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Designer protein disaggregases to counter neurodegenerative disease

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Protein misfolding and aggregation unify several devastating neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. There are no effective therapeutics for these disorders and none that target the reversal of the aberrant protein misfolding and aggregation that cause disease. Here, I showcase important advances to define, engineer, and apply protein disaggregases to mitigate deleterious protein misfolding and counter neurodegeneration. I focus on two exogenous protein disaggregases, Hsp104 from yeast and gene 3 protein from bacteriophages, as well as endogenous human protein disaggregases, including: (a) Hsp110, Hsp70, Hsp40, and small heat-shock proteins; (b) HtrA1; and (c) NMNAT2 and Hsp90. I suggest that protein-disaggregase modalities can be channeled to treat numerous fatal and presently incurable neurodegenerative diseases.

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Introduction

Deleterious protein misfolding and aggregation underpin several invariably fatal and age-related neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) [1]. Typically, in each disease specific proteins misfold, aggregate, and wreak havoc on the nervous system [1]. In AD, amyloid-β (Aβ) peptides form extracellular, neuritic plaques and tau forms intracellular neurofibrillary tangles in afflicted neurons [1]. By contrast, in PD, α-synuclein (α-syn) forms cytoplasmic Lewy bodies in degenerating dopaminergic neurons [1]. In most ALS cases, RNA-binding proteins with prion-like domains, such as TDP-43 or FUS, mislocalize from the nucleus to cytoplasmic aggregates in degenerating motor neurons and glia [1,2]. Current treatments for these disorders are palliative and ineffective. No therapeutics exist that reverse the aberrant protein misfolding and aggregation that underlie disease. The lack of effective therapies is a cause of immense angst as these diseases are increasing in prevalence as our population ages.

Complexity of protein misfolding

Protein misfolding is a complex, multistate process [3,4]. The specific proteins that misfold in neurodegenerative disease are often intrinsically disordered, harbor an intrinsically disordered domain, or passage through partially unfolded states that enables them to morph into an eclectic menagerie of misfolded structures with variable toxicities [1–5]. These structures include self-templating amyloid fibrils with cross-β architecture, disordered aggregates, and small soluble oligomers [1,3]. For example, in PD, a small intrinsically-disordered protein, α-syn, forms amyloid fibrils that self-template or 'seed' their own assembly via recruiting soluble forms of α-syn to their elongating ends where α-syn is conformationally converted to the cross-β structure [1,6–8,9•,10•]. α-Syn amyloid can spread from cell to cell, thereby propagating pathology [1,6,8,11,12••,13,14]. Indeed, amyloid fibrils formed by recombinant α-syn in the test tube can induce a PD-like disease when injected into the brain of a mouse [6,11,13]. This transforming principle establishes that the self-replicating structure of α-syn amyloid can encode the PD phenotype, which develops via the ongoing conversion of endogenous α-syn to the amyloid state as α-syn fibrils spread through the brain [1,8,13,15]. Moreover, α-syn can form fibrils with different cross-β structures, termed 'strains', which encode distinct neurodegenerative phenotypes [9•,10•,16,17•,18,19]. The lateral face of α-syn amyloid provides a surface where α-syn oligomers can nucleate [20]. α-Syn populates diverse soluble, oligomeric species before, during, and after α-syn amyloidogenesis, which can be on or off pathway for amyloid formation [7,21–25,26•]. α-Syn oligomers are typically more toxic than mature fibrils [7,23]. Small soluble oligomers or short, fragmented amyloid fibrils are more toxic than very large aggregated species, which due to their low surface-area-to-volume-ratio shield damaging surfaces inside the aggregate [7,23,27]. A major challenge for any therapeutic aimed at mitigating protein misfolding is the ability to remodel diverse, toxic misfolded conformers, including soluble oligomers and amyloid fibrils into benign species [28,29].

Protein disaggregases as potential therapeutics

I have postulated that protein disaggregases could be uniquely suited to meet this challenge as they can safely deconstruct self-templating amyloid and toxic soluble oligomers, and recover soluble protein with restored functionality from these structures [28,29]. Thus, protein disaggregases could mitigate any toxic gain-of-function or toxic loss-of-function connected with protein misfolding, and simultaneously could eradicate self-templating species that propagate disease [28,29]. Protein disaggregation might also be coupled to protein degradation, which could also be beneficial to eliminate toxic and self-templating conformers, and subsequent translation of new protein could antagonize any toxic loss-of-function [29]. However, protein disaggregases remain among the least understood components of the proteostasis network, and we are only at the inception of realizing their existence and potential [28,29]. Here, I highlight recent advances to define, engineer, and apply protein disaggregases to reverse deleterious protein misfolding in neurodegenerative disease.

