MOLECULAR CHAPERONES AND NEURODEGENERATION

EDITED BY: Cintia Roodveldt, Tiago F. Outeiro and Janice E. Braun
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MOLECULAR CHAPERONES AND NEURODEGENERATION

Topic Editors:
Cintia Roodveldt, Andalusian Center for Molecular Biology and Regenerative Medicine-CABIMER, Spain
Tiago F. Outeiro, University Medical Center Goettingen, Germany
Janice E. Braun, University of Calgary, Canada

Molecular chaperones or heat-shock proteins (HSPs) play essential roles in safeguarding structural stability, preventing misfolding and aggregation of proteins, and maintaining the proteome functionality in the cell. For over two decades until the present time, new functions have been discovered and several molecular mechanisms have been elucidated for many chaperones, while the field is being continuously challenged by new open questions. Probably as a consequence of the increasing research on the molecular bases of neurodegenerative diseases, and the realisation that many such disorders are linked to protein misfolding processes, unleashing the roles and mechanisms of chaperones in the context of neurodegeneration has become a prime scientific goal. This e-book contains a diversity of reviews, perspective and original research articles highlighting the importance and potential of this emerging subject.

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EDITORIAL
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Editorial: Molecular Chaperones and Neurodegeneration

Cintia Roodveldt[1]*, *Tiago F. Outeiro*[2] *and Janice E. A. Braun*[3]

[1] *Andalusian Center for Molecular Biology and Regenerative Medicine (CABIMER)-Spanish National Research Council (CSIC), Universidad Pablo de Olavide, University of Seville, Seville, Spain,* [2] *Department of Experimental Neurodegeneration, Center for Nanoscale Microscopy and Molecular Physiology of the Brain, Center for Biostructural Imaging of Neurodegeneration, University Medical Center Göttingen, Göttingen, Germany,* [3] *Hotchkiss Brain Institute, Department of Biochemistry and Molecular Biology, Cumming School of Medicine, University of Calgary, Calgary, AB, Canada*

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Editorial on the Research Topic

Molecular Chaperones and Neurodegeneration

Molecular chaperones, including heat-shock proteins (HSPs), or stress proteins, are highly conserved proteins that play a critical role in the regulation of cellular protein homeostasis (proteostasis). Proteostasis is essential for the maintenance of the functionality of the proteome and, ultimately, of cells. Disruption of proteostasis leads to the accumulation of aberrantly folded proteins that typically lose their function. The accumulation of misfolded and aggregated proteins, due to genetic mutations, posttranslational modifications, or due to an age-related decline in cellular functions, can be also cytotoxic and has been linked to the pathogenesis of various degenerative diseases including those affecting the nervous system, such as Alzheimer's (AD), Parkinson's (PD) and Huntington's diseases (HD), or amyotrophic lateral sclerosis (ALS).

In addition to essential roles in *de novo* protein folding and the refolding of misfolded proteins, molecular chaperones are functionally diverse and participate in a myriad of cellular processes. These functions include preventing or resolving protein aggregation, and regulating proteostasis through fundamental processes such as the unfolded protein response (UPR), the heat shock response (HSR), chaperone-mediated autophagy (CMA), and the ubiquitin-proteasome system (UPS). Chaperones are important components of multiple cellular networks, as they form functional complexes with each other, with numerous co-chaperones regulating their function, and with hundreds of other cellular proteins. Therefore, they promote the crosstalk between various signaling pathways and regulate transcriptional networks. Finally, certain stress proteins display diverse roles in immunity. Given their ubiquitous cellular roles, the potentially common mechanisms of action that may apply, and the widespread consequences of their dysfunction, there is great interest in understanding how molecular chaperones function and how they may be manipulated to prevent or resolve protein aggregation linked to degenerative diseases, and particularly, in neurodegenerative disorders.

In this special issue, we have gathered 14 articles covering novel and significant aspects about the connection between chaperones and neurodegeneration, providing a series of updated and insightful views of the mechanisms and functions of a wide variety of molecular chaperones in the context of health and disease. Furthermore, this compilation offers a comprehensive overview of the most recent findings, advances, and implications as putative targets for therapeutic intervention. More specifically, we have arranged this special issue into three broad subjects, as follows: (A) General mechanisms of molecular chaperones in the maintenance of cellular proteostasis in health and disease; (B) Heat-shock proteins in aging, amyloid disease and cancer; (C) Functions and mechanisms of molecular chaperones within the nervous system.

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Edited by:
Barry Boland,
University College Cork, Ireland

Reviewed by:
Rafael Linden,
Federal University of Rio de Janeiro, Brazil

***Correspondence:**
Cintia Roodveldt
cintia.roodveldt@cabimer.es

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A. GENERAL MECHANISMS OF MOLECULAR CHAPERONES IN THE MAINTENANCE OF CELLULAR PROTEOSTASIS IN HEALTH AND DISEASE

Ciechanover and Kwon provide an updated general overview of the protein quality control mechanisms in the cell, with a focus on HSP functions in refolding and degradation of terminally misfolded proteins. Their review carefully covers the main protein degradation mechanisms, including the UPS, macroautophagy, and CMA. They also analyse our current understanding of the protective roles of chaperones and recent therapeutic studies in various neurodegenerative diseases. Lackie et al. focus on the HSP70/HSP90 HSPs, their structures, molecular mechanisms, and their cellular functions in combination with other chaperones, co-chaperones, and other protein partners, including the FKBP family members, the PPIase Cyp40, p23, and CHIP. Special attention is paid to the HSP90 co-chaperone Sti/Hop and its multiple roles in HSP90/HSP70-mediated functions in modulating protein misfolding, followed by an update on the main players and mechanisms of the chaperone machinery in the context of the synucleinopathies, HD, ALS, prion disease, and AD. On the other hand, Dubnikov and Cohen provide an insightful review on the emerging links between failure in early protein folding and maturation of secreted proteins and the development of neurodegerative disorders. They carefully analyse the early maturation events and the quality control mechanisms for terminally misfolded proteins within the ER, including ER-associated degradation (ERAD), the unfolded protein response (UPR), the formation of aggresomes, and other types of protein deposition sites, and their emerging association with neurodegenerative disease. Finally, Jackrel and Shorter describe our current knowledge and recent discoveries of molecular chaperones as protein-remodeling factors, to prevent or even reverse, protein aggregation processes in metazoans. In particular, they give a detailed overview of the recently characterized HSP110/HSP70/HSP40 disaggregase system, the NMNAT2 NAD-synthesizing enzyme, and the HtrA1 serine protease, from recent studies with misfolding disease models, while evaluating the therapeutic potential of yeast Hsp104 disaggregase for treating human misfolding diseases.

B. HEAT-SHOCK PROTEINS IN THE CONTEXT OF AGING, AMYLOID DISEASE AND CANCER

The review by Stroo et al. provides a comprehensive account of our current understanding of amyloid aggregates formation and its cellular regulators in health, disease and aging, including chaperone "disaggregases," the role of chaperones in the main protein degradation pathways (UPS, macroautophagy, CMA), UPR, and protein compartmentalization. It also focuses on key aspects linking amyloidogenesis and neurodegeneration, such as amyloid formation modulators, the dysregulation of protein homeostasis processes, and the mechanisms of amyloid toxicity in neurodegenerative disease. The mini-review by Kuiper et al. focuses on polyglutamine (polyQ) protein aggregation associated to certain neurodegenerative disorders such as HD, as well as on the various factors affecting the aggregation process, particularly binding partners and molecular chaperones, e.g., CHIP and members of the DnaJ family. In addition to analyzing the multifactorial and complex nature of the process leading to disease initiation, the authors discuss the possibility that proper assessment of the different factors could help predict the age of onset of disease. Buxbaum and Johansson discuss the puzzling anti-amyloid activity of amyloidogenic transthyretin (TTR) protein and the BRICHOS domain on the AD-linked Aβ peptide, with a structural and mechanistic focus. The review also analyses the emerging links between TTR and BRICHOS-containing proteins and disease through amyloid formation, and also discusses potential therapeutic avenues for these amyloid precursors based on their anti-Aβ oligomerization properties. The review by Casas is a comprehensive and updated characterization of the ER stress-related chaperone GRP78 (also known as Bip), its multiple intracellular locations, interacting partners, and newly discovered functions including its key participation in ERAD, macroautophagy and the UPR. Furthermore, the author also elaborates a comparative analysis between the roles of Bip/GRP78 in tumor cytoprotection and neuroprotection in the context of neurodegenerative disease and aging. Finally, Calderwood and Murshid analyse the exacerbation or decline of HSP expression levels in cancer and AD, in the context of disease pathogenesis. In addition, their review provides an updated overview of the regulation mechanisms of the heat-shock response by HSF1 transcription factor, the key effector of HSP expression. The authors further discuss the emerging evidence of the HSF1-based dysregulation that might contribute to explain the intriguing negative epidemiologic correlation observed between cancer and AD.

C. FUNCTIONS AND MECHANISMS OF MOLECULAR CHAPERONES WITHIN THE NERVOUS SYSTEM

López-Ortega et al. report that long-term moderate reduction of the essential co-chaperone, cysteine string protein (CSPα/DnaJC5), reduces neuromusclar function. CSPα is essential for synapse maintenance and severe functional and structural changes occur in its absence. Through careful and detailed analysis, they demonstrate that 1 year old CSPα heterozygous mice, previously considered to be phenotypically normal, have impairment in neuromuscular function. Their findings imply that challenges lie ahead in identifying reduced levels of chaperones (like CSPα) that lead to mild synaptic impairment in patients. Gorenberg and Chandra provide an insightful review of four main players in synaptic proteostasis: CSPα/DnaJC5, auxilin/DnaJC6, RME-8/DnaJC13, and HSP110. Their review covers the unique features of proteostasis at the synapse highlighting both the current knowledge and current questions. Special attention is paid to the HSP110 disaggregase system and mutations in synaptic chaperones

that cause human neurodegenerative diseases. On the other hand, Deane and Brown provide new information on HSPA6, a member of the HSPA chaperone family that is induced in neurons following heat shock. They carefully document the unique induction and localization of neural HSPA6 in comparison to two other HSPA family members, HSPA1A and HSP8. Given this differential targeting, they highlight the possible role of HSPA6 in human neurodegenerative disorders, emphasizing that while HSPA6 is present in the human genome it is absent from current mouse and rat models of neurodegenerative disease thereby creating a gap in our current understanding of HSPA6 function. Jung et al. provide an insightful review on the links between neurodegeneration and endoplasmic reticulum lipidostasis, proteostasis and calcium homeostasis. Special attention is paid to the role of calnexin, PDDIA3, BiP/GRP78 and cholesterol in the pathological sequence of events underlying neurodegeneration. Finally, Ousman et al. review the current knowledge of molecular chaperones following nerve injury, focusing on neuron survival, myelination, neuropathic pain, axon regeneration, and inflammation. They address how changes in molecular chaperone expression play an active role in injury or repair processes and highlight the therapeutic challenges involved in harnessing the beneficial properties while reducing the injurious functions of chaperones to enhance CNS and PNS recovery.

AUTHOR CONTRIBUTIONS

CR was invited to prepare this Research Topic and invited TO and JB to be co-editors in it. We all prepared and discussed a list of guest authors, invited them, revised their manuscripts, and handled revisions performed by peer-reviewers.

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REVIEW
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Protein Quality Control by Molecular Chaperones in Neurodegeneration

Aaron Ciechanover[1,2] *and Yong Tae Kwon*[1,3]*

[1] Department of Biomedical Sciences, Protein Metabolism Medical Research Center, College of Medicine, Seoul National University, Seoul, South Korea, [2] Technion Integrated Cancer Center, Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Haifa, Israel, [3] Ischemic/Hypoxic Disease Institute, College of Medicine, Seoul National University, Seoul, South Korea

Protein homeostasis (proteostasis) requires the timely degradation of misfolded proteins and their aggregates by protein quality control (PQC), of which molecular chaperones are an essential component. Compared with other cell types, PQC in neurons is particularly challenging because they have a unique cellular structure with long extensions. Making it worse, neurons are postmitotic, i.e., cannot dilute toxic substances by division, and, thus, are highly sensitive to misfolded proteins, especially as they age. Failure in PQC is often associated with neurodegenerative diseases, such as Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), and prion disease. In fact, many neurodegenerative diseases are considered to be protein misfolding disorders. To prevent the accumulation of disease-causing aggregates, neurons utilize a repertoire of chaperones that recognize misfolded proteins through exposed hydrophobic surfaces and assist their refolding. If such an effort fails, chaperones can facilitate the degradation of terminally misfolded proteins through either the ubiquitin (Ub)-proteasome system (UPS) or the autophagy-lysosome system (hereafter autophagy). If soluble, the substrates associated with chaperones, such as Hsp70, are ubiquitinated by Ub ligases and degraded through the proteasome complex. Some misfolded proteins carrying the KFERQ motif are recognized by the chaperone Hsc70 and delivered to the lysosomal lumen through a process called, chaperone-mediated autophagy (CMA). Aggregation-prone misfolded proteins that remain unprocessed are directed to macroautophagy in which cargoes are collected by adaptors, such as p62/SQSTM-1/Sequestosome-1, and delivered to the autophagosome for lysosomal degradation. The aggregates that have survived all these refolding/degradative processes can still be directly dissolved, i.e., disaggregated by chaperones. Studies have shown that molecular chaperones alleviate the pathogenic symptoms by neurodegeneration-causing protein aggregates. Chaperone-inducing drugs and anti-aggregation drugs are actively exploited for beneficial effects on symptoms of disease. Here, we discuss how chaperones protect misfolded proteins from aggregation and mediate the degradation of terminally misfolded proteins in collaboration with cellular degradative machinery. The topics also include therapeutic approaches to improve the expression and turnover of molecular chaperones and to develop anti-aggregation drugs.

Keywords: proteolysis, protein aggregation, ubiquitin-proteasome system, autophagy-lysosome system, chaperon-mediated autophagy, macroautophagy, protein quality control

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Edited by:
Cintia Roodveldt, Centro Andaluz de Biología Molecular y Medicina Regenerativa, Spain

Reviewed by:
Harm Kampinga, University Medical Center Groningen, Netherlands
Fred van Leeuwen, Maastricht University, Netherlands

***Correspondence:**
Yong Tae Kwon
yok5@snu.ac.kr

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INTRODUCTION

Proteins may lose their folding when cells are exposed to stresses, such as oxidative stress, heat, and toxic chemicals. Misfolded proteins and their aggregates grow into intracellular or extracellular amyloid plaques or neurofibrillary tangles (Taylor et al., 2002). Eukaryotic cells operate the PQC system to remove these cytotoxic agents in a timely fashion. The excessive formation of protein aggregates and their fibrillar structures are universally observed in at least 30 different human diseases (Taylor et al., 2002; Broersen et al., 2010). These protein misfolding disorders include various neurodegenerative diseases, such as Alzheimer' disease (AD), Parkinson' disease (PD), Huntington disease' (HD), transmissible spongiform encephalopathies (TSE), and amyotrophic lateral sclerosis (ALS) (Moreno-Gonzalez and Soto, 2011; Doyle et al., 2013; Hetz and Mollereau, 2014; Valastyan and Lindquist, 2014).

One essential component of PQC is molecular chaperones that enhance the refolding of misfolded proteins and, thus, counteract their aggregation (Hartl et al., 2011; Kim et al., 2013). Molecular chaperones constitute up to 10% of the proteome and play important functions in proteostasis under normal conditions as well as during cellular stress responses (Kastle and Grune, 2012). The majority of molecular chaperones are called heat-shock proteins (HSPs) because they are induced by various stresses such as heat shock, oxidative stress, toxic chemical, and inflammation (Garrido et al., 2001). HSPs are divided into several subgroups based on their sizes, such as Hsp70, Hsp90, Hsp60, Hsp40 (DnaJ), and small HSPs. These molecular chaperones can assist the refolding of misfolded proteins through three distinct action modes. *First*, most chaperones such as Hsp70 can hold the clients in an unfolded state until spontaneous fold is achieved (Rudiger et al., 1997; Hartl et al., 2011; Kastle and Grune, 2012). *Second*, some molecular chaperons such as Hsp70 and Hsp60s can use ATP to unfold stable misfolded proteins and convert them into natively refoldable species (Ranford et al., 2000; Itoh et al., 2002; Tutar and Tutar, 2010). *Third*, some chaperones, such as yeast Hsp104 and human Hsp70 in complex with Hsp40 and Hsp110, can act as "disaggregases" because they use the energy of ATP hydrolysis to forcefully unfold and solubilize preformed aggregates into natively refolded proteins (Mosser et al., 2004; Shorter and Lindquist, 2004; Arimon et al., 2008; Lo Bianco et al., 2008; DeSantis et al., 2012). Despite distinct action modes, they share general properties to recognize and bind the hydrophobic sequences which are not normally exposed in the native folding (Buchner, 1996). Their binding to and dissociation from clients can be driven by adenosine-5′-triphosphate (ATP) hydrolysis. The ATPase and chaperone activity are typically regulated through their cooperation with cochaperones. In addition to ATP-dependent chaperones, neurons express ATP-independent chaperons that bind misfolded proteins and promote refolding (D'Andrea and Regan, 2003). These chaperones typically form a coordinated network with cochaperones and the machinery in proteolytic pathways.

While the primary function of molecular chaperones is to assist misfolded or unfolded proteins to regain or acquire the normal folding, they can facilitate the degradation of terminally misfolded proteins in collaboration with proteolytic machinery (Hoffmann et al., 2004; Ellis, 2006, 2007; Ellis and Minton, 2006; Pauwels et al., 2007). Eukaryotic cells operate two major proteolytic systems, the UPS and autophagy. In principle, terminally misfolded proteins are ubiquitinated by E3 Ub ligases and processively degraded by the proteasome. If the substrates are prone to aggregation or escape the surveillance of the UPS, however, they are redirected to macroautophagy in which cargoes are separated in the double membrane structure, called the autophagosome, and degraded by lysosomal hydrolases (Cha-Molstad et al., 2015). Some misfolded proteins carrying the KFERQ pentapeptide sequence can be sorted out by molecular chaperones and directly delivered to the lysosome through chaperone-mediated autophagy (CMA) (Chiang et al., 1989; Dice, 1990; Cuervo et al., 1997).

The UPS is an intracellular proteolytic system that mediates the degradation of more than 80% of normal and abnormal intracellular proteins (Wang and Maldonado, 2006). The importance of molecular chaperones in the UPS was initially proposed and demonstrated by Ciechanover and colleagues who showed that the molecular chaperone Hsc70 is required for Ub-dependent degradation of several substrates (Ciechanover et al., 1995; Bercovich et al., 1997). The UPS involves a cascade of E1, E2, and E3 enzymes whose cooperative activities mediate the conjugation of Ub to target proteins (Pickart, 2001). In this cascade, Ub with a size of 76 residues is activated by the Ub activating enzyme E1 and transferred to the Ub conjugating enzyme E2. The Ub moiety carried by E2 is conjugated to substrates, which requires the ubiquitination activity of the Ub ligase E3. In PQC, most E3s cannot recognize misfolded proteins and rather depend on molecular chaperones for substrate recognition. Ubiquitinated substrates are degraded by the proteasome into short peptides, typically with sizes of 8–12 amino acids. These peptides are displayed on the cell surface for immunosurveillance (Kloetzel and Ossendorp, 2004) or degraded into free amino acids by aminopeptidases. The UPS plays a pivotal role in proteostasis during neurodegeneration and prevents protein misfolding and aggregation (Morawe et al., 2012). In addition to PQC, the UPS regulates a variety of biological processes, including cell cycle, transcription, DNA repair, and apoptosis (Eldridge and O'Brien, 2010; Xie, 2010).

Autophagy is a process by which cytosolic materials are degraded by the lysosome. Depending on the mechanism of cargo delivery to the lysosome, autophagy can be divided into three pathways: microautophagy, CMA, and macroautophagy.

Abbreviations: 17-AAG, 17-N-allylamino-17-demethoxygeldanamycin; Aβ, amyloid-β; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; BBB, blood brain barrier; C-domain, C-terminal dimerization domain; CHIP, C-terminus of Hsc70-interacting protein; CMA, chaperone-mediated autophagy; DUBs, deubiquitination enzymes; ERAD, ER-associated degradation; HD, Huntington's disease; Hsc70, heat shock cognate 70; HSF1, heat-shock factor 1; HSP, heat-shock protein; HSR, heat-shock response; M-domain, mid-domain; mHTT, mutant huntingtin; N-domain, N-terminal ATP-binding domain; NEF, nucleotide exchange factor; PD, Parkinson's disease; PDI, protein disulfide isomerase; PQC, protein quality control; RING, really interesting new gene; RIP-1, receptor interacting protein 1; SBD, substrate binding domain; TDP-43, transactive response DNA binding protein; TRiC, TCP-1 Ring Complex; TSE, transmissible spongiform encephalopathies; Ub, ubiquitin; UBL, Ub-like; UCHL5, ubiquitin C-terminal hydrolase L5; UPS, Ub-proteasome system; VAPB, vesicle associated protein B.

In macroautophagy, terminally misfolded proteins in complex with molecular chaperones are collected by autophagy adaptors, such as p62 and NBR1. Cargo-loaded p62 undergoes self-polymerization and are deposited to the autophagosome through the interaction of p62 with LC3 (Lamark et al., 2009; Stolz et al., 2014). The autophagosome is fused with the lysosome to form the autolysosome wherein cargoes along with p62 are degraded by lysosomal hydrolases. Virtually all the misfolded proteins including those prone to aggregation in neurodegenerative diseases can be degraded by macroautophagy. In contrast to macroautophagy, CMA targets a subset of misfolded cytosolic proteins, especially those containing the KFERQ pentapeptide sequence (Fuertes et al., 2003; Massey et al., 2006; Kaushik and Cuervo, 2012). The substrates of the CMA are recognized by the molecular chaperone Hsc70 belonging to the Hsp70 family (Chiang et al., 1989). The cargo-Hsc70 complex is translocated into the lysosomal lumen and degraded by lysosomal hydrolases (Cuervo and Dice, 1996). Overall, lysosomal proteolysis through macroautophagy and CMA plays an important role in the removal of misfolded proteins that cannot be readily degraded by the UPS.

Misfolded proteins that survive the attempts of molecular chaperones to refold or degrade eventually form aggregates. As the last defense mechanism of PQC, molecular chaperons can directly resolve, i.e., disaggregate the already formed aggregates (Parsell et al., 1994; Mogk et al., 1999; Doyle et al., 2013). The disaggregation activity has been characterized in yeasts and mammals (Weibezahn et al., 2005; Hodson et al., 2012; Winkler et al., 2012). In yeasts, Hsp104 in collaboration with Hsp70, Hsp40, Hsp110, and sHSPs can directly disaggregate and reactivate proteins deposited in high order aggregates (Shorter, 2011; Torrente and Shorter, 2013). In mammals, Hsp110, Hsp105, Hsp100, and Hsp70/Hsp40 have been implicated in disaggregation (Lindquist and Kim, 1996; Glover and Lindquist, 1998; Doyle and Wickner, 2009). Through these multi-step defense processes, molecular chaperones play a key role in proteostasis.

Recent studies using mouse models suggest that molecular chaperones play a protective role in the pathogenesis of neurodegenerative disorders (Wyatt et al., 2012; Carman et al., 2013; Witt, 2013). By using disease models, HSPs have been shown to inhibit the aggregation of aggregation-prone proteins, such as Aβ, tau, HTT, and α-synuclein, and facilitate their degradation by the UPS or autophagy (Wyttenbach, 2004). As such, small molecule compounds that can modulate HSPs and proteolytic machinery are emerging as a means to treat neurodegenerative diseases. Below, we discuss the current understanding on the functions of HSPs in neurodegenerative diseases, including the recent results obtained from animal models of neurodegeneration.

REFOLDING OF MISFOLDED PROTEINS BY MOLECULAR CHAPERONES

Neurons express various molecular chaperones which forms a complicated network of PQC to prevent aggregation. Their primary function is to assist the folding and assembly of newly synthesized polypeptides and the refolding of misfolded or damaged proteins. Depending on their sizes and action modes, molecular chaperones can be divided into several classes based on their sizes, such as Hsp70, Hsp90, Hsp60, Hsp40 (DnaJ), and small HSPs. Although the majority of molecular chaperones share similarity in action modes, such as substrate recognition and ATP hydrolysis-driven substrate binding, they are also different in substrate specificity, localization, and mechanistic details.

The Hsp70 Family

The cytosolic chaperone Hsp70 is evolutionarily conserved and one of the most abundant chaperones. The homologs of Hsp70 are found in various subcellular compartments, including heat shock cognate 70 (Hsc70) in the cytosol and BiP/GRP78 in the ER. Hsp70 shows a broad range of activities in folding newly synthesized polypeptides, refolding misfolded proteins, the degradation of terminally misfolded proteins, and directly resolving already formed aggregates (Kastle and Grune, 2012). They commonly recognize diverse misfolded proteins through the interaction with a four to five residue stretch of hydrophobic amino acids exposed on the surface (Rudiger et al., 1997). The hydrophobic signatures occur on average every 30–40 residues in most misfolded proteins. Central to the chaperone activity of Hsp70 proteins is the transition between open and closed conformations of their substrate binding domain (SBD). In the ATP-bound open conformation, the SBD has low affinity to the client (Hartl et al., 2011). Once ATP hydrolysis is induced by cochaperones, Hsp70 acquires high affinity to the clients. The resulting ADP-bound form of Hsp70 facilitates the client' refolding by holding them in an unfolded state until spontaneous fold is achieved. The client that achieved the correct folding no longer has the exposed hydrophobic patches and, thus, is released from Hsp70. Extensive studies have shown that Hsp70 directly binds various pathogenic misfolded proteins in neurodegenerative diseases and facilitate their refolding. Such substrates of Hsp70 proteins include mutant huntingtin (mHTT) in HD and other polyQ diseases, α-synuclein in PD, amyloid-β (Aβ) and hyperphosphorylated tau in AD, and mutant SOD1 in ALS (Choo et al., 2004; Dedmon et al., 2005; Liu et al., 2005; Evans et al., 2006; Dompierre et al., 2007; Luk et al., 2008).

The Hsp40 (DnaJ) Family

The Hsp40 proteins, also called J-proteins, form a large cochaperone family composed of 49 members (Odunuga et al., 2003). Amongst these, DnaJB6 and DnaJB8 are mainly expressed in neurons and can suppress polyglutamine aggregation and toxicity (Cheetham et al., 1992; Hageman et al., 2010). Although these cochaperones have the activity to bind and counteract protein aggregates or refold them, they also can modulate the ATP hydrolysis of Hsp70. The 70-residue J domain of Hsp40 binds misfolded proteins and interacts with the ATPase domain of Hsp70, which induces the ATP hydrolysis of Hsp70. ATP hydrolysis, in turn, brings the Hsp40-bound substrate close to the SBD of Hsp70 and increases Hsp70 affinity to the substrate, leading to Hsp40 release from the substrate and Hsp70 (Summers et al., 2009). As a consequence of this allosteric conformational

change, the substrate is transferred from Hsp40 to Hsp70. Besides the conserved J domain, Hsp40 proteins carry diverse domains that mediate specific biological processes, such as intracellular localizations and client binding for proteolysis (Cheetham and Caplan, 1998; Kampinga and Craig, 2010). In neurodegenerative disease, Hsp40 proteins can act as cochaperones for Hsp70 proteins to assist the refolding of soluble misfolded proteins (Choo et al., 2004; Dedmon et al., 2005; Liu et al., 2005; Evans et al., 2006; Dompierre et al., 2007; Luk et al., 2008).

The Hsp90 Family

The ATP-dependent chaperone Hsp90, which forms a dimer, is universally present in various cellular compartments, such as the cytosol, nucleus, ER, and mitochondria (Lindquist, 2009). Hsp90 is constitutively expressed in normal conditions, accounting for 1–2% of cellular proteins, and its level can increase to 4–6% if cells are exposed to stresses (Picard, 2002; Whitesell and Lindquist, 2005; Taipale et al., 2010; Finka and Goloubinoff, 2013). The activity of Hsp90 can be regulated by the HSR (heat-shock response) regulator HSF1 (heat-shock factor 1) (McLean et al., 2004; Putcha et al., 2010). Human neurons have a stress-inducible Hsp90α (Hsp90AA1) and a constitutively expressed Hsp90β (Hsp90AB1) that share 86% identity in protein sequence (Ammirante et al., 2008). These Hsp90 proteins bind a variety of clients and hold their folding, including kinases, nuclear receptors, transcription factors and cell surface receptors (Kastle and Grune, 2012). Remarkably, Hsp90 is thought to interact with approximately 2,000 proteins (Garnier et al., 2002), which accounts for up to 10% of total cellular proteins (Ratzke et al., 2010). Structural studies have shown that Hsp90 is composed of an N-terminal ATP-binding domain (N-domain), a mid-domain that binds the substrate (M-domain), and a C-terminal dimerization domain (C-domain) (Picard, 2002; Whitesell and Lindquist, 2005; Taipale et al., 2010; Finka and Goloubinoff, 2013). The substrate binding-release cycle of Hsp90 is regulated by ATP hydrolysis, which induces a large conformational transition between an open vs. closed form. In a free form, Hsp90 is in an open V-shaped conformation and, thus, binds clients. The ATP binding to the N-domain of client-loaded Hsp90 induces a conformational transition (Pearl and Prodromou, 2006). This results in a closed conformation where the N-domains of two Hsp90 molecules dimerize with each other. Following ATP hydrolysis, the substrate is released, and Hsp90 returns to an open conformation. The conformational transition of Hsp90 is regulated by various cochaperones, such as Hop, p23/Sba1, and Cdc37 (Picard, 2002; Whitesell and Lindquist, 2005; Taipale et al., 2010; Finka and Goloubinoff, 2013). Overall, the ability of Hsp90 to support the folding/refolding and stability of proteins is a double edge blade in neurodegeneration because it can also favor the accumulation of toxic protein aggregates (Schulte and Neckers, 1998; Boland et al., 2008; Eskelinen and Saftig, 2009; Chouraki and Seshadri, 2014).

The Hsp60 Family

Hsp60, also called chaperonins, is a 60 kDa mitochondrial chaperone (Ranford et al., 2000; Itoh et al., 2002; Tutar and Tutar, 2010). GroEL, a well characterized bacterial chaperone, also belongs to this class. Although the primary location of Hsp60 is mitochondria, it can migrate to the cytosol under certain cellular stresses (Ranford et al., 2000; Itoh et al., 2002; Tutar and Tutar, 2010). Hsp60 forms a double ring complex, in which each rich is composed of seven subunits. Clients are fed into the central cavity of the double ring complex, in which their exposed hydrophobic residues are sequestered during refolding process (Ranford et al., 2000; Tutar and Tutar, 2010). The folding process by Hsp60 is modulated by a lid, which are formed by cochaperones such as Hsp10 in mitochondria. Following ATP hydrolysis, the unfolded client is released through the opening of the Hsp10 lid, now with a native folding (Ranford et al., 2000; Tutar and Tutar, 2010). Hsp60 works together with Hsp70 for protein folding of unfolded proteins. Neurons contain another type of chaperonins in the cytosol, which do not depend on cochaperones. They form a homotypic or heterotypic double ring complex, each of which is composed of eight subunits. The members of this group include the TCP-1 Ring Complex (TRiC), alternatively called TCP1 complex (CCT) (Lopez et al., 2015). Although, studies have shown that Hsp60 interacts with mutant α-synuclein in PD (Irizarry et al., 1998; Spillantini et al., 1998), the physiological importance of Hsp60 proteins in the refolding of pathogenic misfolded proteins in neurodegeneration remains poorly characterized.

The Small HSP Family

Different from other types of HSPs, small HSPs are ATP independent. To date, 10 small HSPs with sizes ranging from 12 to 42 kDa are known in humans. In mouse brain, five small HSPs are prominently expressed (Quraishe et al., 2008). Amongst these, the neuronal expression of Hsp27 and αB crystallin is selectively induced under stresses (Quraishe et al., 2008). Members of this family are characterized by a 100-residue α-crystallin domain flanked by variable N-terminal and C-terminal extensions. These extensions are responsible for substrate recognition and mediates the formation of oligomers. As holding factors, small HSPs bind to unfolded or misfolded proteins and prevent their aggregation until the clients are delivered to other chaperones, such as Hsp70 and Hsp40 system (Carra et al., 2012). Amongst these, Hsp27 is the most abundant and well characterized. Their expression is selectively induces by various stresses that perturb proteostasis (Sarto et al., 2000; Sun and MacRae, 2005).

DEGRADATION OF MISFOLDED PROTEINS BY MOLECULAR CHAPERONES THROUGH THE UPS

While the primary functions of molecular chaperones relate refolding and unfolding of nascent and misfolded proteins, they can facilitate the degradation of terminally misfolded clients, either through the UPS or autophagy (Lanneau et al., 2010). The majority of these clients are tagged with Ub chains for degradation by the proteasome complex. However, the substrates prone to aggregation are redirected to autophagy. In either case, molecular chaperones are involved in the recognition and/or delivery of terminally misfolded substrates.

Ub-Dependent Selective Proteolysis through the Proteasome

The UPS mediates selective proteolysis of short-lived proteins by the proteasome and accounts for more than 80% of intracellular proteolysis (Hershko and Ciechanover, 1998). In the UPS, Ub is first activated by its conjugation to the ubiquitin activating enzyme E1. This conjugation involves the ATP-dependent formation of a thioester bond between the C-terminal glycine residue of Ub and an active site cysteine of E1 (Hershko and Ciechanover, 1998; Ciechanover, 2013). The activated Ub is transferred to the Ub conjugating enzyme E2 through a thioester bond. The Ub ligase E3 promotes the transfer of Ub from E2 to the lysine (Lys) residue of substrates. This generates an isopeptide bond between the C-terminal glycine residue of Ub and lysine residues on the substrate (Hershko and Ciechanover, 1998; Ciechanover, 2013). The human genome encodes more than 500 E3s. These E3s can be divided into three groups depending on their ubiquitination domains, including the really interesting new gene (RING) finger, the homologous to E6-AP (HECT) domain, and the U-box domain (Qian et al., 2006). Occasionally, E4 enzymes enhance the conjugation of additional Ub molecules to form a Ub chain, typically through the K48 linkage (Upadhya and Hegde, 2007). Once the first Ub is attached to substrate, subsequent Ub conjugations may use any of its seven Lys residues (Peng et al., 2003). This can generates a Ub chain with many different topologies, each of which has distinct functions. Amongst these topologies, the most widely used Lys48 linkage typically leads to proteasomal degradation. The Lys63 linkage mediates nonproteolytic processes, such as Ub-dependent protein-protein interactions (Hadian et al., 2011). Human cells also use the Lys11 linkage for cell cycle regulation and cell division as well as ERAD (Matsumoto et al., 2010) and K27 for ubiquitin fusion and degradation (Morawe et al., 2012). Ub moieties conjugated to substrates are reversible and can be detached and adjusted by deubiquitination enzymes (DUBs). The substrates conjugated with polyubiquitin are degraded by the 26S proteasome (Hershko and Ciechanover, 1998). This 2.5-MDa protease complex is composed of the 20S core particle with a size of 700 kDa associated with two 19S regulatory particles (Ravikumar et al., 2008; Douglas et al., 2009; Ciechanover and Kwon, 2015). The Ub chains conjugated to substrates are recognized by RPN10 and RPN13 of the 19S particle and stripped off by DUBs such as RPN11, USP14, and UCHL5 (ubiquitin C-terminal hydrolase L5; Ravikumar et al., 2008; Douglas et al., 2009; Ciechanover and Kwon, 2015). Deubiquitinated substrates are unfolded into a nascent polypeptide through ATP hydrolysis in the 19S particle and fed into the 20S particle for degradation, generating short peptides with average sizes of 8–12 amino acids (Hershko and Ciechanover, 1998). These peptides are degraded into amino acids by aminopeptidases, which are recycled for protein synthesis, or presented on the cell surface for immunosurveillance (Kloetzel and Ossendorp, 2004).

Molecular Chaperones and Ub Ligases Work Together in the UPS

Several cytosolic or nuclear Ub ligases are known to be involved in degradation of misfolded proteins in collaboration with molecule chaperones, including UBR1, UBR2, San1, Hul5, E6-AP, C-terminus of Hsc70-interacting protein (CHIP) and Parkin (Gardner et al., 2005; Heck et al., 2010; Kettern et al., 2010; **Figure 1**). CHIP is a 35 kDa protein that has dual functions, one as a cochaperone of Hsp70 and Hsp90, and the other as a Ub ligase that mediates ubiquitination of misfolded proteins using its the RING-like U-box domain (Ballinger et al., 1999; McDonough and Patterson, 2003). The E3 activity of CHIP requires the interaction with the E2 Ub conjugating enzyme UBCH5 (Cyr et al., 2002). When interacting with the Hsp70- or Hsp90-client complex, CHIP in collaboration with UBCH5 captures and ubiquitinates the misfolded clients for proteasomal degradation (Demand et al., 2001). To facilitate the delivery of the ubiquitinated substrates to the proteasome, CHIP also interacts with the S5a component (also known as Rpn10) of the 19S proteasome particle (Connell et al., 2001). In this process, CHIP indirectly recognize misfolded proteins through the interaction

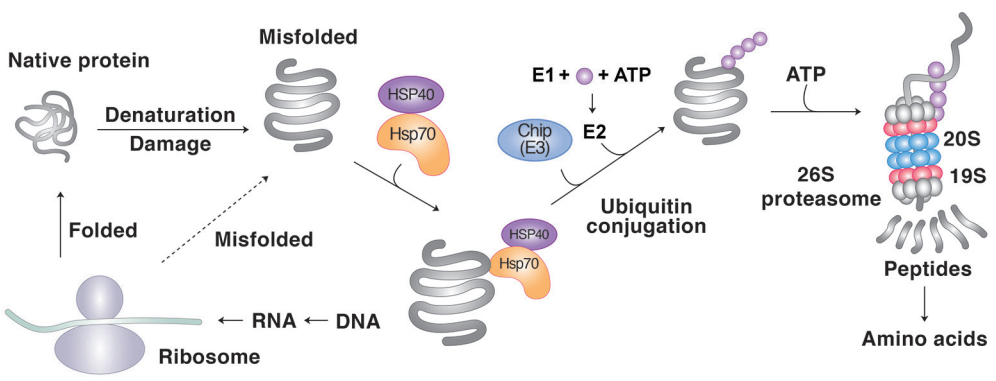

FIGURE 1 | Protein degradation by molecular chaperones through the UPS. Molecular chaperones such as Hsp70 recognizes the hydrophobic sequences of misfolded proteins as degrons. The Ub ligase CHIP guides the chaperone-client complexes to the UPS and mediates the clients' ubiquitination. The UPS involves a cascade of E1, E2, and E3 enzymes whose cooperative activities mediate the conjugation of Ub to target proteins. In PQC, most E3s cannot recognize misfolded proteins and rather depend on molecular chaperones for substrate recognition. Ubiquitinated substrates are degraded by the proteasome into short peptides, typically with sizes of 8–12 amino acids.

between its TPR (tetratricopeptide repeat) domain with Hsp70 or Hsp90 (Lanneau et al., 2010). The substrates of CHIP include hyperphosphorylated tau and mutant SOD1 (Lanneau et al., 2010). CHIP-mediated degradation of Hsp70 clients is further facilitated by the cochaperone BAG-1 (Takayama et al., 1997). BAG-1 uses its C-terminal region to bind the ATPase domain of Hsp70 and acts as a nucleotide exchange factor (NEF), inducing the release of substrates from Hsp70 (Takayama et al., 1997). On the other hand, BAG-1 has also a Ub-like (UBL) domain at its N-terminal region that supports the interaction with the proteasome (Alberti et al., 2003). BAG-1 directly interacts and cooperates with CHIP to guide the terminally misfolded clients to the UPS. In addition to BAG-1, Hsp27 belonging to the small HSP family can directly interact with the proteasome to modulate the ubiquitination of clients (Garrido et al., 2006). Hsp27 also binds the Ub chain of clients and, thus, increase the degradation of ubiquitinated proteins (Garrido et al., 2006).

The clients of Hsp90 can also be degraded through the UPS if they are no longer chaperoned by Hsp90, for example, owing to misfolding. These misfolded clients dissociated form Hsp90 are ubiquitinated by E3 ligases, such as CHIP, and degraded by the proteasome (Didelot et al., 2007). However, CHIP is mainly associated with Hsp70, and there should be additional E3 ligases that target the misfolded clients of Hsp90. One such candidate is the E3 ligase Triad3A which forms a complex with Hsp90 and receptor interacting protein 1 (RIP-1) and mediates the ubiquitination of RIP-1 and proteasomal degradation following Hsp90 inhibition by geldanamycin (Fearns et al., 2006).

The N-end rule pathway is a proteolytic system in which a single N-terminal residue acts as an essential component of a class of degrons, called N-degrons (Bachmair et al., 1986; Tasaki and Kwon, 2007; Sriram and Kwon, 2010; Sriram et al., 2011; Varshavsky, 2011). In mammals, these N-terminal degrons are recognized by the N-recognin family, including UBR1, UBR2, UBR4, UBR5, and p62 (Kwon et al., 1999a, 2002; Tasaki et al., 2005, 2009; An et al., 2006). Amongst these, the Ub ligases UBR1 and UBR2 have been shown to mediate the ubiquitination of misfolded cytosolic proteins, leading to proteasomal degradation (Eisele and Wolf, 2008; Heck et al., 2010; Prasad et al., 2010). These RING finger E3 ligases indirectly recognize misfolded proteins through molecular chaperones such as Hsp110 and Hsp70 (Heck et al., 2010; Nillegoda et al., 2010). Misfolded proteins targeted by N-recognins include TDP43 in ALS and tau and amyloid β in AD (Brower et al., 2013). Interestingly, in addition to the exposed hydrophobic residues, some of their misfolded clients are post-translationally conjugated with the amino acid L-Arg of Arg-tRNA^{Arg} by *ATE1*-encoded R-transferases (Grigoryev et al., 1996; Balogh et al., 2000, 2001; Kwon et al., 2000; Lee et al., 2005). The resulting N-terminal Arg residue acts as N-degron which is recognized by N-recognins such as UBR1 and UBR2 (Kwon et al., 1999b; Lee et al., 2008; Sriram et al., 2009; Meisenberg et al., 2012). In yeasts, the cytosolic E3 ligase Ubr1 has been shown to work with the nuclear E3 ligase San1 if cytosolic misfolded proteins overwhelm the capacity of E3 ligases (Heck et al., 2010; Prasad et al., 2010). In this collaboration between cytosolic and nuclear PQC systems, San1 associated with Hsp70 brings excessive cytosolic misfolded proteins to the nucleus for proteasomal degradation (Heck et al., 2010; Prasad et al., 2010). Different from other E3 ligases, San1 has many disordered structures and stretches of hydrophobic residues and, thus, can directly bind misfolded proteins (Rosenbaum et al., 2011). In mammals, the nuclear Ub ligase UHRF2 has been proposed to be a functional homolog of the yeast San1 (Nielsen et al., 2014).

Eukaryotic cells operate various degradative machinery designated to specific types of misfolded proteins. In yeasts, misfolded proteins generated by heat shock are specifically ubiquitinated by the E3 ligase Hul5 that has a HECT ubiquitination domain (Fang et al., 2011). In mammals, mislocalized membrane proteins are ubiquitinated by the Ub ligase RNF126 (RING finger 126) in collaboration with the BAG6 chaperone system (Rodrigo-Brenni et al., 2014). Proteins synthesized from aberrant mRNAs without stop codons are ubiquitinated by Listerin/Ltn1 (Bengtson and Joazeiro, 2010). Moreover, in the ER, membrane-associated misfolded proteins are ubiquitinated by the Ub ligase DOA10 (Nielsen et al., 2014). By contrast, misfolded proteins in the ER lumen are ubiquitinated by the Ub ligases Hrd1 and Gp78 mediates through a process called ERAD (ER-associated degradation) (Vembar and Brodsky, 2008). Except for San1 and Hul5, most of these E3s indirectly recognize misfolded proteins through cooperating molecular chaperones. Overall, the mechanistic details and clinical importance of these various PQC machinery in neurodegenerative diseases remains largely unexplored.

Deubiquitination Enzymes (DUBs) in the Degradation of Misfolded Proteins

DUBs detach Ub molecules from substrates and, thus, can modulate the proteasomal degradation of Ub-conjugated substrates. The proteasome is associated with DUBs such as RPN11, UCHL5, and USP14. RPN11 is a stoichiometric subunit of the proteasome and detaches Ub molecules *en bloc* from substrates (Hao et al., 2013). The free, unanchored Ub chains are deposited to aggresomes and recognized by HDAC which brings misfolded protein aggregates to aggresomes (Hao et al., 2013). The interaction between HDAC and unanchored Ub chains is essential for cargo-loaded HDAC to see where aggresomes are (Hao et al., 2013). In contrast to RPN11, USP14 is a conditionally recruited to the proteasome through its UBL domain. This enhances its activity up to 800-folds and, thus, modulates the degradation rate of substrates (Crosas et al., 2006). The treatment of the USP14 inhibitor IU1 has been shown to facilitate the degradation of aggregation-prone misfolded proteins such as tau and polyQ-expanded mutant ataxin-3 (Lee et al., 2010). The functions of DUBs in neurodegenerative diseases remain largely unexplored.

The UPS Is Impaired during Neurodegeneration

The pathogenesis of most neurodegenerative diseases, such as AD, PD, ALS, HD, and prion diseases commonly involves the downregulation of the components of the UPS. One prominent

risk factor is aging. The activities of UPS components, such as the proteasome, are often progressively declined as neurons age (Keller et al., 2000; Hwang et al., 2007; Tydlacka et al., 2008; Low, 2011). This may reduce the ability to degrade misfolded proteins, contributing to the accumulation of pathological protein aggregates. Making it worse, the accumulated aggregates as a consequence of reduced UPS activities now further inhibit the activities of UPS components, including the proteasome. The proteasome is particularly vulnerable to protein aggregates because its narrow chamber has a diameter of as small as 13 angstroms. Therefore, proteasome cannot digest protein aggregates that cannot be easily unfolded. For example, β-sheet-rich PrP aggregates were shown to block the opening of the 20S proteasome particle, further reducing proteasomal activity (Andre and Tabrizi, 2012). Following ubiquitination and aggregation, tau in AD binds the recognition site of the 19S catalytic particle and block its gate (Dantuma and Lindsten, 2010; Tai et al., 2012). Aggregates of many other pathogenic proteins in neurodegenerative disorders can directly inhibit proteasome activity (Gregori et al., 1995; Snyder et al., 2003; Lindersson et al., 2004; Kristiansen et al., 2007). The resulting proteotoxicity has adverse effects on neurons (Hegde and Upadhya, 2011). Indeed, the reduced UPS activity has been associated with neuronal damage in AD, HD, PD, ALS, ataxia, Angelman syndrome, Wallerian degeneration, and gracile axonal dystrophy (Hegde, 2010; Hegde and Upadhya, 2011).

DEGRADATION OF MISFOLDED PROTEINS BY AUTOPHAGY

Autophagy is a process by which cytosolic materials are degraded by the lysosome. Depending on the mechanism of cargo delivery to the lysosome, autophagy can be divided into three pathways: microautophagy, CMA, and macroautophagy. Terminally misfolded proteins in neurodegenerative diseases can be degraded through macroautophagy or CMA (**Figure 2**). The role of autophagy in proteostasis is vitally important for postmitotic neurons with long extensions, in which cytotoxic proteins cannot be diluted by cell division.

Macroautophagy

Misfolded proteins prone to aggregation can be directed to macroautophagy for lysosomal degradation. These substrates, typically as a Ub-conjugated form, are collected by autophagy adaptors, such as p62 and NBR1 (Cha-Molstad et al., 2015). P62 is normally inactive and can be activated by binding to the N-terminally arginylated form of the molecular chaperone BiP/GRP78, the ER counterpart of cytosolic Hsp70 (Cha-Molstad et al., 2015). Upon the accumulation of non-degradable autophagic cargoes, BiP and other ER-residing chaperones, such as calreticulin and protein disulfide isomerase (PDI), are N-terminally arginylated by *ATE1*-encoded R-transferase. The resulting N-terminally arginylated BiP, R-BiP, locates in the cytosol where R-BiP binds the ZZ domain of p62

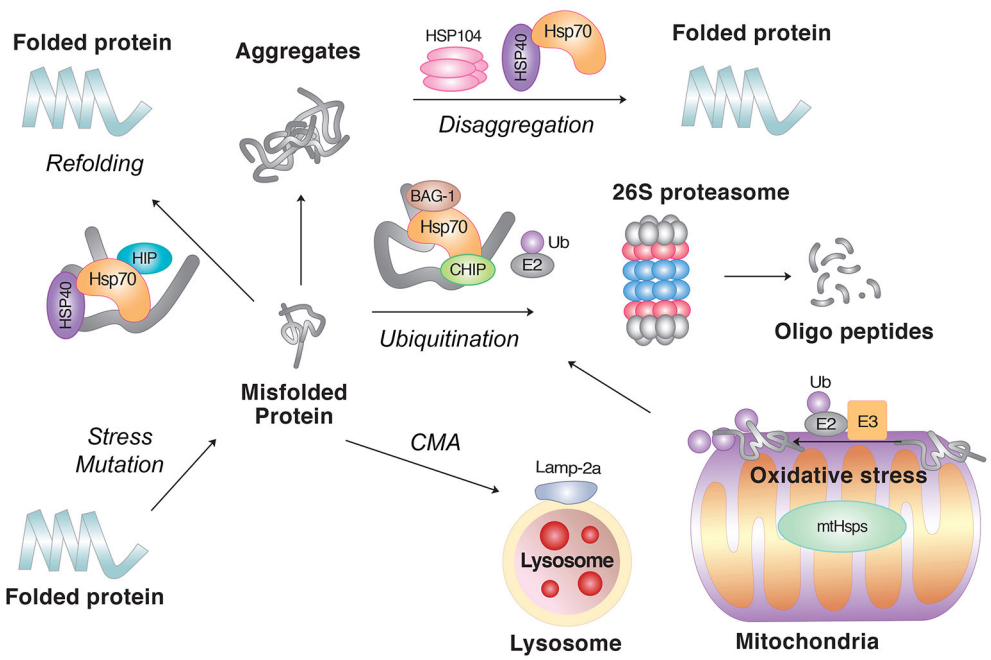

FIGURE 2 | The role of molecular chaperones in PQC. Molecular chaperones, such as Hsp70 in combination with the cochaperone Hsp40, facilitate the refolding of misfolded proteins. If the clients fail to refold, molecular chaperones can also mediate their degradation in collaboration with cellular proteolytic pathways. In principle, soluble misfolded proteins are targeted by the UPS, in which the clients are ubiquitinated by E3 Ub ligases followed by degradation through the 26S proteasome. However, if the clients are prone to aggregation or escape the surveillance of the UPS, they can be degraded by lysosomal hydrolases, either through macroautophagy or CMA. As the last step of PQC, molecular chaperones can disaggregate already formed aggregates. Also shown are misfolded proteins induced by oxidative stress in mitochondria.

through its N-terminal arginine residue. This binding induces a conformational change in p62, facilitating the polymerization of p62 as well as the interaction of p62 with LC3-II which is anchored on the membrane of autophagosomes (Cha-Molstad et al., 2015). It is generally assumed that p62 and other autophagic adaptors recognize the Ub moieties conjugated to misfolded proteins and delivers them to the autophagosome through specific interaction with LC3 (Lamark et al., 2009; Stolz et al., 2014). The cargo-loaded autophagosome is fused with the lysosome to form the autolysosome wherein cargoes along with p62 are degraded by lysosomal hydrolases.

CMA

CMA is a selective proteolytic system and does not involve vesicle formation (Chiang et al., 1989; Dice, 1990; Cuervo et al., 1997). The selectivity enables the degradation of misfolded or damaged cytosolic proteins without interfering with the same kinds of proteins with normal functions (Fuertes et al., 2003; Massey et al., 2006; Kaushik and Cuervo, 2012). The target substrates of the CMA include cytosolic proteins that carry the KFERQ pentapeptide which functions as a degron. The CMA degron, found in approximately 30% of cytosolic proteins (Chiang and Dice, 1988; Dice, 1990), is recognized by chaperones associated with cochaperones such Hsc70 belonging to the Hsp70 family (Chiang et al., 1989) (**Figure 3**). The function of Hsc70 requires cochaperones, such as Hsp40, Hsp90, HIP, HOP, and BAG-1 (Agarraberes and Dice, 2001). The substrates associated with the Hsc70 chaperone system are translocated to the lysosomal membrane through the interaction of Hsc70 with LAMP2A, a single-span membrane protein (Cuervo and Dice, 1996). The stability of LAMP2A requires its association with Lys-Hsc70, a lysosomal homolog of Hsc70. Once the substrate is targeted to the lysosome, Lys-Hsc70 assists the active LAMP2A complex to be disassembled into the inactive monomeric form, which is now available for the next round of the CMA process (Bandyopadhyay et al., 2008). The levels of LAMP2A and Lys-Hsc70 are important underlying the rate of CMA degradation (Cuervo et al., 1995; Agarraberes et al., 1997; Cuervo and Dice, 2000). Although a large number of cytosolic proteins contain the CMA degron sequence, only a limited number of these proteins were demonstrated to be degraded by the CMA (Wing et al., 1991). Post-translational modifications can generate the substrates of the CMA (Chiang and Dice, 1988; Dice, 1990). Studies have shown that CMA is essential for the survival of neurons by degrading misfolded or damaged cytosolic proteins (Cuervo et al., 2004). The misregulation of the CMA has been shown to correlate to the pathogenesis of neurodegeneration (Cuervo et al., 2004).

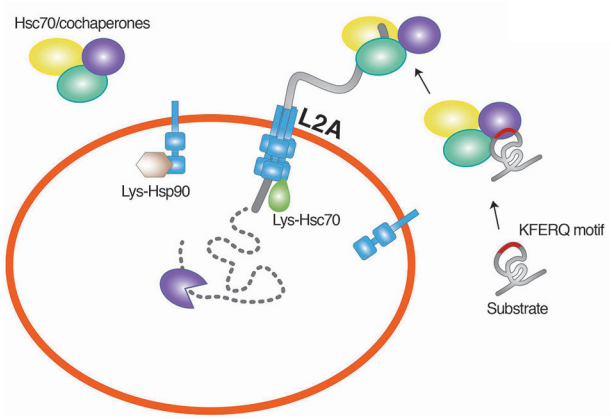

FIGURE 3 | Chaperone-mediated degradation of misfolded proteins. CMA is a selective proteolytic system in which cytosolic proteins carrying the KFERQ pentapeptide are targeted by Hsc70. The function of Hsc70 requires cochaperones, such as Hsp40, Hsp90, HIP, HOP, and BAG-1. The substrates associated with the Hsc70 chaperone system are translocated to the lysosomal membrane through the interaction of Hsc70 with LAMP2A, a single-span membrane protein. L2A, LAMP2A.

DISAGGREGATION OF AGGREGATES BY MOLECULAR CHAPERONES

Misfolded proteins may form aggregates if the PQC system is overwhelmed, for example, under severe stress conditions or by genetic mutations that allows the accumulation of non-degradable polypeptides. Yeast and mammalian cells have molecular chaperones (disaggregases) that can disaggregate the already formed aggregates (Parsell et al., 1994; Mogk et al., 1999; Doyle et al., 2013). Proteins recovered from aggregates are either refolded or degraded (Ravikumar et al., 2008; Douglas et al., 2009; Ciechanover and Kwon, 2015).

Yeast Hsp104 belonging to the Hsp100 family is a powerful AAA^+ ATPase that has a hexameric ring structure with a central channel (Shorter, 2011; Torrente and Shorter, 2013). Once guided to protein aggregates by Hsp70, Hsp104 retrieves proteins from aggregates and threads them into nascent polypeptides (Seyffer et al., 2012; Lee et al., 2013; Lipinska et al., 2013; Carroni et al., 2014). During threading, Hsp70 and Hsp40 assist the unfolding of substrates to generate surface loops that are fed into the core of Hsp104 (Zietkiewicz et al., 2006). This disaggregation activity of Hsp104 was demonstrated to be effective for various aggregates (Mosser et al., 2004; Shorter and Lindquist, 2004; Arimon et al., 2008; Lo Bianco et al., 2008; DeSantis et al., 2012). Despite the disaggregase activities, Hsp104 exhibited the modest efficacy for the pathogenic misfolded proteins in human neurodegenerative diseases (DeSantis et al., 2012). The introduction of a few point mutations markedly increased its disaggregase activity for the preformed aggregates of α-synuclein in PD (Jackrel and Shorter, 2014), and TDP-43 and FUS in ALS (Jackrel and Shorter, 2014; Jackrel et al., 2014). Compared with wild-type Hsp104, the engineered form had increased ATPase activity with reduced dependence on the Hsp70/Hsp40 and, thus, exhibited enhanced activities in protein translocation and remodeling (Jackrel and Shorter, 2014; Jackrel et al., 2014).

In humans, Hsp70 and Hsp40 interact with the cochaperone Hsp110 to facilitate the disaggregation of protein aggregates (Gao et al., 2015; Nillegoda et al., 2015). Hsp110 belonging to the conserved Hsp70 superfamily has structural and functional similarity to Hsp70 including the nucleotide binding domain and acts as an NEF of Hsp70 (Polier et al., 2008). Although

the action mechanism of the Hsp110-containing disaggregase complex remains unclear, it appears that the NEF activity of Hsp110 facilitates ADP release from Hsp70 (Rampelt et al., 2012). This ability to facilitate disaggregation *in vitro* has been equally observed with all three types of human Hsp110 isoforms: Hsp105α/HSPH1, Apg-2/HSPH2, and Apg-1/HSPH3 (Rampelt et al., 2012). The importance of Hsp110 in disaggregation has been demonstrates with various amyloids and prefibrillar oligomers and reactivate proteins from aggregates (Lo Bianco et al., 2008). Loss-of-function studies of Hsp110 have also shown its disaggregation activity in *C. elegans* (Rampelt et al., 2012), mouse cells (Yamagishi et al., 2010) and *Plasmodium falciparum* (Ziningа et al., 2016).

PROTECTIVE ROLE OF CHAPERONES IN NEURODEGENERATION

Many neurodegenerative diseases are directly caused by the excessive accumulation of misfolded proteins and their aggregates. During the pathogenesis, molecular chaperones play a central role in the refolding, degradation, and disaggregation of these pathogenic protein species. Extensive studies have shown that molecular chaperones promote the removal of pathogenic misfolded proteins and their aggregates.

HD and Other PolyQ Diseases

HD is a progressive neurodegenerative disease associated with the accumulation of mutant huntingtin (mHTT) that has the excessive repetition of glutamine residues, called polyQ, which causes misfolding (Shastry, 2003; Lee et al., 2011). These misfolded mHTT causes selective neuronal damage and death, leading to cognitive and motor defects (Gusella and MacDonald, 1998; Ramaswamy et al., 2007; Roos, 2010). Studies have shown that Hsp70 in a complex with Hsp40 plays a major role in inhibiting the formation of mHTT aggregates. Specifically, the Hsp70/Hsp40 machinery binds misfolded mHTT and holds its folding state to attenuate the formation of mHTT oligomers (Jana et al., 2000). The neuroprotective role of Hsp70 in the pathogenesis of PD is highlighted by a genetic screening of *Drosophila* PD model, which identified Hsp70 and Hsp40 as two major suppressors of the neurotoxicity caused by mHTT (Kazemi-Esfarjani and Benzer, 2000). Consistently, the knockout of Hsp70 in R6/2 transgenic HD mice has been shown to aggravate the symptoms in neurodegeneration (Wacker et al., 2009). A similar neuroprotective efficacy was observed with the neuronal chaperone HSJ1a (DNAJB2a) belonging to the Hsp40 family (Labbadia et al., 2012). In addition to the Hsp70-Hsp40 machinery, other members of the Hsp70 family have also been shown to counteract mHTT cytotoxicity. Specifically, the cytosolic chaperone Hsc70 binds and directly delivers mHTT to the lysosome via CMA, leading to selective degradation of mHTT and reduced toxicity (Bauer et al., 2010). This finding is further supported by *in vivo* studies using mice (Bauer et al., 2010) as well as flies (Gunawardena et al., 2003) overexpressing Hsc70. An ER counterpart of Hsp70, BiP/GRP78, has also been shown to counteract the accumulation of mHTT aggregates and apoptosis (Jiang et al., 2012). Besides the Hsp70 family members and their cochaperones, several other chaperones have been implicated in the refolding and/or degradation of polyQ protein aggregates, including Hsp84 (Mitsui et al., 2002), Hsp104 (Vacher et al., 2005), Hsp104/Hsp27 (Perrin et al., 2007), the chaperonin TRiC (Nollen et al., 2004; Behrends et al., 2006; Kitamura et al., 2006), and the cochaperone Prefoldin (Tashiro et al., 2013). Finally, HSPB7 belonging to small HSPs (Vos et al., 2010) and CHIP (Al-Ramahi et al., 2006) were shown to counteract the formation of polyQ aggregates in disease models.

PD

PD is the second most common neurodegenerative disease after AD, affecting up to 10% of humans over 65 years. This protein misfolding disorder is associated with the loss of dopaminergic neurons in the substantia nigra pars compacta of brain (Wirdefeldt et al., 2011). PD is characterized by the formation of insoluble α-synuclein aggregates which are deposited as nuclear inclusions (Goedert, 2001; Ross and Poirier, 2004; Hasegawa et al., 2016) as a ubiquitinated form (Hasegawa et al., 2002). These inclusion, called Lewy bodies, are mainly composed of α-synuclein aggregates (Irizarry et al., 1998; Spillantini et al., 1998) together with various components of PQC, including Ub (Kuzuhara et al., 1988) and molecular chaperones such as Hsp70, Hsp90, Hsp60, Hsp40, Hsp27, and CHIP (McLean et al., 2002). This co-aggregation pattern indicates that α-synuclein aggregates deposited in Lewy bodies are the remnants that survived the attempts of molecular chaperones to maintain proteostasis. Specifically, Hsp70 recognizes the hydrophobic degron of misfolded α-synuclein through its substrate binding domain (Dedmon et al., 2005; Luk et al., 2008). By holding the folding status, Hsp70 facilitates the refolding of misfolded α-synuclein and inhibits the formation of its oligomers (Outeiro et al., 2008). The *in vivo* efficacy of Hsp70 was demonstrated with overexpressed Hsp70 in flies (Auluck and Bonini, 2002; McLean et al., 2004; Zhou et al., 2004; Opazo et al., 2008; Danzer et al., 2011) and mice (Klucken et al., 2004). Moreover, the depletion of molecular chaperones was shown to aggravate the degeneration of neurons caused by proteotoxicity (Ebrahimi-Fakhari et al., 2011).

The ER chaperone BiP belonging to the Hsp70 family can interact with α-synuclein and reduce its neurotoxicity (Gorbatyuk et al., 2012). Overexpressed BiP has been shown to protect nigral dopaminergic neurons in a rat model of PD, which correlates to reduced ER stress mediators and apoptosis (Gorbatyuk et al., 2012). The anti-aggregation and neuroprotective activity of BiP was further demonstrated with photoreceptor cells expressing aggregation-prone mutant rhodopsin (Gorbatyuk et al., 2010; Athanasiou et al., 2012). In addition to Hsp70 proteins, αB-crystallin belonging to small SHPs can interact with α-synuclein and inhibit the elongation of its fibrillar seeds by forming nonfibrillar aggregates (Kudva et al., 1997; Stege et al., 1999; Rekas et al., 2004; Shammas et al., 2011). Another small HSP, Hsp27, can arrest the aggregation of α-synuclein in the initial phage, perhaps by binding to the partially folded monomers (Rekas et al., 2007; Bruinsma et al., 2011).

AD

AD is the most common neurodegenerative disorder caused by aggregation-prone proteins and selective loss, inactivation, or shrinkage in the mature nervous system (Regeur et al., 1994). The pathogenesis involves the deposit of amyloid-β (Aβ) both outside and inside the neurons as well as intracellular neurofibrillary tangles of hyperphosphorylated tau (Shankar et al., 2008; Honjo et al., 2012). Self-assembly of Aβ, which is not misfolded, generates its neurotoxic oligomers, which, in turn, grows into amyloid fibrils (Shankar et al., 2008; Honjo et al., 2012). Studies have shown that various molecular chaperones interact with intracellular Aβ species, which has been internalized by endocytosis, as well as tau and regulate their degradation. Specifically, αB-crystallin (HSPB5) and DnaJB6 bind to Aβ fibrils and inhibit their elongation and growth (Shammas et al., 2011; Mansson et al., 2014). In addition, Hsp70, Hsp40, and Hsp90 interact with the oligomer form of Aβ peptides (Evans et al., 2006). When overexpressed, Hsp70 and Hsp40 were shown to reduce the formation of Aβ aggregates and redirected it from growing into fibrillar to soluble circular structures (Evans et al., 2006). In contrast to Hsp70, Hsp90 supports the folding of tau and, thus, stabilizes this neurotoxic protein, facilitating tau pathology in AD model (Carman et al., 2013). The cochaperone BAG-1 forms a complex with Hsp70 and tau and can inhibit tau degradation in cultured cells, leading to the accumulation of both tau and APP (Elliott et al., 2007, 2009). Given the opposing roles of Hsp70 and Hsp90, the Hsp90 inhibitor 17-AAG was successfully used to induce the expression of various chaperones, such as Hsp70, Hsp40, and Hsp60 (Chen et al., 2014). The induction of these chaperones reduced Aβ toxicity in neurons (Chen et al., 2014). Another line of evidence supporting the protective role of chaperones in AD is provided by a study with UBB+1, a frameshift mutation of ubiquitin B (Hope et al., 2003). UBB+1 can inhibit the proteasome and, thus, can be deposited into intracellular protein inclusions in AD. The overexpression of UBB+1 induced the expression of HSPs, which, in turn, protected cells against oxidative stress.

ALS

ALS is the most common adult onset motor neuron disease that affects the brainstem, cortex and spinal cord. It is characterized by the atrophy, weakness, and paralysis of muscles, leading to death within 3–5 years post diagnosis (Robberecht and Philips, 2013). The majority of ALS patients are sporadic, whereas 5–10% are familial, i.e., linked to mutations in specific genes. Numerous genetic mutations are linked to ALS, either genetically and/or pathologically. Amongst these, the mutations of SOD1, a free radical scavenger enzyme, accounts for 20% of familial ALS cases (Rosen et al., 1993). Several other ALS-linked mutated proteins form intracellular aggregates, including C9ORF72 (DeJesus-Hernandez et al., 2011), transactive response DNA binding protein (TDP-43), fused in sarcoma/translocated in liposarcoma (FUS), vesicle associated protein B (VAPB), ubiquilin-2, optineurin, and protein disulphide isomerase 1 and 3 (PDIA1 and PDIA3) (Robberecht and Philips, 2013). The majority of ALS cases are considered protein misfolding disorder because these mutations cause the accumulation of misfolded proteins and their aggregates.

Various molecular chaperones are implicated in the formation of these protein aggregates in ALS. For example, Hsp70/Hsp40, Hsp27, Hsp25, and αB-crystalline can form complexes with an ALS-causing mutant form of SOD, SOD1G93A. However, the overexpression of Hsp70 alone was not sufficient to reduce mutant SOD1 toxicity in ALS mouse model (Liu et al., 2005). Instead, PDI proteins exhibit a protective role in ALS models (Walker et al., 2010; Jeon et al., 2014). PDI assists the rearrangement of incorrectly arranged disulfide bonds of ER clients. It can also act as a chaperone that not only counteracts the aggregation of proteins independent of disulfide bonds but also delivers terminally misfolded proteins to ERAD (Quan et al., 1995). Over 15 missense mutations of PDIA1 and ERp57/PDIA3 were linked to ALS (Yang and Guo, 2016). Various *in vitro* and animal studies showed that PDI is deposited to the aggregates formed by the mutant forms of TDP-43 and FUS (Honjo et al., 2011; Farg et al., 2012), TDP-43 (Honjo et al., 2011; Walker et al., 2013), and VAPB (Tsuda et al., 2008). The overexpression of PDI reduces mutant SOD1 inclusions *in vitro* whereas PDI knockdown facilitates the formation of ALS inclusions (Walker et al., 2010).

Hsp27 also plays a protective role in the pathogenesis of ALS. Hsp27 binds mutant SOD1 *in vitro* and inhibits its fibril elongation (Yerbury et al., 2013). The overexpression of Hsp27 was shown to inhibit mutant SOD1-induced cell death (Patel et al., 2005). Hsp27 exhibited a synergistic efficacy when Hsp70 was coexpressed (Patel et al., 2005). In addition to Hsp27, HSJ1a shows a similar protective activity against the formation of mutant SOD aggregates at the late stage of the disease (Novoselov et al., 2013). HSJ1a interacts with SOD1G93 and facilitates its ubiquitination and proteasomal degradation.

THERAPEUTIC APPLICATION TARGETING MOLECULAR CHAPERONES IN NEURODEGENERATION

Given the protective role of molecular chaperones against pathogenic protein aggregates in neurodegenerative diseases, molecular chaperones are logical targets for drug development to modulate aggregation and clearance of the aggregates. Indeed, pharmaceutical induction of molecular chaperones has been demonstrated to effectively inhibit the formation of pathogenic aggregates in disease models.

