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REVIEW

# Walking the tightrope: proteostasis and neurodegenerative disease

Justin J. Yerbury,*,† Lezanne Ooi,*,† Andrew Dillin,‡,§ Darren N. Saunders,¶,** Danny M. Hatters,†† Philip M. Beart,‡‡ Neil R. Cashman,§§ Mark R. Wilson*,† and Heath Ecroyd*,†

*Proteostasis and Disease Research Centre, School of Biological Sciences, Faculty of Science, Medicine and Health, University of Wollongong, Wollongong, New South Wales, Australia

†Illawarra Health and Medical Research Institute, Wollongong, New South Wales, Australia

‡Department of Molecular and Cell Biology, Li Ka Shing Center for Biomedical and Health Sciences, The University of California, California, USA

§Howard Hughes Medical Institute, The University of California, Berkeley, California, USA

¶School of Medical Sciences, Faculty of Medicine, University of New South Wales, Randwick, New South Wales, Australia

**The Kinghorn Cancer Centre, Garvan Institute of Medical Research, Darlinghurst, New South Wales, Australia

††Department of Biochemistry and Molecular Biology and Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, Victoria, Australia

‡‡Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, Victoria, Australia

§§Department of Medicine (Neurology), University of British Columbia and Vancouver Coastal Health Research Institute, Brain Research Centre, University of British Columbia, Vancouver, British Columbia, Canada

**Abstract**

A characteristic of many neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), is the aggregation of specific proteins into protein inclusions and/or plaques in degenerating brains. While much of the aggregated protein consists of disease specific proteins, such as amyloid-β, α-synuclein, or superoxide dismutase1 (SOD1), many other proteins are known to aggregate in these disorders. Although the role of protein aggregates in the pathogenesis of neurodegenerative diseases remains unknown, the ubiquitous association of misfolded and aggregated proteins indicates that significant dysfunction in protein homeostasis (proteostasis) occurs in these diseases. Proteostasis is the concept that the integrity of the proteome is in fine balance and requires proteins in a specific conformation, concentration, and location to be functional. In this review, we discuss the role of specific mechanisms, both inside and outside cells, which maintain proteostasis, including molecular chaperones, protein degra-

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Address correspondence and reprint requests to Justin J. Yerbury or Heath Ecroyd, Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, New South Wales 2522 Australia. E-mails: jyerbury@uow.edu.au, heathe@uow.edu.au

*Abbreviations used*: A2M, alpha-2-macroglobulin; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; Aβ, amyloid – β peptide; CLU, clusterin; E3L, E3 ligase; GWAS, genome-wide association studies; HD, Huntington's disease; Hp, haptoglobin; HSF1, heat-shock factor1; HSP, heat-shock protein; HSR, heat-shock response; INQ, intranuclear quality control compartment; IPOD, insoluble protein deposits; iPSC, induced pluripotent stem cells; JUNQ, juxtanuclear quality control compartment; LTP, long-term potentiation; PD, Parkinson's disease; PolyQ, poly glutamine; Proteostasis, protein homeostasis; sHSP, small heat-shock protein; SOD1, superoxide dismutase1; Ub, ubiquitin; UPS, ubiquitin-proteasome system.

 

dation pathways, and the active formation of inclusions, in neurodegenerative diseases associated with protein aggregation.

**Keywords:** aging, aggregation, autophagy, chaperones, heat-shock proteins, ubiquitin-proteasome system.
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## The proteostasis network safeguards cells and organisms from misfolded and damaged proteins

The term proteome homeostasis or proteostasis refers to the maintenance of all proteins in the proteome in a conformation, concentration, and location that is required for their correct function (Balch *et al.* 2008). Proteostasis is essential to maintain normal cellular metabolic function, and is vital for the dynamic changes required for a cell to respond to a given stimulus. An extensive and complex network of signaling pathways are involved in safeguarding cells and organisms against proteotoxic stress. They do so by maintaining the delicate balance between the production and disposal of proteins. Thus, the combined actions of this extended network that maintains proteostasis, the proteostasis network, act as a quality control department in the demanding world of the cell. The proteostasis network comprises pathways that regulate the biogenesis, folding, trafficking, and degradation of proteins (Fig. 1). Some of the most significant players in the network are the chaperone proteins, the guardians of the cell that ensure the integrity of the proteome. Other protective processes involve compartmentalizing misfolded proteins into specific regions in the cell (e.g., the aggresome) or removal of proteins by autophagy or the ubiquitin-proteasome system (UPS). The careful regulation of these activities is critical in reducing the toxicity associated with mutant, misfolded and/or damaged proteins. Obviously, the removal of toxic forms of misfolded proteins is important for all cells, but it is especially significant in post-mitotic cells, such as neurons, since these cannot be readily replaced. In neurons, the maintenance of proteostasis is central to healthy aging; dysregulation of the proteostasis network can lead to neurodegenerative disease. Thus, the successful maintenance of proteostasis is directly related to cellular and organismal longevity.

## Dysregulation of the proteostasis network in neurodegenerative disease

A common characteristic of many neurodegenerative disorders is the presence of specific and distinct proteinaceous inclusions in or around the affected neuron in hallmark regions of the brain (Fig. 2). These inclusions are primarily composed of misfolded, aggregated, and often toxic forms of one or more specific proteins. As examples, PD is characterized by the deposition of the protein α-synuclein into Lewy bodies (particularly in the substantia nigra), AD is associated with amyloid-β plaques and tangles of phosphorylated tau, and HD is associated with aggregates of huntingtin containing expanded repeats of glutamine in the nucleus and cytoplasm. In contrast, ALS pathology is characterized by a number of different types of inclusions, including Bunina bodies, and ubiquitinated, hyaline and skein-like inclusions (Ciechanover 2005). Thus, the presence of inclusions, the visible products of protein aggregation in a number of neurodegenerative diseases, indicates that a significant disruption to proteostasis has occurred. The specific proteins involved in these diseases form aggregates that can be fibrillar and insoluble, as in the case of amyloid-β (Chiti and Dobson 2006), fibrillar and re-soluble as in the case of the hydrogel type aggregates associated with Fused in Sarcoma (FUS) (Han *et al.* 2012), or amorphous.

Genetic mutations to metastable proteins may increase their propensity to aggregate, causing the proteostasis network to be overloaded. However, there is evidence that expression of aggregation-prone proteins can be dealt with by the proteostasis network in early life and that as organisms age the capacity to deal with this added proteotoxic stress is impaired, facilitating aggregation (Ben-Zvi *et al.* 2009; Wang *et al.* 2009; David *et al.* 2010). In the absence of genetic mutations associated with an increased aggregation propensity of a single protein (i.e., in sporadic cases of these neurodegenerative diseases where there is no genetic cause of disease), the failure of the proteostasis network is likely a requisite for aggregation to occur. How the proteostasis capacity is 'overwhelmed' in these cases is yet to be determined, however, a decline in the levels of proteostasis components during aging is likely to be a key factor. For example, previous work has suggested an age-related attenuation of the ability to activate heat-shock factor1 (HSF1) during stress (Shamovsky and Gershon 2004) (see also 'The role of molecular chaperones in neurodegenerative disease' below). Once aggregation occurs it is probable that a further collapse in proteostasis follows because of the sequestration of chaperones and other proteostasis components (e.g., proteasome subunits) into inclusions (Yu *et al.* 2014). Taken together, these data suggest that in early life, in the absence of genetic mutation, the proteostasis network has sufficient capacity to deal with aggregating proteins and that a reduction and/or dysfunction in proteostasis capacity, as occurs with age, expedites aggregation and thus the onset and progression of disease.

