPS [39]. This region is evolutionarily conserved and exhibits a propensity to form an α-helix within the otherwise disordered PrLD [39]. Mutations that reduce α-helical propensity reduce TDP-43 LLPS, whereas mutations that enhance α-helical propensity increase LLPS, and can be ALS-linked as with G335D [84]. This region is required for TDP-43 LLPS in cells [85]. Intriguingly, perturbed LLPS affects TDP-43 splicing function at some exons but not others, indicating a nuanced role for LLPS in TDP-43 functionality in pre-mRNA splicing [84,86].

4.3. Heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1)

hnRNPA1 is another predominantly nuclear RBP that can mislocalize to cytoplasmic inclusions in the degenerating tissues of multisystem proteinopathy (MSP) patients [35,67,87]. MSP patients sometimes present with motor neuron degeneration similar to ALS patients [63,88]. hnRNPA1 has a C-terminal PrLD, and missense mutations in that region are linked to MSP and ALS [35]. For example, a D262V mutation introduces a potent steric zipper into the PrLD that leads to rapid hnRNPA1 fibrillization [35,67]. hnRNAP1 also forms liquid droplets in vitro in a concentration-dependent and PrLD-dependent manner [3,89]. Importantly, persistent droplet formation leads to aggregate accumulation within the droplet, which is aggravated by the D262V mutation [3,89].

4.4. T-Cell restricted intracellular antigen-1 (TIA1)

TIA1 is a major protein component of stress granules. Condensation of its C-terminal PrLD may contribute to stress granule assembly [90]. Indeed, the TIA1 PrLD can enable spontaneous LLPS in the presence of RNA [89]. Disease-related mutations in the C-terminal PrLD (including P326L, A381T, and E384K) promote phase transitions, decrease dynamics and reversibility of droplets, and alter stress granule dynamics by inhibiting their rate of dissociation [91]. These properties likely combine to enhance the mislocalization and inclusion formation of TDP-43 [91].

4.5. Arginine-rich C9orf72-derived dipeptide repeats (poly-GR, poly-PR)

The most common genetic cause of ALS/FTD is a hexanucleotide repeat expansion in the gene *C9orf72* [92,93]. Although the expansion is found in a non-coding region of the gene, dipeptide repeats can be translated bidirectionally via repeat-associated non-AUG (RAN) translation, resulting in five distinct species: poly-GA, -GP, -GR, -PA, and –PR, as well as chimeric dipeptide fusions [69,94–96]. Although the toxic species and interactions of these dipeptide repeats continue to be unraveled, poly-GR and poly-PR have repeatedly been shown to be the most toxic in cellular and animal models [38,97,98]. Additionally, poly-GR and poly-PR rapidly phase separate in physiological conditions in the presence of RNA, and impair the assembly, dynamics, and function of MLOs [38,69,98–100]. Poly-GR but not poly-GA also accelerate TDP-43 aggregation, which likely drives TDP-43 proteinopathy in c9ALS/FTD [68,101].

4.6. Ataxin 2

Ataxin 2 contains a poly-Q tract in one of its three PrLDs, and when this expansion goes beyond a certain threshold (27–33 glutamines), it is implicated in ALS [102–105]. By contrast, poly-Q tracts in ataxin 2 longer than 33 repeats are connected with spinocerebellar ataxia type 2 [106–108]. An unbiased screen to identify modifiers of TDP-43 toxicity in yeast identified ataxin 2 as a deletion suppressor of TDP-43 toxicity [102]. TDP-43 and ataxin 2 associate in an RNA-dependent complex that mislocalizes to the cytoplasm in spinal cord neurons of ALS patients [102]. Ataxin 2 is also involved in the assembly of higher order MLOs known as mRNP assemblies that contribute to long-term memory and plasticity [33]. These assemblies are made up of RNP granules, which may act as vessels where pathogenic proteins aggregate [36,74,109]. Knockdown of ataxin 2 slows the progression of neurodegenerative pathologies connected to ALS with a targeted reduction in RNP granule formation efficiency [33,110]. Indeed, antisense oligonucleotides that reduce ataxin 2 expression are now entering clinical trials for ALS (NCT04494256).