Hsp90 supports in the folding/refolding and stability of a number of clients, including pathogenic misfolded protein aggregates in neurodegenerative diseases. While these activities are overall beneficial for refolding, however, Hsp90 also assists in the stability of neurotoxic proteins, favoring the accumulation of toxic protein aggregates (Schulte and Neckers, 1998; Boland et al., 2008; Eskelinen and Saftig, 2009; Chouraki and Seshadri, 2014). Therefore, one such strategy is the pharmaceutical inhibition of Hsp90. Geldanamycin competes with ATP and inhibits the folding and stabilization of neurotoxic proteins (Schulte and Neckers, 1998; Boland et al., 2008; Eskelinen and Saftig, 2009; Chouraki and Seshadri, 2014). In addition, upon binding to geldanamycin, Hsp90 releases a HSP-inducing transcript factor, HSF1 (McLean et al., 2004; Putcha et al., 2010). The dissociated

HSF1 which otherwise would be sequestered by Hsp90 move to the nucleus and transcriptionally induces HSPs, such as Hsp70 (McLean et al., 2004; Putcha et al., 2010). Geldanamycin was successfully used to inhibit protein aggregation in the *Drosophila* (McLean et al., 2004; Putcha et al., 2010) and mouse PD model (Shen et al., 2005) and in a primary culture model of familial ALS (Batulan et al., 2006).

Despite its therapeutic efficacy, geldanamycin is toxic and cannot penetrate the blood brain barrier (BBB). A number of geldanamycin derivatives or the compounds that target HSF1 are now available, including geranylgeranylacetaone, celastrol, arimoclomol, withaferin A, 17-N-allylamino-17-demethoxygeldanamycin (17-AAG), and PU-DZ8 (Kieran et al., 2004; Niikura et al., 2006; Hoogstra-Berends et al., 2012; Khan et al., 2012; Kalmar et al., 2014; Sharma et al., 2015). Amongst these, celastrol is an anti-inflammatory and antioxidant compound extracted from a perennial creeping plant belonging to the Celastraceae family (Cleren et al., 2005). The treatment of celastrol in HD model mice resulted in the induction of Hsp70 expression associated with reduced loss of dopaminergic neurons induced by MPTP in the substantia nigra pars compacta (Cleren et al., 2005). Celastrol protected neurons against polyglutamine toxicity *in vivo* and *in vitro* (Zhang and Sarge, 2007) and reduced the β-amyloid level in mouse AD (Paris et al., 2010) and HD (Zhang and Sarge, 2007) models. BBB-permeable Hsp90 inhibitors, 17-AAG and PU-DZ8, were used to decrease the levels of phosphorylated tau in the AD model (Luo et al., 2007) and to inhibit neurodegeneration in a fly HD model (Fujikake et al., 2008). In addition, as Hsp90 inhibition causes undesirable proteotoxicity, HSF1A, a small benzyl pyrazole-based compound, has been developed to activate Hsf1 without inhibiting Hsp90 (Neef et al., 2010). Overall, studies using these HSP-inducing compounds in animal models of neurodegenerative diseases demonstrate that this strategy has potential for therapeutic application.

CONCLUDING REMARKS

Neurodegenerative diseases are caused by failure in PQC, which can be attributed to genetic mutations or alternatively an age-related decline in proteolytic activities. Molecular chaperones are an essential component of PQC in that they recognize unfolded or misfolded proteins, hold their folding status, and release them for spontaneous refolding. These nanoscale molecular machines can also facilitate the degradation of terminally misfolded proteins either through the UPS and autophagy. As the last defense mechanism of PQC, molecular chaperons can disaggregate the already formed aggregates. Thus, molecular chaperones play a pivotal role to protect neurons from the accumulation of pathogenic protein aggregates. It is therefore not surprising that pharmaceutical means are exploited to modulate the activities and functions of molecular chaperones. Indeed, small molecule compounds that target molecular chaperones such as Hsp90 have been successfully demonstrated to be effective in various neurodegenerative diseases. There is now an emerging consensus that proteostasis in diseases could be restored by using small molecule compounds or RNA interference that modulates chaperone expression or activities. A better understanding of chaperone functions in neurons will help the development of therapeutic means to restore proteostasis.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Hsp70/Hsp90 Chaperone Machinery in Neurodegenerative Diseases

*Rachel E. Lackie[1,2], Andrzej Maciejewski[1,3], Valeriy G. Ostapchenko[1], Jose Marques-Lopes[1], Wing-Yiu Choy[3], Martin L. Duennwald[4], Vania F. Prado[1,2,5,6] and Marco A. M. Prado[1,2,5,6]**

[1] Molecular Medicine, Robarts Research Institute, University of Western Ontario, London, ON, Canada, [2] Program in Neuroscience, University of Western Ontario, London, ON, Canada, [3] Department of Biochemistry, University of Western Ontario, London, ON, Canada, [4] Department of Pathology and Laboratory Medicine, University of Western Ontario, London, ON, Canada, [5] Department of Physiology and Pharmacology, University of Western Ontario, London, ON, Canada, [6] Department of Anatomy and Cell Biology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, ON, Canada

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***Correspondence:**
Marco A. M. Prado
mprado@robarts.ca

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The accumulation of misfolded proteins in the human brain is one of the critical features of many neurodegenerative diseases, including Alzheimer's disease (AD). Assembles of beta-amyloid (Aβ) peptide—either soluble (oligomers) or insoluble (plaques) and of tau protein, which form neurofibrillary tangles, are the major hallmarks of AD. Chaperones and co-chaperones regulate protein folding and client maturation, but they also target misfolded or aggregated proteins for refolding or for degradation, mostly by the proteasome. They form an important line of defense against misfolded proteins and are part of the cellular quality control system. The heat shock protein (Hsp) family, particularly Hsp70 and Hsp90, plays a major part in this process and it is well-known to regulate protein misfolding in a variety of diseases, including tau levels and toxicity in AD. However, the role of Hsp90 in regulating protein misfolding is not yet fully understood. For example, knockdown of Hsp90 and its co-chaperones in a *Caenorhabditis elegans* model of Aβ misfolding leads to increased toxicity. On the other hand, the use of Hsp90 inhibitors in AD mouse models reduces Aβ toxicity, and normalizes synaptic function. Stress-inducible phosphoprotein 1 (STI1), an intracellular co-chaperone, mediates the transfer of clients from Hsp70 to Hsp90. Importantly, STI1 has been shown to regulate aggregation of amyloid-like proteins in yeast. In addition to its intracellular function, STI1 can be secreted by diverse cell types, including astrocytes and microglia and function as a neurotrophic ligand by triggering signaling via the cellular prion protein (PrP^C). Extracellular STI1 can prevent Aβ toxic signaling by (i) interfering with Aβ binding to PrP^C and (ii) triggering pro-survival signaling cascades. Interestingly, decreased levels of STI1 in *C. elegans* can also increase toxicity in an amyloid model. In this review, we will discuss the role of intracellular and extracellular STI1 and the Hsp70/Hsp90 chaperone network in mechanisms underlying protein misfolding in neurodegenerative diseases, with particular focus on AD.

Keywords: STIP1, HOP, Alzheimer's disease, tau, ALS, Parkinson's disease, Huntington's disease, TDP-43

BRIEF INTRODUCTION TO CHAPERONES AND CO-CHAPERONES

A major requirement for cellular growth, function, and survival is the proper folding, maturation, and degradation of proteins. These activities are carried out by molecular chaperones, many of which are heat shock proteins (Hsps). The heat shock response was first discovered in the early 1960s in *Drosophila* that displayed changes in salivary gland transcriptional activity in response to different incubation temperatures (Ritossa, 1962). It was not until 1974 that Hsps were discovered and interest in this field of biology became widespread (Tissieres et al., 1974). Transcription of heat shock genes is mostly regulated by heat shock factor 1 (HSF1). Inactive HSF1 is localized in the cytosol, but upon heat stress translocates to the nucleus and binds to promoters of heat shock elements, inducing transcription and leading to an increase in Hsp expression (Morimoto, 1998). Activation of HSF1 and subsequent shuttling to the nucleus is a typical stress response and also allows for control of cell cycle, protein translation and glucose metabolism (Dai et al., 2007). It is now well-accepted that Hsps not only aid in mediating cellular responses to stress, but are also critical in general protein quality control. Some of the major roles of molecular chaperones include the regulation of the unfolding protein response due to stress, degradation of misfolded or aggregated proteins, regulation of macromolecular complexes, and protein-protein interactions.

There are several major classes of Hsps involved in the protein quality control machinery: Hsp60, Hsp70 and Hsp90, Hsp40, Hsp100, Hsp110, as well as the ATP-independent small heat shock proteins (sHsps) such as Hsp20, αA-crystallin, and αB-crystallin. Hsp40, also known as DnaJ, is commonly found acting as a co-chaperone for Hsp70 and regulates ATP-dependent polypeptide binding to Hsp70, prevention of premature polypeptide folding, and ATPase activity of Hsp70 (Cyr et al., 1992; Frydman et al., 1994; Tsai and Douglas, 1996). In yeast, the family of Hsp100 proteins protect cells from extreme physiological and environmental stress (Sanchez et al., 1992; Glover and Lindquist, 1998) and have the unique ability to re-solubilize aggregated insoluble proteins (Parsell et al., 1994). In metazoans disaggregase activity is carried out by the tricomplex of Hsp70, a J Protein and Hsp110 (Shorter, 2011; Rampelt et al., 2012; Gao et al., 2015). For the purpose of this review, we will focus mainly on the roles of Hsp70 and Hsp90 as well as of the critical co-chaperone stress-inducible phosphoprotein I (STI1, STIP1) and their regulation of protein misfolding and signaling in neurodegenerative diseases. Comprehensive discussion of different chaperones including their roles in the ER can be found in excellent recent reviews elsewhere (McLaughlin and Vandenbroeck, 2011; Marzec et al., 2012; Melnyk et al., 2015; Ellgaard et al., 2016).

Hsp70 and Hsp90 and homologs are both widely expressed in some lower order prokaryotes and in all eukaryotes, with Hsp90 constituting ∼1% of all cellular proteins in eukaryotes (Borkovich et al., 1989). Hsp90 activity is regulated through interactions with a large network of co-chaperones providing quality control of a wide range of client proteins. Initially, client proteins are recruited by Hsp40 and Hsp70 and then transferred to Hsp90 by the co-chaperone STI1 (the human homolog is also known as Hsp-organizing protein or HOP; Lassle et al., 1997; Chen and Smith, 1998; Johnson et al., 1998; Taipale et al., 2010). Recent studies suggest that Hsp90 has an important role in neurodegeneration. Pharmacological inhibition of Hsp90 results in Hsp70 and Hsp40 upregulation, which can control the expression of several synaptic proteins, but it can also channel misfolded protein for degradation by the proteasome (Luo et al., 2007; Chen et al., 2014; Wang et al., 2016). Protein aggregation is a major hallmark of several neurodegenerative diseases, including Alzheimer's disease (AD), Amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Huntington's disease (HD), and Creutzfeldt-Jakob disease (CJD). Therefore, the chaperone machinery is becoming a major therapeutic target across these diseases.

HSP70

The Hsp70 family of proteins is a class of highly abundant and ubiquitously expressed chaperones that participate in many biological processes, including protein trafficking, early stages of nascent polypeptide folding and the refolding or degradation of aggregated peptide products (Bukau et al., 2006). There are several eukaryotic Hsp70 isoforms (reviewed in Kabani and Martineau, 2008). Hsp70 is composed of two distinct domains, a 40 kDa N-terminal nucleotide-binding domain (NBD) that regulates client association and a 25 kDa C-terminal substrate-binding domain (SBD), which recognizes exposed hydrophobic stretches in the early stages of client protein folding (Rudiger et al., 1997; Bukau et al., 2006). A short, flexible hydrophobic linker joins both domains (Jiang et al., 2005). ATP binding and hydrolysis is coupled to allosteric changes in Hsp70, which influence protein-client interactions in the Hsp70 chaperone cycle (Mayer et al., 2000). Post-translational modification of Hsp70 by phosphorylation at T504 and/or acetylation at several serine residues promotes dimerization of Hsp70 (Morgner et al., 2015). Furthermore, presence of a client and Hsp40 supports dimerization of Hsp70 and substrate binding (Morgner et al., 2015).

ATP binding to the NBD results in the opening of a SBD α-helical lid, stimulating binding of substrate proteins through interactions with the NBD and SBD (Jiang et al., 2005). The ADP-bound state results in the closing of the α-helical lid over the substrate-binding cleft and stabilizes client association (Schlecht et al., 2011). The Hsp70 chaperone cycle is inherently slow due to the low ATPase activity of Hsp70 (Swain et al., 2007). Thus, a family of J proteins recruits the client to Hsp70 and stimulates the Hsp70 ATPase activity (Misselwitz et al., 1998). The J protein then dissociates from the ternary complex and a nucleotide-exchange factor releases the bound ADP from Hsp70 returning it to the apo-conformation. This leaves the NBD available for recruitment of ATP, upon which the

α-helical lid can "open" and release the client peptide (Misselwitz et al., 1998; Schlecht et al., 2011). The cycle repeats in an interactive process until the client peptide adopts its native structure or is passed on to another part of the chaperone machinery.

Hsp70 class of chaperones typically recognizes client proteins in the early stages of folding through short hydrophobic sequences rich in leucine residues (Rudiger et al., 1997). Such hydrophobic stretches are greatly exposed during the early stages of protein translation and folding, leading to unfavorable intra- and inter-molecular interactions (Hartl and Hayer-Hartl, 2009). Hsp70 binding to client proteins in the early stages of protein folding controls the availability of such regions, facilitating formation of the proper protein fold, while inhibiting aggregate formation. If proper folding of the client is not possible, Hsp70 association with additional co-chaperones promotes degradation of the misfolded protein (Meacham et al., 2001; Petrucelli et al., 2004; Jana et al., 2005; Dickey et al., 2007; Muller et al., 2008). The abundance of exposed hydrophobic stretches in proteins prone to self-association in neurodegenerative diseases draws parallel to proteins in early stages of folding, which indicates a potential role of the Hsp70/Hsp90 machinery in modulating pathogenic aggregate formation (Muchowski and Wacker, 2005; Hartl and Hayer-Hartl, 2009).

HSP90

Hsp90 is a highly conserved molecular chaperone from yeast to mammals and it is essential for proper folding and maintenance of its client proteins in eukaryotic cells (see **Figure 1**). Over 500 physical and genetic interactions have been identified in yeast spanning diverse families of cellular proteins, which include transcription factors, steroid hormone receptors, and protein kinases (Zhao et al., 2005; McClellan et al., 2007). Through concerted efforts involving other chaperones and co-chaperones it drives the final maturation of client proteins. There are two major isoforms of Hsp90 identified in the cytosol, inducible Hsp90α and constitutively expressed Hsp90β, as well as Grp94 in the endoplasmic reticulum (Csermely et al., 1998).

Hsp90 forms a homodimeric biologically functional unit composed of three distinct regions connected by flexible linker. Each protomer contains a highly-conserved N-terminal domain (NTD) responsible for nucleotide binding, a middle domain (MD) important for client recognition and ATP hydrolysis and a C-terminal domain (CTD), which is the primary site responsible for dimerization (Pearl and Prodromou, 2006; Taipale et al., 2010). Additionally, the CTD contains a conserved "MEEVD" sequence used for interactions with the large tetratricopeptide repeat domain class of co-chaperone proteins, which regulate Hsp90 protein activity (Young et al., 1998).

The NTD contains a deep ATP/ADP binding pocket evolutionarily conserved amongst the GHKL (gyrase, Hsp90, histidine kinase, MutL) ATPase superfamily (Meyer et al., 2003). The binding pocket is composed of multiple α-helices and a β-sheet flanking the bound ATP molecule (Prodromou et al., 1997). Intramolecular recruitment of the MD on each protomer brings critical residues into contact forming the required split ATPase active site necessary for ATP hydrolysis (Ali et al., 2006). The N-terminal region contains an additional molecular lid mechanism, which crosses over the nucleotide-binding pocket in the ATP-bound state and holds the nucleotide in place, but remains in the open conformation upon ADP-binding (Ali et al., 2006). See **Figure 1** for an overview of the Hsp90 conformations when bound to ADP or ATP.

Nucleotide binding and hydrolysis are directly coupled to large structural rearrangements in Hsp90 that are regulated through interactions with client and co-chaperone proteins (Csermely et al., 1993; Kirschke et al., 2014; Lavery et al., 2014). Apo-Hsp90 presents an "open" conformation where the two protomers form a V-shaped dimer (Vaughan et al., 2010). Nucleotide binding induces an intermediate-state where the N-terminal lid cradles ATP within the nucleotide-binding pocket. This results in a second repositioning of the N-terminal region favoring its dimerization and recruitment of the MD for ATP hydrolysis through a conserved arginine (R380 in yeast) that contacts the γ-phosphate of ATP (Cunningham et al., 2012). Finally, ATP is hydrolyzed and Hsp90 reverts to the "open" conformation where the N-terminal domains dissociate, allowing for the repetition of the cycle. ATP hydrolysis and the structural rearrangements in Hsp90 during client refolding are influenced and regulated by a diverse set of co-chaperones (Zuehlke and Johnson, 2010). These co-chaperones have varying effects on the ATPase activity and conformational rearrangements in Hsp90. Post-translational modifications of Hsp90 include acetylation, nitrosylation, phosphorylation, and methylation, which has been elegantly reviewed by Li and Buchner (2013).

While ATPase induced conformational rearrangements in Hsp90 are well-understood, their influence on client protein folding remains enigmatic. Hsp90-directed folding occurs late during the folding of nascent peptides. Structural studies involving binding to intrinsically disordered tau suggest that substrate recognition occurs through low-affinity hydrophobic interactions between the client protein and a large substrate-binding interface on Hsp90 (Zuehlke and Johnson, 2010; Karagoz et al., 2014). This mechanism allows for detection of scattered exposed hydrophobic patches in protein folding intermediates. Interestingly, Hsp90 affinity for tau appears to be independent of ATP binding as both the apo and ATP-bound forms possess equal affinities for tau (Karagoz et al., 2014).

The prominent role and poor prognosis of Hsp90 overexpression in various cancers has led to the development of a number of Hsp90 inhibitors (Roe et al., 1999; Trepel et al., 2010). Hsp90 inhibition results in the downregulation of many oncogenic client proteins that require Hsp90 for maturation. The most prominent drugs inhibit the ATPase activity of Hsp90 and are based on the geldanamycin, radicicol, or purine derivatives, which function as competitive inhibitors of ATP binding to Hsp90 (Roe et al., 1999; Sidera and Patsavoudi, 2014). Initial geldanamycin and radicicol derivatives proved to be potent inhibitors of Hsp90; however,

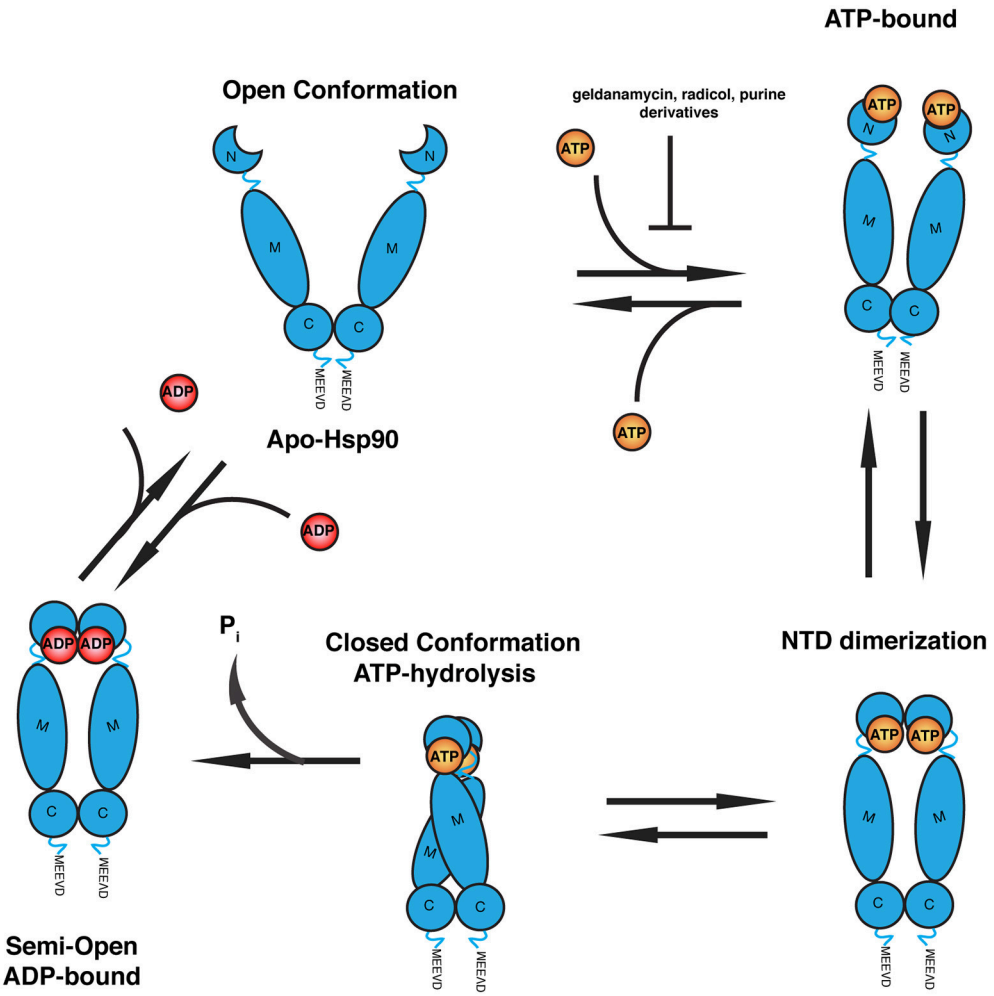

FIGURE 1 | Schematic of the ATPase cycle of Hsp90. Hsp90 homodimer initially adopts an open V-shaped conformation. Binding of ATP to the N-terminal ATPase domain induces a conformational change where the N-terminal lids close and ATP is cradled in the nucleotide-binding pocket. This induces dimerization of the N-terminal domains of each homodimer followed by closure of Hsp90 and recruitment of the M domain for ATP hydrolysis. The dimers dissociate into a semi-open intermediate state with ADP bound. Release of ADP dissociates the N-termini to allow repetition of the ATPase cycle. Common Hsp90 inhibitors (geldanamycin, radicicol, and purine derivatives) bind to the N-terminus of Hsp90 and compete with ATP for binding.

their therapeutic value is low due to severe toxicity by targeting Hsp90 in normal cells (Jhaveri et al., 2012; Trendowski, 2015).

Co-chaperones along with individual Hsps comprise a chaperone network that is altered in malignancy and neurodegeneration (Workman et al., 2007; Moulick et al., 2011; Lindberg et al., 2015; Rodina et al., 2016). Targeting specific co-chaperones or chaperone complexes may help to avoid cytotoxicity by directly targeting Hsp90 activity (Yi and Regan, 2008; Moulick et al., 2011; Rodina et al., 2016). The purine derivative PU-H71 possesses unique selectivity amongst Hsp90 inhibitors, preferentially targeting high-molecular-weight complexes composed of Hsp70/90 and various co-chaperones and client proteins, which are enriched in numerous malignant cell models, but absent in non-oncogenic tissue (Moulick et al., 2011; Rodina et al., 2016). The formation of these large stable chaperone species appears to be cancer specific and diagnostic proteomic approaches may serve as a method to clinically screen patients that are most likely to benefit from targeting such species. Whether large and stable chaperone complexes with misfolded proteins occur in different neurodegenerative diseases is currently unclear. Indeed, therapeutic approaches targeting chaperones in neurodegeneration still fall behind from those in cancer cells.

Hsp70 and Hsp90 both interact with many co-chaperones containing tetratricopeptide repeat (TPR) domains, which consist of three or more 34-amino acid residues (Lamb et al., 1995). These motifs form anti-parallel α-helices (Allan and Ratajczak, 2011) that bind to the C-terminus of the chaperone and are the main interaction site for co-chaperones (Smith, 2004), along the EEVD peptide motif on Hsp70 and Hsp90 (Kajander

et al., 2009). Proteins containing TPR domains typically share no other sequence homology, but are commonly found to be involved in regulation of cell cycle, protein trafficking, phosphate turnover, and transcriptional events (Blatch and Lassle, 1999). TPR domain-containing co-chaperones regulate the ATP cycle of chaperones and aid in client transport to binding pockets, where they are folded. Hsp40 may help coordinate other co-chaperones in binding Hsp70, such as Hsp70-interacting protein (Hip; Hohfeld et al., 1995) in the early stages of the chaperone cycle, as well as STI1 and SGT (Allan and Ratajczak, 2011). STI1 is also a co-chaperone for Hsp90, along with p23, Cdc37, and the immunophilins peptidyl-prolyl cis-trans (PPIases) isomerases FKBP51 and FKBP52, phosphatase PP5 and the cyclophillin Cyp40 (Allan and Ratajczak, 2011). Some of these co-chaperones inhibit Hsp90 ATP turnover (Rehn and Buchner, 2015). C-terminal Hsp70-interacting protein (CHIP) is also a co-chaperone for both Hsp70 and Hsp90. In this review, we will focus mainly on the roles of Hsp70, Hsp90, and the co-chaperone STI1 in protein misfolding. We will also discuss the unique cytokine-like activities of STI1. Importantly, both the extracellular and intracellular activities of STI1 seem to converge to increase cellular resilience (Beraldo et al., 2013). Moreover, we will briefly describe some of the co-chaperones that may also have a role in protein misfolding diseases, such as CHIP and high molecular weight immunophilins.

HSP70/HSP90 PARTNERS IN NEURODEGENERATIVE DISEASES

There is a number of Hsp70 and Hsp90 co-chaperones that have implications for neurodegenerative diseases. High molecular weight FK506-binding proteins (FKBPs) FKBP51 and FKBP52 impose a variety of effects on tau structure and function, which will be discussed further in the AD subsection of this review. Another co-chaperone, a high molecular weight PPIase Cyp40, can bind Aβ and regulate import of Aβ into mitochondria. Interestingly, inhibiting Cyp40 was found to be protective against Aβ-toxicity in mitochondria and neurons in an amyloid-precursor protein (APP) transgenic mouse model (Du and Yan, 2010). This suggests that Aβ may be regulated by an Hsp90/PPIase complex, but further investigation is required. For a more extensive review on PPIases in regulating the levels and toxicity of proteins in AD, see excellent review by Blair et al. (2015).

The Hsp90 co-chaperone p23 typically comes into play in a mature Hsp90-client complex (Felts and Toft, 2003), whereby it inhibits ATP turnover on Hsp90 (Rehn and Buchner, 2015). In the context of AD, p23 has been found to bind γ-secretases and to promote the non-amyloidogenic pathway of amyloid precursor protein (ultimately reducing production of Aβ species; Vetrivel et al., 2008) and silencing of p23 gene expression reduced the levels of total tau and phosphorylated tau (Dickey et al., 2007).

Hsp70 and Hsp90 are critical to folding and maturation of a number of clients, but they are also key players in regulation of the proteasome, a simplified representation of this activity can be found in **Figure 2**. CHIP, a Hsp70-Hsp90 co-chaperone, is an E3 ubiquitin ligase (Ballinger et al., 1999; McDonough and Patterson, 2003) that promotes client degradation via the proteasome. CHIP contains three TPR motifs at its N-terminus, a middle domain of charged residues (Ballinger et al., 1999) and a C-terminus containing a U-Box domain (Zhang et al., 2005). U-Box domains are characteristic of proteins involved in ubiquitination (Aravind and Koonin, 2000). Within this pathway, ubiquitin molecules are covalently attached to the protein of interest, and then recruited to the proteasome complex for degradation. CHIP interacts with Hsp70 and Hsp90 via TPR domain and then can bind with the substrate protein of interest, promoting substrate ubiquitination. Of note, CHIP is capable of binding tau and is responsible for its ubiquitination (Petrucelli et al., 2004), a critical activity that may be of importance for tauopathies.

Transcriptional regulation of the heat shock response is mediated by HSF1. HSF1 activity is dependent upon levels of chaperones and misfolded proteins, and other environmental stressors such as heat, aging, and changes in osmosis. Alternatively, Hsp70 and Hsp90 can negatively regulate the activation of HSF1, suppressing the heat shock response, allowing for a recovery period after the stressor is no longer present. Once in the nucleus, HSF1 trimerizes and binds Hsp gene promoters to activate transcription. Dai et al. (2003) further showed that this trimerization of HSF1 is mediated by CHIP, since both localize to the nucleus in response to cellular stress and form a complex once HSF1 is bound to DNA. Moreover, once degradation of misfolded proteins is complete, CHIP begins to degrade Hsp70 (unbound to any client). Therefore, CHIP stimulates HSF1 upregulation of Hsp70 in stress conditions and mediates its degradation to basal levels in the recovery period from stress.

STI1/HOP Co-chaperone Structure and Function

STI1 was discovered in 1989, as a protein that is upregulated during cellular stress in yeast (Nicolet and Craig, 1989). STI1 is a modular protein composed of three TPR domains (TPR1, TPR2A and TPR2B) and two domains rich in aspartate and proline residues (DP1 and DP2, see **Figure 3**). The TPR domains of STI1 bind Hsp70 and Hsp90 to facilitate client protein transfer. While the C-terminal domain of Hsp90 is critical for the binding of TPR domains, additional contacts are made with the middle domain of Hsp90 (Lee et al., 2012; Schmid et al., 2012). Binding of Hsp90 by STI1 results in non-competitive inhibition of its ATPase activity through interaction with TPR2A-TPR2B fragment and stabilizes Hsp90 in an open conformation. However, the human homolog of STI1, HOP, appears to be ∼10-fold less potent as an inhibitor of the Hsp90 ATPase activity (Prodromou et al., 1999; Richter et al., 2008). Hsp70 and Hsp90 engagement is facilitated through sequential interactions with the individual TPR domains of STI1 (Rohl et al., 2015b). The function of the DP domains (DP1 and DP2) is less clear. The minimal fragment of STI1 that supports client activation is composed of TPR2A-TPR2B-DP2 (Schmid et al., 2012; Rohl et al., 2015b). The *Caenorhabditis elegans*

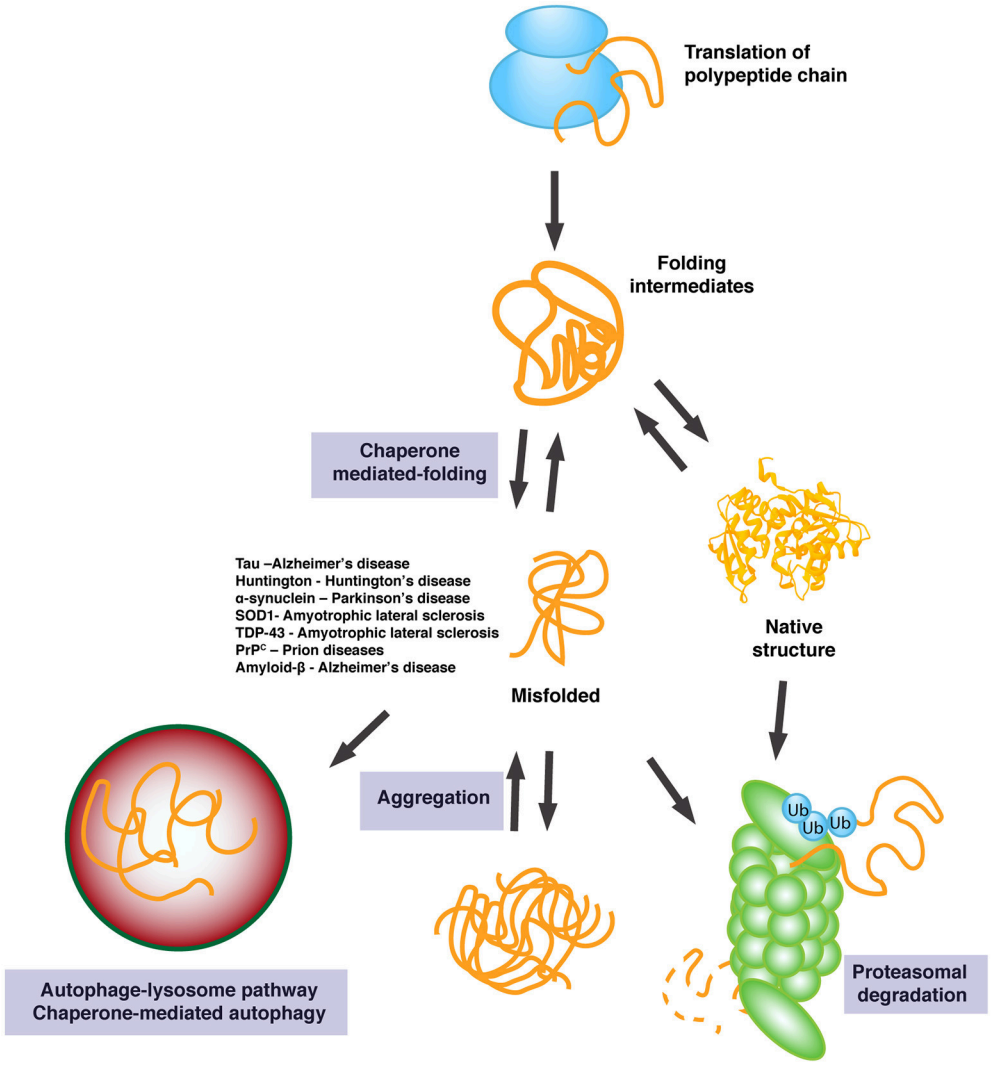

FIGURE 2 | Outline of the chaperone response in protein folding. Chaperones facilitate proper folding of a diverse array of client proteins and prevent oligomer and aggregate formation. If folding is not possible, misfolded proteins are targeted for protein degradation to maintain proper protein homeostasis. Degradation is achieved through the ubiquitin-proteasome system (UPS) or the autophagy-lysosome pathway. A list of diseases and the associated aggregate discussed in this review are outlined.

STI1 homolog, CeHOP, contains only the TPR2A-TPR2B-DP2 module, but is still capable of binding Hsp70 and Hsp90, though not simultaneously (Gaiser et al., 2009). Deletion of CeHOP is not lethal, but mutant worms have deficits in sexual development and reduced resistance to heat stress (Gaiser et al., 2009; Song et al., 2009). Functionality of CeHOP suggests the TPR1-DP1 module may be dispensable for client activation *in vivo* (Gaiser et al., 2009).

Interestingly, STI1-based constructs lacking the DP2 domain did not support glucocorticoid receptor activation, one of STI1 functions in yeast cells (Schmid et al., 2012). X-ray crystallographic structures of the TPR2A-TPR2B domain revealed the linker between these domains is quite rigid. As a result, the TPR domains adopt an S-shaped form with their hydrophobic clefts responsible for binding the C-terminal Hsp90 residues oriented in opposite directions (Schmid et al., 2012). Complementary NMR spectroscopy experiments revealed that additional inter-domain contacts are formed between the C-terminal helix of TPR2B, the linker connecting TPR2B to the DP2 domain and α-helices 1 and 2 of the DP2 domain (Rohl et al., 2015b). These additional contacts form a rigid C-terminus composed of TPR2A-TPR2B-DP2. These results indicate that DP2 contributes to the quaternary structure of STI1, underlying its importance in STI1 function.

STI1 possesses two Hsp70 binding sites located in TPR1 and TPR2B; however, it binds to Hsp70 in a 1:1 stoichiometry (Scheufler et al., 2000; Rohl et al., 2015b). The current model for STI1 function in Hsp70 and Hsp90 coordination proposes

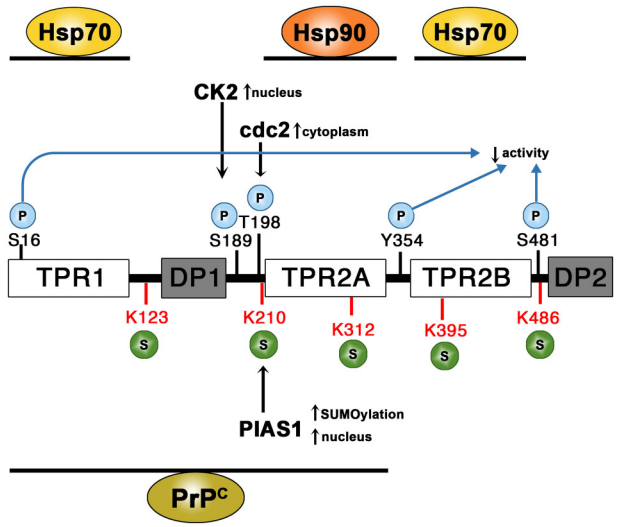

FIGURE 3 | Domain structure of STI1 and sites of post-translational modifications (PTM). STI1 is composed of three structurally similar tetratricopeptide repeat domains (TPR1, TPR2A, and TPR2B) and two regions rich in aspartate and proline residues (DP1 and DP2). The protein is subject to phosphorylation (S16, S189, T198, Y354, and S481). CK2 phosphorylation at S189 induces STI1 accumulation in the nucleus. In contrast phosphorylation by cdc2 at T198 localizes STI1 to the cytoplasm. Five possible SUMOylation sites have been identified (K123, K210, K312, K395, and K486). SUMOylation by PIAS1 at K210 may stimulate SUMOylation at the alternate sites. Association of PIAS1 with STI1 by a SUMO-independent mechanism increases STI1 nuclear accumulation. Regions that bind HSP70, HSP90, and PrP^C are illustrated.

that in the absence of Hsp90, the TPR2B domain represents the high-affinity binding site for Hsp70, these interaction sites are shown in **Figure 3**. Hsp90 binding induces a more "open" conformation between TPR1-DP1 fragment and the functional C-terminal TPR2A-TPR2B-DP2 domains. Hsp90 binding to TPR2A reduces accessibility of Hsp70 binding to TPR2B, thus the TPR1 domain becomes the predominant binding site for Hsp70 in the ternary complex (Rohl et al., 2015b). Binding of Hsp90 may also reorient the TPR1-DP1 module into close proximity to TPR2B, presumably facilitating transfer of client protein (Rohl et al., 2015b). Thus, the length of the linker bridging TPR1-DP1 to TPR2A-TPR2B-DP2 impacts STI1 function in client refolding (Rohl et al., 2015b). Deletion of the linker results in decreased formation of ternary complexes of STI1-Hsp70-Hsp90 and decreased protein client activation *in vivo* (Rohl et al., 2015b).

STI1 is widely expressed in most tissues and is typically localized in the cytoplasm, but can be found associated with the Golgi (Honore et al., 1992) and in the nucleus (Longshaw et al., 2004; Beraldo et al., 2013; Soares et al., 2013). Nuclear accumulation of STI1 is characteristic of stressed cells (Beraldo et al., 2013).

STI1 is subject to posttranslational modifications, which regulate its co-chaperone activity (Rohl et al., 2015a). Five different phosphorylation sites have been identified in the human STI1 homolog HOP corresponding to S16, S189, T198, Y354, and S481, see **Figure 3** for simplified STI1 structure and respective phosphorylation sites. Phosphomimetic mutations resulted in decreased glucocorticoid receptor activation *in vivo* and Hsp70 binding affinities indicating that phosphorylation regulates STI1 co-chaperone function (Rohl et al., 2015a). Interestingly, Y354E phosphomimetic variant located in the loop joining TPR2A-TPR2B appeared to disrupt the rigid linker joining the two domains and promotes a more dynamic flexibility causing loss of function (Rohl et al., 2015a).

STI1 is typically found in the cytosol, but it can shuttle between the nucleus and cytoplasm (Longshaw et al., 2004), due to the presence of a nuclear localization signal (NLS) at amino acids 222–239 (of mouse STI1). Specifically, phosphorylation of STI1 by casein kinase II (CKII) and cell division cycle kinase II (cdc2) at S189 and T198 (respectively), contiguous to STI1 NLS (Longshaw et al., 2000) regulates nuclear localization of STI1 (Longshaw et al., 2004). CKII stimulates cellular growth by promoting cells to enter G1 phase of cell cycle (Pyerin, 1994), whereas cdc2 favors cell division (Matsumoto and Fujimoto, 1990). The rate of STI1 export from the nucleus is much higher than import, which can be inhibited with leptomycin B, a nuclear export inhibitor (Longshaw et al., 2004). Of particular interest, STI1 interacts with the nuclear small ubiquitin-like modifier (SUMO) E3 ligase family protein inhibitor of activated STAT (PIAS) and many other components of the SUMOylation machinery (Soares et al., 2013), suggesting potential STI1 regulation of genotoxic stress responses. STI1 was retained in the nucleus of astrocytes overexpressing PIAS1 (Soares et al., 2013) and Hsp90 was also found to be appreciably colocalized with STI1 and PIAS1 in the nucleus. Specifically, PIAS1 can poly-SUMOylate STI1 at several lysine residues: K123, K210, K312, K395, and K486 (depicted in **Figure 3**; Soares et al., 2013) and it is proposed that K210 hierarchically regulates SUMOylation of the other sites. Co-transfection of HEK293 cells with SUMO, PIAS1, and STI1 increased SUMOylation of STI1, but it was the interaction between PIAS1 and STI1 that supported nuclear retention, not SUMOylation. This suggests that nuclear localization of STI1 may alter its co-chaperone activities with Hsp90 and can help with nuclear protein crowding in particular sites that facilitate protein-protein interactions, such as PML nuclear bodies (Soares et al., 2013). However, STI1 has also been shown to function as a scaffold to recruit Hsp90 for other nuclear functions, including regulation of canalization or developmental robustness, by controlling Piwi and regulating Piwi-interacting RNA and impacting transposons (Gangaraju et al., 2011; Karam et al., 2017).

Deletion of STI1 is not lethal in yeast (Flom et al., 2007) and in *C. elegans* elimination of STI1 caused reduced lifespan (Song et al., 2009). Interestingly, knockout of STI1 in mice is embryonically lethal by E10.5 (Beraldo et al., 2013). Half of STI1-null blastocysts also failed to thrive, suggesting a key role for STI1 early in development (Beraldo et al., 2013). Homozygous embryos have a 50% reduction in Hsp90 client protein expression (p53, GRK2, STAT3—all clients that when knocked out are embryonically lethal; Beraldo et al., 2013). Mouse embryonic fibroblast lacking STI1 also failed to thrive in culture. Moreover, astrocytes derived from STI1 haplo-sufficient mice are also less resilient to irradiation (Soares et al., 2013), and neurons are less

resistant to oxygen-glucose deprivation (Beraldo et al., 2013) or β-amyloid toxicity (Ostapchenko et al., 2013). This indicates that in higher organisms, STI1 is essential for regulating cellular resilience.

Cytokine-Like Activity of STI1

STI1 is a critical co-chaperone in the functionality of the Hsp70/Hsp90 machinery in cells. However, there is also a large body of literature that focuses on the extracellular effects of STI1, specifically the consequences of interaction with the cellular prion protein (PrP^C; Zanata et al., 2002; Lopes et al., 2005; Caetano et al., 2008; Arantes et al., 2009; Beraldo et al., 2010, 2013; Roffe et al., 2010; Hajj et al., 2013; Ostapchenko et al., 2013; Maciejewski et al., 2016), which is illustrated in **Figure 4**. PrP^C is anchored to cell membranes by a glycosylphosphatidylinositol (GPI) moiety and it is thought to serve as a molecular scaffolding protein organizing signaling complexes (Linden et al., 2008).

STI1 must be secreted in order to interact with PrP^C at the cell surface. Like Hsp70 and Hsp90 (Clayton et al., 2005; Lancaster and Febbraio, 2005), STI1 can be secreted via exosomes (Hajj et al., 2013). PrP^C is also secreted by exosomes and both PrP^C and STI1 can be found on the surface of the exosomes derived from astrocytes (Hajj et al., 2013). Secreted STI1 can then interact with and bind to the surface of neurons (Hajj et al., 2013), having a variety of effects on cell growth and survival, in a PrP^C-dependent manner.

Martins, Linden and Brentani (Chiarini et al., 2002; Zanata et al., 2002) were the first to report the interaction between STI1 and PrP^C and to describe STI1 as a signaling molecule. Notably, interaction between STI1 and PrP^C reduced apoptotic cell death induced by the protein synthesis inhibitor anisomycin. These effects were confirmed to be PrP^C-dependent using PrP-null neurons or exogenous treatment with a truncated form of STI1 lacking a critical PrP^C binding site (STI1Δ230–245). Lopes et al. (2005) found that cultured neurons treated with exogenous STI1 are more resilient to protein synthesis inhibitors, but this increased neuronal resilience required activation of cAMP-PKA pathway. STI1-PrP^C engagement is also capable of stimulating neuronal differentiation and this was dependent upon activation of the mitogen-activated protein kinase (MAPK/ERK) signaling pathway (Lopes et al., 2005). Roffe et al. (2010) showed that STI1-PrP^C interaction increased protein synthesis in hippocampal neurons via mTOR and this was dependent upon the MAPK/ERK and PI3K signaling pathways. STI1 and PrP^C interact briefly at the cell surface and are quickly internalized by distinct cellular pathways, limiting the levels of signaling activation by STI1 (Caetano et al., 2008). Activation of PKA and ERK signaling cascades by STI1-PrP^C interaction is in part due to calcium signaling (Beraldo et al., 2010). STI1-PrP^C signaling in hippocampal neurons requires α7 nicotinic acetylcholine receptors and inhibition of these receptors with α-bungarotoxin or use of knockout neurons eliminates STI1-PrP^C neuroprotection (Beraldo et al., 2010; Ostapchenko et al., 2013). Finally, STI1-PrP^C association supports proliferation and pluripotency of neural stem cells and promotes neurosphere formation (Santos et al., 2011). These findings altogether suggest that STI1 is critical for proper growth, development, and resilience to cellular stress.

Decreased STI1 levels significantly reduces cellular tolerance and cells display stress phenotypes, such as increased STI1 nuclear localization and nuclear labeling for γ-H2AX, a marker for double stranded DNA breaks, as seen in germ-line knockdown of STI1 in Drosophila (Karam et al., 2017) and in mouse embryonic fibroblasts (Beraldo et al., 2013). Astrocytes derived from STI1 haplo-insufficent mice also secrete 50% less STI1, which has consequences on PrP^C-dependent resilience. Exogenous treatment with recombinant STI1 in neuronal cultures subjected to oxygen-glucose deprivation (OGD) reduced levels of cellular death in a PrP^C and α7 nicotinic receptor-dependent manner (Beraldo et al., 2013), further supporting the

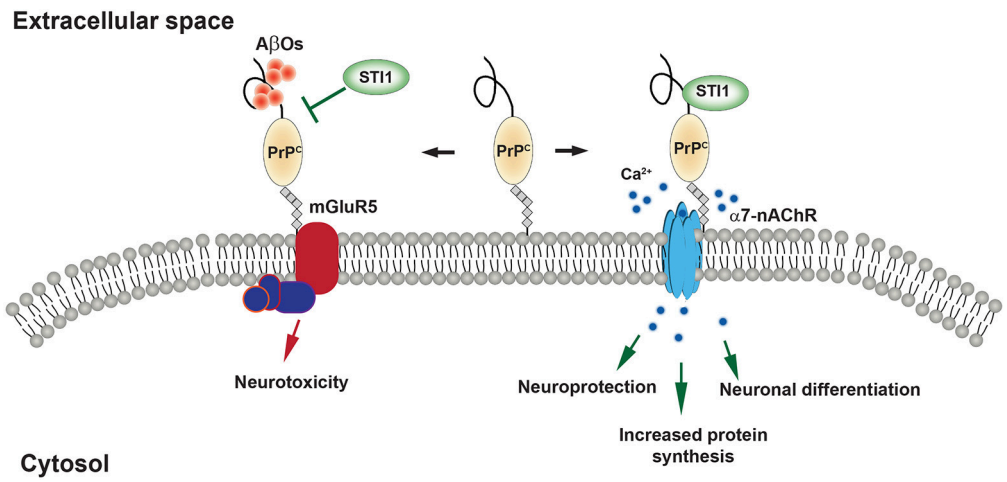

FIGURE 4 | STI1 signaling mediated by the cellular prion protein (PrP^C). (Right) PrP^C binding to extracellular STI1 induces neuroprotective and neuro-differentiation through Ca^{2+} influx via α7-nAChR. **(Left)** Aβ oligomers transmit toxic signaling events through PrP^C. STI1 inhibits Aβ oligomer binding to PrP^C and/or activate protective signaling events.

notion that extracellular STI1 is responsible for inducing some of these neuroprotective effects. Neuronal cultures from PrP-null animals were not protected from OGD upon STI1 treatment, further indicating that these effects are indeed PrP^C-dependent.

Middle cerebral artery occlusion, a model for ischemic stroke was conducted on STI1-haplosufficient mice (Beraldo et al., 2013), which had increased mortality and a reliably larger infarct volume compared to control littermates. Additionally, Lee et al. (2013) found an upregulation of STI1 immunoreactivity in brains from MCAO rats and post-mortem tissue of stroke patients. STI1 contains a hypoxia response element within its promoter region, which can be activated by hypoxia-inducible factor 1-α (HIF1-α; Lee et al., 2013). This binding event is responsible for the elevated STI1 levels post-stroke, as knockout for HIF1-α or lentiviral shRNA administration to mice inhibited the increase in STI1 immunoreactivity around the infarct. Lee et al. (2013) also showed that STI1 increases proliferation of bone-marrow derived cells and recruits these cells to the areas of damage, as a means to promote and accelerate recovery. Together, these studies provide strong evidence that extracellular STI1 is required for recovery of post-ischemic insult and that exogenous treatment with STI1 could potentiate the recovery process.

It is educational also to learn how STI1 can signal in cancer cells, as some of the signaling pathways may be similar to those in neurons. In the context of cancer, increased proliferative capacity due to STI1 is a major problem. Wang et al. (2010) found that levels of STI1 were much higher in malignant vs. benign ovarian tumors. Additionally, serum levels of STI1 were ∼6 times higher in ovarian cancer patients (Wang et al., 2010) and were being secreted by the cancerous cells. Activation of the ERK signaling pathway by secreted STI1 was responsible for the increased proliferative capacity of these cancerous cells. Elevated levels of STI1 were correlated with worsened prognosis in ovarian cancer patients (Chao et al., 2013), making this a useful biomarker for this type of cancer. Recently, Wang et al. (2017) found increased intracellular and extracellular levels of STI1 in renal cell carcinoma (RCCs) tumor cells. Increased proliferation was mediated by the activin A receptor, type II-like kinase 2 (ALK2), and the receptor regulated SMAD1/5 protein signaling cascade, independent of PrP^C. Only differentiation of the osteoclasts from the RCCs was dependent upon STI1-PrP^C signaling. These studies provide further evidence that STI1 acts as a cytokine-like signaling molecule, promoting cellular growth by activating PrP^C dependent and independent pathways.

PROTEIN QUALITY CONTROL IN NEURODEGENERATIVE DISEASES

Protein misfolding can lead to the formation of aggregates in diverse neurodegenerative diseases, such as AD, PD, ALS, frontotemporal dementia (FTD), HD, and prion diseases (Knowles et al., 2014). Protein aggregates are formed by highly ordered filamentous inclusions with β-sheet conformation in the core. Deposits can be fibrillar and insoluble (Chiti and Dobson, 2006), fibrillary but with some degree of solubility (Han et al., 2012), or amorphous. The composition of these deposits is often specific for each disease and composed of one predominant protein in each of these diseases, such as β-amyloid, tau, huntingtin, α-synuclein or prion protein (Goedert and Spillantini, 2006). These proteins will be further discussed in following subsections with relation to the disease the misfolded species are typically found in.

Degradation of misfolded proteins by the ubiquitin-proteasome system (UPS) or the autophagy-lysosome pathway (ALP; Labbadia and Morimoto, 2015; Yerbury et al., 2016) have been regarded as potential therapeutic targets for the treatment of neurodegenerative diseases, as aging leads to decreased efficiency of protein quality control (Ben-Zvi et al., 2009; Wang J. et al., 2009). Ubiquitination of misfolded proteins ultimately leads to degradation of proteins by the 26S proteasome and release of the ubiquitin chain (Pickart, 2001).

Proteins with longer half-life are predominantly degraded via the ALP. The role of chaperone-independent macroautophagy (which leads to the formation of autophagosomes) in the central nervous system of mammals is well-documented (Grant and Donaldson, 2009; He and Klionsky, 2009). In addition, chaperone-mediated autophagy (CMA) has been shown to protect against accumulation of tau, α-synuclein, and polyQ-huntingtin (Htt) in models of tauopathy, PD, and HD, respectively (Wang Y. et al., 2009; Qi et al., 2012; Xilouri et al., 2013). As shown by Agarraberes and Dice (2001), CMA requires the formation of complexes of chaperones and co-chaperones, including Hsp40, Hsp70, STI1/HOP, Hsp70-interacting protein (Hip), and Bcl2-associated athanogene 1 protein (BAG-1). Increasing evidence has shown that disrupted autophagy—either through autophagosomes or CMA—and lysosomal mechanisms contribute to the pathogenesis of AD, PD, HD, ALS, and FTD (Anglade et al., 1997; Nagata et al., 2004; Yu et al., 2005; Morimoto et al., 2007; Hu et al., 2010; Cuervo and Wong, 2014; Nah et al., 2015).

The following subsections will briefly discuss PD, HD, ALS, prion diseases and AD, and the effects of Hsp70, Hsp90, and STI1 (if investigated) on the misfolded protein species in each of these diseases and it is also summarized briefly in **Table 1**. We will also discuss how modulating the levels or activities of these chaperones affects protein toxicity and aggregative-capacity.

Chaperones in Synucleinopathies

PD is the second most common neurodegenerative disease characterized mainly by loss of dopaminergic neurons that project from the Substantia nigra, although several other neuronal groups are also affected (Davie, 2008). Several genes have been linked to genetic forms of PD, and amongst them *SNCA*, that codes for α-synuclein is of particular interest. α-synuclein, a protein associated with neuronal membranes (Maroteaux and Scheller, 1991), can aggregate in plaques in AD, termed the non-amyloid component (Ueda et al., 1993). However, α-synuclein is more commonly associated with PD, where it forms filamentous intraneuronal inclusions composed of ubiquitinated and phosphorylated α-synuclein, a component of Lewy bodies (Trojanowski et al., 1998; Goedert, 1999). This results in loss of neurons, and of importance to symptoms in PD, dopaminergic neurons in the Substantia nigra are particularly

TABLE 1 | Overview comparing Hsp70, Hsp90, STI1, and/or Hsp40 protein quality control in various model organisms of neurodegenerative disease.

Disorder	Model	Hsp70	Hsp90	STI1	Hsp40
Parkinson's disease	*In vitro* or cell line or yeast	↓ α-synuclein fibril formation *in vitro* (Roodveldt et al., 2009). Heat shock-induced ↑ Hsp70, ↓ α-synuclein inhibition of proteasome in human fibroblasts (Lindersson et al., 2004); Hsp70 overexpression in neuroglioma cells ↓ α-synuclein oligomerization (Outeiro et al., 2008)	Hsp90 ↓ α-synuclein fibril formation *in vitro* (Falsone et al., 2009); ↑ ROS in Hsp90 haploid deletion yeast mutants by α-synuclein (Liang et al., 2008); Hsp90 inhibition in neuroglioma ↓ α-synuclein oligomerization and toxicity (Putcha et al., 2010)	STI1 ↓ monomeric A53T α-synuclein aggregation *in vitro* (Daturpalli et al., 2013)	
	Animal model	↑ Synuclein inclusions in Hip knockdown (Roodveldt et al., 2009), dependent on Hsp70 Hsp70 deletion in *D. melanogaster* ↑ α-synuclein (Auluck et al., 2002); ↑ Hsp70 in mice ↓ α-synuclein oligomerization (Klucken et al., 2004); Hsp70 injection into s.nigra ↓ dopaminergic cell loss in rats (Dong et al., 2005)			
Huntington's disease	*In vitro* or cell line or yeast	Hsp70 overexpression ↓ Htt aggregates and toxicity in yeast and various cell lines (Warrick et al., 1999; Carmichael et al., 2000; Jana et al., 2000; Krobitsch and Lindquist, 2000; Wacker et al., 2004)		STI1 overexpression in yeast ↓ Htt toxicity, promoted reorganization to foci, Hsp70/TPR1-dependent (Wolfe et al., 2013)	Hsp40 overexpression ↓ Htt aggregates and toxicity in yeast and various cell lines (Warrick et al., 1999; Carmichael et al., 2000; Jana et al., 2000; Krobitsch and Lindquist, 2000; Wacker et al., 2004)
	Animal model	Deletion of Hsp70 in mice ↑ inclusion body size (Wacker et al., 2009); Hsp70 overexpression ↓ Htt aggregates and toxicity; Hsp70 silencing in *C. elegans* ↑ Q35 aggregation (Brehme et al., 2014)	Hsp90 silencing in *C. elegans* ↑ Q35 aggregation (Brehme et al., 2014)	STI1 silencing in *C. elegans* ↑ Q35 aggregation (Brehme et al., 2014)	Hsp40 silencing in *C. elegans* ↑ Q35 aggregation (Brehme et al., 2014); Hsp40 expression in *D. melanogaster* ↓ Htt toxicity (Kazemi-Esfarjani and Benzer, 2000)
ALS	*In vitro* or cell line or yeast	Hsp70 binds and regulates TDP43 nuclear accumulation in HeLa (Freibaum et al., 2010; Udan-Johns et al., 2014); Hsp70 knockdown in N2a ↑ phospho-TDP43 and C-terminal fragment (Zhang et al., 2010); Heat shock-induced Hsp70 in HEK293 ↓ insoluble and hyperphosphorylated TDP-43 species (Chen et al., 2016)	Pharmacological Hsp90 inhibition in HeLa ↓ levels of full length TDP43 (Lotz et al., 2003)		Hsp40 binds and regulates TDP43 nuclear accumulation in HeLa (Freibaum et al., 2010; Udan-Johns et al., 2014); ↑ DnaJC5 in HEK293 ↑ disease-associated cleaved TDP43 secretion (Fontaine et al., 2016); Heat shock-induced Hsp40 in HEK293 ↓ insoluble and hyperphosphorylated TDP-43 species (Chen et al., 2016); ↓ Hsp40 in N2a ↑ phospho-TDP43 and C-terminal fragment (Zhang et al., 2010)
	Animal model	↓ Hsp70 levels in TDP-43 transgenic mouse line (Chen et al., 2016)			↓ Hsp40 levels in TDP-43 transgenic mouse line (Chen et al., 2016)

(Continued)

TABLE 1 | Continued

Disorder	Model	Hsp70	Hsp90	STI1	Hsp40
	Humans	↓ Hsp70 in sporadic cases *post-mortem* (Chen et al., 2016)			↓ Hsp40 in sporadic cases *post-mortem* (Chen et al., 2016)
Prion diseases	*In vitro* or cell line or yeast	↓ PS^+ replication and propagation in yeast with mutation in Hsp70 allele (Jones et al., 2004)		STI1 deletion in yeast ↑ PS^+ propagation (Jones et al., 2004; Reidy and Masison, 2010); STI1 reduces RNQ^+ prion toxicity (Wolfe et al., 2013)	
	Animal model	↑ Astrocytic Hsp70 in scrapie injected mice (Diedrich et al., 1993); Overexpression of human Hsp70 in mice did not ameliorate prion pathology (Tamguney et al., 2008)			
	Humans	↑ Inducible Hsp70 in CJD, regions with less atrophy have ↓PrP^{Sc} and ↑Hsp70 (Kovacs et al., 2001)			
Alzheimer's disease	*In vitro* or cell line or yeast		*In vitro* Hsp90 competes with PrP for STI1 binding (Maciejewski et al., 2016); in primary murine neurons Hsp90 decreased STI1 neuroprotection against Aβ oligomers (Ostapchenko et al., 2013)	STI1 ↓ PrP^C-AβO binding *in vitro* (Maciejewski et al., 2016); in HEK cells and primary neurons (Ostapchenko et al., 2013); STI1 protected primary murine neurons from AβO toxicity in PrP^C/α7nAChRs-dependent way (Ostapchenko et al., 2013)	
	Animal model	Toxicity buffering against Aβ in *C. elegans* (Brehme et al., 2014)	Toxicity buffering against Aβ in *C. elegans* (Brehme et al., 2014); treating AD mice with 17-AAG improved synaptic marker density and memory Hsp90 inhibitors ↓ hyperphosphorylated tau (Chen et al., 2014; Wang et al., 2016)	Toxicity buffering against Aβ in *C. elegans* (Brehme et al., 2014); STI1 downregulation ↑ tau-induced neuron loss in *D. melanogaster* (Ambegaokar and Jackson, 2011); ↑ in hippocampus in APPswe/PS1dE9 mice (Ostapchenko et al., 2013)	Toxicity buffering against Aβ in *C. elegans* (Brehme et al., 2014)
	Humans			↑ In cortex *post-mortem* (Ostapchenko et al., 2013)	

affected (de Lau and Breteler, 2006; Dickson, 2012). Augmented levels of α-synuclein or α-synuclein-containing aggregates are also characteristic of other neurodegenerative diseases including Lewy body dementia, multiple system atrophy and AD (Halliday et al., 2011; Serrano-Pozo et al., 2011; Ingelsson, 2016), forming a group of diseases termed "synucleinopathies." The involvement of molecular chaperones in PD was first suggested by the observation that Hsp90, Hsp70, Hsp60, Hsp40, and Hsp27 were localized in Lewy bodies (McLean et al., 2002; Uryu et al., 2006; Leverenz et al., 2007). Increasing evidence has shown protective actions of molecular chaperones against α-synuclein-induced toxicity both *in vitro* and *in vivo*. Lindersson et al. (2004) showed that filaments of α-synuclein are able to bind to a component of the proteasome (the 20S subunit) and selectively impede the chymotrypsin-like activities of the proteasome *in vitro*. Recombinant human Hsp70 was capable of binding these α-synuclein filaments and attenuating their chymotrypsin-like inhibitory activity. Furthermore, heat shock of fibroblasts expressing α-synuclein lead to a significant increase in Hsp70 levels, which also reduced the inhibitory effects of α-synuclein on the proteasome (Lindersson et al., 2004). Roodveldt et al. (2009) found that Hsp70 and Hsp70-interacting protein (Hip) prevented formation of α-synuclein fibrils *in vitro* and in *C. elegans* knockdown of Hip increased synuclein inclusions in an Hsp70-dependent manner. Interestingly, Hsp70 overexpression in human neuroglioma cells transfected with mutant α-synuclein led to 50% less oligomeric α-synuclein species (Outeiro et al., 2008). Transgenic expression of familial PD mutations (A30P and A53T in α-synuclein) in fruit flies causes a significant degeneration of dopaminergic neurons, but co-expression of human Hsp70 abrogated this loss (Auluck et al., 2002). Overexpression of Hsp70 in yeast and mouse models,

and Hsp90 inhibition with geldanamycin in human cell lines has been shown to counteract formation and accumulation of α-synuclein oligomers and alleviate α-synuclein-induced toxicity (Klucken et al., 2004; McLean et al., 2004; Flower et al., 2005; Luk et al., 2008). Conversely, Auluck et al. (2002) also reported acceleration of α-synuclein toxicity after inducing a dominant negative mutation of fruit fly Hsp70, which further confirmed the critical role of Hsp70 in α-synuclein regulation. Similarly, injection of Hsp70 into the Substantia nigra of MPTP-treated rats (a toxin that results in similar pathologies to those seen in PD) prevented dopaminergic cell loss (Dong et al., 2005).

Much less is known about the role of Hsp90 in regulating α-synuclein aggregation. *In vitro* experiments show that Hsp90 can both abolish α-synuclein binding to vesicles and promote fibril formation in an ATP-dependent manner (Falsone et al., 2009). More recent i*n vitro* experiments investigating Hsp90 interaction with the A53T mutant of α-synuclein revealed that all three Hsp90 domains bind to and prevent A53T α-synuclein aggregation. However, Hsp90 could not bind to monomeric or fibrillary synuclein species in this model (Daturpalli et al., 2013). Interestingly, haploid deletion of yeast Hsp90 (Hsp82) enhanced α-synuclein toxicity, specifically, by increasing reactive oxygen species accumulation (Liang et al., 2008). However, Putcha et al. (2010) showed that Hsp90 inhibition with 17-AAG (a geldanamycin derivative), which leads to upregulation of Hsp70, prevented α-synuclein oligomer formation and toxicity in the H4 neuroglioma cell line. Due to STI1 ability to modulate Hsp70/Hsp90 activity, Daturpalli et al. (2013) conducted an *in vitro* experiment to assess if STI1 could have its own effect on α-synuclein aggregation. STI1 could only attenuate monomeric A53T α-synuclein aggregation *in vitro* (Daturpalli et al., 2013). This suggests that STI1 is capable of having some of its own chaperone-like activity, but interaction with Hsp70 or Hsp90 would have a greater effect on reorganization of toxic α-synuclein species. The literature thus far suggests that increasing Hsp70 levels by activating the heat shock response or by genetic manipulation would be a suitable method for reducing α-synuclein toxicity. This could prove beneficial in reducing toxicity-related symptoms.

Chaperones in Huntington's Disease

Excess CAG repeats in the IT15 gene is a heritable mutation that causes HD and leads to the accumulation of huntingtin protein [Huntingtons's Disease Collaborative Research Group (1993)]. Protein deposits of mutated huntingtin form inclusion bodies within motor neurons in the spinal cord, as well as neurons in the cerebellum, cortex and striatum (Davies et al., 1997). Recent work suggests that the sequestering of toxic huntingin (Htt) into inclusion bodies may be a way to remove this toxic species as increased formation correlated with a reduction in levels of toxicity and neuronal death (Arrasate et al., 2004; Miller et al., 2010).

HD is part of the polyQ group of neurodegenerative diseases, which includes spinocerebellar ataxias, spinal and bulbar muscular atrophy (also known as Kennedy's disease), and dentatorubral-pallidoluysian atrophy (Williams and Paulson, 2008). In a fly model, Kazemi-Esfarjani and Benzer (2000) showed transgenic vector-mediated suppression of Htt toxicity by the molecular chaperones dHDJ1, a homolog of human Hsp40, and dTPR2, a homolog of human tetratricopeptide repeat protein 2. Likewise, deletion of Hsp70 in mice increased the size of polyQ inclusion bodies (Wacker et al., 2009). In addition, overexpression of Hsp40 and/or Hsp70 suppressed polyQ-dependent aggregation and neurodegeneration in cell cultures, yeast, fly, and mouse models (Warrick et al., 1999; Carmichael et al., 2000; Jana et al., 2000; Krobitsch and Lindquist, 2000; Wacker et al., 2004). STI1 overexpression in yeast suppressed Htt toxicity and drove the re-organization of small Htt103Q foci into larger assemblies through interaction with Hsp70, whereas STI1 deletion aggravated Htt toxicity and hampered foci formation (Wolfe et al., 2013). Specifically, point mutations (A31T or G76N) in the TPR1 domain of yeast STI1 (Hsp70 interacting domain) prevented reorganization of Htt to STI1 foci, which resulted in a significant reduction in cell growth. This indicates that functional STI1 interaction with Hsp70 is required for Htt103Q reorganization and toxicity buffering. In *C. elegans* expressing the Q35 aggregate prone-protein, siRNA for Hsp40, Hsp70, Hsp90, or STI1 significantly increased the number of Htt aggregates (Brehme et al., 2014). Therefore, there is strong evidence for an important role of chaperones and co-chaperones as therapeutic targets in HD.

Chaperones in Amyotrophic Lateral Sclerosis

ALS is a fatal neurodegenerative disorder that affects motor neurons of the brainstem, cortex and spinal cord, and results in weakness and atrophy of voluntary skeletal muscles (Paez-Colasante et al., 2015). ALS can be divided into two major classes—either the disease presents sporadically, which is 90% of cases, or it can be inherited. There are a number of proteins, RNAs and miRNAs dysregulated in ALS. The first aggregated protein to be identified was Cu/Zn superoxide dismutase (SOD1; Rosen et al., 1993), then trans-active DNA binding protein-43 (TDP-43; Arai et al., 2006; Neumann et al., 2006), along with fused in sarcoma/translocated in liposarcoma (FUS; Kwiatkowski et al., 2009; Vance et al., 2009), see Blokhuis et al. (2013) for a more extensive review on toxic protein accumulation in ALS.

However, in most familial or sporadic cases of ALS, the RNA binding protein TDP-43 shows signs of mislocalization and aggregation. TDP-43 is capable of binding to DNA and RNA, making it a key regulator of transcription, translation and cellular growth (Ayala et al., 2011; Polymenidou et al., 2011). TDP-43 can mislocalize to the cytoplasm, be ubiquitinated, hyperphosphorylated and ultimately form aggregates (Neumann et al., 2006; Mackenzie et al., 2007; Sreedharan et al., 2008; Brettschneider et al., 2013). Mutations in TDP-43 have been linked to ALS and FTD (Arai et al., 2006; Neumann et al., 2006). All together these results suggest the need to better understand the relationship between TDP-43 and chaperones.