The most significant universal risk factor for neurodegenerative diseases is age. Neurons are seemingly more suscep-

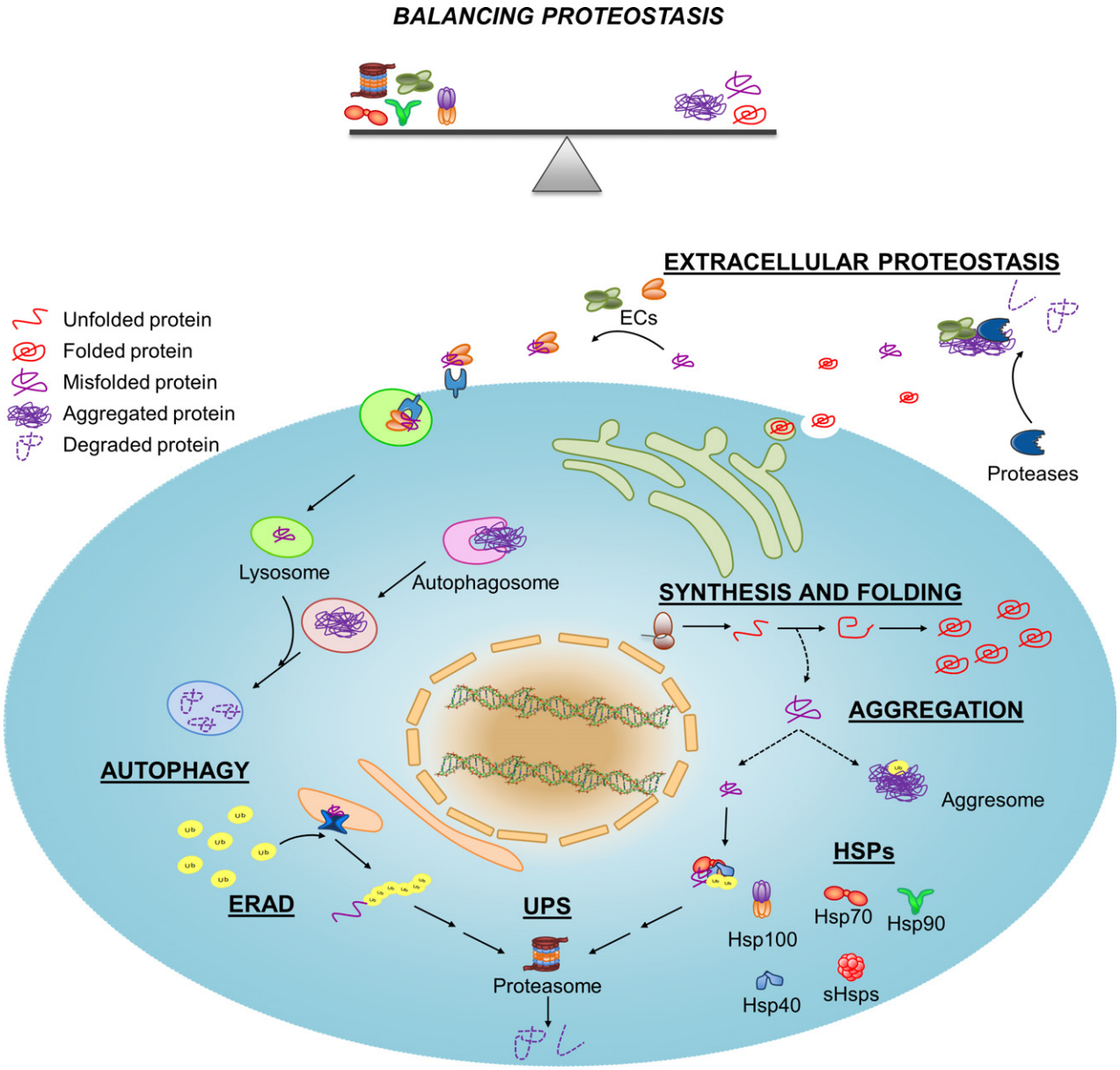


**Fig. 1** Schematic representation of the proteostasis network. Protein homeostasis pathways function to maintain the proteome in a native conformation, in the correct location and concentration. During nascent protein synthesis (cytoplasmic and ER) and folding a significant proportion of the proteome requires attention of molecular chaperones and/or the ubiquitin-proteasome system (UPS). Cytosolic misfolded proteins are bound and triaged by molecular chaperones such as the HSPs, and ER proteins are sorted by the endoplasmic reticulum associated degradation (ERAD) pathways. Any protein that cannot be degraded may be directed into aggresome-like structures. Aggregates that form in the cell can be degraded by macroautophagy. Secreted proteins are monitored by extracellular proteostasis pathways. Misfolded proteins in the extracellular space are recognized by extracellular chaperones (ECs) which bind and direct misfolded proteins to cell surface receptors for degradation in lysosomes. Protein aggregates can also trigger activation of extracellular proteases such as plasminogen activators, which can result in degradation of aggregates.

tible to detrimental age-related metabolic changes than other cell types and several inter-related factors could contribute to this susceptibility. In most regions of the adult brain, damaged neurons are not replaced and central axons regenerate poorly after injury (reviewed in Liu *et al.* 2011). Although neurogenesis can take place in certain areas of the hippocampus and olfactory bulb, this process slows with aging and the integration of new neurons into functional networks becomes limited (Kuhn *et al.* 1996; Overstreet-Wadiche *et al.* 2006). In addition, neurons are highly

