

MODULATION OF NEURODEGENERATION BY MOLECULAR CHAPERONES

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Abstract | Many neurodegenerative disorders are characterized by conformational changes in proteins that result in misfolding, aggregation and intra- or extra-neuronal accumulation of amyloid fibrils. Molecular chaperones provide a first line of defence against misfolded, aggregation-prone proteins and are among the most potent suppressors of neurodegeneration known for animal models of human disease. Recent studies have investigated the role of molecular chaperones in amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease and polyglutamine diseases. We propose that molecular chaperones are neuroprotective because of their ability to modulate the earliest aberrant protein interactions that trigger pathogenic cascades. A detailed understanding of the molecular basis of chaperone-mediated protection against neurodegeneration might lead to the development of therapies for neurodegenerative disorders that are associated with protein misfolding and aggregation.

PROTEIN AGGREGATE

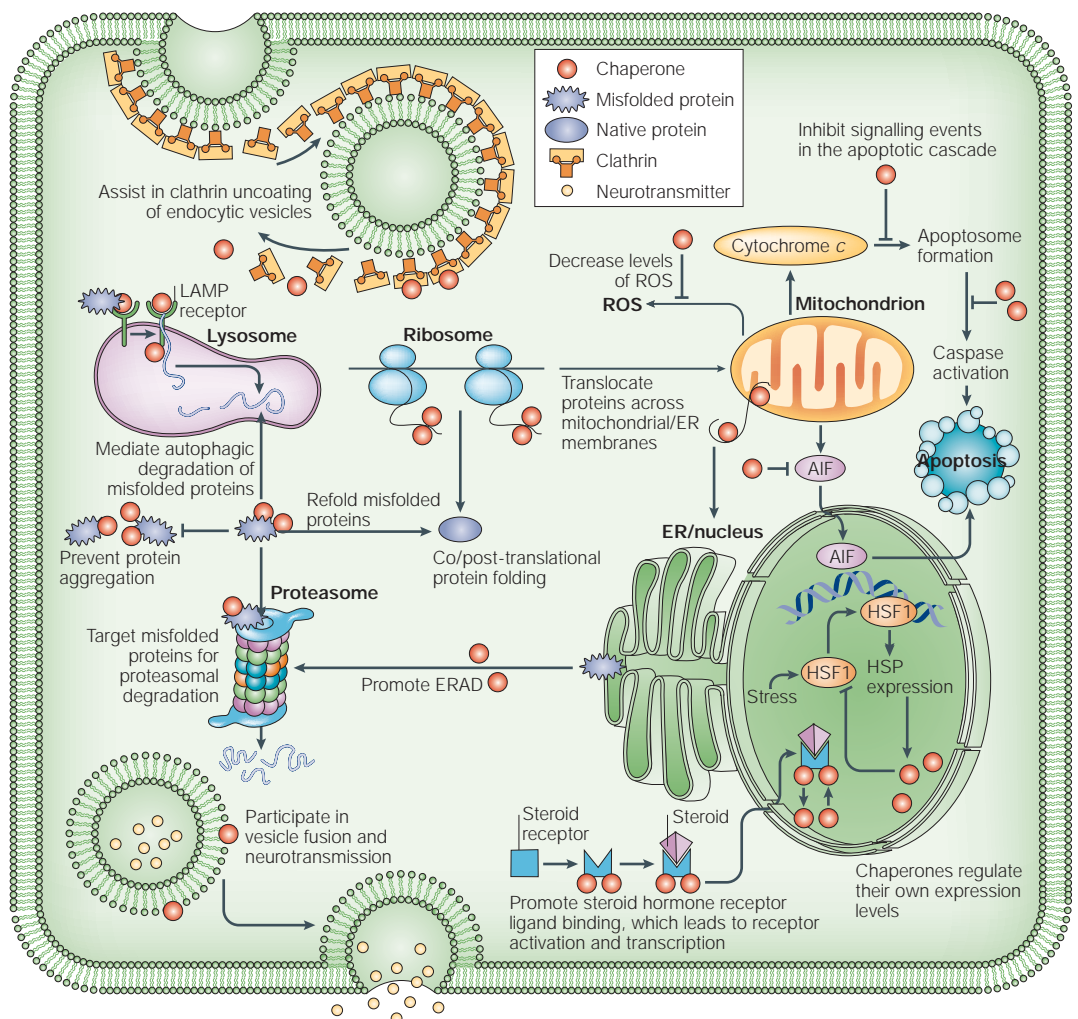
An abnormal protein assembly that results from the cohesion of two or more misfolded monomeric proteins. Protein aggregates that form amyloid fibrils are often resistant to solubilization with ionic detergents after boiling.

Many systemic and neurodegenerative disorders, termed 'protein-misfolding disorders', or perhaps more appropriately 'protein-conformational disorders', are characterized by the accumulation of intracellular or extracellular **PROTEIN AGGREGATES**. Post-mitotic cells, such as neurons, are particularly vulnerable to the detrimental effects of misfolded and/or aggregated proteins because they cannot dilute potentially toxic species through cell division. To make matters worse, misfolded proteins are thought to accumulate in neurons and in other cells as a result of normal ageing — concomitant with a decrease in proteasome activity and with alterations in the induction and/or functional capacity of **MOLECULAR CHAPERONES**¹. Collectively, these factors might account for the late onset of neurodegenerative diseases that are linked to protein aggregation². This review focuses on studies that have investigated the function of molecular chaperones in neurodegenerative disorders that are characterized by the accumulation of aggregated protein, including Alzheimer's disease (AD), Parkinson's disease (PD), familial amyotrophic lateral sclerosis (FALS),

Huntington's disease (HD) and related polyglutamine (polyQ) expansion diseases. Recent evidence indicates that chaperones are potent suppressors of neurodegeneration and are, therefore, promising therapeutic targets for protein conformational disorders.

Protein aggregation and molecular chaperones. Although the amino acid sequence of a protein contains all the information that is required to dictate proper folding into a functional, three-dimensional structure³, the crowded intracellular milieu places constraints on the folding of polypeptides, thereby promoting misfolding and aggregation. As a consequence, protein folding *in vivo* is typically not spontaneous⁴, and organisms from archaea to eukaryotes have evolved a highly conserved class of proteins called molecular chaperones that prevent inappropriate interactions within and between non-native polypeptides, enhance the efficiency of *de novo* protein folding and promote the refolding of proteins that have become misfolded as a result of cellular stress⁵. Importantly, chaperones only

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MOLECULAR CHAPERONES
Several families of highly conserved proteins that mediate the folding or assembly of other proteins, but are not components of the final functional structures. Chaperones target misfolded proteins and prevent protein aggregation in all cell types.