4.7. UBQLN2

UBQLN2 is implicated in the degradation of misfolded proteins through the ubiquitin-proteasome system and macroautophagy [111]. In some patients with ALS, it is found in cytosolic inclusions of degenerating motor neurons [112]. Several missense mutations in UBQLN2 are connected with ALS and all are found around an unusual Pxx repeat, which is present in UBQLN2 but not other UBQLNs [113]. Importantly, mutations in the Pxx region of UBQLN2 alter its LLPS behavior [34]. Additionally, Pxx mutants that enhanced the oligomerization of UBQLN2 decreased the concentration of the protein needed for LLPS and promoted solid-like assemblies [34]. These findings raise the question of whether ALS-associated mutations in the Pxx region potentially contribute to the aberrant stress granule dynamics, which may promote formation of the characteristic inclusions found in motor neurons of ALS patients.

5. Pathological proteins that undergo LLPS in Alzheimer's disease

Alzheimer's disease (AD) is a progressive fatal neurodegenerative disease with no cure or effective treatments [114]. The hallmarks of disease include extracellular amyloid-β plaques and intracellular tangles of aggregated tau [114]. Tau is a microtubule-associated protein and preventing its aggregation and spreading may be an important therapeutic approach to treat disease [115]. However, the initial events which seed the pathological aggregation of tau are not well understood. In addition to tau aggregation, there may also be a pathological contribution from RBP aggregates, which associate with tau aggregates in the brain of AD patients [116]. One such example is the RBP, U1 small nuclear ribonucleoprotein 70 kDa (U1-70K), but the co-aggregation mechanisms are incompletely understood [117].

5.1. Tau

Tau aggregation is a hallmark of AD and promotes neuronal loss and cognitive decline [118–122]. Several other disorders are characterized by aberrant tau aggregation, which are referred to as tauopathies, one example of which is FTD [123,124]. It has long been recognized that hyperphosphorylation of tau can cause it to dissociate from microtubules and specific tau phosphoforms can facilitate self-interaction and stimulate aggregation [125–127]. However, the exact mechanism that causes tau to become pathological has remained enigmatic. Strategies to reduce tau aggregation and increase degradation of aberrant species can restore proteostasis and mitigate cognitive decline in AD mouse models [128,129]. Tau can also undergo LLPS, which may help promote local nucleation of microtubule bundles [130]. However, tau LLPS can also initiate its aggregation and cause it to seed the aggregation of other proteins [44,131–133]. Phosphorylation can also increase the propensity of tau to undergo LLPS [44]. On the other hand, tau acetylation reduces tau LLPS [134].

5.2. U1-70K-RNA-binding protein (U1-70K)

U1-70K aggregates are also found in the brains of AD patients [117,135]. Repetitive basic-acidic motifs located in two low-complexity domain (LCD) fragments of U1-70K induce phase separation, which leads to accelerated aggregation in vitro [136,137]. Interestingly, whereas LLPS of many proteins depends on the presence of uncharged, polar residues, U1-70K condenses in a charge-driven manner [136].

6. Pathological proteins that undergo LLPS found in Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease and is characterized by a progressive loss of dopaminergic neurons and accumulation of intracellular inclusions of aggregated α-synuclein [138]. The α-synuclein-rich inclusions, termed Lewy bodies, are associated with cytotoxicity and lead to neuronal loss and concomitant motor impairments [139–147]. Prevention or elimination of pathological α-synuclein aggregates may serve as a therapeutic approach for PD treatment [148,149].

6.1. α-Synuclein

α-Synuclein is a presynaptic protein with roles in neuronal survival and synaptic vesicle trafficking regulation [150]. Under physiological conditions in vitro, α-synuclein spontaneously forms amyloid fibrils, which are grossly similar to the amyloids found in Lewy bodies [151]. However, α-synuclein fibrils isolated from disease tissue can have distinct structures from those formed in vitro [152]. Preventing pathological α-synuclein aggregation rescues cells from death and restores cognitive and motor functions [153], but the exact mechanism leading to aggregation is not well understood. The LLPS behavior of α-synuclein may offer clues into how pathological aggregation is initiated. α-Synuclein undergoes spontaneous LLPS in the presence of molecular-crowding agents such as polyethylene glycol, and this process is highly dynamic and reversible at early stages [37]. However, sustained α-synuclein LLPS leads to a loss of dynamics and reversibility, and eventually aggregation [37]. Additionally, PD-related factors, including mutations (A53T, E46K, and S129E) and disease-relevant conditions, accelerate α-synuclein LLPS and subsequent fibril formation [37]. Thus, α-synuclein may populate liquid states prior to converting to fibrils in Lewy bodies. Indeed, in a *C. elegans* model of PD, α-synuclein forms liquid droplets that convert into amyloid-rich hydrogels, in a process

