

AAA+ Protein-Based Technologies to Counter Neurodegenerative Disease

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ABSTRACT Protein misfolding and overloaded proteostasis networks underlie a range of neurodegenerative diseases. No cures exist for these diseases, but developing effective therapeutic agents targeting the toxic, misfolded protein species in disease is one promising strategy. AAA+ (ATPases associated with diverse cellular activities) protein translocases, which naturally unfold and translocate substrate proteins, could be potent therapeutic agents to disassemble toxic protein conformers in neurodegenerative disease. Here, we discuss repurposing AAA+ protein translocases Hsp104 and proteasome-activating nucleotidase (PAN) to alleviate the toxicity from protein misfolding in neurodegenerative disease. Hsp104 effectively protects various animal models from neurodegeneration underpinned by protein misfolding, and enhanced Hsp104 variants strongly counter neurodegenerative disease-associated protein misfolding toxicity in yeast, *Caenorhabditis elegans*, and mammalian cells. Similarly, a recently engineered PAN variant (PAN^{et}) mitigates photoreceptor degeneration instigated by protein misfolding in a mouse model of retinopathy. Further study and engineering of AAA+ translocases like Hsp104 and PAN will reveal promising agents to combat protein misfolding toxicity in neurodegenerative disease.

INTRODUCTION

Proper folding of proteins is essential for their functions (1). However, protein folding *in vivo* is challenging because of high macromolecular concentrations (~300–400 mg/mL), fluctuating microenvironmental conditions, and constraints imposed by cotranslational folding (1). To ward against these challenges, cells have evolved various molecular chaperones to help proteins achieve and maintain their native conformations and several protein-degradation machineries to remove terminally misfolded protein (2,3). Collectively, these protein quality control factors comprise the protein homeostasis (proteostasis) network (2,3).

The efficacy of the proteostasis network declines with age, and misfolded and aggregated proteins form and persist (2,3). Misfolded proteins can gain toxicity by impeding the ubiquitin-proteasome system and have even been shown to directly inhibit proteasomes (4). This aspect is especially pronounced for postmitotic cells such as neurons, which cannot dilute cytoplasmic aggregates through cell division. Indeed, compromised proteostasis is now widely recognized

as a unifying feature of several degenerative disorders (5). Diseases such as Parkinson's disease and amyotrophic lateral sclerosis, despite having diverse clinical symptoms, are united in that the most prominent pathological feature of both disorders is the accumulation of misfolded protein (6). The situation is further complicated by the fact that proteins implicated in each disease adopt a range of misfolded conformations (7). Thus, in Parkinson's disease, α -synuclein accumulates in toxic soluble oligomers and amyloid fibers that are the major component of cytoplasmic Lewy bodies in degenerating dopaminergic neurons (8–12). Likewise, in amyotrophic lateral sclerosis, the normally nuclear RNA-binding proteins TDP-43 and FUS accumulate in toxic oligomeric structures and cytoplasmic inclusions (13–20). Therefore, effective therapeutic strategies must be able to recognize and eradicate each of these misfolded structures (21).

Protein translocases from the AAA+ (ATPases associated with diverse cellular activities) superfamily have emerged as interesting candidates to antagonize protein misfolding. AAA+ proteins are defined by the presence of conserved AAA+ domains containing Walker A and B motifs necessary for ATP binding and hydrolysis (22). AAA+ proteins deploy the power generated from ATP binding and hydrolysis to effect a diverse array of biological functions, including

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substrate protein unfolding and translocation (22–25). This protein unfolding mechanism has fueled hypotheses that AAA+ protein translocases may be applied to counteract protein misfolding and the aggregation that underpins neurodegenerative disease (21,26–31). In this perspective, we highlight efforts to apply two AAA+ protein translocases, Hsp104 from yeast and proteasome-activating nucleotidase (PAN) from archaea, to mitigate protein misfolding in animal models of neurodegenerative disease.