TDP-43 carries out most of its functions in the nucleus, but it can be transported to the cytosol due to the nuclear export sequence near its N-terminus (Ayala et al., 2008). TDP-43 contains two RNA recognition motifs in the core

of the protein and a C-terminal domain that contains a glutamine/asparagine-rich prion-like region that cooperates in protein-protein interactions (Ou et al., 1995; Budini et al., 2012a,b; Mackness et al., 2014). This prion-like domain allows for association with other TDP-43 molecules (Budini et al., 2012b) and is becoming a major area of research in neurodegenerative diseases involving TDP-43 associated pathology. Deletion of the prion-like domain of TDP-43 in HeLa cells eliminated heat shock induced nuclear aggregation, and further deletion of a glycine-rich region in this domain significantly reduced cytosolic mislocalization of the toxic 25 kDa TDP-43 variant (Udan-Johns et al., 2014). Recent studies in HeLa cells showed that Hsp40 and Hsp70 constitutively bind to and regulate the nuclear aggregation of TDP-43 (Freibaum et al., 2010; Udan-Johns et al., 2014).

Using HEK293T cells Fontaine et al. (2016) investigated the roles of the constitutively expressed Hsp70 homolog Hsc70 and its co-chaperone DnaJC5 in the secretion of neurodegenerative-disease associated proteins. Secretion of these proteins by unconventional mechanisms is thought to contribute to their spreading in the brain. DnaJC5 supports secretion via the Soluble NSF Attachment Protein Receptor (SNARE) complex at synapses in a calcium-dependent manner (Jacobsson and Meister, 1996; Chamberlain and Burgoyne, 1997, 1998; Umbach and Gundersen, 1997; Weng et al., 2009; Sharma et al., 2012). Overexpression of DnaJC5 lead to significant secretion of WT and disease associated mutants of TDP-43 from HEK cells (Fontaine et al., 2016) and this was dependent upon functional Hsc70. Interference with this mechanism could potentially regulate the spreading of misfolded TDP-43 in the brain. However, to date there has been limited experiments in neuronal cells, neurons or in animal models to test these findings obtained in non-neuronal cells.

Both Hsp70 and Hsp90 can be co-immunoprecipitated with TDP-43. Moreover, knockdown of Hsp70 or Hsp90 in human neuroblastoma cells lead to a significant increase in C-terminal and phosphorylated TDP-43, which are toxic TDP-43 species known to aggregate in the cytoplasm (Zhang et al., 2010). Treating HeLa cells with celastrol, an Hsp90 inhibitor, reduced levels of full length TDP-43, specifically by impairing Cdc37 (an Hsp90 co-chaperone which aids in client docking; Lotz et al., 2003)—Hsp90 interaction with TDP-43 (Jinwal et al., 2012). Recent work by Chen et al. (2016) further supported the role of Hsps in TDP-43 regulation, whereby activation of the heat shock response, by overexpression of HSF1 in HEK cells increased levels of Hsp70 and Hsp40, which lead to increased clearance of insoluble and hyperphosphorylated TDP-43. Interestingly, TDP-43 misregulation has also been found in a proportion of patients with AD (Amador-Ortiz et al., 2007; Wilson et al., 2011). hnRNP A2/B1 and A1, which are RNA-binding proteins that interact with TDP-43, have been shown to be decreased in AD, due to abnormal regulation of cholinergic signaling (Berson et al., 2012; Kolisnyk et al., 2013, 2016a,b). Although we have started to understand how chaperones and co-chaperones may regulate TDP-43, their role in neurons and other brain cells has not yet been examined in detail.

A significant reduction in Hsp70 and Hsp40 protein levels is observed in the brains of TDP-43Q331K transgenic mouse model of ALS and patients with sporadic ALS (Chen et al., 2016). HSF1 protein levels were also reduced in mice, but not in human brains (Chen et al., 2016). This suggests that, in disease, the heat shock response may be compromised and thus contribute to the accumulation of insoluble TDP-43 protein aggregates.

Chaperones and Prions

In prion diseases, PrP^{C} is converted into PrP^{Sc}, which can work via template-mediated misfolding to further convert host PrP^{C} protein into a variety of misfolded forms that aggregate and accumulate within the nervous tissue (Will and Ironside, 1999; Budka, 2003; Soto and Castilla, 2004; Linden et al., 2008). Misfolding of PrP^{C} results in a class of diseases called transmissible spongiform encephalopathies (TSEs). Prion disease can arise sporadically, from genetic mutation or through transmission, such as by consumption of prion-infected tissues. TSEs include bovine spongiform encephalopathy in cattle, as well as sheep scrapie and variant CJD in humans (Linden et al., 2008). PrP^{C} contains a disordered N-terminal domain and a globular C-terminal domain that is largely α-helical (Riek et al., 1996, 1997).

In order to better understand prion propagation and chaperone regulation in a simple eukaryotic model, yeast prions have been extensively studied. Yeast prions are self-propagating amyloid forms of soluble proteins that can function as protein-based inheritable elements. The yeast prion, PSI^{+}, is a transmissible, self-replicating, and aggregation prone mutant of yeast translation termination factor Sup35p (Glover et al., 1997; King et al., 1997). Jones et al. (2004) found that a mutation in the SSA1 Hsp70 allele (SSA1–21p) significantly impaired PSI^{+} self-replication and propagation. Interestingly, in this cell line, deletion of yeast STI1 regenerated PSI^{+} propagation. Conversely, overexpression of STI1 reduced the mitotic capacity of PSI^{+} prions (Jones et al., 2004). Additionally, overexpression of Hsp104 is capable of eliminating PSI^{+} prions (Chernoff et al., 1995), but this is dependent upon expression of STI1 (Reidy and Masison, 2010). Deleting STI1 had no effect on levels of Hsp104, but eliminated Hsp104 "curing" activity (Reidy and Masison, 2010). Specifically, STI1 coordination of Hsp70 and Hsp90 was responsible for this prion elimination activity, as mutations in the TPR1 and TPR2 domains of STI1 lead to a drastic increase in PSI^{+} propagation. This suggests that STI1 coordination of Hsp70-Hsp90 as well as Hsp104 activity is required for disaggregation of yeast prions. Furthermore, STI1 expression and activity was also found to reduce toxicity of Rnq1 (a yeast protein with a glutamine-rich prion domain) prions, RNQ^{+} (Wolfe et al., 2013). STI1 recruited RNQ^{+} prions to foci containing Hsp104, amyloid like proteins and Hsp40, ultimately buffering toxicity by these prions.

As with many of the misfolded proteins that cause neurodegenerative diseases, knowledge of the physiological functions of PrP^{C} is still not complete. Knockout of PrP^{C} in mice affects synaptic transmission (Maglio et al., 2006), causes gross demyelination in the sciatic nerve specifically due to PrP^{C} depletion in neurons (Bremer et al., 2010), and alterations in sleep pattern (Tobler et al., 1996). Elimination of PrP^{C} also protects mice against infection with PrP^{Sc} (Bueler et al., 1993).

C57BL6 mice injected with 22L strain of scrapie had a significant increase in protein levels of inducible Hsp70 in active astrocytes (Diedrich et al., 1993). Similarly, mice infected with forms of scrapie known to induce plaques and increased vacuolation, had a significant increase in Hsp70 RNA expression toward the terminal phases of infection (Kenward et al., 1994). Kovacs et al. (2001) found increased immunoreactivity of inducible Hsp70 in Purkinje cells from CJD patients, and regions with higher levels of Hsp70 had less spongiform-like atrophy and increased levels of PrP^{C} rather than PrP^{Sc}. This suggests a potential neuroprotective effect of Hsp70.

Tamguney et al. (2008) conducted a seminal study on 20 potential gene candidates that could regulate the replication of prions in mice infected with scrapie or cow 301V prions. Genes were selected based upon known interactions with PrP^{C} in a diseased or non-diseased state, significant upregulation in prion disease, post-translational modification of PrP, or involvement in PrP^{C}-related signal transduction (Tamguney et al., 2008). Interestingly, overexpression of human Hsp70 had no effect on prion disease onset.

To our knowledge there have been almost no studies investigating the role of Hsp90 and its co-chaperones in prion diseases. STI1 can signal via the prion protein as discussed above, and prion infection in cells abolishes STI1 signaling via the prion protein (Roffe et al., 2010). Interestingly, interaction of Hsp90 with STI1 also decreases PrP^{C}-dependent STI1 neuroprotection (Maciejewski et al., 2016), which suggests that secreted Hsp90 may interfere with STI1 interaction with PrP^{C}. Given that STI1 regulates protein aggregates via its co-chaperone activity (Wolfe et al., 2013), and also has extracellular cytokine-like neurotrophic function, it is likely that its effects on prion diseases and other neurodegenerative diseases are complex. By further understanding the cause and mechanism of the aggregation of these proteins, interventions targeting the chaperone machinery toward refolding or degradation could be utilized.

Chaperones in AD

AD is the most common form of dementia, particularly affecting the aging population. Pathologically, it is defined by accumulation of two types of protein aggregates in the forebrain; extracellular plaques of Aβ, and intraneuronal neurofibrillary tangles (NFT) of microtubule-associated protein tau (Selkoe, 1991). As is the case with other diseases associated with misfolded proteins, analysis of AD brains and AD animal models revealed increased levels of Hsps and their co-chaperones, including Hsp27 (Renkawek et al., 1994), Hsp70 (Perez et al., 1991), and STI1 (Ostapchenko et al., 2013). A significant effort was made by a number of researchers to test whether chaperone system participates, directly or indirectly, in the pathogenic processes of Aβ and tau misfolding.

Aβ Peptide Generation and Toxicity

Aβ peptides consisting of around 39–43 residues are formed by proteolytic cleavage of its precursor, APP, by beta-site APP cleaving enzyme (BACE, β-secretase) and by a γ-secretase complex, formed by several proteins including presenilins (O'Brien and Wong, 2011). Aβ peptide toxicity was originally thought to be related mainly to the amyloid plaques that form throughout cortex and hippocampus in AD. However, during the last two decades it has also become recognized that soluble Aβ oligomers (AβOs) are toxic to synapses (Lambert et al., 1998; Walsh et al., 2002; Ferreira and Klein, 2011; Benilova et al., 2012; Mucke and Selkoe, 2012; Lesne et al., 2013). Aβ oligomers are thought to increase early before plaque formation and correlate with the onset of the neurotoxic events, such as excitotoxicity, synaptic loss as well as impairment of LTP and learning/memory in rodent models (Lambert et al., 1998; Klein et al., 2001; Klein, 2002; Walsh et al., 2002; Wang et al., 2005). Synaptotoxicity by AβOs depends on their interaction and corruption of multiple neuronal receptors, an effect that seems to depend on the initial interaction with PrP^{C} (Lauren et al., 2009; Gimbel et al., 2010; Caetano et al., 2011; Kudo et al., 2012; Um et al., 2012; Ostapchenko et al., 2013; Beraldo et al., 2016). AβO/PrP^{C} can engage metabotropic glutamate receptor 5 (Um et al., 2012, 2013; Beraldo et al., 2016) to activate pathogenic intracellular pathway that leads to activation of Fyn kinase, NMDA receptor mistrafficking, excitotoxicity and LTP inhibition.

Significant effort by several research groups were aimed to prevent AβO toxicity, employing anti-$Aβ_{42}$/AβO (reviewed in Wisniewski and Drummond, 2016) and anti-PrP^{C} (Chung et al., 2010; Barry et al., 2011) antibodies and N-terminal fragment of PrP^{C} (Beland et al., 2014). However, to our knowledge, none of these potential therapies passed or even reached clinical trials yet. Remarkably, extracellular STI1, can bind to PrP^{C} and activate α7 nicotinic acetylcholine receptor (nAChR), which mediate STI1-PrP^{C} neurotrophic effects, efficiently preventing the binding of AβOs to PrP^{C} on the neuronal surface, as well as general binding of these oligomers to neurons (Ostapchenko et al., 2013). Due to this effect, as well as protective signaling via α7 nAChR/PrP^{C} complex, STI1 completely blocks AβO/PrP^{C} toxicity *in vitro* (Ostapchenko et al., 2013). Whether Hsp70/Hsp90/STI1 exist extracellularly in AD brain separately or as a complex is unknown, but one may expect complex effects of extracellular chaperones on Aβ aggregation and toxicity in AD brain. To start with, we found in a biochemical assay that Hsp90 modulates formation of the STI1/PrP^{C} complex, possibly resulting in decreased STI1 neurotrophic signals (Maciejewski et al., 2016).

Many *in vivo* AD models, including those that employ invertebrate and mammal species, are based on Aβ toxicity. In *C. elegans* Aβ expression leads to formation of peptide deposits and decreased motility (Link, 1995). This toxicity can be rescued by blocking the insulin growth factor-like signaling pathway, with a major role being played by HSF1 (Cohen et al., 2006). In this study, Cohen and colleagues showed that treatment with HSF1 RNAi increased Aβ toxicity in worms, probably due to increased amount of neurotoxic Aβ aggregates. The question remains, which mechanism activated by HSF1, plays a role in increased Aβ toxicity. Morimoto and colleagues approached this question by analyzing the various chaperones in worms expressing Aβ (Brehme et al., 2014). Systematic knockdown of Hsps and co-chaperones showed that Hsp40, Hsc70, Hsp90, and STI1, while not affecting motility in young animals, seem to normally buffer Aβ toxicity in *C. elegans*, as well as to alleviate age-related decrease in worm motility. Of interest, these chaperones and

co-chaperones form an expression network in human brains, but the connecting links are significantly weakened in both AD and normal aging (Brehme et al., 2014), suggesting dysfunctional chaperone activity with age and disease.

The role of HSF1 in AD suggested by the results in *C. elegans* was recently supported by work with AD mouse models studying the effect of Hsp90 inhibitors on Aβ synaptotoxicity and behavioral impairment. Treatment of AD mice with 17-AAG (Chen et al., 2014) or OS47720 (Wang et al., 2016), Hsp90 inhibitors improved synaptic markers and density, *in vivo* LTP and memory loss and these effects were mediated by HSF1 activation and upregulation of synaptic genes. In contrast to the effect of HSF1, it is remarkable that whereas in *C. elegans* knockdown of Hsp90 is deleterious, in mammals inhibition of Hsp90 can actually improve Aβ-mediated toxicity.

Interestingly HSF1, which under stress conditions induces expression of Hsp70, Hsp90 and other chaperones, also upregulates production of APP (Dewji and Do, 1996). HSF1, besides its role in upregulation of heat shock machinery, is known as a major factor facilitating synaptic fidelity (Hooper et al., 2016). It is possible that HSF1-induced APP upregulation may be due to the pro-synaptogenic activity of this transcription factor. Indeed, APP has been shown to serve as a cell adhesion molecule (Small et al., 1999). On the other hand, synaptic activity by itself affects APP trafficking, routing it toward synapses (Tampellini et al., 2009). Besides, the same study showed that in neurons overexpressing APP with the Swedish familial mutation, synaptic activity also decreases intraneuronal Aβ. This result is paralleled by findings that activation or de-activation of synaptic activity, increases or decreases Aβ secretion, respectively (Kamenetz et al., 2003; Cirrito et al., 2005; Bero et al., 2011; Li et al., 2013; Yuan and Grutzendler, 2016). Considering the deleterious effect of Aβ on synaptic activity and integrity, HSF1, APP, and Aβ may form a self-regulating mechanism for controlling neuronal function.

Tau

Significant influence of Hsp70/90 machinery on AD pathology is implemented via microtubule associated protein tau. Physiologically, tau acts as a major regulator of microtubule formation (Weingarten et al., 1975) and in the CNS, tau is typically found in the cytoplasm or axons (Binder et al., 1985), where it promotes outgrowth and stabilizes microtubule formation. Tau is abnormally phosphorylated in AD due to increased activity of GSK-3 and other tau kinases (Alvarez et al., 1999; Avila et al., 2010; Tremblay et al., 2010; Cavallini et al., 2013), likely as a result of initial Aβ toxicity (Tamagno et al., 2003; Ryan et al., 2009; Hernandez et al., 2010). Hyperphosphorylated tau forms paired helical filaments, which are the main component of neurofibrillary tangles (Grundke-Iqbal et al., 1986a,b; Cao and Konsolaki, 2011), a critical pathological hallmark in AD. A number of studies have shown that these hyperphosphorylated tau species can be recognized by Hsps and their co-chaperones, including Hsp27, Hsp70, CHIP, and αB crystalline, in order to repair malignant tau or proceed with its recycling (Dou et al., 2003; Dabir et al., 2004; Petrucelli et al., 2004; Shimura et al., 2004; Luo et al., 2007). Recently, the structure of Hsp90-tau complex has been resolved (Karagoz et al., 2014). It explained how Hsp70 and Hsp90 can simultaneously bind to the intrinsically unstructured tau, making use of the atypically large substrate-binding site on Hsp90, which is rather open and accessible to clients such as tau. Of interest, low affinity hydrophobic connections in the Hsp90 substrate binding site could explain a general principle of Hsp90 interaction with disordered substrates or folded proteins.

Interestingly, inhibitors of Hsp90 decrease levels of phosphorylated tau, suggesting that Hsp90 may protect hyperphosphorylated tau from degradation (Dickey et al., 2006). Inhibition of Hsp90 in HeLa cells transfected with mutant tau (P301L) increased CHIP complex formation with phosphorylated tau (p-tau) and CHIP selectively degraded these p-tau species, essentially preventing aggregation of p-tau (Dickey et al., 2007). CHIP is also highly colocalized with p-tau and neurofibrillary tangles (aggregates of hyperphosphorylated tau; Dickey et al., 2007). These findings make CHIP a suitable candidate for modulating tau activity in neurodegenerative tauopathies, especially due to its ubiquitin enzyme activity. On the other hand, a complex of Hsp90 with the co-chaperone FKBP51 protected tau from proteasomal degradation and correlated with the neurotoxic tau species (Jinwal et al., 2010; Blair et al., 2013). FKBP51 overexpression decreased the amount of tau tangles in P301L tau transgenic mice, but increased soluble tau, including oligomeric and hyperphosphorylated species. This in turn led to increased tau toxicity, reflected in neuronal loss in P301L mice hippocampus and in decreased proliferation of tau-expressing neuronal cultures (Blair et al., 2013). Dickey and colleagues also found that FKBP51 expression is increased with age and in AD (Blair et al., 2013). This led them to hypothesize that Hsp90 interaction with FKBP51 is altered in aging and AD brains, allowing for the preservation of soluble, but possibly neurotoxic protein species. Another member of FKBP family, FKBP52, may also be involved in tau-related neurodegeneration. Recent evidence suggests that FKBP52 is a key regulator of tau association with microtubules, specifically in inhibiting this function (Chambraud et al., 2010). Moreover, a significant reduction in tau-mediated neurite outgrowth was observed in cells overexpressing FKBP52 (Chambraud et al., 2010).

Alternatively, Hsp70 promotes tau stability and association with microtubules at high levels of expression (Dou et al., 2003; Jinwal et al., 2009). STI1 may also be important for protection against aberrant tau species, as its downregulation in fruit flies worsened tau-induced retinal degeneration (Ambegaokar and Jackson, 2011). Upregulation of both Hsp70 and Hsp90 increases tau association with microtubules (Dou et al., 2003). Of note, this study used geldanamycin-induced Hsp90 inhibition, which resulted in increased Hsp70/90 expression due to HSF1 activation. As HSF1 activates multiple members of Hsp machinery, it is difficult to draw conclusions as to which particular chaperone affected tau-microtubule coupling. Soluble levels of tau correlate with those of Hsps and their co-chaperones, while in tauopathies where total levels of tau increase, Hsp70/90 decrease (Dou et al., 2003). Overall, tau regulation by the Hsp machinery is very complex and careful analysis of all possible effects on tau is needed

when considering an anti-AD therapy that modulates this machinery.

CONCLUSION

In summary, the common observation of misfolded and aggregated proteins in neurodegenerative disease suggests dysregulation of chaperone activity. The balance between levels of Hsp70 and Hsp90 are becoming a major area of investigation, as both upregulation of Hsp70 and inhibition of Hsp90 in mammals reduce protein aggregation and toxicity. STI1 should be further investigated in models of protein aggregation, as STI1-PrP^C interaction results in neuroprotection, attenuates AβO toxicity, and STI1 is an irreplaceable co-chaperone for the Hsp70/Hsp90 machinery. Ultimately, much is still unknown about how to effectively control protein misfolding and prevent aggregation by targeting chaperones and co-chaperones in neurodegenerative disease. Further, investigation of chaperones and their partners using new mouse models, could help to elucidate the underlying mechanisms of these proteinopathies and allow for generation of effective and unambiguous pharmacological therapies.

AUTHOR CONTRIBUTIONS

RL, AM, JML, and VO literature review, wrote the manuscript; WC, MD, and VP edited the manuscript; MP Literature review, wrote, and provide editing and scientific direction for the manuscript.

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The Emerging Roles of Early Protein Folding Events in the Secretory Pathway in the Development of Neurodegenerative Maladies

*Tatyana Dubnikov and Ehud Cohen **

Department of Biochemistry and Molecular Biology, The Institute for Medical Research Israel—Canada, The Hebrew University School of Medicine, Jerusalem, Israel

Although, protein aggregation and deposition are unifying features of various neurodegenerative disorders, recent studies indicate that different mechanisms can lead to the development of the same malady. Among these, failure in early protein folding and maturation emerge as key mechanistic events that lead to the manifestation of a myriad of illnesses including Alzheimer's disease and prion disorders. Here we delineate the cascade of maturation steps that nascent polypeptides undergo in the secretory pathway to become functional proteins, and the chaperones that supervise and assist this process, focusing on the subgroup of proline *cis/trans* isomerases. We also describe the chaperones whose failure was found to be an underlying event that initiates the run-up toward neurodegeneration as well as chaperones whose activity impairs protein homeostasis (proteostasis) and thus, promotes the manifestation of these maladies. Finally, we discuss the roles of aggregate deposition sites in the cellular attempt to maintain proteostasis and point at potential targets for therapeutic interventions.

Keywords: chaperone, aggregation, endoplasmic reticulum stress, neurodegeneration, aggresome

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****Correspondence:***
Ehud Cohen
ehudc@ekmd.huji.ac.il

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The generation of a polypeptide by the ribosome is the first step of the long and complex process that leads to the formation of a mature, functional protein. Cytosolic proteins maturate at the cytosol (Hartl and Hayer-Hartl, 2002) while secreted and membrane proteins are processed at the secretory pathway (Ellgaard and Helenius, 2003). Here we focus on protein folding and maturation within the secretory pathway and delineate how failures in this process underlie the manifestation of certain cases of late-onset neurodegenerative disorders.

PROTEIN MATURATION AND QUALITY CONTROL WITHIN THE ENDOPLASMIC RETICULUM

The first domain of many nascent chains of a secretory proteins to exit the ribosome is a hydrophobic signal sequence of 20–30 amino acids that targets the polypeptide into the endoplasmic reticulum (ER; Hegde and Bernstein, 2006). The appearance of the signal peptide, and its recognition by the signal recognition particle (SRP; Lauffer et al., 1985), leads to the binding of the translating ribosome to the ER channel protein complex Sec61p (Sanders et al., 1992) and to the co-translational insertion of the nascent polypeptide into the ER lumen. This translocation mechanism is not exclusive as newly synthesized polypeptides can enter the ER lumen

through alternative mechanisms that have been discovered in recent years. A subgroup of "tail-anchored (TA) proteins" bear a C-terminal hydrophobic trans-membrane domain that interacts with the family of GET proteins (guided entry of TA proteins). In yeast, the TA protein-GET interactions promote the post-translational entry into the ER by a SRP-independent mechanism (Schuldiner et al., 2008). Mammalian TA proteins enter the ER through an analogous mechanism that requires the GET orthologue Asna1/TRC40. Interestingly, under certain circumstances, the TRC40-dependent ER translocation mechanism cooperates with the canonical Sec61p apparatus to orchestrate proper post-translational entry of polypeptides into the ER (Johnson et al., 2012).

An additional ER translocation mechanism that functions independently of SRP and GET proteins but depends upon the activity of the Snd1, Snd2 and Snd3 proteins has been discovered recently (Aviram et al., 2016).

Upon entry into the ER, the ER-localization signal is cleaved (Haeuptle et al., 1989), and many of the newly synthesized polypeptides are anchored to the ER membrane through their transmembrane domains. Some other proteins, including the prion protein (PrP), acquire a glycophosphatidylinositol (GPI) lipid tail (Muñiz and Zurzolo, 2014). Oligosaccharides are attached to asparagine residues of many newly synthesized proteins. These modifications render the polypeptides recognizable by lectin folding chaperones which play key roles in the folding and maturation of glycosylated proteins (Noack et al., 2014). These chaperones and folding-assisting enzymes (foldases) catalyze the molecule's maturation by a series of sequential events that help it attain its desired spatial structure (**Figure 1**). Examples of some well-characterized ER foldases are Calnexin (CNX), Calreticulin (CRT), the protein disulfide isomerase (PDI) ERp57 (Oliver et al., 1999), and cyclophilin B (CypB) (Jansen et al., 2012; for a comprehensive overview on ER-resident chaperones see (Gidalevitz et al., 2013). The interactions of the nascent polypeptide with CNX, CRT, and additional chaperones such as the Hsp70 family member BiP/GRP78 (Haas and Wabl, 1983), initiate the folding process and can recruit ERp57 (Kozlov et al., 2006) that catalyzes the formation of disulfide bonds. CypB, a member of the peptidylprolyl cis/trans isomerases (PPIases) family of chaperones, which utilizes specific proline residues to convert the maturating polypeptide from *cis* to *trans* position (Barik, 2006), emerges as a pivotal coordinator of the folding process (Jansen et al., 2012). FKBP10, a member of the FK506-binding proteins (FKBPs), an additional subgroup of PPIase chaperones, is needed to mediate the entry of certain nascent polypeptides into the ER (Stocki et al., 2016).

Despite the assistance of the intricate nexus of expert folding chaperones, subsets of newly synthesized proteins fail to fold properly and expose hydrophobic domains that lead to the aggregation of the protein. These terminally misfolded molecules are recognized by specialized ER-chaperones which impede their shuttling to the Golgi (Ellgaard and Helenius, 2003), and promote their destruction by the ER-associated degradation (ERAD). This process is executed by a conserved set of ERAD components, including the membrane integral, ERAD E3 ubiquitin ligase HRD1 (Bordallo et al., 1998) and the ATPase VCP/p97 that mediate the retro-translocation of unfolded polypeptides to the cytosol (Ye et al., 2004), and confer their degradation by the proteasome (Ruggiano et al., 2014). It is important to note that macro-autophagy emerges as an additional mechanism that play roles in the degradation of ERAD substrates (Lipatova and Segev, 2015). Under regular conditions the orchestrated activities of protein folding, quality control, and degradation mechanisms maintain proper protein homeostasis (proteostasis; Balch et al., 2008) however, in the face of stress, mutations or aging, subsets of proteins that bear the propensity to misfold, escape the cellular surveillance system, and form aggregates within the ER.

ER STRESS RESPONSES

The accumulation of protein aggregates within the crowded environment of the ER lumen impairs its functionality and has the potential to be hazardous to the cell. Thus, highly conserved cellular mechanisms that refold unfolded polypeptides, clear aggregates, and restore functional proteostasis have been developed. One such well-defined mechanism is the unfolded protein response (UPR^{ER}), a signaling cascade which has at least four arms that have similar principles of activity. When specialized ER proteins sense an accumulation of misfolded proteins, a sequence of events activates the UPR^{ER} which initiates the migration of transcription factors to the nucleus. The ATF6(N) fragment is cleaved from ATF and enter the nucleus, XBP1 is activated by IRE1 and modulates gene expression and ATF4 is shuttled to the nucleus by the PERK downstream mechanism. These transcription factors elevate the expression of genes that encode for ER chaperones (such as BiP) to enhance folding capacity and reduce the expression of genes that encode proteins that require the assistance of ER chaperones to fold properly, aiming to lower the aggregation challenge within the ER lumen (reviewed in Walter and Ron, 2011).

Nevertheless, under certain circumstances, typically in late stages of life, the aggregation challenge exceeds the ER's protein folding and clearance capacities hindering the restoration of proteostasis. When potentially hazardous aggregates accumulate within the ER, they are actively convoyed to designated deposition sites (**Figure 2**). Such aggregated proteins are deposited next to the nucleus when proteasomes are inhibited. The formation of these cellular deposition foci was impeded by the inhibition of protein synthesis (Wójcik et al., 1996). These findings proposed that the juxta-nuclear, cellular foci are quality control compartments, where aggregates are temporarily stored to enable their degradation when conditions allow. A similar phenomenon of protein deposition in a cytosolic, nucleus-adjacent location was later reported. These sites, which were termed "aggresomes" (Johnston et al., 1998), contain aggregated membrane proteins and are positioned at the Micro Tubule Organizing Center (MTOC). We discovered that aggresomes which contain aggregated PrP are dynamic quality control compartments that attract molecular chaperones and

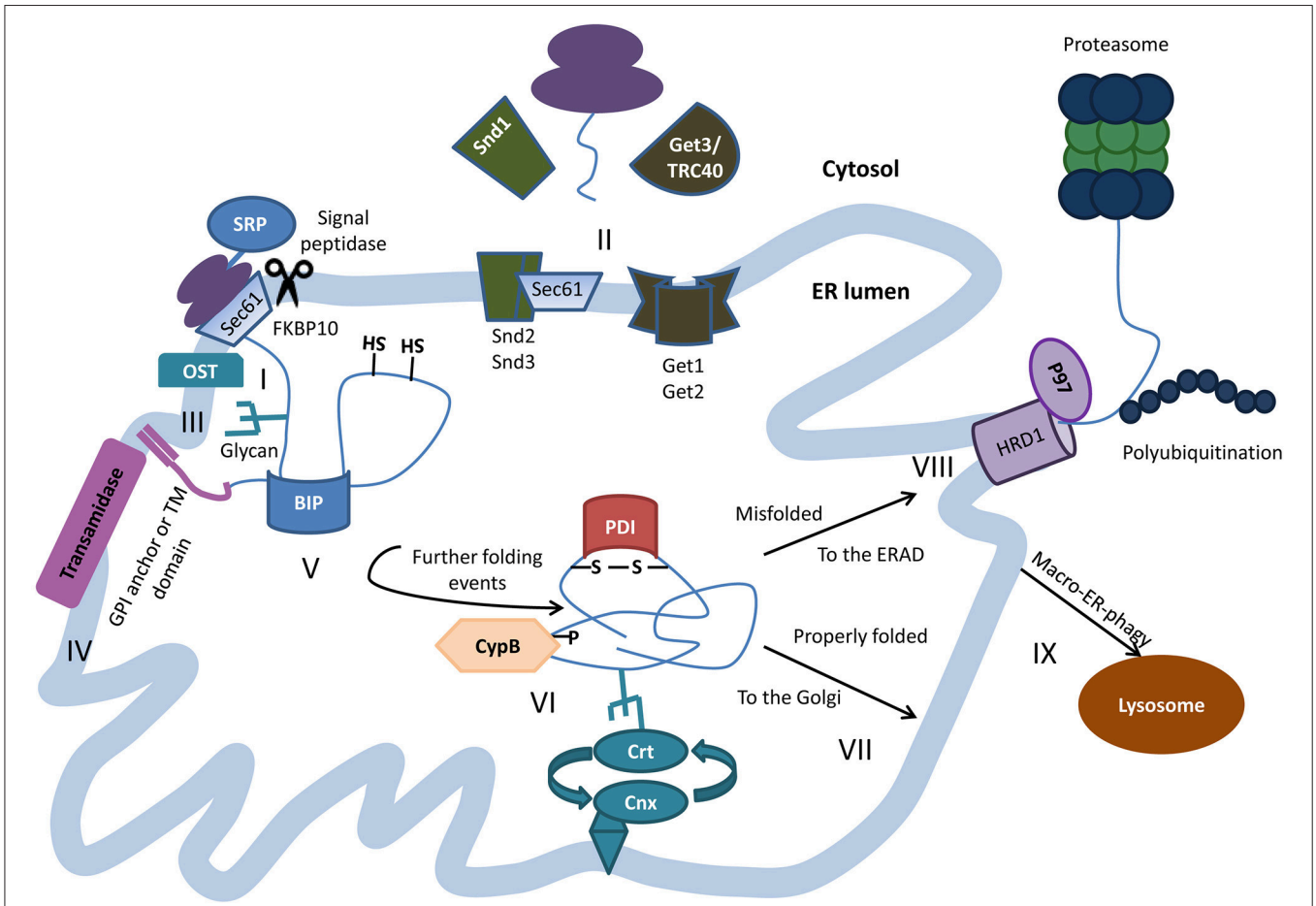

FIGURE 1 | Protein folding within the ER lumen. Newly synthesized polypeptides are co-translationally translocated into the ER through the translocon Sec61p complex. Targeting and insertion of the signal peptide is mediated by signal recognition particle (SRP) and at least in the case of PrP, is assisted by FKBP10 (I). In some cases post translational insertion of secretory proteins occurs in an SRP-independent manner, as in case of the GET-mediated insertion of tail-anchored proteins or by the SND mechanism in other cases (II). As the nascent polypeptide exits the translocon, the signal peptide is cleaved and N-linked glycans are added co-translationally by oligosaccharyltransferase (OST) (III). Transmembrane proteins are inserted into the ER membrane and some proteins get anchored to the membrane through the addition of glycophosphatidylinositol (GPI) lipid tail by the transamidase complex (IV). The highly conserved chaperone BiP binds nascent polypeptides as they are translocated into the ER, and maintains them in a state competent for subsequent folding and oligomerization. (V). The peptide undergoes further processing with the help of additional chaperones and folding enzymes, among them the calnexin/calreticulin lectin chaperones, protein disulfide isomerase (PDI) that oxidizes cysteine residues to induce disulfide-bond formation, and the ER resident cyclophilin B (CypB) that catalyzes cis/trans isomerization on the axis of certain proline residues (VI). Properly folded proteins are shipped for further processing in the Golgi (VII). Terminally misfolded proteins are retro-translocated to the cytosol to be degraded by the proteasomes through the ER-associated protein degradation (ERAD), often mediated by the E3 ubiquitin ligase HRD1 and the ATPase VCP/p97 (VIII). Sometimes, ERAD substrates and excess membranes and membrane proteins are shuttles for degradation in the lysosome via macro-ER-phagy (IX).

proteasomes to mediate the degradation of their content (Ben-Gedalya et al., 2011). Additional types of cytosolic deposition sites where characterized in yeast and mammalian cells including the aggresomes-like, Juxta Nuclear Quality control compartment (JUNQ), and Insoluble Protein Deposit (IPOD) (Kaganovich et al., 2008). It is not entirely clear whether the JUNQ is cytosolic or nuclear, as a recent study claimed that the aggregates that were previously reported to accumulate in the cytosol are deposited within the nucleus (Miller et al., 2015). An additional type of deposition site is the "ER quality control compartment" (ERQC) which accumulates protein aggregates within the ER lumen (Kamhi-Nesher et al., 2001). Why certain protein aggregates accumulate in cytosolic sites while others are deposited within the ER is not known however, it is plausible that the cell fails to retro-translocate certain molecules to the cytosol and thus, deposits them in the ERQC.

In the face of aging-associated decline in protein quality control capabilities (Carvalhal Marques et al., 2015), or due to mutations that severely destabilize the three dimensional structures of aggregation-prone proteins, the cell fails to maintain proteostasis, and protein aggregates accumulate. Such uncontrolled protein aggregation can be toxic and underlie the development of proteinopathies (Paulson, 1999). Neurodegenerative maladies including Alzheimer's disease, Parkinson's disease (Selkoe, 2003), and prion disorders (Aguzzi

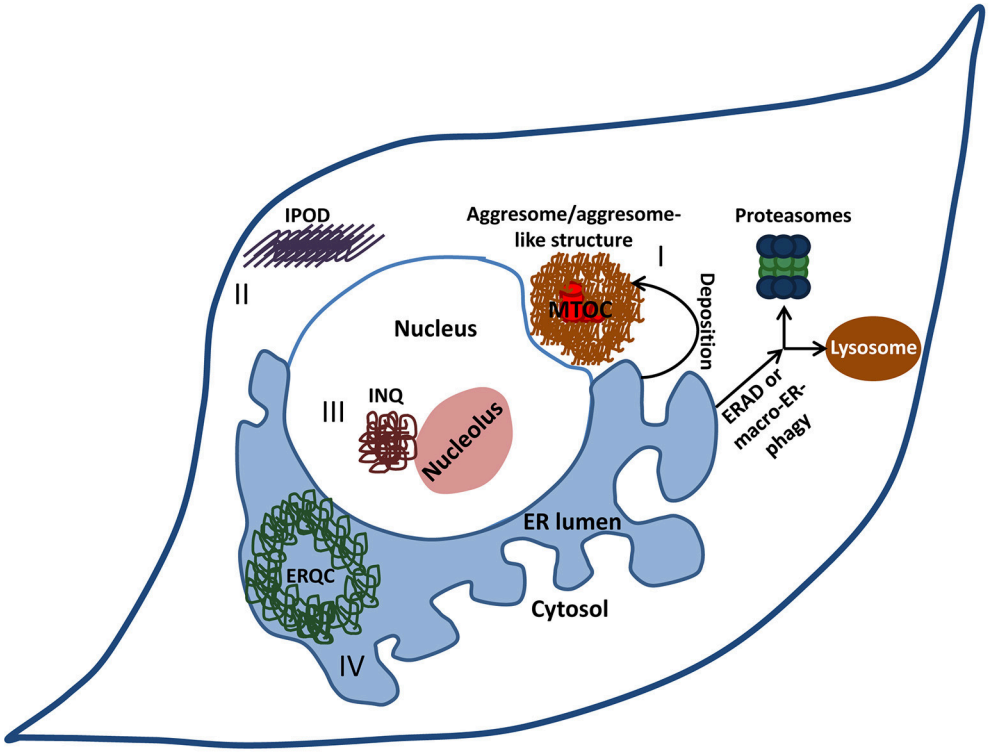

FIGURE 2 | Cellular deposition sites. When the burden of misfolded proteins exceeds the cell's clearance and refolding capacity, potentially hazardous aggregates accumulate within the ER. Under certain circumstances, these aggregates are actively convoyed to designated deposition sites. Aggresomes or aggresome like-structures (I) are cytosolic juxta-nuclear inclusion bodies that serve as quality-control centers. Another type of a cytosolic deposition site is the insoluble protein deposit (IPOD) (II) where terminally aggregated proteins tend to accumulate. Intra-nuclear quality control compartment (INQ) (III) resides in the nucleus next to the nucleolus and harbors nuclear as well as cytosolic misfolded proteins. Some proteins that aggregate within the ER are deposited in the ER-derived quality-control compartment (ERQC) (IV).

and Calella, 2009) are a subgroup of proteinopathies. Late onset (Amaducci and Tesco, 1994) and the deposition of aggregated proteins in the brain are common features of these illnesses (Soto, 2003).

ALZHEIMER'S DISEASE AND PRION DISORDERS

Alzheimer's disease (AD), the most common type of dementia, is characterized by two pathological hallmarks; the deposition of small hydrophobic peptides known as β amyloid (Aβ) in plaques and the formation of Neurofibrillary Tangles (NFTs) of aggregated, hyper-phosphorylated microtubule-associated protein tau in the brain (Selkoe, 2011). According to the amyloid hypothesis (Hardy and Higgins, 1992), AD develops as a result of a dual cleavage of the Amyloid Precursor Protein (APP) by two proteases, the β and γ secretases. This digestion releases the family of Aβ peptides which form various types of oligomers and high molecular weight aggregates. Oligomers were found to be the most toxic Aβ species (Cohen et al., 2006; Shankar et al., 2008). The amyloid hypothesis proposes hyper Aβ production as a key mechanistic condition for the development of this devastating disorder (Hardy and Higgins, 1992). Similarly to other neurodegenerative disorders AD exhibits more than one pattern of occurrence. While most AD cases onset sporadically, individuals who carry mutations in the sequence of APP or of Presenilin 1 or 2 (both are components of the γ secretase complex) develop early-onset familial AD (fAD).

The misfolding and aggregation of the prion protein (PrP) underlies the development of several conformational diseases. Creutzfeldt-Jakob disease (CJD) is a fatal prion disorder that can onset sporadically, as a familial, mutation-linked malady as well as an infectious disease. It is well-documented that individuals who consumed contaminated beef developed CJD (Prusiner, 1998). Similarly to the case of Aβ, small oligomeric PrP structures are the most infectious prion species (Silveira et al., 2005). Interestingly, different mutations in the sequence of PrP lead to the development of distinct disorders. While certain mutations are accountable for the development of CJD, others underlie the manifestation of Gerstmann-Straussler-Scheinker disease (GSS; Salmona et al., 2003) or of Fatal Familial Insomnia (FFI; Medori et al., 1992). Unlike CJD, GSS, and FFI solely emerge as mutation-linked, familial disorders and exhibit much slower etiology.

FAILURE OF EARLY MATURATION EVENTS UNDERLIE THE ONSET OF CERTAIN CASES OF NEURODEGENERATION

Recent studies challenge the amyloid hypothesis suggesting that at least some fAD cases emanate from the attenuation of γ secretase activity that is inflicted by mutations in the sequence of presenilin 1. Analysis of γ secretase activity in brain samples of individuals who carried various fAD-associated mutations or developed sporadic AD (sAD) unveiled that in most cases the total levels of Aβ were lower than those observed in brains of individuals who showed no signs of dementia. Unexpectedly, no significant difference in total Aβ levels was detected among brains of individuals who had sAD and those of unaffected people (Szaruga et al., 2015). Another interesting avenue of research unveiled that in some cases, the loss of γ secretase endopeptidase function is associated with fAD. For instance, transgenic mice that harbor two copies of mutated presenilin 1 carrying either the L435F or C410Y fAD-linked mutations, exhibit near complete loss of γ secretase function but develop neurodegeneration (Xia et al., 2015). These findings clearly show that increased Aβ production is not a prerequisite for AD development and raise the question of what the mechanisms that underlie fAD are. Why these and other mutations lead to the loss of the γ secretase proteolytic function and whether protein maturation within the secretory pathway plays any role in the pathogenic process that leads to the development of fAD are largely unanswered questions.

To identify common mechanisms that initiate the development of neurodegenerative maladies, we searched for similar mutational patterns in different proteins which cause distinct neurodegenerative illnesses. This approach is based on the rationale that since folding chaperones of the secretion pathway assist the maturation process of many nascent polypeptides, it is probable that analogous mutations which impede chaperone-client interactions can lead to the development of distinct maladies. We identified similar PXXP motifs in the sequences of PrP and of presenilin 1. Previous reports indicated that the substitution of either proline in these motifs of PrP (Hsiao et al., 1989; Yamazaki et al., 1999) and presenilin 1 (Campion et al., 1995) cause GSS or fAD, respectively. To explore why the substitution of these prolines leads to misfolding and disease we used cultured cells and the cyclophilin-specific inhibitor cyclosporin-A (CsA) and found that when CypB is prevented from assisting presenilin 1 to fold properly, the nascent polypeptide misfolds, and forms aggregates that accumulate in the ERQC. This leads to severe impairment of γ secretase activity and to aberrant mitochondrial distribution and function. Similarly, the expression of presenilin 1 molecules carrying the fAD-linked mutations, P264L or P267L/S, resulted in the same phenotypes (Ben-Gedalya et al., 2015).

A similar mechanism triggers the pathogenic process that causes GSS. The inhibition of CypB by CsA or by the substitution of proline 102 or of 105 in the sequence of PrP resulted in the protein's aggregation and deposition in aggresomes (Cohen and Taraboulos, 2003).

Our studies show that hindering the interaction of CypB, a key ER-resident folding chaperone, with PrP and presenilin 1, results in the manifestation of certain cases of fAD or GSS. It is important to note that additional studies illustrate failures in other stages of protein maturation as the source of neurodegenerative disorders. The levels of CNX and CRT were found to be reduced in Parkinson's disease cellular model (Kuang et al., 2014) and ER stress response was found to be activated in amyotrophic lateral sclerosis (ALS)-model mice that express an ALS-linked mutated SOD 1 in their muscles (Chen et al., 2015). In addition, one of the early modifications that PrP undergoes upon entry to the ER is the attachment of GPI. Individuals who carry mutations, such as Q227Stop, that prevent GPI attachment to the newly synthesized molecule, express anchorless PrP and develop GSS (Jansen et al., 2010). It is plausible that anchorless PrP molecules cannot be recognized by ER chaperones and thus form aggregates within this organelle. This possibility may be supported by the report that similarly to CypB, CNX interacts with PrP and reduces PrP-mediated toxicity (Wang et al., 2010). Nevertheless, further research is needed to examine this hypothesis.

The accumulation in aggresomes of molecules that failed to fold properly within the ER, raises the question of whether these deposition sites (Ben-Gedalya et al., 2011) are cytosolic components of an ER-resident protein quality control mechanism or whether they also serve other cellular organelles as aggregate disposal centers.

AGGRESOMES ARE CYTOSOLIC COMPONENTS OF THE ER PROTEIN QUALITY CONTROL MECHANISM

The association of aggresomes with neurodegenerative maladies was first demonstrated by the accumulation of the fAD-causing, presenilin-1 which carries the A246E mutation in these structures (Johnston et al., 1998). Toxic PrP species (Kristiansen et al., 2005), disease causing PrP mutants (Cohen and Taraboulos, 2003; Mishra et al., 2003), and Parkinson's disease-associated, aggregated α-synuclein (Tanaka et al., 2004; Wong et al., 2008) were also shown to be deposited in aggresomes of mammalian cells, further linking these sites with a myriad of human neurodegenerative illnesses. To test whether aggresomes are mechanistically linked to the ER quality control machinery we created fluorescently-tagged PrP constructs that either efficiently enter the ER or stay entirely cytosolic and tested whether these molecules are deposited in aggresomes upon exposing the cell to CsA treatment. We found that PrP must enter the ER in order to be deposited in the aggresome (Dubnikov et al., 2016) defining these sites as remote cytosolic components of the ER. Interestingly, the attachment of a GPI anchor is needed for the direction of PrP to the aggresome but the Golgi apparatus appears to have no role in shuttling aggregated PrP to this structure. This insight further associates the ER protein folding mechanism with deposition sites, a key hallmark of neurodegenerative diseases, and shows that proper activity of folding chaperones

protects from these disorders. However, is foldase activity always protective?

Recent studies have shown that counter-intuitively, knocking down the activity of certain chaperones protects from neurodegeneration. The PPIase FKBP51 acts in collaboration with Hsp90 to prevent the clearance of aggregated tau enhancing the protein's oligomerization. Accordingly, mice over-expressing FKBP51 suffered from neurotoxicity (Blair et al., 2013). Recently, it was shown that reducing the rate of PrP entry into the ER by the inhibition of the ER-resident PPIase FKBP10, reduces toxicity in mammalian cell systems (Stocki et al., 2016), suggesting that under these specific circumstances this foldase enhances PrP toxicity. It is possible that FKBP51 and FKBP10 enhance proteotoxicity not by the modulation of protein folding but by exhibiting other functions and that the activities of certain chaperones may be protective in the face of certain proteotoxic challenges but deleterious in the face of others.

THERAPEUTIC OPPORTUNITIES

Accumulating information point at the ER lumen as an important arena where neurodegenerative-causing events occur. Accordingly, interventions that modulate the activity of ER components can help cells maintain functional proteostasis, prevent the accumulation of hazardous aggregates within the lumen and delay the manifestation of neurodegenerative maladies. Nevertheless, the emerging understanding that various mechanisms can underlie the development of the same malady and the apparent damaging functions of certain folding chaperones requires a careful characterization and classification of neurodegeneration-causing mechanisms. The approval of proteostasis-enhancing compounds and their high efficacy for the treatment of Cystic Fibrosis (Van Goor et al., 2009; Carter et al., 2015) and the counter-proteotoxic effects of aging-modulating compounds (El-Ami et al., 2014) are very encouraging developments which indicate that a comprehensive understanding of the mechanisms that underlie proteinopathies is the basis for the development of novel therapies for hitherto incurable, devastating disorders.

AUTHOR CONTRIBUTIONS

EC and TD wrote the manuscript. TD prepared the figures.

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REVIEW
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Protein-Remodeling Factors As Potential Therapeutics for Neurodegenerative Disease

Meredith E. Jackrel and James Shorter**

Department of Biochemistry and Biophysics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA

Protein misfolding is implicated in numerous neurodegenerative disorders including amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, and Huntington's disease. A unifying feature of patients with these disorders is the accumulation of deposits comprised of misfolded protein. Aberrant protein folding can cause toxicity through a loss or gain of protein function, or both. An intriguing therapeutic approach to counter these disorders is the application of protein-remodeling factors to resolve these misfolded conformers and return the proteins to their native fold and function. Here, we describe the application of protein-remodeling factors to alleviate protein misfolding in neurodegenerative disease. We focus on Hsp104, Hsp110/Hsp70/Hsp40, NMNAT, and HtrA1, which can prevent and reverse protein aggregation. While many of these protein-remodeling systems are highly promising, their activity can be limited. Thus, engineering protein-remodeling factors to enhance their activity could be therapeutically valuable. Indeed, engineered Hsp104 variants suppress neurodegeneration in animal models, which opens the way to novel therapeutics and mechanistic probes to help understand neurodegenerative disease.

Keywords: protein-remodeling factors, protein-misfolding disease, neurodegeneration, Hsp104, Hsp70, Hsp110, NMNAT, HtrA1

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****Correspondence:***
Meredith E. Jackrel
mjackrel@mail.med.upenn.edu
James Shorter
jshorter@mail.med.upenn.edu

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INTRODUCTION

There are numerous devastating, and incurable, neurodegenerative disorders that are increasing in prevalence as our population ages (Dobson, 2003; Forman et al., 2004; Morimoto, 2006). These disorders include: Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and frontotemporal dementia (FTD) (Dobson, 2003; Forman et al., 2004; Morimoto, 2006; Lagier-Tourenne et al., 2010; Robberecht and Philips, 2013). Treatments for these disorders remain palliative, and no therapeutics are available that address their underlying cause (Forman et al., 2004; Robberecht and Philips, 2013). Furthermore, each of these disorders manifests in different ways in patients. For instance, AD patients have impaired memory yet their movement is preserved, while ALS patients' memory is preserved while their control of movement becomes impaired (Forman et al., 2004; Lagier-Tourenne et al., 2010; Robberecht and Philips, 2013). Yet, at the fundamental level, these neurodegenerative disorders are linked by the presence of insoluble proteinaceous inclusions in the brain (Dobson, 2003; Forman et al., 2004; Lagier-Tourenne et al., 2010; Robberecht and Philips, 2013).

It is important to note that these neurodegenerative diseases are not due to mass protein misfolding, but instead the misfolding of specific proteins are implicated in each disease (Dobson, 2003; Cushman et al., 2010). For instance, α-synuclein misfolds into amyloid fibrils that accumulate in Lewy bodies in the dopamine neurons of PD patients, while in ALS patients TDP-43 or FUS misfold into cytoplasmic aggregates in degenerating motor neurons and glia (Spillantini et al., 1997; Neumann et al., 2006; Chen-Plotkin et al., 2010; Mackenzie et al., 2010; Robberecht and Philips, 2013; Dehay et al., 2015). These proteins, as well as many others that underpin diverse neurodegenerative disorders, are expressed in nearly all cells. Yet it remains perplexing what initiates and drives the misfolding of specific proteins in specific neuronal subtypes, leading to subtype-specific neurodegeneration (Saxena and Caroni, 2011). Additionally, it remains unclear if neuronal degeneration is always a direct consequence of aggregate accumulation. Indeed, many of these proteins serve essential functions, and so a loss of function due to aggregation could alternatively lead to toxicity (Winklhofer et al., 2008; Yang et al., 2014; O'Rourke et al., 2016).

In each of these neurodegenerative disorders, the protein homeostasis (proteostasis) network ultimately fails to combat the accumulation of misfolded conformers, consequently leading to disease (Balch et al., 2008; Shorter, 2016). To address the protein-misfolding problem, there are several avenues that could be explored. First, degradation of the toxic, misfolded conformers might be beneficial. For instance, in some PD patients, an increase in α-synuclein levels is implicated, and thus degradation of this excess α-synuclein might be beneficial (Ebrahimi-Fakhari et al., 2012). A similar strategy might be useful in Huntington's disease patients (Yamamoto et al., 2000). Alternatively, stalling the protein-misfolding process is an effective means of therapeutically treating patients with familial amyloid neuropathy (FAP) (Bulawa et al., 2012; Cho et al., 2015; Ankarcrona et al., 2016). FAP is caused by the misfolding of transthyretin, which forms amyloid fibrils that accumulate in various tissues and organs, ultimately leading to organ failure. To combat FAP, the drug Tafamidis was developed to stabilize the native tetrameric form of transthyretin, thus blocking further misfolding and stalling the amyloid cascade. Tafamidis is approved for use by the European Medicines Agency, and is the only therapeutic in use that mitigates neurodegenerative disease by preventing protein misfolding (Ruberg and Berk, 2012). Additionally the drug Tolcapone, which is FDA-approved for PD, was found to also stabilize transthyretin and block aggregation (Sant'Anna et al., 2016). A similar strategy to pharmacologically stabilize α-crystallins may effectively block their misfolding and aggregation and treat cataracts (Makley et al., 2015). The success of Tafamidis provides strong proof of concept that targeting protein misfolding can be therapeutically effective (Bulawa et al., 2012; Cho et al., 2015; Ankarcrona et al., 2016). Additionally, clinical trials are ongoing to assess the efficacy of antibodies aimed at clearing plaques comprised of Aβ that accumulate in AD patients (Sevigny et al., 2016), though notably one trial recently failed. Indeed, an additional intriguing possibility would be to remodel the misfolded species such that the protein regains its functional, native conformation, which would simultaneously mitigate toxicity due to loss-of-function or gain-of-function (Jackrel and Shorter, 2014b, 2015; Mack and Shorter, 2016; Shorter, 2016). However, many of the proteins that misfold in these disorders adopt a cross-beta fibrillar form, termed amyloid, which is a highly stable and self-templating structure (Dobson, 2003). Nonetheless, protein-remodeling factors that have evolved to antagonize protein misfolding could be harnessed to reverse deleterious protein misfolding in disease (**Table 1**).

The proteostasis network ultimately collapses in neurodegenerative disease (Shorter, 2016). This network is comprised of many molecular chaperones that normally promote the proper folding of disease-associated proteins, as well as the entire proteome. Thus, an intriguing way to address the collapse of the proteostasis network would be to remedy or rewire this network (Jackrel et al., 2014a; Jackrel and Shorter, 2014b, 2015). This approach could be pursued by either enhancing and tuning the activity of endogenously expressed protein-remodeling factors, or by introducing new protein-remodeling factors that are not normally expressed (Warrick et al., 1999; Auluck et al., 2002; Jackrel et al., 2014a). Many protein-remodeling factors have been proposed to function in alleviating protein misfolding, including: Hsp104, Hsp110/Hsp70/Hsp40, NMNAT, and HtrA1 (Zhai et al., 2008; Jackrel and Shorter, 2014b, 2015; Poepsel et al., 2015; Ali et al., 2016; Mack and Shorter, 2016; Shorter, 2016). Some of these proteins are capable of actively disaggregating and restoring the solubility of the misfolded conformers (Warrick et al., 1999; Auluck et al., 2002; Jackrel and Shorter, 2014a; Jackrel et al., 2014a). Thus, the application of protein-remodeling factors in a therapeutic setting is a highly promising avenue to address neurodegenerative disease. In this review, we discuss the potential application of these molecular chaperones and protein disaggregases in the development of therapeutics for neurodegenerative disorders. These agents might be harnessed for therapeutic purposes through upregulation or through the introduction of exogenous protein through either gene therapy using adeno-associated viral vector technologies or direct injection. Alternatively, protein-remodeling factors could be therapeutically modulated using small molecules or even potentiated via engineering. We focus on efforts to reformulate a robust protein disaggregase from yeast, Hsp104, which has several unique properties that make it a particularly promising protein-remodeling factor for further exploration and application to reverse the protein misfolding implicated in numerous devastating neurodegenerative diseases (Lo Bianco et al., 2008; DeSantis et al., 2012; Cushman-Nick et al., 2013; Jackrel and Shorter, 2014a,b, 2015; Jackrel et al., 2014a). We also discuss several other protein-remodeling factors that have been recently assessed for their capacity to suppress or reverse protein misfolding connected to neurodegenerative disease.

Hsp70 BLOCKS PROTEIN MISFOLDING

One of the first molecular chaperones to be explored as a possible therapeutic for combating neurodegenerative disease was Hsp70. The Hsp70 family of proteins serves diverse functions in protein folding. Hsp70 promotes the refolding of aggregated or misfolded

TABLE 1 | Protein-remodeling factors can remodel diverse substrates.

Protein remodeling factor	Activity	Substrates remodeled
Hsp70	Blocks misfolding	Polyglutamine, α-syn, Aβ
Hsp110/Hsp70/Hsp40	Dissolves preformed aggregates	SOD1, α-syn
NMNAT	Dissolves preformed aggregates	Tau
Htra1	Dissolves and degrades preformed aggregates	Aβ and tau
Hsp104	Dissolves preformed aggregates, amyloid, and pre-amyloid oligomers	α-syn, TDP-43, FUS, Aβ, tau, polyglutamine

proteins (Mayer and Bukau, 2005; Mack and Shorter, 2016). It also serves to ensure the proper folding of newly synthesized proteins (Mayer and Bukau, 2005). To do so, Hsp70 functions cooperatively with its co-chaperone, Hsp40, to bind and thus protect hydrophobic stretches harbored by its clients (Mayer and Bukau, 2005; Mashaghi et al., 2016). This function is crucial during protein synthesis, but is also important following cellular stresses that partially denature mature proteins, because by binding exposed stretches on these partially denatured proteins, Hsp70 can block protein aggregation (Mayer and Bukau, 2005; Mack and Shorter, 2016). Thus, in disease, upregulation of Hsp70 might prevent protein aggregation and promote the restoration of proteostasis. A *Drosophila* model of polyglutamine misfolding has been established in which overexpression of polyglutamine leads to neurodegeneration (Warrick et al., 1998). In this model, overexpression of Hsp70 suppressed polyglutamine-induced neurodegeneration (Warrick et al., 1999). Similarly, in a *Drosophila* model of α-synuclein misfolding, Hsp70 suppressed neurodegeneration (Auluck et al., 2002). However, it is important to note that while Hsp70 inhibited neurodegeneration in these models, it was not found to solubilize aggregates (Warrick et al., 1999; Auluck et al., 2002; Cushman-Nick et al., 2013). Nonetheless, in a mouse model of ALS, intraperitoneal injection of human Hsp70 increased lifespan, delayed the onset of symptoms, arrested denervation, preserved axonal function, and prolonged motor neuron viability (Gifondorwa et al., 2007, 2012).

Elevating Hsp70 expression can slow neurodegeneration in fly and mouse models (Warrick et al., 1999; Auluck et al., 2002; Gifondorwa et al., 2007, 2012). Hsp70 likely becomes overwhelmed in neurodegenerative disease. Thus, it may be important to enhance Hsp70 activity via potentiating mutations or small molecules (Mack and Shorter, 2016; Shorter, 2016). Indeed, using protein-engineering techniques the activity of the bacterial homolog of Hsp70, DnaK, has been enhanced and these variants demonstrate elevated luciferase refolding activity (Aponte et al., 2010; Schweizer et al., 2011). Recently, Hsp70 engineering has been extended to human Hsp70 and neurodegenerative disease-associated substrates (Aprile et al., 2015). Here, Hsp70 was tuned through rational design to more potently bind α-synuclein and Aβ42. Peptides complementary to target epitopes in α-synuclein and Aβ42 were developed, and these peptides were introduced into the C-terminal region of Hsp70 (Aprile et al., 2015). While introduction of these peptides enhanced the binding affinity of Hsp70 to α-synuclein and Aβ42, binding to other client proteins was unaffected (Aprile et al., 2015). Thus, tuning Hsp70 to broaden its substrate specificity does not come at the cost of restricted capacity to regulate its diverse client pool (Aprile et al., 2015). Additionally, small molecules have been identified that can enhance specific aspects of Hsp70 activity. For instance, four small molecules: MKT-077, JG-98, YM-1, and YM-8 bind the nucleotide-binding domain of Hsp70 in the ADP, but not ATP-bound state. This binding stabilizes the ADP-bound state resulting in increased affinity of Hsp70 for its clients, which can under some circumstances lead to their enhanced folding (Rousaki et al., 2011; Miyata et al., 2013; Wang et al., 2013; Shorter, 2016). In the cellular environment, YM-1 promotes clearance of polyglutamine oligomers and aggregates (Wang et al., 2013). All four of these molecules promote the clearance of tau and are therapeutically beneficial in tauopathy models (Abisambra et al., 2013; Miyata et al., 2013; Fontaine et al., 2015).

THE METAZOAN PROTEIN-DISAGGREGASE SYSTEM: Hsp110/Hsp70/Hsp40

It has long been hypothesized that humans might possess a protein disaggregase similar to those in the Hsp100 family of proteins that are highly conserved in bacteria, fungi, and plants (Shorter, 2008, 2011; Torrente and Shorter, 2013). However, the discovery of such a protein disaggregase has been elusive until it was discovered that Hsp110 in collaboration with Hsp70 and Hsp40 can disaggregate and reactivate protein (Shorter, 2011; Mattoo et al., 2013; Torrente and Shorter, 2013; Finka et al., 2015; Gao et al., 2015; Nillegoda and Bukau, 2015; Nillegoda et al., 2015). Hsp110 is an Hsp70 family member that in collaboration with Hsp70 and Hsp40 can disaggregate preformed aggregates and amyloid (Shorter, 2011; Duennwald et al., 2012; Gao et al., 2015; Nillegoda et al., 2015). Hsp110 collaborates and synergizes with Hsp70 and two classes of Hsp40 cochaperones to resolve large protein aggregates (Nillegoda and Bukau, 2015; Nillegoda et al., 2015). It is hypothesized that due to the large number of possible complexes that could form between different Hsp70s and Hsp40s, distinct and specific complexes might be harnessed to dissolve different protein aggregates (Nillegoda and Bukau, 2015; Nillegoda et al., 2015). Perhaps one specific combination might be employed in specific neuronal subtypes, or a given combination might specifically disaggregate α-synuclein while another might specifically disaggregate tau.

Ultimately, failure of the Hsp110/Hsp70/Hsp40 system might underpin numerous protein-misfolding disorders, and restoration or specific activation of this system might be therapeutically useful (Nillegoda and Bukau, 2015; Shorter, 2016). Indeed, overexpression of Hsp110 with Hsp40 suppressed the toxicity induced by polyglutamine overexpression in *Drosophila*, though it is not apparent if Hsp110 modulates polyglutamine aggregation (Kuo et al., 2013). Additionally, transgenic overexpression of Hsp110 in neurons enhanced survival in ALS model mice, but again, the effects of Hsp110 on SOD1 aggregation were not assessed in these experiments (Nagy et al., 2016). It remains unclear if upregulation of Hsp110 levels will be sufficient to restore normal functionality in animal models, and ultimately in humans. It may be useful to tune the activity of the Hsp110/Hsp70/Hsp40 system using protein-engineering techniques, or alternatively, small-molecule modulators could be developed to enhance the activity of this system. Small heat-shock proteins can also enhance the disaggregase activity of this system (Duennwald et al., 2012), and might also be targeted therapeutically (Makley et al., 2015). However, determining precisely how to therapeutically boost the activity of this system comprised of several components may prove challenging.

NMNAT

Nicotinamide mononucleotide adenylyl transferases (NMNATs) are nicotinamide adenine dinucleotide (NAD)-synthesizing enzymes. NAD is an important cofactor that mediates numerous cellular processes. NMNATs are important in neuronal maintenance, thus NMNAT knockdown leads to axonal degeneration, while NMNAT overexpression is neuroprotective in several animal models of neurodegeneration (Zhai et al., 2008; Gilley and Coleman, 2010; Ali et al., 2016). NMNAT2 is highly expressed in the mammalian brain, and NMNAT2 mRNA levels are reduced in PD, HD, AD, and tauopathy patients (Ali et al., 2016). Furthermore, elevating NMNAT2 levels in tauopathy model mice suppressed neurodegeneration (Ljungberg et al., 2012). Additionally, NMNAT2 mRNA levels correlate positively with cognitive function and negatively with the pathological features of AD (Ali et al., 2016). In AD brains, NMNAT2 mRNA and protein levels are greatly reduced relative to controls, and NMNAT2 co-localizes with aggregated tau (Ali et al., 2016). NMNAT2 overexpression can reduce the pathological accumulation of hyperphosphorylated tau without altering total tau levels (Ljungberg et al., 2012; Ali et al., 2016). NMNAT2 can prevent protein denaturation and promote protein refolding with similar activity to Hsp70 (Ali et al., 2016). Surprisingly, this activity is maintained even in enzymatically-dead NMNAT2 mutants that lack NAD synthetic activity (Ali et al., 2016). These enzymatically-dead NMNAT2 mutants also reduced hyperphosphorylated tau levels (Ali et al., 2016).

NMNAT2 has been demonstrated to form a complex with Hsp90 to solubilize and refold aggregated substrates (Ali et al., 2016). Moreover, deletion of NMNAT2 increases the vulnerability of cortical neurons to proteotoxic stress (Ali et al., 2016). Thus, therapeutically upregulating NMNAT or enhancing NMNAT activity via small-molecule modulation might be effective in regulating tau levels. It will be interesting to assess the protein-remodeling activity of NMNATs against the many other substrates implicated in protein-misfolding disorders. Given the fundamental role NMNATs play in neuronal maintenance, failure of NMNATs to combat protein misfolding might be common to many other disease-associated substrates in addition to tau.

HtrA1 CAN DISAGGREGATE AND DEGRADE TOXIC CONFORMERS

HtrA1 is a PDZ serine protease that disassembles tau and Aβ fibrils, which are linked to AD, and then degrades them (Poepsel et al., 2015). Intriguingly, HtrA1 is found in the cytoplasm and is also secreted (Poepsel et al., 2015). Correlating with this pattern, Aβ42 fibrils are found in the extracellular space while tau fibrils are found in the cytoplasm. Thus, it has been hypothesized that HtrA1 might be a system that naturally disaggregates and degrades Aβ42 and tau (Poepsel et al., 2015; Shorter, 2016). Indeed, HtrA1 activity might be insufficient in AD patients (Shorter, 2016). Therefore, boosting and fine-tuning the activity of HtrA1 might be valuable in combating AD. It has been demonstrated that HtrA1 activity can be tuned through protein engineering. The disassembly and degradation activities of HtrA1 can be separated, as protease-defective HtrA1 variants dissolve but do not degrade Aβ fibrils, providing HtrA1 variants that can either dissolve the aggregates or dissolve and degrade the aggregates (Poepsel et al., 2015). The ability to separate or combine disassembly and degradation activities in a single protein is very valuable, and might find utility in certain situations. Therefore, it will be very interesting to engineer substrate-specific HtrA1 variants that can target substrates beyond Aβ and tau. These substrate-specific variants could be constructed in both the disaggregate-only or disaggregate-and-degrade backgrounds. This advance would allow for the flexibility to reactivate proteins that serve beneficial functions. Alternatively, subsets of PD patients show increased α-synuclein levels (Ebrahimi-Fakhari et al., 2012), and thus for these patients it may be beneficial to not just solubilize α-synuclein, but also to degrade it. Additionally, small-molecule enhancers of HtrA1 have been identified, and so it will be important to test the effects of these compounds in various models of protein-misfolding disorders (Jo et al., 2014). It will also be important to assess if HtrA1 can clear highly toxic pre-amyloid oligomeric forms of Aβ and tau, or only the fibrils.

Hsp104 VARIANTS SUPPRESS PROTEIN MISFOLDING, MISLOCALIZATION, AND TOXICITY IN YEAST AND ANIMAL MODELS

Hsp104 is a ring-shaped hexameric AAA+ protein from yeast that serves two distinct functions (Sweeny and Shorter, 2016; Yokom et al., 2016). First, it solubilizes proteins that aggregate

following cellular stress to promote yeast survival (Parsell et al., 1991, 1994; Glover and Lindquist, 1998; Glover and Tkach, 2001; Wallace et al., 2015). Second, it regulates yeast prion formation and dissolution (Chernoff et al., 1995; Shorter and Lindquist, 2004, 2005, 2006; Sweeny and Shorter, 2008, 2016; Sweeny et al., 2015). In serving these two roles, Hsp104 recognizes and regulates a diverse milieu of substrates, comprised of the entire yeast proteome, as well as yeast prions (Newby and Lindquist, 2013). While Hsp104 is highly conserved in bacteria, fungi, and plants, Hsp104 has no metazoan homolog (Erives and Fassler, 2015).

The amyloid fold is a highly conserved protein structure, thus it was hypothesized that the natural capacity of Hsp104 to recognize and solubilize yeast prions might translate to a capacity to recognize and solubilize diverse amyloid species associated with human disease (DeSantis et al., 2012; Jackrel and Shorter, 2014b, 2015; Jackrel et al., 2014a). Indeed, using purified proteins, Hsp104 has been shown to solubilize diverse amyloid species implicated in human disease including: Aβ, α-synuclein, polyglutamine expansions, prion protein, tau, and amylin (Liu et al., 2011; DeSantis et al., 2012; Jackrel and Shorter, 2014a; Jackrel et al., 2014a). Additionally, Hsp104 suppresses proteotoxicity in animal models (Satyal et al., 2000; Vacher et al., 2005; Lo Bianco et al., 2008; Cushman-Nick et al., 2013; Jackrel et al., 2014a). In a transgenic mouse model of HD, Hsp104 extended lifespan and decreased aggregate load (Vacher et al., 2005). Furthermore, Hsp104 has been demonstrated to be neuroprotective in a rat model of PD (Lo Bianco et al., 2008). Here, lentiviral vectors coding for α-synuclein were injected into the substantia nigra of rats, and following 6 weeks of expression, brain slices were stained for dopaminergic markers. In this system, Hsp104 co-expression was neuroprotective and no off-target effects were observed (Lo Bianco et al., 2008). Additionally, Hsp104 has been shown to directly clear preformed oligomeric forms of α-synuclein as well as eliminate self-templating α-synuclein conformers. These experiments have provided strong evidence that Hsp104 may have therapeutic value. However, the activity of Hsp104 in suppressing degeneration in these animal models is limited as complete neuroprotection is not achieved (Vacher et al., 2005; Lo Bianco et al., 2008).