that may mimic Lewy body assembly in PD [154]. Additionally, ultrastructural analyses of Lewy bodies from PD patient tissue indicate that they do not always contain cross-β fibrillar forms of α-synuclein [155–157]. Thus, other higher-order forms of α-synuclein (such as liquid states) might contribute to the spontaneous assembly of Lewy bodies [37,142,154]. Lewy body formation that is seeded by preformed α-synuclein fibrils may bypass these liquid states or accelerate their conversion to solid states [144,146].

7. Pathological proteins that undergo LLPS in Huntington's disease

Huntington's disease (HD) affects the central nervous system and is a fatal neurodegenerative disease. Within brain cells of patients, aggregates of a mutant form of polyQ-expanded huntingtin protein cause toxicity [158]. For the disease to occur, the length of the poly-Q tract must be beyond a threshold of 36 [159]. The length of the repeat expansion has a strong inverse correlation to the age of disease onset as well as disease severity [160]. Curiously, pathogenic polyQ expansions (range, 40–64 repeats) in huntingtin have also been found in rare ALS/FTD cases that do not display any features of HD [161].

7.1. Huntingtin

Mutant polyQ-expanded fragments of huntingtin can undergo LLPS and form reversible liquid droplets [43]. The LLPS behavior is driven by the length of the polyQ tract and proline-rich regions in the protein, with a correlation between the length of the repeat and the ability to undergo LLPS [43]. Additionally, sustained population in the liquid state drives polyQ fibrillization [43].

8. Strategies to combat pathological protein phase transitions

8.1. Molecular chaperones and protein disaggregases

In mammalian cells, the abundance of proteins must be carefully regulated to ensure proteostasis [162]. Proteostasis is especially essential in neurons as they do not divide, and thus they are highly vulnerable to accumulation of toxic misfolded conformers that cannot be diluted out by cell division [163]. To maintain proteostasis, the cell depends on an extensive network of molecular chaperones and proteolytic systems [164]. There are over 150 molecular chaperones that come in many different forms. These chaperones assist with maintaining proteostasis through several mechanisms, including aiding in protein folding, protein degradation, translation of nascent peptides, and in helping misfolded proteins recover their native form [165]. In some specialized cases, molecular chaperones can possess protein-disaggregase activity where they recover natively folded proteins from aggregated species [54,57,61,166–168]. Several neurodegenerative diseases are associated with mutations in chaperone proteins suggesting that their normal function may be protective [169–172]. Additionally, since neurodegenerative diseases are associated with an inability to maintain proteostasis, harnessing the power of molecular chaperones could be a promising therapeutic strategy [59,61,173–176].

Several strategies are being developed to harness molecular chaperones and improve their activity so that they can be leveraged as effective therapeutics for neurodegenerative diseases. Many of these strategies could also antagonize aberrant protein LLPS associated with neurodegenerative diseases. Below we will discuss the strategies for using protein disaggregases and other agents to combat aberrant LLPS.

8.2. Potentiation of protein disaggregases

Molecular chaperones and protein disaggregases lose the battle against protein misfolding in the context of neurodegenerative disease [173]. One strategy to shift the balance toward protein folding has been to overexpress individual protein disaggregases or molecular chaperones, which can partially mitigate protein aggregation or neurodegenerative phenotypes in model systems [148,149,177–181]. We have hypothesized that WT protein disaggregases may not be optimally adapted to reverse deleterious phase transitions in the environment of the aging brain [57,174,182]. Thus, we suggest that engineering potentiated protein disaggregases could be an effective strategy to increase their activity and specificity for their target clientele [57,174,182]. This strategy has been used primarily on the Hsp104 disaggregase to increase its activity to reverse pathological aggregation that is characteristic of neurodegenerative diseases [183–185].