THE UBIQUITIN-PROTEASOME PATHWAY
A major cellular pathway for protein catabolism that is important for the 'housekeeping' and turnover of many regulatory proteins. Degradation by the proteasome occurs by conjugation of multiple ubiquitin moieties to a substrate and degradation of the tagged protein by the 26S proteasome complex. Molecular chaperones cooperate with this pathway to mediate the degradation of misfolded proteins.

LYSOSOME-MEDIATED AUTOPHAGY
An important pathway for intracellular protein degradation that involves an acidic cellular compartment, the lysosome vacuole. This pathway mediates the bulk degradation of cytosol and organelles and might degrade aggregated proteins. Chaperones mediate one form of autophagy.

Figure 1 | **Molecular chaperones regulate several important cellular processes.** It is well known that the molecular chaperones facilitate protein folding and prevent protein aggregation. However, molecular chaperones also regulate several other cellular processes, such as autophagy, vesicle fusion, signal transduction, apoptosis and proteasomal degradation. AIF, apoptosis-inducing factor; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; HSF1, heat shock transcription factor 1; HSP, heat shock protein; LAMP, lysosomal-associated membrane protein; ROS, reactive oxygen species.

transiently stabilize and mediate the folding or assembly of unfolded protein substrates and are not present in the native protein. Molecular chaperones have essential roles in many cellular processes, including protein folding, targeting, transport, degradation and signal transduction⁵ (FIG. 1).

Conditions of stress, including (but not limited to) temperature elevation, activate a cellular programme known as the heat shock response, which is characterized by a robust increase in the synthesis of a subset of heat shock proteins (HSPs) that are crucial for recovery from stress-induced protein damage⁶. Almost all HSPs function as molecular chaperones, and they have been classified into six main families on the basis of their approximate molecular mass (in kDa): HSP100, HSP90, HSP70, HSP60, HSP40 and the small HSPs (sHSPs), which weigh less than 40 kDa (BOX 1). The number of diseases that are known to be caused by mutations in chaperones is large, and increasing (BOX 2).

In addition to molecular chaperones, cells have evolved two mechanisms for the degradation of misfolded proteins — the UBIQUITIN-PROTEASOME PATHWAY and LYSOSOME-MEDIATED AUTOPHAGY. Under certain conditions, when chaperones cannot repair misfolded proteins, chaperone-mediated targeting to the ubiquitin-proteasome system or to lysosomes results in selective degradation. CHIP (carboxy terminus of HSC70-interacting protein) is a protein that binds heat shock cognate 70 (HSC70) or HSP70 in the mammalian cytosol, attenuates the HSP40-stimulated ATPase and refolding activities of HSP70 (REF. 7) and acts as an E3 ligase to facilitate the transfer of a polyubiquitin chain to misfolded substrates⁸. CHIP also mediates crosstalk between molecular chaperones and the ubiquitin-proteasome system by associating with BCL2-associated athanogene 1 (BAG1), a protein that binds to the 26S PROTEASOME and assists in the degradation of specific chaperone substrates⁹. HSP70 also contributes to the

Box 1 | Families of molecular chaperones

HSP100

Heat shock protein 100 (HSP100) chaperones are members of the AAA+ protein family (adenosine triphosphatases with diverse activities) that share a common ATPase domain and form large ring-shaped structures. In yeast, **Hsp104**, the best-characterized Hsp100, regulates protein aggregation, disaggregation and thermotolerance, but no mammalian homologue has been identified so far^{97–99}.

HSP90

HSP90 chaperones are an essential component of the eukaryotic cytosol¹⁰⁰, where they stabilize misfolded proteins and regulate the activity of various signalling proteins, including steroid hormone receptors, tyrosine kinases, nitric oxide synthase and calcineurin¹⁰¹.

HSP60

HSP60 chaperones are heptameric complexes of identical subunits stacked back to back in a double-ring structure that contains a large central cavity in which protein folding is thought to occur¹⁰². In eukaryotes, HSP60 family members (also called Group I chaperonins) are found in the mitochondria, and cooperate with a cofactor of the HSP10 family. A second class of chaperonins (Group II chaperonins) is found in the eukaryotic cytosol but has no HSP10 cofactor. The best-characterized Group II chaperonin is TRiC, which comprises eight subunits per ring encoded by different genes. TRiC is thought to be crucial for the folding of actin and tubulin in the eukaryotic cytosol.

HSP70

HSP70 chaperones (with HSP40s, their co-chaperones) assist in the stabilization and folding of many substrates and are found in most cellular compartments¹⁰². In humans, 11 genes that encode HSP70 family members have been identified¹⁰³, including the constitutive cytosolic member heat shock cognate 70 (HSC70), the stress-induced cytosolic HSP70, the endoplasmic reticulum-localized glucose-regulated protein 78 (GRP78) and the mitochondrial GRP75 (REF. 104). All HSP70 proteins have a conserved amino-terminal ATPase domain that binds and hydrolyses ATP, and a carboxy (C)-terminal substrate-binding domain.

HSP40

HSP40 co-chaperones bind HSP70 through a conserved J-domain and stimulate ATP hydrolysis, resulting in a conformational switch that closes the substrate-binding pocket of HSP70 and facilitates the capture of non-native protein substrates^{105–107}. HSP40s also bind protein substrates and target these substrates to HSP70, enhancing the efficiency of the HSP70/HSP40 refolding cycle¹⁰⁸. Higher eukaryotes have many HSP40 family members, whose differential expression or localization might regulate the substrate specificity of conserved HSP70 family members¹⁰⁹.

Small heat shock proteins

sHSPs have a molecular mass of less than 40 kDa and assemble into large, oligomeric structures that resemble a hollow ball. All sHSPs contain a conserved, C-terminal α -crystallin domain of about 100 residues that mediates oligomeric assembly. Similar to HSP90 chaperones, sHSPs transiently interact with and stabilize misfolded substrates, conceivably until the HSP70/HSP40 system can actively refold them^{100,110,111}.

delivery of protein substrates to lysosomes, a process known as chaperone-mediated autophagy¹⁰.

Protein aggregation and neurodegeneration
The collective activities of the molecular chaperones, the ubiquitin–proteasome system and lysosome-mediated autophagy are normally sufficient to prevent the accumulation of misfolded proteins. However, under certain pathological conditions, the capacity of this protein quality control machinery is exceeded and misfolded proteins accumulate to dangerous levels. AD, PD, ALS and the polyQ diseases are all characterized by the accumulation of distinct aggregated proteins, mutations of which cause severe, inherited forms of disease (TABLE 1). Remarkably, although the proteins that aggregate in

these disorders are unrelated in size or primary amino acid sequence, the characteristic lesions of each disease typically contain fibrillar, amyloid-like structures with common biochemical characteristics such as detergent-insolubility, high β -sheet content and a cross β -structure, protease resistance and the ability to bind lipophilic dyes such as congo red¹¹. These commonalities indicate that a conserved mechanism of pathogenesis might connect these phenotypically diverse diseases. Early in the disease process a crucial conformational change in the disease protein might lead to a toxic gain of function for the monomer, and/or promote the formation of toxic aggregates. The toxic species, whether monomeric or higher-order, might subsequently initiate a cascade of pathogenic protein–protein interactions that culminates in neuronal dysfunction. Precisely when in the disease process such interactions occur is unclear, as is any structural understanding of how altered protein conformations or aggregates trigger neuronal dysfunction.