We have enhanced the activity of Hsp104 via engineering (Jackrel et al., 2014a,b, 2015; Jackrel and Shorter, 2014a). We have constructed large libraries of randomized Hsp104 variants and developed screening techniques to isolate enhanced variants. When overexpressed in yeast, the proteins TDP-43, FUS, and α-synuclein all form cytoplasmic foci and are toxic (Outeiro and Lindquist, 2003; Johnson et al., 2008; Sun et al., 2011). These yeast models have also empowered the identification of genetic risk factors for these disorders (Elden et al., 2010; Ju et al., 2011; Sun et al., 2011). Deletion or overexpression of Hsp104 does not suppress the toxicity or aggregation of these proteins in yeast (Jackrel et al., 2014a). Thus, these yeast models provide an ideal screening platform to isolate Hsp104 variants with a gain of therapeutic function (Jackrel et al., 2014a,b, 2015). Using these yeast assays, we have identified numerous Hsp104 variants that potently suppress TDP-43, FUS, and α-synuclein toxicity (Jackrel and Shorter, 2014a,b, 2015; Jackrel et al., 2014a,b, 2015). In addition to their suppression of toxicity, these variants also dissolved cytoplasmic foci of TDP-43, FUS, and α-synuclein (Jackrel and Shorter, 2014a; Jackrel et al., 2014a, 2015). Furthermore, the potentiated variants restored alpha-synuclein to the plasma membrance and TDP-43 to the nucleus (Jackrel et al., 2014a). These results are very promising. TDP-43 must shuttle to the nucleus to fulfill its roles in RNA homeostasis, and restoration of nuclear TDP-43 suggests that solubilization of TDP-43 can restore natively folded and functional TDP-43 (Jackrel et al., 2014a). These potentiated Hsp104 variants clear preformed TDP-43, FUS, and α-synuclein fibrils at concentrations where Hsp104 is ineffective (Jackrel et al., 2014a).

To assess the therapeutic utility of potentiated Hsp104 variants, they have been tested in a *C. elegans* model of PD (Jackrel et al., 2014a). Here, the potentiated Hsp104 variants were robustly neuroprotective, while Hsp104 and an ATPase-dead negative control showed no activity (Jackrel et al., 2014a). To further demonstrate the therapeutic possibilities of potentiated Hsp104 variants, it will be essential to demonstrate their activity in additional neuronal models including mammalian neurons. It will also be important to develop additional potentiated Hsp104 variants with improved properties. To do so, it will be crucial to apply additional protein engineering techniques to enhance the activity and substrate specificity of the potentiated variants. There are numerous examples of proteins that are believed to have evolved from roles as generalists to specialists. Thus, it will be very interesting to see if laboratory techniques can accelerate this process for Hsp104 and produce finely-tuned variants. Additionally, perhaps Hsp104 variants can be produced to target pre-amyloid oligomers vs. fibrils and vice versa. In addition to being potentially of direct therapeutic benefit, these variants may hold great value in unraveling the key contributors and drivers of neurodegenerative disease. For instance, engineered disaggregases that can solubilize and reactivate oligomers but not fibrils may be employed as precise mechanistic probes to investigate the effects of resolving specific protein species. Also, the discovery that very subtle modification of natural protein-remodeling factors can confer dramatic alterations in chaperone activity (Jackrel et al., 2014a, 2015) suggests that subtle modification of other protein-remodeling factors, including Hsp110/Hsp70/Hsp40, NMNAT, and HtrA1 might also be amenable to potentiation.

CONCLUSIONS AND FUTURE DIRECTIONS

Protein misfolding is an enormously challenging issue that underpins many of the most devastating diseases facing society. As the population continues to age, the toll of neurodegenerative disease will continue to rise. Unfortunately, while substantial efforts have been mounted to counter these disorders, there are no treatments available for any of these diseases (with the exception of Tafamidis for FAP). Thus, in the development of new therapeutics to combat these disorders, it will be important to employ innovative approaches. While it is unknown

what specifically causes proteins to misfold and cause disease, the accumulation of misfolded aggregates, amyloid, and pre-amyloid species are key contributors to pathogenesis. Therefore, if protein-misfolding trajectories could be reversed, perhaps so could these diseases. Protein-remodeling factors, which have the capacity to block and even reverse protein misfolding might be uniquely positioned as potential therapeutics. Many protein-remodeling factors have been assessed and demonstrated to be potentially useful in combating these disorders. For instance, increased levels or activity of the protein HtrA1 might be employed to dissolve and degrade both tau and Aβ aggregates in AD patients. However, as HtrA1, as well as NMNAT and Hsp110/Hsp70/Hsp40, are all present in humans, it appears that these systems are either insufficient to prevent pathogenesis or are compromised in certain individuals. Thus, it will be important to continue to focus not just on the application of these chaperones directly in disease models, but also to continue to develop approaches to boost and nuance these protein-remodeling systems.

While highly promising, the idea of modulating the proteostasis network is not without caveats. For instance, upregulation of protein-remodeling factors might be beneficial to enhance protein folding and combat neurodegenerative disorders, yet enhanced protein folding might also enable cell proliferation which could promote cancers. Nonetheless, protein-remodeling factors present a unique opportunity to restore proteins to their native fold and function, thus simultaneously alleviating both a loss or gain of function. As with all therapeutics, it will be important to assess for possible off-target effects. For instance, in developing new disaggregase technologies, it will be important to harness protein disaggregation to avoid the unfolding of functional protein complexes. However, it is important to note that Hsp104 does not unfold natively folded proteins. Regardless, it will be important to continue to engineer protein-remodeling factors with desired traits, such as enhanced substrate specificity. New approaches to develop small-molecule modulators of protein-remodeling systems, as well as the engineering of tailored protein-remodeling systems, will prove invaluable in our efforts to rewire and restore the proteostasis network and thus combat neurodegenerative disease.

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REVIEW
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Cellular Regulation of Amyloid Formation in Aging and Disease

*Esther Stroo, Mandy Koopman, Ellen A. A. Nollen * and Alejandro Mata-Cabana **

European Research Institute for the Biology of Aging, University of Groningen, University Medical Center Groningen, Groningen, Netherlands

As the population is aging, the incidence of age-related neurodegenerative diseases, such as Alzheimer and Parkinson disease, is growing. The pathology of neurodegenerative diseases is characterized by the presence of protein aggregates of disease specific proteins in the brain of patients. Under certain conditions these disease proteins can undergo structural rearrangements resulting in misfolded proteins that can lead to the formation of aggregates with a fibrillar amyloid-like structure. Cells have different mechanisms to deal with this protein aggregation, where the molecular chaperone machinery constitutes the first line of defense against misfolded proteins. Proteins that cannot be refolded are subjected to degradation and compartmentalization processes. Amyloid formation has traditionally been described as responsible for the proteotoxicity associated with different neurodegenerative disorders. Several mechanisms have been suggested to explain such toxicity, including the sequestration of key proteins and the overload of the protein quality control system. Here, we review different aspects of the involvement of amyloid-forming proteins in disease, mechanisms of toxicity, structural features, and biological functions of amyloids, as well as the cellular mechanisms that modulate and regulate protein aggregation, including the presence of enhancers and suppressors of aggregation, and how aging impacts the functioning of these mechanisms, with special attention to the molecular chaperones.

Keywords: neurodegeneration, protein aggregation, amyloid, protein quality control, SERF

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Eva Zerovnik, Jožef Stefan Institute, Slovenia

****Correspondence:***
Ellen A. Nollen
e.a.a.nollen@umcg.nl
Alejandro Mata-Cabana
matacabana@gmail.com

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INTRODUCTION

The process of aging is defined as a time-dependent functional decline eventually resulting in an increased vulnerability to death (reviewed in López-Otín et al., 2013). Gaining knowledge about the molecular events that occur in the cell during aging is important in order to understand the disease process of age-related diseases. Some neurodegenerative diseases, including Alzheimer (AD), Parkinson (PD), and Huntingtin disease (HD), share as hallmark the appearance of protein aggregates with fibrillary amyloid-like structures in the brain. These amyloid fibrils are composed of aggregation-prone proteins, such as mutant huntingtin (HTT) in Huntington disease, α-synuclein in Parkinson disease, and amyloid-beta (Aβ) in Alzheimer disease (Scherzinger et al., 1999; Chiti and Dobson, 2006; Goedert and Spillantini, 2006; See **Table 1** for a list of aggregation-prone proteins involved in neurodegenerative diseases). The role of these aggregates in disease is not fully understood: the most prevalent hypothesis is that aggregation intermediates—single or complexes of aggregation-prone proteins—are toxic to cells and that the aggregation process represents a cellular protection mechanism against these toxic intermediates (Lansbury and Lashuel, 2006; Hartl and Hayer-Hartl, 2009).

TABLE 1 | Neurodegenerative diseases associated with protein aggregation.

	Identified disease genes	Protein that aggregates	Location of aggregates	Affected brain region
Alzheimer disease (AD)	*APP* (Chartier-Harlin et al., 1991; Goate et al., 1991; Murrell et al., 1991) *PS1* (Sherrington et al., 1995) *PS2* (Levy-Lahad et al., 1995; Rogaev, 1995)	Amyloid-beta, Tau	Extracellular Intracellular	Cortex and Hippocampus
Huntington disease (HD)	*HD* (Hess et al., 2016)	Huntingtin	Intracellular	Striatum
Parkinson disease (PD)	*SNCA* (Polymeropoulos et al., 1997) *Parkin* (Kitada et al., 1998) *PINK1* (Valente et al., 2001) *DJ1* (Bonifati et al., 2003) *LRRK* (Zimprich et al., 2004) e.a.	Alpha synuclein	Intracellular	Substantia Nigra
Dementia with Lewy bodies (DLB)	*SNCA* (Higuchi et al., 1998) *SNCB* (Ohtake et al., 2004)	Alpha synuclein	Intracellular	Cortex and hippocampus
Frontotemporal dementia (FTA)	*MAPT* (Wilhelmsen et al., 1994)	Tau	Intracellular	Frontal and temporal cortex
Prion disease (PrD)	*PRNP* (Oesch et al., 1985)	Prion protein	Extracellular	Brain and spinal cord
Amyotrophic lateral sclerosis (ALS)	*SOD1* (Rosen et al., 1993) *FUS* (Kwiatkowski et al., 2009) *C9orf72* (DeJesus-Hernandez et al., 2011; Renton et al., 2011) e.a.	SOD, FUS, TDP-43	Intracellular	Upper and lower Motor neurons

The familial forms of many neurodegenerative diseases appear to involve toxic gain-of-function mutations in disease-specific proteins that increase their misfolding and aggregation properties. The resulting misbalance in protein homeostasis can speed up the process of amyloid formation, thereby often provoking an early-onset of several neurodegenerative disorders.

In this review, we address the involvement of aggregation-prone proteins in the development of different age-related disease. We describe how different cellular regulators impact on protein aggregation and how they are affected by aging, with special focus on the molecular chaperone machinery and other pathways involved in maintaining protein homeostasis. We also discuss different mechanisms that may underlie the toxicity of amyloid-forming proteins and we highlight some new findings in the amyloid field.

Abbreviations: Aβ, amyloid-beta; AD, Alzheimer disease; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; APR, aggregation prone region; ATTR, transthyretin amyloidosis; CMA, chaperone mediated autophagy; CJD, Creutzfeldt-Jakob disease; CPEB, cytoplasmic polyadenylation element-binding protein; DLB, dementia with Lewy bodies; ER, endoplasmic reticulum; FTD, frontal temporal dementia; HD, Huntington disease; HSF-1, heat shock factor 1; HSP, heat shock protein; HTT, huntingtin; IAPP, islet amyloid polypeptide; IIS, insulin/insulin-like growth factor 1 signaling; IPOD, insoluble protein deposit; JUNQ, juxtanuclear quality control compartments; LLPS, liquid-liquid phase separation; MOAG-4, modifier of aggregation 4; NPC, nuclear pore complex; PD, Parkinson disease; PolyQ, polyglutamine; PQC, protein quality control; PrD, prion disease; PrP, prion protein; RNP, ribonucleoprotein; SAA, serum amyloid protein; SERF, small EDKR rich factor; UPR, unfolded protein response; UPS, ubiquitin-proteasome system.

CELLULAR REGULATORS OF PROTEIN AGGREGATION

Protein Quality Control

Cells have a protein quality control (PQC) system to maintain protein homeostasis. Preserving protein homeostasis involves the coordinated action of several pathways that regulate biogenesis, stabilization, correct folding, trafficking, and degradation of proteins, with the overall goal to prevent the accumulation of misfolded proteins and to maintain the integrity of the proteome.

Chaperones

One of the cellular mechanisms that copes with misfolded proteins is the chaperone machinery. A molecular chaperone is defined as a protein that interacts with, stabilizes or assists another protein to gain its native and functionally active conformation without being present in the final structure (Ellis, 1987). Many members of the chaperone protein family are referred to as heat shock proteins (HSP), as they are upregulated during stress conditions such as heat shock (Ellis and Hartl, 1999; Kim et al., 2013). In addition to folding of misfolded proteins, molecular chaperones are also involved in a wide range of biological processes such as the folding of newly

synthesized proteins, transport of proteins across membranes, macromolecular-complex assembly or protein degradation and activation of signal transduction routes (Kim et al., 2013; Kakkar et al., 2014). Under the denomination of "molecular chaperones" there are a variability of proteins that have been classified into five different families according to sequence homology, common functional domains or subcellular localization: the HSP100s, the HSP90s, the HSP70/HSP110, HSP60/CCTs, and the a-crystallin-containing domain generally called the "small HSPs" (Lindquist and Craig, 1988; Sharma and Priya, 2016). Typically, molecular chaperones recognize exposed hydrophobic domains in unfolded or misfolded proteins, preventing their self-association and aggregation (Hartl et al., 2011; Kim et al., 2013). The regulation of chaperones can be divided into three categories, (1) constitutively expressed, (2) induced upon stress, and (3) constitutively expressed and induced upon stress (Morimoto, 2008). Under normal conditions the HSP levels match the overall level of protein synthesis, but during stress when mature proteins are unfolded the chaperone machinery is challenged and the expression of specific HSPs increases (Kakkar et al., 2014).

Next to their function under normal cellular conditions, chaperones play an important part during neurodegeneration when there is an overload of the PQC system by unfolded proteins (Kim et al., 2013; Kakkar et al., 2014; Lindberg et al., 2015). Each neurodegenerative disease is associated with a different subset of HSPs that can positively influence the overload of unfolded proteins (Kakkar et al., 2014). One example is the molecular chaperone DNAJB6b that can suppress polyglutamine (polyQ) aggregation and toxicity in a cell model for polyQ diseases (Hageman et al., 2010; Gillis et al., 2013), and suppress the primary nucleation step by a direct protein-protein interaction with polyQ proteins (Månsson et al., 2014b) and Aβ42 (Månsson et al., 2014a). Overexpression of DNAJB6 in a mouse model for HD results in reduction of the disease symptoms and increase life span (Kakkar et al., 2016). In PD, the overexpression of HSP70 can prevent α-synclein-induced cell death in yeast, *Drosophila* and mouse models of this disease (Auluck and Bonini, 2002; Klucken et al., 2004; Flower et al., 2005; Sharma and Priya, 2016). HSP70 has been shown to bind prefibrillar species of α-synclein and to inhibit the fibril formation (Dedmon et al., 2005). There is also a role for molecular chaperones in AD, where the overexpression of heat shock factor 1 (HSF-1), main regulator of HSPs expression, in an AD mouse model diminished soluble Aβ levels (Pierce et al., 2013), and multiple HSPs alleviated Tau toxicity in cells (Kakkar et al., 2014).

Additionally to the inhibition of protein aggregation of misfolded proteins, a disaggregase activity has been described for some molecular chaperones that can solubilize aggregated proteins (Glover and Lindquist, 1998; Tyedmers et al., 2010; Winkler et al., 2012). In bacteria, yeast, fungi and plants the HSP100 disaggregases are highly conserved (Tyedmers et al., 2010; Torrente and Shorter, 2013). In yeast, HSP104 collaborates with the other HSPs, to effectively disaggregate and reactivate proteins trapped in disordered aggregates (Glover and Lindquist, 1998; Shorter, 2011; Torrente and Shorter, 2013; Lindberg et al., 2015). Metazoans entirely lack HSP100 disaggregases in the cell, however, it has recently shown that in mammalians the disaggregase function is performed by the HSPH (Hsp110) family in cooperation with the HSP70-40 machine (Rampelt et al., 2012; Gao et al., 2015; Nillegoda and Bukau, 2015). This machinery has been shown to fragmentize and depolarize large α-synclein fibrils within minutes into smaller fibrils, oligomers and monomeric α-synclein in an ATP-dependent fashion (Gao et al., 2015).

Chaperones are also involved in other pathways of PQC. As discussed below they can mediate the degradation of misfolded proteins or their sequestration in cellular compartments.

Together, this shows the important direct role chaperones play in the formation of amyloids and thereby making chaperones an interesting therapeutic target for neurodegenerative diseases.

Protein Degradation

Protein degradation is another key mechanism to deal with misfolded proteins. Three pathways have been described, i.e., the ubiquitin (Ub)-proteasome system (UPS), chaperone mediated autophagy (CMA), and macroautophagy (Ciechanover, 2006; Ciechanover and Kwon, 2015). Soluble misfolded proteins are degraded by the UPS, a system that is dependent on a cascade of three enzymes E1, E2, and E3 ligase that conjugate ubiquitin to the misfolded proteins. The ubiquitinated protein is transported by molecular chaperones to the proteolytic system, where the protein is unfolded and passed through the narrow chamber of the proteasome that cleaves it into short peptides (Ciechanover et al., 2000). The CMA degrades proteins that expose KFERQ-like regions, these regions are recognized by the chaperone heat-shock cognate 70 (Hsc70) and delivered to the lysosomes and degraded by lysosomal hydrolases into amino acids (Kiffin et al., 2004; Rothenberg et al., 2010). Protein aggregates or proteins that escape the first two degradation pathways are directed to macroautophagy, a degradation system where substrates are segregated into autophagosomes which in turn are fused with lysosomes for degradation into amino acids (Koga and Cuervo, 2011). The proteins involved in neurodegenerative disease can rapidly aggregate and can thereby escape degradation when they are still soluble, the aggregates, and intermediate forms are partly resistant to the known degradation pathways (reviewed in Ciechanover and Kwon, 2015).

Unfolded Protein Response

In the endoplasmic reticulum (ER), the unfolded protein response (UPR), induced during periods of cellular and ER stress, aims to reduce unfolded protein load, and restore protein homeostasis by translational repression. ER stress can be the result of numerous conditions, including amino acid deprivation, viral replication and the presence of unfolded proteins, resulting in activation of the UPR. The UPR has three pathways activated through kinases, (1) protein kinase RNA (PKR)-like ER kinase (PERK), (2) inositol-requiring enzyme 1 (IRE1), and (3) activating transcription factor 6 (ATF6; Halliday and Mallucci, 2015). These kinases are kept in their inactive state by the binding immunoglobulin protein (BiP), during ER stress this protein binds to exposed hydrophobic domains of

unfolded proteins and thereby allowing activation of these factors (Gething, 1999). In neurodegenerative diseases markers of the UPR, like PERK-P and eIF2α-P, have been reported in the brain of patients with neurodegenerative disease and in mouse models of neurodegeneration (Hetz and Mollereau, 2014; Scheper and Hoozemans, 2015).

Protein Compartmentalization

In the cell, misfolded proteins can be sequestered in distinct protein quality control compartments by chaperones and sorting factors. These compartments function as temporary storage until the protein can be refolded or degraded by the proteasome. Different compartments have been described in the literature that sequester different kind of misfolded proteins at various conditions, these include JUNQ, IPOD, Q-body, and aggresome (Sontag et al., 2014). Insoluble proteins are sequestered into insoluble protein deposit (IPOD) compartments that are located near the periphery of the cell (Kaganovich et al., 2008; Specht et al., 2011). If the proteasome is impaired these insoluble proteins can also be sequestered in aggresomes (Johnston et al., 1998), whereas, soluble misfolded proteins can be sequestered into ER-anchored structures named Q-bodies (Escusa-Toret et al., 2013). However, when the proteasome is impaired soluble ubiquitinated misfolded proteins are sequestered into ER-associated juxtanuclear quality control compartments (JUNQ) compartments (Kaganovich et al., 2008; Specht et al., 2011).

The JUNQ and Q-bodies concentrate misfolded proteins in distinct compartments together with chaperones and clearance factors, which makes processing them easier and more efficient. The IPOD and aggresomes are thought to protect the cell from toxic misfolded species, they do however also contain some disaggregases and autophagy related proteins and might therefore be recovered from these compartments (Kaganovich et al., 2008; Specht et al., 2011).

Drivers of Amyloid Formation

Most studies on neurodegenerative diseases focus on either the toxic mechanisms or on the PQC system as possible targets for treatment. Only a few studies so far have focused directly on modifiers of the protein aggregation pathway. One example is the study that focused on a reduced insulin/insulin-like growth factor 1 signaling (IIS), which induces the assembly of Aβ into densely packed and larger fibrillar structures (Cohen et al., 2009). The exact mechanisms behind the formation of these tightly packed amyloid structures by IIS signaling remains to be unraveled.

MOAG-4 (modifier of aggregation 4) was found in a forward genetic screen using *C. elegans* models for neurodegenerative diseases, as an enhancer of aggregation and toxicity of several aggregation-prone disease proteins, including polyQ, α-synuclein, and Aβ (van Ham et al., 2010). MOAG-4 is a small protein of unknown function that is evolutionarily highly conserved. It contains a 4F5 domain of unknown function and is predicted to have a helix-loop-helix secondary structure. MOAG-4 itself was excluded from the polyQ aggregates in the *C. elegans* model. Based on biochemical experiments with worm extracts, MOAG-4 has been suggested to act on the formation of a compact aggregation intermediate. Furthermore, *in vitro* studies with mutant HTT exon 1 and MOAG-4 show a direct increase in aggregation (Unpublished data). Moreover, it was shown that the effect on aggregation works independent from DAF-16, HSF-1, and chaperones.

The human orthologs of MOAG-4 were found to be a two small proteins with unknown function, i.e., Small EDKR Rich Factor (SERF) 1A and 2. These two orhologs are 40% identical and 54% similar to MOAG-4 (van Ham et al., 2010). It was found that SERF1a (Falsone et al., 2012) is able to directly drive the amyloid formation of mutant HTT exon 1 and alpha-synuclein in an *in vitro* assay. It has been suggested that SERF1a directly affects the amyloidogenesis of alpha-synuclein by catalyzing the transition of an alpha-synuclein monomer into a amyloid-nucleating species (Falsone et al., 2012). From cell culture experiments we know that overexpression of SERF1a or SERF2, together with mutant HTT exon 1 results in an increase in toxicity and aggregation of the polyQ protein. Whereas, knock down of SERF using siRNA results in reduced toxicity and aggregation (van Ham et al., 2010).

PROTEIN HOMEOSTASIS IN AGING

Under normal conditions, the PQC can rapidly sense and correct cellular disturbances, by e.g., activating stress-induced cellular responses to restore the protein balance. During aging or when stress becomes chronic, the cell is challenged to maintain proper protein homeostasis (**Figure 1**; Koga et al., 2011; Labbadia and Morimoto, 2015; Radwan et al., 2017). Eventually, this can lead to chronic expression of misfolded and damaged proteins in the cell that can result in the formation of protein aggregates. The presence of aggregation-prone proteins contributes to the development of age-related diseases (Chiti and Dobson, 2006; Kakkar et al., 2014). The decline of protein homeostasis during aging is a complex phenomenon that involves a combination of different factors.

In line with the decreased protein homeostasis, there appears to be an impairment of the upregulation of molecular chaperones during aging (reviewed in Koga et al., 2011). This has been reported for HSP70 in senescent fibroblasts and in different tissues from different species, including monkeys (Fargnoli et al., 1990; Pahlavani et al., 1995; Hall et al., 2000). The importance to regulate the expression of HSPs is seen in flies and worms, where upregulation of HSPs leads to increase in lifespan (Walker et al., 2001; Hsu et al., 2003; Morley and Morimoto, 2003). Furthermore, lymphocytes from human centenarians show chaperone-preserved upregulation during heat shock (Ambra et al., 2004). It has been proposed that inability of the transcription factor HSF-1 to bind the chaperone gene promoter could explain the failure of *hsp70* to respond to stress during aging (Ambra et al., 2004; Singh et al., 2006). The functional decline of chaperones during aging also impairs proper folding of proteins in the ER resulting in activation of the UPR (reviwed in Taylor, 2016). Moreover, it has been shown that the capacity of some elements of the UPR, like PERK or IRE-1 also decline with age (Paz Gavilán et al., 2006; Taylor and Dillin, 2013).

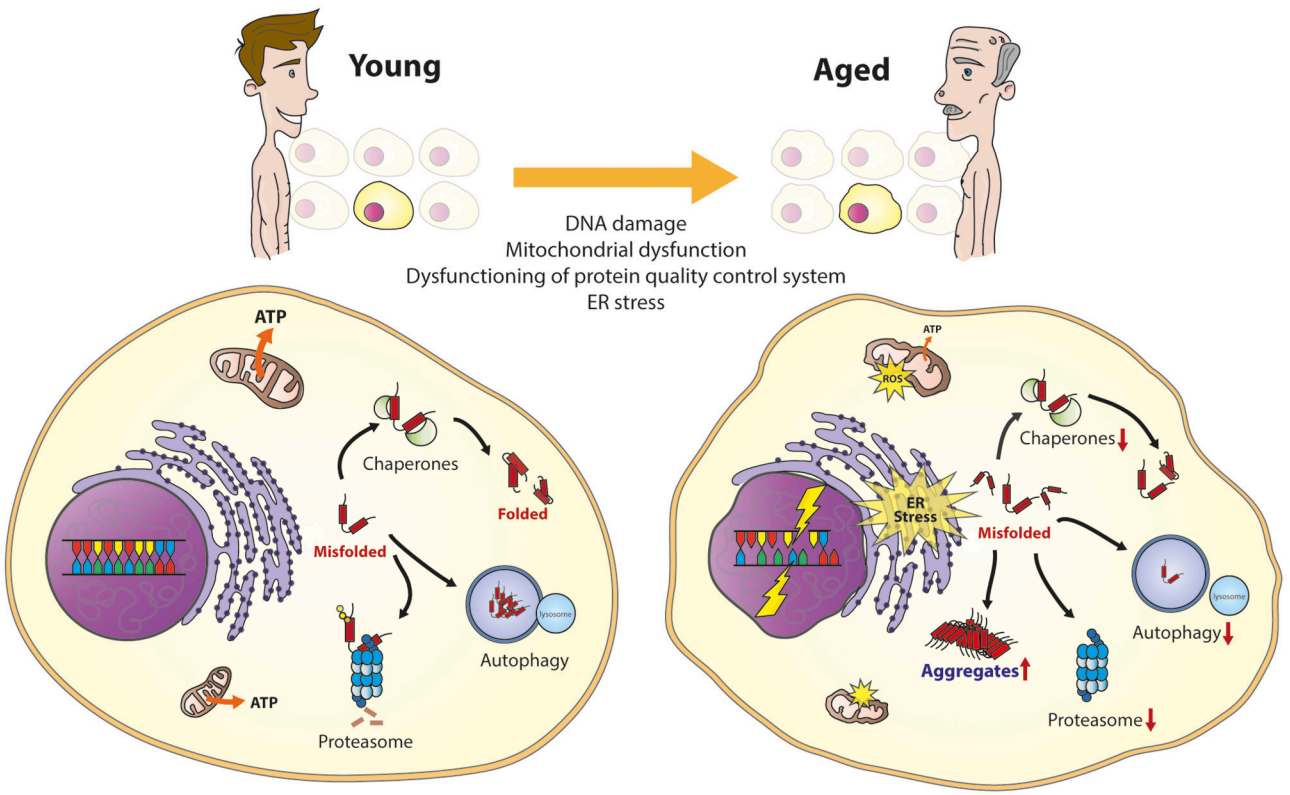

FIGURE 1 | The aging cell. Important cellular processes are affected during aging. This will result in several cellular phenotypes, including the overload of the protein quality control system, DNA damage, mitochondrial dysfunction, and ER stress, together resulting in vulnerability to cell death.

Since all major classes of molecular chaperones, with the exception of the small HPSs, are ATPases it has been suggested that the depletion of ATP levels during aging due to mitochondria dysfunction would affect their activity (Kaushik and Cuervo, 2015; Yerbury et al., 2016). This is reflected by the repression of ATP-dependent chaperones and the induction of ATP-independent chaperones in the aging human brain (Brehme et al., 2014). This may contribute to the decline of chaperoning function during aging.

The activity of the degradation pathways of the PQC, autophagy and the proteasome, are also reduced during aging (reviewed in Koga et al., 2011 and Kaushik and Cuervo, 2015). The proteasome decline is caused by a down-regulation or deregulation of different proteasomal subunits and regulatory factors (Keller et al., 2000; Ferrington et al., 2005). In autophagy, fusion between the vesicles carrying the cytosolic cargo and lysosomal compartments is severely impaired. The chaperone-mediated autophagy is reduced due to progressively lower levels of receptors at the lysosomal membrane with age (Cuervo and Dice, 2000; Koga et al., 2011). Furthermore, a more active proteasome has been found in fibroblasts from centenarians (Chondrogianni et al., 2000; Koga et al., 2011) and reactivation of the proteasome and/or autophagy pathways increases lifespan of yeast, worms, and flies (Chondrogianni et al., 2015; Kaushik and Cuervo, 2015; Madeo et al., 2015). Altogether, showing the importance to remain a functioning PQC during aging.

MECHANISMS OF PROTEIN TOXICITY IN NEURODEGENERATIVE DISEASES

Neuronal loss is one of the hallmarks of neurodegenerative diseases, where the neurons that are vulnerable to disease pathology differ for each disease. Initially it was thought that the protein aggregates that are observed in post-mortem brain material of patients were toxic (Davies et al., 1997; Kim et al., 1999). But this view shifted toward the hypothesis that the protein aggregates may actually be neuroprotective and that intermediate species are toxic. Indeed, the presence of diffuse protein resulted in higher toxicity compared to the presence of protein aggregates only (Arrasate et al., 2004). Furthermore, overexpression of HSF-1 in a cell model for HD leads to fewer but larger aggregates and increased viability (Pierce et al., 2010). The toxicity of intermediate species may arise from the presence of hydrophobic groups on their surface, that under normal physiological conditions would not be accessible within the cellular environment (Campioni et al., 2010). Accessible hydrophobicity in proteins can result in inappropriate interactions with many functional cellular components like the plasma membrane (Bucciantini et al., 2012). Therefore, aggregation might be a mechanism to assist in the clearance of misfolded proteins. In this regard, it has been described that chaperones can supress the toxicity of the oligomeric intermediate species by promoting the formation of

larger aggregates (Lindberg et al., 2015). The question remains why these intermediate species are toxic. Different mechanisms have been suggested.

The increase of misfolded proteins during aging or disease can interfere with the PQC system by overloading the system (**Figure 2**), which in turn, can result in a propagation of folding defects and eventually protein aggregation (Labbadia and Morimoto, 2015). In polyQ worm models disruption of the PQC system by the polyQ aggregates resulted in the loss of function of several metastable proteins with destabilizing temperature-sensitive mutations, which also enhanced the aggregation of polyQ proteins (Gidalevitz et al., 2006). Furthermore, polyQ aggregates also impair the ubiquitin-proteasome system in cellular models for disease (Bence et al., 2001).

A "gain of function" mechanism is another form of cellular toxicity. Due to misfolding, hydrophobic residues of the protein can be located at the surface, permitting uncommon interactions with a wide range of cellular targets (**Figure 2**; Stefani and Dobson, 2003), including molecular chaperones (Park et al., 2013). Using cytotoxic artificial β-sheet protein aggregates it was found that the endogenous proteins that are sequestered by these aggregates share many physicochemical properties, including their relatively large size and enriched unstructured regions. Many of these proteins play essential roles in the several pathways, including translation, chromatin structure, and cytoskeleton. A loss of these proteins might results in a collapse of essential cellular functions and consequently may induce toxicity (Olzscha et al., 2011).

Recently, an effect of protein aggregation on the nuclear pore complex (NPC) was described. The GGGGCC (G_4C_2)repeat expansion in the non-coding region of the C9orf72 protein is the most common cause of sporadic and familial forms of amyotrophic lateral sclerosis (ALS) and frontal temporal dementia (FTD), (DeJesus-Hernandez et al., 2011; Renton et al., 2011). However, the exact mechanism of how the C9orf72 mutations contribute to the disease remains elusive. Two hypotheses are proposed, the first describes that the repeat containing transcripts can form intra-nuclear RNA foci that sequester various RNA-binding proteins (Donnelly et al., 2013), and the second describes the production of toxic dipeptide repeat proteins (DPRs; Ash et al., 2013). New insights have shown that mutant C9orf72 RNA affects nuclear transport of proteins and RNA (**Figure 2**). Loss of NPC proteins were found to enhance G_4C_2 repeat toxicity in fly and human cell models for disease (Freibaum et al., 2015; Zhang et al., 2015). Moreover, a screen to identify modifiers of toxicity by PR_{50}DPR identified an enrichment in nucleocytoplasmic transport proteins, in which the six strongest hits were members of the karyopherin family of

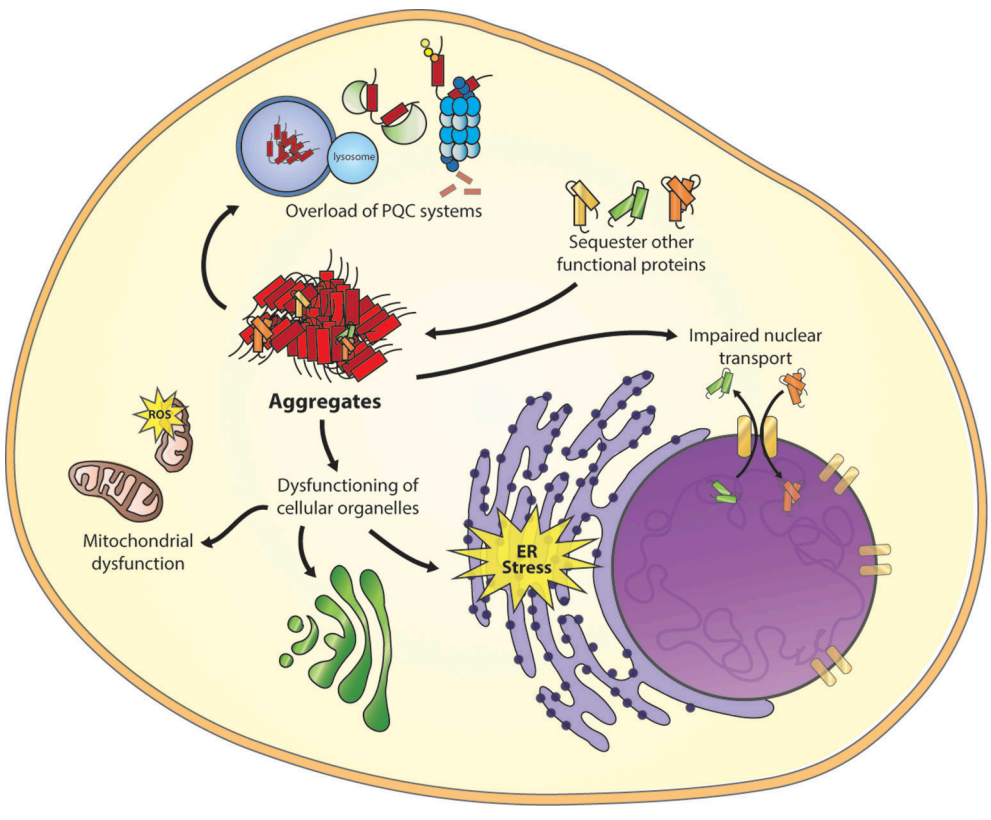

FIGURE 2 | Toxic mechanism of misfolded proteins. Important cellular processes are affected as a result of misfolded proteins, including overload of the protein quality control (PQC) system, sequestering of functional proteins, disruption of the nuclear core complex and dysfunction of other cellular organelles as mitochondria, ER stress, and trans-Golgi network (the figure focuses on only one intermediate species, other species can be toxic too).

nuclear-import proteins (Jovičić et al., 2015). Furthermore, it was shown that nuclear localization of artificial β-sheet-, HTT-, and TDP-43 aggregates reduces toxicity in comparison to cytoplasmic aggregates. Because the cytoplasmic aggregates interfere with both import and export of proteins through the nuclear pore complex, they specifically affect proteins containing disordered and low complexity domains including many nuclear transport factors (Woerner et al., 2016). These studies show that reduced nuclear transport, as a result of protein aggregates, results in cellular toxicity. However, a better understanding of the exact mechanism behind these observations could provide us with a new therapeutic target to restore nuclear transport. In addition, several studies described toxic effects of protein aggregates on the functioning of other cellular organelles as the ER (Duennwald and Lindquist, 2008), mitochondrion (Rhein et al., 2009), and the trans-Golgi network (Cooper et al., 2006). Identifying different toxic consequences of misfolded proteins gives possibilities for treatments options.

Another mechanism of toxicity has been proposed in the literature, in which oligomeric aggregation intermediates bind and disrupt lipid membranes (Lashuel and Lansbury, 2006). Annular oligomeric structures have been identified for different amyloidogenic proteins, such as Aβ (Lashuel et al., 2002a,b), α-synuclein (Lashuel et al., 2002b,c), PrP (Sokolowski et al., 2003), or polyQ proteins (Wacker et al., 2004). These are pore-like structures that can embed into lipid bilayers and permeabilize membranes allowing the transit of small molecules. Diseases-associated mutations in Aβ (E22G) and α-synuclein (A53T and A30P) promote the formation of amyloid pores (Lashuel et al., 2002b,c; Lashuel and Lansbury, 2006). This is known as the amyloid pore hypothesis (Lashuel and Lansbury, 2006; Stöckl et al., 2013). Alternatively, a different explanation has been proposed for the permeabilization of membranes by α-synuclein, in which oligomers of this protein would not form pores, but they rather decrease the lipid order by incorporating between the tightly packed lipids, facilitating the diffusion of molecules through the membranes (Stöckl et al., 2013). Whether this alternative hypothesis can also be applicable to other amyloidgenic proteins still needs to be revealed. Furthermore, recent studies on non-pathological (Oropesa-Nuñez et al., 2016) and pathological proteins (Di Pasquale et al., 2010; Fukunaga et al., 2012; Mahul-Mellier et al., 2015) show that negatively charged ganglioside rich lipid rafts mediate toxicity of the prefibrillar oligomers.

Probably the toxicity of the disease proteins cannot be wholly explained by one of these mechanisms but rather by a combination of them.

Gliosis

Neuroinflammation or gliosis, a reactive change of the glial cells in response to damage, is a common pathological feature in neurodegenerative diseases like AD and HD (Perry et al., 2010). However, whether inflammation plays an active or consequential role in disease is still a topic for debate. Glial cells are divided into two major classes: microglia and macroglia, where microglia are the phagocytes that are ubiquitously distributed in the brain and are mobilized after injury, disease, or infection. Pathological triggers, such as neuronal death or protein aggregates, activate the migration of microglia, which accumulate at the site of injury. This migration and recruitment is followed by the initiation of an innate immune response, which is a non-specific reaction resulting in the release of pro-inflammatory chemo- and cytokines (Gordon and Taylor, 2005; Hanisch and Kettenmann, 2007; Perry et al., 2010). The importance of glial cells in neurodegeneration is supported by the association found in genome wide association studies of immune receptors like TREM2 (Guerreiro et al., 2013; Jonsson et al., 2013) and CD33 (Griciuc et al., 2013) in AD. Gliosis has also been described for other neurodegenerative diseases as PD (Gerhard et al., 2006) and HD (Shin et al., 2005), but as the main aggregates are intracellular the response from microglia is not as strong as in AD.

Spreading

Prion diseases (PrD) are a group of fatal neurodegenerative disorders caused by infectious proteins called prions. In humans most PrD can be identified under the name Creutzfeldt-Jakob disease (CJD), and in animals under the name bovine spongiform encephalopathy (BSE; Collinge, 2001). In PrD, the cellular form of the prion protein (PrP^{C}) undergoes a conformational conversion into a β-sheet enriched isoform denoted as PrP^{Sc}. This occurs when the PrP^{Sc} comes in contact with the mostly α-helical PrP^{C}, as a result the PrP^{C} is misfolded into pathogenic PrP^{Sc}, which in turn can become a template for conversion of other PrP^{C}. The PrP^{Sc} form can form protein aggregates, prion deposits, often present as amyloid structures, which can propagate and possibly cause cell death (Collinge and Clarke, 2007; Collinge, 2016). PrDs are well-known to be able to spread throughout the brain via infectious prions. By the conversion of the protein into "seeds" due to stress, mutations or when PrP^{C} comes in contact with PrP^{Sc}, it incites a chain reaction of PrP misfolding (Halliday et al., 2014). Prions are out of scope for this review, although they are one of the most relevant topics in neurodegenerative diseases especially due to their infectivity. This "prion-like" character of other neurodegenerative disease proteins has been proposed.

Spreading of Aβ in AD was first observed in a marmoset injected with brain extract from AD patients or AD affected marmosets, leading to AD pathology 6–10 years after injection (Baker et al., 1993; Ridley et al., 2006). Injection with only cerebrospinal fluid of AD patients or synthetic Aβ did not result in AD pathology in the marmoset (Ridley et al., 2006). As studies with marmosets are limited, these studies were replicated in mice to further investigate the spreading of Aβ. Brain extracts from AD patients or transgenic mouse models can initiate AD pathology in the brains of transgenic mice overexpressing the Swedish-mutated human APP (Meyer-Luehmann et al., 2006). Injection of synthetic human Aβ fibrils can induce AD pathology in mice, however the potency is lower than with AD brain extract (Stöhr et al., 2012). In mice depleted of amyloid-beta precursor protein (APP) there is no spreading of the disease, however if you take brain extracts of APP depleted mice inoculated with Aβ seeds, this can lead to propagation after second transmission for up to 180 days,

suggesting extreme longevity of the Aβ "seeds" (Ye et al., 2015). Infectiousness of AD in humans has not yet been proven, though possible spreading of Aβ in humans was observed in two individual studies. The first study described four individuals with infectious Creutzfeldt-Jakob disease (CJD) who also showed moderate to severe AD pathology, they were injected as children with human growth hormone from cadaveric pituitary glands that contained PrP (Jaunmuktane et al., 2015). Another study observed infectious CJD in patients who received a dura mater transplant as a result of brain trauma or tumor, in five patients AD pathology was observed (Frontzek et al., 2016). As the patients in both studies did not carry pathogenic AD mutations or risk alleles and were too young to develop sporadic AD, the studies suggested that the treatment samples contained Aβ peptides.

Spreading of the PD pathology was first suggested when healthy dopaminergic neurons injected into the brain of PD patients showed Lewy body formation 11–16 years after transplantation (Kordower et al., 2008; Li et al., 2008). Follow-up studies in PD mouse models show that injection of brain extracts of PD transgenic mice results in the formation of PD pathology and increased mortality (Luk et al., 2012b; Mougenot et al., 2012). Furthermore, injection of synthetic α-synuclein (Luk et al., 2012a) or dementia with Lewy bodies (DLB) patient brain extract (Masuda-Suzukake et al., 2013) also results in PD pathology and neuronal death in healthy mice.

PROTEIN TOXICITY IN NON-NEURODEGENERATIVE DISEASES

Protein aggregation is also involved in non-neurodegenerative diseases, and can be distinguish into two groups: non-neuropathic systemic amyloidosis and non-neuropathic localized disease (reviewed in Chiti and Dobson, 2006; **Figure 3**). Similar to neurodegenerative diseases they arise from the failure of a specific protein or peptide to acquire its native functional conformational state resulting in aggregation of the protein.

In non-neuropathic localized disease, the protein aggregation occurs in a single cell type or tissue other than the brain. The most well-known disease is type II diabetes, an age-related disease in which the glucose homeostasis is disturbed due to pancreatic islet β-cell dysfunction and death caused by aggregation of the islet amyloid polypeptide (IAPP; Abedini and Schmidt, 2013; Westermark and Westermark, 2013; Knowles et al., 2014). The amyloid deposits in the islet β-cells were first described over 100 years ago (Opie, 1901), and are a common feature in the pancreas of post-mortem material of type II diabetes patients. Pancreatic β-cells normally secrete insulin to regulate glucose uptake and metabolism in the body, mature IAPP is stored in the insulin secretory granule and co-secreted with insulin (Marzban et al., 2005). The exact role of IAPP is still unknown, although many functions have been suggested including regulation of glucose homeostasis (Abedini and Schmidt, 2013). The human IAPP is extremely amyloidogenic *in vitro*, and amyloids accumulate in the pancreatic islet in the majority of the type II diabetes patients (Westermark et al., 1989; Betsholtz et al., 1990).

Another common non-neuropathic localized disease is cataracts, a common form of blindness affecting more than 50% of the individuals over the age of 70. Normally, the lens can stay transparent throughout life, as there is no protein turnover or synthesis. In cataracts soluble proteins of the lens accumulate into amyloids, resulting in reduced transparency and thus reduced sight. Thirty percent of the lens is made up of the molecular chaperones αA-crystallin and αB-crystallin that maintain the solubility of other lens proteins. However, during aging damaged proteins accumulate which can lead to aggregation of the crystalline proteins (Bloemendal et al., 2004). Furthermore, the R120G mutation in αB-crystallin causes early onset cataracts (Vicart et al., 1998; Perng et al., 1999).

The non-neuropathic systemic amyloidosis are rare diseases caused by protein aggregation in multiple tissues (Falk et al., 1997). The most common non-neuropathic systemic amyloidosis is AL amyloidosis, a mainly sporadic disease that is characterized by aggregation of fragments of the misfolded monoclonal immunoglobin light chains in various organs (Comenzo, 2006; Chaulagain and Comenzo, 2013). The fragment can form β-sheets that are prone to form amyloids. The protein is produced by a plasma cell clone in the bone marrow and after internalization it can cause severe organ dysfunction and failure. The main organs affected by AL amyloidosis are the heart and kidneys, however, also other organs such as the liver, nervous system, and spleen can be affected (Falk et al., 1997; Comenzo, 2006). The treatment of the disease is aimed at eliminating the plasma cell clone, but a delay in the diagnosis of the disease often results in irreversible organ damage and thus poor prognoses (Chaulagain and Comenzo, 2013). Two other common non-neuropathic systemic amyloidosis are caused by transthyretin amyloidosis (ATTR) and serum amyloid A protein (SAA), both proteins are produced in the liver and affect various organs, however in ATTR heart failure is most common whereas SAA often results in renal failure (reviewed in Chiti and Dobson, 2006).

STRUCTURAL AND FUNCTIONAL PROPERTIES OF AMYLOID

The first amyloid was observed and described in 1854 by Rudolph Virchow for systemic amyloidosis (Sipe and Cohen, 2000). Since then, many diseases have been associated with amyloids. The proteins associated with protein aggregation diseases have no obvious similarity in sequences, native structures, or function. They do however, share characteristics in their amyloid state as they can undergo structural rearrangements leading to the formation of amyloid fibrils (**Figure 4A**). The amyloid fibrils have a highly organized and stable structure composed of proteins with a cross β-sheet structure oriented vertically to the fibril axis. They appear under the electron microscope as unbranched filamentous structures of just a few nanometers in diameter while up to micrometers in length. The cross β-sheet structure of amyloid fibrils provides a stable structure for the formation

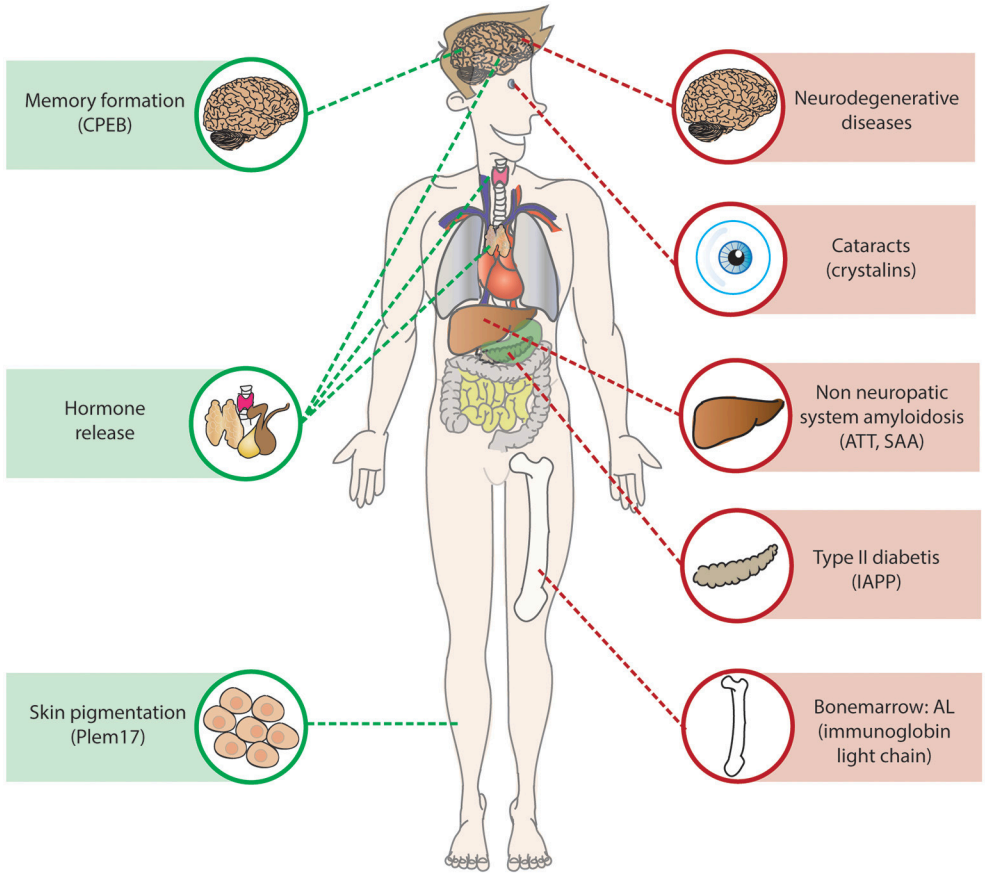

FIGURE 3 | Amyloids in health and disease. Amyloids are present throughout the body in health and diseases, in green examples of functional amyloids described in the section is called "Functional Amyloid". In red examples of amyloids resulted causing disease, the non-neuropathic systemic amyloidosis AL, ATT, and SAA are located at the point where they are produced, they do however affect multiple organs as the heart and kidney.

of continuous arrangement of hydrogen bonds between fibrils, eventually resulting in the formation of amyloids. The amyloid structures can be characterized by their following properties: insolubility to detergents like SDS and NP40, binding to specific dyes such as Thioflavins and Congo Red and resistance to proteases (reviewed in Chiti and Dobson, 2006). To learn more about intermediate species of the aggregation process the kinetics of aggregation can be studied *in vitro*. Using purified protein and a amyloid dye in a test tube, three phases of aggregation can be distinguished (**Figure 4B**). During the first lag phase there are mainly protein monomers and oligomers, this is followed by a rapid growth phase in which protein fibrils are formed, followed by a plateau phase in which the reaction is ended due to depletion of soluble proteins (Blanco et al., 2012).

The aggregation propensity of a protein is determined by short aggregation prone regions (APR) that are generally buried in the hydrophobic core of the protein. However, due to misfolding or mutations, these regions can be exposed and therefore self-assemble into aggregates. APRs are typically short sequence segments between 5 and 15 amino acids with high hydrophobicity, low net charge, and have a high tendency to form β-sheet structures (Ventura et al., 2004; Esteras-Chopo et al., 2005). Different algorithms have been generated to predict protein aggregation propensity of proteins or the effect of disease mutations, for example WALTZ an algorithm to determine amyloid forming sequences (Maurer-Stroh et al., 2010) and TANGO an algorithm that identifies the β-sheet regions of a protein sequence (Fernandez-Escamilla et al., 2004). Disease associated variants, not only related with neurodegenerative diseases, but also for cancers and immune disorders, tend to increase the predicted aggregation propensity of proteins (De Baets et al., 2015).

Amyloid in Disease

Proteins or peptides of most neurodegenerative diseases are intrinsically disordered in their free soluble form, like the Aβ peptide in AD and α-synclein in PD (Chiti and Dobson, 2006, 2009). Mutations in these disease proteins can make the protein even more prone to aggregate. For example, the A53T and A30P mutation of α-synclein found in early onset PD, promotes the acceleration of amyloid fibrils *in vitro* (Conway et al., 1998, 2000).

Furthermore, having too many copies of an aggregation-prone protein itself can lead to disease by increasing protein concentrations in the cell (Chiti and Dobson, 2006, 2009). This

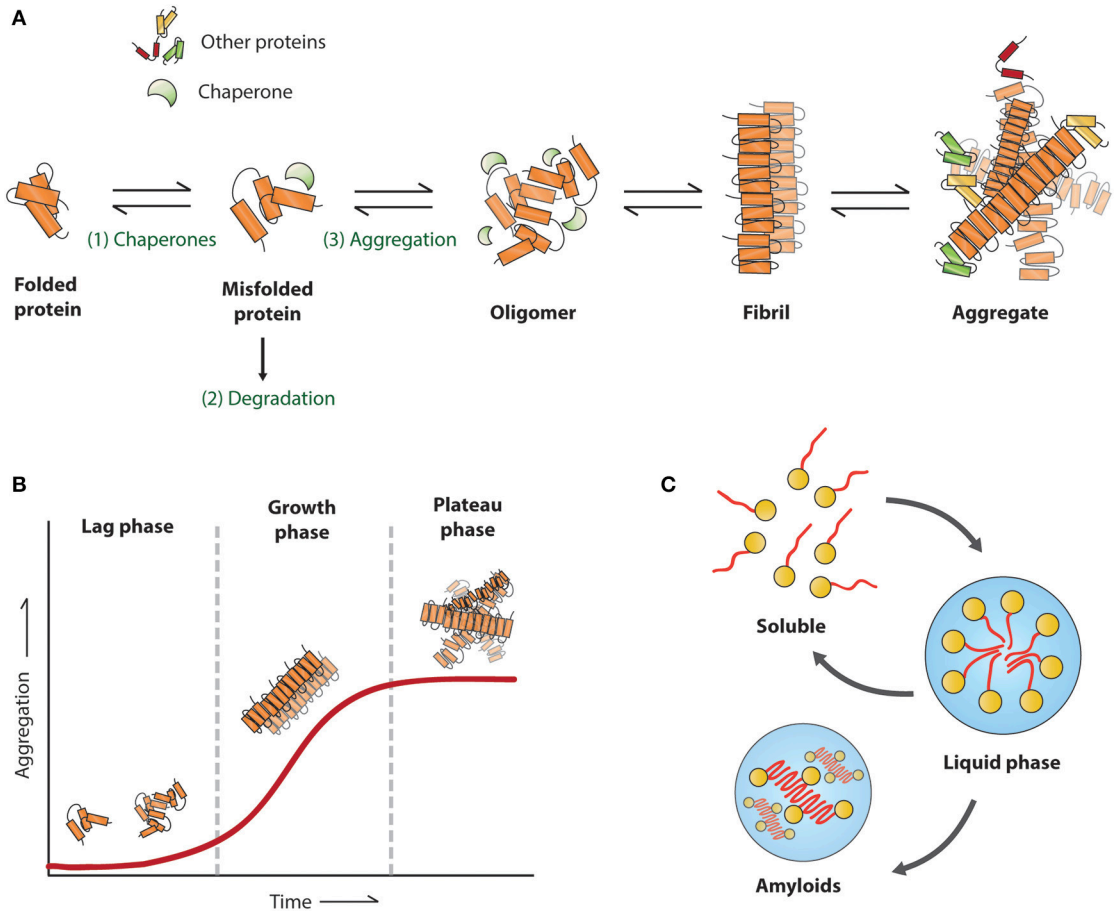

FIGURE 4 | Proposed mechanism for amyloid formation. (A) A misfolded protein can be refolded (1), degraded (2), or aggregated (3), the first step in the aggregation pathway involves oligomers, followed by fibril formation around the fibril axis until the initial aggregates. **(B)** Schematic view of an *in vitro* assay with the corresponding aggregation stages for each phase **(C)** formation of liquid droplets.

increase in protein concentration can switch the stability of the soluble state toward the amyloid state. For examples trisomy 21 patients (Down's syndrome) who have an extra copy of the APP protein and a highly increased risk of developing early onset AD (Wiseman et al., 2015). In addition, duplication or triplication of the α-synuclein gene (*SNCA*) results in early onset PD (Singleton et al., 2003), besides, the onset, progression, and severity of the disease phenotype increases with the number of copies of the *SNCA* gene (Chartier-Harlin et al., 2004). To this end, also proteins that regulate expression levels of disease proteins can cause or influence diseases, an example is the RNA binding protein Pumilio1 that regulates the mRNA levels of *Ataxin1* RNA. Pumilio1 haploinsufficiency accelerates the SCA1 disease progression in a mouse model for disease due to increase of the Atxn1 mRNA and protein levels (Gennarino et al., 2015). If protein levels strongly influence the toxicity and disease phenotype this would suggests that lowering the protein load could be a therapeutic strategy. This was shown in an AD mouse model where the APP transgenes could be turned off with a tet-off system, when the APP levels were halted there was an arrest of the AD pathology without clearance of the excising plaques (Jankowsky et al., 2005), resulting in a significant effect on cognitive function (Fowler et al., 2014). Indicating that the concentration of disease proteins influences the disease progression, thereby affecting the development of disease.

That structural differences between amyloid "strains" can influence disease phenotype was first described for PrD, where isolated strains of PrP aggregates from different sources propagated different in mice showing distinct incubation times and patterns of neuropathology (Fraser and Dickinson, 1973). Furthermore, different human PrP strains have been associated with differences in proteinase K digestion and distinct phenotypes of neuropathology (reviewed in Collinge and Clarke, 2007). More recently, investigation of two familial human AD patients with different disease symptoms, showed a structural difference in amyloid fibril structure (Lu et al., 2013). Furthermore, Arctic and Swedisch familial AD patients brain homogenate results in distinct disease phenotypes in transgenic mice even after serial passage (Watts et al., 2014). Comparable results were found for Tau, another aggregation-prone protein

involved in AD. Injection of two distinct *in vitro* generated Tau strains into transgenic mice resulted in distinct pathologies up to three generations (Sanders et al., 2014). These studies suggest that variations in the properties of amyloid fibrils could affect disease pathology and symptoms. How these different strains are formed and how they contribute to the disease pathology is still unknown. It was however found that reduced IIS signaling in the APP/PS1 AD mouse model induces the assembly of Aβ into densely packed and larger fibrillar structures later in life, resulting in reduced AD symptoms (Cohen et al., 2009). Suggesting that altering the structure of the amyloid fibrils could be beneficial for patients, as certain structures appear to be more toxic than others.

Functional Amyloid

Amyloids structures are known to have biological functions in *Escherichia coli*, silkworms, fungi, and mammals (Fowler et al., 2007). One example in mammals is Pmel17 (**Figure 3**), a highly aggregation-prone protein that forms functional amyloid structures that are the main component of melanosome fibrils, membrane-bound organelles in pigment cells that store and synthesize melanin. Plem17 contains a partial repeat sequence that is essential for amyloid formation that can only be formed in the mildly acid pH of melanosomes (McGlinchey et al., 2009). The exact function of Pmel17 in melanosomes is unknown, although a role in protection against oxidative damage has been suggested, as well as a role in concentrating melanins to facilitate intra- and extracellular transport (Watt et al., 2013).

More functional amyloids in mammals can be found in hormone release, it was shown that certain hormones can be stored in amyloid-like aggregates in the secretory granules of the cell. These secretory granules have a β-sheet rich structure that is Thioflavin S and Congo Red positive and are able to release functional monomeric hormone structures upon dilution, and show only moderately toxicity on cell cultures, possibly due to their membrane-encapsulated state in the granules (Maji et al., 2009).

Interestingly, the formation of amyloids has recently been associated with long-term memory. The cytoplasmic polyadenylation element-binding protein (CPEBs) is a regulator of activity dependent synthesis in the synapse. The fly homolog Orb2 (Majumdar et al., 2012) and mouse homolog CPEB3 (Fioriti et al., 2015) are present in the brain as a monomer and SDS-resistant oligomer. Activation of the fly or mouse brain results in increase of the oligomeric Orb2/CPEB3 species. Selectively disrupting the oligomerization capacity of Orb2 by a genetic mutation resulted in long-term memory loss in flies (Majumdar et al., 2012) and loss of CPEB3 in the mouse brain resulted in impaired long term memory (Fioriti et al., 2015). Orb2 alters protein composition of the synapse by a mechanism in which the oligomeric Orb2 stimulates translation by elongation and protection of poly(A) tail, whereas the monomeric Orb2 does the contrary (Khan et al., 2015).

These functional amyloids point toward the origin of amyloid-prone sequences and their suppressors and enhancers. Even though, these functional amyloids have not been linked to human diseases, a functional role might be the case for the amyloid domains of disease proteins with unknown functions. More studies toward understanding the functionality of these amyloids and the difference with the disease amyloids are required to have a better understanding of why certain amyloids are toxic while others are not.

Liquid Droplets/Liquid-to-Solid-Phase Transition

It was recently found that proteins with prion-like domains can form functional non-membrane-bound organelles like ribonucleoprotein (RNP) bodies, that behave like liquid droplets which can rapidly assemble and disassemble in a response to changes in the cellular environment (Han et al., 2012; Kato et al., 2012). The RNP bodies include processing bodies and stress granules in the cytoplasm, and nucleoli, Cajal bodies and PML bodies in the nucleus. Due to the dynamic structures of RNPs there is free diffusion within the bodies and rapid exchange with the external environment. Like in liquid-liquid phase separation (LLPS) the RNP bodies exhibit liquid-like behaviors such as wetting, dripping, and relaxation to spherical structures upon fusion (Chong and Forman-Kay, 2016; Uversky, 2017). These properties can facilitate their function, by allowing for high concentration of molecular components that nonetheless remain dynamic within the droplet. Interestingly many of the proteins known to segregate into RNP bodies contain repetitive putatively prion-like domains, that can reversibly transform from soluble to a dynamic amyloid-like state (Kato et al., 2012). Furthermore, dysregulation of these RNP bodies by RNA-binding proteins have been associated with neurodegenerative diseases as ALS (Ramaswami et al., 2013).

The link for these RNP bodies in disease was first found for the FUS protein, mutations in the N-terminal prion-like domain have been associated with ALS, and FTD. This protein plays an important role in RNA processing and localizes to both cytoplasmic RNP bodies and transcriptionally active nuclear puncta, the prion-like domain is essential for forming these liquid-like compartments (Shelkovnikova et al., 2014). The N-terminus of FUS is structurally disordered both as a monomer and in its liquid state (Burke et al., 2015). *In vitro*, these droplets convert with time from a liquid to an aggregated state (**Figure 4C**), and this conversion is accelerated by patient-derived mutations (Patel et al., 2015). Furthermore, concentrated liquid droplets increase the probability of aggregation events of RNA-binding proteins in the RNP bodies in a concentration dependent manner (Molliex et al., 2015). mRNA itself can drive its phase transition of the disordered RNA binding-protein Whi3, and thereby altering the droplet viscosity, dynamics, and propensity to fuse. Suggesting that, mRNA contains biophysical properties of phase-separated compartments. Like FUS droplets the Whi3 droplets mature over time and appear to be fibrillar (Zhang et al., 2015).

This new line of research indicates another possible function for prion-like domains of various proteins and the proteins it interacts with. Furthermore, research to these RNP bodies shows possible reasons why these proteins form amyloids. However,

much is still unknown about the exact mechanisms of the amyloid like domains and the RNP bodies that have to be investigated.

CONCLUSION

Protein aggregation is a complex process influenced by many factors, pathways, and mechanisms. Under the right conditions any protein could form amyloid-like structures (Chiti and Dobson, 2006). Although amyloids have been traditionally related to diseases, they also have diverse functions in organisms from bacteria to human that may underlie their nature. Nevertheless, the toxicity of amyloid intermediate species associated with disease makes protein aggregation a process that has to be under tight control and regulation. In this context, aging is a key risk factor due to the progressive decline of protein homeostasis, which leads to increased protein misfolding and aggregation. This can eventually result in the onset of age-related diseases characterized by protein aggregation. Mutations or duplications that lead to the appearance of aggregation-prone proteins that are constitutively expressed in the cell, creating a chronic stress situation, leads to an early onset of those diseases due to the deregulation of the protein homeostasis balance.

As the human population becomes older, it is essential to understand the processes underlying age-related diseases that are the result of protein aggregation and its associated toxicity. This is a very broad research field, ranging from biophysics to clinical trials. Every year discoveries are made that involve the identification of factors affecting protein aggregation. Examples include the discovery of modifiers of protein aggregation such as MOAG-4/SERF, or the processes where protein aggregation and amyloid structure are involved, like RNA granules and liquid droplets formation. It can be concluded that the overall knowledge of the aggregation process is improving, which will allow for the development of new and accurate treatments against aggregation-linked diseases.

AUTHOR CONTRIBUTIONS

ES wrote the review with the contribution and substantial intellectual input from MK, EN, and AM. MK did the figure design.

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MINI REVIEW
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Chaperones in Polyglutamine Aggregation: Beyond the Q-Stretch

E. F. E. Kuiper[1†], Eduardo P. de Mattos[1,2,3†], Laura B. Jardim[2,3,4], Harm H. Kampinga[1] and Steven Bergink[1]*

[1] Department of Cell Biology, University Medical Center Groningen, University of Groningen, Groningen, Netherlands, [2] Programa de Pós-Graduação em Genética e Biologia Molecular, Department of Genetics, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, [3] Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil, [4] Departamento de Medicina Interna, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

Expanded polyglutamine (polyQ) stretches in at least nine unrelated proteins lead to inherited neuronal dysfunction and degeneration. The expansion size in all diseases correlates with age at onset (AO) of disease and with polyQ protein aggregation, indicating that the expanded polyQ stretch is the main driving force for the disease onset. Interestingly, there is marked interpatient variability in expansion thresholds for a given disease. Between different polyQ diseases the repeat length vs. AO also indicates the existence of modulatory effects on aggregation of the upstream and downstream amino acid sequences flanking the Q expansion. This can be either due to intrinsic modulation of aggregation by the flanking regions, or due to differential interaction with other proteins, such as the components of the cellular protein quality control network. Indeed, several lines of evidence suggest that molecular chaperones have impact on the handling of different polyQ proteins. Here, we review factors differentially influencing polyQ aggregation: the Q-stretch itself, modulatory flanking sequences, interaction partners, cleavage of polyQ-containing proteins, and post-translational modifications, with a special focus on the role of molecular chaperones. By discussing typical examples of how these factors influence aggregation, we provide more insight on the variability of AO between different diseases as well as within the same polyQ disorder, on the molecular level.

Keywords: aggregation, Huntington's disease, Machado-Joseph disease, molecular chaperones, polyglutamine disease

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Tatiana Rosado Rosenstock, Santa Casa de São Paulo School of Medical Sciences, Brazil

***Correspondence:**
Steven Bergink
s.bergink@umcg.nl

[†]These authors have contributed equally to this work.

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INTRODUCTION

Polyglutaminopathies are a family of diseases characterized by CAG trinucleotide expansions in the coding regions of at least nine unrelated genes, resulting in proteins with an abnormally long polyglutamine (polyQ) stretch, which have a high aggregation propensity. PolyQ aggregates can impede cellular protein homeostasis, loss of which is also observed in many other neurodegenerative diseases (Soto, 2003). These mutant proteins lead to one recessive inherited, X-linked spinal and bulbar muscular atrophy (SBMA), and eight dominantly inherited neuronal dysfunctions, Huntington's disease (HD), dentatorubral pallidoluysian atrophy (DRPLA), and the spinocerebellar ataxias (SCAs) type 1, 2, 3, 6, 7, and 17 (Margolis and Ross, 2001). All known polyglutaminopathies show a strong inverse correlation between expansion size and age at onset (AO) of the disease, with longer repeats significantly correlating with earlier onset of symptoms and

higher aggregation proneness of the affected protein, indicating that an expanded polyQ is tightly related to the diseases. There are two main features that are striking in the association between polyQ length and AO. First, there is marked variability between polyQ diseases in expansion thresholds that determines the pathogenicity, indicating that AO has only a partial dependence on the polyQ stretches and their absolute lengths (**Figure 1A**). Second, there is also CAG-length independent phenotypic variation within a given polyQ disease (**Figure 1B**). Both these findings imply that factors beyond the polyQ stretch are co-determining disease onset (Ranum et al., 1994; DeStefano et al., 1996; Hayes et al., 2000; Wexler et al., 2004; van de Warrenburg et al., 2005; Kaltenbach et al., 2007; Branco et al., 2008; Lessing and Bonini, 2008; Bettencourt et al., 2011; Tezenas du Montcel et al., 2014; Bečanović et al., 2015). It was hypothesized that the differential effects of distinct polyQ proteins with polyQ tracts of similar lengths could be, at least in part, due to the sequences flanking the polyQ expansion (Nozaki et al., 2001).