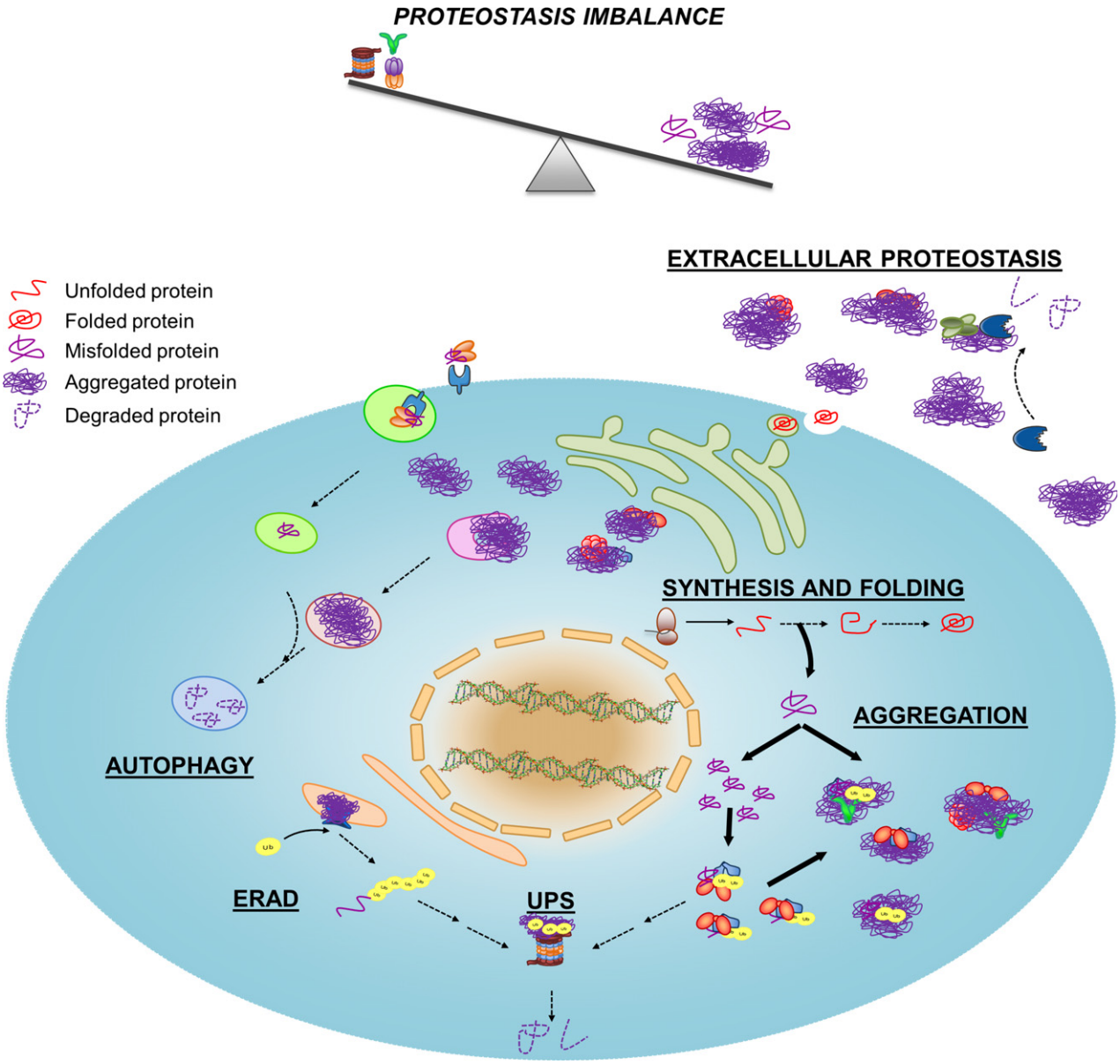


**Fig. 2** Proteostasis imbalance during aging. The proteostasis network may be reduced in its capacity during aging. For example, HSPs may reduce in number meaning that misfolded proteins generated from synthesis may not be directed to the UPS, but instead aggregate. A reduction in the efficiency of autophagy may allow the accumulation of these aggregates. Similarly, an overloading of the extracellular proteostasis systems may also result in the accumulation of protein aggregates in the extracellular space.

compartmentalized, with long axons and dendrites that cover large distances and form thousands of connections with other cells. Local protein synthesis at synapses carries a high-energy demand and neuronal ATP requirements increase with aging (Gabbita *et al.* 1997). These increased ATP requirements with aging are possibly because of a reduction in the efficiency of energy utilization by aged neurons, however, the precise reasons for this remain unclear. Moreover, the generation of ATP by oxidative phosphorylation leads to the release of oxidative radicals that damage neuronal DNA, proteins and lipids. In the absence of neuron turnover, oxidative damage accumulates over the life span of the cell/organism. Lastly, chaperones, including the heat-shock proteins (HSPs) appear to have a higher threshold for activation and/or lower overall expression in neurons compared to other cells. For example, induction of HSP70 by HSF1 is lower in neurons than non-neuronal cells (Marcuccilli *et al.* 1996; Manzerra *et al.* 1997). Protein degradation via the UPS is also highly dependent on ATP (Hershko *et al.* 1980). Hence, age-related changes in neuronal ATP balance

or ATP requirements are likely to have significant effects on the capacity of this system (Huang *et al.* 2013). Conversely, mitochondrial proteins and components of energy metabolic pathways (glycolysis and oxidative phosphorylation) are substrates of the UPS, indicating the potential for significant crosstalk between neuronal energy metabolism and protein turnover (Heo and Rutter 2011; Livnat-Levanon and Glickman 2011).

Overall it thus appears that neurons are more susceptible to cellular stressors than other cell types. Collectively these factors can lead to substantial overload of the proteostasis network, because of (i) the inability of neurons to dilute aggregates via cell division, (ii) the extreme lengths of axons, and the requirement for protein turnover far from the nucleus, and (iii) signaling pathways of the proteostasis network being ATP-dependent. The overload of the proteostasis network can lead to widespread protein misfolding in specific neuronal types in the brain. The inability of neurons to maintain proteostasis does not appear to be random; there are temporal and spatial patterns of inclusion formation during disease progression and the overall 'inclusion load' correlates very well with the advancement and increased severity of pathological changes. It is thus vital to understand the components of the network that govern the proteostasis capacity of the brain.

The following sections of this review detail the central systems in the proteostasis network within the context of neurodegenerative disease, including the molecular chaperones, compartmentalization of misfolded proteins, the ubiquitin proteasome system, autophagy, and extracellular proteostasis.

## The role of molecular chaperones in neurodegenerative disease

Exposure to acute or chronic proteotoxic stress results in the induction of highly conserved and ubiquitous stress response pathways in cells (Voisine *et al.* 2010; Morimoto 2011). Induction of the heat-shock response (HSR) is dependent on the activation of the transcription factor, HSF1, which involves its transition from an inactive monomer into an active hyperphosphorylated trimer with high affinity for DNA (Akerfelt *et al.* 2010). DNA-binding of trimeric HSF1 to heat-shock elements in promoter regions of target genes leads to the rapid and robust expression of a variety of stress response genes, in particular the HSP molecular chaperones (Anckar and Sistonen 2011; Morimoto 2011; Baird *et al.* 2014).

Of the proteostasis pathways responsible for combating protein aggregation, molecular chaperones are arguably one of the most important, as they form a cell's first line of defense against protein misfolding and subsequent aggregation (Hartl *et al.* 2011). Hence, the presence of protein inclusions in neurodegenerative diseases can be interpreted as a failure of chaperones to prevent aggregation in the context of these disorders. Molecular chaperones represent a structurally diverse class of proteins that support correct folding by preventing unproductive interactions between partially folded protein intermediates (Voisine *et al.* 2010; Hartl *et al.* 2011; Wyatt *et al.* 2013; Treweek *et al.* 2015). A recent comprehensive analysis of the human intracellular 'chaperome' (this study did not consider extracellular chaperones which are discussed below) identified 332 chaperone genes (88 of which are functionally classified as chaperones and 244 are classified as co-chaperones) that were placed into nine families: HSP90, HSP70, HSP60, HSP40, Prefoldin, small HSPs (sHSPs), tetratrico peptide repeat-domain containing, and chaperones of the endoplasmic reticulum and mitochondria (Brehme *et al.* 2014). Five of these chaperone families correspond to heat-shock proteins (HSP90, HSP70, HSP60, HSP40, and sHSPs), a family of evolutionarily conserved proteins with diverse functions in proteostasis. As a result of their well-characterized molecular chaperone functions, HSPs play a role (directly or indirectly) in most cellular processes. For example, molecular chaperones play important roles in stabilizing the cytoskeleton, regulating stress responses, trafficking proteins into and out of cellular compartments, mitigating apoptotic signaling and shuttling damaged proteins for degradation by the proteasome or by autophagy. It is beyond the scope of this work to provide a comprehensive summary of the role of individual HSPs in proteostasis, however, there have been a number of recent reviews by others in this area (e.g., Kim *et al.* 2013; Brandvold and Morimoto 2015; Duncan *et al.* 2015; Finka *et al.* 2015; Labbadia and Morimoto 2015; Pratt *et al.* 2015; Treweek *et al.* 2015).