Historically, lesions that contain AMYLOID FIBRILS were thought to mediate neurodegeneration directly, but there is increasing evidence that fibrillar aggregates are inert — or perhaps even protective — rather than being directly pathogenic. Amyloid plaques have been found in individuals who lack any clinical symptoms of AD¹², and, in cases of AD, the severity of dementia does not correlate well with plaque density^{13,14}. In PD, neuropathological analyses indicate that neurons that contain fibrillar protein deposits known as Lewy bodies are healthier than surrounding cells¹⁵, indicating that INCLUSION BODIES might be protective. In support of this hypothesis, the formation of inclusion bodies by proteins with expanded polyQ repeats is a regulated cellular process that requires an intact microtubule cytoskeleton and might have evolved as a protective mechanism to sequester toxic, misfolded protein entities that could otherwise disrupt cellular homeostasis^{16–18}. Strong evidence for this hypothesis was described recently in an elegant study by Arrasate *et al.*, which showed that in a cell culture model of HD, inclusion body formation predicts neuronal survival, whereas the level of diffuse huntingtin correlates significantly with cell death¹⁹.

If amyloid fibrils and inclusion bodies are not directly pathogenic, what is the misfolded species that mediates neurotoxicity? The assembly of misfolded protein into amyloid fibrils (and subsequently into inclusion bodies) is a complex process that might be conserved among the various disease-associated proteins. ATOMIC FORCE MICROSCOPY (AFM) of the aggregation of amyloid- β (A β) and α -synuclein proteins has been used to characterize several metastable structures, such as SPHERICAL OLIGOMERS, protofibrils and pore-like ANNULAR structures, which might be components of a pathway to fibril formation²⁰ (FIG. 2). Many studies indicate that these small, potentially diffusible assemblies, rather than mature amyloid fibrils, might trigger neuronal dysfunction by initiating a cascade of events that culminates in neuronal death. Microinjection of soluble A β oligomers into the rat hippocampus inhibits the late phase of LONG-TERM POTENTIATION (LTP), indicating that soluble A β oligomers are potent neurotoxins *in vivo*²¹.

26S PROTEASOME

A multicatalytic protease that is found in the cytosol, perinuclear regions and nucleus of eukaryotic cells.

AMYLOID FIBRILS

Structures formed by many disease-causing proteins when they aggregate. Amyloid fibrils share common biochemical characteristics such as detergent-insolubility, high β -sheet content and a cross β structure, protease resistance and the ability to bind lipophilic dyes, such as congo red.

Box 2 | Mutations in molecular chaperones that are responsible for human disease

Small heat shock proteins

Mutations that disrupt the structure of and/or decrease the activity of small heat shock proteins (sHSPs)¹¹¹ cause several autosomal dominant genetic disorders. α -crystallin, a sHSP that is composed of two subunits, α A-crystallin and α B-crystallin, is a crucial protein constituent in the lens. A cysteine for arginine substitution at position 116 in α A-crystallin causes cataracts, whereas a glycine for arginine substitution at position 120 in α B-crystallin (also expressed at high levels in muscle and other tissues) causes desmin-related myopathy (DRM). Although the cause of cataract formation is unclear, DRM is characterized by the accumulation of aggregates of the intermediate filament protein desmin, which co-localize with α B-crystallin, indicating a failed attempt by this sHSP to prevent the accumulation of aggregated proteins^{112–114}. Mutations in HSP27 (REF. 115) cause Charcot-Marie-Tooth disease and distal hereditary motor neuropathy, two diseases that are characterized by a degeneration of peripheral nerves. Mutations that target the α -crystallin domain of HSP22 (REF. 116) cause distal hereditary motor neuropathy. Although the molecular mechanisms that underlie these diseases are unknown, it has been suggested that mutant sHSPs could cause improper neurofilament assembly¹¹⁵.

HSP60

A missense mutation (isoleucine substituted for valine at position 72) in the mitochondrial chaperonin HSP60 (REF. 117) is responsible for an autosomal dominant form of hereditary spastic paraplegia, a neurodegenerative disorder that is characterized by progressive spastic weakness in the lower extremities. Although the molecular mechanism that underlies this disease has not been characterized, the V72I mutation in HSP60 appears to cause a loss of chaperone function¹¹⁷. Loss-of-function mutations in several other chaperones, including the tubulin-specific chaperone E and the putative chaperonin hMKKS, are also implicated in the aetiology of other heritable diseases¹¹⁸.

INCLUSION BODIES

Cellular structures found inside neurons that are composed of aggregated proteins, including amyloid fibrils, molecular chaperones and components of the ubiquitin–proteasome pathway. Recent studies indicate that the formation of inclusion bodies correlates with neuronal survival and is a protective response.

ATOMIC FORCE MICROSCOPY

(AFM). A type of scanning probe microscopy, in which a cantilever with a sharp tip scans over a sample, such as an amyloid fibril, on an inorganic surface. The repulsive force between the sample and the tip is transduced into a three-dimensional profile of the sample on the surface.

SPHERICAL AND ANNULAR OLIGOMERS

Metastable structures observed in many amyloid-forming proteins that might be on a pathway to fibril formation. These structures have been proposed to be the principal toxic entities that mediate neuronal dysfunction.

LONG-TERM POTENTIATION

(LTP). An enduring increase in the amplitude of excitatory postsynaptic potentials as a result of high-frequency (tetanic) stimulation of afferent pathways. It is measured both as the amplitude of excitatory postsynaptic potentials and as the magnitude of the postsynaptic cell population spike. LTP is most frequently studied in the hippocampus and is often considered to be the cellular basis of learning and memory in vertebrates.

LIPID RAFTS

Membrane microdomains, formed by high concentrations of sphingolipids and cholesterol immersed in a phospholipid-rich environment, that are involved in specialized pathways of protein/lipid transport and signalling.

Although provocative, these experiments must be interpreted with caution, as the tools that allow us to determine whether A β retains its oligomeric structure after injection have only recently become available.

The universality of the ‘toxic oligomer’ hypothesis is supported by the finding that a single monoclonal antibody can recognize a common conformational epitope that is displayed by several disease-associated proteins, including A β , α -synuclein and polyQ-containing peptides²². Surprisingly, co-incubation of the anti-oligomer antibody with oligomers of the aforementioned disease proteins blocks their toxicity when applied to cultured cells, indicating that oligomeric structures formed by distinct disease proteins might confer toxicity through a similar mechanism²². Although these *in vitro* results are intriguing, the development of new methods must continue to allow us to determine whether such intermediates exist *in vivo*, and, if so, whether the appearance of such intermediates correlates with neuronal dysfunction and/or death.