Hsp104, a protein disaggregase from yeast

Hsp104 is an asymmetric ring-shaped translocase and hexameric AAA+ protein found in yeast [30,31••]. Hsp104 couples ATP hydrolysis to the rapid dissolution and reactivation of diverse proteins trapped in disordered aggregates, ordered stress-induced assemblies, preamyloid oligomers, amyloids, and prions [32–37,38••]. Optimal Hsp104 disaggregase activity can require collaboration with the Hsp70 chaperone system [30]. In yeast, Hsp104 performs critical functions in stress tolerance, prion inheritance, asymmetric partitioning of aggregates during cell division, and promoting longevity [30]. Hsp104 rapidly disaggregates Sup35 prions within a few minutes [35–37,39]. Moreover, Hsp104 effectively dissolves amyloids formed by diverse human degenerative disease proteins, including: Aβ42, tau, polyglutamine, α-syn, prion protein (PrP), and amylin [34,40–42]. Hsp104 also rapidly remodels amyloid fibrils formed by fragments of prostatic acid phosphatase (PAP248-286 and PAP85-120) [43••], which are abundant in human seminal fluid and promote HIV infection [44]. This rapid and broad-spectrum amyloid-disaggregase activity of Hsp104 is unusual and might represent a therapeutic opportunity [28].

Intriguingly, Hsp104 is absent from metazoa, but is found in all non-metazoan eukaryotes, all eubacteria, and some archaebacteria [45]. Thus, Hsp104 could be developed into a vital disruptive technology that retools proteostasis to combat neurodegenerative disease and HIV infection [28,43••,46]. Indeed, we have established Hsp104 as the only factor known to dissociate α-syn oligomers and amyloids connected with PD *and* rescue α-syn-induced neurodegeneration in the substantia nigra of a rat PD model [34,40,41]. Moreover, Hsp104 rescues polyglutamine toxicity and neurodegeneration in *Caenorhabditis elegans*, fly, mouse, and rat [28,47]. Hsp104 even rescues polyglutamine toxicity after degeneration has begun [47]. Hsp104 expression is not detrimental in metazoa and can be broadly and safely expressed in worm, fly, mouse, and rat, as well as in mammalian cell and neuronal cultures [28,34,47]. These findings make it difficult to understand why Hsp104 was lost from metazoa, but also emphasize that Hsp104 might be safely introduced and developed as a therapeutic agent [28,43••,46].

Despite these encouraging activities, very high Hsp104 concentrations are needed for optimal disaggregation of human disease proteins, such as α-syn, which may restrict efficacy [34,40,41]. Thus, we have engineered potentiated Hsp104 variants, which rescue aggregation and toxicity of proteins associated with neurodegenerative disease such as TDP-43, FUS, TAF15, and α-syn, and mitigate neurodegeneration in the metazoan nervous system at concentrations where Hsp104 is ineffective [46,48••,49,50,51••,52]. Hsp104 activity can be potentiated by single missense mutations at specific positions in the middle domain or nucleotide-binding domain 1 of Hsp104 [46]. Potentiating mutations reconfigure how Hsp104 subunits collaborate, alter substrate discrimination, alleviate any stringent requirements for Hsp70, and enhance Hsp104's ATPase, translocase (rate at which substrates are translocated across the central channel of Hsp104), unfoldase, and disaggregase activity [48••,49,50]. These combined properties enable potentiated Hsp104 variants to outperform Hsp104 under conditions where an aggregation-prone protein such as TDP-43, FUS, or α-syn has exceeded proteostatic buffers and is undergoing widespread misfolding and aggregation [46]. Potentiated Hsp104 variants can have off-target effects [48••,49], and may require further engineering to minimize these via increasing substrate selectivity [46]. Importantly, substoichiometric concentrations of potentiated Hsp104 variants can remodel amyloid [43••]. For example, nanomolar concentrations of an enhanced Hsp104 variant, $Hsp104^{A503V}$, can remodel micromolar concentrations of PAP248-296 sequestered in SEVI fibrils [43••]. The challenge ahead is to determine whether Hsp104 or enhanced variants can confer increased therapeutic benefits in mammalian cells, patient-derived neurons, and additional animal models of neurodegeneration.