One reason that chaperones may fail to prevent protein aggregation associated with neurodegenerative diseases is highlighted by recent work demonstrating that there is, in general, a decrease in chaperone levels (in particular of ATP-dependent chaperones) in the human brain during aging, and in AD, HD, and PD patients; the main exceptions to this being the sHSPs and tetratrico peptide repeats which increase with age and in the context of these neurodegenerative diseases (Brehme *et al.* 2014). Thus, changes to the relative balance of the 'chaperome' network may facilitate the cascade of events that manifest as the age- and neurodegenerative disease-associated collapse in proteostasis. Moreover, cellular senescence, as occurs in neurons, results in a decline in proteostasis capacity which could, at least in part, also be accounted for by the general decrease in chaperone levels seen with age and in neurodegenerative diseases. As such, exogenous application of HSPs, or therapeutic up-regulation of existing proteostatic pathways that regulate their levels, represents a promising avenue for the treatment of neurodegenerative diseases.

There have been a wide range of studies that have examined the protective role (or otherwise) chaperones have

on protein aggregation and inclusion formation in cell-based and mouse models of diseases, with varying results. One often overlooked aspect of such work is the interdependence and compensatory mechanisms that exist within the chaperome. For example, the chaperone-mediated degradation of aggregation-prone proteins via autophagy requires the concerted actions of HSC70, HSP40, and co-chaperones such as Hip and Hop (Agarraberes and Dice 2001) and there are multiple members (often with overlapping expression and function) of each chaperone class (Brehme *et al.* 2014). Thus, over-expression or knock-down/out studies of individual HSPs can be an over-simplification. The impact increasing (or decreasing) the levels of one chaperone isoform has on the levels of others, is often not taken into account. Nevertheless, various studies have demonstrated that over-expression of the HSPs can prevent the aggregation and associated toxicity of disease-related proteins, for example, amyloid-β (Hoshino *et al.* 2011), polyglutamine-expanded proteins (Wyttenbach *et al.* 2002; Jiang *et al.* 2012; Ormsby *et al.* 2013); α-synuclein (Auluck *et al.* 2002; McLean *et al.* 2002; Tue *et al.* 2012; Cox *et al.* 2014) (Auluck *et al.* 2002; McLean *et al.* 2002; Tue *et al.* 2012; Cox *et al.* 2014), and superoxide dismutase 1 (Koyama *et al.* 2006; Crippa *et al.* 2010; Novoselov *et al.* 2013); proteins implicated in the pathogenesis of AD, HD, PD, and ALS, respectively. However, others have reported that over-expression of some HSPs has no significant effects on disease progression in mouse models of disease (e.g., Liu *et al.* 2005; Krishnan *et al.* 2008; Shimshek *et al.* 2010; Xu *et al.* 2015). Thus, it remains to be determined whether up-regulating chaperone levels is a valid therapeutic approach for the treatment of these neurodegenerative diseases.

In studies that have reported a neuroprotective effect of HSPs, these effects have been primarily attributed to their ability to (i) mitigate the earliest aberrant protein interactions that trigger pathogenic aggregation (and promote subsequent re-folding of the misfolded proteins) or (ii) shuttle proteins for degradation. The co-localization of HSPs with plaques and inclusions in the brains of neurodegenerative disease patients (e.g., Wilhelmus *et al.* 2006; Leverenz *et al.* 2007) and in mouse models of these diseases (e.g., Basso *et al.* 2009; Zetterstrom *et al.* 2011) has led to the interpretation that HSPs are 'overwhelmed' by the increasing levels of protein aggregation associated with these diseases. This results in their subsequent deposition into the inclusion itself (and loss of chaperone function as a result). However, recent work has demonstrated that many chaperones can interact with the aggregated form of proteins and, in doing so, may even mediate their packaging into inclusions (Dedmon *et al.* 2005; Falsone *et al.* 2009; Waudby *et al.* 2010; Danzer *et al.* 2011; Binger *et al.* 2013). Indeed, it has been recently shown that sequestering proteins into chaperone-enriched aggregates prevents an age-related decline in proteostasis and prolongs lifespan in *C. elegans* (Walther *et al.* 2015). Thus, HSPs may play an active role in the formation of the inclusions, rather than merely being a part of them as a fall-out of their formation. It is likely that this action plays two important roles: (i) inhibiting aggregate fragmentation and secondary nucleation events, both of which can be the source of cytotoxic oligomers associated with protein aggregation (Knowles *et al.* 2009); and (ii) facilitating the degradation of the aggregated protein via the UPS and chaperone-mediated autophagy (see below).

## Maintaining proteostasis by aggregation

Although it is still not clear whether protein aggregation into inclusions is a cause or consequence of neurodegenerative diseases, aggregation alone provides strong evidence that proteostasis has been disrupted in these diseases. Moreover, while each neurodegenerative disease is specifically associated with the aggregation of particular proteins into inclusions, there are many other proteins also present in these protein inclusions. Characteristically, these proteins are expressed at levels that exceed their predicted aggregation propensity (i.e., they are super-saturated) (Ciryam *et al.* 2013). There is still some debate about whether the formation of protein inclusions in cells is a protective mechanism (Saudou *et al.* 1998; Miller *et al.* 2011), or toxic to the cells. In any case it is clear that protein aggregation and the formation of inclusions are closely associated with neuronal degeneration (Leigh *et al.* 1991; Chiti and Dobson 2006; Ticozzi *et al.* 2010; Ormsby *et al.* 2013).

Classically, inclusions were thought to be purely the result of the misfolding and random self-assembly of proteins into amorphous and/or fibrillar structures in a process replicating the well-characterized aggregation of proteins *in vitro* (Chiti and Dobson 2006; Knowles *et al.* 2014). Indeed, it is possible that the chronic misfolding and aggregation of a disease-associated protein, such as SOD1, 'overwhelms' the proteostasis capacity of the cell and that this explains the widespread protein aggregation of metastable proteins (Gidalevitz *et al.* 2006). Given the observed three-dimensional spread of neurodegeneration throughout the nervous system, proteostatic collapse may spread throughout the nervous system in conjunction with the misfolding and aggregation of proteins, such as observed for SOD1 in ALS (Grad *et al.* 2014).

In contrast, it is now clear, that in an effort to maintain proteostasis, cells are able to actively sequester misfolded and aggregating proteins into subcellular compartments. These compartments are not organelles but spatially distinct regions in the cell. This process minimizes the risk that misfolded proteins pose to cells (e.g., even small aggregates can nucleate further aggregation and be toxic to cells) by clustering proteins so that they can be resolubilized or degraded, as appropriate.

Several mechanisms for forming inclusions in mammalian cells have been described, including those associated with the structures known as aggresomes (Johnston *et al.* 1998), insoluble protein deposits (IPOD) and juxtanuclear quality control (JUNQ) (Kaganovich *et al.* 2008), aggresome-like structures (Szeto *et al.* 2006), RNA interactor specific compartments/inclusions (Farrawell *et al.* 2015), ER-associated degradation-associated vesicles (Kamhi-Nesher *et al.* 2001) and intranuclear protein quality control compartments (Nollen *et al.* 2001; Miller *et al.* 2015a,b). The almost ubiquitous nature of these types of inclusions in neurodegenerative diseases, and the diverse and specific mechanisms that underpin their formation, reveals the importance of inclusions to cellular protein quality control and human pathology.