Molecular chaperones and neurodegeneration
In addition to sharing common morphological and biochemical features, the plaques/inclusion bodies that are characteristic of AD, PD, FALS and the polyQ diseases all co-localize with several of the same proteins, including various molecular chaperones (TABLE 1) and components of the ubiquitin–proteasome degradation system²³. Co-localization of the protein quality control machinery with inclusion bodies might reflect an irreversible sequestration and subsequent loss of function, and/or a failed attempt to refold or degrade aggregated proteins. To begin to investigate this possibility, fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) were used in cellular models of polyQ aggregation to characterize polyQ inclusion bodies. Mutant ataxin 1, which causes the autosomal dominant disorder spinocerebellar ataxia 1 (SCA1; TABLE 1) shows

fast and slow association kinetics with inclusion bodies, indicating that it is not irreversibly sequestered²⁴. By contrast, mutant huntingtin fragments and ataxin 3 (which causes SCA3) show low mobility in inclusion bodies, consistent with their physical and possible functional sequestration^{25,26}. Interestingly, HSP70 has rapid association and dissociation kinetics with mutant huntingtin inclusion bodies and might actively regulate their formation^{25,26}. This regulation could involve efforts by HSP70 to refold, solubilize or enhance the degradation of aggregating proteins in the inclusion body, or to promote the spatial sequestration of small, potentially toxic misfolded assemblies into an inclusion body. Below, we review studies that have investigated the role of molecular chaperones in specific neurodegenerative diseases.

Alzheimer’s disease and related dementias. The accumulation of molecular chaperones in extracellular senile plaques found in the brains of patients with AD is perplexing, as most cytosolic HSPs, including HSP70, do not contain a secretory signal sequence, and release through the classic exocytic pathway is not likely. In cultured cells, the extracellular accumulation of HSP70 might be facilitated by a calcium-induced interaction with LIPID RAFTS²⁷. It is tempting to speculate that, in response to the generation of intracellular A β oligomers, a small fraction of cytosolic chaperones could be targeted to lipid rafts and become associated with A β oligomers before their eventual secretion into the extracellular space. Irrespective of the mechanism, once outside neurons, chaperones might also have a role in the phagocytic digestion of amyloid plaques by microglia^{28,29}, although whether chaperone-mediated microglial activation could suppress neuronal dysfunction and/or death in an animal model of AD remains to be investigated.

Table 1 | Protein misfolding diseases associated with molecular chaperones

Neurodegenerative disease	Disease genes	Lesion	Main aggregate component	Co-localization of chaperones with lesions (human brain tissue)	References
Alzheimer's disease	<i>APP</i> Presenilin 1 Presenilin 2	Extracellular senile plaques	A β peptides (A β 40, A β 42)	HSP72 HSP28 α B-crystallin, HSP27 GRP78, HSP90	119 120 121 28
		Intracellular neurofibrillary tangles	Hyperphosphorylated tau	HSP27 HSP90	122 43
Parkinson's disease	α -synuclein Parkin <i>UCHL1</i> <i>PINK1</i> <i>DJ1</i>	Intracellular Lewy bodies	α -synuclein	HSP70, HSP40	50
Dementia with Lewy bodies		Intracellular Lewy bodies	α -synuclein	HSP90, HSP70, HSP60, HSP40, HSP27	47
Familial amyotrophic lateral sclerosis	<i>SOD1</i>	Intracellular inclusions	Mutant SOD1	HSC70	123
Polyglutamine diseases		Cytoplasmic and nuclear inclusions			
Huntington's disease	<i>IT15</i> (huntingtin)		Mutant huntingtin		
Spinocerebellar ataxias (SCA1–3, 7)	ataxins		Mutant ataxin	HSP40, HSP70 HSP40	59 124
Spinal and bulbar muscular atrophy (SBMA)	Androgen receptor		Mutant androgen receptor		

A β , amyloid- β ; *APP*, amyloid precursor protein; *DJ1*, Parkinson disease (autosomal recessive, early onset) 1; GRP78, glucose-regulated protein 78; HSC70, heat shock cognate 70; HSP, heat shock protein; *PINK1*, phosphatase and tensin induced putative kinase 1; *SOD1*, superoxide dismutase 1; *UCHL1*, ubiquitin carboxyl-terminal hydrolase L1.

There is substantial evidence from transgenic mouse models that intracellular A β initiates cellular dysfunction before it accumulates in extracellular plaques^{30–33}. A β peptides are generated by the sequential action of β - and γ -secretases on amyloid precursor protein (APP) in intracellular compartments, including lipid rafts, the endoplasmic reticulum (ER)/Golgi apparatus and endosomes^{34–37}. Cell culture experiments have shown that GRP78, an HSP70 that is found in the ER, binds APP and decreases the secretion of amyloid- β 40 (A β 40) and A β 42, indicating that GRP78 might retain APP in the ER and/or shield APP from β / γ -secretase cleavage³⁸. Although the effect of GRP78 on toxicity was not evaluated in this study, an independent study subsequently showed that overexpression of cytosolic HSP70 rescues neurons from intracellular A β 42-mediated toxicity³⁹. It is not clear how cytoplasmic HSP70 protects neurons in this system, but it might involve chaperone-stimulated ER-associated degradation of A β , a process by which misfolded secretory and/or membrane proteins are recognized by a quality control mechanism in the ER, retro-translocated into the cytosol and degraded by the 26S proteasome⁴⁰. Direct interactions between chaperones and A β — either intracellular or extracellular — might regulate the formation of toxic A β assemblies. This is supported by the fact that, *in vitro*, α B-crystallin maintains A β peptides in a non-fibrillar form that is highly toxic when applied to cell cultures⁴¹.

Crosstalk between molecular chaperones and the ubiquitin–proteasome system might be pivotal in regulating the deposition and toxicity of tau. Tau is a neuronal microtubule-binding protein that normally enhances

microtubule stability, but it can be hyperphosphorylated in pathogenic conditions and be released from microtubules and accumulate in the neurofibrillary tangles that are a second neuropathological hallmark of AD. HSP27 interacts preferentially with a hyperphosphorylated tau variant in human brain samples⁴², and in cell culture it decreases hyperphosphorylated tau levels, increases the abundance of dephosphorylated tau and suppresses tau-mediated cell death⁴². A functional interaction between tau, HSP70 and HSP90 has also been established *in vitro*⁴³. RNA-mediated interference (RNAi) of HSP70 and HSP90 in cell culture increases the accumulation of insoluble, aggregated tau and impairs the association of tau with microtubules. GELDANAMYCIN, an ansamycin antibiotic that normally induces HSP70/HSP90 expression, has the opposite effect in this system, indicating that the activity of these chaperones maintains the native fold and function of tau⁴³. Co-transfection of CHIP and HSP70 facilitates tau ubiquitylation⁴⁴ and suppresses toxicity⁴⁵ in cell culture, but paradoxically enhances the levels of insoluble, ubiquitylated tau^{44,45}, indicating that under certain pathological conditions CHIP-mediated ubiquitylation might partition soluble, toxic forms of phosphorylated tau into an insoluble compartment.