An issue that is often raised about introducing any exogenous protein as a therapeutic is whether the patient might mount a deleterious immune response against the therapeutic protein. However, it is important to note that the central nervous system (CNS) exhibits immune privilege [53]. Thus, immune responses to CNS antigens are very slow to develop [53], which could provide a therapeutic window for exogenous agents, such as Hsp104 [29], tetanus and botulinum toxin variants [54], or even

CRISPR-Cas9 [55] to be delivered to the CNS. Indeed, we observed no deleterious side effects of expressing Hsp104 for 6 weeks in the rat substantia nigra when delivered via lentivirus [34]. Transient expression or delivery of an exogenous therapeutic protein to the CNS may thus be well tolerated (particularly if combined with an immunosuppressant), although significant caution is highly warranted.

Gene 3 protein, a protein disaggregase from bacteriophage M13

Hsp104 must hydrolyze ATP to disaggregate proteins [30], which would limit disaggregase activity in ATP-depleted environments such as the extracellular space, where the ATP concentration is ~10 nM compared to ~3–10 mM inside cells [56]. The extracellular space is where Aβ deposits accumulate in AD and prions accumulate in Creutzfeldt–Jakob Disease [1]. Thus, to antagonize these extracellular protein-misfolding events Hsp104 would need to be engineered to operate effectively at limiting ATP concentrations. An alternative strategy is to define ATP-independent protein disaggregases, which may couple binding energy to disaggregation and are operational outside cells. Select small molecules, including CLR01, a lysine- and arginine-specific molecular tweezer, and the green tea polyphenol, EGCG, can safely disaggregate or remodel diverse amyloids [57,58,59••,60]. A human Aβ oligomer- and fibril-specific monoclonal antibody, aducanumab, promotes clearance of Aβ plaques and may retard clinical decline in AD patients and is now in phase 3 trials [61••]. However, whether this Aβ-plaque clearance is due to fibril disaggregation (as with select anti-Aβ antibodies that bind the N-terminal region of Aβ [62,63]), phagocytic clearance by microglia, or both is uncertain [61••]. Several other ATP-independent protein disaggregases have emerged including a subunit of the chloroplast signal recognition particle [64–66], cyclophilin [67–69], HtrA1 [70••], and gene 3 protein (g3p) [71••].

G3p is a minor capsid protein from filamentous bacteriophage M13, which enables viral entry into the bacterial host [72]. Remarkably, g3p enables M13 bacteriophages to slowly remodel diverse amyloids, including those formed by Aβ42, α-syn, tau, and NM (the prion domain of Sup35) [71••,73]. This amyloid-remodeling activity may enable phages to infect bacteria by penetrating protective amyloid-based biofilms [74]. The amyloid-binding activity of g3p was mapped to its two N-terminal domains, which are separated by a glycine-rich hinge [71••]. A recombinant soluble g3p fragment, termed G3P, which comprises the two N-terminal domains and the hinge could bind diverse amyloids but could not disaggregate them [71••]. However, 3-5 copies of g3p form an oligomer at the tip of the filamentous phage capsid [72]. Thus, g3p multivalency may enable amyloid remodeling [71••]. Indeed, amyloid-disaggregation activity was restored by engineering G3P to be dimeric via an immunoglobulin (Ig) Fc-G3P fusion protein (Ig-G3P) [71••]. Importantly, Ig-G3P selectively bound and disaggregated diverse amyloids, including Aβ42 and tau fibrils, and did not interact with various disordered aggregates or monomeric Aβ42 [71••]. It is not yet clear whether Ig-G3P remodels soluble toxic oligomers. Although the mechanism by which Ig-G3P dissociates diverse amyloids is uncertain, multiple binding events to different regions of assembled fibrils seems likely to be important [71••]. Importantly, weekly intraperitoneal injection of Ig-G3P reduced both Aβ and tau pathologies and improved cognition in mouse models [75•]. Thus, Ig-G3P is an interesting therapeutic candidate for AD that targets Aβ and tau misfolding, and is now in Phase 1B clinical trials [75•].