Originally, cell driven, microtubule-dependent juxtanuclear inclusions of the cystic fibrosis transmembrane conductance regulator, that colocalized with the microtubule organizing center, were discovered and termed aggresomes (Johnston *et al.* 1998). Aggresomes were found to organize and sequester aggregated proteins into a specific region of the cell, and to have chaperones and proteasome subunits associated with them (Kopito 2000). Aggresomes are ensheathed by a cage of vimentin, however, it is not clear if this event indicates an attempt to contain the aggregate or if it represents the collapse of intermediate filaments in the cell (Johnston *et al.* 1998). While there is still little known about the formation of these structures, their formation is dependent on dynein microtubules and HDAC6 (Kawaguchi *et al.* 2003).

IPOD inclusions have been observed in yeast and mammalian cells and are characterized as dense, immobile compartments consisting of insoluble protein aggregates that (at least initially) are not ubiquitinated (Kaganovich *et al.* 2008; Hipp *et al.* 2012). Proteins targeted to IPODs end up as insoluble aggregates or amyloid fibrils. The most common experimental model of IPOD is huntingtin with pathogenic polyQ expansions (Kaganovich *et al.* 2008; Polling *et al.* 2014). In yeast, HSP104 and the autophagy protein Atg8 colocalize with IPODs, consistent with a cellular effort to degrade the aggregated proteins (Kaganovich *et al.* 2008). In primary neuronal cultures the huntingtin-polyQ IPOD structures are extremely stable with no loss of protein from the inclusion over the period of a week (Tsvetkov *et al.* 2013).

Aggresomes may be related to the compartment later referred to as JUNQ in studies of yeast and mammalian cells (Kaganovich *et al.* 2008). JUNQ-like inclusions are enriched in ubiquitylated proteins, proteasome subunits, and chaperones (such as HSP70); proteins are able to freely diffuse into and out of the aggregate in mammalian cells (Kaganovich *et al.* 2008; Weisberg *et al.* 2012; Polling *et al.* 2014). As a result, JUNQ has been proposed to be a cellular quality control center in which soluble misfolded proteins or aggregates from the cytosol accumulate for proteasomal degradation and refolding (Kaganovich *et al.* 2008; Weisberg *et al.* 2012). The precise mechanisms that underpin JUNQ formation still remain a mystery, as does the cell's method of containing the aggregated proteins in the inclusion. However, it is clear that in mammalian cells, proteins can be targeted to JUNQ via their ubiquitination, and require transport via microtubules (Kaganovich *et al.* 2008; Farrawell *et al.* 2015). While it was originally thought that JUNQ inclusions possessed the same characteristics in all eukaryotic cells, recently it has been demonstrated that JUNQ-like structures in yeast can be found within the nucleus, rather than the cytosol (Miller *et al.* 2015a,b). It has been proposed that these structures in yeast should be referred to as the intranuclear quality control compartment (INQ). Indeed, additional work demonstrated that the intranuclear compartment contains a distinct set of proteins that are ubiquitylated and sumoylated in response to genotoxic stress (Gallina *et al.* 2015). The relationship (if any) these structures have to JUNQ aggregates in the mammalian cytosol, such as those formed by SOD1, remains unclear. In any case, these findings highlight the need to further characterize the processes in cells that act to organize aggregated proteins into specific regions and to identify a set of criteria to properly define and identify various types of inclusions in cells.

Clearly, we are only just beginning to understand the intricate cellular processes that actively promote the formation of inclusions in cellular systems. Further understanding of the molecular properties of these structures, their organization and their involvement in cellular proteostasis may uncover important biological processes critical to the understanding of many neurodegenerative diseases. For example, given that cells actively partition aggregating proteins, under some conditions it may be detrimental to suppress this process with anti-aggregation small molecules.

## Balancing proteostasis via protein degradation

### The ubiquitin–proteasome system

Ubiquitination is one of the most abundant protein modifications in cellular signaling, regulating numerous cellular pathways including transcription, translation, vesicle transport, apoptosis, cell cycle, and DNA damage repair. Ubiquitin (Ub) labels substrate proteins via a highly ordered multi-step enzymatic cascade, with specific differences in the length and topology of poly-Ub chains resulting in a range of signaling outcomes (e.g., allosteric regulation, intracellular trafficking) or proteolytic degradation via the proteasome.

In the nervous system, Ub contributes to the regulation of neuronal growth and development, excitability, neurotransmission, long-term potentiation, and synapse formation and elimination (Mabb and Ehlers 2010; Kawabe and Brose 2011). Maintenance of the UPS is central to neuronal health as neurons are very sensitive to prolonged Ub deficiency, which leads to cell death (Tan *et al.* 2000, 2001). Mounting

genetic and functional evidence supports a role for compromised ubiquitin homeostasis in the pathophysiology of neurodegenerative diseases, and the ubiquitination machinery is emerging as a highly promising target for human therapy (Jankowska *et al.* 2013).

Ubiquitin exists in a dynamic cellular equilibrium, partitioning into four major pools; (i) immobile in the nucleus (primarily bound to histones), (ii) immobile in the cytoplasm (bound to organelles and cytoskeleton), (iii) in soluble polyUb chains, and (iv) a small fraction as free monomeric Ub (Dantuma *et al.* 2006). Ubiquitin homeostasis is tightly regulated at multiple levels. *De novo* replenishment of Ub is achieved through transcription of four separate mammalian genes (UBB, UBC, UBA52, and UBA80) and Ub is degraded by the 26S proteasome (Ciechanover 2005). However, the key regulator of the balance between free and protein-conjugated ubiquitin pools is the opposing actions of E3 ubiquitin ligases and deubiquitinating enzymes (DUBs). Reminiscent of the coordinated action of kinases and phosphatases, there is emerging evidence that E3 ubiquitin ligases and deubiquitinating enzymes coordinate to achieve dynamic fine-scale regulation of substrate ubiquitination (e.g., Diefenbacher *et al.* 2014). An example of this dynamic Ub equilibrium is the competition between histones and other Ub substrates for a limited pool of free ubiquitin in an 'Ub Cycle' coupled to other cellular process requiring Ub (Dantuma *et al.* 2006; Groothuis *et al.* 2006). Kimura *et al.* (2009) used the example of a rapid loss of free Ub following heat shock to propose that free Ub chains act as a cellular reservoir for maintenance of monomeric Ub under normal conditions and provide a capacity for rapid response to stress conditions requiring substrate conjugation (analogous to the glucose/glycogen cycle).

Accumulation of ubiquitylated proteins in insoluble inclusions is another potential mechanism for loss of free Ub, but removal of Ub from the aggregated protein is possible though Ub-specific proteases. Free Ub was recently shown to be increased in cells containing huntingtin aggregates by overexpression of the de-ubiquitylation enzyme USP14 (Hyrskyluoto *et al.* 2014). Not only did this protect cells from aggregate-induced toxicity, but it also reduced ER stress, which is thought to precede inclusion formation in ALS models (Atkin *et al.* 2014). For example, proteasome inhibition depletes free Ub levels to as low as 5% of basal in less than 2 h (Mimnaugh *et al.* 1997; Patnaik *et al.* 2000). Inhibition of translation also depletes free Ub through reduced production, while toxicity can be rescued by overexpression of Ub (Hanna *et al.* 2003).