Parkinson's disease. Familial PD is characterized by accumulation of the α -synuclein protein in Lewy bodies, and several studies have investigated the effects of molecular chaperones on α -synuclein aggregation and toxicity. Because transfection of neuronal cell lines or primary cultures with wild-type or mutant α -synuclein does not

GELDANAMYCIN

An ansamycin antibiotic that induces the expression of chaperones. This drug was protective in a fly model of Parkinson's disease.

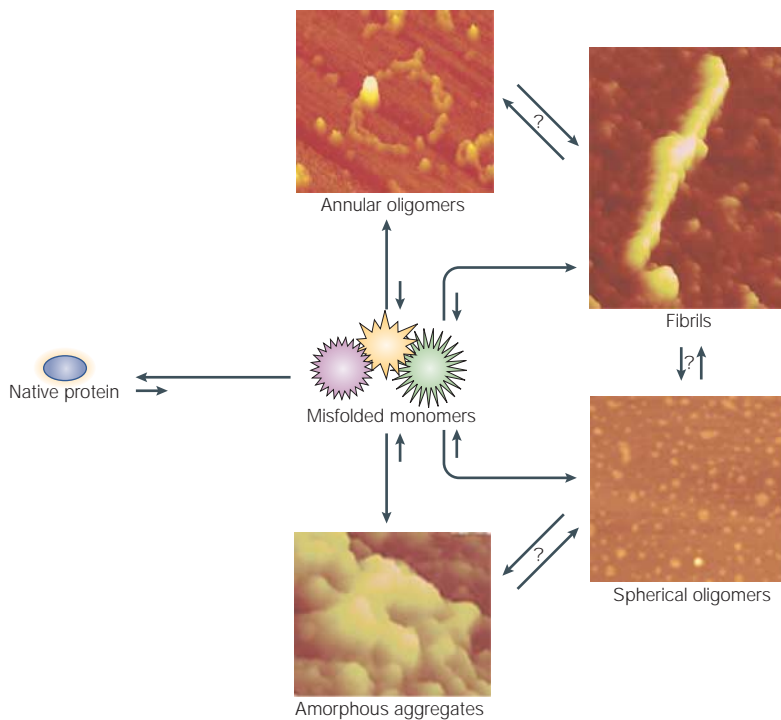


Figure 2 | **A general model of amyloid assembly.** When a disease protein becomes misfolded, an assembly process follows that results in the production of several distinct structures such as spherical oligomers, annular oligomers, amorphous aggregates and/or fibrils. It is not clear whether oligomeric structures seed the assembly of higher-order structures such as fibrils or amorphous aggregates, or vice versa. Atomic force microscopy images of the *in vitro* aggregation of a mutant huntingtin fragment are shown here.

cause robust inclusion body formation, cell culture models have been developed whereby treatment with protease inhibitors, transfection of a truncated, tagged α -synuclein, or co-transfection of a tagged α -synuclein and synphilin 1 (an α -synuclein-interacting protein that localizes to Lewy bodies) facilitates the formation of inclusion bodies⁴⁶. Overexpression of HDJ1 (an HSP40) or HSP70 in an α -synuclein/synphilin 1 cell model markedly decreases (by more than 50%) the number of cells that contain inclusion bodies, although no effect on cell viability was reported⁴⁷. In a subsequent study of the same cells, HSP70 overexpression caused a decrease in detergent-insoluble, high molecular mass α -synuclein species, as well as a decrease in total α -synuclein protein, indicating that HSP70 might enhance refolding and/or promote degradation of α -synuclein⁴⁸. Furthermore, overexpression of HSP70 caused a decrease of about 20% in the toxicity of transfected α -synuclein, indicating that, *in vitro*, the molecular chaperones mediate a biochemical change in α -synuclein that suppresses its toxicity⁴⁸. It was recently suggested that mutant α -synuclein might antagonize the receptor that is involved in chaperone-mediated autophagy, thereby enhancing the accumulation of toxic misfolded proteins and resulting in cellular dysfunction⁴⁹.

Recent studies in *Drosophila melanogaster* indicate that HSP70 might have a protective role in PD^{50,51}. Expression of wild-type or mutant α -synuclein in dopaminergic neurons results in inclusion body formation and causes pronounced (~50%) neuronal loss. Co-expression of human HSP70 prevents α -synuclein-mediated toxicity, but, paradoxically, has no visible effect on the inclusion body phenotype at the level of light microscopy. The protective effect of HSP70 might be attributed to the destabilization of toxic, misfolded α -synuclein monomers and/or small micro-aggregates that are not visible using light microscopy. Co-expression of a dominant-negative form of *D. melanogaster* HSP70 with α -synuclein accelerates the loss of dopaminergic neurons, indicating that endogenous chaperones modestly suppress α -synuclein-mediated neurodegeneration⁵⁰. Consistent with these results, administration of geldanamycin protects against α -synuclein toxicity in this fly model⁵¹. Although the effect of HSP70 on neurodegeneration has not been investigated in mouse models of PD, overexpression of HSP70 in mice that are transgenic for α -synuclein significantly reduces the formation of high molecular mass, detergent-insoluble material by α -synuclein⁴⁸.

Familial amyotrophic lateral sclerosis. For reasons that remain unclear, motor neurons have a high threshold for induction of the heat shock response, which might contribute to the selective degeneration of motor neurons observed in FALS. Cellular stress, such as the accumulation of misfolded proteins, normally stimulates the binding and activation of transcription factors to elements on heat shock promoters, increasing the synthesis of HSPs⁵². Remarkably, primary spinal cord cultures fail to upregulate HSP70 in response to heat shock, glutamate excitotoxicity or expression of mutant superoxide dismutase 1 (SOD1)⁵³, whereas cerebellar, cortical and pyramidal neurons, as well as astrocytes, efficiently upregulate HSP70 in response to heat shock⁵³⁻⁵⁵.