Hsp110, Hsp70, and Hsp40 disaggregases in humans

Bafflingly, Hsp104 is absent from metazoa [28,45], and whether metazoa even possess a protein disaggregation and reactivation machinery had endured as a long-standing enigma [41,52]. It is now clear that human Hsp110, Hsp70, and Hsp40 synergize to dissolve and reactivate model proteins trapped in disordered aggregates and depolymerize amyloid fibrils formed by α-syn [41,52,76–80]. Small heat-shock proteins can further enhance the disaggregase activity of this system [76]. Proteins disaggregated by Hsp110, Hsp70, and Hsp40 can be refolded [41,80] or passed to the proteasome to be degraded [81••]. This latter pathway is mediated by Ubiquilin 2, which recognizes client-bound Hsp70 and enables transfer of client to the proteasome [81••]. Interestingly, ALS-linked mutations in Ubiquilin 2 impair this activity, which may contribute to disease [81••].

Hsp110, Hsp70, and Hsp40 drive disaggregation by exerting pulling forces on aggregated polypeptides, which are forcibly extracted from the aggregate [52,82,83]. This system might also remodel toxic soluble oligomers formed by various proteins [37,84]. Hsp70 must engage substrate and Hsp110, and hydrolyze ATP to drive protein disaggregation [41,52]. Hsp40 must harbor a functional J domain (which stimulates Hsp110 and Hsp70 ATPase activity) to promote protein disaggregation, but the J domain alone is insufficient [41,52]. Whether Hsp110 acts simply as a nucleotide exchange factor (NEF) for Hsp70 or whether it must also bind substrate, hydrolyze ATP, or both as part of the disaggregation reaction is debated [52,82,83]. Likewise, whether Hsp70 must act as a NEF for Hsp110 to drive protein disaggregation is debated [52,78,83]. I suspect there is plasticity in disaggregase mechanism with respect to the exact role of Hsp110, which may depend on aggregate structure as with Hsp104 [40,52]. Indeed, Hsp70 (and likely Hsp110) exhibits functional plasticity via alternative modes of client engagement, which can promote

protein folding or unfolding [85•]. Regardless, humans express a variety of Hsp110, Hsp70, and Hsp40 chaperones, and the precise combination and ratio of components can enhance or inhibit disaggregase activity against different substrates [41,77–80].

The Hsp110, Hsp70, and Hsp40 disaggregase machinery must become overwhelmed in neurodegenerative disease. Indeed, Hsp110 knockout mice develop age-dependent tau hyperphosphorylation, early accumulation of insoluble Aβ, and neurodegeneration [86]. Moreover, Hsp110 (or another Hsp70 NEF, Bag3), Hsp70, Hsp40, and small heat-shock proteins collaborate to dissolve stress granules [87,88,89••,90], dynamic RNP assemblies that accumulate upon stress (and incorporate TDP-43 and FUS), and are connected to ALS and formation of pathological aggregates [2,4]. Upregulation or stimulation of Hsp110, Hsp70, and Hsp40 disaggregase activity, perhaps with small-molecule drugs [91], could have key therapeutic applications [29]. Importantly, Hsp104 can greatly enhance the disaggregase activity of Hsp110, Hsp70, and Hsp40 [41,76]. Co-expression of Hsp110 and Hsp40 in *Drosophila* suppresses polyglutamine toxicity [92]. Moreover, increased expression of Hsp110 extends lifespan of ALS-linked SOD1 transgenic mice [93••].

Engineering Hsp110, Hsp70, and Hsp40 to have enhanced disaggregase activity may enable robust neuroprotection [94]. Hsp70 variants with enhanced chaperone or disaggregase activity against specific model substrates have been uncovered [94–97], but whether these can be translated into neuroprotective agents *in vivo* is unknown. Engineering a secreted form of Hsp70 rescued a *Drosophila* model of AD, although in this case Hsp70 promoted clustering of Aβ42 into larger aggregates with reduced neurotoxicity [98•]. This clustering activity appears to be a general feature of molecular chaperones that must operate under ATP-limited conditions such as the extracellular space, and can be protective by minimizing exposure of reactive surfaces by confining them within the aggregate interior [43••,99].