Probably the two most compelling lines of evidence for the role of Ub in neurodegenerative diseases come from the widely observed enrichment of Ub in cytoplasmic inclusion bodies defining many of these diseases, and from the loss of function phenotypes in mouse and human. For example, Ub is a pervasive feature of inclusions in ALS and motor neuron death in all forms of ALS is associated with the formation of aberrant Ub-positive protein inclusions (Giordana *et al.* 2010). Ub can also accumulate in inclusions without the aggregation of Transactive response DNA binding protein of 43 kDa (TDP-43) in sporadic ALS (Giordana *et al.* 2010), suggesting that aggregation of proteins such as TDP-43, FUS, and SOD1 may not be necessary for Ub depletion-induced toxicity. Ub accumulation is also linked to impaired function of the cellular proteostasis network in models of HD (Hipp *et al.* 2012).

Several mouse models of Ub deficiency lead to protein aggregation and neurodegeneration (Hallengren *et al.* 2013). For example, Usp14-null mice display ataxia caused by reduced levels of monomeric Ub, resulting from decreased DUB-mediated enzymatic recycling (Anderson *et al.* 2005). Mice with loss-of-function mutations in the PD-associated gene UCHL1 (encoding a DUB) also have reduced neuronal monoubiquitin (Osaka *et al.* 2003). Furthermore, disruption of the *Ubb* poly-ubiquitin gene in mice causes hypothalamic neurodegeneration through decreased total Ub levels (Ryu *et al.* 2008). Interestingly, compensatory expression from the *Ubc* gene was later shown to be significantly up-regulated in $Ubb^{-/-}$ mice in other brain regions to maintain free Ub and protect neuronal function (Park *et al.* 2012). Together, these data suggest that maintenance of Ub homeostasis (particularly the pool of free Ub) is necessary for neuronal protection.

### Autophagy

Autophagy involves formation of double-membrane cytosolic vesicles known as autophagosomes, which engulf long-lived proteins or particular organelles (e.g., mitochondria) and transport them to lysosomes for degradation (Ravikumar *et al.* 2010). Still controversial is the topic of autophagic cell death (Clarke and Puyal 2012), which certainly occurs in dying neurons subjected to oxidative stress (Higgins *et al.* 2011, 2012), excitotoxicity and acute ischemic-like injuries (Puyal *et al.* 2012). While current evidence indicates the contribution of autophagy to neuronal death is caspase-independent, this needs careful scrutiny in the context of brain pathologies since autophagy can recruit compensatory apoptosis and the two processes are intertwined, sharing common mediators such as Beclin 1 (Higgins *et al.* 2011; Puyal *et al.* 2012).

Much of our understanding of the mechanistic and genetic regulation of autophagy (and the UPS) comes from work in yeast, Drosophila and non-mammalian cells. Indeed, although autophagy was discovered essentially concurrently with lysosomes (in the 1950s), and was initially believed to be a response linked to nutrient and energetic availability and cellular homeostasis, it was not until some 10 years ago that its importance in the brain was recognized. Basal autophagic flux proved hard to detect in healthy neurons because the brain is at least partially protected by tightly controlled

mechanisms that regulate its nutrient and energetic supply (Boland and Nixon 2006). However, the essential role of autophagy in brain has been convincingly demonstrated by evidence such as the accumulation of abnormal organelles and ubiquitinated proteins in the autophagy-deficient Atg7 (autophagy related protein 7) knockout mouse (Komatsu *et al.* 2005). Now extensive data have shown autophagy is essential for neuronal health, maintenance of axonal function and morphology, and that compromised autophagy likely contributes to neurodegenerative conditions where toxic aggregates cause the increased abundance of autophagosomes and autolysosomes (Rubinsztein *et al.* 2005).

Recent genome wide association studies have suggested that compromised autophagic and/or lysosomal mechanisms may underlie (and thus contribute to) the pathology of most neurodegenerative diseases. For example, in frontotemporal degeneration, a clinically heterogeneous syndrome in which the major protein accumulating in dystrophic neurons is tau, two proteins (Rab38 and cathepsin C) involved in lysosomal biology were recently identified in a genome wide association studies (Ferrari *et al.* 2014), however, these are yet to be experimentally confirmed. Nevertheless, these finding do support earlier evidence for decreased function of autophagic processes in frontotemporal dementia (Hu *et al.* 2010; Brady *et al.* 2013). Systems biology approaches have also revealed the pivotal role of autophagy in frontotemporal dementia (Caberlotto and Nguyen 2014).

Autophagy also appears to be mechanistically linked to disease progression in AD (Moreau *et al.* 2014) and PD (Westbroek *et al.* 2011). Misfolded proteins can be degraded by chaperone-mediated autophagy and autophagy activation appears to protect against α-synuclein-induced neurodegeneration (Xilouri *et al.* 2013). This exciting observation represents part of a growing awareness that the autophagy/lysosomal cascade may contain therapeutic targets for the treatment of neurodegenerative diseases (Friedman *et al.* 2015). However, while there is great hope for the clinical management of neurodegenerative diseases via up-regulation of degradative pathways, many unknowns still need to be addressed before these pathways can be therapeutically targeted. These include determining (i) the role(s) of different neuronal populations and glia in degrading aggregated protein, (ii) the cellular 'load' (be it damaged organelles or aggregates) and its relationship with total protein degradation mechanisms, and (iii) the timing of potential treatments relative to overall pathology.

Mitochondrial dysfunction with consequent oxidative stress also occurs in most neurodegenerative diseases (Gibson *et al.* 2010; Higgins *et al.* 2010). Mitophagy (mitochondrial autophagy) can be negatively impacted upon by the presence of protein inclusions (Ashrafi and Schwarz 2013; Baker *et al.* 2014). In this context, collapse of the mitochondrial membrane potential initiates recruitment of two proteins, PTEN-induced kinase 1 and the Ub ligase Parkin, which orchestrate autophagic degradation of the mitochondria. Mutations in both PTEN-induced kinase 1 and Parkin have been associated with familial early-onset PD (Ashrafi and Schwarz 2013). An important component of mitochondrial dynamics is the active transport of mitochondria by microtubules to all morphological compartments of the neuron and, as a result, dysfunction can result in dieback of the neuritic tree during injury or dysregulation of axonal transport (Van Laar and Berman 2013). Components of the UPS have also been implicated in regulating microtubule dynamics (Bheda *et al.* 2010).

## Extracellular proteostasis, thinking outside the cell

Decades of research have led to the current awareness of the importance of the many cellular processes that act to counter inappropriate misfolding and aggregation of intracellular proteins. Like most cells in the body, neurons are bathed in protein-rich extracellular fluids which, relative to the cell cytoplasm, are oxidizing and subject to shear stress as a result of pressure differentials between different body compartments and fluid flows. The extracellular fluid environment is therefore 'stressful' to proteins and over time will result in cumulative damage to protein structure leading potentially to protein misfolding and aggregation. Presumably this is one reason why all secreted proteins in the body are 'turned-over' with different half-lives (to replace 'worn' proteins with new ones before any pathology results) (Wyatt *et al.* 2013). Given this background, it is obvious how serious pathologies can result from the inappropriate misfolding and aggregation of proteins in body fluids (e.g., in AD where extracellular aggregating Aβ may be the primary toxic species causing extensive brain cell loss).