Intranuclear co-microinjection of expression vectors for HSP70 and mutant SOD1 into primary motor neurons reduces the toxicity of mutant SOD1, decreases SOD1 aggregation and enhances survival⁵⁶ compared with the injection of the SOD1 vector alone. A physical interaction between HSP70 and mutant SOD1 probably mediates neuroprotection; HSP70, HSP40 and α B-crystallin co-immunoprecipitate with SOD1 in cell lines that express mutant, but not wild-type, SOD1 (REF. 57). The interaction between HSP70 and mutant SOD1 is evident in total cell extracts, but barely discernible in an isolated supernatant fraction of soluble proteins, indicating that HSP70 interacts specifically with detergent-insoluble SOD1 complexes⁵⁷. Overexpression of both HSP70 and HSP40 in the presence of mutant SOD1 in an N2a (neuroblastoma-like) cell model results in a synergistic reduction of aggregate formation, but only ameliorates toxicity to a similar extent to HSP70 alone⁵⁸.

Polyglutamine expansion diseases. The effect of chaperones on the aggregation and toxicity of proteins with polyQ expansions has been intensely investigated in a diverse range of models, including *in vitro* systems, yeast, worms, flies and mice. Many studies have analysed the effect that chaperone overexpression has on inclusion body formation and toxicity of pathogenic polyQ fragments in cell culture¹. Overexpression of HSP40 consistently suppresses the formation of polyQ inclusion bodies and their toxicity^{59–62}. Overexpression of HSP70 also suppresses polyglutamine toxicity, and typically correlates with a decrease in inclusion body formation^{59–61}, although one study reported no change in the formation of inclusion bodies, and indicated that the ability of HSP70 to suppress apoptosis depends on inhibition of the pro-apoptotic factors caspase 3 and caspase 9 (REF. 63). In a similar study, overexpression of HSP27 was seen to decrease polyQ toxicity without altering inclusion body formation, but in a manner that correlates with a decrease in free radical production⁶². These studies highlight the possibility that chaperones facilitate neuroprotection through several distinct mechanisms, but because small, diffusible, potentially toxic polyQ assemblies could not be evaluated, an essential role for the refolding activity of chaperones cannot be ruled out. Furthermore, the stoichiometry of chaperones relative to the polyQ proteins might determine their effect on aggregation, and it is difficult to control expression levels in experiments that use transient transfection.

Several models of polyQ aggregation have been developed in *Saccharomyces cerevisiae* because chaperones are well characterized in this organism and are highly conserved with their human counterparts, and also because genetic tools are readily available to study the effects of chaperone mutations on polyQ aggregation^{64–66}. Overexpression of Ssa1 (HSP70) or Ydj1 (an HSP40 homologue) inhibits the formation of large, detergent-insoluble inclusion bodies and facilitates the accumulation of smaller aggregates⁶⁶. Mutations in the yeast HSP40 homologue *SIS1* or deletion of *HSP104* inhibits the NUCLEATION of huntingtin aggregates, whereas mutations in *SSA1*, *SSA2* and *YDJ1* inhibit the polymerization of huntingtin aggregates⁶⁵, indicating that these chaperones might be required for certain stages of aggregate formation. Consistent with these results, RNAi of two HSP70 homologues in a *Caenorhabditis elegans* model of polyQ disease delays the formation of inclusion bodies⁶⁷.

Studies in *D. melanogaster* have provided some insight into the possible mechanisms of chaperone suppression of polyQ-mediated neurodegeneration *in vivo*. Expression in the eye of a truncated form of ataxin 3 that contains a polyQ tract expansion (MJDtr-Q78) causes severe degeneration that is potentiated by a dominant-negative HSP70 and is rescued by co-expression of human HSP70 (REFS 68,69). Furthermore, although pan-neuronal expression of MJDtr-Q78 is lethal, co-expression of HSP70 increases male survival by 2% and female survival by 30%. Most intriguingly, HSP70 overexpression causes no identifiable differences in the

onset, size or number of inclusion bodies, indicating that the protective effect of HSP70 does not require inclusion body clearance⁶⁹. Overexpression of *D. melanogaster* HDJ1, but not *D. melanogaster* HDJ2, suppresses the MJDtr-Q78 degenerative eye phenotype, and, not surprisingly, overexpression of HDJ1 together with HSP70 suppresses neurodegeneration synergistically without having a visible effect on inclusion body formation⁷⁰. Importantly, however, the chaperones do enhance inclusion body solubility, indicating that, as in the studies with α -synuclein⁴⁸, neuroprotection might correlate closely with biochemical, rather than morphological, alterations in inclusion bodies^{66,70}. More recently, it has been shown that, in flies, HSC70 overexpression restores neuronal transport and suppresses the cell death that is caused by polyQ proteins⁷¹.

Overexpression of HSP70 in mouse models of polyQ disease has yielded mixed results. The R6/2 transgenic mouse model of HD expresses the first exon of the human huntingtin gene with 150 CAG repeats under the control of its own promoter, and has a progressive neurological phenotype with many of the characteristics of HD, including tremor, weight loss and neuronal inclusion bodies⁷². With the exception of a minor decrease in weight loss, overexpression of HSP70 in these mice to about 5–15 times normal levels has no effect on the behavioural or neuropathological phenotype⁷³. As chaperone levels progressively decrease in this mouse model⁷⁴, these results indicate that perhaps even higher levels of HSP70 or co-expression of HSP40 and HSP70 are necessary to ameliorate the severe R6/2 phenotype.

However, overexpression of HSP70 alone can modulate neurodegeneration in other mouse models of polyQ disease. Crossing the B05 line of spinocerebellar ataxia 1 homologue (SCA1) transgenic mice, which express ataxin 1 82Q (ataxin 1 with an expanded polyglutamine stretch of 82 residues) at 50–100 times endogenous levels, with an HSP70 transgenic line that has a 10-fold increase in HSP70 expression levels significantly improves the behavioural and neuropathological phenotypes without a corresponding change in inclusion body morphology⁷⁵. HSP70 had a dose-dependent effect, as mice that are homozygous for HSP70 showed a more robust improvement than hemizygous mice. Finally, overexpression of HSP70 in a mouse model of spinal and bulbar muscular atrophy (SBMA) causes a dose-dependent improvement in motor coordination that correlates with a decrease in the nuclear localization of the mutant androgen receptor and in high molecular mass and monomeric protein in Western blots, indicating that HSP70 overexpression enhances degradation of the mutant protein⁷⁶.

Unbiased genetic screens in *S. cerevisiae*, *D. melanogaster* and *C. elegans* models of polyQ aggregation have further validated the importance of molecular chaperones and protein folding in regulating polyQ aggregation and toxicity. In fruitfly and yeast screens several HSP40 homologues have been identified as potent suppressors of polyQ-mediated toxicity^{77–79}. In a genome-wide RNAi screen designed to look for modifiers of polyQ aggregation in *C. elegans*, two members of

NUCLEATION

A process by which the addition of a small amount of pre-aggregated protein to a monomeric preparation of the same protein robustly accelerates the assembly of amyloid fibrils.