HtrA1, an ATP-independent human protein disaggregase

HtrA1 is a ubiquitously expressed chaperone and homo-oligomeric PDZ serine protease abundant in human brain [100,101], which is also an ATP-independent protein disaggregase [70••]. HtrA1 is localized to the cytoplasm and extracellular space, and selectively degrades misfolded substrates while leaving their folded counterparts alone [29,70••,100,101]. HtrA1 disassembles and degrades tau and Aβ42 fibrils connected to AD [70••]. An engineered protease-defective HtrA1 variant dissolves tau and Aβ42 fibrils without degrading them [70••]. Thus, HtrA1 could be tailored to dissolve inclusions in AD or tauopathies, which could be important to rapidly restore tau loss-of-function [29]. A significant inverse correlation exists between HtrA1 and tau levels in AD patient brains [101]. These data suggest that HtrA1 functions as a tau disaggregase and protease *in vivo* [70••,101]. Whether HtrA1 can dissociate and degrade toxic soluble oligomers is uncertain. Nonetheless, HtrA1 could be a valuable ATP-independent protein disaggregase against AD, and like Ig-G3P targets both tau and Aβ42 [70••,71••]. It will be important to test whether elevating HtrA1 activity is protective in animal models of AD and tauopathy. Intriguingly, defects in the mitochondrial isoform, HtrA2, have been connected to PD [102].

NMNAT2 and Hsp90 combine to refold aggregated proteins

Nicotinamide mononucleotide adenylyl transferases (NMNATs) synthesize nicotinamide adenine dinucleotide (NAD^+) [103], a critical co-enzyme that performs key electron-transfer events in metabolism and is an essential substrate for sirtuins and poly(adenosine diphosphate-ribose) polymerases [104]. Humans express three NMNATs, with NMNAT2 being abundant in the brain, whereas *Drosophila* express a single NMNAT [103]. Remarkably, NMNATs can be neuroprotective in several models of neurodegenerative disease, and can function as chaperones that prevent aggregation of disease proteins, including polyglutamine-expanded ataxin 1 and tau [103,105,106]. NMNAT2 collaborates with Hsp90 to disaggregate and refold previously aggregated proteins [107••]. NMNAT2 disaggregase activity is independent from NAD^+ biosynthesis, but requires a unique C-terminal ATP-binding site that may be activated by Hsp90 [107••]. Thus, NMNAT2 chaperone and disaggregase activity may reduce proteotoxicity, whereas its NAD^+ biosynthetic activity may protect neurons from excitotoxicity [107••]. Indeed, NAD^+ replenishment strategies may confer neuroprotection in prion diseases [108]. In the absence of NMNAT2, Hsp90 can depolymerize TDP-43 fibrils *in vitro* [109]. The precise mechanism by which NMNAT2 and Hsp90 combine to promote protein disaggregation and reactivation remains unclear. Nonetheless, methods to stimulate the disaggregase activity or upregulate expression of NMNAT2, Hsp90, or both, perhaps with small-molecule drugs, could be important to combat neurodegenerative disease.

Conclusions and future directions

Endogenous human protein disaggregases fail to counter neurodegenerative disease, perhaps due to reduced expression or activity in selectively vulnerable neurons [93••,101,107••,110]. Thus, augmenting or stimulating their activity could have therapeutic utility [29]. Here, I have highlighted several natural and engineered protein-disaggregase modalities that could be appropriated for therapeutic purposes. Excitingly, Ig-G3P is now in clinical trials for AD, and Hsp104 [34,37,47,48••], Hsp110, Hsp70, and Hsp40 [92,93••], and NMNAT2 plus Hsp90 [106,107••] have shown efficacy in animal models of

neurodegenerative disease. Additional fine tuning of disaggregase activity for specific substrates may help optimize each system for specific disorders [29]. There is also great interest in defining small-molecule drugs that increase expression or directly enhance the activity of endogenous human protein disaggregases [29]. It will also be important to determine whether natural polymorphisms in endogenous human molecular chaperones or protein disaggregases [111] enhance their activity and render individuals more resistant to developing a neurodegenerative disease. We are still only beginning to realize the existence and therapeutic potential of protein disaggregases and many challenges lie ahead in translation to therapeutics [29]. Nonetheless, protein disaggregases represent a valuable opportunity to develop treatments for several devastating neurodegenerative diseases.

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