Here we discuss that, next to aggregation of the core polyQ stretch, which is common to all polyglutaminopathies (**Figure 2A**), the context around the cores can modulate aggregation in several ways and may be linked to differential handling of the protein quality control systems, including molecular chaperones, the ubiquitin proteasome system, and autophagy. These degradation processes, and their relationship with the chaperone system, are of importance and greatly influence the aggregation process (Rubinsztein, 2006). Certain chaperones act together with the protein degradation machineries to effectively clear aggregation-prone polypeptides, such as polyQ-containing proteins (Dekker et al., 2015). The molecular details of these downstream events are still unclear and will not be discussed here; instead we will focus on the impact of molecular chaperones on the aggregation process itself. Molecular chaperones are known to influence aggregation of polyQ proteins. This could either be directly by preventing the polyQ stretch from aggregating or via the flanking sequences. For only a few of the molecular chaperones the direct interaction with the polyQ proteins has been shown, although many chaperones are found to co-localize with polyQ inclusions (Cummings et al., 1998; Kazemi-Esfarjani and Benzer, 2000; Schmidt et al., 2002; Helmlinger et al., 2004; Bilen and Bonini, 2007; Hageman et al., 2010; Gao et al., 2011; Kakkar et al., 2014; Matilla-Dueñas et al., 2014; Reis et al., 2016; Zhao et al., 2016). However, co-localization of chaperones does not provide information on their mode of interaction and does not distinguish whether chaperones are truly interacting with the polyQ protein, or whether the presence of chaperones in the aggregates is a mere secondary effect due to a collapse of other cellular components with the inclusions. In this review, we will discuss: first, how polyQ tracts drive aggregation; second, how their flanking sequences could directly affect the aggregation proneness of the polyQ protein; and third, how polyQ proteins can be modified, changed in conformation, or fragmented, inducing aggregation (**Figure 2B**). We will not focus on the function, or loss of function, of the affected polyQ proteins, since this was so far not shown to be causative for disease, even though the native function of the protein might be important for normal cellular function. Furthermore, we will not go into the discussion on the toxicity of aggregation. For instance, it is still unclear whether the presence of aggregates contributes to SCA2 pathology (Huynh et al., 2000), even though aggregates are found in affected brain areas (Pang et al., 2002; Seidel et al., 2016). Finally, we will highlight the role of chaperones in the aggregation process and include only studies that provide insight in direct interaction of chaperones with the polyQ proteins. Rather than providing a complete overview, molecular mechanisms of typical examples will be discussed, aiming at providing general principles affecting polyQ aggregation on

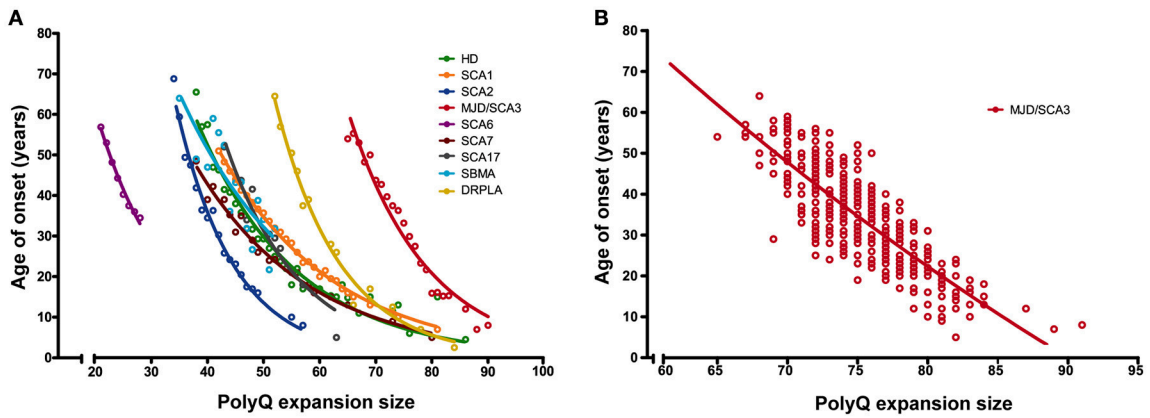

FIGURE 1 | Age of onset of disease inversely correlates with the size of the expanded polyQ tract in all known polyQ diseases. (A) Correlation between age of onset (AO) and CAG expansion size for all nine polyQ diseases identified so far. Circles depict mean AOs for a given expansion size based on multiple reported cohorts of patients. Lines represent the fitted data according to an exponential decay model. **(B)** Age of onset of disease is not completely determined by the expanded polyQ tract alone. Data on the variability of AO for a particular polyQ expansion size is shown as in **(A)** and was based on the large cohort of MJD/SCA3 patients reported by Saute and Jardim (2015). Circles represent single patients. Please refer to Supplementary File 1 for a complete list of references of the original cohort descriptions. Note that graph **(A,B)** are not drawn to the same scale.

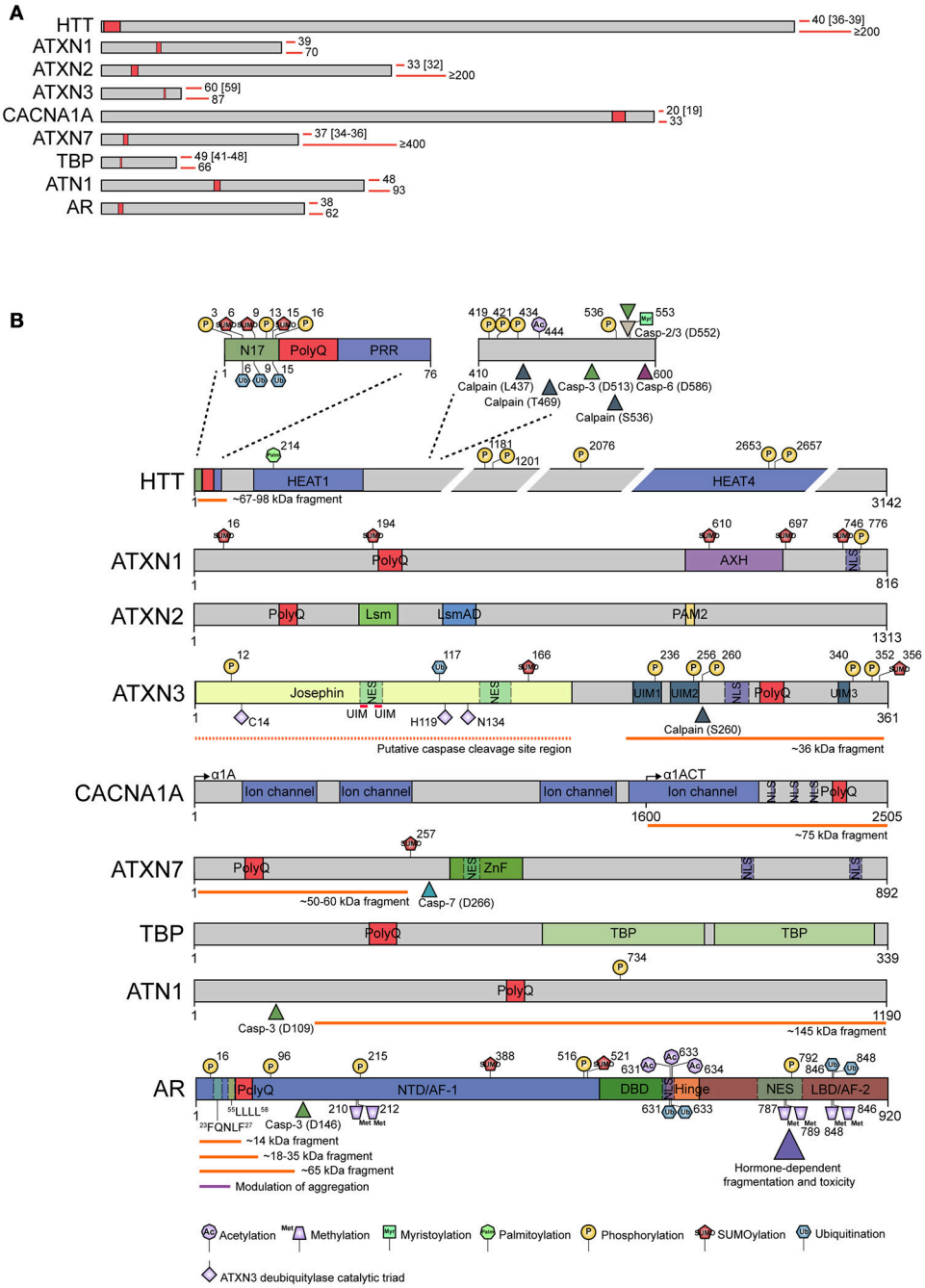

FIGURE 2 | Representation of pathogenic polyQ proteins and known modulating events associated with aggregation. (A) Schematic representation of the nine disease-related polyglutamine proteins drawn to scale. In each case, a polyQ stretch of fixed length is depicted at the approximate position (red boxes). Red bars on the right side of each protein show the smallest and largest number of glutamine repeats identified in patients of each polyQ disease to date. Numbers between brackets represent polyQ expansion sizes that have been reported to behave as incomplete penetrance alleles. **(B)** Detailed representation of all nine polyQ proteins. Domain organization is indicated. Known post-translational modifications associated with disease, caspase/calpain cleavage sites, and fragments identified are indicated. For ataxin-3, the long isoform with 3 ubiquitin-interacting motifs is shown. Residues C14, H119, and N134 depict the catalytic triad of the deubiquitylase activity of the Josephin domain. The CACNA1A locus encodes two proteins: α1A (full-length α1A) and α1ACT (C-terminal fragment of α1A) using a bicistronic mRNA with a cryptic internal ribosomal entry site. The polyQ is found in both. Many studies report a C-terminal fragment which probably represents α1ACT. For the androgen receptor, the only phosphorylation sites depicted are those with biochemical evidence of modulation of polyQ aggregation, cleavage and/or toxicity. Similarly, amino acid sequences 23FQNLF27 and 55LLLL58 highlight motifs shown to influence polyQ behavior. For simplicity, most huntingtin cleavage products are omitted and only the major N-terminal polyQ containing fragment is indicated. Amino acid numbering is based on Uniprot accession numbers P42858 (HTT), P54253 (ATXN1), Q99700 (ATXN2), P54252 (ATXN3), O00555 (CACNA1A), O15265 (ATXN7), P20226 (TBP), P54259 (ATN1), and P10275 (AR). However, for clarity, some residues

(Continued)

FIGURE 2 | Continued
are numbered according to their original publication, which might differ from the numbering according to the reference protein sequence (due to the expanding nature of polyQ proteins). AR, androgen receptor; ATN1, atrophin-1; ATXN1, ataxin-1; ATXN2, ataxin-2; ATXN3, ataxin-3; ATXN7, ataxin-7; AXH, ataxin-1/high-mobility group box containing protein-1; CACNA1, α1A subunit of the P/Q-type or CaV2.1 voltage-gated calcium channel; Casp, caspase; DBD, DNA binding domain; HTT, huntingtin; PolyQ, polyglutamine stretch; NTD/AF-1, amino-terminal domain/ activation function-1; LBD/AF-2, ligand-binding domain/ activation function-2; NES, nuclear export signal; NLS, nuclear localization signal; HEAT, huntingtin/elongation factor 3/PR65/A subunit of protein phosphatase 2A/ lipid kinase TOR domain; PRR, proline-rich region; N17, first 17 amino acids of huntingtin; TBP, TATA-binding protein (domain); UIM, ubiquitin-interacting motif; Ub-1/Ub2, ubiquitin-binding sites; Lsm, Like RNA splicing domain Sm and Sm2; LsmAD, Like-Sm-associated domain; PAM2, poly (A)-binding protein interacting motif 2; ZnF, SCA7-like zinc finger domain. For references to specific domains or post-translational modifications, please refer to Supplementary File 1.

the molecular level that may partially explain the individual differences between patients and steer future studies.

AGGREGATION PROPERTIES OF THE POLYQ STRETCH

Aggregates formed by polyQ stretches contain identical β-strand-based cores. Already in 1994, Perutz et al. described the ability of elongated polyQ stretches to form β-sheets (Perutz et al., 1994). Like many other amyloidogenic proteins (Sawaya et al., 2007), the polyQ chains can form β-sheets that are connected through interdigitating extended side chains and contain intramolecular β-hairpins (Hoop et al., 2016). Formation of β-hairpins allows for hydrogen bonding between the stacked side chains, providing a strong interaction (Hoop et al., 2016). The β-hairpins play an important role in the aggregation process. Q-stretches with a range up to 25Q are not able to form stable β-hairpins and therefore are not able to induce aggregation, except when mutations known to enhance β-hairpin formation are introduced (Kar et al., 2011, 2013). It is hypothesized that longer polyQ stretches can form more stable intramolecular β-hairpins, providing a critical monomeric nucleus necessary for inducing aggregation (Kar et al., 2011). The high affinity of the β-sheets affects interactions between molecules and might not only do so for the same pathogenic polyQ protein, but also as a secondary effect for other endogenous polyQ containing proteins (Nóbrega et al., 2015). For example, the endogenous, non-expanded TATA-box binding protein (TBP) was found to sequester into aggregates formed by other pathogenic polyQ proteins, such as huntingtin (HTT; Perez et al., 1998; Kim et al., 2002; Matsumoto et al., 2006). Similarly, inclusions containing ataxin-2 (ATXN2), ataxin-3 (ATXN3) and TBP are observed in SCA1, SCA2, SCA3, and DRPLA (Uchihara et al., 2001). Whether these secondary co-aggregating events contribute to disease is currently not clear (Kampinga and Bergink, 2016).

The crucial role for the formation of β-hairpins in the aggregation process is nicely illustrated by findings on missense CAG to CAT mutations. These mutations, coding for histidine, were found in the CAG-repeat in ATXN1, leading to insertion of one or more other amino acids and interrupting the Q-stretch (Sobczak and Krzyzosiak, 2004; Jayaraman et al., 2009; Menon et al., 2013). The AO is in these cases inversely correlated to the longer uninterrupted CAG stretch which, rather than a specific interruption pattern, dictates also the aggregation propensity *in vitro* (Menon et al., 2013). The structure of the polyQ-stretches is not changed because of the histidine-interruptions but the polyQ aggregation rates are decreased due to the Q-length dependent ability of the protein to form a critical nucleus to initiate aggregation (Jayaraman et al., 2009; Menon et al., 2013).

From all the different intracellular chaperones, so far the only ones described that could act on the β-sheets or β-hairpins formed by the Q-stretch are DNAJB6 and its closest homolog DNAJB8, two members of the DNAJ family of Hsp70 co-chaperones. In a screen for suppressors of aggregation of huntingtin (HTT-119Q) both DNAJB6 and DNAJB8 were superior suppressors of aggregation with a specificity for the polyQ tract, since they were similarly effective in the suppression of aggregation of HTT, ATXN3, the androgen receptor (AR), and polyQ alone (Hageman et al., 2010; Månsson et al., 2013). These DNAJ chaperones have a unique region containing 18 residues of the polar hydroxyl group amino acids serine and threonine, that is exposed on one face of the DNAJB6 monomer where it is predicted to interact with the hydrogen bonds in the polyQ β-hairpins (Månsson et al., 2013; Kakkar et al., 2016).

AGGREGATION INITIATION BY FLANKING DOMAINS IN POLYQ-CONTAINING PROTEINS

A longer Q-stretch not only has a higher aggregation propensity, but also affects the conformation of other parts of the protein. This can cause exposure of other regions in the proteins that have aggregation-prone properties by themselves (Ellisdon et al., 2006; Kelley et al., 2009; Tam et al., 2009). The intrinsic aggregation propensity leads to a two-stage aggregation mechanism (Ellisdon et al., 2006) in which the first aggregation step is actually thought to be a nucleation step of the non-polyQ-containing flanking domains. The formed nucleus can speed up the aggregation of the polyQ-stretch, which is then the second aggregation step. Aggregation of the flanking region and the polyQ stretch may enhance each other in a positive feedback loop accelerating aggregation and AO (Ellisdon et al., 2007; Saunders et al., 2011). The most striking examples of this process are known for HTT and ATXN3.

HTT is a relatively large protein with the polyQ stretch located in the first exon of the protein. The polyQ tract in HTT is flanked by a 17 amino acid long N-terminal (N17) domain and a polyproline domain on its C-terminus (Dehay and Bertolotti, 2006; Rockabrand et al., 2007; **Figure 2**). The N17 domain is highly soluble by itself and has an intrinsic tendency to collapse into an aggregation-resistant compact coil state (Thakur et al., 2009; Crick et al., 2013). When the Q-stretch is expanded,

the N17 domain undergoes a conformational change going into a more α-helical extended state (Tam et al., 2009; Thakur et al., 2009; Sivanandam et al., 2011), exposing a hydrophobic face through which self-association is induced (Kelley et al., 2009; Liebman and Meredith, 2010). Self-association provides an initial nucleus that increases the local concentration of the adjacent polyQ, promoting polyQ aggregation (Kelley et al., 2009; Liebman and Meredith, 2010; Sahoo et al., 2016). Aggregation of HTT can be prevented by modifying the hydrophobic face of the α-helix (Tam et al., 2009), confirming the important role of the N17 domain in initial aggregation. Moreover, synthetic polyQ peptides lacking the N17 domain show much slower aggregation kinetics (Månsson et al., 2013; Monsellier et al., 2015; Sahoo et al., 2016).

The exposed hydrophobic face on the N17 domain was identified as an interaction site for several chaperones amongst which the chaperonin TRiC, specifically the subunit CCT1 (Tam et al., 2006). CCT1 can suppress HTT aggregation by binding via its apical substrate-binding domains to the hydrophobic motifs in the N17, preventing the initial step of aggregation (Spiess et al., 2006; Tam et al., 2009; Shahmoradian et al., 2013; Sahl et al., 2015). The constitutively expressed Hsp70 (Hsc70/HSPA8) was found to co-localize, like many other Hsp70s including the prokaryotic DnaK and yeast Ssa1 (Jana et al., 2000; Muchowski et al., 2000; Novoselova et al., 2005; Tam et al., 2006), and interact with the N17 domain of HTT via its client protein binding domain (Monsellier et al., 2015). HSPA8 is not able to delay aggregation of a Q-stretch lacking flanking sequences (Månsson et al., 2013) and acts, similar to CCT1, by disrupting the interaction between N17 domains of HTT, slowing down aggregate formation (Monsellier et al., 2015).

Another example of a polyQ protein that undergoes a similar two-stage aggregation mechanism is ATXN3, causative for SCA3. ATXN3 is involved in proteostasis by editing specific ubiquitin sidechains that are targeting proteins to the proteasome (Kuhlbrodt et al., 2011). ATXN3 has an unstructured C-terminus containing the polyQ expansion and multiple ubiquitin interacting motifs (UIMs), and an N-terminus containing the Josephin domain (JD), which is a structured monomeric domain that folds into a globular conformation (Chow et al., 2004; Masino et al., 2004; **Figure 2**). The JD is the catalytic domain responsible for the deubiquitinating (DUB) properties of ATXN3 and has a high α-helical content forming a groove with two additional UIMs for recognition of the polyubiquitin chains of different linkages, and positioning them for cleavage (Masino et al., 2004; Nicastro et al., 2009, 2010). Sequence motifs on the helices in the groove are functionally important for binding conjugated ubiquitin but are predicted to be highly amyloidogenic and therefore responsible for the aggregation propensity of the JD itself (Masino et al., 2011; Lupton et al., 2015). Indeed, *in vitro* the isolated JD shows fibrillogenic behavior even under physiological conditions (Masino et al., 2004, 2011; Ellisdon et al., 2006), but when ubiquitin is added, the aggregation propensity of ATXN3 is lowered (Masino et al., 2011). Expansion of the polyQ stretch influences the conformation of the JD in such a way that the molecular mobility of two α-helices is increased and the amyloidogenic motif gets more exposed (Lupton et al., 2015; Scarff et al., 2015), providing a nucleus through which the first aggregation step of ATXN3 is initiated. This can in turn accelerate aggregation of the polyQ stretch (Gales et al., 2005; Ellisdon et al., 2007). In a dedicated screen, several modifiers of ATXN3 were identified that all fell into the canonical chaperone and ubiquitin pathways (Bilen and Bonini, 2007). Amongst the chaperones was alphaB-crystallin (HSPB5), which was found to interact with the JD in the distorted ubiquitin interacting groove, possibly masking the amyloidogenic motives, and having an effect on the initial nucleation step of ATXN3 (Robertson et al., 2010).

Flanking regions can also suppress aggregation of the polyQ stretch. For example, the proline-rich flanking domain (C38) in HTT has an opposite effect compared to the N17 domain. The C38 is also highly soluble, but actually lowers the rate of aggregation (Bhattacharyya et al., 2006; Dehay and Bertolotti, 2006; Duennwald et al., 2006; Crick et al., 2013). Other polyQ-containing proteins apart from HTT, also have a proline-rich region adjacent to the Q-stretch, like TBP, AR, and ATXN2 (Kim, 2014). It is tempting to speculate that these regions confer an evolutionary benefit and co-evolved with Q stretches to modulate their aggregation.

BINDING PARTNERS THAT CAN INFLUENCE AGGREGATION

As we have now seen, the opening up of physiologically needed hydrophobic, aggregation-prone, motifs in non-polyQ-containing parts of the protein, can lead to the unwanted formation of an initial nucleus for aggregation. These motifs are normally buried or in interaction with binding partners (or substrates), like ubiquitin in the case of ATXN3, which prevents exposure of the hydrophobic regions (Masino et al., 2011). Binding partners of polyQ-containing proteins can influence the aggregation to a great extent, also for ataxin-1 (ATXN1). ATXN1 is the protein that underlies SCA1, and has a Q-stretch in the N-terminal part of the protein and an AXH domain in the C-terminus (**Figure 2**). Just like the JD in ATXN3, the AXH domain in ATXN1 has aggregation-prone properties that are needed for its normal functioning, but therefore can be detrimental in the presence of an expanded polyQ stretch (De Chiara et al., 2013a). The AXH domain is responsible for transcriptional repression, RNA-binding activity, and is necessary for interacting with other proteins, mostly transcriptional regulators. For the domain to be able to bind all its different substrates, it has a remarkable conformational plasticity (Chen et al., 2004; De Chiara et al., 2013b; Deriu et al., 2016). Moreover, the AXH domain is responsible for ATXN1 self-association. Multimerization can bring polyQ stretches together, associated with aggregation and amyloid formation (De Chiara et al., 2005b, 2013a; Lasagna-Reeves et al., 2015). *In vivo* ATXN1 forms oligomers and interestingly the interaction partner transcriptional repressor Capicua (CIC) is found in these complexes (Lam et al., 2006; Lasagna-Reeves et al., 2015). The interaction of CIC with the AXH domain of ATXN1 stabilizes toxic soluble prefibrillar oligomers of ATXN1. When CIC levels are reduced, ATXN1

forms more fibrillar oligomers that are less toxic (Lasagna-Reeves et al., 2015). Also when the AXH domain is deleted, aggregate formation is reduced (De Chiara et al., 2005a,b). There are chaperones known to prevent ATXN1 aggregation and reduce toxicity, but the exact mechanism of action of the chaperones on ATXN1 is not known (Cummings et al., 1998; Zhai et al., 2008). A possible mechanism of action could be that chaperones bind to the AXH domain of ATXN1 to prevent complex formation or to prevent CIC from binding.

CLEAVAGE/FRAGMENTATION

Fragmented polyQ proteins have been found in patients and proteolytic processing of polyQ proteins into smaller, highly aggregation-prone fragments that are more toxic than the full-length protein has been described for most polyQ diseases, HD (Mangiarini et al., 1996; Martindale et al., 1998), DRPLA (Igarashi et al., 1998; Wellington et al., 1998), SBMA (Butler et al., 1998; Kobayashi et al., 1998; Wellington et al., 1998), and SCAs (Ikeda et al., 1996; Paulson et al., 1997; Zander et al., 2001; Goti et al., 2004; Helmlinger et al., 2004; Kordasiewicz et al., 2006; Matos et al., 2016a; **Figure 2B**). However, for SCA1, SCA2, and SCA17 the evidence for the presence of fragments is limited (Matos et al., 2016a). Proteases play a key role in the generation of these polyQ fragments, and inhibition of proteases or mutation of their cleavage sites can modulate the disease AO (Ona et al., 1999; Chen et al., 2000; Graham et al., 2006; Aharony et al., 2015). Importantly, expression of these fragments containing the polyQ stretch can already give rise to aggregation and the disease phenotype (Ikeda et al., 1996), although it is still not entirely clear why the polyQ fragments display enhanced toxicity when compared to their respective full-length proteins. Cleavage may lead to changes in aggregation propensity, conformation of the protein, localization, and molecular interactions (Matos et al., 2016a). For SBMA, it has been reported that a conformational change exposing the polyQ tract is already sufficient to drive aggregation (Heine et al., 2015) and cleavage might expose the polyQ stretch in a similar way as such a conformational change does. Protein domains that would otherwise prevent, or enhance, the aggregation may be removed, exposing the Q-stretch itself for aggregation. Finally, recognition sites and binding of molecular chaperones could be changed, exemplifying once more the importance of regions outside the polyQ tract in the modulation of aggregation.

For ATXN3, a cleavage product containing the C-terminal fragment from amino acid 221 with the 71Q expansion was found in mice showing the disease phenotype, but rarely in mice not showing the phenotype (Goti et al., 2004). This polypeptide was also found in SCA3 patients (Goti et al., 2004) indicating that fragmentation of the polyQ protein ATXN3 has a strong correlation with disease. Interestingly, while full-length ATXN3 with an expanded polyQ was mostly non-aggregating, co-expression with truncated ATXN3 makes the full-length protein co-localize with the truncated version in perinuclear aggregates (Paulson et al., 1997). More putative cleavage sites in ATXN3 were identified (Haacke et al., 2006; Colomer Gould et al., 2007) and it was shown that caspases are not the sole contributors to the fragmentation of ATXN3, but also the activity of calpains, such as calpain-2, is involved (Simões et al., 2012; Hübener et al., 2013). ATXN3 cleavage and translocation to the nucleus, and thus also aggregation, can be prevented by inhibiting calpains through overexpression of calpastatin in mice (Simões et al., 2012). Conversely, knocking down calpastatin worsened aggregation (Hübener et al., 2013). These data clearly show that under non-stressed conditions *in vivo*, fragmentation is both required and sufficient for aggregation of polyQ containing ATXN3. Similar data has been found for HTT. In almost all studies on HD, a fragment containing the first exon of HTT with the polyQ stretch is being used, since this fragment already gives rise to the HD phenotype. Toxic N-terminal fragments are found to be generated through cleavage by caspases, both in animal models and in patients (Wellington et al., 2002; Sawa et al., 2005; Graham et al., 2006; Maglione et al., 2006). Like in SCA3, fragmentation of HTT is crucial for disease progression, since the HD disease phenotype can be rescued by either mutating the cleavage site of caspase-6 in exon 13 (Graham et al., 2006), genetically ablating caspase-6 (Wong et al., 2015), or pharmacologically inhibiting caspases 1, 3, or 6 (Ona et al., 1999; Chen et al., 2000; Aharony et al., 2015). We have already discussed the ability of certain chaperones to bind to the N17 domain, which is present in the cleaved fragments.

POST-TRANSLATIONAL MODIFICATIONS

Post translational modifications (PTMs) like phosphorylation, ubiquitination, and SUMOylation, can affect the aggregation propensity of many polyQ proteins (Humbert et al., 2001; Steffan et al., 2004; Luo et al., 2005; Warby et al., 2005; Menon et al., 2012; Matos et al., 2016b; **Figure 2**). The transient nature of the PTMs usually indicates differential regulation of proteins and they can provide an interesting extra layer of modulation, possibly influencing all of the above-mentioned features of polyQ aggregation. PTMs can create alternative binding surfaces, affecting the affinity to binding partners like proteases and chaperones, and can lead to conformational changes to expose the Q-stretch. Therefore, either increased or decreased PTMs are associated with aggregation.

For most of the polyQ proteins there are several residues known to be modified (see **Figure 2B** for PTMs that impact aggregation). For ATXN3 six phosphorylation sites have been described, in the catalytic JD and in the UIMs (Fei et al., 2007; Mueller et al., 2009; Matos et al., 2016b; **Figure 2**). Phosphorylation of serine (S)340 and S352 in the third UIM did not change aggregation propensity, but shifted the localization of the aggregates from the cytoplasm to the nucleus (Mueller et al., 2009). Phosphorylation of S256 in the second UIM was shown to inhibit the formation of large insoluble polyQ complexes (Fei et al., 2007), and phosphorylation of S12 in the JD also reduces aggregation (Matos et al., 2016b). The protective effect of constitutive phosphorylation of S12 might be dependent on its close proximity to the catalytic sites in the JD, causing hindrance of the intramolecular aggregation. Phosphorylation of HTT on

S421 (Humbert et al., 2001) and S434 (Luo et al., 2005), leads to a decrease in polyQ aggregation due to a reduction in caspase-mediated cleavage thus preventing the formation of fragments (Luo et al., 2005; Warby et al., 2009). For ATXN1, S776 is the most studied phosphorylation site since it leads to reduced aggregate formation (Emamian et al., 2003; Orr, 2012). Another interesting PTM on ATXN1 is ubiquitination of K589 in the AXH domain. Mutating this residue leads to reduced degradation and, hence, more aggregation of ATXN1 (Kang et al., 2015), suggesting that PTMs may also affect the degradation of polyQ proteins resulting in a higher concentration of proteins at risk for aggregation.

Chaperone-dependent degradation of still soluble polyQ proteins could therefore be another important aspect in ameliorating disease. Interestingly, the co-chaperone CHIP (C-terminus of Hsp70-interacting protein), an E3 ligase that can interact with and modulate Hsp70 activity (Ballinger et al., 1999; Scheufler et al., 2000), has been implicated as a modulator in many polyQ diseases (Jana et al., 2005; Choi et al., 2007; Gao et al., 2011). CHIP interacts with ATXN1 via the phosphorylated S776 and the phospho-dead S776A mutation reduced this interaction. The CHIP-ATXN1 interaction is likely mediated via Hsp70, since the tetratricopeptide repeat (TPR) domain of CHIP, with which it interacts with Hsp70, is needed for the interaction and for promotion of ATXN1 degradation (Choi et al., 2007). A similar model of CHIP and Hsp70 interaction with HTT and ATXN3 was proposed, although no single modified residue was identified as a recognition site (Jana et al., 2005).

Members of DNAJ family of Hsp70 co-chaperones were also shown to play a role in the PTM dependent degradation of polyQ proteins, like in ATXN3 (Gao et al., 2011). DNAJB1 was identified to suppress aggregate formation of ATXN3 (Chai et al., 1999), but aggregation of the S256A mutant of ATXN3 could not be prevented by DNAJB1 (Fei et al., 2007), it is still unclear whether DNAJB1 has preferential affinity for phosphorylated ATXN3. Interestingly, Hsp70 can prevent S256A aggregation (Fei et al., 2007). Next to DNAJB1, DNAJB2 was found to suppress polyQ protein aggregation via two UIMs that were shown to be crucial for its interaction with K63-linked ubiquitination of HTT (Labbadia et al., 2012). Intriguingly, all the PTMs on HTT are less present in polyQ-expanded HTT, especially in the regions in the brain that are mostly affected, abolishing the possible protective effect of the modifications (Luo et al., 2005; Warby et al., 2005; Aiken et al., 2009). Currently it is unclear whether the drop in modification is causal or a consequence of aggregation.

PERSPECTIVES

The expanded polyQ stretches in the different disease-associated proteins are the determining factor of disease onset and progression in all of the polyglutaminopathies. Above a certain threshold, Q-stretches are prone to aggregate. However, more often than not, the Q-stretch and its aggregation propensities are modulated by secondary events that we categorized here; flanking regions, which have modulating capacity due to intrinsic stability issues, binding of partners (including chaperones), modification by PTMs, and cleavage of the Q-stretch. The examples of molecular interactions described, clearly indicate that polyQ protein aggregation is a multifactorial and likely multistep process that not always has to go through the same sequence of events toward aggregate formation. For example, the intrinsic fibrillogenic behavior of the JD and cleavage of ATXN3 (leading to a fragment not containing the JD) can both trigger aggregation independently. It could very well be that initial aggregation can be triggered via different mechanisms leading to secondary events that stimulate aggregation further. Thus, *in vivo* aggregation of the JD might stimulate ATXN3 cleavage and, vice versa, cleavage might destabilize the JD domain resulting in a fast forward feedback loop of aggregation. Modulating events, together with the unique expression pattern and level of each polyQ protein, could explain the variation in AO between the nine diseases.

Moreover, the modulating events acting on the flanking regions might also explain the variation of AO among patients with a similar Q length within a given polyQ disease. By combining information on Q length (CAG repeat), expression levels of the chaperone DNAJB6, which modulates Q aggregation directly, and the expression levels of chaperones that act on the disease-specific flanking regions, with the PTM and fragmentation status, perhaps a better predication of AO could be made. A strategy targeting chaperones acting on the Q-stretch with those acting on the flanking regions might provide a synergistic approach for delaying AO, benefiting individuals diagnosed with an expanded polyQ tract. There is little information on the factors influencing progression of disease after onset and it would also be of interest to know whether progression of disease is influenced by the same factors that modulate aggregation propensity. If so, these could be used as a therapeutic modality as well.

AUTHOR CONTRIBUTIONS

EK and ED compiled all the data and contributed equally to this work. EK, ED, LJ, HK, and SB gave intellectual feedback and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2017.00145/full#supplementary-material

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REVIEW
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Transthyretin and BRICHOS: The Paradox of Amyloidogenic Proteins with Anti-Amyloidogenic Activity for Aβ in the Central Nervous System

Joel N. Buxbaum[1,2]* *and Jan Johansson*[3]*

[1] Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA, [2] Scintillon Institute, San Diego, CA, USA, [3] Division of Neurogeriatrics, Department of Neurobiology, Care Sciences, and Society (NVS), Center for Alzheimer Research, Karolinska Institutet, Huddinge, Sweden

Amyloid fibrils are physiologically insoluble biophysically specific β-sheet rich structures formed by the aggregation of misfolded proteins. *In vivo* tissue amyloid formation is responsible for more than 30 different disease states in humans and other mammals. One of these, Alzheimer's disease (AD), is the most common form of human dementia for which there is currently no definitive treatment. Amyloid fibril formation by the amyloid β-peptide (Aβ) is considered to be an underlying cause of AD, and strategies designed to reduce Aβ production and/or its toxic effects are being extensively investigated in both laboratory and clinical settings. Transthyretin (TTR) and proteins containing a BRICHOS domain are etiologically associated with specific amyloid diseases in the CNS and other organs. Nonetheless, it has been observed that TTR and BRICHOS structures are efficient inhibitors of Aβ fibril formation and toxicity *in vitro* and *in vivo*, raising the possibility that some amyloidogenic proteins, or their precursors, possess properties that may be harnessed for combating AD and other amyloidoses. Herein, we review properties of TTR and the BRICHOS domain and discuss how their abilities to interfere with amyloid formation may be employed in the development of novel treatments for AD.

Keywords: transthyretin, BRICHOS, Alzheimer's disease, amyloidosis, neurodegeneration, protein-protein interactions, chaperones

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***Correspondence:**
Joel N. Buxbaum
jbux@scripps.edu
Jan Johansson
janne.johansson@ki.se

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INTRODUCTION

The amyloidoses are a set of human diseases (and their animal models) in which the precursors, synthesized as soluble proteins, aggregate and become insoluble under physiologic conditions. They ultimately form ultrastructural non-branching fibrils 10 nm in diameter with a characteristic cross-β sheet structure on x-ray diffraction analysis (Glenner, 1980). In tissues the fibrillar aggregates are seen as extracellular plaque-like structures in the affected organs, which bind the dye Congo red displaying green birefringence under polarized light (Linke, 2006). Amorphous pre-fibrillar or "off-pathway" aggregates may be present in the same tissues. Thus far 36 proteins have been noted to be associated with local or systemic amyloid deposition (Sipe et al., 2016).

In recent years with the ability to make large amounts of recombinant proteins from virtually any source, it has been noted that many proteins unrelated to disease will form fibrils under amyloid forming conditions and generate oligomeric species that are cytotoxic *in vitro* (Bucciantini et al., 2002). In mammalian cells several examples of physiologically functional amyloids have been

described (Fowler et al., 2005; Maji et al., 2009). Approximately ∼0.2% of all human proteins (36/20,000) form disease associated or functional amyloids. Further, in prokaryotes amyloid structures function as critical elements in biofilm formation (Blanco et al., 2012). One explanation for the apparent propensity of only some proteins to be fibrillogenic *in vivo* is that potentially amyloidogenic protein domains are "self-chaperoned" in the context of their intact native conformations. Perhaps evolution has recognized both the functional usefulness of sequences that can lead to amyloid formation and the risk of aggregation *in vivo* and embedded them in structures that do not allow the aggregation prone domains to form the homotypic interactions ("stearic zippers") required for fibrillogenesis under physiologic conditions (Goldschmidt et al., 2010). Alternatively it may be that all proteins have the capacity to form amyloid under the appropriate conditions with those conditions rarely found in biology (Dobson, 1999). In eukaryotes, apart from so-called "normal" amyloids, it is rare for wild type proteins to form amyloid *in vivo* unless they undergo some modification, e.g., cleavage, as in the case of the Aβ protein precursor (AβPP) of Alzheimer's disease (AD) or the serum amyloid A (SAA) protein precursor in inflammation associated amyloidosis (Haass and Selkoe, 1993; Kluve-Beckerman et al., 2002). More likely is the occurrence of variants encoded by a mutation in the germline gene, as is the case with transthyretin (TTR) and other precursors responsible for human autosomal dominant hereditary amyloidoses (Rowczenio et al., 2014). It has also been argued that evolution has resulted in cells/organisms constraining the synthesis of some potentially amyloidogenic molecules to ensure that quantitatively they do not exceed the critical concentrations sufficient to nucleate the aggregation process in the presence of adequate cellular chaperone activity (Tartaglia et al., 2007).

In humans, while many of the amyloidoses are systemic in distribution, a disproportionate number are represented in the neurodegenerative diseases associated with aging. The prototype is AD, a disorder characterized by progressive memory loss and behavioral changes. Current thinking, based on genetic, biochemical and *in vivo* observations, favors the notion that cleavage fragments of the normal single pass transmembrane molecule AβPP, i.e., $A\beta_{1-38-43,}$ which are aggregation prone, form oligomers, which can be shown to be cytotoxic in tissue culture and synaptotoxic in hippocampal slices (Selkoe and Hardy, 2016). It is not yet clear if the toxic oligomers are on the same folding pathway as the fibrils found in the Congophilic deposits in the brain or whether the fibrils are a less toxic form of aggregate (Wu et al., 2013). The sequence of AβPP cleavage followed by aggregation of Aβ fragments is causal in the rare autosomal dominant forms of AD and is likely to participate in the pathogenesis of the sporadic disease, but may not be the sole etiology in the latter. In both forms there appears to be a multiplicity of contemporaneous or downstream events involving other cell types (microglia, astrocytes) and proteins, particularly Tau, which contribute to the development of the characteristic dementia.

During the last decade studies in transgenic models of human Aβ deposition have shown that many human genes and the silencing of a number of mouse genes may have profound impacts on the pathogenesis of the AD-like changes seen in the murine models and have suggested roles for these molecules in inhibiting or facilitating the process of amyloidogenesis *in vivo*. The salutary effects of two such genes (*TTR* and *ITMB2*) were quite unexpected since by themselves both protein products were clearly amyloidogenic and responsible for distinct forms of clinically relevant human amyloidosis.

Wild type and mutant forms of human TTR cause a spectrum of human systemic amyloid syndromes including Familial Amyloidotic Cardiomyopathy (FAC), Familial Amyloidotic Polyneuropathy (FAP) and Senile Systemic Amyloidosis (ATTRwt). Mutant forms of *ITM2B*, encoding the BRICHOS (see below) domain containing Bri2 protein are etiologic in Familial British Dementia and Familial Danish Dementia, while mutations in the gene (*SFTPC*) encoding another protein with a BRICHOS domain, lung surfactant C precursor (proSP-C), cause interstitial lung disease (ILD) with pulmonary amyloid deposits. We will discuss the available information describing the biologic and biophysical findings that are apparently involved in the prevention of one form of amyloid, i.e., that formed by the Aβ protein seen in the plaques in human AD, by BRICHOS and TTR, molecules that are direct precursors of other distinct forms of human amyloidosis and what this may mean in the universe of protein-protein interactions in complex organisms.

BRICHOS STRUCTURE

The BRICHOS domain is found in several different precursor proteins. In proSP-C and Bri2 these precursors also contain segments that are amyloidogenic (**Figure 1**). The transmembrane (TM) part of the mature lung surfactant protein C (SP-C) is an archetypical discordant α-helix composed of a long poly-Val segment, i.e., the most β-sheet prone sequence possible (Johansson et al., 1994). As expected from the predicted β-sheet structure, native SP-C can convert into amyloid fibrils *in vitro* (Gustafsson et al., 1999), and expression of the SP-C part only, i.e., without the rest of proSP-C (including the BRICHOS domain) in transgenic mice generates severe SP-C aggregation and toxicity (Conkright et al., 2002). As predicted from the high α-helix propensity of Leu, poly-Val to poly-Leu replacements result in a non-aggregating and functional SP-C analog, (SP-CLeu), which, like SP-C, inserts in surfactant phospholipid membranes, but unlike SP-C, does not form amyloid fibrils, since its α-helical conformation is thermodynamically stable (Kallberg et al., 2001). A synthetic surfactant, presently in clinical trials, has been developed based on synthetic SP-CLeu, (Johansson et al., 2003), demonstrating that modulation of β-sheet and amyloid propensity is a feasible means of designing stable proteins for biologic drug development.

Under physiological conditions, the poly-Val segment in native (pro) SP-C is intrinsically prone to form toxic β-sheet aggregates and amyloid fibrils, and we have proposed that the BRICHOS domain present in proSP-C prevents the poly-Val segment from folding into β-sheet aggregates, and promotes formation of a stable α-helix (Conkright et al., 2002; Johansson

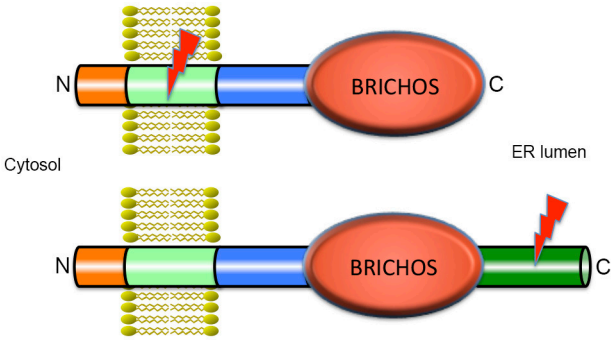

FIGURE 1 | Architecture of BRICHOS containing proteins. All proteins that contain a BRICHOS domain have a similar modular structure with a short N-terminal part in the cytosol, a transmembrane (TM) region, a linker region, the BRICHOS domain, and in most cases, a C-terminal region. ProSP-C is depicted in the upper part of the figure and is the only example where the BRICHOS domain is not followed by a C-terminal region. All other BRICHOS containing proteins, depicted in the lower part of the figure, (i.e., the Bri family, chondromodulin, and tenomodulin, gastrokines, and group C, see Hedlund et al., 2009) contain a C-terminal region of varying length. In spite of their similar architectures, only the amino acid sequence of the BRICHOS domain is conserved between BRICHOS containing proteins, while the other parts are structurally unrelated. The lightning symbols denote the regions that are amyloidogenic, the TM region in proSP-C and the C-terminal region in Bri2, see text for details.

et al., 2006). Intriguingly, mutations in the proSP-C BRICHOS domain result in amyloid formation of SP-C and ILD with lung fibrosis, apparently the first described amyloid disease that occurs as result of a mutation in an intramolecular chaperone domain (Willander et al., 2012a).

The BRICHOS domain is found in species ranging from humans to simple marine organisms. It is small (about 100 amino acid residues), has a unique fold and is present in a diverse set of pro-proteins that generate bioactive peptides after proteolytic processing (Hedlund et al., 2009). BRICHOS has been identified in 10 human protein families and the name is derived from BRI2, CHOndromodulin-I and Surfactant protein C (SP-C). The proteins containing a BRICHOS domain have a wide range of functions, and disease associations, including ILD with amyloid deposits (proSP-C), dementia (Bri2), and cancer (Chondromodulin-I) (Sánchez-Pulido et al., 2002; Willander et al., 2012a). There are low pairwise sequence identities between different BRICHOS domain families (∼15–25%) but all have similar predicted secondary structures. The precursor proteins have a common overall architecture, and are predicted to be type II TM proteins (Sánchez-Pulido et al., 2002; Hedlund et al., 2009; Knight et al., 2013) with the N-terminus located in the cytosol (**Figure 1**). All BRICHOS containing proproteins have a cytosolic segment, a hydrophobic TM region, a linker region followed by a BRICHOS domain, and a C-terminal region except proSP-C, in which there is no C-terminal region following the BRICHOS domain. All the proproteins except proSP-C have a segment with high β-sheet propensity, the C-terminal region. In proSP-C the high β-sheet propensity is found in the TM region (Sánchez-Pulido et al., 2002; Hedlund et al., 2009). In all BRICHOS containing pro-proteins the regions that are prone to form β-sheets are well conserved, and are likely to be the BRICHOS client regions that are destined to aggregate in the absence of functional BRICHOS.

The only BRICHOS crystal structure thus far determined is that of proSP-C BRICHOS (pdb code 2yad). It has a unique fold composed of five β-strands arranged in a mixed anti-parallel and parallel fashion, and two flanking α-helices (**Figure 2**). Helix 1 packs against face A of the β-sheet and helix 2 packs against the opposite side of the β-sheet, face B. Molecular dynamic simulations suggest that helix 1 can translocate exposing the underlying face A of the β-sheet. This implicates face A as the binding site for possible substrates (Willander et al., 2012a), but direct evidence of binding of any peptide to face A has not been found. Homology models of the human BRICHOS domains from each family showed that they are compatible with the proSP-C BRICHOS structure with respect to the secondary structural elements, but the loop regions are highly variable among different BRICHOS domains. An interesting observation was that face A of the proSP-C BRICHOS contains mainly hydrophobic residues, which are apparently complementary to its hydrophobic target sequence—the TM region of proSP-C. Bri2, and Bri3 BRICHOS instead have a face A that contains several residues with charged side-chains, and the proposed Bri2 and Bri3 BRICHOS target sequences, i.e., the respective Bri2 and Bri3 C-terminal regions that are prone to form β-sheets, contain multiple charged residues. This suggests that the properties of face A reflect its binding preferences in the respective BRICHOS domain (Knight et al., 2013). There are only three strictly conserved residues in all BRICHOS domains, two cysteines and one aspartic acid. The cysteines form a disulfide bridge in proSP-C BRICHOS and their strict conservation suggests that a corresponding disulfide bridge is present in all BRICHOS domains.

TRANSTHYRETIN (TTR) STRUCTURE

In contrast to BRICHOS, which represents a domain common to a diverse family of proteins, some of which are known to be amyloidogenic, TTR is a unique human protein synthesized in hepatocytes, retinal pigment epithelial cells, choroid plexus epithelium, pancreatic α-cells, Schwann cells, and neurons under some conditions. It is the major carrier of retinol binding protein (RBP) charged with retinol in serum and a minor carrier of the thyroid hormone precursor thyroxine (T4) prior to its conversion to the physiologically more active tri-iodo—thyronine (T3) by tissue deiodinases (Buxbaum, 2007). Only a small fraction of the circulating TTR carries T4 while 25–50% is loaded with RBP-retinol. However, in cerebrospinal fluid choroid plexus synthesized TTR is the major T4 carrier. TTR is a non-disulfide linked homo-tetramer in which the mature polypeptide monomer, after cleavage of the leader sequence, contains 127 amino acids. The tetramer is thermodynamically and kinetically quite stable with a Ka $= 1.1 \times 10^{24} M^{-3}$ (Hurshman et al., 2004). The crystal structure shows a twofold axis of symmetry (Blake et al., 1978). It is assembled as a dimer of dimers around a central channel, which is primarily hydrophobic and contains the two T4 binding sites (**Figure 3**). T4 binding in the first site induces an

allosteric change that makes the second site less accessible to its natural ligand (Neumann et al., 2001). While different portions of the protein bind T4 and RBP, both stabilize the tetrameric structure reducing its tendency to dissociate (White and Kelly, 2001).

proSP-C BRICHOS IN INTERSTITIAL LUNG DISEASE

The proSP-C gene (*SFTPC*) is located on chromosome 8 and contains 6 exons encoding a 197 amino acid protein. The protein is expressed exclusively in the secretory pathway of pulmonary alveolar type II cells (Mulugeta and Beers, 2006). Proteolytic cleavage of proSP-C eventually generates the 35-residue SP-C, consisting of an α-helical poly-Val TM region plus an 8-residue N-terminal segment located outside the membrane (Johansson et al., 1994, 1995). The SP-C peptide is secreted as part of lung surfactant, into the alveolar space (Beers et al., 1994; Whitsett and Weaver, 2002). SP-C is unique in that although the primary translation product is a TM protein it is ultimately secreted as a lipophilic, mature peptide (Russo et al., 1999). Mutations in the proSP-C gene lead to ILD, a form of fibrosis (Nogee et al., 2001, 2002; Beers and Mulugeta, 2005; Willander et al., 2012a) with Congophilic deposits containing the mature SP-C segment (Willander et al., 2012a). Both inherited and spontaneous proSP-C mutations have been implicated in ILD (Hamvas, 2006) and a systematic search revealed 91 *SFTPC* disease-causing mutations (Litao et al., 2017). Roughly two thirds of the resulting residue exchanges are localized to the BRICHOS domain, but the most frequent mutation (I73T) is localized in the linker region in between the TM region and BRICHOS. A majority of the ILD associated mutations are located in the linker region or in the BRICHOS domain, and these mutations are proposed to lead to amyloid formation of the SP-C peptide (Willander et al., 2012a). The TM α-helix of SP-C has very high β-sheet propensity, since it is composed of mainly valine residues (Kallberg et al., 2001; Johansson et al., 2010; see above under BRICHOS Structure). It has been hypothesized that proSP-C BRICHOS promotes correct folding and insertion into the membrane of the α-helical TM part of SP-C, preventing the formation of amyloid and ILD (Hedlund et al., 2009; Willander et al., 2012a). Consistent with that notion is the observation that native SP-C isolated from lung surfactant, aggregates into amyloid fibrils *in vitro* that can be visualized by electron microscopy (EM), but co-incubation with proSP-C BRICHOS abrogates SP-C fibril formation (Nerelius et al., 2008).

BRI2 IN FAMILIAL BRITISH AND DANISH DEMENTIA

Integral membrane proteins 2B (ITM2B) and 2C (ITM2C) also called Bri2 and Bri3 respectively are part of the BRI family. The Bri2 gene (*ITM2B*) is located on chromosome 13 and contains 6 exons. The Bri3 gene (*ITM2C*) is located on chromosome 2 and contains 7 exons. Bri2 and Bri3 proteins share 42% overall sequence identity, and their BRICHOS domains have 60% identical residues. The BRI family may be the most ancient family

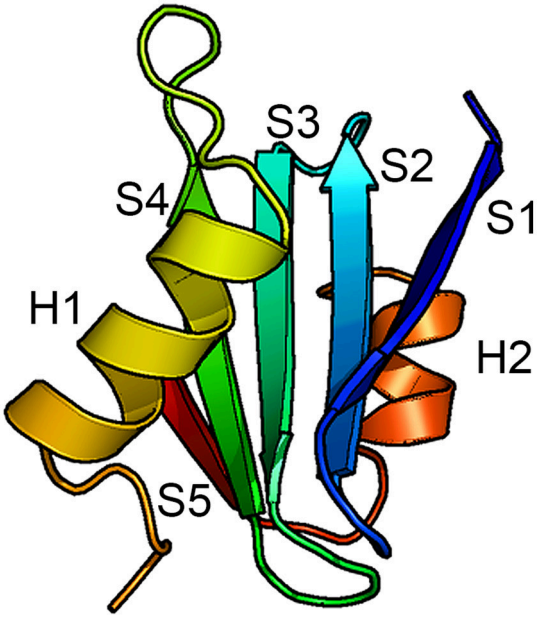

FIGURE 2 | Structure of the BRICHOS domain. Backbone conformation of proSP-C BRICHOS (Willander et al., 2012a) with the five β-strands (S1–S5) and the two α-helices (H1 and H2) labeled. Face A of the β-sheet is localized toward H1 and face B is localized toward H2.

of BRICHOS containing proteins, considering it has members in the most ancient species (flies and worms; Sánchez-Pulido et al., 2002). The Bri2 protein is a 266-residue long, type II TM proprotein consisting of an N-terminal cytosolic part (residues 1–54), a TM region (residues 55–75), a linker (residues ∼76–130), a BRICHOS domain (residues ∼130–231) and a C-terminal region (residues 232–266). Bri2 has an *N*-glycosylation site at asparagine, Asn170 (Tsachaki et al., 2011), and is expressed at high levels in brain, heart, placenta, and pancreas (Vidal et al., 1999). Processing of Bri2 by furin releases a 23-residue peptide referred to as Bri23 (corresponding to residues 244–266 of Bri2) from the C-terminal region. Mutations in Bri2 give rise to release of extended, 34-residue, C-terminal peptides, ABri, or ADan, that deposit primarily in the CNS in two rare amyloid diseases, familial British dementia (FBD) and familial Danish dementia (FDD), respectively (Cantlon et al., 2015a). After the discovery of the pathogenic FBD and FDD mutations and Bri2 as the precursor to the ABri and ADan peptides (Vidal et al., 1999, 2000), furin was identified as the major protease responsible for the proteolytic cleavage releasing the C-terminal peptides (Kim et al., 1999), but other proprotein-like convertases may also process Bri2, releasing C-terminal peptides (Kim et al., 1999; Vidal et al., 2000). Moreover the Bri2 BRICHOS domain can be shed by ADAM10 cleavage and released into the extracellular space, but Bri3 is apparently not processed by ADAM10 (Martin et al., 2008). The remaining membrane associated N-terminal fragment of Bri2, is cleaved by intramembranous proteolysis by signal peptide peptidase-like (SPPL) proteases, SPPL2a and SPPL2b. This cleavage releases

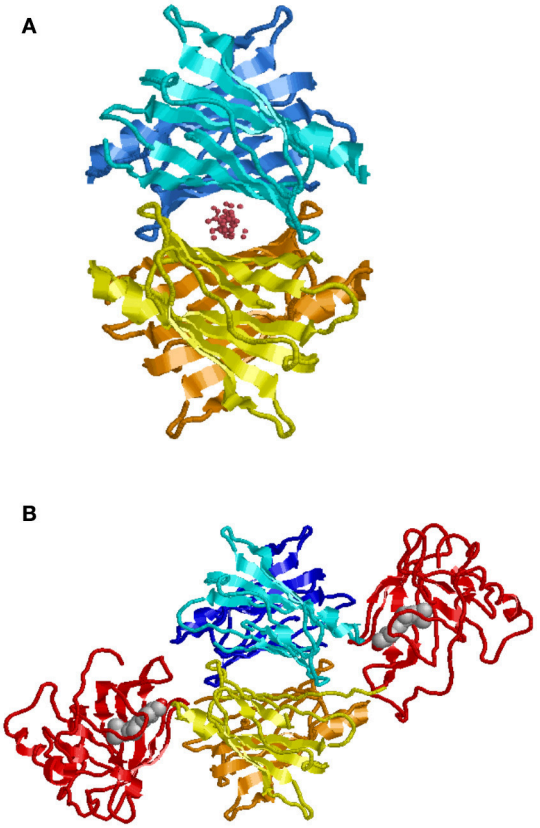

FIGURE 3 | Model of TTR tetramer. Shown with ligand in T4 binding pocket **(A)**. Shown with two molecules of retinol binding protein (RBP) charged with retinol **(B)** (Monaco, 2002). While there are two potential T4 binding sites, binding of the first results in an allosteric change restricting access to the second. In humans, generally only one of the RBP binding sites is occupied.

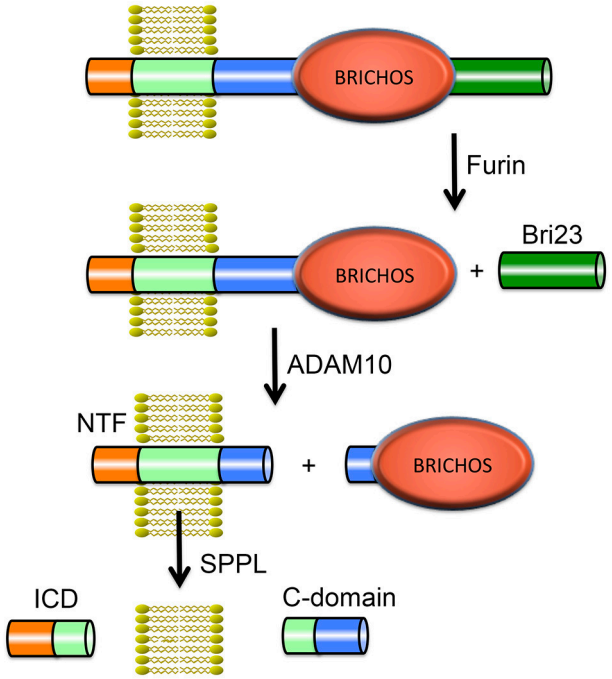

FIGURE 4 | Proteolytic processing of Bri2. The full-length Bri2 protein is cleaved by furin resulting in release of the Bri23 peptide, and subsequent cleavage by ADAM10 releases the BRICHOS domain and generates an N-terminal fragment (NTF) that can be cleaved by signal peptide peptidase like (SPPL) proteases into an intracellular domain (ICD) and a released C-domain.

a Bri2 intracellular domain (ICD) as well as a secreted so called C-domain (Martin et al., 2008). SPPL cleavage of Bri2 likely takes place secondary to ADAM10 mediated shedding. Moreover ADAM10 processing is not sequence specific but rather occurs at specific distances from the plasma membrane (Sisodia, 1992). Together, the available data are most consistent with ADAM10 releasing the BRICHOS domain by cleavage in the linker region of Bri2. See **Figure 4** for an overview of Bri2 processing.

FBD is a rare disease that shares many similarities with AD with memory loss and dementia (Mead et al., 2000). Typical histological findings are amyloid deposition of ABri, cerebral amyloid angiopathy (CAA) and neurofibrillary tangles (NFT's) (Vidal et al., 1999). FDD shares similarities with FBD but patients also show cataracts and deafness. Histological findings in FDD include CAA, NFT's and hippocampal ADan amyloid plaques (Vidal et al., 2000). The FBD pathogenic mutation converts the stop codon in the Bri2 gene to a codon for arginine, extending the open reading frame to include 11 additional amino acids, giving rise to the 34 residue long extended peptide, ABri. The FDD mutation is different and leads to a 10-nucleotide duplication, causing a frame shift replacing the stop codon of Bri2, extending the peptide to another 34 residue peptide, ADan. Both ABri and ADan are thus 11 residues longer than the non-pathogenic Bri23, but the additional residues share no sequence homology (Cantlon et al., 2015b). Aβ has been found in both fibrillar and non-fibrillar deposits of ADan in FDD (Tomidokoro et al., 2005), and Bri2, and/or parts thereof, have been found to deposit with Aβ plaques in AD (Del Campo et al., 2014). These observations suggest possible links between the events underlying the two diseases. It has been suggested that FBD and FDD are caused by the aggregation of ABri and ADan respectively, and/or by a loss-of function of mature Bri2 (Cantlon et al., 2015a). Experimental support for both theories can be found. Data from FBD-Bri2 and FDD-Bri2 knock-in mice as well as human patients show a reduction in Bri2 levels (Tamayev et al., 2010a; Matsuda et al., 2011), and knocking in Bri2 in FDD-Bri2 knock-in mice rescues negative effects on cognition (Tamayev et al., 2010a,b). Moreover, studies show that ABri and ADan aggregation *in vitro* causes cell toxicity (El-Agnaf et al., 2001, 2004) and effects on synaptic plasticity (Cantlon et al., 2015b) similar to Aβ.

THE TTR AMYLOIDOSES

TTR is encoded by a single gene on chromosome 18 that encompasses approximately 19 Kb of DNA with 4 exons included within 7 Kb, 6 Kb of upstream (5′) sequence

and 6 Kb downstream containing the conventional 3′ non-coding sequence that allows normal mRNA processing after transcription. The promoter proximal 2 Kb appears to contain all the sequences required for tissue specific expression of the gene (Sasaki et al., 1985; Costa et al., 1986; Sparkes et al., 1987; Li et al., 2011; Wang et al., 2014). Despite extensive screening of human populations there have been no reports of a complete absence of a functional TTR protein. However, mouse knockouts survive, are fertile but have a persistent behavioral abnormality with neuronal loss and mild gliosis in the cortex and CA3 region of the hippocampus (Buxbaum et al., 2014).

Amyloidogenic protein variants causing the autosomal dominant clinical disorders Familial Amyloidotic Polyneuropathy (a sensori-motor and autonomic polyneuropathy) and Familial Amyloidotic Cardiomyopathy have been found in 77 of the 127 amino acids in the protein (**Figure 5**). Forty residues have been found to have a single amyloidogenic mutation while fifteen have 2, six have 3, five have 4, and one has 5. Fifty amino acids have none and 12 mutations have been described that did not lead to clinically detectable amyloidosis, although two of the involved residues had both amyloidogenic and non-amyloidogenic substitutions (Rowczenio et al., 2014) (**Figure 5**). There is an increasing frequency of wild type TTR amyloid deposition in the heart, carpal tunnel and gut associated with increasing age currently thought to be related to post-synthetic (perhaps oxidative) changes that may render the wild type protein less stable although other, as yet undefined, mechanisms may be responsible. In the case of the mutations it appears that they all form tetramers which are kinetically or thermodynamically unstable under physiologic conditions resulting in enhanced dissociation releasing monomers which are susceptible to rapid misfolding, aggregation, and fibril formation (Johnson et al., 2012). These observations suggest that the monomers functionally "chaperone" each other.

Recombinant TTR monomers (M-TTR) have been engineered by replacing residues involved in the interaction between monomers required to form the dimers required for tetramerization (F87ML110M) (Jiang et al., 2001). While these monomers can form a functional tetramer they are highly aggregation prone and have provided a useful model to examine the process of aggregation and fibril formation. Such experiments have revealed that aggregation requires the structured monomer to become denatured prior to misfolding and fibril formation (Hurshman et al., 2004). Each monomer contains eight regions of β-sheet, which may explain their inherent tendency to form the β-sheet rich amyloid fibrils. Aggregation appears to primarily involve interaction between the F and H beta strands (Lim et al., 2013). Interestingly murine TTR, which is 80 percent identical to the human protein, is orders of magnitude more kinetically stable and is essentially non-amyloidogenic under physiologic conditions. The crystal structures of the wild type and mutant human proteins and the normal mouse protein are very similar (Hörnberg et al., 2000; Reixach et al., 2008). It is also interesting to note that mouse Aβ and islet amyloid polypeptide are apparently non-amyloidogenic.

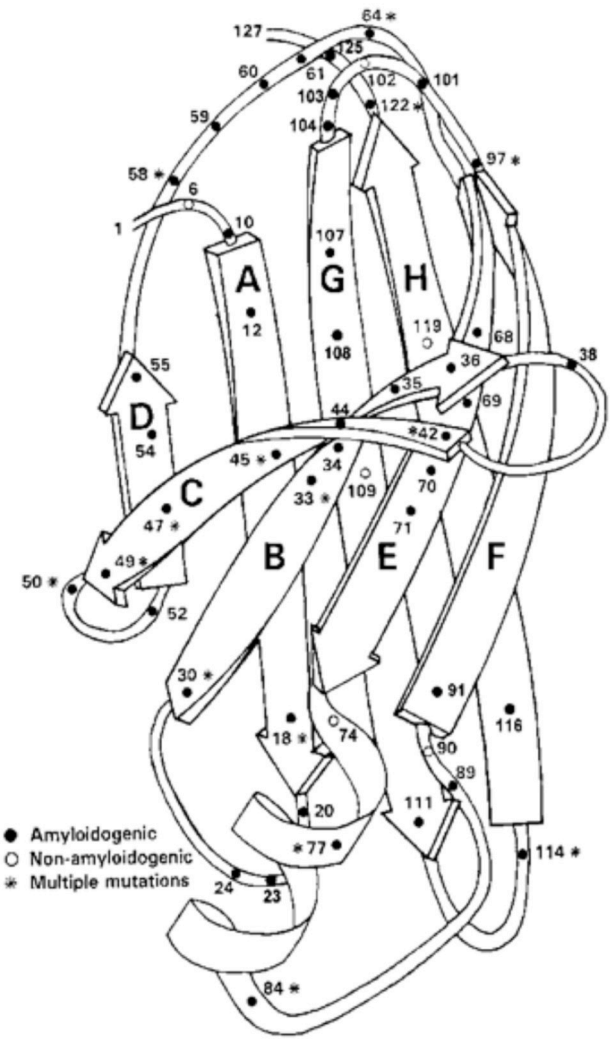

FIGURE 5 | Ribbon Diagram of TTR Monomer: The figure shows the location of many of the identified amyloidogenic and non-amyloidogenic amino acid substitutions, demonstrating that almost no region of the protein is spared (structure after Blake et al., 1978).

Tissue culture studies have shown that exposing target cells to the engineered TTR monomer or amyloidogenic tetramers can induce cytotoxicity. However, the tetramer has to dissociate to release monomer, which appears to bind to cells in a manner consistent with a receptor ligand like interaction, with aggregates forming on the cell surface (within a 3–6 h period) with cell death 48–72 h later (Reixach et al., 2004; Manral and Reixach, 2015). Non-amyloidogenic, non-toxic tetramers are endocytosed directly by the target cells with little evidence of aggregation and no apparent effect on cell viability or function.

In vivo it appears that both oligomeric and fibrillar TTR aggregates deposit with an apparent hierarchy of tissue tropism favored by particular mutants. It has been known for some time that limited proteolytic digestion may accelerate amyloid formation by a given precursor and both intact TTR and

fragments have been found in the tissue deposits with the relative proportions of each possibly playing a role in the cardiac deposition phenotype (Thylén et al., 1993; Bergström et al., 2005). The mechanism of tissue selectivity, i.e., predominantly peripheral nerve or myocardium, is unclear and may be more apparent than real since at autopsy deposits of fibrils formed from the mutant proteins can be found in most tissues. However, it is possible that different tissues vary in their capacity to digest TTR or in the nature of their extracellular matrix either of which may be related to where fibril deposition occurs.

BRICHOS INTERACTIONS WITH ALZHEIMER'S DISEASE PEPTIDES AND ITS EFFECTS ON AMYLOIDOGENESIS

Bri2 binds AβPP and modulates its processing leading to a reduction of secreted Aβ, both *in vitro* (Fotinopoulou et al., 2005; Matsuda et al., 2005) and *in vivo* (Matsuda et al., 2008; Tamayev et al., 2012), likely because Bri2 restricts the access of the secretases involved in AβPP cleavage, but it has also been shown that Bri2 modulates β-secretase levels (Tsachaki et al., 2013), providing an alternative explanation for how Bri2 affects AβPP processing. Moreover, residues 46–106 (the TM region and parts of the linker) are apparently responsible for binding the juxtamembrane and membrane spanning domains of AβPP (Fotinopoulou et al., 2005), i.e., the BRICHOS domain is not necessary for Bri2 interactions with AβPP in these experimental models (Fotinopoulou et al., 2005; Matsuda et al., 2005). However, an interesting observation is that while the TM region of AβPP is involved in binding to Bri2 (Fotinopoulou et al., 2005), the proSP-C BRICHOS domain and linker region are implicated in correct folding and incorporation of the metastable TM α-helix of SP-C into the membrane (Willander et al., 2012a). It is conceivable that the Bri2 linker together with its BRICHOS domain could have a similar role in incorporating the TM region of AβPP into the membrane. Bri3, which has been less extensively studied than Bri2, is expressed mainly in the brain and co-localizes with AβPP in neurites, and co-immunoprecipitates with BACE1 (β-amyloid converting enzyme 1). Similarly to Bri2, it is cleaved by furin (Wickham et al., 2005). Overexpression of Bri3 reduces cleavage of AβPP, and Bri3 knockdown by RNA interference results in increased levels of Aβ(Kim et al., 1999).