Hsp104 is a hexameric AAA+ ATPase and protein disaggregase found in all non-metazoan eukaryotes [59,166,186,187]. It has successfully been engineered to have potentiated disaggregase activity [183–185,188]. Many genetically engineered versions of Hsp104 have now been shown to have enhanced activity and ability to revert TDP-43, FUS, and α-synuclein aggregation, suppress toxicity and eliminate protein aggregates in yeast [183–185,188]. Additionally, enhanced Hsp104 variants can dissolve cytoplasmic ALS-linked FUS and TDP-43 aggregates in mammalian cells [189,190], and antagonize α-synuclein-mediated dopaminergic neurodegeneration in *C. elegans* [188,191].

While metazoan systems lack Hsp104, they do encode other AAA+ proteins that can function as protein disaggregases, including Skd3 [168] and VCP [192,193]. Thus, Skd3 and VCP may also be candidates for engineering. Likewise, the human protein-disaggregase system, comprised of Hsp110, Hsp70, Hsp40, and small heat-shock proteins might also be targeted [174]. This system can disaggregate and reactivate proteins trapped in disordered aggregates and amyloid, but fails in the context of neurodegenerative disease [167,194–200]. Hsp70 also maintains liquid forms of TDP-43 in the nucleus and prevents their solidification [201]. The bacterial version of Hsp70 (DnaK) has been successfully engineered to outperform the WT version in refolding denatured proteins with an improved initial refolding rate [202]. Likewise, engineering efforts are also beginning to uncover mildly enhanced versions of human Hsp70 [174,203–205]. It will be interesting to define how potentiated disaggregases affect LLPS of protein clients, and discern if further genetic engineering could improve their ability to modulate LLPS.

Another human protein-disaggregase system that has recently come to light is comprised of the tripartite-motif (TRIM) proteins [149,206]. TRIM proteins are defined by the TRIM/RBCC motif consisting of a RING domain, one or two B-boxes, and a coiled-coil region [207]. These proteins are found only in metazoans and exist as a large number of distinct variants, with ~20 in *C. elegans* and over 70 in mice and humans [207]. Recent studies have established that TRIM11 prevents the formation of protein aggregates and dissolves pre-existing protein deposits, including amyloid fibrils [149]. These molecular chaperone and disaggregase activities are ATP independent [149]. The chaperone and disaggregase activity of TRIM11 enhances the folding and solubility of normal proteins and cooperate with TRIM11 SUMO-ligase activity to degrade aberrant proteins [149]. TRIM11 reverses α-synuclein fibrillization and mitigates α-synuclein toxicity in cell models of PD [149]. Intracranial delivery of TRIM11 mitigates α-synuclein-mediated pathology, neurodegeneration, and motor impairments in a PD mouse model [149]. Other TRIMs can also function as ATP-independent molecular chaperones and disaggregases, including TRIM19 and TRIM21 [149,206]. Importantly, TRIM19 can be engineered to display enhanced activity against polyQ [208]. Thus, TRIMs are a potent and multifunctional protein quality-control system in metazoa, which might be applied and engineered to treat neurodegenerative diseases.

Conventional pharmacological treatments have proven difficult to develop for the treatment of neurodegenerative diseases. This difficulty reflects, at least in part, the challenges for drugs to cross the blood-brain barrier and the risk of direct administration to the central nervous system. One approach that has been successfully conceptualized for the treatment of such disorders is through gene transfer mediated by an

adeno-associated virus (AAV) [209]. After a single administration of AAV, non-dividing cells like neurons can be successfully transduced allowing for the permanent expression of a therapeutic gene of interest [210]. The safety of this delivery method has been consistently demonstrated in clinical trials for neurodegenerative diseases [211–213], but most transgenes have shown limited efficacy. This limited efficacy highlights the need for a transgene that can address the root underlying cause of disease. Protein disaggregases such as TRIMs or engineered variants may be promising targets for delivery by AAV [149,206–208].

8.3. Drugs that upregulate chaperone expression

Another possible method to induce the expression of chaperones or disaggregases is through stimulation by brain-penetrant small-molecule drugs. Here, rather than overexpressing an individual disaggregase or enhanced variants, the strategy is to elicit transcription of a battery of chaperones or specific chaperones as might occur in a stress response [214,215]. However, this strategy assumes that transcriptional stress responses can still occur productively under the conditions of neurodegenerative disease or aging, which may not be the case [214,216,217]. Likewise, chronic overactivation of transcriptional stress responses can also be damaging, and there may also be merit in inhibiting their overactivation [218–223]. Nonetheless, several drugs have been developed for boosting chaperone expression and some are undergoing clinical trials for treatment of ALS [224], including arimoclomal and colchicine.