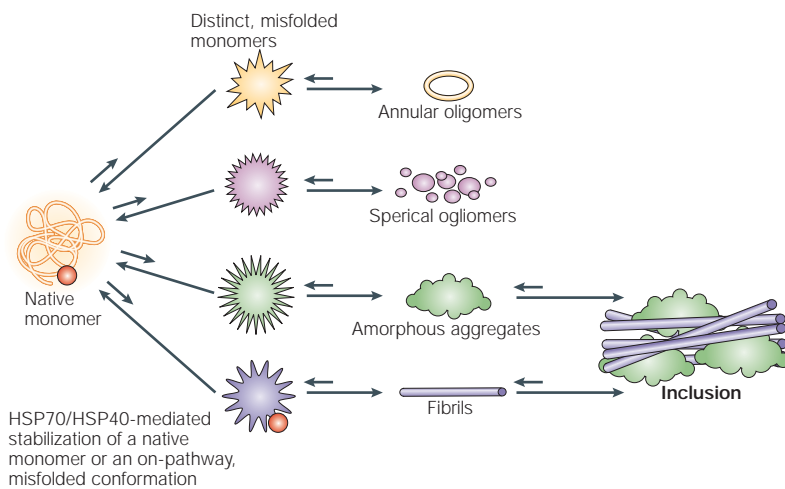


Figure 3 | Proposed actions of heat shock protein 70 and heat shock protein 40 chaperones on amyloid assembly. A native monomer might misfold into several distinct conformations (depicted by the rightward-pointing arrows that oppose a high-energy barrier for misfolding), each of which could give rise to a distinct higher-order assembly, such as an annular oligomer, a spherical oligomer, an amorphous aggregate or a fibril. By stabilizing a native or misfolded monomeric conformation, HSP70/HSP40 (depicted as red spheres) might prevent the intramolecular transition that gives rise to spherical and annular oligomers, and simultaneously stabilize a conformation that promotes inclusion body formation.

the HSP70 family and one DnaJ domain protein (an HSP40 homologue) were identified as suppressors of aggregation⁸⁰.

Mechanisms of neuroprotection

Although many studies have shown that chaperones are protective in models of neurodegenerative disease, the molecular basis of this protection is not known. Because a fundamental cellular role of chaperones is to recognize misfolded proteins, it is logical to investigate the direct effects of chaperones on amyloid assembly. With this aim, we analysed the effects of chaperones in an *in vitro* model of huntingtin aggregation. Using AFM, we showed that, similar to A β and α -synuclein, a mutant huntingtin fragment assembles into spherical oligomers, annular structures and amorphous aggregates that might be on a pathway to fibril assembly⁸¹. HSP70 and HSP40 attenuate the formation of spherical and annular mutant huntingtin oligomers, and facilitate the accumulation of fibrillar and amorphous aggregates. Biochemical analyses have indicated that HSP70 and HSP40 concomitantly stabilize a monomeric huntingtin conformation. Consistent with these results, Schaffar *et al.* used fluorescence resonance energy transfer (FRET) to show that HSP70 and HSP40 prevent an intra-molecular conformational change in a mutant huntingtin fragment that probably occurs before fibril assembly⁸². In doing so, the chaperones also suppress heterotypic interactions between the mutant huntingtin fragment and transcription factors with a polyQ stretch. Although AFM analyses of the aggregation reactions were not reported, the experimental conditions used indicate that this conformational change occurs before or concomitant with the formation of spherical structures. We propose a model in which HSP70 and HSP40 facilitate the folding

of a specific misfolded monomeric conformation that allows ON-PATHWAY ASSEMBLY to occur by monomer addition to fibril nuclei, while decreasing the likelihood of interactions that promote the formation of spherical and annular oligomers that might be OFF-PATHWAY for fibril formation but still important for neurotoxicity (FIG. 3). Consistent with our model, Collins *et al.* have recently shown that the formation of amyloid fibrils by the yeast prion protein Sup35 can occur exclusively as the result of monomer addition, and suggested that amyloid polymerization and oligomer assembly are competitive reactions⁸³. A future challenge will be to determine the mechanism of amyloid assembly *in vivo* and how this pathway is modulated by molecular chaperones.

It is likely that molecular chaperones facilitate neuroprotection by functioning at several levels that might not be linked exclusively to their direct effects on protein aggregation (FIG. 4). For example, clearance of toxic misfolded assemblies by chaperone-mediated autophagy or proteasomal degradation might help to decrease or prevent aberrant interactions with key cellular proteins such as transcription factors and signalling molecules. Chaperones might also help to prevent neuronal dysfunction by assisting intracellular trafficking, including synaptic transmission. The ability of molecular chaperones to interfere with oxidative stress and block apoptotic signalling pathways should also not be overlooked. Ultimately, molecular genetic analyses in animal models will be required to determine which actions of chaperones mediate their protective effects against neurodegeneration, and it is possible that several direct and indirect chaperone actions might be required to impede disease pathogenesis *in vivo*.

Molecular chaperones as potential drug targets
Chemical chaperones. Several low molecular mass compounds, such as the organic solvent dimethyl sulphoxide and the cellular osmolytes glycerol, trimethylamine N-oxide and trehalose, increase the stability of native proteins *in vitro*^{84–86}. The synthesis of trehalose in yeast, like that of HSPs, is upregulated in response to cellular stress⁸⁷, and enhances protein stability and maintains aggregation-prone proteins in a partially folded state that facilitates subsequent refolding by the molecular chaperones⁸⁸. Trehalose was identified in an *in vitro* screen for inhibitors of polyQ aggregation, and its efficacy was subsequently evaluated in the R6/2 transgenic model of HD⁸⁹. Although its effects were mild, administration of 2% trehalose in the drinking water of transgenic mice reduced brain atrophy, improved motor dysfunction and extended the lifespan of the mice. Consistent with these findings, deletion of a gene that encodes a protein involved in trehalose synthesis enhances α -synuclein toxicity in yeast⁷⁹.

Drugs that induce the expression of molecular chaperones. It is clear from animal studies that overexpression of molecular chaperones might prove beneficial for the treatment of neurodegenerative diseases. However, excessive upregulation of chaperones might lead to

ON-PATHWAY ASSEMBLY
 A misfolded protein monomer or higher-order aggregate that is an obligate intermediate in the formation of amyloid fibrils.

OFF-PATHWAY ASSEMBLY
 A misfolded protein monomer or higher-order aggregate that is not an obligate intermediate in the assembly of amyloid fibrils, and might actually compete with on-pathway interactions to suppress amyloid fibril polymerization.