Hsp104 protects dopaminergic neurons from α -synuclein toxicity in worms and rats

Hsp104 is an AAA+ protein translocase (32–34) first discovered in yeast as an essential factor for yeast survival during thermal stress (35). In addition, Hsp104 plays a role in the inheritance and maintenance of several yeast prions (26,36–40). These activities are due to ATPase-driven protein unfolding and disassembly of protein aggregates, amyloids, and preamyloid oligomers (26,27,38,41,42), which it can perform on its own (26,27,38,43) or in collaboration with Hsp70 and Hsp40 (41,44). Indeed, Hsp104 was the first protein factor discovered to have these unique activities (26,38,41,42). Typically, proteins processed by Hsp104 are restored to active soluble states and are not degraded (42,45–47). However, Hsp104 can also promote the proteasomal degradation of some select clients (48,49). Although Hsp104 is conserved among eubacteria, nonmetazoan eukaryotes, and some archaea, it is lost in metazoa (50). Perhaps unsurprisingly then, much speculation has arisen over why Hsp104 was lost and whether it might be added back to enhance the proteostasis network in mammalian lineages (21,50,51). Indeed, Hsp104 is well tolerated in mammalian cells, enhances their thermal tolerance, disaggregates heat-denatured luciferase aggregates, and reduces the aggregation and toxicity of various disease-linked proteins, suggesting a gain-of-function in the proteostasis network of these cells (51–53). Hsp104 also collaborates effectively with human chaperones Hsp70, Hsp40, Hsp110, and small Hsps (28,54–56).

Of particular interest is the possibility that the ability of Hsp104 to disassemble amorphous aggregates, preamyloid oligomers, and amyloid fibers might be useful in combating neurodegenerative diseases, in which formation of these protein conformers are widely believed to underpin disease (5). In vitro, Hsp104 can dissolve fibrils composed of tau, amylin, polyglutamine, amyloid β , prion protein, and α -synuclein that are associated with human diseases (27,55,57). Hsp104 has also been applied in several animal disease models. Hsp104 expression reduces polyglutamine toxicity in *Caenorhabditis elegans*, fly, and rodent models (29,58–60). Hsp104 reduces dopaminergic neuron degeneration in a rat model of Parkinson's disease (55). Indeed, Hsp104 is the only protein factor known to eliminate α -synuclein oligomers and fibrils and exhibit protective effects in

the rat substantia nigra (55). Furthermore, the substantia nigra of rats expressing Hsp104 and α -synuclein have a decreased load of phosphorylated α -synuclein inclusions, suggesting a link between the protein-disaggregase activity of Hsp104 and the protection of dopaminergic neurons (55). Thus, Hsp104 can indeed buffer proteotoxicity in diverse animal systems and is a therapeutic candidate for several neurodegenerative disorders.

However, Hsp104 activity against neurodegenerative disease proteins can be limited, and typically high concentrations of Hsp104 are required for modest disaggregation (27). Thus, strategies to enhance the disaggregase activity of Hsp104 against non-native substrates have been investigated (61). We engineered potentiated Hsp104 variants to potently suppress toxicity associated with TDP-43, FUS, and α -synuclein in yeast (28,62–68). Potentiated Hsp104 variants have a substantially increased intrinsic disaggregase activity and, like wild-type Hsp104, can synergize with human Hsp70 and Hsp40 (28,43,62,63,65). Potentiated Hsp104s also efficiently eradicate preformed fibrils composed of TDP-43, FUS, and α -synuclein (28,62); protect dopaminergic neurons in *C. elegans* (28); and disassemble FUS inclusions in mammalian cells (69). Recently, we also discovered an Hsp104 homolog, from the thermophilic fungus *Calcarisporiella thermophila*, that naturally suppresses proteotoxicity arising from TDP-43, α -synuclein, and polyglutamine misfolding (70). This finding raises the possibility that natural sequence variation among Hsp104 homologs may be harnessed for therapeutic protein-disaggregase modalities (70). Going forward, it will be very interesting to discover whether additional Hsp104 homologs have therapeutic activities, to understand the basis of these activities, and to apply these and other potentiated Hsp104 variants to more sophisticated animal neurodegenerative disease models to determine their effects.