Arimoclomol is an analog of bimoclomol, a compound with stress protein-inducing activity and cytoprotective effects that is non-toxic [225,226]. Specifically, arimoclomol is a co-inducer of Hsp70 and Hsp90, and it is able to delay disease progression in ALS and inclusion body myopathy mice [227,228]. Moreover, a randomized, double-blind, placebo-controlled trial of arimoclomol treatment in rapidly progressive SOD1 ALS patients concluded that the drug was safe and well tolerated, but not powerful enough for a therapeutic effect [229]. However, it was noted that a larger sample size may show a therapeutic effect because a positive, but not significant, trend was observed [229]. Furthermore, the identification of superior biomarkers that can be used to diagnose ALS earlier may also improve the prospects for arimoclomol. Earlier administration of arimoclomol may enable greater neuroprotection.

Colchicine is a microtubule-destabilizing drug that leads to an upregulation of a chaperone protein. Colchicine greatly enhances expression of HspB8, a small heat shock protein, as well as the expression of several other proteins involved in autophagy [230]. In neurons, the expression of HspB8 is normally induced when proteasomal impairments occur [231,232]. HspB8 is also part of the Hsp70-Bag3-HspB8 complex. This complex recognizes and responds to misfolded proteins in stress granules and removes them for refolding or degradation [233]. Interestingly, HspB8 mRNA levels are significantly higher in the spinal cord of ALS patients than in age-matched controls, leading to the hypothesis that its expression is involved in the cellular response to molecular insults associated with ALS [234]. Indeed, when HspB8 is induced by colchicine, it facilitates the removal of insoluble accumulations of TDP-43 [235]. Moreover, in a transgenic ALS SOD1-G93A mouse model, there was a robust increase of HspB8 protein levels in the anterior horn cells of the spinal cord that survived to end-stage of disease [232]. This increase was associated with more diffuse, non-aggregated mutant SOD1 [232]. The effects of upregulating HspB8 expression with colchicine may be promising, and there is an ongoing phase II multicenter clinical trial to assess colchicine as a potential therapeutic for ALS (NCT03693781).

8.4. Nuclear-import receptors (NIRs)

When macromolecules pass from the cytoplasm to the nucleus, they are typically transported through the nuclear pore complex [236]. Small molecules are able to pass through via passive transport, but large proteins (>20 kDa) must usually engage nuclear-import receptors (NIRs) via a nuclear-localization signal (NLS) to be effectively transported across the nuclear pore complex [236]. NIRs are typically members of the Karyopherin-β (Kap-β) family, which drive most cytoplasm-to-nucleus transport events [236].

NIRs have been shown to drive nuclear localization of RBPs that are connected with neurodegenerative disease pathology [237] and, importantly, can chaperone and disaggregate disease-linked RBPs with an NLS [25,52,101,238–243]. In fact, by binding to the NLS of its cargo, Karyopherin-β2 (Kapβ2) can prevent and reverse fibrillization of FUS, hnRNPA1, hnRNPA2, TAF15, EWSR1, and several disease-linked variants [52]. Kapβ2 can also prevent poly-GR from stimulating TDP-43 aggregation, and NIRs can also directly antagonize poly-GR condensation [101]. Moreover, importin α and Kapβ1 prevent and reverse TDP-43 condensation and fibrillization [52,101]. Nuclear magnetic resonance spectroscopy suggested that after Kapβ2 binds the NLS of FUS it then engages with sequence elements and structural domains distributed throughout FUS that contribute to FUS phase separation [243]. Thus, the chaperone activity of Kapβ2 is achieved without ATP hydrolysis, and occurs by competing with multivalent cargo-cargo interactions that facilitate oligomerization [243]. In this way, Kapβ2 and other NIRs prevent aberrant accumulation of RBPs containing NLSs into cytoplasmic inclusions, and re-establish their nuclear localization and function, rescuing the degeneration caused by mislocalized RBPs in disease [52,61]. Identifying small-molecule drugs to increase expression or stimulate NIR activity (e.g. by stabilizing NIR interactions with specific cargo such as TDP-43 or FUS) is another potential approach to treating diseases associated with aberrant phase transitions [52,61].