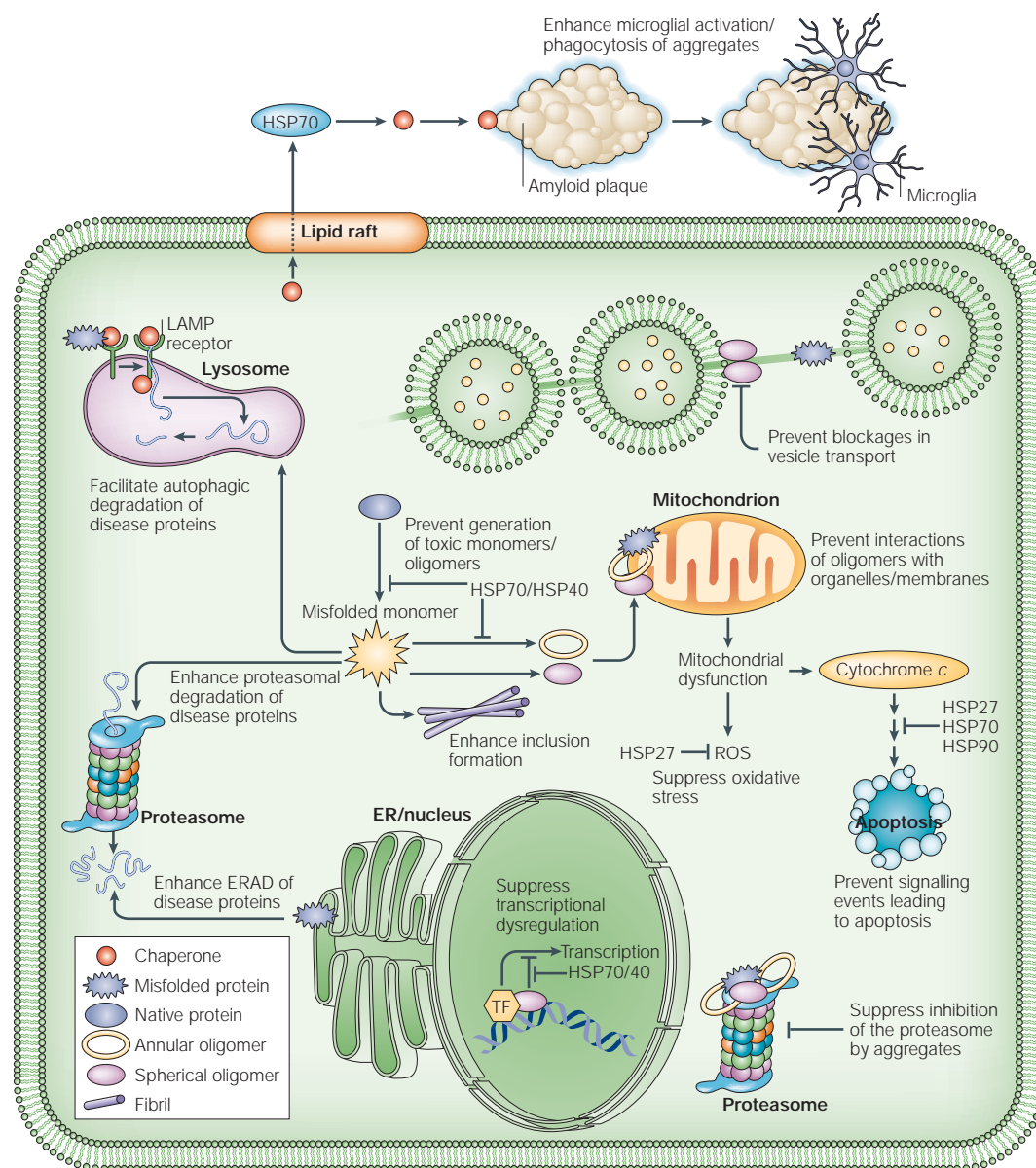


Figure 4 | **Direct and indirect effects of molecular chaperones on disease protein toxicity.** Molecular chaperones might prevent toxicity by blocking inappropriate protein interactions, by facilitating disease protein degradation or sequestration, and by blocking downstream signalling events that lead to neuronal dysfunction and apoptosis. ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; HSP, heat shock protein; LAMP, lysosomal-associated membrane protein; ROS, reactive oxygen species; TF, transcription factor.

undesirable side effects, such as alterations in cell cycle regulation and cancer⁹⁰. A delicate balance of chaperones is likely to be required for a beneficial, neuroprotective effect. The exposure of stressed cells to a non-native protein can activate heat shock transcription factor 1 (HSF1)⁹¹. HSP90 is a part of a complex that negatively regulates the activity of HSF1 (REF. 92), and is therefore an attractive therapeutic target. Geldanamycin binds to an ATP site on HSP90 and blocks its interaction with HSF1, promoting HSF1 activation and the synthesis of HSPs^{92,93}. The investigation of chaperone-inducing compounds such as the antibiotic radicicol, which has the same functional

mechanism as geldanamycin but a 50-fold greater affinity for HSP90 (REFS 94,95), might yield similar results. An exciting study by Kieran *et al.* has shown that treatment of transgenic ALS mice with arimoclomol, a small molecule that acts as a co-inducer of the heat shock response by prolonging the activity of HSF1, improved behavioural phenotypes, prevented neuronal loss and extended survival rates by 22% (REF. 96). Ultimately, chemical chaperones, used in combination with a pharmacological agent that upregulates the synthesis of molecular chaperones, might be a valid therapeutic approach for treating protein conformational disorders.

Conclusions

There is overwhelming experimental evidence that molecular chaperones are crucial modulators of protein aggregation and neurodegeneration. However, the mechanism by which chaperone activity facilitates neuroprotection remains poorly understood. A proximal step in the pathogenesis of neurodegenerative diseases linked to protein aggregation probably stems from aberrant protein interactions due to altered conformations in disease-causing proteins, effectively triggering a cascade of pathogenic events that culminates in neuronal dysfunction before the appearance of inclusion bodies. Which proteins are involved in these aberrant protein interactions, how do they mediate neuronal dysfunction and how do molecular chaperones rescue this effect? Ultimately, molecular genetic approaches in mice and other models will be required to enable us to answer these questions. Although not extensive, animal studies

have shown that chaperones facilitate neuroprotection without any visible effect on inclusion body formation, indicating that protective interactions might occur at the level of small, diffusible aggregate assemblies, or perhaps of misfolded conformations of monomers. It will be important to develop tools and techniques that allow *in vivo* detection of these disparate misfolded conformations and/or assemblies and to determine how interaction of these abnormal structures with other cellular proteins, such as the molecular chaperones, alters pathogenesis. Effective therapies will probably require the simultaneous modulation of several components of the protein quality control apparatus, and molecular chaperones will have a key role in these types of approach. As molecular chaperones provide a first line of defence against misfolded proteins and probably function at the earliest stages of disease pathogenesis, they are a particularly exciting prospect for therapeutic intervention.

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Competing interests statement

The authors declare no competing financial interests.

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