PAN ameliorates proteotoxicity in rod photoreceptors that causes blindness

PAN is another interesting AAA+ protein unfoldase that has recently been applied to counteract toxic protein misfolding. PAN is homologous to the six AAA+ proteins that comprise the 19S regulatory particle in the eukaryotic proteasome (71). The subunit diversification that occurred during evolution from the archaeal PAN to the eukaryotic 19S proteasome may reflect the adaptation to a more complex proteasome, in analogy with putative adaptive diversification that has been proposed for the evolution of the eukaryotic chaperonin TRiC from the ancestral GroEL (72). Like the 19S particle, PAN associates with the 20S catalytic particle and unfolds substrates before their degradation (71). In archaea, proteins marked for proteasomal degradation are conjugated with ubiquitin-like small archaeal modifier proteins, which are engaged by PAN and shuttled to the proteasome (73). PAN can also recognize substrates with

surface-exposed hydrophobic residues and translocate them into the proteasome for degradation (71,74,75). Moreover, PAN displays chaperone activity and prevents the aggregation of certain model substrates (76). These characteristics, in addition to its AAA+ architecture, draw parallels between PAN and Hsp104, including the possibility that PAN may counter protein misfolding in neurodegenerative disease. Indeed, a PAN variant was recently developed by adding a C-terminal FLAG epitope tag (PAN^{ct}), which impedes PAN interaction with the 20S proteasome by obstructing a conserved HbYX proteasome-interacting motif (77). Thus, PAN^{ct} retained the ability to unfold GFP tagged with an unstructured ssrA tag (GFP-ssrA), but lost the ability to stimulate gate opening of the 20S proteasome (77). PAN^{ct} could be functionally expressed in mouse rod photoreceptors and did not affect retinal morphology, number of rod photoreceptors, or visual response to light compared to wild-type mice (77). Thus, PAN^{ct} decouples substrate unfolding from proteasome degradation and is unlikely to have widespread off-target effects and aberrantly unfold essential endogenous proteins in rod photoreceptors.

Having established that PAN^{ct} is well tolerated in mice, PAN^{ct} was deployed in a mouse model of inherited blindness (77). In this model, mice lacking the γ subunit of the G-protein coupled receptor transducin ($G\gamma_1^{-/-}$ mice) fail to form functional transducin complexes in rod photoreceptors, which in turn overwhelms the proteasome with mistargeted β ($G\beta_1$) and α transducin subunits and ultimately causes severe toxicity and loss of photoreceptors (78–80). Excitingly, $G\gamma_1^{-/-}$ mice that expressed PAN^{ct} were protected against rod photoreceptor degeneration. Whereas almost all rods in $G\gamma_1^{-/-}$ mice degenerated by 7 months, over half of the rods in $G\gamma_1^{-/-}$ mice expressing the PAN^{ct} transgene survived (77). Furthermore, $G\gamma_1^{-/-}$;PAN^{ct}(+) mice maintained strong visual responses at 7 months as measured by the a-wave amplitude from electroretinogram analysis (77). Indeed, 7-month-old $G\gamma_1^{-/-}$;PAN^{ct}(+) mice showed similar magnitudes in visual response to 1-month-old $G\gamma_1^{-/-}$;PAN^{ct}(-) mice (77). Taken together, these studies establish that the PAN^{ct} unfoldase is an effective tool to mitigate protein misfolding-mediated photoreceptor degeneration.

Although the phenotypic effects of PAN^{ct} expression in $G\gamma_1^{-/-}$ mice are undoubtedly promising, the molecular basis of how PAN^{ct} interacts with the photoreceptor proteome to achieve those effects is unclear. We suggest four possible mechanisms here. One possibility is that PAN^{ct} expression mitigates the rod cell degeneration chiefly by limiting nonproductive interactions of $G\beta_1$ with other members of the proteostasis network. For instance, previous work reported that in $G\gamma_1^{-/-}$ mice, more $G\beta_1$ copurified with the chaperonin TRiC than in wild-type mice (80). Thus, $G\beta_1$ may exert proteotoxicity by competitively inhibiting the folding of other TRiC clients. It will be interesting to determine whether this elevation in TRiC-associated $G\beta_1$ is alleviated upon PAN^{ct} expression. A second possibility is that $G\beta_1$ may also form soluble oligomers that inhibit the proteasome, as has been determined previously (4), and PAN^{ct} may prevent or reverse this oligomerization. This possibility could be explored by generating $G\beta_1$ oligomers from a recombinant protein and determining whether PAN^{ct} prevents or reverses oligomer formation. A third possibility is that PAN^{ct} could facilitate the formation of hybrid transducin complexes. Indeed, a recent study demonstrated that in $G\gamma_1^{-/-}$ mice, $G\beta_1$ can associate with $G\gamma_2$ and $G\gamma_3$ (81). Thus, it would be interesting to determine whether the formation of noncanonical $G\beta\gamma$ complexes is more efficient in PAN^{ct}(+) mice. That $G\gamma_1^{-/-}$;PAN^{ct}(+) mice have robust visual responses suggests that this may be possible. It would be interesting to measure the abundance of the transducin α_1 and β_1 subunits in PAN^{ct}(+) mice, which previous studies showed are depleted in $G\gamma_1^{-/-}$ mice (79), to assess whether they are protected from degradation in a PAN^{ct}-dependent manner. A fourth possibility is that endogenous quality