BRICHOS domains exhibit potent anti-amyloidogenic chaperone activity for Aβ peptides and are thought to protect these aggregation prone client peptide sequences from forming amyloid. The two homolog BRICHOS-containing proteins Bri2 and Bri3 are expressed in the human central nervous system (CNS) and are of particular interest in relation to AD. A range of studies suggests that BRICHOS domains can significantly reduce the level of toxic oligomeric amyloid species *in vivo*. For example, co-expression of $A\beta_{1-42}$ and proSP-C or Bri2 BRICHOS in the CNS of transgenic *Drosophila melanogaster* results in delayed $A\beta_{1-42}$ aggregation and dramatic improvements in both lifespan and locomotor function compared to flies expressing $A\beta_{1-42}$ alone (Hermansson et al., 2014; Poska et al., 2016). Results from transgenic mice overexpressing $A\beta_{1-42}$ as a fusion protein with the Bri2 protein support the possibility that BRICHOS prevents Aβ toxicity although it only delays amyloid fibril formation. In this study (Kim et al., 2013), the normal C-terminal peptide of Bri2, Bri23, was substituted for the $A\beta_{1-42}$ sequence, which resulted in generation of free $A\beta_{1-42}$ by proteolytic release. Surprisingly, these mice were not cognitively affected, even though they had high $A\beta_{1-42}$ expression and eventually developed amyloid plaques. The authors suggested that high $A\beta_{1-42}$ levels and aggregates are not sufficient to induce memory dysfunction, and that AβPP processing derivatives (which were obviously not generated from the Bri2-Aβ construct used) are contributing to the toxicity seen in AβPP transgenic mouse models (Kim et al., 2013). However, since the Bri2 BRICHOS domain has been shown to be released from its proprotein by proteolysis ((Martin et al., 2008), **Figure 4**), an alternative explanation for the lack of toxic effects seen in the Bri2- $A\beta_{1-42}$ expressing mouse model (Kim et al., 2013) could be that liberated BRICHOS domain delays Aβ aggregation and prevents toxicity, in a manner similar to that seen in the fly model. This possibility is further supported by the fact that the Bri2-Aβ expressing mice formed fewer Aβ oligomers than AβPP expressing mice (Kim et al., 2013), a finding which is difficult to explain by the absence of AβPP processing products, but which fits well with the BRICHOS mechanism of action that involves dramatic reduction in oligomer formation (see below).

Studies of the kinetics of *in vitro* Aβ aggregation in the presence or absence of recombinant proSP-C or Bri2 BRICHOS show that sub-stoichiometric amounts of BRICHOS domain significantly slow down fibril formation. It was demonstrated that BRICHOS inhibits the formation of $A\beta_{1-42}$ oligomers by binding to Aβ fibrils and suppressing surface catalyzed secondary nucleation (Cohen et al., 2015; **Figure 6**). This redirects the reaction pathway toward elongation events, thus minimizing the level of toxic Aβ intermediates. ProSP-C BRICHOS binds to Aβ fibrils with low nM affinity but does not bind to Aβ monomers (Cohen et al., 2015). From the X-ray structure of proSP-C BRICHOS (**Figure 2**) a mechanism by which BRICHOS domains may prevent amyloid formation by specifically targeting a β-hairpin structure was proposed (Willander et al., 2012a), but it remains to be determined how this mechanism could be applied in the context of binding to fibrils. The detailed mechanism(s) used by proSP-C and Bri2 BRICHOS domains to inhibit Aβ amyloid formation as well as non-fibrillar aggregation apparently differ (see below) and it is possible that BRICHOS exerts different effects toward different client peptides as well as depending on its location, i.e., whether it is intra- or extracellular.

The anti-amyloid activity of BRICHOS extends beyond the physiological client peptides; recombinant BRICHOS from proSP-C and Bri2 efficiently delay fibril formation, and more importantly $A\beta_{1-42}$ toxicity *in vivo* in the CNS of *Drosophila* (Hermansson et al., 2014; Poska et al., 2016). Endogenous Bri2 BRICHOS is found in amyloid plaques in human AD brains (Del Campo et al., 2014), BRICHOS binds with high affinity to $A\beta_{1-42}$ fibrils *in vitro* and this dramatically reduces the formation of toxic Aβ oligomers via a novel and specific mechanism. Taken together, available results suggest that BRICHOS binds to Aβ plaques, and thereby shuts down generation of toxic Aβ

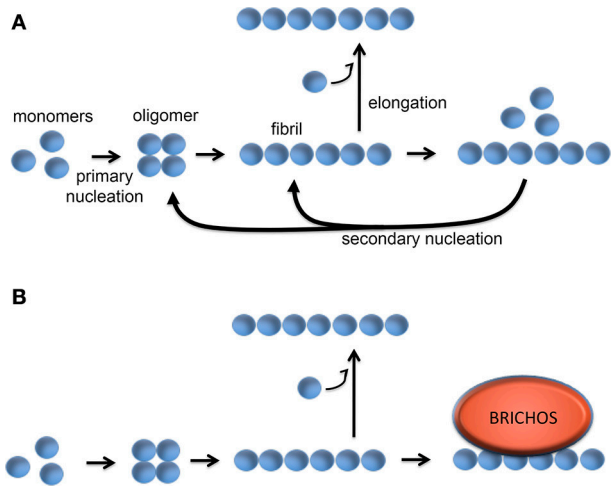

FIGURE 6 | Molecular mechanism of BRICHOS against Aβ42 fibril formation and toxicity. (A) Aβ42 forms oligomers via a primary nucleation reaction, which thereafter can convert into fibrils. The fibrils can be elongated via addition of monomers, while binding of Aβ monomers to the fibril surface can catalyze formation of oligomers via a secondary nucleation event. **(B)** BRICHOS domain binds to the surface of the Aβ fibrils and thereby markedly reduces the contribution of the secondary nucleation event to the formation of oligomers (Cohen et al., 2015). See text for details.

species, a mechanism that might be harnessed for AD treatment. The natural expression of proSP-C is restricted to the alveolar epithelium, which makes it an unsuitable to target for treatment of AD and hence ongoing efforts to target AD are mainly focused on the Bri2 BRICHOS, which is expressed in the CNS.

Bri2 is produced in several peripheral tissues and in the brain, with significant expression in neurons of the hippocampus and cerebellum in humans (Vidal et al., 1999; Akiyama et al., 2004). *In vitro*, Bri2 BRICHOS was found to be much more efficient than proSP-C BRICHOS against both $A\beta_{1-40}$ and $A\beta_{1-42}$ fibrillation, and even *in vivo* the Bri2 BRICHOS domain seems to inhibit $A\beta_{1-42}$ toxicity in *Drosophila* central nervous system or eyes more efficiently than pro-SP-C BRICHOS (Willander et al., 2012b; Poska et al., 2016). Bri2 BRICHOS not only blocks the secondary nucleation pathway during $A\beta_{1-42}$ fibrillation, but also affects the elongation process, which could be the reason why Bri2 BRICHOS is more efficient than proSP-C BRICHOS (Arosio et al., 2016). Moreover, proSP-C BRICHOS, like TTR, (*vide infra*) has low "general" molecular chaperone activity (traditionally defined as ability to prevent non-fibrillar aggregation of destabilized model substrate proteins), while Bri2 BRICHOS efficiently suppresses aggregation of destabilized proteins (Poska et al., 2016). Recombinant proSP-C BRICHOS is predominantly a trimer while Bri2 BRICHOS forms mainly large complexes (Poska et al., 2016), and therefore it was suggested that different quaternary structures mediate molecular chaperone and anti-amyloid activities, respectively, analogous to the situation for small heat shock proteins (sHSPs) (Roman et al., 2016). BRICHOS, like many other molecular chaperones, is apparently "stored" in an inactive form in which the binding surface is buried, thus avoiding inadvertent interactions with non-clients. For proSP-C BRICHOS the inactive form is a homotrimer, and consequently, dissociation of the trimer into monomeric subunits should release active BRICHOS. Experimental data support this possibility; addition of low molecular mass ligands increases both the ratio of proSP-C BRICHOS monomer/trimer and the anti-amyloid activity (Biverstål et al., 2015). Bri2, in contrast to proSP-C, BRICHOS forms mainly polydisperse oligomers but also monomers and dimers are found, and it remains to be established which species mediate the ability to prevent Aβ fibril formation and toxicity.

TTR INTERACTIONS WITH Aβ CONFORMERS AND ITS EFFECTS ON *IN VIVO* AND *IN VITRO* AMYLOIDOGENESIS

An *in vivo* relationship between TTR and AD was suggested when it was shown that TTR in the CSF could bind Aβ. It was the third CSF protein to display this activity, the other two being ApoE and Clusterin (ApoJ) (Ghiso et al., 1993; Strittmatter et al., 1993; Schwarzman et al., 1994). Early *in vitro* studies indicated that TTR could inhibit the formation of Congo red binding fibrils by Aβ (Schwarzman et al., 2004). *In vivo* studies demonstrated that *C. elegans* transgenic for an Aβ construct driven by the unc-54 muscle promoter displayed abnormalities in motility which were abrogated when they were co-transfected with a TTR cDNA driven by the same promoter (Link, 1995). Tg2576 mice transgenic for a mutant human Aβ gene had increased TTR expression in the cerebral cortex and unilateral injection of an anti-TTR antibody resulted in increased deposition of Aβ on the ipsilateral compared with the contralateral side of injection (Stein et al., 2004). Most definitively, when APP23 AD model mice were crossed with mice over-expressing a wild type human TTR gene the behavioral and neuropathologic features of Aβ deposition were suppressed (Buxbaum et al., 2008). Cortical and hippocampal $A\beta_{1-40/42}$ deposits were reduced by 60–75%. The hyper-phosphorylation of Tau seen in the APP23 mice was diminished and the mice did not develop the defect in spatial learning seen in mice expressing only human Aβ. When the same APP23 mice were crossed with *Ttr* knockout mice amyloid lesions appeared at 4.5 months of age compared with 9 months in wild type APP mice. Similar findings were seen in a different Aβ transgenic strain that were hemizygous for the *Ttr* knockout, only with a somewhat less accelerated phenotype, suggesting a TTR gene dose effect (Choi et al., 2007). The development of pathology did not seem to be accelerated in the absence of *Ttr* in transgenic models that rapidly developed Aβ deposition (Doggui et al., 2010).

In parallel studies it was shown that 70% of cortical and hippocampal neurons in human AD brains (vs. 10% in brains of age matched non-demented subjects) stained with an antibody specific for TTR as did virtually all the cortical and hippocampal neurons in the APP23 mice. TTR was also noted to be present in the Aβ plaques and in vessel walls that contained Aβ deposits. AβTTR-Aβ complexes were isolated from some human AD brains and the brains of the APP23 mice (Li et al., 2011).

MRNA analysis of cultured primary neurons from APP23 mice revealed that the TTR staining was a function of increased neuronal synthesis not uptake from the extracellular space (Li et al., 2011). This was the first demonstration of TTR synthesis by primary neurons and that neuronal TTR transcription was increased in the presence of a human AβPP gene. Chromatin immunoprecipitation (ChIP) studies showed that in neurons (in contrast to hepatocytes) TTR is up-regulated by the general stress response regulator Heat Shock Factor 1 (HSF1) which binds to specific sequences in both the human and mouse promoter region (Wang et al., 2014). The parallel increases in transcription of HSP's 40, 70, and 90, supported the notion that TTR behaves as a neuronal stress protein. Detailed molecular studies of CHO cells stably transfected with either wild type or mutant human AβPP genes, then transfected with a human TTR construct, revealed that TTR also bound to the intact APP protein and the C99 β-secretase cleavage product. The latter interaction (between the T4 binding pocket of TTR and amino acids A665, T668, and G659 of C99) resulted in reduced Aβ in the culture medium presumably because TTR binding interfered with the γ-secretase cleavage required for the generation of Aβ either because of an allosteric effect (as suggested for Bri2, *vide supra*) or the reduction in phosphorylation of T668 (Li et al., 2016).

Several laboratories have studied the interaction of TTR with Aβ *in vitro* in attempts to define the biophysical basis of its apparent salutary effect *in vivo*. The earliest studies of the effect of various TTR variants on *in vitro* Aβ aggregation were somewhat difficult to interpret because the only assay utilized was the inhibition of Congo red binding and the puzzling result that some TTR variants actually seemed to enhance Aβ aggregation as measured in this assay (Schwarzman et al., 2004). Since the molecular nature of the starting Aβ material was not stringently analyzed the nature of the interaction could only be hypothesized. Subsequent studies of the inhibition of red blood cell lysis and neuroblastoma cell apoptosis by a highly aggregation prone (but not naturally occurring) sub-fragment of $A\beta_{25-35}$ by TTR also lacked biophysical or structural studies examining the nature of the interaction (Giunta et al., 2005).

The first rigorous biophysical analysis of the interaction utilized TTR isolated from human plasma and a synthetic form of $A\beta_{1-40}$ and showed that TTR sub-stoichiometrically slowed the rate of Aβ aggregation. A mathematical model suggested that TTR both slowed protofibril elongation and the lateral association of protofilaments to produce fibrils. The authors hypothesized that TTR interacted with aggregated rather than monomeric Aβ (Liu and Murphy, 2006). Subsequent studies comparing the effects of recombinant TTR and TTR isolated from plasma were reported as showing that the plasma protein slowed aggregation but did not inhibit cytotoxicity while the recombinant protein increased aggregation and was an effective inhibitor of cytotoxicity. The investigators attributed the differences in activity to the previously described sulfonylation of Cys10 in the plasma protein that was not seen in the recombinant molecule, however there are other possible explanations and a clear explanation is not yet available (Liu et al., 2009).

Other investigators used ^{125}I-labeled recombinant TTR to study the interaction between TTR and $A\beta_{1-42}$ in a competition binding assay (Costa et al., 2008a). In contrast to the earlier work they reported that TTR binding was similar with Aβ monomers, oligomers, and fibrils. They also reported that binding of different TTR variant tetramers to Aβ was proportional to the stability of the tetramers, findings that were not consistent with the results of studies from other laboratories examining the capacity of tetramers of different stabilities to inhibit fibril formation, suggesting that binding and the inhibition of aggregation may not be directly related (Du and Murphy, 2010; Li et al., 2013). The same group reported that TTR could behave as a disaggregase of Aβ oligomers and fibrils via its intrinsic cryptic protease activity, an observation that has not been reproduced by others (Costa et al., 2008b; Li et al., 2013).

Several laboratories have shown that recombinant M-TTR bearing mutations (F87ML110M) that do not allow it to form stable tetramers is a very efficient inhibitor of Aβ aggregation, appearing to interact with Aβ oligomers in a sub-stoichiometric fashion. This coupled with the observation that the ability to inhibit Aβ fibril formation was inversely related to tetramer (Du and Murphy, 2010; Li et al., 2013) stability suggested the hypothesis that tetramer dissociation was required for inhibition of Aβ aggregation, a similar scenario has been shown for BRICHOS, for which trimer dissociation into monomers increased the ability to prevent Aβ fibril formation (Biverstål et al., 2015). However, the ability of highly stable variant TTR tetramers such as TTR T119M and TTR K15A and murine TTR (which do not dissociate under the conditions of the *in vitro* experiments) to inhibit fibril formation indicates that tetramer dissociation is not required to inhibit Aβ fibril formation (Li et al., 2013). Further, although the monomer is a more potent inhibitor of fibrillogenesis *in vitro, in vivo* TTR tetramer concentration is one thousand fold that of the monomer hence unless there is a biologic compartment enriched in monomeric TTR and Aβ, it is likely that the tetramer is the active inhibitor *in vivo* (Sekijima et al., 2001, **Figure 7**).

Cross-linking and alanine scanning mutagenesis suggested that TTR strand G and the EF helix and amino acids L17, L82, S85, and L110 were directly involved in Aβ binding (Liu et al., 2009; Du et al., 2012). More detailed precise analysis of binding using nuclear magnetic resonance spectrometry confirmed the involvement of amino acids L17 and L110 but not L82 and S85 (Li et al., 2013). Almost 20 amino acids showed resonance shifts when TTR interacted with the Aβ monomer all located in and around the T4 binding site, suggesting that this region behaves as a predominantly hydrophobic pocket reactive with small T4-like ligands and hydrophobic stretches of some proteins (Li et al., 2013). These data were reinforced by experiments showing that tafamidis, a resveratrol related molecule with high affinity for the T4 binding site, inhibited the inhibition of fibril formation by TTR tetramers but had no effect on the inhibition of fibrillogenesis by the engineered monomeric TTR, which does not form a T4 binding pocket (Johnson et al., 2012).

Isothermal titration calorimetric (ITC) analysis of the interaction between WT TTR tetramer and the $A\beta_{1-40}$ monomer (controlled for the possible self-aggregation of the reactants) had a K_D in the micromolar range and a stoichiometry of 1, differing considerably from those reported using other techniques (Li

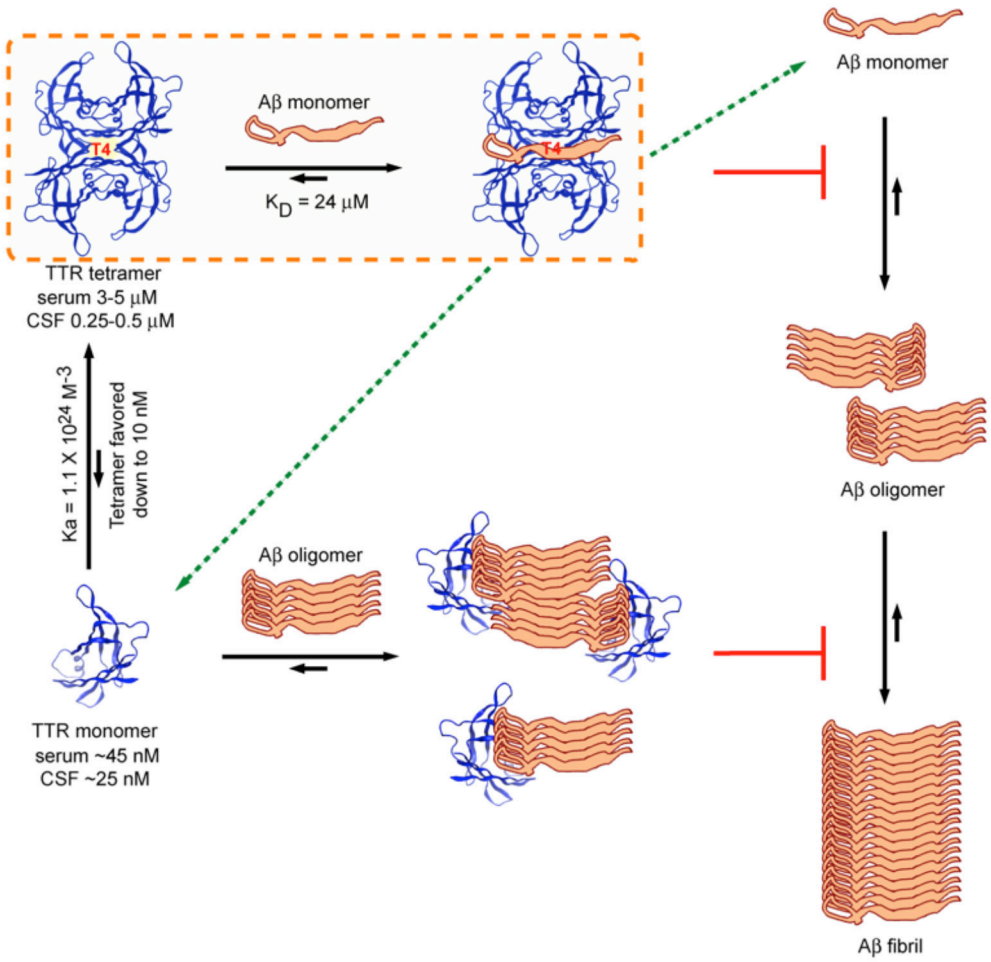

FIGURE 7 | Model of Interactions between TTR (tetramer) and $A\beta_{1-40/42}$ and M-TTR (engineered monomer) and $A\beta_{1-40/42}$. The tetramer clearly binds Aβ monomers and pulls them out of the aggregation reaction. It is also capable of binding oligomers and fibrils. M-TTR primarily binds oligomers. What is not clearly shown here is that it is likely that M-TTR is in an oligomeric state when it engages the Aβ oligomers (from Li et al., 2013).

et al., 2013). Interestingly the stoichiometry and K_D of the interaction between the human and mouse tetramers and the Aβ monomer were very similar even though the human protein was a much better inhibitor of fibrillogenesis, suggesting, as did other data, that binding and inhibition of fibril formation are not equivalent. The stoichiometry of interaction between tetrameric human TTR and Aβ monomers (approximately 1:1) was much higher than that determined for the inhibition of fibril formation by TTR tetramers (sub-stoichiometric,1:40–1:100) indicating that in the latter experiments it was likely that TTR also interacted with oligomeric species (**Figure 7**).

The TTR binding site of Aβ involved the 4G8 epitope, i.e., amino acids 17–21 (Li et al., 2013). Based on western blots, which show interaction of oligomeric M-TTR with Aβ tetramers and octamers, NMR, which showed no resonance shifts on MTTR-Aβ monomer interaction and ITC, which did not detect heat gain or release on incubation of MTTR with monomeric Aβ, the interaction of the recombinant monomeric MTTR appears to require oligomerization of the monomer prior to an interaction with Aβ oligomers rather than the Aβ monomer in a sub-stoichiometric fashion. Similar observations have been made examining the interaction between M-TTR and $A\beta_{1-42}$ using fluorescence correlation spectroscopy (Verghese et al., 2013). It is possible that some of the discrepancies in results between the early studies and the most recent work with respect to the efficacy of inhibition of Aβ by the various mutant TTR tetramers reflects the presence of variable amounts of TTR oligomers formed from monomers generated during the period of incubation of Aβ with different TTR preparations. It was also quite evident that binding studies using conformers bound to fixed surfaces gave different results than experiments performed in liquid phase, e.g., M-TTR bound $A\beta_{1-42}$ monomers fixed to nitrocellulose but no interaction was seen in the liquid phase NMR or ITC experiments (Li et al., 2013).

A series of experiments examining the mechanism of inhibition of $A\beta_{1-40/42}$ cytotoxicity by TTR using both pre-formed $A\beta_{42}$ and HypF-N (a model amyloidogenic bacterial

protein that behaves similarly to $A\beta_{42}$) cytotoxic aggregates compared the inhibitory effects of various TTR conformers and showed that M-TTR was the best with human tetramer being less effective and the murine tetramer the least inhibitory, a rank order similar to that seen with inhibition of fibril formation as an assay (Cascella et al., 2013). More interesting was the demonstration that the various TTR species all worked by interacting with the toxic HypF-N oligomeric aggregates to make them larger and less toxic. In a recent follow up analysis the inhibitory process utilized by M-TTR was compared with that seen in the inhibition of HypF-N cytotoxicity by the extracellular chaperone clusterin and the small heat shock protein αB-crystallin (Cappelli et al., 2016). Like the chaperones M-TTR increased oligomer size and reduced the structural order of the aggregates. These data with HypF-N and correlation spectroscopy results with $A\beta_{1-42}$ strongly suggest that M-TTR does not inhibit aggregation, rather it changes the nature of the aggregates allowing them to supersaturate the solution forming non-fibrillar, non-toxic structures. Interestingly while clusterin and the other "extracellular chaperones" haptoglobin and α_2 macroglobulin also inhibit non-fibrillar protein aggregation, TTR (like proSP-C BRICHOS *vide supra*) only inhibits amyloidogenesis. The mechanism of the latter may reflect the fact that fibrillogenesis requires precise homotypic alignment of β-strand structures ("stearic zippers") (Goldschmidt et al., 2010). It is possible that while pre-amyloid oligomers form internally homotypic aggregates, those formed by one precursor, e.g., TTR, are not precisely in register with those of the second, i.e., Aβ, perhaps forming "hetero-zippers" (Eisenberg, D, personal communication). The resulting heterotypic aggregates are less structured and incapable of attaining the order required for protofibril formation. This would be different in the case of pairs of amyloid precursors in which cross-seeding occurs in which the heteromeric oligomeric fit does not disrupt the parallel or anti-parallel β-sheet structures required for protofibril formation (Solomon et al., 2007; Oskarsson et al., 2015).

In summary tetrameric human TTR appears to inhibit Aβ aggregation by binding Aβ monomers and removing them from the fibril forming (elongation) pool. It also binds oligomers and fibrils contributing to the formation of larger, non-cytotoxic amorphous aggregates. Since the tetramer is far and away the most prevalent form of TTR *in vivo* it is likely that this represents a significant component of its protective effect in the mouse models. What happens to the TTR-Aβ complexes is unclear, perhaps as hypothesized elsewhere, it enhances Aβ transport out of the brain (Alemi et al., 2016). The recent data that it is also capable of decreasing Aβ production (at least in cultured cells) suggests a possible additional mechanism.

While monomeric TTR may not be functionally protective *in vivo* the comparison of the biophysics of its interaction with Aβ and HypF-N with that seen in molecules long known to be members of the proteostatic network, i.e., clusterin and αB-crystallin has given some insight into how those molecules may function *in vivo*.

FIGHTING FIRE WITH FIRE: DO AMYLOID PRECURSORS HAVE THERAPEUTIC POTENTIAL IN AD?

From the forgoing it is evident that BRICHOS domains and TTR tetramers and monomers can inhibit Aβ oligomerization, fibril formation and cytotoxicity *in vitro*. It also appears that genetically induced over-expression of the two parent molecules can suppress or delay the eventual development of the neuropathologic and behavioral abnormalities seen in transgenic fly or mouse models of human Aβ deposition (**Table 1**). At this moment such genetic approaches are not possible in human AD. However, it is likely for TTR at least, that human neurons may already be utilizing this molecule as a defender of neuronal integrity in the face of proteotoxic and perhaps other forms of cellular stress. As noted above, the majority (70%) of neurons in human AD brains make TTR, compared with 10% in age matched non-demented control brains, a finding also seen in transgenic models of human Aβ deposition. Further both TTR and Bri2 have been found in human AD plaques. The latter may reflect either that they are behaving as co-conspirators or more likely is that those molecules represent failed (or successful, if the fibrils are the least toxic form of Aβ aggregates) chaperones. These observations pose the question does AD represent an age related failure of host neuronal defense mechanisms? If that is the case can the failure be overcome therapeutically? While current approaches to AD focus on reducing Aβ production, might we do better by enhancing host resistance or some combination of the two?

Gene Therapy

Direct administration of a *TTR* or BRICHOS containing gene to humans is currently not feasible, however it may become possible to isolate totipotent stem cells from subjects with AD, differentiate them to neurons or astrocytes, engineer them to contain a wild type human TTR or BRICHOS containing gene regulated by either their own or an inducible promoter and administer those cells to the patient from whom they were obtained. Currently there are many unknowns surrounding this approach. It assumes that a mode of administration will be available to insure that the cells reach the nervous system and home to regions of pathology. The gene must include all the regulatory sequences required for tissue specific expression and protein production in the secretory pathway so that the encoded protein will be available to interact with Aβ and its oligomers secreted by the endogenous neurons. If the gene is driven by an inducible promoter the inducing agent must be able to cross the blood brain barrier. Further the long term behavior of cells differentiated from pluripotential precursors is currently unclear.

Protein or Peptide Therapy

A large number of proteins including TTR and BRICHOS and peptides derived from them have been shown to inhibit Aβ aggregation *in vitro* (e.g., Mangrolia et al., 2016). Currently it is difficult to deliver these molecules to the central nervous system, although there have been some successes administering molecules such as insulin by nasal spray (Hölscher, 2014).

TABLE 1 | Alzheimer's disease related activities of Transthyretin and BRICHOS—containing proteins.

Feature	Transthyretin (TTR)	BRICHOS (Bri2)
Clinical amyloidosis human	FAP, FAC, SSA	FBD, FDD
Amyloid formation *in vitro*	+	+
Protein topology	Unique Homotetramer	Domain in many proteins
Interaction with Aβ *in vitro*	Monomers, oligomers, fibrils	+
Transgene interaction with AD model *in vivo*	suppresses	suppresses
Effect of knockout on AD model	accelerates	?
Presence human AD plaques	+	+
Effect of knockout on CNS function (no AD gene)	Behavioral abnormality	?
Increased neuronal synthesis AD	+	?
Inhibits primary nucleation Aβ	+	–
Inhibits secondary nucleation Aβ	?	+
Inhibits elongation Aβ	+	+

One way to circumvent the problems inherent in protein or peptide delivery has been to use Adenoviral, AAV or lentiviral vectors as carriers for sequences encoding the therapeutic protein or peptide (e.g., Kim et al., 2004; Bourdenx et al., 2014; Parr-Brownlie et al., 2015; Blessing and Déglon, 2016; Saraiva et al., 2016). Vectors have been developed that will preferentially target neurons although most still require intracerebral inoculation. This approach may be more appropriate for a BRICHOS based reagent than for a TTR related molecule since the quaternary structural demands of the TTR tetramer may be constraining with respect to having excess monomers available to misfold and aggregate rather than interact with some form of Aβ.

A third approach being explored is based on the observation that both systemic and neuronal TTR production go down with increasing age and the assumption that maintaining neuronal TTR production throughout life will continue to make TTR available to bind Aβ and its soluble aggregates in the environment in which they appear to be neurotoxic. If small molecules can be identified which specifically induce neuronal TTR (or BRICHOS- containing domain) synthesis and can cross the blood brain barrier after systemic administration, they might be able to slow or arrest the progression of neurodegeneration.

All of these approaches present practical problems with respect to delivery, specificity of the cellular and molecular targets and intrinsic amyloidogenicity of the therapeutic agent. In the case of small molecule therapeutics the potential for off-target or mechanism related toxicities is always an issue that cannot be ignored. The delivery and specificity issues are no different than they are for any therapeutic biological. The intrinsic amyloidogenicity of molecules as TTR or BRICHOS-containing proteins, whether encoded by a naked gene, produced by an engineered differentiated pluripotent stem cell or induced by a small molecule is a real risk. It would not be good to either enhance amyloid oligomer formation by endogenously produced Aβ or induce the synthesis of a sufficient amount of the therapeutic anti-amyloid to exceed the critical concentration required to nucleate its own fibrillogenesis. Based on observations in mice carrying many copies of the wild type human *TTR* gene with all the known elements required for tissue specific expression, it appears that neurons regulate TTR production quite tightly, perhaps precluding the possibility of local TTR aggregation and oligomer formation even while systemic amyloid deposition of liver synthesized TTR goes on (Buxbaum et al., 2008).

In recent years much has been made of the phenomenon of "templated misfolding" as a mechanism for spreading of both Parkinson's and Alzheimer's diseases (Walker et al., 2006; Kordower et al., 2008). It has also been possible to "seed" Aβ aggregation in mice and rats by the intracerebral or parenteral administration of homologous brain or fibril fragments (Brouillette et al., 2012; Heilbronner et al., 2013). In at least one instance the seeding has been inhibited by pre-incubation with human TTR (Brouillette et al., 2012).

In other systems it has also been possible to nucleate murine AA amyloid *in vivo* by the administration of other amyloid aggregates raising the notion of cross species seeding by the ingestion of Foie gras (Solomon et al., 2007). We have described two examples in which two discrete human amyloid precursors rather than "cross seeding" inhibit the formation of cytotoxic Aβ aggregates and fibrils *in vitro* and *in vivo* in transgenic murine models of human Aβ deposition. Further human data suggest that the production of these molecules is increased in the course of human AD, perhaps in the context of neuronal defense. We have reviewed the structural features of these molecules that appear to be responsible for the "protective" heterotypic interactions that prevent the homotypic formation of toxic oligomers and fibrils *in vivo* and speculate that these interactions may not be coincidental but represent an evolutionarily conserved mode of neuroprotection.

AUTHOR CONTRIBUTIONS

JJ wrote the sections on the BRICHOS proteins. JB wrote the sections on transthyretin. Both contributed to the introduction and the potential therapeutics sections and both edited and reviewed the entire manuscript.

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REVIEW
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GRP78 at the Centre of the Stage in Cancer and Neuroprotection

Caty Casas *

Department of Cell Biology, Physiology and Immunology, Institut de Neurociències, Universitat Autònoma de Barcelona, Barcelona, Spain

The 78-kDa glucose-regulated protein GRP78, also known as BiP and HSP5a, is a multifunctional protein with activities far beyond its well-known role in the unfolded protein response (UPR) which is activated after endoplasmic reticulum (ER) stress in the cells. Most of these newly discovered activities depend on its position within the cell. GRP78 is located mainly in the ER, but it has also been observed in the cytoplasm, the mitochondria, the nucleus, the plasma membrane, and secreted, although it is dedicated mostly to engage endogenous cytoprotective processes. Hence, GRP78 may control either UPR and macroautophagy or may activated phosphatidylinositol 3-kinase (PI3K)/AKT pro-survival pathways. GRP78 influences how tumor cells survive, proliferate, and develop chemoresistance. In neurodegeneration, endogenous mechanisms of neuroprotection are frequently insufficient or dysregulated. Lessons from tumor biology may give us clues about how boosting endogenous neuroprotective mechanisms in age-related neurodegeneration. Herein, the functions of GRP78 are revealed at the center of the stage of apparently opposite sites of the same coin regarding cytoprotection: neurodegeneration and cancer. The goal is to give a comprehensive and critical review that may serve to guide future experiments to identify interventions that will enhance neuroprotection.

Keywords: GRP78, BiP, neuroprotection, endogenous mechanisms, neurodegeneration, ER stress, autophagy, ERAD

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Toru Hosoi,
Hiroshima University, Japan
Rafael Linden,
Federal University of Rio de Janeiro, Brazil

***Correspondence:**
Caty Casas
caty.casas@uab.cat

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Several systems, including the nervous system, have a remarkable ability for repair under stressful conditions. Conserved intrinsic mechanisms counteract damaging effects of endogenous and/or exogenous toxic agents. Under circumstances of damage, intrinsic pro-survival pathways, that collectively are termed endogenous neuroprotective mechanisms, are activated. Endogenous protective mechanisms have been mainly investigated in diverse pathological states such as vascular diseases, trauma, and cancer. The question of why neurodegenerative diseases occur even when beneficial mechanisms have been triggered deserves in-depth analysis. GRP78 appears to orchestrate several of these endogenous mechanisms. We herein describe the characteristics and known functions of GRP78, explore its roles in tumor cell survival, proliferation, and chemoresistance and reflect on how this knowledge should guide investigations into its functions in neuroprotection.

GRP78, A VERY IMPORTANT PROTEIN WITH MULTIPLE FUNCTIONS IN MULTIPLE LOCATIONS

Transcriptional and Post-translational Regulation of GRP78 Levels

GRP78 has multiple functions in maintaining cell viability. Its expression is highly regulated at different points. At the transcription level, GRP78 is encoded by the gene *Hsp5a*. It is the most abundant protein within the heat shock protein-70 (Hsp70) family, but, unlike the other members of this family, it is not induced by heat shock because the promoter of *GRP78* lacks the heat shock element. Levels of GRP78 are maintained at relatively low levels within the cell and are increased considerably under stresses that affect the endoplasmic reticulum (ER) and calcium homeostasis. Indeed, GRP78 was initially discovered in 1977 as a 78-kDa protein strongly induced in chicken embryo fibroblasts cultured in glucose-free medium (Shiu et al., 1977). Later, it was observed that *GRP78* expression can be induced by other stimuli such as calcium ionophore A23187 (Resendez et al., 1985), calcium depletors or chelators such as thapsigargin and BAPTA-AM (Suzuki et al., 1991), and inhibitors of the protein secretory pathway such as tunicamycin (Lee, 1987). The upregulation of GRP78 expression under such a variety of stressful stimuli is mainly due to the presence of conserved elements in the promoter of the *Hsp5a* gene (Li and Lee, 2006) such as a CCAAT box (Resendez et al., 1988), a cAMP responsive element CRE-like (CREB; Alexandre et al., 1991), and the ER stress response element (ERSE; Resendez et al., 1988). Transcription factors that bind to these regulatory elements, including CBF/NF-Y (Roy and Lee, 1995), CREB, activating transcription factor 2 (ATF-2; Chen et al., 1997), YY1, YB1, Sp1 (Li et al., 1997), ATF4 (Luo et al., 2003), TFII (Parker et al., 2001), ATF6 (Yoshida et al., 2001b), and XBP1 (Yoshida et al., 2001a), participate in the regulation of *Hsp5a* gene (**Figure 1**).

The post-transcriptional regulation of GRP78 is mediated by the activation of internal ribosome entry sequence (IRES) in the 5′ untranslated region of *GRP78* mRNA (Macejak and Sarnow, 1991). IRESs are often present in mRNAs that encode proteins crucial for cell survival and stress recovery. Thus, in circumstances where repression of global protein synthesis is promoted, *GRP78* mRNA is selectively translated (Yang and Sarnow, 1997). In some situations, the presence of the IRES serves to amplify translation of *GRP78* mRNA. For instance, after infection of foreskin fibroblasts with human cytomegalovirus, activation of the *GRP78* IRES by the viral machinery results in a 3–4-fold increase of at the mRNA level but about a 50-fold increase at the protein level (Buchkovich et al., 2010). Other viral infections, including herpes simplex virus type 1 and poliovirus, have also been reported to activate the *GRP78* IRES (Kim et al., 2001; Saffran et al., 2010). Several cellular proteins are implicated in the translational activation of the GRP78 IRES including NS1-associated protein NSAP1, SSB/La autoantigen, p50, and p95 (Yang and Sarnow, 1997; Kim et al., 2001; Cho et al., 2007; **Figure 1**).

Another post-transcriptional regulatory mechanism acts on protein stability. It has been shown that activation of PI3K/AKT pathway in ER-stressed HEK-293 cells leads to an increase in GRP78 protein stability through unknown mechanisms (Dai et al., 2010). Regulation is also mediated through the action of specific microRNAs (miRNAs) such as miR-181 (Ouyang et al., 2012), miR-181a (Ji et al., 2017), miR-181b (Peng et al., 2013), miR-376a (Iwamune et al., 2014), and miR-30a (Wang P. et al., 2015) that bind to the *GRP78* mRNA 3′-untranslated region (**Figure 1**).

GRP78 Localization Reflects Multiple Functions

GRP78 acts as a molecular chaperone (Haas and Wabl, 1983) and binds to nascent polypeptides. Like cytosolic HSP70, it contains an N-terminal ATPase domain and a C-terminal peptide binding domain (Määttänen et al., 2010). GRP78 is also a calcium binding protein. It is inhibited by a high concentration of calcium ions, and its ATPase activity is activated by calcium depletion. Due to the presence of an ER signaling peptide, GRP78 is mainly found in the ER lumen, although under some circumstances it is redistributed to the cytosol, nucleus, mitochondria, or the plasma membrane or is secreted (Suzuki et al., 1991). Thus, different locations prime GRP78 to trigger different molecular signaling events.

GRP78 Multifunction Associated with the Endoplasmic Reticulum

At the ER, GRP78 has diverse functions and relies on a number of interaction partners and co-chaperones, nucleotide exchange factors, and signal transducers for its various activities. The diversity of functions include translocating nascent polypeptides, facilitating *de novo* protein folding and assembly, targeting misfolded proteins to endoplasmic-reticulum-associated protein degradation (ERAD) machinery, and maintaining calcium homeostasis (since it is as a luminal calcium ER binding protein; Gardner et al., 2013). GRP78 is usually the first chaperone to bind a nascent polypeptide chain and prefers to bind surfaces with alternating aromatic and hydrophobic amino acids. GRP78 shifts to its tighter affinity substrate binding conformation after ATP

Abbreviations: GRP78, 78-kDa glucose-regulated protein; UPR, unfolded protein response; ER, endoplasmic reticulum; PI3K, phosphatidylinositol 3-kinase; Hsp70, heat shock protein-70; ERSE, ER stress response element; CREB, cAMP responsive element CRE-like; IRES, internal ribosome entry sequence; NSAP1, NS1-associated protein; miRNA, microRNA; ERdj, ER resident J-domain co-chaperones; ERAD, endoplasmic-reticulum-associated protein degradation; AMPK, AMP-activated protein kinase; IRE1, inositol-requiring enzyme 1; ATF, activating transcription factor; PERK, protein kinase R-like endoplasmic reticulum kinase; XBP1, X-box binding protein; Sig1R, sigma receptor 1; IP_3R, inositol trisphosphate receptor; KDEL, carboxyl-terminal ER-retention signal; OxPAPC, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; α2-M, α2-macroglobulin; CPT1a, carnitine palmitoyltransferase 1a; ROS, reactive oxygen species; ApoE, apolipoprotein E; SREBP1-c, sterol regulatory element-binding protein 1c; MAM, mitochondria-associated ER membrane; SNc, substantia nigra pars compacta; SOD1, superoxide dismutase 1; AD, Alzheimer's disease; sCJD, sporadic Creutzfeldt-Jakob disease; vCJD, variant CJD; BSE, bovine spongiform encephalopathy; PD, Parkinson's disease; PrP^{Sc}, pathological prion protein; IPC, ischemic preconditioning; PrP^{C}, prion protein; ALS, amyotrophic lateral sclerosis; FAD, autosomal-dominant familial AD; PS, presenilin; APP, amyloid beta precursor protein; Aβ, amyloid-β peptides; α-syn, α-synuclein.

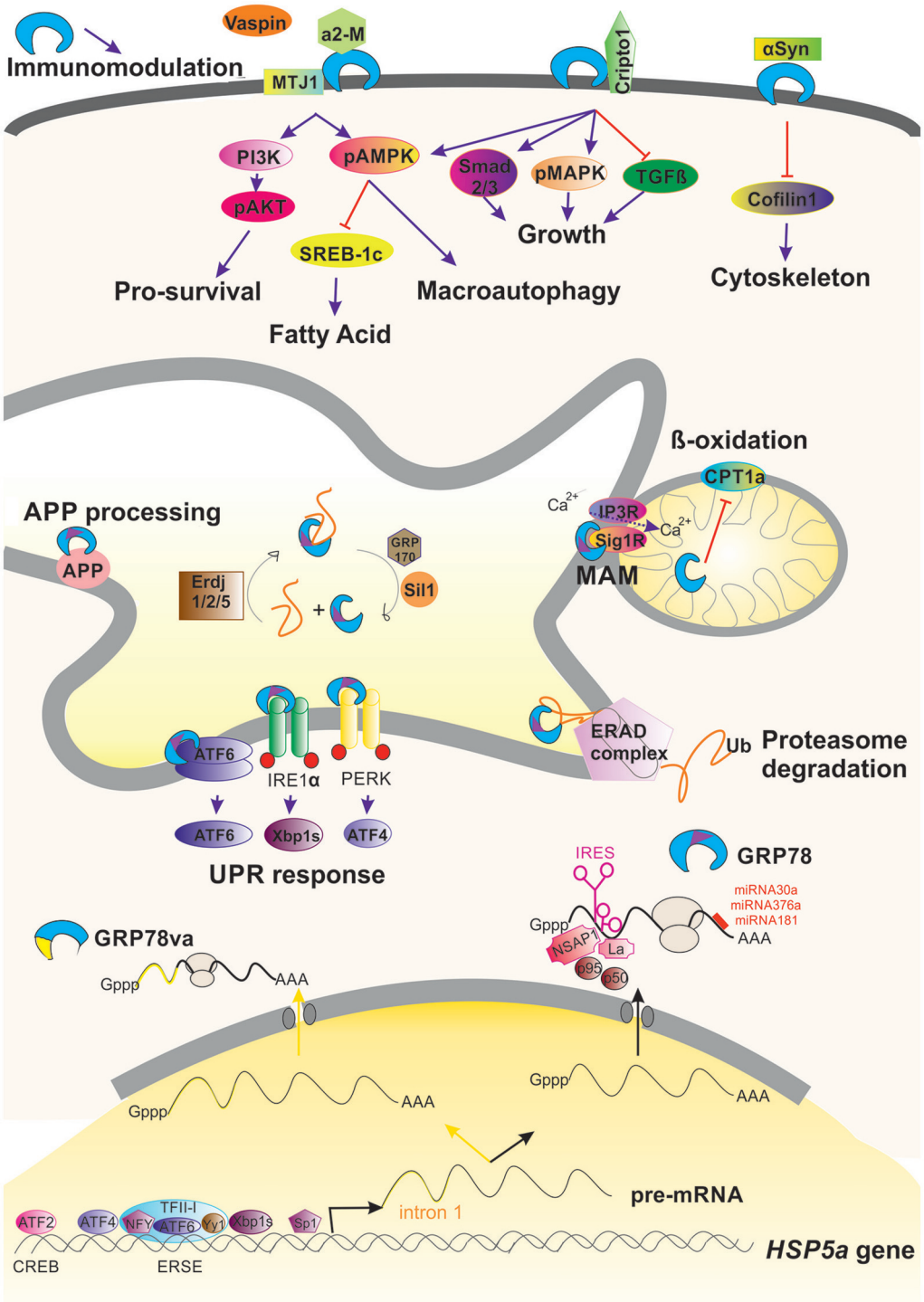

FIGURE 1 | Graphical summary of the regulation and activities promoted by GRP78 within a cell. Induction and regulation of the transcription of the *HSP5a* gene is mediated by several transcription factors that bind to ERSE or CREB motifs in the promoter of the gene. Alternative processing of its pre-mRNA can occur under stressful conditions leading to retention of intron 1 (yellow line) that advance an stop codon, giving to GRP78va truncated protein that is retained in the cytosol because it lacks the ER-signaling motif (purple triangle). Commonly processing GRP78 is submitted under post-transcriptional regulation either due to the action of factors on its IRES motif or by the action of different miRNAs. GRP78 is found mainly in the luminal ER where it can promote the activation of the UPR, ERAD, or MAM regulation. In some circumstances, GRP78 can be translocated to the cell surface where it can interact to multiple partners and hence modulate different pathways. It is also be secreted where it can immunomodulate.

hydrolysis to ADP (Blond-Elguindi et al., 1993). Several partners participate in this process. The hydrolysis of ATP by GRP78 is stimulated by ER resident J-domain co-chaperones (ERdj), ERdj1 and 2, homologs of yeast Sec63 (Otero et al., 2010), and also to co-chaperones such as P58(IPK) (Tao and Sha, 2011). In addition, the ADP-bound closed state of GRP78 is re-opened by exchange of ADP for ATP, and this process is enhanced by the nucleotide exchange factors GRP170 and Sil1, also known as BiP-associated protein (BAP; for a review see Määttänen et al., 2010; **Figure 1**).

Newly synthesized proteins in the ER are subjected to a rigorous quality control system and misfolded proteins are retrotransported back into the cytoplasm to be degraded by the ubiquitin-proteasome system. GRP78 associates with nascent chains immediately and for properly folded proteins, transiently upon synthesis. However, its association with misfolded or mutant proteins is prolonged (Sörgjerd et al., 2006). This prolonged association might be a signal for degradation of the bound protein (Petrova et al., 2008). The multistep process of ERAD, is initiated by GRP78 and other ER-resident chaperones that recognize the misfolded protein. Together, these chaperones facilitate deglycosylation and disassembling of misfolded proteins. The chaperones drive substrates to the translocon channel where they are pulled out of the membrane by a complex of proteins with ATPase activity. The emerging substrate is most likely ubiquitinated and addressed to the proteasome for degradation (review in Printsev et al., 2016; **Figure 1**). ERAD in combination with the ubiquitin-proteosome system (UPS) is thought to be the mechanism for quality control in long-lived cells such as neurons; hence, GRP78 is likely a critical component of the endogenous neuroprotective program.

Evidence from studies carried out in yeast indicates that when the ERAD system is saturated, macroautophagy removes both soluble and aggregated forms of unfolded proteins and dysfunctional organelles. Macroautophagy can be induced by various forms of cellular stress including nutrient or growth factor deprivation, hypoxia, reactive oxygen species, DNA damage, protein aggregates, damaged organelles, or intracellular pathogens (Klionsky et al., 2016). GRP78 plays a role in autophagic protein quality control, participating in the destruction of misfolded proteins in the cytosol. The autophagic process can be roughly divided into three steps: autophagosome formation, autophagosome-lysosome/late endosome fusion (autophagosome maturation), and degradation. The formation of autophagosomes necessitates the concerted and sequential action of autophagy related (ATG) proteins, originally identified in yeast (Itakura and Mizushima, 2010; Klionsky et al., 2016).

ATG proteins are regulated by conserved nutrient and energy-dependent signaling cascades that crucially involve the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase belonging to the phosphatidylinositol kinase-related (PIKK) family, and AMP-activated protein kinase (AMPK). Starvation, amino acid deprivation, and growth factor withdrawal inhibit mTOR activity and lead to autophagy induction. AMPK is a major positive regulator of autophagy that is activated by low ATP availability (Kroemer et al., 2010). Both mTOR and AMPK control the cascade of events leading to the activation of the phosphatidylinositol 3-kinase class III (PI3KC3 also known as VPS34; Russell et al., 2013). PI3KC3, together with beclin 1, p150, and ATG14L, translocates to the initiation site of autophagosome formation (Matsunaga et al., 2010). At the ER, PI3KC3-mediated phosphatidylinositol 3-phosphate production (Axe et al., 2008; Hayashi-Nishino et al., 2009) fosters the formation of the phagophore. The phagophore sequesters cargo before closing in on itself to form the autophagosome. Phagophore expansion requires the conjugation of microtubule-associated protein 1A/1B-light chain 3 (LC3) to phosphatidylethanolamine, a process also called LC3 lipidation (Kabeya et al., 2000; Hamasaki et al., 2013). The LC3-positive autophagosome sequesters cytoplasmic material by binding to sequestosome 1 SQSTM1/p62. The autophagosome then fuses with an endosome or lysosome for cargo breakdown, and the degraded material is transported to the cytoplasm. SQSTM1/p62 binds LC3 and recruits proteins into autophagosomes for final degradation by lysosomal hydrolases.

GRP78 acts on the autophagic process at several points. Evidence for a role in the initiation and formation of the autophagosome is based on the finding that GRP78 overexpression increases autophagic signaling by stimulating AMPK (Cook and Clarke, 2012; Wen et al., 2012; **Figure 1**). In addition, GRP78 can interact to VPS34 and GRP78 overexpression activates the Class III PI3K-mediated autophagy pathway (Li et al., 2015). When GRP78 expression is inhibited, AMPK signaling activation does not occur (Cook and Clarke, 2012) and formation of autophagosomes is blocked (Li et al., 2009), although GRP78 deficiency does not prevent LC3 lipidation. GRP78 also acts at the final steps of macroautophagy since GRP78 binds to misfolded proteins and to SQSTM1/p62 in cells under stress. GRP78 binding induces a conformational change in SQSTM1/p62 that favors cargo delivery into the autophagosome for its subsequent degradation into amino acids (Jin et al., 2014; Kim et al., 2014; Abdel Malek et al., 2015; Cha-Molstad et al., 2015, 2016). Thus GRP78 acts as a chaperone for aggregation-prone misfolded proteins leading to their degradation by macroautophagy.

Macroautophagy is a pro-survival mechanism activated within the cell under stressful conditions. As it does in macroautophagy, GRP78 has a role in another cytoprotective process, the unfolded protein response (UPR) as well GRP78 (Paschen, 2004). The UPR is well-conserved from yeast to mammalian cells. Impaired processing and folding reactions that lead to an accumulation of misfolded proteins or potentially toxic aggregates, ATP depletion, and disturbances in calcium homeostasis, produce ER stress and UPR activation. To cope with ER stress, UPR activation coordinates the increase in ER-folding capacity through a broad transcriptional upregulation of ER folding, lipid biosynthesis, and ERAD machinery components with a decrease in folding load through selective mRNA degradation and translational repression (Gardner et al., 2013). GRP78 orchestrates the UPR by functionally regulating three ER transmembrane proteins that act as the main effectors: inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF-6), and protein kinase R-like endoplasmic reticulum kinase (PERK; Schröder and Kaufman, 2005; Wang and Kaufman,

2016). GRP78 binds to IRE1, PERK, and ATF6 in unstressed cells and dissociates from these UPR sensors during acute ER stress (Bertolotti et al., 2000; Okamura et al., 2000; Shen et al., 2002; **Figure 1**).

IRE1 can also be directly activated by binding to unfolded proteins. Although ligand-induced oligomerization activates IRE1 (Shamu et al., 1994), GRP78 association stabilizes the inactive, monomeric form of IRE1 preventing its over response to low levels of ER stress (Korennykh et al., 2009; Pincus et al., 2010; Gardner and Walter, 2011). The RNase activity of IRE1 generates spliced mRNA encoding the X-box binding protein (XBP1), and XBP1 protein upregulates the expression of GRP78. ATF6 is cleaved by site 1 protease (S1P) and site 2 protease (S2P) to generate a p50-ATF6 fragment that has transcriptional activity. Upon cleavage, the p50-ATF6 fragment upregulates the expression of GRP78 through an ERSE in the promoter region of the *GRP78* gene as mentioned above. PERK has a kinase domain that phosphorylates the translation factor eIF2a, thereby suppressing most of the *de novo* protein synthesis during ER stress but stimulating the translation of certain mRNAs, including that encoding ATF4.

All of these processes are necessary to attenuate the accumulation of unfolded proteins during ER stress. IRE1 and ATF6 are especially critical in the prevention of ER stress-induced apoptosis via their upregulation of GRP78 expression (Gardner et al., 2013). Prolonged activation of IRE1 and CHOP can trigger apoptosis in cells under certain physiologic and pathophysiologic conditions (Szegezdi et al., 2006). In normal physiology, UPR-induced apoptosis may be a means to eliminate the few cells in an ER-stressed environment that remain uncorrected despite the actions of the UPR. Overexpression and antisense approaches in cell systems show that GRP78 can protect cells against cell death caused by disturbance of ER homeostasis (Morris et al., 1997; Yu et al., 1999; Jeon et al., 2016). Overexpression of GRP78 attenuates ER stress, both by enhancing protein folding and by helping to maintain IRE1, ATF6, and PERK in their inactive states (Bertolotti et al., 2000; Laybutt et al., 2007) and preventing *CHOP* induction to avoid apoptosis (Wang et al., 1996; Oyadomari and Mori, 2004).

GRP78 at the Mitochondria and the Mitochondria-Associated ER Membrane

GRP78 has also been observed in the mitochondria in association with co-chaperones known to be involved in calcium-mediated signaling between the ER and mitochondria that is important for bioenergetics and cell survival. ER stress and UPR signaling induce the overexpression of GRP78, which results in its mitochondrial localization. Sub-mitochondrial fractionation studies showed that GRP78 is mainly localized in the intermembrane space, inner membrane, and mitochondria matrix (Sun et al., 2006). GRP78 plays a direct role in controlling efflux of calcium ions from the ER by closing the Sec61 channel during protein translocation and in the absence of translocation (Hamman et al., 1998; Haigh and Johnson, 2002; Alder et al., 2005). In addition, upon calcium depletion from the ER via the inositol trisphosphate receptor IP_3R, the calcium-sensitive co-chaperone sigma receptor 1 (Sig1R) dissociates from GRP78 and associates with IP_3R, thereby protecting the otherwise unstable IP_3R from ERAD and prolonging calcium signaling to the mitochondria (Hayashi and Su, 2007; **Figure 1**).

Secreted and Cell-Surface GRP78

Finally, GRP78 can be located at the plasma membrane where it is cytoprotective. In cultured cells, the ER stress agent, thapsigargin, actively promotes cell surface expression of GRP78, as the increase in cell surface GRP78 is several fold higher than the increase in intracellular GRP78 induced by thapsigargin (Zhang et al., 2010). Nonetheless, ER stress is not required for cell-surface localization of GRP78. Ectopic expression of GRP78 can induce its translocation in the absence of ER stress as indicated by the lack of CHOP induction. Moreover, deletion of the carboxyl-terminal ER-retention signal (KDEL) alters GRP78 relocation. This suggests that the KDEL retrieval system plays a significant role in regulating how much GRP78 leaves the ER.

Although GRP78 translocation have been studied mainly in cancer cell lines and have been found to be cell context-dependent (Tsai et al., 2015), there exist some common details for its mechanism of action. GRP78 can be translocated and anchored to the cell surface by binding to the ER-co-chaperone HTJ-1/MTJ-1 (Birukova et al., 2014; **Figure 1**). The translocation is promoted by accumulation of oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC), a phospholipid that directly interacts with GRP78, induces membrane accumulation of the GRP78/HTJ-1 complex and its targeting to caveolin-enriched microdomains (Birukova et al., 2014). Once the complex is at the membrane, it activates Src/Fyn kinase leading to assembly of the PI3K complex and activation of mTOR and sphingosine-1-phosphate receptor 1. This in turn results in cortical actin cytoskeletal remodeling in endothelial cells. Thus, GRP78 regulates OxPAPC-mediated cytoskeletal remodeling.

In the plasma membrane, GRP78 functions as a signal-transducing receptor or co-receptor for soluble ligands such as α2-macroglobulin (α2-M; Misra et al., 2005b), tumor differentiation factor (Sokolowska et al., 2012), and vaspin (Nakatsuka et al., 2012). Other molecules that bind to GRP78 include glycosylphosphatidylinositol-anchored proteins, for example, T-cadherin (Philippova et al., 2008) and Cripto, the teratocarcinoma-derived growth factor (Shani et al., 2008), among others (Ni et al., 2011). In-depth details of activated downstream signaling due to these interactions have been extensively reviewed by Ni et al. (2011). The binding of GRP78 to most of these ligands activates the AKT/PI3K pro-survival pathway (Misra et al., 2004, 2006; Philippova et al., 2008; **Figure 1**). Soluble Cripto has also been shown to bind cell-surface GRP78/BiP initiating PI3K and MAPK signaling via Src activation (Gray and Vale, 2012) or binding directly to c-Src (Gu et al., 2015). Indeed, cell-surface GRP78 is also involved in cell-matrix adhesion by α1-integrin interaction and focal adhesion kinase (FAK) regulation. This interaction has been related to cell migration and invasion process, an effect partly mediated through its association with uPA–uPAR protease system (Li et al., 2013). The interaction with α1-integrin, considered also important for axonal regeneration, might be interesting to be

further explored since GRP78 was found to promote neurite outgrowth *in vitro* (Satoh et al., 2000). Other interacting partners have been recently described that appear to be related to neurodegeneration which will be discussed below in other sections.

A recent study using a combination of biochemical, mutational, FACS, and single molecule super-resolution imaging approaches, reports that GRP78 mainly exists as a peripheral protein on plasma membrane via interaction with other cell surface proteins including glycosylphos-phatidylinositol-anchored proteins since it lacks a true transmembrane domain (Tsai et al., 2015). In addition, the authors discovered that cell-surface GRP78 expression requires its substrate binding activity but is independent of ATP binding.

Accordingly, GRP78 has also been observed as a secreted protein even in the human peripheral circulation (Delpino and Castelli, 2002). Secreted GRP78 can be found as well in the oviduct where apparently modulates sperm-zona pellucida binding (Marín-Briggiler et al., 2010). In a totally different context, the extracellular GRP78 has been proofed to have powerful immunomodulatory and anti-inflammatory properties by increasing IL-10 and reducing TNF-α (Corrigall et al., 2004; Panayi and Corrigall, 2014; **Figure 1**). This observation suggests that it would be relevant to determine such immunomodulatory property within the central nervous system.

Alternative Variants of GRP78 in the Cytoplasm

In addition to its localization in membrane-associated structures and organelles, GRP78 is observed in the cytoplasm. GRP78 can be relocated from the ER to the cytoplasm through several mechanisms: (i) via the ERAD pathway (Duriez et al., 2008), (ii) via a Bax/Bak-dependent change in membrane permeability produced during ER stress-induced apoptosis that allows luminal proteins to flow out (Wang et al., 2011), (iii) through *GRP78* alternative splicing of *GRP78* nuclear pre-RNA. The alternative processing results in retention of intron 1, which leads to an mRNA with an alternative translation initiation site and a premature stop codon that causes the loss of the ER signaling peptide in the encoded truncated isoform termed GRP78va (Ni et al., 2009; **Figure 1**).

LESSONS FROM CANCER

Cancer cells are characterized by altered glucose metabolism, and the tumor microenvironment is marked by impaired blood flow and hypoxia, all of which can cause ER stress. GRP78 is involved in several aspects of cancer development including tumor survival and proliferation, chemoresistance, angiogenesis, and metastasis. Many tumor cells overexpress GRP78 on the outer plasma membrane. In addition, in different types of cancer, such as those of prostate, breast, and melanoma origins, abnormally high GRP78 expression is correlated with tumor resistance, greater risk for cancer recurrence, and an overall decrease in patient survival (reviewed in Pfaffenbach and Lee, 2011). Thus, GRP78 at the cell surface has been postulated to be a promising target for cancer therapeutics and a useful prognostic marker.

The utilization of knockdown and overexpression techniques and genetic mouse models has furthered our understanding of the role of GRP78 in cancer. In a transgene-induced endogenous mammary tumor model, GRP78 haploinsufficiency resulted in delayed tumor latency, decreased tumor proliferation, and increased apoptosis (Wang et al., 2010). Strikingly, in mice harboring bi-allelic conditional knockouts of both *GRP78* and *PTEN* in the prostate epithelium, prostate tumorigenesis was potently arrested, providing the first evidence that GRP78 is required for tumorigenesis driven by loss of *PTEN* and activation of the PI3K/AKT oncogenic pathway (Fu et al., 2008). Indeed, ligation of cell-surface GRP78 by antibody slowed growth rate and blocked PI3K/AKT signaling (Misra and Pizzo, 2010b).

Through formation of complexes with other proteins on the cell surface such as α2-M or Cripto, GRP78 is reported to mediate tumor cell signal transduction. Autoantibodies from serum of prostate cancer patients against a segment of GRP78 (Leu 98-Leu115) induces cell proliferation, suggesting that these antibodies serve as agonists of activated α2-M, which recognizes the same site of GRP78 (Gonzalez-Gronow et al., 2006). The interaction of α2-M with cell-surface GRP78 promotes cell proliferation by activating ERK1/2, p38 MAPK, and PI3K and enhances cell survival by inducing the AKT and NF-kB signaling cascades (Misra et al., 2004, 2006). In addition, in highly metastatic and invasive 1-LN prostate cancers, cell-surface GRP78 acts as a receptor for activated α2-M leading to activation of PAK-2, and together with LIMK and cofilin phosphorylation, increases motility enhancing metastasis (Misra et al., 2004, 2005a). Another pathway is triggered by binding to Cripto oncoprotein. The complex of Cripto and GRP78 enhances tumor growth via inhibition of TGF-β signaling. Furthermore, blockade of Cripto binding to cell-surface GRP78 by an antibody against the N-terminus of GRP78 inhibits oncogenic Cripto signaling and this involves the MAPK/PI3K and Smad2/3 pathways (Kelber et al., 2009). A commercial polyclonal antibody directed against the C-terminus of GRP78 was reported to induce apoptosis in melanoma cells (A375) and prostate cancer cells (1-LN, DU145) but not in the PC-3 prostate cancer cell line. GRP78 expression was undetectable on the surface of the PC-3 cells but was present on the other cell types (Misra et al., 2009). The proposed mechanism is that this antibody leads to suppression of Ras/MAPK and PI3K/AKT signaling (Misra et al., 2009; Misra and Pizzo, 2010a,b).

A different pathway has also been revealed recently. Katherine L. Cook and collaborators showed that GRP78 specifically inhibits *de novo* fatty acid synthesis in breast cancer cells and reduces mitochondrial β-oxidation through inhibition of mitochondrial carnitine palmitoyltransferase 1a (CPT1a), which catalyses the primary regulated step in overall mitochondrial fatty acid oxidation (Cook et al., 2016).

It has been suggested that GRP78 acts in concert to coordinate tumor cell growth to accommodate cancer cells to nutritional changes through facilitation of macroautophagy (Li et al., 2015). In agreement, one study showed that functional blockade of the proteasome induces GRP78, promoting autophagosome formation and enhancing myeloma survival (Abdel Malek et al., 2015). In tumor cells this activation can lead to autophagic

degradation of IκB kinase, which caused inactivation of NF-κB pathway, an important mediator of apoptotic signaling.

The alternative cytosolic form, GRP78va is also important in tumorigenesis. This isoform is overexpressed in leukemic cells and leukemia patient samples. In the cytosol, GRP78va may associate with P58(IPK), which acts as inhibitor of PERK during UPR, antagonizing it and increasing cell survival under ER stress (Rutkowski et al., 2007). This study suggested that GRP78va has the potential to influence survival of cancer cells in adaptation to ER stress through modulating UPR signaling.

In summary, tumor cells use GRP78 to orchestrate the stimulation of processes such as macroautophagy, to combat the presence of reactive oxygen species (ROS), and to activate pro-survival signaling pathways.

GRP78 IN NEURODEGENERATIVE PROCESSES

Age-related neurodegenerative diseases are commonly associated with the accumulation of misfolded and aggregated proteins and the presence of oxidative stress, calcium dysregulation, and mitochondrial dysfunction, particularly at the mitochondria-associated ER membrane (MAM). Neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and prion-related diseases, have different clinical manifestations, but all present common events that also occur in neurodegenerative processes triggered by brain ischaemia or trauma. Aging, which is a risk factor for most neurodegenerative diseases, is accompanied by decreases in activity of several endogenous neuroprotective mechanisms that certainly may contribute to their etiopathogenesis.

GRP78 in Alzheimer's Disease

AD is a neurodegenerative disease characterized by cognitive alterations and memory loss. Early-onset cases of autosomal-dominant familial AD (FAD) are often caused by mutations in the genes encoding amyloid beta precursor protein (*APP*) or presenilin proteins (*PS1*, *PS2*). Aspartyl proteases PS1 and PS2 are components of the γ-secretase complex that, together with β-secretase, process APP to produce amyloid-β peptides (Aβ) of 40 and 42 amino acids ($A\beta_{40}$, $A\beta_{42}$). Hallmark lesions in AD are amyloid plaques and neurofibrillary tangles, both arising from protein misfolding. In plaques there is an abnormal increase in the $A\beta_{42}$:$A\beta_{40}$ ratio, whereas neurofibrillary tangles are composed of the aberrantly phosphorylated tau protein (Mattson, 1994).

The bulk of immature APP associates with GRP78 in the ER. GRP78 facilitates correct folding of APP and modulates intracellular APP maturation and processing (Yang et al., 1998; Kudo et al., 2006). Under ER stress, overexpression of GRP78 retains APP in the early secretory compartments resulting in a reduction of Aβ generation because β/γ-secretase activity itself is thought to be located in late secretory compartments, such as the Golgi apparatus and endo-lysosomal system (Kudo et al., 2006). In other way, GRP78 is a key player in APP processing also through ERAD. Some authors have found that another ER-resident protein dnj-27 (the ortholog of mammalian ERdj5), which works as an enhancer of ERAD together with GRP78 and EDEM, protects against the aggregation of both Aβ and α-synuclein (α-syn), involved in PD pathogenesis, in *C. elegans* (Muñoz-Lobato et al., 2014).

GRP78 levels are two-fold higher in AD temporal cortex and hippocampus compared to non-demented control cases as shown by immunohistochemistry. This increase was found in neurons in AD brains that were still healthy and that do not co-localize with neurofibrillary tangles indicating that GRP78 overexpression may slow down neurodegeneration (Hoozemans et al., 2005). Intriguingly, in the triple transgenic mice bearing FAD-linked mutations in APP and presenilins (3xTg-AD), which serve as an AD model, GRP78 levels are increased only by 1.5–2-fold in 2 month-old 3xTg-AD mice compared to controls, and this increase is associated with the presence of accumulated toxic Aβ peptide (Soejima et al., 2013). It is remarkable that this level of overexpression of GRP78, reported *in vivo* in this animal model and similar to those observed in post-mortem human AD tissue, is minor compared to the levels induced by ER stress (e.g., by using tunicamycin) in a wild-type animal, which can be more than 3-fold in several tissue types (Li et al., 2012; Galán et al., 2014). This observation suggests that the degree of GRP78 level increased in AD models and human AD neurons might be insufficient to cope with sustained ER stress. This observation is supported by age-related difficulties for GRP78 increase after ER stress as described further down in the aging section. In addition, it would be interesting to know where GRP78 is located within the neurons in AD tissues, as its functions depend on localization as discussed above. Importantly, the extracellular chaperone α_2-M, a ligand of GRP78 at the plasma membrane, is co-localized with plaques in AD (Yerbury and Wilson, 2010), and it has been shown both to protect cells from Aβ toxicity and to favor Aβ removal from the brain (reviewed in Yerbury and Wilson, 2010). It is likely that some of these beneficial effects occur through the intervention of GRP78, although this has not been demonstrated yet.

Tau hyperphosphorylation is another pathological hallmark in AD brain and other Tauopathies. In a recent study, it was found that overexpression of GRP78 induced tau hyperphosphorylation via activating glycogen synthase kinase-3β (GSK-3β), an important tau kinase in AD brain, and increased the association with tau and GSK-3β. This was concurrent with SIL1 down regulated expression (Liu et al., 2016). However, when the authors forced the expression of both proteins prevented ER stress-induced tau hyperphosphorylation and GSK-3β activation suggesting the importance of ATP binding activity for beneficial effects promoted by GRP78.

In addition to APP processing, other abnormalities have been found associated to AD pathology where GRP78 can have also an opportunity for neuroprotection. Calcium level is dysregulated in AD brains, although its role in pathology is not well-understood. Calcium signaling may act even upstream of APP processing, as elevations in Ca^{2+} can increase production of oligomeric Aβ peptides (Itkin et al., 2011). Indeed, stabilizing ER calcium with dantrolene, a ryanodine receptor antagonist, restores normal synaptic function and

plasticity and reduces amyloid load in the brains of 3xTg AD mice and knock-in FAD mice (reviewed in Frazier et al., 2017). A recent review by Area-Gomez (Area-gomez and Schon, 2016) proposed that the pathogenesis of AD might be mediated by increased ER-mitochondrial communication, which may cause aberrant increases in calcium trafficking between the two organelles, unusual phospholipid profiles, perturbed cholesterol homeostasis, changes in mitochondrial function and morphology, and an increased $A\beta_{42}$:$A\beta_{40}$ ratio. In particular, the authors argue that the altered ER membrane topology at the MAM in AD could explain the shift in the location of the γ-secretase cleavage toward $A\beta_{42}$. In this regard, GRP78 localized at the MAM might have an important role in neuroprotection as a calcium binding protein.