8.5. RNA and oligonucleotides

Anti-sense oligonucleotides (ASOs) to modulate expression of specific proteins represent attractive therapeutic strategies in treating neurological diseases [56,244–246]. Indeed, ASOs are now approved therapeutics for spinal muscular atrophy [56,244]. Several possibilities for ASO interventions in neurodegenerative disease are apparent. For example, TIA1 is a key protein involved in stress granule assembly [91]. It also has been shown to interact with tau, and interestingly, knockdown of TIA1 via ASOs prevented tau pathology and toxicity in primary neurons and AD rodent models [247,248]. Another example of successful treatment by ASOs is through the knock-down of ataxin 2, another key protein involved in the regulation of stress granules [110]. Targeting ataxin 2 via ASOs led to reduced TDP-43 pathology in a transgenic mouse model of TDP-43 proteinopathy and increased survival [110]. Additionally, the deletion of a key region of ataxin 2 involved in LLPS prevented neurodegeneration induced by both FUS and c9orf72 dipeptide repeats [33]. Ataxin 2 ASOs are now in phase 1/2 clinical trials (NCT04494256). Likewise, several other neurodegenerative disease proteins are being targeted by therapeutic ASOs, including SOD1 [245,249], huntingtin [250], prion protein [251,252], α-synuclein [253,254], and c9orf72 [245,255–257]. In these cases, it is thought that knockdown of these genes will not have damaging side effects. The situation is more complex for FUS and TDP-43, which serve more essential functions. Thus, severely reduced expression of TDP-43 or FUS could have negative effects on neurons [70]. However, in some genetic forms of disease it may still be possible to specifically knockdown a mutant form of TDP-43 (e.g. M337V) [258] or FUS (e.g. P525L) [259] which is causing disease. Here, selectively knocking down the mutant but not WT version of the RBP may be therapeutic. Alternatively, moderate reductions in WT TDP-43 or FUS expression could enable therapeutic effects while maintaining enough TDP-43 or FUS to perform essential functions.

More recently it has been shown that RNAs themselves can act as chaperones to prevent aberrant phase transitions [21,260,261]. Indeed, many of the pathological proteins involved in neurodegenerative

disorders (particularly in ALS/FTD) are RBPs, and their propensity for self-association varies as a function of the RNA in their environment [260]. For instance, abnormal interactions between residues in the PrLD of TDP-43 facilitate its pathological accumulation [81,262]. However, in the presence of RNA, this self-interaction is antagonized, while mutations in the RNA-recognition motifs of TDP-43 that reduce RNA binding also ablate the protective function of RNA [21]. The chaperone activity of RNA occurs both in vitro and in cells, as aberrant and toxic LLPS of TDP-43 in the cytoplasm of neurons could be rescued with an intervention of oligonucleotides designed to bind to TDP-43 RRMs [21]. This "bait RNA" was able to prevent aberrant TDP-43 inclusions and delay cell death in multiple cell models [21]. These results beg the question as to whether this bait RNA strategy can be used for other RBPs [263,264].

8.6. Anti-aggregation molecules

Small molecules that specifically bind to and prevent the aggregation of disease-linked proteins may be promising targets for the intervention of aberrant LLPS. One such example is AIM4, an acridine derivative, which interacts with the C-terminal PrLD of TDP-43 and prevents its aggregation [265]. Akin to the effects of RNA on TDP-43 LLPS, AIM4 can inhibit LLPS of a C-terminal TDP-43 fragment bearing an A315T familial ALS mutation [265]. Therefore, anti-aggregation molecules that prevent aggregation of target proteins by binding to regions also important for LLPS may be promising therapeutic leads. More studies are needed to explore the effects of other small molecules with this mechanism of action. Often an aggregation-prone protein will escape inhibition by a single small-molecule inhibitor by forming alternative aggregated structures or 'strains' [266–268]. However, this process can be mitigated by combinations of two or more small molecules that synergize to antagonize aggregation [266–268]. Thus, it will also be of great interest to find small-molecule combinations that synergize to antagonize aberrant LLPS to ensure maximal inhibition and minimize formation of alternative structural polymorphs.