control mechanisms may be upregulated in response to the misfolding. For instance, the expression of chaperones and proteasome components may be increased in response to the misfolding. This possibility could be explored by measuring the abundance of these proteins in PAN^{ct}(+) mice. A fifth possibility is that PAN^{ct} may directly interact with the proteasome and facilitate its activity. This possibility could be explored by generating PAN^{ct} variants that are unable to interact with the proteasome and determining whether they are still able to protect against rod cell degeneration. A sixth possibility is that PAN^{ct} may interact with other members of the proteostasis network, such as the chaperonin TRiC, and facilitate its activity. This possibility could be explored by generating PAN^{ct} variants that are unable to interact with TRiC and determining whether they are still able to protect against rod cell degeneration. A seventh possibility is that PAN^{ct} may interact with other members of the proteostasis network, such as the chaperone Hsp70, and facilitate its activity. This possibility could be explored by generating PAN^{ct} variants that are unable to interact with Hsp70 and determining whether they are still able to protect against rod cell degeneration. A eighth possibility is that PAN^{ct} may interact with other members of the proteostasis network, such as the chaperone Hsp90, and facilitate its activity. This possibility could be explored by generating PAN^{ct} variants that are unable to interact with Hsp90 and determining whether they are still able to protect against rod cell degeneration. A ninth possibility is that PAN^{ct} may interact with other members of the proteostasis network, such as the chaperone Hsp104, and facilitate its activity. This possibility could be explored by generating PAN^{ct} variants that are unable to interact with Hsp104 and determining whether they are still able to protect against rod cell degeneration. A tenth possibility is that PAN^{ct} may interact with other members of the proteostasis network, such as the chaperone Hsp110, and facilitate its activity. This possibility could be explored by generating PAN^{ct} variants that are unable to interact with Hsp110 and determining whether they are still able to protect against rod cell degeneration.

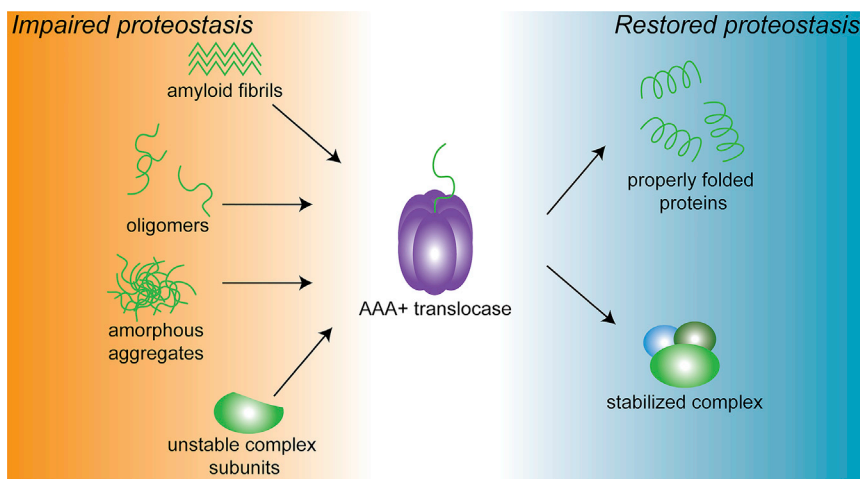


FIGURE 1 AAA+ protein translocases transform misfolded proteins. AAA+ proteins like Hsp104 or PAN recognize a range of misfolded conformers, including amyloid fibers, amorphous aggregates, oligomers, or unstable monomers, and unfold and remodel these substrates to their native fold and function.

control factors fail to recognize orphan $G\beta_1$ subunits in $G\gamma_1^{-/-}$ mice (possibly because of aberrant chaperone interactions or the occlusion of degrons within oligomers) and target them for degradation. However, PAN^{ct}-mediated unfolding of $G\beta_1$ may facilitate recognition via downstream quality control pathways for protein orphans and enable $G\beta_1$ degradation (82). These studies raise exciting questions for future studies concerning how PAN^{ct} averts rod degeneration at the molecular level.