One mechanism through which Aβ peptides cause cytotoxicity is by production of ROS via facile copper-redox cycling (Barnham et al., 2004), which can, in turn, result in oxidative damage to neuronal proteins and lipids (Mark et al., 1997). Imbalances in ROS production and detoxification are strongly implicated in AD neurodegeneration, as reflected by cerebral elevations in oxidized lipids and proteins (Sayre et al., 1997; Greilberger et al., 2008). According to recent studies revealing important roles of GRP78 in regulation of lipid content and inhibition of lipotoxicity resulting from lipid peroxidation and ROS generation (Cook et al., 2016) it is possible that overexpression of GRP78 can have neuroprotective properties against ROS as well.

Finally, sporadic AD (SAD) comprises the vast majority of AD cases. Mutations in the gene encoding apolipoprotein E (ApoE), particularly the *ApoEε4* allele, are the strongest genetic risk. ApoEε4 promotes transient membrane cholesterol loading, which increases $A\beta_{42}$ secretion and its accumulation in plaques in patients with AD and in cognitively normal people (reviewed in Sato and Morishita, 2015). Cholesterol and phospholipids have been shown to modulate the activity of APP-related secretases (Di Paolo and Kim, 2011). ER function is also affected by lipid composition and lipid biosynthetic enzymes (Lagace and Ridgway, 2013). Exogenous expression of GRP78 by adenoviral administration reduces liver lipogenesis by inhibiting activation of the central lipogenic regulator, the sterol regulatory element-binding protein 1c, SREBP1-c (Kammoun et al., 2009). Further, supporting the hypothesis that GRP78 modulates lipid metabolism, GRP78 heterozygous mice are resistant to obesity when placed on a high fat diet (Ye et al., 2010). Overexpression of GRP78 reduces the expression of lipogenic genes and plasma triglycerides and rescues the levels of the ER-processed ABCG5-G8 heterodimer transporter of cholesterol in the liver of obese mice lacking the receptor of leptin (db/db mice; Wang Y. et al., 2015). The mechanisms by which GRP78 functions in lipid and cholesterol management are far from clear, particularly in the brain, in light of these results in other tissues, it would be very interesting to further investigate its involvement in the lipid-related pathophysiology of neurodegenerative diseases such as AD.

GRP78 in Parkinson's Disease

Parkinson's disease is an idiopathic movement disorder characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) and the presence of Lewy bodies. Lewy bodies are distinct protein inclusions composed of aggregated α-syn. Studies on post-mortem brain samples have revealed immunoreactivity for UPR activation markers (Bellucci et al., 2011). Indeed, α-syn induces ER-stress and activates the UPR pathway in dopaminergic neurons in the SNc (Gorbatyuk et al., 2012).

In cell and animal models of α-syn accumulation, there is evidence that GRP78 forms a complex with α-syn (Bellucci et al., 2011; Colla et al., 2012; Gorbatyuk et al., 2012). Interestingly, both the level and localization of GRP78 are altered in different models of PD. For instance, in a rabbit model of PD, it has been demonstrated that GRP78 translocates from the ER to the nucleus and cytosol in response to treatment with MPP+, which causes a marked reduction in Tyrosine Hydroxylase-positive cells in the SNc (Ghribi et al., 2003). In cultured neurons, extracellular α-syn binds to GRP78 located at the cell surface, triggering a signaling cascade leading to cofilin 1 inactivation and stabilization of microfilaments, thus affecting morphology and dynamics of actin cytoskeleton. Inactivation of cofilin 1 and stabilization of actin cytoskeleton also occurs in fibroblasts derived from PD patients, suggesting that extracellular GRP78 might be the responsible. Dysregulation of actin turnover has been shown to lead to deficits in synaptic function that normally precede neurodegeneration in PD models. In addition, the interaction with extracellular α-syn renders GRP78 sequestered and clustered at the cell surface, which impedes its proper recycling toward the ER and results in a virtual depletion from the ER. Accordingly, overexpression of GRP78 was found to be neuroprotective, through a mechanism that involves decreases in the levels of UPR target genes, preventing the loss of dopaminergic neurons and dopamine in the SNc (Ni et al., 2011).

GRP78 in Amyotrophic Lateral Sclerosis

ALS is a progressive neurodegenerative disease, involving the selective degeneration of motoneurons in the spinal cord, most of the brainstem, and the cerebral cortex. Many different mutations are associated with familial ALS, but all lead to protein misfolding and aggregation. These mutations are in genes encoding superoxide dismutase 1 (SOD1), TAR DNA-binding protein 43-KDa, FUS, and other proteins. SOD1 aggregates have been observed in patients with sporadic ALS (Ezzi et al., 2007; Chattopadhyay et al., 2008; Bosco et al., 2010). Mutant SOD1 aggregates, but not wild-type SOD1, forms high molecular weight species that interact with GRP78 as observed in microsomal fractions of spinal cords derived from mouse models of ALS (Kikuchi et al., 2006).

Saxena's group investigated the pattern of expression of the ER folding network in vulnerable and resistant motoneurons and found that the ER folding network has a relevant role in ALS (Maharjan and Saxena, 2016). Remarkably, the knock-in mice that express mutant GRP78 lacking the KDEL sequence have age-related motor problems concomitant with loss of

selective vulnerable motoneurons and aggregation of wild-type SOD1 reminiscent of ALS symptoms (Bosco et al., 2010; Jin et al., 2014). Several co-chaperones of GRP78, such as SIL1 and Sig1R, are important in ALS. SIL1 is mostly expressed in resistant motoneurons, suggesting it is involved in neuroprotection. Accordingly, SIL1 deficiency enhances ALS pathology, whereas SIL1 overexpression affords significant neuroprotection related to improved ER proteostasis and reduced SOD1 aggregation (reviewed in Rozas et al., 2017). Chronic treatment with PRE084, an agonist of Sig1R lead to increase neuroprotection of motoneurons in a mouse model of ALS (Mancuso et al., 2012). For all these reasons, it is possible that overexpression of GRP78 would mediate neuroprotection in ALS patients.

GRP78 in Prion-Related Diseases

Human prion diseases are rare, rapidly progressive, invariably lethal neurodegenerative diseases, symptomatically characterized by severe memory impairment and a general decline in cognitive functions, which may include motor, linguistic, executive, and social skills (Wadsworth et al., 2003). Most often, human prion diseases have a sporadic etiology [e.g., sporadic Creutzfeldt-Jakob disease (sCJD)], but hereditary (e.g., fatal familial insomnia and Gerstmann-Sträussler-Scheinker syndrome), and infectiously acquired [e.g., iatrogenic CJD, kuru, and variant CJD (vCJD)] forms of the disease also exist. Prion diseases have also extensively been described in animals; these include bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep.

At the neuropathological level, human prion diseases are characterized by the accumulation of pathological prion protein (PrP^{Sc}), neuronal loss, astrogliosis, and spongiosis. During human prion disease progression, normal prion protein (PrP^{C}) is converted into insoluble, β-sheet rich PrP^{Sc} aggregates. Once formed this pathological PrP^{Sc} conformer ensures conversion of native PrP^{C} into PrP^{Sc} and propagation of pathology to neighboring cells (reviewed in Wadsworth et al., 2003). One study reported increased expression of GRP78 and several other ER chaperones in post-mortem brain samples of sCJD and vCJD patients, although signal was not compared to controls (Hetz et al., 2003). In brain tissue samples from animals naturally infected with BSE, GRP78 is upregulated only by up to 2.3-fold (Tang et al., 2010). Increases in UPR markers such as GRP78 are thought to be an attempt of the neurons to cope with ER stress and are essentially markers of neuroprotective processes as mentioned. In a recent study, Jin and collaborators showed that GRP78 interacts transiently with PrP^{c} in the ER, in agreement with its involvement in the folding of nascent PrP^{c} polypeptides (Jin et al., 2000). GRP78 might remain associated for an extended period of time with some isoforms of mutant PrP causing its subsequent retrotranslocation for proteasomal degradation and so preventing the formation of homo-aggregates (Jin et al., 2000). It will be interesting to determine whether boosting GRP78 expression further will lead to neuroprotection as was demonstrated for another chaperone GRP58 (Hetz et al., 2005).

GRP78 in Neurodegenerative Processes after Ischemia or Trauma to the Nervous System

Neurodegeneration is a secondary event after traumatic brain injury and ischaemia. Ischemic preconditioning (IPC) is a sublethal ischemic episode that engages endogenous cytoprotective mechanisms to protect cells from subsequent severe ischemia (Zhang et al., 2015). As suggested by researchers in the field, uncovering the mechanisms of brain ischemic preconditioning might lead to the development of effective treatments for ischemic cerebrovascular disease that could be exploited therapeutically. Several studies have observed that IPC leads to upregulation of GRP78, which activates autophagy. Accordingly, specific suppression of GRP78 with pharmacological and genetic approaches inhibits autophagic activation and abolishes ischemic tolerance (reviewed in Zhang et al., 2015).

Overexpression of GRP78 is important for protection of astrocytes after ischemic injury as it reduces the flux of Ca^{2+} from the ER to the mitochondria, increases Ca^{2+} uptake capacity in isolated mitochondria, reduces free radical production, and preserves respiratory activity and mitochondrial membrane potential after stress (Ouyang et al., 2011).

After trauma, it has been demonstrated that GRP78 plays a relevant role. After abrupt proximal axotomy or avulsion of the nerve root, a retrograde neurodegenerative process occurs in spinal motoneurons. In contrast to root avulsion, after distal axotomy, motoneurons can engage signaling pathways that allow them to survive and regenerate. In these conditions, GRP78 is downregulated during neurodegenerative processes but overexpressed in the regenerative condition (Penas et al., 2009, 2011a). Indeed, forced expression of GRP78 or pharmacological activation of its co-chaperone Sig-R1 in a root avulsion model leads to neuroprotection (Guzmán-Lenis et al., 2009; Penas et al., 2011a,b). These observations suggested that GRP78 plays a relevant role activating endogenous neuroprotection and that its effects can be mimicked to exert neuroprotection in different conditions.

GRP78 during Aging

A commonality in neurodegenerative diseases is that the UPR is not correctly activated. In *ex vivo* human diseased brain tissue and *in vivo* models, there is significant depletion of ER molecular chaperones involved in the UPR despite ER stress (Lee et al., 2010; Gorbatyuk et al., 2012; Drake, 2015). Although, the mechanisms that underlie UPR dysfunction are unclear, aging might be a determinant factor. It has been reported that during aging, the quality control mechanism becomes inefficient since ER chaperones are less responsive to ER stress, as evidenced by decreased levels and activities of ER chaperones in aged tissue (Nuss et al., 2008). This defect has been attributed to increased oxidation of several key ER chaperones (Rabek et al., 2003), which would agree with the mitochondrial free radical theory of aging (Cadenas and Davies, 2000).

In particular, a reduction in GRP78 levels has been observed during aging and throughout progression of degenerative

disorders (Paz Gavilán et al., 2006). Old mice (20–24 months old) have 20% less GRP78 ATPase activity than young mice (3–5 months old), which is consistent with a 2-fold higher level of GRP78 carbonylation in old mice. Such findings support the hypothesis that loss of ER or other cellular functions, often seen in age-related diseases, is caused by the life-long accumulation of oxidative damage to key proteins (Nuss et al., 2008; Salganik et al., 2015). Another study reported that there was about 73% less *GRP78* mRNA in old (900 days old) compared to young (21 days old) rats, suggesting that loss of GRP78 activity and the associated physiological declines occur at both the protein and transcript levels (Erickson et al., 2006). This suggests that the loss of GRP78 function could be a predisposing factor for neurodegenerative disorders associated with age (Brown and Naidoo, 2012).

A decrease in macroautophagy with age has also been reported in a variety of systems (Martinez-Lopez et al., 2015). The exact mechanisms by which protein aggregation contributes to neuronal degeneration remain to be fully elucidated; however, accumulating evidence suggests that defects in autophagy-related pathways contribute substantially to premature aging (Rajawat et al., 2009) and neurodegeneration (Ravikumar et al., 2004). Indeed, landmark studies have demonstrated that enhancing autophagy confers a protective effect in AD, PD, and Huntington's disease (reviewed in Ntsapi and Loos, 2016), whereas genetic suppression of basal autophagy causes neurodegeneration (Hara et al., 2006; Komatsu et al., 2006).

Successful and precise targeting of the autophagy process in the clinical setting has thus far not been accomplished, but it would be very interesting to know whether restoring GRP78 levels after ER stress in an aged-brain improve autophagy efficiency, reduces the extent of mitochondria dysregulation and protein aggregation.

CONCLUDING REMARKS

GRP78 or BiP is a very important protein. It has a relevant role to promote survival in tumor cells by activating potent endogenous cytoprotective mechanisms. Regarding these lessons, it is possible that engaging the same mechanisms in the nervous system this would be capable to cope with multiple stressful situations in the course of a disease. Multifunctional GRP78 can elicit neuroprotection by attenuating ER stress, managing misfolded proteins to avoid its accumulation, inducing macroautophagy, buffering calcium unbalance, facilitating mitochondria-ER crosstalk and activating pro-survival signaling pathways. Thus, GRP78 is an excellent target to take into consideration for neuroprotective therapeutical strategies targeting specifically neurons to avoid any putative undesirable side effect although GRP78 itself is not proto-oncogenic.

AUTHOR CONTRIBUTIONS

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REVIEW
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Molecular Chaperone Accumulation in Cancer and Decrease in Alzheimer's Disease: The Potential Roles of HSF1

Stuart K. Calderwood and Ayesha Murshid*

Molecular and Cellular Radiation Oncology, Beth Israel Deaconess Medical Center, Center for Life Sciences 610, Harvard Medical School, Boston, MA, USA

Molecular chaperones are required to maintain the proteome in a folded and functional state. When challenges to intracellular folding occur, the heat shock response is triggered, leading to increased synthesis of a class of inducible chaperones known as heat shock proteins (HSP). Although HSP synthesis is known to undergo a general decline in most cells with aging, the extent of this process varies quite markedly in some of the diseases associated with advanced age. In Alzheimer's disease (AD), a prevalent protein folding disorder in the brain, the heat shock response of some critical classes of neurons becomes reduced. The resulting decline in HSP expression may be a consequence of the general enfeeblement of many aspects of cell physiology with aging and/or a response to the pathological changes in metabolism observed specifically in AD. Cancer cells, in contrast to normal aging cells, undergo *de novo* increases in HSP levels. This expansion in HSP expression has been attributed to increases in folding demand in cancer or to the evolution of new mechanisms for induction of the heat shock response in rapidly adapting cancer cells. As the predominant pathway for regulation of HSP synthesis involves transcription factor HSF1, it has been suggested that dysregulation of this factor may play a decisive role in the development of each disease. We will discuss what is known of the mechanisms of HSF1 regulation in regard to the HSP dysregulation seen in in AD and cancer.

Keywords: molecular chaperone, heat shock protein, cancer, Alzheimer's disease, proteotoxic stress

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Cintia Roodveldt, Centro Andaluz de Biología Molecular y Medicina Regenerativa, Spain

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Diego Ruano, University of Seville, Spain
Catarina Oliveira, University of Coimbra, Portugal
Justin John Yerbury, University of Wollongong, Australia

****Correspondence:***
Stuart K. Calderwood
scalderw@bidmc.harvard.edu

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INTRODUCTION

Cancer and Alzheimer's disease (AD) each affect large proportions of the population and, at least in their sporadic forms are much more prevalent in older people. In both diseases, there is strong evidence for loss of regulation of molecular chaperone function that may contribute to the morbidity of the diseases (Calderwood et al., 2009; Calderwood and Gong, 2016). However, these changes take different forms in the two disease types. In short, cancer is associated with expansion in molecular chaperone expression, while onset of AD and other neurodegenerative diseases has been associated with reduced HSP levels and a decrease in the ability to deal with proteotoxic stress (Batulan et al., 2003; Calderwood and Gong, 2016). These findings are confluent with numerous epidemiological studies that show a negative correlation between the risk of cancer in persons with AD or other neurodegenerative diseases (Roe et al., 2005; Driver, 2014). We will explore here

regulation of molecular chaperone synthesis and how it may become modified during development of AD and cancer.

HEAT SHOCK PROTEINS- AN INDUCIBLE CLASS OF MOLECULAR CHAPERONES

The most intensely studied property of HSPs is their facilitation of the pathways of protein folding (Lindquist and Craig, 1988; Kayser et al., 2013; Kityk et al., 2015). They thus belong to the families of molecular chaperones. HSPs possess the capacity to recognize structures commonly found in the interior of proteins and to bind such structures. Their major role appears to deter the formation of quasi-stable conformations during folding (Ellis, 2007). With this aid, nascent proteins or polypeptides being rescued from denaturation will then collapse into their low-energy, native conformations in which they can direct cell metabolism. In order to be released from client proteins after folding, and take part in further rounds of activity, Hsp70 and other chaperones utilizes an intrinsic ATPase domain to hydrolyze ATP and assume a free conformation (Kityk et al., 2015). The low molecular weight chaperone Hsp27, lacking an ATPase domain, requires rescue by Hsp70 to release from its clients. Hsp27, Hsp70, and Hsp90 can act in relay to permit unfolded proteins to achieve functional activity in a stepwise manner (Calderwood and Gong, 2016). Although there are a number of other HSP families, we have concentrated on Hsp27, Hsp70, and Hsp90, as they are the most intensely studied HSPs in cancer and AD. In some cases, proteins with fragile conformations remain associated with Hsp90 in order to maintain a functional conformation (Kirschke et al., 2014). Thus, most cells contain high concentrations of these proteins, particularly Hsp90 to chaperone the proteome. During proteotoxic stress, a subclass of inducible chaperones is induced at the transcriptional level to boost chaperoning capacity, and these are the HSPs (Richter et al., 2010). For a more detailed overview of molecular chaperone function and interaction with protein co-factors (co-chaperones), readers are referred to a previous review (Calderwood, 2013).

REGULATION OF THE HEAT SHOCK RESPONSE BY HSF1

Exposure of almost any cell to heat shock leads to the almost immediate transcription, translation and accumulation of a cohort of HSPs that increase to quite remarkable levels when the stress is pronounced (Richter et al., 2010). Such cells then become resistant to further stress by heat shock (Li and Hahn, 1980). We now know that a major property of HSPs is to facilitate the folding of a large proportion of intracellular proteins toward functional conformations (Ellis, 2007). Heat shock triggers the unfolding and aggregation of many intracellular proteins—hence the development of the heat shock response early in evolution to cope with environmental stress (Zhang and Calderwood, 2011). The key effector of *HSP* gene transcription is heat shock factor 1 (HSF1), a sequence specific factor that binds upstream of all *HSP* genes at the onset of stress (Wu, 1995). There is currently no unique hypothesis as to the mechanism by which HSF1 senses the heat shock and becomes activated. HSF1 may be able to both sense the change in temperature shift directly, as with a thermometer, or may have evolved to respond to the toxic effects of the heat shock such as protein unfolding within the cytoplasm (Zhong et al., 1998; Zou et al., 1998). In all eukaryotes, HSF1 responds to stress by undergoing a monomer to trimer transition and becomes heavily phosphorylated, leading to its acquiring ability to rapidly bind to DNA and activate transcription (Akerfelt et al., 2010). It became clear quite early that transcriptional activation by mammalian HSF1 required more than its trimerization and DNA binding (Price and Calderwood, 1991). Recent studies also suggested a similar two stage activation in yeast (Zheng et al., 2016). In fact, some stimuli- such as exposure to high levels of sodium salicylate could lead to almost quantitative HSF1 binding to DNA without increasing HSP transcription (Jurivich et al., 1992; Housby et al., 1999). Heat shock sensitivity of HSF1 would seem to require at least two regulatory events. The Voellmy lab were able to recreate HSF1 activation in the absence of heat shock by combining: (1) sodium salicylate stimulation (which produces trimerization and DNA binding) with (2) exposure to phosphatase inhibitors- strongly suggesting that the second stimulus for HSF1 induction involves direct or indirect effects of phosphorylation (Voellmy, 1994; **Figure 1**).

Work from the Kingston lab next provided a structural basis for the two-stimulus hypothesis by the discovery of a new temperature sensitive domain in HSF1 remote from the trimerization domain (Green et al., 1995; Newton et al., 1996). This region—called the regulatory domain was shown to be in the middle of the HSF1 protein sequence, C-terminal to the trimerization and DNA binding domains and upstream from the *trans*-activation domains and to control HSP gene transcription independently from DNA binding. Interestingly, the regulatory domain appeared to repress *trans* activation in the absence of stress but transmitted the inducing effects of heat shock to the trans activation domains (Green et al., 1995; Newton et al., 1996). Interestingly, this domain contains both positively acting and repressive phosphorylation sites (Calderwood et al., 2010). Serine 303 is phosphorylated by glycogen synthase kinase 3 (GSK3) under non-stress conditions and represses HSF1 through mechanisms including induction of repressive SUMO modifications as well as nuclear export (Wang et al., 2003; Anckar et al., 2006). Heat shock overrides this form of repression as well as leading to activation of positively acting phosphorylation on serines 320 and 326 (Zhang et al., 2011; Chou et al., 2012). One could thus envisage a scenario in which stress triggered the first activating stimulus, leading to trimerization and rapid localization of HSF1 on *HSP* genes, and in a similar time frame activated a second signal through the regulatory domain to render the chromatin-bound HSF1 able to positively regulate *trans*-activation. Many questions still remain of course- such as the nature mechanism by which the regulatory domain led to the induction of the remote *trans*-activation domains and whether the activities of the trimerization region and the regulatory domain were in some way coordinated. In addition, there are important PTMs outside the regulatory domain. Serine 121 is

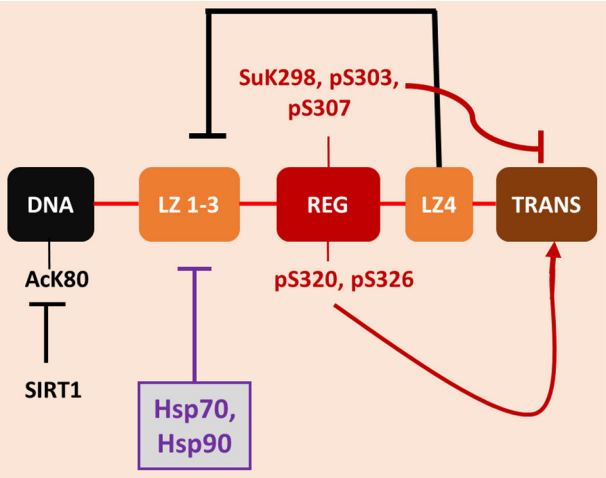

FIGURE 1 | Regulatory mechanisms governing HSF1 activity. The figure depicts the major functional domains within HSF1 in their linear organization along the protein structure. (note the domains are not drawn to scale). There are, N terminally to C-terminally, a DNA binding domain (DNA), a trimerization domain (leucine zipper 1-3 or LZ1-3), a central regulatory domain (REG), a fourth region of leucine zipper or hydrophobic heptad repeat sequence (LZ4) and the C-terminal double *trans*-activation domains (TRANS). The primary mechanism for HSF1 regulation appears to the intramolecular coiled coil interaction between LZ4 and LZ 1-3 that prevent trimerization and DNA binding under basal conditions, which is severed during heat shock. A second regulatory mechanism is feedback repression exerted by Hsp70 and Hsp90 that can bind at various positions in the HSF1 molecule to effect inhibition. The regulatory domain contains an array of phosphorylation sites that can govern activity. Most notable among these is serine 303 (pS303) whose phosphorylation mediates inhibition of HSF1, and sumoylated lysine 298 (SuK298). In addition, other PTMs have been found elsewhere in the molecule, with lysine 80 (K80) undergoing repressive acetylation (AcK80) that can be relieved by the deacetylase sirtuin 1.

another repressive phosphorylation site that acts to suppress DNA binding (Wang et al., 2006). In addition, HSF1 is modified by acetylation, most notably at lysine 80, an effect that reduces the duration of DNA binding (Westerheide et al., 2009). HSF1 activation can be effected by a range of deacetylases, including sirtuin 1, HDAC7 and HDAC9 that each remove the acetyl group from K80 and increase the lifetime of association of HSF1 trimers with DNA (Westerheide et al., 2009, 2012; Zelin and Freeman, 2015). It is of note that histone acetylase p300 is recruited by HSF1 to heat shock genes along with positive transcriptional elongation factor b (pTEFb) and is involved in *trans* activation by acetylating key histones (Zhang et al., 2011). This event could also be involved in HSF1 switch off, in the resolution of the response through K80 acetylation.

However, at some time within the aging organism, these complex regulatory mechanisms go wrong and the heat shock response loses its ability to switch on or off at the appropriate moment, effects that may differ in different organs and different diseases (Calderwood et al., 2009). The multiple molecular inputs into HSF1 regulation may each play distinct roles in the dysregulation of the factor in disease. It is also apparent that different triggers may activate HSF1 by alternative mechanisms. Fast activation in heat shock may involve the intrinsic capacity to sense temperature, while more gradual triggers as in cancer may be mediated through reversal of repressive pathways and activating PTMs (Zhong et al., 1998; Khaleque et al., 2005).

HSPS AND THE PROMOTION OF CANCER

It is now accepted that the levels of HSPs are relatively high in most types of human cancer compared to their normal tissues of origin (Ciocca and Calderwood, 2005; Ciocca et al., 2013). Most such studies have concerned themselves with a select group of HSPs—Hsp27, Hsp70, and hsp90 - and we will concentrate mostly on these proteins in this discussion (Calderwood and Gong, 2016). The proteins shown to play causal roles in carcinogenesis are by definition effector proteins in the processes that define malignancy, such as growth factor independent growth, escape from cell death and senescence pathways, promotion of angiogenesis and metastasis (Hanahan and Weinberg, 2011). It is evident that the canonical functions of HSPs do not appear to include the ability to directly effect these properties (Ellis, 2007). Their best-known properties exclusively involve protein folding, as described above. However, HSPs facilitate the properties that give cancer its morbidity (Calderwood and Gong, 2016). Indeed, HSPs are required for stimulus-independent growth, escape from apoptosis and senescence, angiogenesis, invasion and metastasis (Garrido et al., 2006; Yaglom et al., 2007; Thuringer et al., 2013; Calderwood and Gong, 2016). Many of these effects involve the chaperoning of oncoproteins involved in each of these cell behaviors. Interestingly, HSPs may contribute in different ways to transformation and tumor progression, depending on the driver oncogene that mediates tumorigenesis. For instance, knockout of Hsp70 reduced the growth of mammary cancers transformed by the oncogene Her2 due to cell senescence (Meng et al., 2011). However, in mammary epithelial cells transformed by the Polyoma Middle T antigen, the effects of hsp70 inactivation was to reduce tumor cell invasion and metastasis (Gong et al., 2015). Although an increasing role for HSPs in cancer is emerging, much needs to be learned regarding their multiple roles in disease progression.

One hypothesis that has arisen to explain the aberrantly high concentrations of HSPs in cancer is known as "addicted to chaperones." Tumor cells are thought to be in some ways similar to mildly heat shocked cells, with oncogene overexpression and mutation, polyploidy and exaggerated levels of translation generating an intracellular folding demand (Workman et al., 2007). This hypothesis draws heavily upon the proposed mechanism for HSF1 activation involving reversal of its feedback inhibition by HSPs such as Hsp90 and Hsp70 (Zou et al., 1998; Gómez et al., 2008; **Figure 1**). Such HSPs are thought to act by suppressing HSF1 trimerization or recruiting co-repressors to HSF1, while activation involves the sequestration of the HSPs in protein aggregates and release of free HSF1 to pursue its transcriptional role (Zou et al., 1998). Hsp90 targeted drugs indeed have the predicted property of causing the degradation of unchaperoned oncoproteins while activating HSF1 and HSP

synthesis (Workman et al., 2007; Conde et al., 2009). However, this hypothesis is difficult to prove conclusively and is not universally supported; it is not clear that the tumor environment can be shown to be in a state of folding demand (Colvin et al., 2014). It also seems apparent that that HSF1 can be coupled directly to some of the cancer signaling pathways. In cancer, signal transduction pathways that are normally coupled tightly to the occupation of growth receptors can operate independently, due to mutations or increases in expression in signaling intermediates (Hanahan and Weinberg, 2011). As mentioned above, HSF1 is repressed by phosphorylation on serine 303, within the regulatory domain, through the kinase GSK3 (Chu et al., 1996). In breast cancer HSF1 repression can be relieved by exposure to the ligand heregulin that binds cell surface receptor tyrosine kinase HER3. HER3 activation then activates HSF1 through its triggering of the kinase Akt that can in turn lead to inhibition of GSK3 and relief of repression (Khaleque et al., 2005). Feedback regulation through HSPs does not seem to be involved in this case and HSP synthesis and treatment resistance can thus be coupled directly to mammary cancer signaling pathways known to promote malignancy. Phosphorylation at an adjacent domain, serine 326 causes HSF1 activation (Chou et al., 2012). It is notable that HSF1 in mammary cancer stem cells, the cells that govern tumorigenicity, invasion and metastasis, is constitutively phosphorylated on serine 326 and dephosphorylated on serine 303, suggesting a causal role for activated HSF1 in stemness (Chou et al., 2015; Gong et al., 2015). Indeed, activated HSF1 induces the stem cell renewal factor beta-catenin (Chou et al., 2015). Thus, HSF1 activation and HSP expression in cancer may involve relief of the repressive effects of both HSPs and GSK3 as well as positive input through serine 326 (**Figures 1**, **2**). This mechanism appears to resemble the two-signal model of HSF1 activation by heat shock developed by Voellmy et al and others, with HSP sequestration permitting step (1), HSF1 escape from chaperone repression and trimerization and (2) phosphorylation in the regulatory domain providing the second signal for activation (Voellmy, 1994; Calderwood et al., 2010). It is also notable that HSF1 can exert tumorigenic effects through non-HSP chromosomal targets, including metastasis associated protein 1 and others (Khaleque et al., 2008). Therefore, HSF1 regulates both HSP expression as well as non-HSP targets that may go some way to explaining its potency in carcinogenesis (Ciocca et al., 2013).

DECLINE OF THE HEAT SHOCK RESPONSE IN ALZHEIMER'S DISEASE

It is now widely accepted that Alzheimer's disease (AD) and other neurodegenerative diseases are, at least partially, protein folding disorders (López-Otin et al., 2013; Cardinale et al., 2014). In this review, we have concentrated on three chaperones-Hsp27, Hsp70, and Hsp90 in cancer and AD. The rationale for this choice was that the vast majority of publications regarding the roles of HSPs in cancer deal with these three chaperones and we thus concentered on these when comparing the diseases (Ciocca and Calderwood, 2005; Calderwood and Gong, 2016). However, readers are referred to a recent publication showing a complex pattern of multiple chaperones and co-chaperones that are modulated over time in age-dependent neurodegenerative disease- some of which are increased while others decline (Brehme et al., 2014). Two of the major lesions associated with AD have been identified as large protein aggregates, known as senile plaques that accumulate in the cerebral cortex during disease progression, leading to loss of neurons and synapses and ultimately dementia. These two lesions are: (1) Beta amyloid plaques, derived from the amyloid precursor protein APP in the neuronal plasma membrane. Peptides derived from APP of 39-43 amino acids spontaneously form oligomers and ultimately highly insoluble myeloid fibrils (Sipe and Cohen, 2000). Such beta amyloid fibrils are toxic to adjacent neurons due to their disruption of calcium homeostasis, leading to apoptosis. (2) AD also belongs to the tauopathy family of diseases, each characterized by progressive formation of tangled aggregates containing the microtubule stabilizing protein Tau (Holmes et al., 2014). The mechanisms of Tau aggregation are not fully understood, but involve its hyperphosphorylation by GSK3. Hyperphosphorylated Tau can then recruit healthy, normally phosphorylated Tau into aggregates and tangles (Alonso et al., 1996; Wang et al., 2013). Tau aggregation is toxic to cells, leading to the disruption of microtubules essential for molecular transport along axons, a key aspect of neuronal survival.

HSPs have been shown to be involved in the healthy processing of amyloid beta, which can be found associated with Hsp10, Hsp27, Hsp60, Hsp70, and Hsp90 (Maiti et al., 2014). Although it is unlikely that intracellular chaperones could directly interact with extracellular amyloid beta plaques, they may influence the chaperoning of APP in neurons (Maiti et al., 2014) or they could influence the proteolytic processing of amyloid plaques phagocytosed by brain resident macrophages. Hsp70 binds to Tau reducing its hyperphosphorylation, decreasing aggregation and promoting Tau binding to microtubules (Sarkar et al., 2008). Hsp90 plays a similar role in Tau homeostasis (Sarkar et al., 2008). Degradation of Tau was observed after treatment with Tau-derived synthetic peptides and appeared to involve the substitution of Hsp70 associated with tau oligomers by Hsp90 (Thompson et al., 2012). Hsp90 rather than Hsp70 appeared to play a primary role in Tau degradation through the proteasome (Thompson et al., 2012). In addition, Hsp27 was shown to rescue neuronal deficits in *tau* knockout mice. These effects of Hsp27 appeared to involve its phosphorylation-dependent chaperoning capacity, as mutation of the key phosphorylation sites in the chaperone ablated its ability to rescue the *tau* transgenics (Abisambra et al., 2010). Each HSP family member then can participate in the deterrence of AD lesions in cells or rodent models of the disease.

It is notable that the most common neurodegenerative diseases: AD, Huntington's disease and Parkinson's disease, each associated with protein folding disorder, occur as the organism ages (Calderwood et al., 2009). In addition, although each disease type involves distinct proteins with dominantly aggregating properties (respectively—Tau, huntingtin, alpha-synuclein), neurodegenerative symptoms develop with aging in each disease (Calderwood et al., 2009). This time-dependent

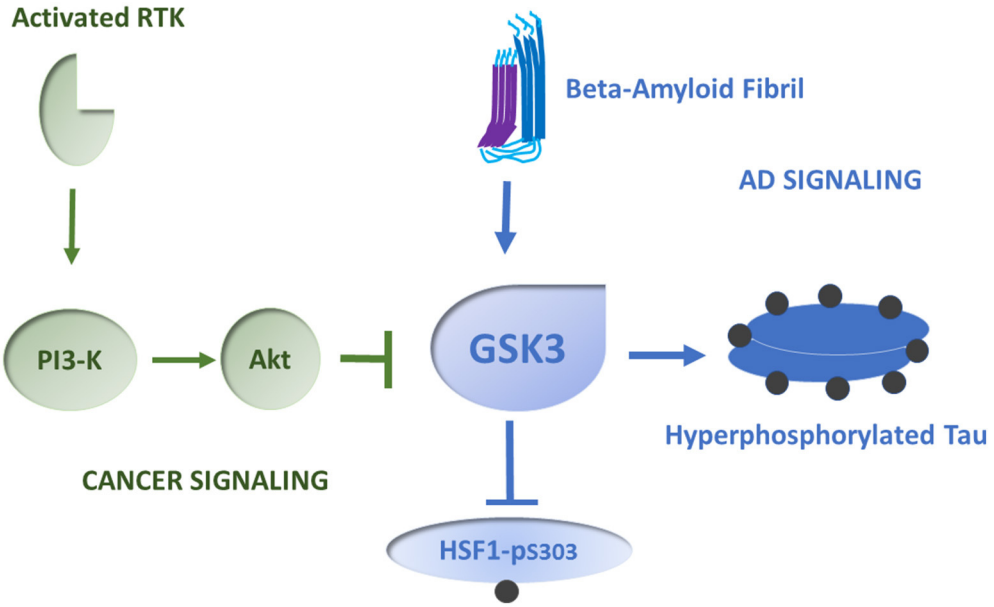

FIGURE 2 | Role of GSK3 in HSF1 regulation in cancer and AD. We depict GSK3 as a key HSF1 repressor that may govern its activity in cancer and AD. In many types of cancer, GSK3 becomes inhibited when the enzyme Akt is induced by a cascade response involving activated receptor tyrosine kinases (RTK), and phosphatidylinositol-3-kinase (PI-3K). Akt phosphorylates and inactivates GSK3. Thus, HSF1 is relieved of repression and can induce HSPs in tumors. In AD, GSK3 shows increased levels of activity, leading to HSF1 repression. This mechanism may involve activation of GSK3 downstream of aggregated amyloid beta fibrils. Concomitantly Tau is hyperphosphorylated by a mechanism involving active GSK3, leading to Tau aggregation. Phosphorylated residues on HSF1 and Tau are suggested by dark spheres.

window for induction of neurodegenerative diseases has been attributed to a decline in the proteotoxic stress response with age, permitting the phenotype of the dominantly aggregating proteins to become revealed (Sherman and Goldberg, 2001; Hands et al., 2008; Winklhofer et al., 2008).

HSP REGULATION IN THE BRAIN AND ENSUING DYSREGULATION IN AD

There appears to be an overall decline in protein quality control pathways and HSP synthesis with aging in many tissues, including the brain, muscle and liver, as reviewed in Calderwood et al. (2009). Studies in invertebrates have identified HSF1 as a significant longevity factor and, for instance this factor plays a key role in the enhanced longevity mediated by dietary restriction and its inactivation reduces lifespan in *C. elegans* (Hsu et al., 2003; Steinkraus et al., 2008). HSF1 mediated chaperone synthesis may thus promote longevity by maintaining protein folding capacity (Hsu et al., 2003). It has been shown that HSF1 is of limited activity in neuronal cells in tissue culture, suggesting that these tissues may be critically sensitive to age-dependent declines in HSP inducibility and ability to respond to folding deficits (Batulan et al., 2003). Interestingly these effects appear to be mediated by the regulatory domain of HSF1 (**Figure 1**) and deletion of this region led to renewed ability of the factor to promote transcription in a neuronal cell setting (Batulan et al., 2003). Interestingly the regulatory domain contains a repressive phosphorylation site for GSK3 and removal of this site would lead to relief of repression (**Figure 2**). GSK3 is increased in activity in the aging brain and is of critical importance in AD, as this is the principal kinase that leads to Tau phosphorylation and tangle formation (Hooper et al., 2008). Thus, elevated GSK3 activity may offer double jeopardy for AD, in promoting Tau pathology while repressing HSF1 and the heat shock response. In both HSF1 and Tau, GSK3 phosphorylation is associated with recruitment of 14-3-3 adapter proteins that leads in the case of HSF1 to transcriptional repression and in Tau to increased fibril formation (Wang et al., 2003; Qureshi et al., 2013). Another kinase that impacts HSF1 is mTOR (Chou et al., 2012). It was shown that, although mTOR can activate HSF1, increased HSF1 activity seemed to lead, conversely to decreases in mTOR signaling (Bandhakavi et al., 2008). Increases in mTOR activity are regarded as important in AD pathology and HSF1 may thus be important in protection against such mTOR-mediated morbidity (Wang C. et al., 2014). In addition, HSF1, Hsp60, Hsp70, and Hsp90 were each expressed at low levels in the cerebella of AD rats (Jiang et al., 2013). Overexpression of HSF1 was shown to increase HSP levels in the cerebellum and lead to an increase in the number of Purkinje cell bodies in the brains of mouse models of AD (Jiang et al., 2013). Another key HSF1 regulator is the histone deacetylase sirtuin 1, a key longevity factor which deacetylates HSF1 and increases its binding to HSP promoters (**Figure 1**). Sirtuin 1 mRNA and protein levels are known to be reduced in the brains of AD patients concomitantly with Tau accumulation, suggesting a further route to HSF1

malfunction in AD (Julien et al., 2009). Another mechanism involved in loss of HSF1 activity could be increased HSF1 degradation due to the ubiquitin E3 ligase NEDD4. Degradation of HSF1 through the NEDD4 pathway was antagonized when HSF1 was deacetylated by sirtuin1 (Kim et al., 2016). Aggregated alpha-synuclein was shown to trigger this pathway targeting HSF1 in transfected neuroblastoma cells. It might be rewarding to study the potential role of this pathway in tauopathies such as AD, particularly in light of the known reduction in Sirt1 levels in clinical AD. (Julien et al., 2009). HSF1 is also known to have non-HSP transcriptional targets and these may be important in AD (Khaleque et al., 2008). HSF1 activates transcription of transthyretin (TTR) a protein that can impact symptoms in Mouse AD model by inhibiting beta-amyloid aggregation and detoxifying the amyloid oligomers (Wang X. et al., 2014).

All in all then, the balance of evidence suggests that the activity of HSF1 and the levels of HSPs are depleted in AD. It would however, be desirable to obtain a fuller picture of dysregulation of the heat shock response in the AD brain.

DISCUSSION- COMPARING THE RECIPROCAL DYSREGULATION OF THE HEAT SHOCK RESPONSE IN CANCER AND AD

Although cancer and AD are diseases encountered later in life, HSP metabolism is altered in different directions in each case. These findings are consonant with epidemiological studies showing a negative correlation between the risk of cancer in persons with AD (Roe et al., 2005; Driver, 2014). These differences may in some ways reflect the gulf between terminally differentiated neurons in AD brains, accumulating a lifetime's damage to DNA and proteins, and de-differentiating, evolving cancer cells that maintain the ability to proliferate and renew. However, in each case there appears to be an acquired folding deficit, with increases in unfolded and aggregated proteins (Sherman and Goldberg, 2001; Winklhofer et al., 2008; Calderwood and Gong, 2016). Despite this, HSP levels respond by increasing in cancer and declining in AD. Thus, the simple hypothesis of HSF1 induction due to loss of feedback repression by Hsp70 or Hsp90 does not help us with understanding the changes in chaperone expression encountered in AD. One potential unifying thread explaining some of the differences in cancer and AD might be the kinase GSK3, the repressor of HSF1 (Chu et al., 1998; **Figure 2**). In malignant diseases, many tumorigenic pathways, such as those conferred by oncogenic receptors PDGF-R, EGF-R, and HER2, by widespread increases in PI-3 kinase and loss of the phosphatase PTEN often encountered in cancer, converge on the kinase Akt that is a GSK3 repressor (Yuan and Cantley, 2008; Chalhoub and Baker, 2009). Indeed, HSF1 can be activated directly in mammary cancer through the Her2 pathway by activated Akt which mediates GSK3 inhibition (Khaleque et al., 2005). Highly malignant mammary cancer stem cells contain very low levels of the GSK3 target HSF1-phospho-S303, consonant with the findings of that levels of Hsp70 are required for stemness (Chou et al., 2015). GSK3 is also intimately involved in the pathology of AD, with increases in GSK3 activity contributing to the multiple areas of neuronal pathology associated with the disease (Hooper et al., 2008; Kremer et al., 2011). GSK3 plays the key role in Tau phosphorylation and aggregation and may be involved in coupling the APP / beta amyloid pathway to Tau pathology (Kremer et al., 2011; **Figure 2**).

The pharmacological targeting of HSP activity in each disease is currently under investigation. In cancer, Hsp90 inhibitors have been extensively tested as therapeutics, although problems with normal tissue toxicity currently limit their clinical application (Workman et al., 2007). There is also interest in targeting HSP interactions with Tau in AD, and both Hsp70 and Hsp90 have recently been investigated (Jinwal et al., 2010; Thompson et al., 2012). This approach may ultimately have considerable promise for each disease, although it is currently limited by the toxicity to healthy tissues that may accrue when whole families of chaperones are targeted by inhibitors. Drugs specific for individual members of the HSP families would offer the promise of specificity and sparing of healthy tissues, although this approach may also be conceptually difficult due to the high levels of conservation among the chaperone family members (Lindquist and Craig, 1988).

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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CSPα, a Molecular Co-chaperone Essential for Short and Long-Term Synaptic Maintenance

Elena Lopez-Ortega[1†], *Rocío Ruiz*[2] *and Lucia Tabares*[1*]

[1] *Department of Medical Physiology and Biophysics, School of Medicine, University of Seville, Seville, Spain,* [2] *Department of Biochemistry and Molecular Biology, School of Pharmacy, University of Seville, Seville, Spain*

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Janice Eva Arlee Braun, University of Calgary, Canada
Konrad Ernst Zinsmaier, University of Arizona, USA

***Correspondence:**
Lucia Tabares
ltabares@us.es

†Present Address:
Elena Lopez-Ortega, Solomon H. Snyder Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD, USA

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Cysteine string protein α (CSPα) is a vesicle protein located in the presynaptic terminal of most synapses. CSPα is an essential molecular co-chaperone that facilitates the correct folding of proteins and the assembly of the exocytic machinery. The absence of this protein leads to altered neurotransmitter release and neurodegeneration in multiple model systems, from flies to mice. In humans, CSPα mutations are associated with the development of neuronal ceroid lipofuscinosis (NCL), a neurodegenerative disease characterized by intracellular accumulation of lysosomal material. Here, we review the physiological role of CSPα and the pathology resulting from the homozygous deletion of the gene or its mutations. In addition, we investigate whether long-term moderate reduction of the protein produces motor dysfunction. We found that 1-year-old CSPα heterozygous mice display a reduced ability to sustain motor unit recruitment during repetitive stimulation, which indicates that physiological levels of CSPα are required for normal neuromuscular responses in mice and, likely, in humans.

Keywords: cysteine string protein, co-chaperone, motor neurons, synaptic transmission, neuromuscular junction, CSPα

PROTEIN DESCRIPTION

Cysteine string protein α (CSPα) (*Dnajc5*) is a highly conserved protein (**Figure 1A**) typically associated with the membrane of synaptic vesicles and secretory granules (Zinsmaier et al., 1990). It contains a DNA-J domain characteristic of Hsp40 co-chaperones. This domain interacts with the 70 kDa heat shock cognate protein (Hsc70) (Braun et al., 1996) and regulates the refolding of client proteins (Hennessy et al., 2005). A linker region connects the DNA-J domain with the cysteine string domain. The cysteine string domain is approximately 25-amino-acids long and contains 13–15 cysteines, most of them palmitoylated. Palmitoylation is essential to target CSPα to synaptic vesicles and to promote neurotransmitter release (Arnold et al., 2004). CSPα also contains a C-terminal domain, the function of which is not well-understood.

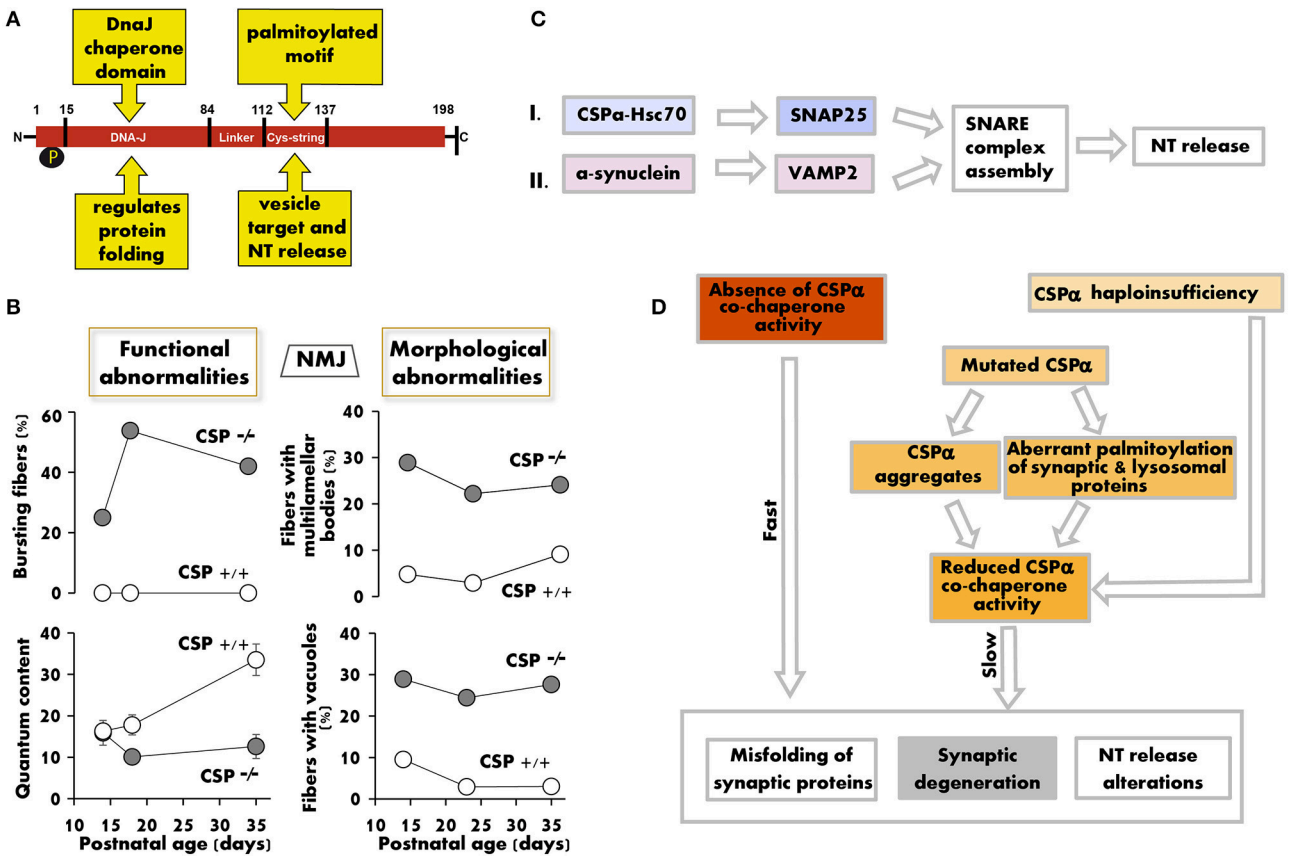

FIGURE 1 | The lack/decrease of CSPα induces structural and functional changes that compromise synaptic maintenance. (A) Functional domains of the CSPα protein. **(B)** Synaptic release defects (left) and fraction of motor nerve terminals with multilamellar bodies and vacuoles in electron microscopy profiles (right) vs. postnatal age in CSPα KO mice. Graphs summarize numerical values in Ruiz et al. (2008, 2014) (left) and in Fernández-Chacon et al. (2004) (right). **(C)** Two parallel chaperone pathways promote efficient SNARE complex formation and normal neurotransmission by regulating SNAP25, through CSPα (pathway I), and synaptobrevin (VAMP2), through α-synuclein (pathway II). The deficit in CSPα produces synaptic dysfunction (Ruiz et al., 2008, 2014; Rozas et al., 2012), whose severity, age of onset, and time course depend on the amount of functional CSPα available. α-synuclein overexpression, however, can avoid the synaptic pathology produced by a defect in pathway I by increasing the formation of SNARE complexes (Sharma et al., 2011, 2012a). **(D)** Hypothetical model of how genetic ablation, gene mutations, or increased CSPα degradation induce a positive loop of neurotransmitter (NT) release deficit, accumulation of misfolded synaptic proteins, and neurodegeneration. In humans, it remains unknown to what extent CSPα haploinsufficiency, sequestration of CSPα in aggregates, and aberrant palmitoylation of CSPα and other proteins contribute to ANCL.

CSPα DEFICIENCY AND SYNAPTIC DYSFUNCTION

CSPα is not essential for synaptogenesis, but it is required for normal neurotransmission and neuronal maintenance in flies (Zinsmaier et al., 1994), worms (Kashyap et al., 2014), mice (Fernández-Chacon et al., 2004), and humans (Nosková et al., 2011). Deletion of the CSPα gene in *Drosophila* produces an embryonic semilethal phenotype, and flies that survive to adulthood present neurotransmitter release alterations and temperature-sensitive paralysis. Synaptic defects in CSPα-null (CSPα KO) mice start early after birth, and death occurs before 3 months of age. Both motor and sensory neurons are affected by the lack of CSPα (Fernández-Chacon et al., 2004; Schmitz et al., 2006). In motor nerve terminals, the first sign of functional alteration appears at 2 weeks of age and consists of repeated bursts of high-frequency spontaneous release (Ruiz et al., 2008) (**Figure 1B**). Only 4 days later, the terminal displays multiple functional alterations such as reduced quantum content, low release probability, increased short-term facilitation during repetitive stimulation, and reduced calcium sensitivity of the secretory machinery (Ruiz et al., 2008, 2014). In addition, the size of the readily releasable pool of synaptic vesicles is decreased (Rozas et al., 2012; Ruiz et al., 2014).

CSPα AS A MOLECULAR CO-CHAPERONE

CSPα interacts with several proteins that participate in exo-/endocytosis, including syntaxin, synaptotagmin, N- and P/Q-type calcium channels, and dynamin 1 (for a review see Burgoyne and Morgan, 2015). The functional significance of many of these interactions is not well established. However,

one of the best-known functions of CSPα is its role as a co-chaperone (Chamberlain and Burgoyne, 2000; Zinsmaier, 2010; Donnelier and Braun, 2014). CSPα interacts with Hsc70 and together refold client proteins such as SNAP25 (Sharma et al., 2011, 2012a; Zhang et al., 2012). SNAP25 is required for the assembly of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment receptor) complex, formed by synaptobrevin, syntaxin, and SNAP25, which in turn is essential for exocytosis and neurotransmitter release (**Figure 1C**). In CSPα KO mice, both SNAP25 and SNARE complex levels are reduced by half (Chandra et al., 2005).

CSPα AND NEURODEGENERATION

In CSPα KO motor nerve terminals, hallmarks of degeneration (i.e., vacuoles and multilamellar bodies) appear very early (Fernández-Chacon et al., 2004), even before the evoked release defects become apparent (**Figure 1B**, left graphs). The degeneration is more prominent in cells with high electrical activity, such as motor neurons, photoreceptors, and GABAergic neurons (Fernández-Chacon et al., 2004; Schmitz et al., 2006; García-Junco-Clemente et al., 2010), suggesting that high synaptic activity potentiates degeneration, which in turn may increase the release deficit (**Figure 1D**).

At the molecular level, it has been proposed that SNAP25 reduction plays a major role in neurodegeneration. This hypothesis is reinforced by the fact that SNAP25 overexpression in CSPα KO mice prevents neurodegeneration (Sharma et al., 2012a). Nevertheless, SNAP25 heterozygous mice, with 50% of the protein, are phenotypically normal and do not develop neurodegeneration (Washbourne et al., 2002), indicating that solely reducing functional SNAP25 is not sufficient to produce the pathology. On the other hand, misfolded SNAP25 could have a dominant negative effect over the normally folded protein copies, or ubiquitinated SNAP25 molecules could accumulate in the proteasome, interfering with its normal function. Surprisingly, however, the fact that pharmacological inhibition of the proteasome increases SNAP25 and SNARE complex levels, and, hence, improves synaptic function in CSPα-depleted cells (Sharma et al., 2012b), has challenged this last hypothesis.

Remarkably, neurodegeneration in CSPα KO mice is prevented when SNARE complexes are increased by the overexpression of α-synuclein (Sharma et al., 2011, 2012a), in spite of the fact that SNAP25 levels are not restored and, presumably, the amount of misfolded SNAP25 is not reduced. Overexpression of the mutated form of α-synuclein A53T in CSPα KO mice restores life span and motor function as well as wild-type (WT) α-synuclein (Chandra et al., 2005). On the other hand, overexpression of A30P α-synuclein does not rescue survival, but can transitorily ameliorate the release deficit and the calcium sensitivity defect in motor nerve terminals of CSPα KO mice (Ruiz et al., 2014). The partial effect of A30P α-synuclein is likely due to its limited ability to increase the formation of SNARE complexes. These findings suggest that two parallel pathways regulate SNARE complex formation (**Figure 1C**), and raise the question of how the neurodegeneration program is activated in the absence of CSPα. The molecular basis of the neurodegeneration is unknown, but a possibility is that neurodegeneration is linked to the reduced ability of the synapse to form SNARE complexes and, therefore, to the mismatch between functional demands and efficient release.

CSPα DEFICIENCY IN HUMANS

Neuronal Ceroid Lipofuscinosis (NCLs) constitute a heterogeneous group of inherited neurodegenerative disorders characterized by lysosomal accumulation of autofluorescent ceroid-lipofuscin aggregates in neurons and other cell types. The clinical symptoms of NCLs include seizures, movement disorders, cognitive deterioration, and progressive dementia, followed by a premature death. The majority of NCL cases affect children, and only 10% of total cases are in adults.

In recent years, two mutations in the gene that encodes CSPα, *DNAJC5*, have been linked to the development of adult-onset NCL (ANCL) (MIM #162350). These mutations consist of a point mutation (p.L115R) and an in-frame codon deletion (p.L116Δ), both affecting dileucine residues located in the cysteine string domain of CSPα (Nosková et al., 2011). This domain is highly palmitoylated and mediates the membrane binding and intracellular targeting of CSPα. Therefore, mutations in this region may explain the diffuse intracellular localization of CSPα observed in the neurons of ANCL patients. Moreover, the mutated forms of CSPα present an increased tendency to self-associate, forming detergent-resistant aggregates. These aggregates interfere with WT CSPα proteins, reducing the co-chaperone function of CSPα in neurons (Nosková et al., 2011; Greaves et al., 2012; Zhang and Chandra, 2014) (**Figure 1D**), which is likely one of the main reasons why the NCL-linked *DNAJC5* gene mutations display an autosomal dominant (AD) inheritance pattern. Additionally, a dominant effect of mutated CSPα on the palmitoylation pattern of lysosomal and synaptic proteins has been suggested as a mechanism for the development of *DNAJC5*-linked ANCL (Henderson et al., 2016).

LONG-TERM MODERATE CSPα DEFICIENCY ALTERS MOTOR RESPONSES

Interestingly, only homozygous CSPα KO mice present synaptic defects, while heterozygous mutant mice appear phenotypically normal up to 3 months of age (Fernández-Chacon et al., 2004). Given the late onset of AD-ANCL (around 30 years of age), we studied the motor function of 1-year-old CSPα heterozygous mice. Motor performance was first assessed with Balance and Grip Strength tests (**Figure 2A**). The balance was measured by placing the mouse on a horizontal pole suspended in the air. The pole was rotated manually at a constant speed of one rotation cycle per second (**Figure 2A** left). The grip strength test consisted of suspending the mouse from the pole by its forelimbs (**Figure 2A** right). In both trials, the amount of time the mouse remained suspended from the pole (maximum 10 s)

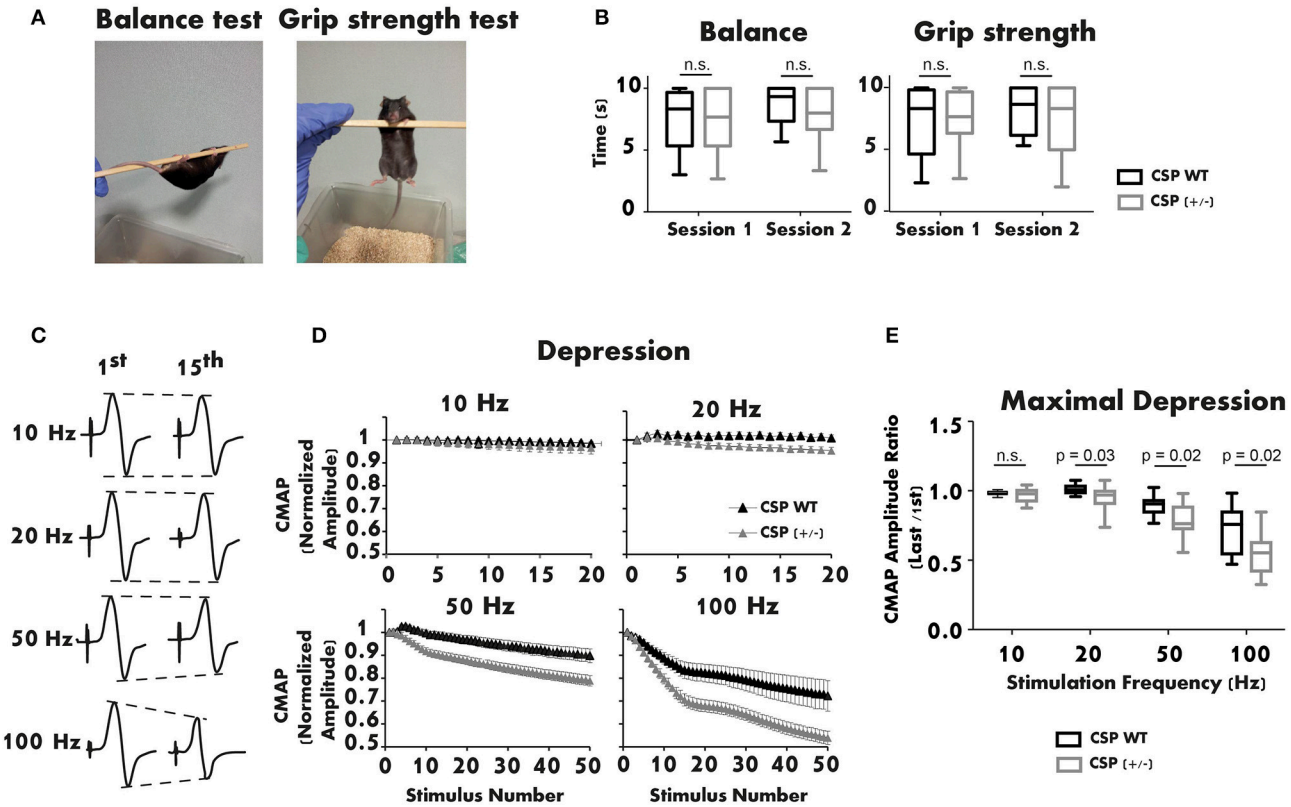

FIGURE 2 | The neuromuscular function of 1-year-old CSPα heterozygous mice is altered. (A,B) Balance and grip strength tests show no significant differences between CSPα heterozygous ($n = 27$) and WT mice ($n = 9$). The plot illustrates the average values obtained in three replicates/session for each genotype (Mann-Whitney U test). **(C)** Representative recordings of CMAPs in a CSPα$^{+/+}$ mouse after supramaximal stimulation of the sciatic nerve (1st and 15th responses at different stimulation frequencies). **(D)** Depression of the neuromuscular responses (CMAP normalized amplitude) is significantly larger in CSPα$^{+/-}$ ($n = 25$) than in WT mice ($n = 7$) during stimulation trains at 20, 50, and 100 Hz. **(E)** Maximal depression of the neuromuscular response (normalized) at different stimulation frequencies in CSPα$^{+/-}$ ($n = 24$–25) and WT mice ($n = 7$) (Mann-Whitney U test). Either sex experimental mice (C57BL/6 background) were used. The mouse line was kindly donated by Dr. Südhof. All experiments were performed according to the guidelines of the European Council Directive for the Care of Laboratory Animals. The protocol was approved by the Ethics Committee for Animal Experimentation of the Junta de Andalucía (ref. 23-11-2015-364).

was measured. Two sessions separated by 1 week were performed for each test (**Figure 2B**). Data obtained from the neurological tests showed no significant differences between WT and CSPα heterozygous mice, in either balance or grip strength.

Next, the neuromuscular response of CSPα heterozygous mice was studied using electromyography (EMG). Evoked Compound Motor Action Potentials (CMAPs) were recorded from the right lateral gastrocnemius of anesthetized mice. Stimulation needle electrodes were placed at the sciatic notch and the head of the fibula (Ruiz et al., 2005). The active recording electrode was placed in the medial region of the recorded muscle. The reference electrode was inserted at the base of the fifth foot phalanx. The ground electrode was located at the base of the tail. Brief supramaximal stimulation pulses were applied at 10 Hz (2 s), 20 Hz (1 s), 50 Hz (1 s), and 100 Hz (0.5 s). Representative recordings of CMAPs registered during the trains are shown in **Figure 2C**. The study revealed enhanced depression with stimulations between 20 and 100 Hz in CSPα heterozygous mice compared to WT littermates (**Figure 2D**). The mean maximal depression was 5% (20 Hz), 12% (50 Hz), and 25% (100 Hz) lower in CSPα heterozygous than in WT mice, while no significant difference was observed at 10 Hz (**Figure 2E**). Remarkably, the depression in the EMG recordings was similar to that seen in 3-week-old CSPα KO mice (Fernández-Chacon et al., 2004), a phenotype not observed in CSPα$^{+/-}$ mice up to 3 month of age. These results indicate that, over time, a moderate reduction of CSPα expression alters the ability of the neuromuscular system to respond normally to stimulation.

FUTURE DIRECTIONS

The multiple functions of CSPα range from acting as a chaperone, participating in the assembly and dissociation of multi-protein complexes, and regulating Ca^{2+} sensitivity for neurotransmitter release. The severe functional and structural changes that take place in the absence of CSPα in invertebrate and vertebrate organism models confirm the importance of this protein in synapse maintenance and neurotransmitter release. In humans, CSPα mutations are associated with the development of AD-ANCL, synaptic degeneration, and neuronal

loss. Therefore, although both the reduction of CSPα expression and the presence of CSPα mutations are pathogenic to the synapse, the severity and time course of the neurological impairments may vary from severe, including premature death, to mild, depending on the amount of functional CSPα in each case. The moderate decrease in CSPα and SNARE complexes in neurons over time could result in motor function impairment and, in addition, influence the evolution of common age-related neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases. Future challenges are to identify patients with reduced levels of molecular chaperones (such as CSPα), decipher the mechanisms responsible for the molecular deficit, understand how the homeostasis of the synapse is altered, and determine to what extent the reduction of the chaperones influences the severity of associated neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

Experiments shown in **Figure 2** were performed by EL. EL, RR, and LT conceived and wrote the manuscript.

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REVIEW
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The Role of Co-chaperones in Synaptic Proteostasis and Neurodegenerative Disease

Erica L. Gorenberg[1] *and Sreeganga S. Chandra*[2,3]*

[1] Interdepartmental Neuroscience Program, Yale University, New Haven, CT, United States, [2] Department of Neurology, Yale University, New Haven, CT, United States, [3] Department of Neuroscience, Yale University, New Haven, CT, United States

Synapses must be preserved throughout an organism's lifespan to allow for normal brain function and behavior. Synapse maintenance is challenging given the long distances between the termini and the cell body, reliance on axonal transport for delivery of newly synthesized presynaptic proteins, and high rates of synaptic vesicle exo- and endocytosis. Hence, synapses rely on efficient proteostasis mechanisms to preserve their structure and function. To this end, the synaptic compartment has specific chaperones to support its functions. Without proper synaptic chaperone activity, local proteostasis imbalances lead to neurotransmission deficits, dismantling of synapses, and neurodegeneration. In this review, we address the roles of four synaptic chaperones in the maintenance of the nerve terminal, as well as their genetic links to neurodegenerative disease. Three of these are Hsp40 co-chaperones (DNAJs): Cysteine String Protein alpha (CSPα; DNAJC5), auxilin (DNAJC6), and Receptor-Mediated Endocytosis 8 (RME-8; DNAJC13). These co-chaperones contain a conserved J domain through which they form a complex with heat shock cognate 70 (Hsc70), enhancing the chaperone's ATPase activity. CSPα is a synaptic vesicle protein known to chaperone the t-SNARE SNAP-25 and the endocytic GTPase dynamin-1, thereby regulating synaptic vesicle exocytosis and endocytosis. Auxilin binds assembled clathrin cages, and through its interactions with Hsc70 leads to the uncoating of clathrin-coated vesicles, a process necessary for the regeneration of synaptic vesicles. RME-8 is a co-chaperone on endosomes and may have a role in clathrin-coated vesicle endocytosis on this organelle. These three co-chaperones maintain client function by preserving folding and assembly to prevent client aggregation, but they do not break down aggregates that have already formed. The fourth synaptic chaperone we will discuss is Heat shock protein 110 (Hsp110), which interacts with Hsc70, DNAJAs, and DNAJBs to constitute a disaggregase. Hsp110-related disaggregase activity is present at the synapse and is known to protect against aggregation of proteins such as α-synuclein. Congruent with their importance in the nervous system, mutations of these co-chaperones lead to familial neurodegenerative disease. CSPα mutations cause adult neuronal ceroid lipofuscinosis, while auxilin mutations result in early-onset Parkinson's disease, demonstrating their significance in preservation of the nervous system.

Keywords: Hsp110, HSP70, neurodegeneration, proteostasis, synapse maintenance, endocytosis, exocytosis

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***Correspondence:**
Sreeganga S. Chandra
sreeganga.chandra@yale.edu

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PROTEIN FOLDING AND CHAPERONES

Proteins must fold into unique three-dimensional structures to fulfill their biological functions. As proteins are inherently structurally dynamic, they are apt to misfold into conformations that prevent their function or lead to toxic aggregates (Bukau et al., 2006; Hartl et al., 2011). This potential for misfolding can be exacerbated by cellular stress, such as that caused by heat shock or hypoxia (Bukau et al., 2006; Hartl et al., 2011). Chaperones are a diverse set of evolutionarily conserved proteins that function to assist in protein folding, assembly, and stability, thereby ensuring homeostasis of the proteome (proteostasis). Chaperones comprise up to 10% of the mass of human cells and unfold, dissociate, refold, or degrade the other 90% of cellular proteins as required (Finka et al., 2016). Chaperones act throughout the lifetime of a given protein, first assisting in folding the nascent polypeptide to its native conformation, then surveilling metastable/misfolded intermediates and disaggregating aggregated proteins, and finally removing terminally aggregated proteins through proteolytic degradation.