8.7. Modulating ATP levels

As humans age, the risk of being diagnosed with a neurodegenerative disease substantially increases. Aging is also accompanied by a reduction in ATP levels [269]. This ATP depletion has an effect on cellular LLPS, as lowering cellular ATP levels decreases the dynamics of stress granules and makes them less reversible [270]. This loss of reversibility is most likely due to the fact that stress-granule disassembly is an ATPase-driven process [193]. Additionally, enhanced levels of ATP in the cell can act as a chemical hydrotrope that directly prevents phase separation and subsequent aggregation [271]. Therefore, strategies to increase the levels of ATP in cells may also be effective at combating aberrant LLPS that is observed in neurodegenerative diseases.

8.8. Alteration of protein post-translational modifications

Post-translational modifications (PTMs) of proteins can have large effects on their activity and structure, including altering protein charge, size, and hydrophobicity. PTMs occur via the addition of functional groups by specialized enzymes or through more subtle changes such as oxidation [272]. Many protein PTMs serve as on/off switches, which regulate cellular processes [273] and LLPS [274]. There are several examples of PTMs on neurodegenerative disease-associated proteins that can alter both their aggregation characteristics as well as their LLPS behavior. Although several PTMs are associated with changes in protein LLPS, a potent example is phosphorylation.

Protein phosphorylation is the process of covalently adding a phosphoryl group to an amino acid residue. The most common residues to be phosphorylated are serine, threonine, and tyrosine in eukaryotes [275]. The addition of a phosphoryl group alters the charge of the protein, which can promote different interactions that, depending on their location, can either promote or inhibit LLPS. It is possible that phosphorylation will promote charge-charge interactions that drive LLPS [276], but alternatively it could cause charge repulsion or steric hindrance which instead inhibits LLPS [277].

The phosphorylation of serine/threonine residues in TIA1 [278] and tau [44] promote their LLPS behavior. Interestingly, phosphorylation of these proteins at the same residues that promote LLPS behavior also promotes their aggregation [44,278]. Additionally, hyperphosphorylation of tau is a key biomarker in the AD patient brain [279], and specific tau phosphoforms exhibit accelerated fibrillization [126,127].

By contrast, phosphorylation of the PrLD of FUS reduces LLPS [280]. In fact, FUS aggregation and LLPS are diminished when the FUS PrLD is phosphorylated or bears phosphomimetic substitutions [76,77,281]. Phosphorylation of the FUS PrLD introduces electrostatic charges that reduce its prion-like nature and multivalent interactions that drive LLPS [76]. Phosphorylation of the TDP-43 PrLD results in significant decreases in the number of cells containing TDP-43 puncta [282]. However, the phase separation of TDP-43 is also regulated by PTMs in its N-terminal structured domain. Phosphorylation of serine 48 in the N-terminal domain, which is highly conserved across species, is enough to suppress TDP-43 LLPS [82].

Collectively, these findings suggest that genetic or pharmacological intervention to upregulate or downregulate specific PTMs may be an attractive therapeutic strategy against aberrant LLPS. However, before this strategy can be realized the target protein in question must be well characterized regarding how PTMs affect its LLPS behavior, propensity to aggregate, and toxicity.

9. Conclusions

It is now established that several of the pathological proteins found in an aggregated or mislocalized state in neurodegenerative diseases are also able to undergo LLPS under physiological conditions. Although these phase transitions are likely important for various physiological functions, neurodegenerative disease-related mutations and conditions can alter the LLPS behavior of these proteins, which can elicit toxicity. Thus, therapeutics that antagonize aberrant LLPS may be able to mitigate toxicity and aggregation that is ubiquitous in neurodegenerative disease. Here, we have discussed the mechanisms by which aberrant protein phase transitions may contribute to neurodegenerative disease. We have also outlined several potential therapeutic strategies to counter deleterious phases. We anticipate that these strategies alone or perhaps in synergistic combination will provide a powerful arsenal to ultimately eliminate neurodegenerative disease.

CRediT authorship contribution statement

A.L.D. and J.S. wrote the manuscript.

Declaration of competing interest

A.L.D. has nothing to declare. J.S. is a consultant for Dewpoint Therapeutics and Maze Therapeutics.

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