Some recent key discoveries have thrown light on processes that have evolved to protect the body from pathological protein species in extracellular fluids. Several proteins abundant in human blood and present at lower levels in cerebrospinal fluid have been shown to have an ATP-independent chaperone action (like the sHSPs). These extracellular chaperones include clusterin (CLU) (Humphreys *et al.* 1999; Poon *et al.* 2000), haptoglobin (Hp) (Yerbury *et al.* 2005) and alpha-2-macroglobulin (A2M) (French *et al.* 2008; Wyatt *et al.* 2014). The best studied of these is CLU which promiscuously interacts with essentially any misfolding protein to potently inhibit its aggregation. In the case of amorphous aggregation, CLU forms stable, soluble high molecular weight complexes with the misfolded client protein. In a rat model, these complexes are cleared from circulation in the blood on a time scale of a few minutes (Wyatt *et al.* 2011). *In vitro*, high molecular weight CLU-client complexes are internalized by rat hepatocytes into lysosomes and proteolytically degraded. Both Hp and A2M also form stable complexes with misfolded client proteins that are bound by a variety of cell types. It has also been

shown that CLU, Hp and A2M can interact with exposed hydrophobicity on client proteins to neutralize their toxicity (Yerbury *et al.* 2009). Thus, it appears very likely that extracellular chaperones are, in a sense, extracellular counterparts to GRP78 in the endoplasmic reticulum or HSP70 in the cytoplasm, binding to exposed regions of hydrophobicity to inhibit aggregation, keep them soluble, and safeguard against toxicity.

The extracellular chaperones form key elements of an integrated model of extracellular proteostasis in which their ability to maintain misfolded proteins in soluble complexes aids their clearance from body fluids via receptor-mediated endocytosis and subsequent lysosomal degradation (Wyatt *et al.* 2013). In both *in vitro* studies and a rat model, scavenger receptors have been implicated in this clearance although the precise receptor(s) involved have yet to be identified. It appears, therefore, that at least one mechanism to dispose of extracellular misfolded proteins utilizes an intracellular proteolytic system, the lysosome, to carry out end-stage degradation. There are also indications that when extracellular misfolded proteins evade the normal defenses of the extracellular chaperones and form insoluble deposits in the body, extracellular proteolytic mechanisms are triggered. For example, it has been shown that amyloid fibrils formed by Aβ can activate the plasminogen system to generate the very active protease plasmin (Kranenburg *et al.* 2005). It is also interesting to note that the plasmin-$\alpha_2$-anti-plasmin (fibrinolytic) system is activated in systemic amyloidosis (Bouma *et al.* 2007). Furthermore, recent studies indicate that when cells die by acute injury, cross-linked proteins are exposed that can activate plasmin, and that this event is involved in helping clear the proteinaceous cell debris *in vivo* (Samson *et al.* 2009, 2012). Thus, extracellular proteolytic systems, perhaps in concert with professional phagocytes, may aid in the clearance of large insoluble deposits in the body. The details of the mechanisms that operate in this context, however, remain to be fully elucidated. From studies relevant to clinical 'stroke', it is known that plasmin-mediated degradation of fibrin clots can generate cytotoxic protein fragments (Guo *et al.* 2009). We do not yet know whether similar pathological protein fragments might be released from extracellularly degraded misfolded protein deposits, but this knowledge could prove invaluable when, for example, designing a therapy to clear amyloid deposits from the brains of AD patients.

There are some critical gaps in knowledge remaining that must be addressed before we can be confident of understanding at least the major processes that monitor and control extracellular protein misfolding. The importance of these processes comes from the fact that they are very likely to protect us from neurodegenerative (and other serious) diseases, and the aging process itself. Future research is needed to identify: (i) the specific receptor(s) that clear chaperone-misfolded protein complexes from extracellular fluids; (ii) the protease systems that act to help clear extracellular protein deposits, and whether these synergize with extracellular chaperones to safely accomplish this task; (iii) whether it is possible to treat disease pathologies arising from inappropriate extracellular protein misfolding by pharmacologically manipulating the *in vivo* expression levels of extracellular chaperones (or perhaps their activities).

## Control of proteostasis at the organismal level

While reductionist approaches that focus on individual cell types have yielded a wealth of information on proteostasis and how it is regulated in the cell, our broader understanding of the interplay between aging, proteostasis, and neurodegeneration has been greatly advanced by studies using more complex cellular models and model organisms, such as *C. elegans*. One of the main strengths of these studies is their ability to elucidate non-cell autonomous mechanisms involved in proteostasis.

There is a strong link between inflammation and neurodegeneration; inflammatory cells, such as microglia, are activated to phagocytose cellular debris and protein aggregates (Roberts *et al.* 2013) that are generated through neurodegeneration. A new wave of data suggests that inflammation may not only be evoked in order to clear toxic protein aggregates but also to initiate metabolic changes in cells that directly influence longevity (Riera *et al.* 2014; Tatum *et al.* 2015). The coordination of a response to proteotoxic stress in one part of the body can be transmitted via sensory neurons to initiate or up-regulate proteostasis pathways in cells of the periphery. For example, the activation of thermosensory neurons enhances serotonin release, which activates the heat-shock response by up-regulating HSF1 expression (Tatum *et al.* 2015). Using optogenetics, Tatum *et al.* (2015) were able to specifically excite serotonergic neurons in *C. elegans* and show that their activation reduces protein misfolding in peripheral tissue. These data provide evidence of a direct link between thermosensory neuron signaling and longevity. Blocking important components in sensory signaling, such as calcitonin gene related peptide or the thermosensing TRPV1 channel, increases metabolic health and prolongs lifespan in *C. elegans* (Riera *et al.* 2014). Together these data suggest that there is potential for (i) inflammation to directly affect longevity and (ii) for pathogens to activate the proteostasis network and therefore affect longevity even prior to inflammatory signaling. This paradigm-shifting research highlights the possibility that infections lead to activation of sensory neurons that can directly affect longevity.

Whether manipulation of sensory neuron activity affects longevity in more complex organisms is currently unclear. However, investigation of the key players in organismal control of proteostasis in *C. elegans* suggests these processes could be conserved in mammals. Inositol-requiring protein 1-

mediated splicing of the transcription factor X box-binding protein 1 (XBP-1) is a major pathway for non-cell autonomous proteostasis in *C. elegans* with the ability to up-regulate the unfolded protein response in the ER of distal cells (Taylor and Dillin 2013). XBP-1 splicing also controls ER signaling between cell types in mouse models, promoting a proinflammatory phenotype that promotes tumor development (Mahadevan *et al.* 2011) and controlling immune system homeostasis (Osorio *et al.* 2014). A number of neurotransmitters that are involved in the coordination of organismal proteostasis in *C. elegans* have counterparts in mammals that may have similar immune consequences. In terms of sensory neuron signaling, the *C. elegans* equivalent of noradrenaline, octopamine, is involved in organismal control of proteostasis (Sun *et al.* 2011). In mammals noradrenaline is responsible for the 'Fight or Flight' response, conditions of acute stress that depress the immune response. It is likely that similar mechanisms in mammals signal the initiation of proteostasis defence mechanisms, following the signaling of proteotoxic events possibly by neurotransmitters such as serotonin (as described above) and dopamine, which is involved in resistance to infection (Anyanful *et al.* 2009), whereas GABA and acetylcholine activate and suppress HSF-1-mediated protection against protein aggregation (Garcia *et al.* 2007). Future studies developing the findings from *C. elegans* into more complex organisms will be a challenge for the next few years in proteostasis research.