Another interesting avenue for future work includes determining the substrate repertoire of PAN^{ct}. PAN^{ct} may be deployed in diverse neurodegenerative diseases, similarly to the broad utility seen with Hsp104 (27). Similarly, PAN variants with augmented unfoldase activity may be engineered or discovered among naturally occurring homologs to more robustly antagonize proteotoxic misfolding (61,83). Indeed, PAN could be deployed against many other neurodegenerative diseases linked to protein misfolding.

Conclusions and future opportunities

AAA+ protein translocases such as PAN and Hsp104 are unique hubs within the proteostasis network that can transform deleterious conformers back to their native form and function (Fig. 1), simultaneously eliminating loss-of-function phenotypes associated with the depletion of functional proteins and any toxic gain-of-function phenotypes associated with the accumulation of misfolded conformers. The foregoing studies establish the potential therapeutic utility of this protein remodeling in antagonizing protein misfolding to impede neurodegeneration. Yet, significant questions remain to be answered. Hsp104 and PAN are both from nonmetazoan lineages and are likely finely adapted to remodel the proteomes of their respective hosts; although the beneficial phenotypes seen upon expressing Hsp104 or PAN in diseased animals is auspicious, there is also undoubtedly room for improvement. Significant opportunity likely lies in protein engineering efforts to augment the therapeutic activities of Hsp104 or PAN to more efficiently detangle misfolded substrates. At the same time, attention must be paid to averting deleterious off-target effects that may arise while engineering these enhanced AAA+ proteins. For instance, Hsp104 expression in *Drosophila* is subject to some dose-dependent toxicity (29), and some potentiated Hsp104 variants are mildly toxic in some yeast genetic backgrounds (28,63). Thus, an important future goal is understanding how AAA+ protein translocases such as Hsp104 and PAN discriminate substrates, and how that might be tailored to create “designer” disaggregases (30,61,83) that safely eradicate deleterious conformers with minimal disruption of natively folded proteins and complexes.

Finally, and perhaps most intriguingly, it may be possible to unlock protein-disaggregase activity of native human AAA+ proteins via pharmacologic modulation, thus obviating the

complex challenges associated with genetic engineering. For instance, pharmacologic upregulation of the cyclic adenosine monophosphate/protein kinase A pathway leads to proteasome phosphorylation that stimulates proteasome activity (84). Remarkably, the same treatment administered to transgenic mice expressing pathogenic tau led to decreased tau deposition and improved cognitive performance (85). A recent study demonstrated that proteasomes directly fragment tau and α -synuclein fibrils, although these fragments are more cytotoxic than untreated fibrils, somewhat confounding a beneficial role for the proteasome in eliminating misfolded protein conformers (86). However, proteasome stimulation through phosphorylation may enable the complete eradication of toxic protein conformers. Similarly, although Hsp104 lacks a direct human homolog, emerging human genetics data implicates several human AAA+ proteins in neurodegenerative diseases, including p97/valosin-containing protein (87,88) and torsin A (89–91), and suggests that these proteins may have some level of protein-disaggregase activity that may become overwhelmed in neurodegeneration. Likewise, another AAA+ protein, RuvBL, might disassemble protein aggregates (92). It will be exciting to delineate how these proteins interface with the human proteostasis network and to explore the potentiation of these endogenous human AAA+ proteins via small-molecule modulation to enhance human proteostasis (30,83). Clearly, a wide range of literature now points to a critical utility of AAA+ proteins to counteract protein misfolding in neurodegenerative disease, and it will be interesting to explore the biochemical basis of protein unfolding by endogenous human AAA+ proteins in the future.

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