## Innovations leading to advances in proteostasis research

In the last few years there have been significant advances in our understanding of how proteostasis collapse is associated with the pathogenesis of neurodegenerative diseases. This progress can be predominately attributed to two major innovations in this research field; (i) advances in information technology that have nurtured and facilitated a systems biology approach to neurodegenerative diseases and proteostasis, and (ii) the use of induced pluripotent stem cells (iPSCs) that have enabled us to model neurodegenerative diseases in a dish (Ooi *et al.* 2013). These developments signal the birth of a revolution in which ever more complex cell biological networks can be investigated *in silico*, *in vitro,* and *in vivo*. For example, we are now in a position to exploit the ability of iPSCs to model disease onset and progression in specific neuronal subtypes and link this with a systems biology approach to investigate how components of the proteostasis network change during the onset of disease phenotypes in these cells. This information can be used to establish generic changes to the proteostasis network that occur during disease (by comparing across iPSCs from different individuals or across different neuronal subtypes) or specific changes that occur within an individual or within a particular neuronal subtype.

Our ability to monitor pathological events in individual cells in real time and to model multiple cell types in three-dimensions are the logical next steps in the development of the field. In the case of an AD model, this has included the simultaneous formation of amyloid plaques and deposits of insoluble tau, rarely seen in classical two-dimensional cell models (Choi *et al.* 2014). The visualization of cell migration in live animal brains with time-lapse magnetic resonance imaging videos is now also providing three-dimensional positional information at the single cell level (Mori *et al.* 2014). The use of advanced imaging techniques to observe single cell effects in the context of neurodegenerative diseases, such as the propagation of misfolded proteins between individual cells in organisms, would be invaluable. These areas will lead to an improved understanding of the molecular players involved in, and the cellular events leading to, protein aggregation, degradation and clearance, and how these pathways mediate degeneration of human neurons. Such techniques will allow us to, address the susceptibility of specific neuronal subtypes in distinct diseases, a central question in many neurodegenerative diseases.

## Potential proteostasis based therapeutic avenues in neurodegenerative diseases

If exceeding proteostasis capacity is a fundamental underlying cause of neurodegenerative conditions then it makes sense that either reducing the misfolded protein burden or increasing the proteostasis capacity are valid therapeutic strategies. A new drug development mindset is required. For over a century, pharmacological science has been highly successful in drugging fixed structures with small molecules: an effective strategy for selective targeting of channels, pores and enzyme surface pockets and pits that can induce allosteric change in activity. However, targets for misfolded proteins are often large and 'floppy,' with little purchase for small molecules. For effective treatment and diagnosis of protein misfolding diseases, a new paradigm is needed, for example, based on the rational identification of selective antibody targets (epitopes) in the misfolded proteins that are key to the disease process. Selective antibody targeting of misfolded proteins is effective by several mechanisms, including the neutralization of cytotoxicity and inhibition of prion-like propagated misfolding, which has been found to participate in Aβ oligomers/fibrils, tau, α-synuclein, and SOD1 operant in AD, PD, ALS, and the tauopathies (reviewed in Guest *et al.* 2011). Moreover, in some conditions, such as infectious prion disease and AD, the specific targeting of misfolded propagating proteins is a 'needle in a haystack' scenario in which the misfolded species is present in a thousand to a million fold lower concentration than the natively folded species – a situation dubbed 'target distraction'. In the context of the prion-like spread of misfolded protein antibody neutralization of released material may block the cell-to-cell spread and slow the progression of disease (Grad *et al.* 2014). Finally,

specific immunotherapy for misfolded proteins would spare normal isoforms from autoimmune recognition, with attendant risks and narrowed therapeutic index.

An alternative strategy is to target the machinery whose capacity is theoretically exceeded, for example, the activity of chaperones and the degradative pathways. As the HSR pathway is responsible for a dramatic up-regulation of chaperone expression under conditions of cellular stress it has been shown to be a promising therapeutic target (Akerfelt *et al.* 2010). For example, inducing the HSR by over-expression of HSF1 can ameliorate disease phenotypes and mitigate neurotoxicity in various models of neurodegenerative diseases (Fujimoto *et al.* 2005; Lin *et al.* 2013; Pierce *et al.* 2013). A variety of HSR-inducing compounds that activate HSF1 are currently under investigation (e.g., geranylgeranylacetaone, celastrol, arimoclomol, withaferin A, and 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) (Kieran *et al.* 2004; Niikura *et al.* 2006; Hoogstra-Berends *et al.* 2012; Khan *et al.* 2012; Kalmar *et al.* 2014; Sharma *et al.* 2015a,b). Moreover, the majority of studies using animal models of neurodegenerative diseases have reported a HSR-mediated reduction in the load of toxic protein and enhanced neuroprotection suggesting that this is a viable therapeutic approach. With regards to targeting protein degradation pathways, it may be beneficial to activate the proteasome as over-expression of the proteasome activator subunit PA28γ enhances survival of neurons in a HD cell culture model (Seo *et al.* 2007). In addition, small molecule stimulation of autophagy enhances cell survival in neuronal ALS models (TDP-43 aggregation) (Barmada *et al.* 2014) suggesting that targeting autophagy may be a reasonable therapeutic target (Fleming *et al.* 2011). It is likely that any therapeutic strategy that increases the proteostasis capacity of organisms will be of benefit to neurodegenerative disease progression.

## Future perspectives

While there is a strong association between protein aggregation, inclusion formation and neuronal dysfunction and death, the mechanistic relationship between each of them remains one of the biggest questions in the field. What is clear is that proteostasis collapse is a critical part of this process that can be caused by the burden of pathogenically misfolded proteins or peptides. In contrast, as was the focus of this review, proteostasis imbalance can be caused by dysfunction in pathways that maintain proteostasis such as molecular chaperones and protein degradation and this may contribute to both protein aggregation and neurodegeneration. Regardless, increasing the capacity of the proteostasis network will be an important area of future research.

It remains a central question as to why certain neurons are especially vulnerable to aggregating proteins in distinct neurodegenerative diseases (e.g., motor neurons of the motor cortex and spinal cord in ALS; dopaminergic neurons of the substantia nigra in PD; cholinergic neurons of the entorhinal cortex and hippocampus in AD; medium spiny neurons of the striatum in HD). Identifying deficiencies in some cell types, and not others, will be a significant advance in our understanding of the vulnerabilities of neurons in the context of these diseases. Differing expression levels of components of the proteostasis network, as identified in proteomic maps of the brain (Sharma *et al.* 2015a,b), could underlie neuronal subtype-specific responses in disease. This should also be considered in the context of the level of expression of the disease specific proteins, such as α-synuclein, SOD1 and huntingtin, in the neuronal subtypes. In essence the goal of future neurodegenerative disease research will be to identify deficiencies in cell- or neuron-specific adaptive responses and try to boost them in susceptible cells.

With an aging population and the corresponding predicted rise in people suffering from neurodegenerative diseases, understanding the dynamics of the proteostasis network in the nervous system is an important task for the research community. Advances in high-throughput single cell analyses will drive our understanding of how and why neurons are different to other cell types, and what makes them so susceptible to defects in the proteostasis network. Unbiased approaches to identify key players in this system will be important for formulating novel therapeutic strategies. Reductionist approaches have identified some elements of proteostasis, but to gain a complete view there is a need to move towards capturing the system as a whole and to consider the impact of different cells and tissue types, working together. A more holistic approach to understanding proteostasis in the context of neurodegenerative diseases will therefore be important for future research.

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