Chaperone members are often referred to as stress proteins or heat shock proteins (Hsps) because they are upregulated in conditions of conformational stress. The major chaperone families are classified by molecular weight (Hsp40, Hsp60, Hsp70, Hsp90, Hsp100, and the small Hsp; reviewed in Jee, 2016). All chaperone families, except for the small Hsps, catalyze ATP-dependent processes. The different classes of chaperones perform diverse cellular functions that are coordinated and integrated into a proteostasis network to achieve proper protein and cellular homeostasis. For instance, Hsp70s work in conjunction with Hsp40 co-chaperones (also known as DNAJ or J proteins) and will be discussed in detail below (Otto et al., 2005). The Hsp90s not only support protein folding, but also assist in conformational maturation and maintenance. Hsp90 coordinates with the Hsp70 system through the Hsp70-Hsp90 Organizing Protein (HOP) co-chaperone (Chen and Smith, 1998). Eukaryotic Hsp60 class chaperone TriC is a nano-cage that aids in the folding of cytoskeletal proteins such as actin and tubulin (Frydman et al., 1992). Disaggregases containing the Hsp100 family member Hsp110 are upregulated during proteomic stress when proteins misfold and aggregate. Small Hsps exhibit chaperone-like activity in preventing aggregation of target proteins, maintaining them in a folding–competent state and refolding them independently or in concert with other ATP-dependent chaperones such as Hsp70s (McGreal et al., 2012). For a more comprehensive review on small Hsps, the authors suggest Strauch and Haslbeck (2016).

Chaperone families are compartmentalized within the cell, with unique members in distinct sub-compartments to most efficiently carry out their protein folding functions. Hsp70s and Hsp90s are ubiquitous cytosolic chaperone proteins whose specific localization is determined by their partner Hsp40 co-chaperones. Mitochondria-specific chaperones include mHsp70 and Hsp60 that are involved in importing mitochondrial proteins into the matrix and folding them (Manning-Krieg et al., 1991). Another complement of chaperones is exclusive to the endoplasmic reticulum (ER), the site of synthesis and folding of membrane and secretory proteins. There, ER chaperones such BiP and the ER Hsp70 function to prevent stress from elevated levels of unfolded proteins. Recent papers have highlighted the importance of the chaperones involved in the unfolded protein response at the ER in neuronal proteostasis and neurodegenerative disease (Hetz et al., 2015). Finally, Hsp100s and small Hsps are nearly ubiquitous in terms of cellular localization (Haslbeck and Vierling, 2015; Zuo et al., 2016).

UNIQUE REQUIREMENTS FOR SYNAPTIC PROTEOSTASIS

Synapses are specialized junctions that connect neurons into circuits. Synapses must be maintained throughout an organism's life for appropriate brain function and behavior. Chemical synapses consist of a presynaptic compartment from which neurotransmitter is released into a synaptic cleft, juxtaposed to a postsynaptic compartment, with receptors for the corresponding neurotransmitters. The postsynaptic neuron transduces neurotransmitter signals and propagates them as action potentials. Synaptic transmission is noted for its speed (on the order of milliseconds), precision, and spatial control. Synaptic functions require a large complement of specialized proteins, such as neurotransmitter receptors, synaptic vesicle exo- and endocytosis proteins, and organizers of the active zone and post synaptic density, all of which are essential for neurotransmission. Synaptic proteins are under constant proteostatic stress, as they execute reactions that require precise conformational changes and protein-protein interactions at high frequencies. It has also been noted that synapses are enriched in proteins susceptible to misfolding and aggregation, such as α-synuclein, tau, and amyloid-β, all of which are linked to neurodegenerative diseases (Schubert et al., 1991; Fortin et al., 2004; Kramer and Schulz-Schaeffer, 2007; Greten-Harrison et al., 2010; Schulz-Schaeffer, 2010). In addition, synapses can be at great distances from the neuronal cell body, where the majority of proteins are synthesized and properly folded. Though there is strong evidence that a small subset of proteins is locally translated at postsynaptic spines (Weiler et al., 2004) and similar emerging data for the presynaptic termini (Shigeoka et al., 2016; Younts et al., 2016), most newly-synthesized proteins must be transported from the cell body along the full length of the axon. The slow rates of axonal transport are not ideal for replacing misfolded or dysfunctional synaptic proteins on demand. Due to the sustained nature of neurotransmission, the synaptic proteome is perilously prone to protein misfolding for the reasons stated above, yet perseveration of the proper structure and function of synaptic proteins is fundamentally important to the health and survival of neurons.

To maintain their specialized structure and function, neurons possess dedicated synapse-specific proteostasis machinery localized to pre- and post-synaptic compartments, of which chaperones are an integral component. In fact, 4% of the synaptic vesicle compartment proteome is composed of chaperones

TABLE 1 | Summary of chaperones identified at the synapse.

Synaptic chaperone	Method of identification	Reference
HSP70s AND 90s		
Hsc71	1-D SDS; PPF extraction	Phillips et al., 2005; Burré et al., 2006
Hsp70	DIGE; PPF extraction	Phillips et al., 2005; Zhang et al., 2012
Hsc70	DIGE/iTRAQ; PPF extraction	Phillips et al., 2005; Zhang et al., 2012
Hsp90	DIGE	Zhang et al., 2012
Hsp70-4	iTRAQ	Zhang et al., 2012
Hsp84	dSDS	Burré et al., 2006
Hspa5	DIGE	Zhang et al., 2012
HspA4/A4L	iTRAQ	Zhang et al., 2012
Hsp8	dSDS; PPF extraction	Phillips et al., 2005; Burré et al., 2006
HSP40s/DNAJs		
DNAJC6 (auxilin)	1-D SDS	Burré et al., 2006
DNACJ5 (CSPα)	iTRAQ	Takamori et al., 2006; Zhang et al., 2012
DNAJ homologs	PPF extraction	Phillips et al., 2005
Hsp40-3 cognate	PPF extraction	Phillips et al., 2005
DNAJA	DIGE/iTRAQ	Zhang et al., 2012
Hsp40 (DNAJB1)	Western Blot	Suzuki et al., 1999
HSP60		
Chaperonin TriC	DIGE	Zhang et al., 2012
HSP100s		
Hsp105/110	iTRAQ	Zhang et al., 2012
OTHER CO-CHAPERONES		
HSF binding protein 1	PPF extraction	Phillips et al., 2005
Calnexin	1-D SDS	Burré et al., 2006
CCT family	1-D SDS	Burré et al., 2006
FLJ10737 cognate	PPF extraction	Phillips et al., 2005
HOP	DIGE	Zhang et al., 2012
HIP	DIGE/iTRAQ	Zhang et al., 2012

Abbreviations: 1-D SDS, One dimensional sodium dodecyl sulfate electrophoresis; PPF, post-synaptic particle fraction; DIGE, 2D fluorescence Difference Gel Electrophoresis; iTRAQ, Isobaric Tag for Relative and Absolute Quantitation.

(Phillips et al., 2005; Burré et al., 2006; **Table 1**). The chaperones found at the synapse include Hsp60, Hsp70, and Hsp90 members: Hsc71, Hsp70, Hsp70-4, Hsc70, Hspa5, Hsp8, Hspa4, Hspa4l, inducible and constitutive Hsp90, as well as Hsp105/110, and heat shock factor binding protein 1 (Phillips et al., 2005; Burré et al., 2006; Zhang et al., 2012; Zhang and Chandra, 2014). The synaptic chaperone complement also includes Hsp40/DNAJ proteins such as DNAJC6 (auxilin), DNAJC5 (CSPα), cognate of Hsp40-3, DNAJA1 homologs, DNAJB1 and DNAJB2 (Stetler et al., 2010; **Table 1**). Autophagy is also a mechanism employed at synapses for the removal of misfolded proteins (Hara et al., 2006; Komatsu et al., 2006; Ariosa and Klionsky, 2016). As such, atg3, Atg16L1, LC3B, and Rab33B, which are essential for autophagy, have been found at synaptic termini (Binotti et al., 2015; Soukup et al., 2016). In this review, we will focus on Hsc70/Hsp40 chaperone complexes that are enriched at the synapse, their modes of action, and the mechanisms by which mutations in these genes cause neurodegenerative disease.

HSP70, HSC70, HSP40 PROTEINS

Stress-inducible Hsp70s and their constitutive relative, heat shock cognate 70 (Hsc70) are first responders to cellular stress and cases of misfolded protein accumulation. In the nervous system, induction of Hsp70 by stress is weak, therefore Hsc70 is the main Hsp70-class chaperone (Pardue et al., 1992; Marcuccilli et al., 1996; Kaarniranta et al., 2002; Batulan et al., 2003).

As the chief cytosolic chaperones, Hsp70/Hsc70s are involved in executing myriad ATP-dependent protein folding reactions. Hsp70/Hsc70s consist of a 44-kD, N-terminal nucleotide binding domain (NBD) that allows them to bind and hydrolyze ATP. The NBD is connected via a hydrophobic linker to a 25 kD substrate binding domain, that binds preferentially to hydrophobic sequences (Flaherty et al., 1990; Blond-Elguindi et al., 1993). Hsp70s can also contain an additional C-terminal domain that allows for interactions with their Hsp40 co-chaperones and refolding of specific substrates (Radons, 2016).

Hsc70 recognizes five- to seven-amino acid segments in protein substrates (also known as clients) that are enriched in hydrophobic residues likely to misfold, or in regions that are susceptible to β aggregation (Flynn et al., 1991; Blond-Elguindi et al., 1993; Behnke et al., 2016). Hsc70 is promiscuous in its binding to clients but has a nucleotide-dependent conformational cycle that regulates its association with clients and its chaperone activity (**Figure 1**; reviewed in Mayer, 2013). Hsc70 has rapid client binding-release activity when bound to ATP, and slow, inefficient binding and release rates when bound to ADP (Nollen et al., 2000; Kampinga and Craig, 2010). Hsc70 is dependent on its Hsp40 co-chaperones to accelerate the rate of ATP hydrolysis to facilitate client binding (discussed further below), and on NEFs to accelerate ADP-ATP exchange for client release (Bracher and Verghese, 2015). NEFs, such as Hsp110, are responsible for restoring Hsc70 to its active form. Each combination of Hsp40 and NEF is required at a different stoichiometric ratio with Hsc70 to stimulate maximum chaperone activity (Rauch and Gestwicki, 2014). For a comprehensive review of the Hsc70 NEFs, see Bracher and Verghese (2015).

Hsp40 co-chaperones (traditionally called DNAJ proteins or J proteins after *DnaJ* in *E. coli*) are crucial players in the Hsc70 conformational cycle (Rassow et al., 1995). Hsp40s stimulate Hsp70 ATP hydrolysis, the rate-limiting step, resulting in as much as a 150,000-fold increase in Hsp70's ATPase activity at 5°C (Russell et al., 1999). In addition, Hsp40s play crucial roles in targeting Hsp70s to distinct sub-compartments and binding clients, thus increasing the regional diversity and specificity of Hsc70/Hsp40 clients. Humans, for example, have 11 Hsp70s that interact with 41 Hsp40s, thereby generating a large cohort of potential combinatorial chaperone complexes (Kampinga and Craig, 2010). Hsp40s are broadly expressed

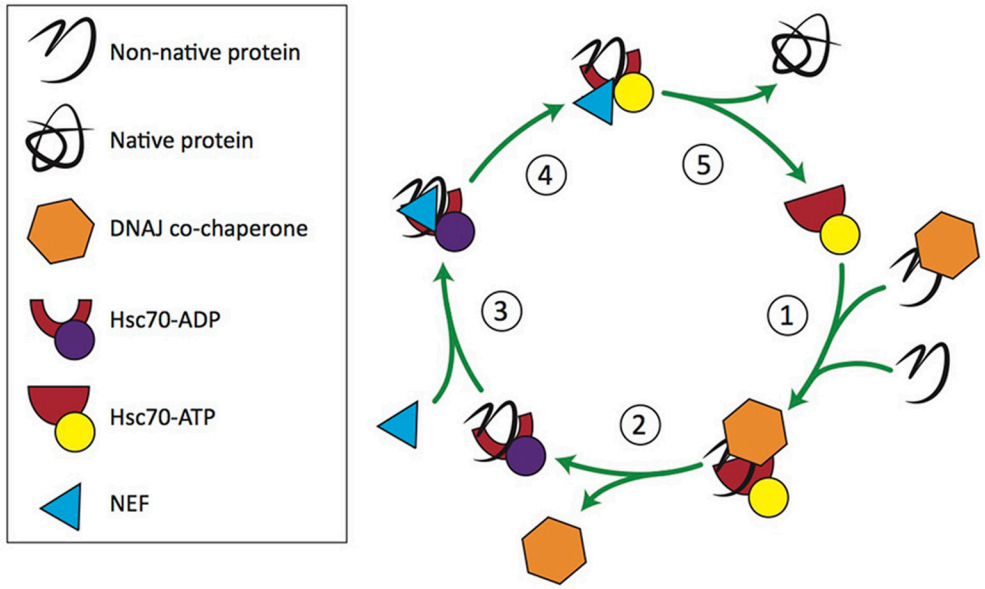

FIGURE 1 | Hsc70/DNAJ co-chaperone cycle. 1. Free clients or those recruited by DNAJ bind Hsc70-ATP. 2. DNAJ stimulates Hsc70's ATPase activity. 3. An Hsc70 nucleotide exchange factor (NEF) binds Hsc70-ADP. 4. NEF stimulates ADP-ATP exchange. 5. Hsc70-ATP releases the client and enters a new round of chaperone activity.

throughout the nervous system, and within neurons in sub-compartments ranging from the cytosol and nucleus, to the ER, to the mitochondria (Finka et al., 2016). For instance, Hsp40 is the postsynaptic co-chaperone, localized to dendritic spines and believed to play a role in synaptic plasticity through its interactions with Hsp70 (Suzuki et al., 1999), while CSPα is a Hsp40 co-chaperone located at the presynaptic terminal.

Structurally, Hsp40s are defined by their conserved J domain and typically have large variation in the rest of the protein (**Figure 2A**). Classical Hsp40s have both a J domain as well as a zinc finger domain that regulates client protein binding. The J domain of Hsp40 proteins consists of a 70-amino acid sequence with four alpha helices (**Figure 2B**), which is conserved in co-chaperones from *E. coli* to humans (Tobaben et al., 2003). Within the J domain, the histidine, proline, aspartic acid (HPD) motif, which falls between the second and third alpha helices, is highly conserved, as it is necessary for stimulation of Hsp70 ATPase activity (Jiang et al., 2007). Despite the conservation of the J domain, Hsp40s range in size from 18 to 520 kD (Koutras and Braun, 2014; Fontaine et al., 2016). The structural diversity outside of the J domain allows the Hsp40 co-chaperones to provide specificity to Hsc70 activity, as these regions function in the recruitment of clients to Hsc70 (Behnke et al., 2016). Hence, Hsp40s are drivers of Hsp70 functional diversity.

The Hsp40/DNAJ proteins are grouped into three classes: A, B, and C, based on motifs and domains present (Hageman and Kampinga, 2009). Class A DNAJ proteins are structurally similar to *E. coli DnaJ* (Goffin and Georgopoulos, 1998) and have an N-terminal J domain, followed by a Gly and Phe-rich region, four repeats of the CxxCxGxG type zinc finger motif and a C-terminal domain, which is now known to bind client proteins. Class B DNAJ proteins also have high homology with the domains of *E. coli* DNAJs, but lack the zinc finger domain. The only conserved feature of members of the DNAJC class is the J domain, but not necessarily in its canonical location at the N-terminus (Kampinga et al., 2009). The greater variation in the DNAJC protein structure provides these proteins with highly specific client interactions as compared to the more nonspecific, promiscuous binding of the A- and B-class DNAJ proteins (**Figure 2C**).

The Hsc70-DNAJ chaperone complex functions in nascent folding, refolding, disaggregation, and degradation of a wide range of proteins (Fontaine et al., 2016). There is increasing evidence for DNAJ dysfunction as a cause for disease, as seven J chaperones have mutations linked to neurological disorders. Besides the three that will be discussed below, *DNAJC19* (TIM14) and *DNAJC29* (sacsin) are linked to forms of ataxia, *DNAJB2* (HSJ1) is linked with distal motor neuropathy, *DNAJC12* is associated with phenylketonuria-related neurodevelopmental deficits, and *DNAJB6* (Mrj) is linked with limb-girdle muscular dystrophy type 1D (Koutras and Braun, 2014; Anikster et al., 2017). *DNAJB2* was also recently identified as a cause of spinomuscular atrophy and Parkinsonism (Sanchez et al., 2016). In this review, we will focus on synaptic Hsc70 chaperone complexes with DNAJC proteins CSPα, auxilin, and RME-8, as well as DNAJA1 and B1 in their interactions with Hsp110.

DNAJC5: CYSTEINE STRING PROTEIN α

CSP was discovered in *Drosophila melanogaster* in a screen of antibodies labeling the nerve terminal (Zinsmaier et al., 1990)

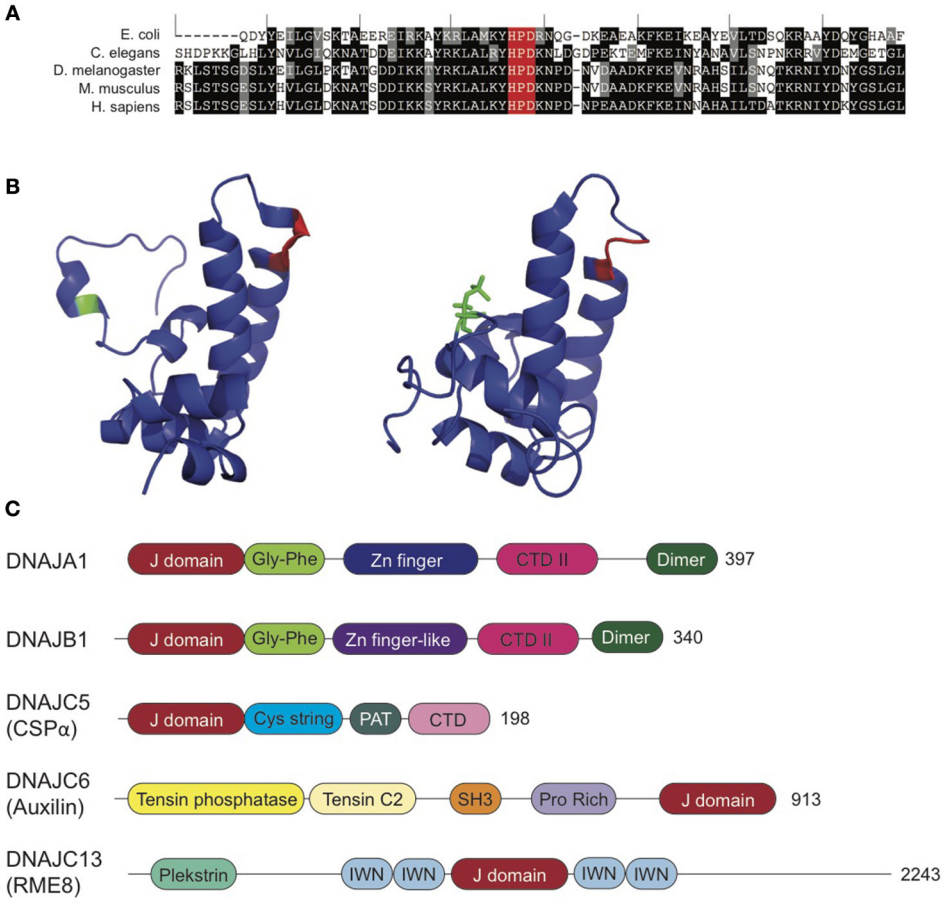

FIGURE 2 | Synaptic Co-Chaperones. (A) DNAJ proteins exhibit high homology within the J domain. J domains from *E. coli* DNAJ, *C. elegans* DNAJ14 and *D. melanogaster*, *M. musculus*, and *H. sapiens* DNAJC5. Black indicates identical residue. Gray indicates amino acids with conserved properties. The conserved HPD motif is highlighted in red. Sequence alignments generated using T Coffee (Notredame et al., 2000; Di Tommaso et al., 2011). **(B)** CSPα J domain exhibits conformational shift with phosphorylation at serine 10 (green). Unphosphorylated (left, PDB: 2N05) and phosphorylated (right; PDB: 2N04) CSPα J domain. HPD motif is highlighted in red. See, Patel et al. (2016) for further details. **(C)** Domain organization of J co-chaperones reviewed in this article: DNAJA1 (PDB: 2M6Y), DNAJB1 (Kampinga and Craig, 2010), DNAJC5 (CSPα; PDB: 2N04 and 2NO5), DNAJC6 (auxilin; PDB: 3N0A), and DNAJC13 (Zhang et al., 2001). Not to scale. See also, Kampinga and Craig (2010). CTD, C-terminal domain; IWN, 90-amino acid conserved motif; PAT, palmitoylacyltransferase recognition region.

and was then shown to be expressed mainly in the brain and retina. Immunohistochemistry analysis revealed that CSP is restricted to presynaptic termini (Zinsmaier et al., 1990; Kohan et al., 1995). The vertebrate homolog of CSP was identified in *Torpedo californica* electric organ (Gundersen and Umbach, 1992). Three mammalian homologs of the single fly CSP gene have been identified—CSPα, CSPβ, and CSPγ (Fernández-Chacón et al., 2004). CSPα is the functional homolog of the fly CSP and is localized to synaptic vesicles at the presynaptic terminal. CSPβ is expressed in both auditory hair cell neurons and testes, while CSPγ is expressed solely in testes (Fernández-Chacón et al., 2004; Schmitz et al., 2006).

CSPα has the domain organization of a DNAJ C-class Hsp40 co-chaperone. It possesses an N-terminal helix (residues 1–13 in rat), followed by a J domain (residues 14–81), an adjacent linker (residues 82–111), and an eponymous cysteine string domain containing 13–15 extensively palmitoylated cysteine residues in a 25-residue motif (residues 112–136; Braun and Scheller, 1995; Patel et al., 2016), which is immediately adjacent a palmitoylacyltransferase (PAT) recognition site responsible for palmitoylation of the protein (residues 136–145). CSPα also possesses a C-terminal domain (residues 146–198; **Figure 2C**).

Through its J domain, CSPα interacts with Hsc70 (Braun et al., 1996). Within alpha helices 1 and 2 of the 4-alpha helix J domain, there is an HPD motif that, when mutated, abolishes CSPα's stimulation of Hsc70 ATPase activity (Chamberlain and Burgoyne, 1997). Yeast two-hybrid studies showed that the cysteine string region may also be responsible for an interaction between CSPα and the small glutamine-rich tetratricopeptide repeat-containing protein (SGT; Tobaben et al., 2003). CSPα recruits SGT and Hsc70 to synaptic vesicles where its interaction leads to maximal Hsc70 ATPase activity. However, a recent study demonstrated the role for the Hsc70/SGT interaction in stimulating protein folding, while inhibiting Hsc70-mediated

synaptic membrane deformation for autophagy of Hsc70 clients (Uytterhoeven et al., 2015). Hence, the physiological relevance of the Hsc70/CSPα vs. Hsc70/CSPα/SGT complex still needs to be resolved.

The N-terminal domain of CSPα is phosphorylated by protein kinase A on a serine at position 10 (phospho-Ser^{10}), effectively reducing accessibility to the α1 helix of the N terminus and inhibiting binding to putative clients, namely syntaxin and synaptotagmin I (**Figure 2B**). However, this does not alter the J domain, HPD motif, or interactions with Hsc70 (Patel et al., 2016). CSPα also has a linker between the cysteine string and J domains that may have an effect on the interaction between CSPα and clients, however this remains to be established. Deletion and point mutations within this region should elucidate the most important residues, and give additional insight into its interactions with client proteins.

CSPα is one of the most heavily palmitoylated proteins known. In the brain, the CSPα cysteine string domain is normally fully palmitoylated by several PATs, most prominently DHHC5/HIP14 (Ohyama et al., 2007; Stowers and Isacoff, 2007). The palmitoylation of cysteine residues in the cysteine string allows for peripheral membrane association and is essential for targeting CSPα to synaptic vesicles. On average, there are 2.8 copies of CSPα per synaptic vesicle (Takamori et al., 2006). CSPα is also found on secretory vesicles in non-neuronal tissues.

Most of our present understanding of CSPα function comes from knockout (KO) studies in flies and mice. In *Drosophila*, CSP nulls result in 95% embryonic lethality, but those flies that do survive to adulthood exhibit progressive sluggishness, spasmic jumping, shaking, uncoordinated movement, and high-temperature paralysis, as well as premature death (Zinsmaier et al., 1990, 1994; Burgoyne and Morgan, 2015). These phenotypes were ascribed to defects in Ca^{2+} dynamics, neurotransmission deficits, progressive deterioration of CSPα null synapses and eventual neurodegeneration, all phenomena that may be explained by the accumulation of incorrectly folded client proteins at presynaptic termini leading to dysfunction in neurotransmitter release (Umbach et al., 1994; Zinsmaier et al., 1994). Mutations in both *Drosophila* CSP and Hsc70 cause a similar temperature-sensitive loss of evoked neurotransmitter release that can be restored by elevating Ca^{2+} levels (Bronk et al., 2001). These common phenotypes are consistent with the idea that Hsc70 and CSP function together to chaperone the synaptic vesicle fusion machinery. Overexpression of *Drosophila* CSP suppresses the decrease of evoked release induced by the overexpression of syntaxin 1A, suggesting that CSP modulates protein-protein interactions of syntaxin, including SNAREs (Nie et al., 1999). In addition, CSP interacts with the SNARE synaptobrevin and the Ca^{2+} sensor synaptotagmin in a phosphorylation dependent manner (Evans and Morgan, 2002; Boal et al., 2004).

CSPα KO mice are normal at birth but exhibit a progressive sensorimotor phenotype and age-dependent synapse deterioration beginning around P20 (Fernández-Chacón et al., 2004). These mice also exhibit degeneration of retinal photoreceptors and an increased susceptibility for degeneration of the most active GABAergic synapses, followed by death around 8 weeks (Schmitz et al., 2006; García-Junco-Clemente et al., 2010). Congruently, hippocampal neuron cultures from CSPα KO mice show selective vulnerability of synaptotagmin-2^{+} GABAergic cells to neurodegeneration, as well as mIPSC (decreased frequency), and mEPSC (decreased amplitude) neurotransmission deficits (García-Junco-Clemente et al., 2010). CSPα also functions in motor neurons at neuromuscular junctions to maintain the readily releasable pool of vesicles (Rozas et al., 2012). In CSPα KO mice, a defect in vesicle recycling at the neuromuscular junction may be partially responsible for the canonical motor phenotypes observed in these animals. Combined, these studies indicate that higher neuronal activity leads to faster synaptic deterioration and neurodegeneration in the absence of CSPα.

Detailed electrophysiological analysis of P10 CSPα KO pups prior to synapse loss and neurodegeneration has revealed normal Ca^{2+} currents and neurotransmission (Fernández-Chacón et al., 2004), suggesting that CSPα is not directly required for neurotransmitter release. At P20-P30, evoked release becomes asynchronous and deteriorates progressively with age, indicating that CSPα clients play key roles in the maintenance of synaptic structure and function (Fernández-Chacón et al., 2004). Congruently, Rozas and colleagues have demonstrated the importance of CSPα in synaptic vesicle exo- and endocytosis; CSPα KOs show decreases in exocytic release sites, as well as endocytosis. CSPα KO synapses do not efficiently recycle membrane back to generate synaptic vesicles (Rozas et al., 2012). This leads to decreases in the levels of releasable vesicles, further impairing the neuron's exocytic capacity.

Interestingly, genetic studies have demonstrated that CSPα KO phenotypes are rescued by α-synuclein overexpression (Chandra et al., 2005), a key gene in the pathophysiology of Parkinson's disease, while α-synuclein KO exacerbates these phenotypes. These genetic crosses suggest that synucleins act downstream of CSPα to stabilize one or more of its clients.

In order to ascribe mouse CSPα KO phenotypes to molecular processes, there has been a concerted effort to identify CSPα clients, mainly through two approaches—binding assays and quantitative proteomics. Via binding assays, SNAP-25, the t-SNARE for synaptic vesicle fusion, was identified as the first CSPα client (Chandra et al., 2005; Sharma et al., 2011). SNAP-25 was confirmed as a client through analysis of CSPα KO brains. These brains exhibit significant decreases in SNAP-25 levels due to its activity-dependent ubiquitination and degradation (Sharma et al., 2011). Interestingly, SNAP-25 directly binds Hsc70 as opposed to binding via CSPα.

To find additional CSPα clients, our lab embarked on a proteomic screen (Zhang et al., 2012). The rationale for the screen design was that clients of CSPα would be misfolded and targeted for degradation in the absence of CSPα, leading to decreases in their levels in CSPα KO brains. Thus, comparing the synaptic proteome of wild-type and CSPα KO would be instructive in identifying clients in an unbiased and systematic manner (Zhang et al., 2012). Through this screen we identified 22 proteins, other than chaperones, that were decreased in the CSPα KO proteome. This list included SNAP-25, affirming our

screen design. We validated many of these proteins by orthogonal methods. Through direct binding in the presence of ADP, we showed that the endocytic GTPase dynamin-1 is also a client of CSPα (Zhang et al., 2012). Furthermore, addition of purified dynamin-1 results in increases in Hsc70 ATPase activity in the presence of CSPα, confirming it as a Hsc70/CSPα client. The oligomerization of dynamin-1 is deficient in CSPα KO brains, implying either dynamin-1 self-assembly or disassembly is regulated by CSPα.

Through direct binding and mass spectrometric approaches, we confirmed that SNAP-25 and dynamin-1 are bona-fide CSPα clients and showed that CSPα has in the range of 5–6 clients. Previously, other known CSPα client proteins include VAMP-1, G-protein subunit, and N-type Ca^{2+} channels (Chamberlain et al., 2001).

CSPα KO phenotypes are likely to be a compound loss- or gain-of-function of CSPα clients. For instance, SNAP-25 knockdown in CSPα KO animals exacerbates the CSPα phenotype, while lentiviral expression of SNAP-25 in CSPα KO mice rescues the neurodegenerative phenotype (Sharma et al., 2012). These data suggest that loss of SNAP-25 function in CSPα KOs results in the synaptic phenotypes and neurodegeneration associated with CSPα KO or dysfunction. These results are consistent with its function in maintenance of synaptic exocytic machinery. Intriguingly, despite the ability of overexpressed α-synuclein to rescue CSPα phenotypes, α-synuclein does not rescue SNAP-25 levels, only SNARE complex levels, indicating that α-synuclein rescue is not mediated by SNAP-25. In the case of dynamin-1, rescue experiments have not been completed to determine its mode of action in CSPα KOs. Identification of CSPα's clients has revealed that neurodegeneration in CSPα mutants and KOs may be due to an exo-endocytosis coupling defect, resulting in the neuron's failure to maintain and recycle synaptic vesicles during prolonged stimulation.

DNAJC6: AUXILIN

Auxilin is the best studied mammalian Hsp40 and was identified in a study of the clathrin-coated vesicle (CCV) uncoating mechanism as a cofactor for Hsc70/Hsp70c (Ungewickell et al., 1995). It is unique among the Hsp40 co-chaperones in that it has only one known client, although research is underway to elucidate its influence on other coat proteins, such as COPII (Ding et al., 2016).

Auxilin is a brain-specific, 970-amino acid protein that is enriched at presynaptic termini. Auxilin has a C-terminal J domain (residues 814–910) that allows for its interaction with Hsc70 (**Figure 2C**; PDB: 3N0A). The auxilin J domain highly divergent, with an extra N-terminal alpha helix as well as an extended loop between the first and second helices of the domain that is important for Hsc70 binding (Jiang et al., 2003). Deletions within auxilin's J domain inhibit its Hsc70 co-chaperone activity, but not its interaction with Hsc70, congruent with its divergent structure (Holstein et al., 1996). Auxilin also has a lipid binding domain (residues 60–387), which contains a PTEN phosphatase-like domain followed by a C2 domain (Guan et al., 2010). *In vitro* studies have shown that the auxilin C2 domain binds specifically to phosphoinositide-containing membranes, and the PTEN domain has affinity to phosphoinositides PI4P and PIP_2. Together, this region is required for recruitment of auxilin to clathrin-coated vesicles following dynamin mediated scission (Guan et al., 2010). In addition, auxilin has a clathrin binding domain (residues 547–814) that facilitates interaction with its client (Haynie and Ponting, 1996; Edvardson et al., 2012). Proper auxilin function necessitates physical association of the clathrin binding domain and the J-domain, as the combination of separate J domain deletion mutants and clathrin binding domain mutants does not rescue the clathrin coated vesicle accumulation phenotype of auxilin KOs (described below).

The structure of auxilin bound to the clathrin coat has been resolved by electron microscopy and has provided great insight into the mechanisms of auxilin-dependent clathrin uncoating. Auxilin was shown to bind clathrin at a 1:3 ratio at the clathrin ankle segment. Ankles from three clathrin triskelia are typically crossed in a stable clathrin coat (**Figure 3**), and auxilin binding causes a shift in ankle orientation that leads to a disruption of coat structure (Fotin et al., 2004b). Auxilin bound to the clathrin ankle recruits Hsc70 to the vertices of the clathrin coat, where it hydrolyzes ATP and binds clathrin, further increasing the strain on clathrin cage interactions (Fotin et al., 2004b; Xing et al., 2010).

Functional studies have established that auxilin and Hsc70 interact in the context of clathrin-mediated endocytosis to disassemble clathrin coats (Ungewickell et al., 1995). As a classical Hsp40, the presence of auxilin increases Hsc70 ATPase activity 5-fold (Jiang et al., 1997). Auxilin binds clathrin independently of nucleotide, initiating a strain on the clathrin coat structure, and then recruits Hsc70 which allows for further disruption of the clathrin cage, ultimately resulting in its disassembly (Fotin et al., 2004b; Xing et al., 2010). Auxilin has a high affinity for Hsc70-ATP, while clathrin has a high affinity for Hsc70-ADP (Jiang et al., 1997). When ATP hydrolysis occurs, auxilin releases Hsc70 and clathrin, but Hsc70 remains bound to clathrin, possibly preventing its re-oligomerization (see **Figure 1**; Holstein et al., 1996). The exact ratio of this reaction *in vivo* is somewhat controversial. Researchers have demonstrated a 1:3:3 ratio of Auxilin:Hsc70: clathrin, as well as a 1:1:>3 ratio (Böcking et al., 2011, 2014). There may be a variety of Auxilin:Hsc70:clathrin ratios dependent on cellular conditions and bond stability, and lower ratios may be possible but energetically unfavorable.

Auxilin KO in mice results in decreased viability and birth weight in a copy number-dependent manner (Yim et al., 2010). In auxilin KO animals, the efficiency of clathrin uncoating is impaired, which leads to an excess of clathrin-coated vesicles at the synapse and impairments in endocytosis (Yim et al., 2010). While auxilin is brain-specific, its homolog, G-associated kinase (GAK; DNAJC26), is broadly expressed and may partially compensate for a loss of auxilin function (Yim et al., 2010) as levels of GAK increase in auxilin KOs. Furthermore, Lee et al. demonstrated that conditional deletion of GAK in adult mice is lethal, demonstrating the essential role of clathrin-mediated endocytosis in all cell types (Lee et al., 2008). Surprisingly, auxilin/GAK double KO with expression of only the clathrin

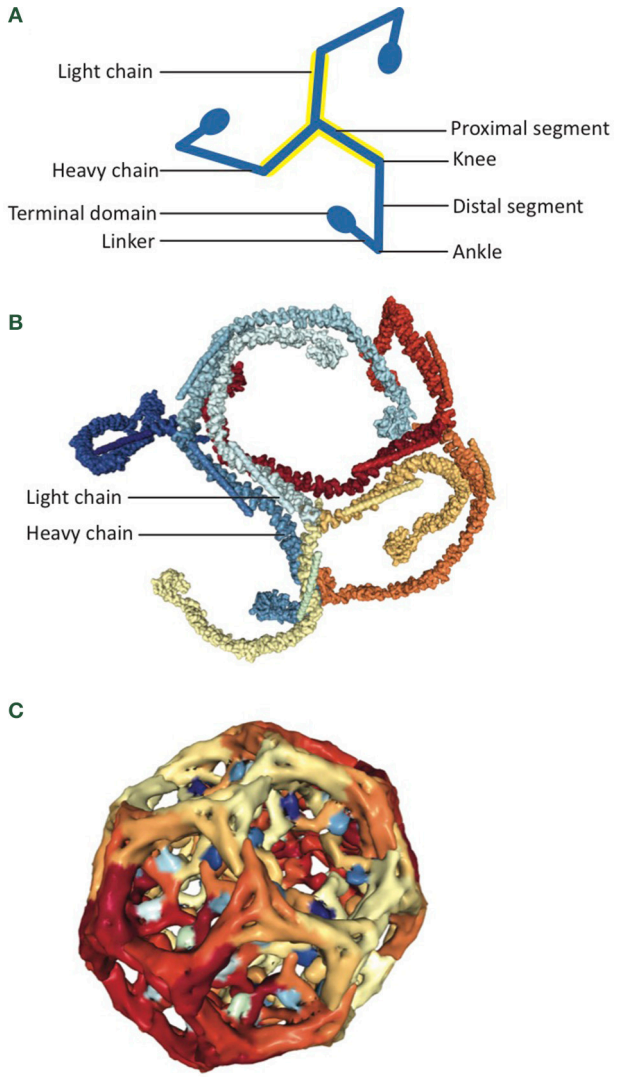

FIGURE 3 | Auxilin is an Hsc70 co-chaperone for clathrin uncoating. (A) Cartoon depicts structure of a clathrin triskelion. Three clathrin ankle segments cross to form a stable clathrin cage. **(B)** Assembled clathrin coat containing clathrin heavy chain and clathrin light chain, as determined by electron microscopy (PDB:3IYV; Fotin et al., 2004b). **(C)** The J domain of auxilin (shades of blue) interacts with the clathrin coat (shades of red and yellow; PDB: 1XI5; Fotin et al., 2004a).

binding and J domains can rescue lethality despite the mice having a body size similar to auxilin KO alone, further confirming that auxilin defects are due to reduced clathrin uncoating and vesicle recycling (Park et al., 2015).

While clathrin cages have been long thought to be the only clients of auxilin, recent data suggest that it functions early in the secretory pathway on COPII vesicles (Ding et al., 2016). In cells depleted of auxilin, trafficking is disrupted in COPII-dependent regions between the ER and Golgi, as well as throughout the Golgi, and COPII-mediated vesicle fusion is disrupted. This finding has raised the intriguing possibility that auxilin has additional clients.

DNAJC13: RECEPTOR MEDIATED ENDOCYTOSIS 8

RME-8 is a 2,000 amino acid protein that was identified in a *C. elegans* screen for organisms defective in the endocytosis of yolk proteins (Zhang et al., 2001) and is required for *C. elegans* survival and development (Fujibayashi et al., 2008). It is expressed in all tissues in varying levels (Ishikawa et al., 1998). RME-8 has a J domain (residues 1,301–1,366) toward the C-terminal half of the protein (**Figure 2C**). Through its J domain, RME-8 interacts and specifically stimulates the ATPase activity of Hsc70-4, with no effect on any other Hsc70 family member (Chang et al., 2004). Flanking its J domain, RME-8 contains four repeats of about 90 amino acids known as IWN repeats, which are conserved among RME-8 homologs but have an as-of-yet undetermined function (Zhang et al., 2001; Chang et al., 2004; Girard et al., 2005; Fujibayashi et al., 2008; Xhabija and Vacratsis, 2015). RME-8 also contains a pleckstrin homology domain (residues 312–350) that allows it to interact with the phosphoinositide PI3P, which aids in the localization of RME-8 to PI3P rich membranes as in endosomes (Xhabija and Vacratsis, 2015).

RME-8 is a peripheral membrane protein localized to early, but not late, endosomes where it appears to associate with the membrane via the PI3P/pleckstrin homology domain (Fujibayashi et al., 2008). In *C. elegans*, RME-8 is required for receptor mediated endocytosis as well as fluid tracer uptake (Chang et al., 2004). Although RME-8 is required for invertebrate survival, its knockdown has minimal effects on endocytosis in mammalian cultured cells. However, C-terminal deletion (lacking the last IWN repeat, but with the J domain) leads to formation of RME-8 puncta and vacuoles, accumulation of ubiquitinated proteins, and changes in early endocytic morphology (Fujibayashi et al., 2008). This defect suggests that the C-terminus of RME-8 plays an integral role in trafficking through early endosomes to late and recycling endosomes. Congruent with such a function, RME-8 is localized to Rab5 positive organelles throughout the cell in mammalian neurons, including synapses.

Two roles have been described for RME-8 in trafficking through early endosomes. First, similar to its role in *C. elegans*, RME-8 is important in clathrin-mediated endocytosis in *Drosophila*, and is essential for the uptake and internalization of membrane ligands and receptors (Chang et al., 2004). Defects in RME-8 in *Drosophila* lead to defects in the uptake of endosomal tracers, as well as disorganization of the endosomal compartment. These phenotypes of RME-8 mutants bear a strong resemblance to mutations in Hsc70-4, suggesting that the two genes act in a common pathway. Additionally, studies in HeLa cells have demonstrated that siRNA-mediated knockdown of RME-8 leads to defects in EGF uptake into the cells (Girard et al., 2005). However, as RME-8 has no clathrin binding site, it must modulate clathrin-mediated endocytosis through a mechanism independent of auxilin. This suggests that RME-8 may exert its effects between the dynamin-1 and auxilin stages of endocytosis, as is has similarities to both. Second, RME-8 has been shown to be a component of the retromer complex, which executes

sorting and retrieval to the trans-Golgi (Girard et al., 2005; Freeman et al., 2014; Seaman and Freeman, 2014). RME-8 aids in localization of the WASH (WASP and Scar homolog) complex to endosomal tubules for retromer-mediated endosomal protein sorting (Perrett et al., 2015). Currently, a thorough mechanism of RME-8 function in the early endocytic pathway is lacking, and identification of additional clients, interactors, and members of the pathway will provide detail on RME-8's role in endocytosis.

DISAGGREGASE: HSC70, HSP110, DNAJA1, AND DNAJB1

Metazoans lack the key Hsp100 disaggregases, such as the yeast Hsp104, which exist in all non-metazoans. Recently, the metazoan disaggregase was identified and shown to consist of Hsc70, Hsp110, and DNAJA and B proteins (Nillegoda et al., 2015). This disaggregase complex functions in the disaggregation of insoluble proteins, including those in amyloid-like structures. The transcription of Hsp110 is stimulated by conditions of stress, and it works in concert with other quality control proteins to restore native protein folding states (Zuo et al., 2016).

Hsp110 was identified as an Hsp70 interactor and NEF (ATP for ADP) to induce client release from Hsc70 (Dragovic et al., 2006). Though Hsp110 on its own possesses chaperone activity *in vitro*, it acts only as a NEF in the presence of Hsc70 (Mattoo et al., 2013).

Several new studies have demonstrated the disaggregative properties of Hsp110 with both model clients such as aggregated luciferase and most significantly with neurodegenerative disease-linked proteins such as α-synuclein and prion protein (Gao et al., 2015; O'Driscoll et al., 2015). Furthermore, studies have demonstrated Hsp110's ability to prevent aggregation-prone proteins from becoming toxic to cells (Eroglu et al., 2010). As a disaggregase, Hsp110 can work with both DNAJA1 and DNAJB1 in conjunction with Hsc70 to disaggregate and refold aggregated proteins (Nillegoda et al., 2015). In the case of the Parkinson's disease linked α-synuclein protein, Hsc70, DNAJB1 and Hsp110 individually have little effect on insoluble α-synuclein fibrils (Gao et al., 2015). However, when the three chaperone proteins are combined *in vitro*, they selectively disaggregate and solubilize α-synuclein fibrils into small polymers and monomers in a concentration-dependent manner. By contrast, DNAJA family members do not effectively resolubilize α-synuclein fibrils, demonstrating the selectivity of DNAJB proteins in this interaction.

This newly discovered disaggregase activity is consistent with prior findings demonstrating that Hsp110 regulates aggregation in several neurodegenerative diseases. Hsp110 mouse KOs show increased tau phosphorylation and amyloid beta accumulation in the brain (Eroglu et al., 2010). Hsp110 also interacts with Hsc70c and DNAJB1 to protect *Drosophila* rhabdomeres against the accumulation and toxicity of polyQ expansion proteins (Kuo et al., 2013). The disaggregase also prevented neurodegeneration caused by polyQ proteins. Furthermore, DNAJB1 and Hsp70 family member HspA1A can function in concert to reduce the levels of aggregated huntingtin (Rujano et al., 2007). Importantly, transgenic overexpression of Hsp110 in SOD1 mutant mice rescues their survival, further highlighting the importance of functional disaggregation machinery in combating misfolding and neurodegenerative diseases *in vivo* (Nagy et al., 2016).

The above data raise the exciting possibility that the canonical protein aggregation occurring in age-related neurodegenerative diseases may be a reversible with appropriate disaggregase chaperone activity. Aggregation of proteins is known to have both loss-of-function mechanisms, due to misfolding and decreased normal activity, and toxic gain-of-function mechanisms, such as the sequestration of neural and synaptic chaperones. If protein aggregates can be disassembled, both of these toxic mechanisms can be prevented. In the future, disaggregase chaperones such as Hsp110 may be a broad-acting therapeutic target for the protein aggregates that occur in a variety of neurodegenerative diseases, most prominently Alzheimer's and Parkinson's disease (Shorter, 2016).

SYNAPTIC CO-CHAPERONES AND NEURODEGENERATIVE DISEASES

Mutations in synaptic Hsp40 co-chaperones are causally related to human neurodegenerative diseases, underscoring the importance of the synaptic proteostasis network for the healthy brain. In CSPα, two mutations in the cysteine string region (L115R and L116Δ) result in autosomal dominant adult neuronal ceroid lipofuscinosis (ANCL), also known as Kufs disease and Parry disease (Benitez et al., 2011; Nosková et al., 2011; Velinov et al., 2012; Cadieux-Dion et al., 2013). ANCL is a hereditary, adult-onset, progressive neurodegenerative disease with variable clinical symptoms. Clinically, patients present with epilepsy, movement disorders, dementia, anxiety, speech changes, and early mortality (Burgoyne and Morgan, 2015). Pathologically, ANCL is characterized by intralysosomal accumulation of lipofuscin containing the protein saposin. ANCL patient brains show decreased CSPα protein levels as well as the presence of CSPα aggregates (Nosková et al., 2011), though this was not evident in early stages of the disease (Benitez et al., 2015). These proteostatic changes can be recapitulated by expression of L115R and L116Δ mutant CSPα in mouse CSPα KO neurons. This leads to overall low CSPα levels, as well as accumulation of the mutant CSPα in the cell body and low expression at synapses. This suggests that the mechanism of neurodegeneration in ANCL patients may be both a loss-of-function at the synapse, and an aberrant gain-of-function at the cell body.

Biochemical characterization of the L115R and L116Δ CSPα mutants revealed that they function as co-chaperones, consistent with the mutations being in the cysteine string domain and not in the J- or C-terminal domains (Zhang and Chandra, 2014). However, both ANCL mutants (L115R and L116Δ) have a high propensity to oligomerize and form aggregates *in vitro*, with mutant CSPα forming ubiquitinated inclusions (Zhang and Chandra, 2014). Presently, it is controversial whether the aggregates of ANCL mutant CSPα are palmitoylated, with contradictory data available (Greaves et al., 2012; Zhang and

Chandra, 2014; Diez-Ardanuy et al., 2017). Regardless, ANCL mutant CSPα can readily co-oligomerize with the wildtype CSPα protein, possibly explaining the autosomal dominant nature of this disease. Significantly, oligomerization (both homo- and hetero-) leads to decreased co-chaperone activity, suggesting a dominant negative mechanism of mutant CSPα, as the protein present in higher molecular weight species cannot function as a co-chaperone for Hsc70 (Zhang and Chandra, 2014).

Remarkably, analysis of L115R and L116Δ ANCL patient brains revealed increased expression, decreased specific activity, and mislocalization of palmitoyl protein thioesterase 1 (PPT1), the enzyme responsible for the removal of palmitoyl groups from proteins, including CSPα (Henderson et al., 2016). Loss-of-function mutations in PPT1 also cause NCL (Vesa et al., 1995), suggesting that the mutations in the cysteine string region of the protein affect CSPα's palmitoylation status, even though the residual mutant CSPα appears fully palmitoylated. This hypothesis is supported by a novel CSPα ANCL mutation resulting in duplication of a segment of the cysteine string region (Jedličková et al., 2016). However, further study is required to understand the mechanism by which CSPα affects PPT1 activity, or vice versa, and PPT1's role in proteostasis. Studies of mutant CSPα palmitoylation and membrane association may provide more insight into the nature of this interaction.

CSPα dysfunction may also be related to other neurodegenerative diseases. CSPα levels have been shown to decrease in the degenerating regions of Alzheimer's disease patient brains (Zhang et al., 2012). Impaired assembly of the SNARE complex, a hallmark of CSPα KO brains, has also been identified in Alzheimer's and Parkinson's diseases (Garcia-Reitböck et al., 2010). Recently, Sambri and colleagues demonstrated decreased CSPα levels in a mouse model of mucopolysaccharidosis type IIIA, a lysosomal storage disease. Importantly, overexpression of CSPα rescued many of the phenotypes in the mucopolysaccharidosis mouse model (Sambri et al., 2017), supporting a functional role for CSPα in this disease. The present idea is that in mucopolysaccharidosis type IIIA, CSPα-mediated lysosomal dysfunction may disrupt the activity of the presynaptic compartment through dysregulation of SNARE proteins.

Auxilin and RME-8 mutations are associated with Parkinson's disease in humans. Loss-of-function mutations in auxilin cause juvenile onset (between 10 and 21 years old) Parkinson's disease. This form of Parkinson's disease is characterized by visual hallucinations, cognitive deterioration, epilepsy, and psychosis, as well as the typical motor symptoms of tremor, rigidity, and bradykinesia (Köroğlu et al., 2013; Elsayed et al., 2016). In a study of an inbred family with high rates of juvenile parkinsonism, Köroğlu et al. identified a combination homozygous nonsense mutation and missense mutation in the DNAJC6 gene that resulted in premature truncation of the auxilin protein (Köroğlu et al., 2013). The nonsense mutation is the result of a C to T substitution at position 2200 of the DNA sequence, resulting in a stop codon within exon 16 of the mRNA and truncation of about 1/5 of the C-terminal portion of auxilin (p.Q734X), including the J-domain. The missense mutation results in the substitution of cysteine at position 61 for serine, a change that is likely deleterious to protein structure and function. Additionally, several other mutations have been identified in auxilin that are linked to juvenile onset Parkinson's disease (**Table 2**). Milder mutations in auxilin lead to later onset, while complete loss-of-function leads to earlier onset forms (Elsayed et al., 2016). Congruently, sequence variants in GAK, the auxilin homolog, have also been linked to Parkinson's disease risk by meta-analysis of GWAS, as well as in a study of sporadic Parkinson's disease in China (Nalls et al., 2014; Zhang et al., 2016). It is presently not clear why clathrin uncoating defects through the loss-of-function of auxilin should lead to Parkinson's disease. One possibility is that deficiency leads to alternative modes of endocytosis that is taxing to dopaminergic neurons. Another possibility is that Hsc70 is sequestered to CCVs leading to a severe synaptic proteostasis defect. Alternatively, auxilin has additional clients in substantia nigra neurons.

TABLE 2 | Mutations in auxilin (DNAJC6) associated with Parkinson's disease.

Mutation	Zygosity	Protein	References
c.801-2 A>G	Homozygote	-Deletion of aa 268-328 -premature stop	Edvardson et al., 2012
c.1468 + 83 del	Compound heterozygote		Olgiati et al., 2016
c.2200C>T	Homozygote	p.Q734X	Köroğlu et al., 2013
c.2038 + 3 A>G	Compound heterozygote		Olgiati et al., 2016
c.2223A>T	Homozygote	p.Thr741*	Olgiati et al., 2016
c.2365C>T	Homozygote	p.Gln789*	Elsayed et al., 2016
c.2371C>T		p.Gln791*	
c.2779A>G	Homozygote	p.Arg927Gly	Olgiati et al., 2016

*Bold denotes mutations associated with double heterozygote forms of Parkinson's disease. *Denotes premature stop codon.*

A mutation in RME-8 was identified in a large pedigree with autosomal dominant Parkinson's disease (p.Q734X). In this Mennonite family, a heterozygous N855S mutation in RME-8 was discovered by exome sequencing of the proband and relatives with Parkinson's disease (Vilariño-Güell et al., 2014). The mutation falls between the first two IWN domains (Zhang et al., 2001). However, the N855S mutation was not always associated with disease in this pedigree. A recent publication using the same family found a mutation in an uncharacterized ORF encoding a protein TMEM230, which is localized to synapses, using positional mapping (Deng et al., 2016). This new study casts the role of RME-8 in Parkinson's disease in doubt and requires further examination to identify the contribution of each protein to the disease.

Clients of CSPα, auxilin, and RME-8 all participate in either synaptic vesicle exocytosis, endocytosis and/or recycling of synaptic vesicles and membrane components (**Figure 4**). Thus, the association of these co-chaperones with neurodegenerative diseases highlights the importance of efficient functional synaptic exocytic and endocytic machinery in synapse- and

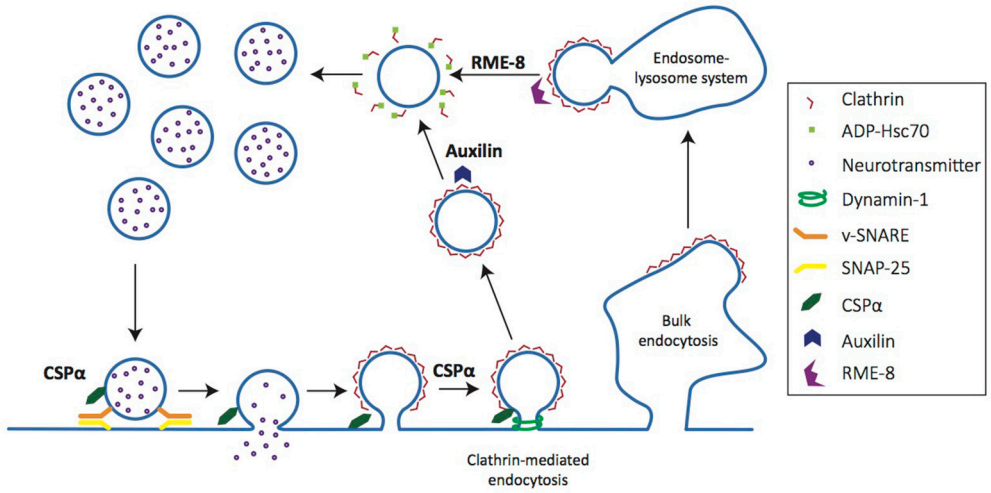

FIGURE 4 | Co-chaperones regulate distinct steps of the synaptic vesicle cycle. Cartoon depicts synaptic vesicle cycle. CSPα chaperones SNAP-25, a t-SNARE involved in vesicle fusion for neurotransmitter release. CSPα also chaperones dynamin-1, a GTPase necessary for vesicle scission in clathrin-mediated endocytosis. Auxilin acts on clathrin-coated vesicles, uncoating them to generate nascent vesicles that are returned to the recycling pool. RME-8 regulates clathrin uncoating at the early endosome.

neuroprotection. In particular, the synaptic vesicle endocytosis deficits seen in co-chaperone mutants raise an important question: when clathrin mediated endocytosis is impaired, do the alternative modes of synaptic vesicle cycling that ensue lead to protein sorting inefficiencies that further disrupt synaptic proteostasis and cause synaptic dysfunction and loss? Hence, understanding synaptic vesicle cycling in co-chaperone mutants will be very instructive to understanding early steps in neurodegeneration.

SYNAPTIC PROTEOSTASIS, NEURODEGENERATION, AND AGING

It is well established that Aβ in Alzheimer's disease and α-synuclein in Parkinson's disease aggregate to form defining pathologies—amyloid plaques and Lewy bodies, respectively. Recent evidence suggests that their aggregation begins at the synapse and/or that synapses are sites of action of these pathological aggregates. In line with this idea, aggregation of α-synuclein has been demonstrated to begin at synaptic termini where the protein normally resides (Kramer and Schulz-Schaeffer, 2007; Scott et al., 2010; Spinelli et al., 2014), even though Lewy bodies are present in soma. These synaptic aggregates are likely to contribute to the synaptic dysfunction and synapse loss observed in Parkinson's disease (Nemani et al., 2010; Scott and Roy, 2012). Therefore, there is new interest in understanding why the synaptic proteostasis network is unable to deal with neurodegenerative disease aggregates and is overwhelmed during disease processes. One possible explanation is that the accumulation of disease-related proteins can not only cause a toxic gain-of-function, but may lead to a loss-of-function of specific proteins at the synapse through the sequestration of bystander proteins, especially chaperones (Rampelt et al., 2012). For example, aggregation of expanded polyglutamine repeat proteins such as those implicated in Huntington's disease and spinocerebellar ataxia can inhibit clathrin-mediated endocytosis by competitive binding and sequestration of Hsc70, and decreased Hsc70 expression (Yamanaka et al., 2008; Yu et al., 2014).

Age is the single biggest risk factor for neurodegenerative disease. New studies examining the reason behind this have uncovered several probable links to changes in the synaptic proteostasis network (Labbadia and Morimoto, 2015). Analysis of the human chaperome of distinct brain regions showed that Hsp60s, Hsp40s, and Hsp70s were consistently repressed with age. Among repressed genes, the Hsp40s exhibited the most significant change, with 62% of 48 Hsp40 genes repressed in aging. Pertinent to synaptic proteostasis, DNAJC5 and DNAJA1 levels are also decreased with aging (Brehme et al., 2014) and in Alzheimer's disease (Zhang et al., 2012). This analysis of the chaperome in aged human brain revealed a concordant exacerbation of responses in neurodegenerative disease, and provides evidence for similar changes in the synaptic proteostasis network in both aging and neurodegenerative disease. The causal relationship between age-related decrement in proteostasis and protein aggregation has been examined in worm. In aging *C. elegans,* dysregulation of proteostasis has been demonstrated to lead to the accumulation of aggregation-prone proteins, thus starting a vicious cycle (Walther et al., 2015). From a therapeutic standpoint, in *C. elegans,* the demonstrated correlation between age and decreased synaptic integrity can be prevented by increased expression of the master heat shock transcription factor *hsf*-1 (Toth et al., 2012).

FUTURE DIRECTIONS

While recent years have seen progress in understanding the role of synaptic chaperone systems in maintaining functional synaptic connections, more research is required to deepen our understanding of synaptic chaperone clients, the relationship between changes in synaptic proteostasis and normal aging as well as neurodegenerative diseases.

In the case of CSPα, a complete characterization of its clients will help delineate their importance to CSPα KO phenotypes. This analysis can lead to testable hypothesis as to why α-synuclein rescues CSPα KO phenotypes. It is presently unclear how ANCL mutations disrupt CSPα co-chaperone activity and what role palmitoylation plays in this disruption *in vivo* due to the paucity of animal models. Although auxilin has been well-characterized, recent evidence of its functions in alternative endocytic pathways prompts further mechanistic study. As COPII is only the second client of auxilin identified, further effort on unearthing additional clients may identify new roles for auxilin's co-chaperone activity. For Parkinson's disease linked to auxilin mutations, it remains to be investigated why clathrin-mediated endocytosis deficits leads to neurodegeneration. Additionally, RME-8 is the least-studied co-chaperone discussed in this review. The clients of RME-8 need to be identified, and this will address the mechanisms by which RME-8 regulates endocytosis at the synapse. Studies have already made it clear that RME-8 plays a distinct role from auxilin, but its specific function in clathrin-mediated endocytosis requires more investigation. Finally, additional investigation is required to identify the effect of RME-8 in familial Parkinson's disease, and to determine whether its dysfunction works independently or synergistically with the mutation in TMEM230 to influence disease progression.

CONCLUSIONS

The importance of co-chaperones in the maintenance of synapse function and the nervous system is becoming clear given that their dysfunction leads to loss of synapses and neurodegenerative disease.

AUTHOR CONTRIBUTIONS

ELG and SSC prepared the manuscript.

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Differential Targeting of Hsp70 Heat Shock Proteins HSPA6 and HSPA1A with Components of a Protein Disaggregation/Refolding Machine in Differentiated Human Neuronal Cells following Thermal Stress

Catherine A. S. Deane and Ian R. Brown *

Department of Biological Sciences, Centre for the Neurobiology of Stress, University of Toronto Scarborough, Toronto, ON, Canada

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***Correspondence:**
Ian R. Brown
ibrown@utsc.utoronto.ca

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Heat shock proteins (Hsps) co-operate in multi-protein machines that counter protein misfolding and aggregation and involve DNAJ (Hsp40), HSPA (Hsp70), and HSPH (Hsp105α). The HSPA family is a multigene family composed of inducible and constitutively expressed members. Inducible HSPA6 (Hsp70B') is found in the human genome but not in the genomes of mouse and rat. To advance knowledge of this little studied HSPA member, the targeting of HSPA6 to stress-sensitive neuronal sites with components of a disaggregation/refolding machine was investigated following thermal stress. HSPA6 targeted the periphery of nuclear speckles (perispeckles) that have been characterized as sites of transcription. However, HSPA6 did not co-localize at perispeckles with DNAJB1 (Hsp40-1) or HSPH1 (Hsp105α). At 3 h after heat shock, HSPA6 co-localized with these members of the disaggregation/refolding machine at the granular component (GC) of the nucleolus. Inducible HSPA1A (Hsp70-1) and constitutively expressed HSPA8 (Hsc70) co-localized at nuclear speckles with components of the machine immediately after heat shock, and at the GC layer of the nucleolus at 1 h with DNAJA1 and BAG-1. These results suggest that HSPA6 exhibits targeting features that are not apparent for HSPA1A and HSPA8.

Keywords: HSPA1A (Hsp70-1), HSPA6 (Hsp70B'), HSPA8 (Hsc70), DNAJ (Hsp40), HSPH1 (Hsp105α), human neuronal SH-SY5Y cells

INTRODUCTION

Heat shock proteins (Hsps) are highly conserved proteins that play roles in cellular repair and protective mechanisms (Muchowski and Wacker, 2005; Asea and Brown, 2008; Paul and Mahanta, 2014). They co-operate in multi-protein machines to counteract protein misfolding and aggregation that are characteristic of neurodegenerative diseases (Muchowski and Wacker, 2005; Rampelt et al., 2012; Duncan et al., 2015; Nillegoda and Bukau, 2015; Nillegoda et al., 2015; Smith et al., 2015; Goloubinoff, 2017; Jackrel and Shorter, 2017). Misfolded proteins are detected

by DNAJs (Hsp40s) and refolded into biologically active states by members of the HSPA (Hsp70) family (Rampelt et al., 2012; Mattoo and Goloubinoff, 2014; Clerico et al., 2015; Nillegoda and Bukau, 2015; Nillegoda et al., 2015). While Hsp70 and Hsp40 co-operate to prevent aggregation of misfolded proteins, they cannot dissociate protein aggregates that accumulate during neurodegenerative diseases (Rampelt et al., 2012; Gao et al., 2015; Nillegoda and Bukau, 2015; Nillegoda et al., 2015).

Yeast cells express a well-characterized "disaggregase" (Hsp104) that is able to solubilize aggregated proteins, homologs of which are lacking in mammalian cells (Glover and Lindquist, 1998; Weibezahn et al., 2005; Bösl et al., 2006; Nillegoda and Bukau, 2015). Studies have shown that HSPH1 (Hsp105α), a member of the mammalian Hsp110 family, acts co-operatively with Hsp70/Hsp40 as a "disaggregase" to dissociate aggregated proteins (Rampelt et al., 2012; Nillegoda and Bukau, 2015; Nillegoda et al., 2015). It has been reported that the mammalian disaggregation/refolding machine dissociates amyloid fibrils of α-synuclein that are associated with Parkinson's disease (Gao et al., 2015). Misfolded protein aggregates accumulate during the course of neurodegenerative diseases and upregulation of Hsps is being investigated as a potential protective strategy (Asea and Brown, 2008; Genc and Özdinler, 2014; Kalmar et al., 2014; Paul and Mahanta, 2014; Deane and Brown, 2016; Kampinga and Bergink, 2016).

The HSPA family is a multigene family composed of inducible and constitutively expressed members (Morimoto, 2008). HSPA6 (Hsp70B') is an inducible member that has received little attention compared to the more widely studied HSPA1A (Hsp70-1). HSPA6 has been investigated in cultured human neuronal cells (Chow and Brown, 2007; Chow et al., 2010; Khalouei et al., 2014a,b; Deane and Brown, 2016, 2017; Shorbagi and Brown, 2016; Becirovic and Brown, 2017), and in human cancer cell lines (Noonan et al., 2007, 2008). Interestingly, the HSPA6 gene is found in the human genome but not in mouse and rat, hence it is absent in current animal models of neurodegenerative diseases (Noonan et al., 2007; Deane and Brown, 2016, 2017).

To advance knowledge of HSPA6, we investigated whether it is targeted to stress-sensitive neuronal sites with components of a protein disaggregation/refolding machine in human neuronal SH-SY5Y cells that have been previously used as a model in studies of neurodegenerative diseases (Grynspan et al., 1997; Imamura et al., 2006; Ross and Spengler, 2007; Cheung et al., 2008; Plowey et al., 2008; Krishna et al., 2014). Neurodegenerative diseases affect differentiated neurons of the adult central nervous system, hence SH-SY5Y cells were differentiated in the present study by treatment with retinoic acid which results in inhibition of cell division and stimulates the development of neuronal processes (Jacobs et al., 2006; Ross and Spengler, 2007; Cheung et al., 2008). Retinoic acid is required for adult neurogenesis in the rat brain (Jacobs et al., 2006; Bonnet et al., 2008) and for maintenance of the differentiated state of dopaminergic neurons in the nigrostriatal pathway (Maden, 2007). The present studies suggest that HSPA6 exhibits features in its targeting that are not observed for the widely studied HSPA1A.

MATERIALS AND METHODS

Cell Culture and Differentiation

Human neuronal SH-SY5Y cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Wisent, QC, Canada) with 10% fetal bovine serum (FBS; Wisent) at 37°C in a humidified 5% CO_2 atmosphere. Cells were plated at 4.5×10^4 cells per cm^2 on coverslips placed inside 6-well plates and allowed to settle onto the growth surface and adhere for 24 h. Neuronal differentiation was induced by treatment with 10 μM all-*trans*-retinoic acid (R2625; Sigma Aldrich, St. Louis, MO, USA) for 72 h under serum free conditions.

Induction of Hsps and Heat Shock Treatment

Following 72 h of differentiation, media containing all-*trans*-retinoic acid was removed and replaced with fresh serum free DMEM with 0.3 μM celastrol plus 50 μM arimoclomol to induce Hsps (Deane and Brown, 2016, 2017). Celastrol (70950; Cayman Chemical, Ann Arbor, MI, USA) dissolved in DMSO was added directly to the media. Arimoclomol (gift from Professor Michael Cheetham, Institute of Ophthalmology, University College London, UK) was prepared fresh for each experiment by dissolving in serum free DMEM and filtering. DMSO was used as a vehicle control for celastrol. Following 12 h incubation to facilitate Hsp induction, cells were fixed for immunofluorescence (no HS) or exposed to heat shock (HS). For heat shock, cells were immersed in a water bath calibrated at 43°C (± 0.2°C) for 20 min. Cells were then fixed for immunofluorescence (20 min) or returned to 37°C until being fixed at a later time during recovery (1 or 3 h). The commencement of heat shock represents $t = 0$.

Immunofluorescence

Cells were fixed with 4% paraformaldehye in phosphate buffered saline (pH 7.4) for 30 min, permeabilized with 0.1% triton X-100 and 100 mM glycine for 30 min, and then blocked with 5% FBS for 1 h before being incubated with primary antibodies overnight in 1% FBS. HSPA1A (SPA-810), HSPA6 (SPA-754), HSPA8 (SPA-815), DNAJB1 (SPA-400), and HSPB1 (SPA-803) antibodies were obtained from Enzo Life Sciences (Farmingdale, NY, USA). DNAJA1 [clone KA2A5.6] (ab3089), HSPH1 (ab109624), BAG-1 (ab7976), SC35 (ab11826), nucleophosmin (ab37659), and RNA polymerase II CTD repeat YSPTSPS (phospho S5) (ab5131) primary antibodies were purchased from Abcam (Toronto, ON, CA). Primary antibody for the nuclear speckle marker SON (HPA023535) was obtained from Sigma Aldrich. Cells were washed and incubated with Alexafluor® Donkey secondary antibodies (Molecular Probes, Life Technologies, Burlington, ON, CA) and then counterstained with 300 nM DAPI (Invitrogen, Life Technologies). Fluorescence images were acquired using a Quorum Wave FX-X1 spinning disk confocal microscope (Quorum Technologies, Guelph, ON, CA) outfitted with a high resolution Humamatsu Orca R2 camera (Humamatsu Photonics, Japan) and a Plan-APO 63x/1.4NA oil objective.

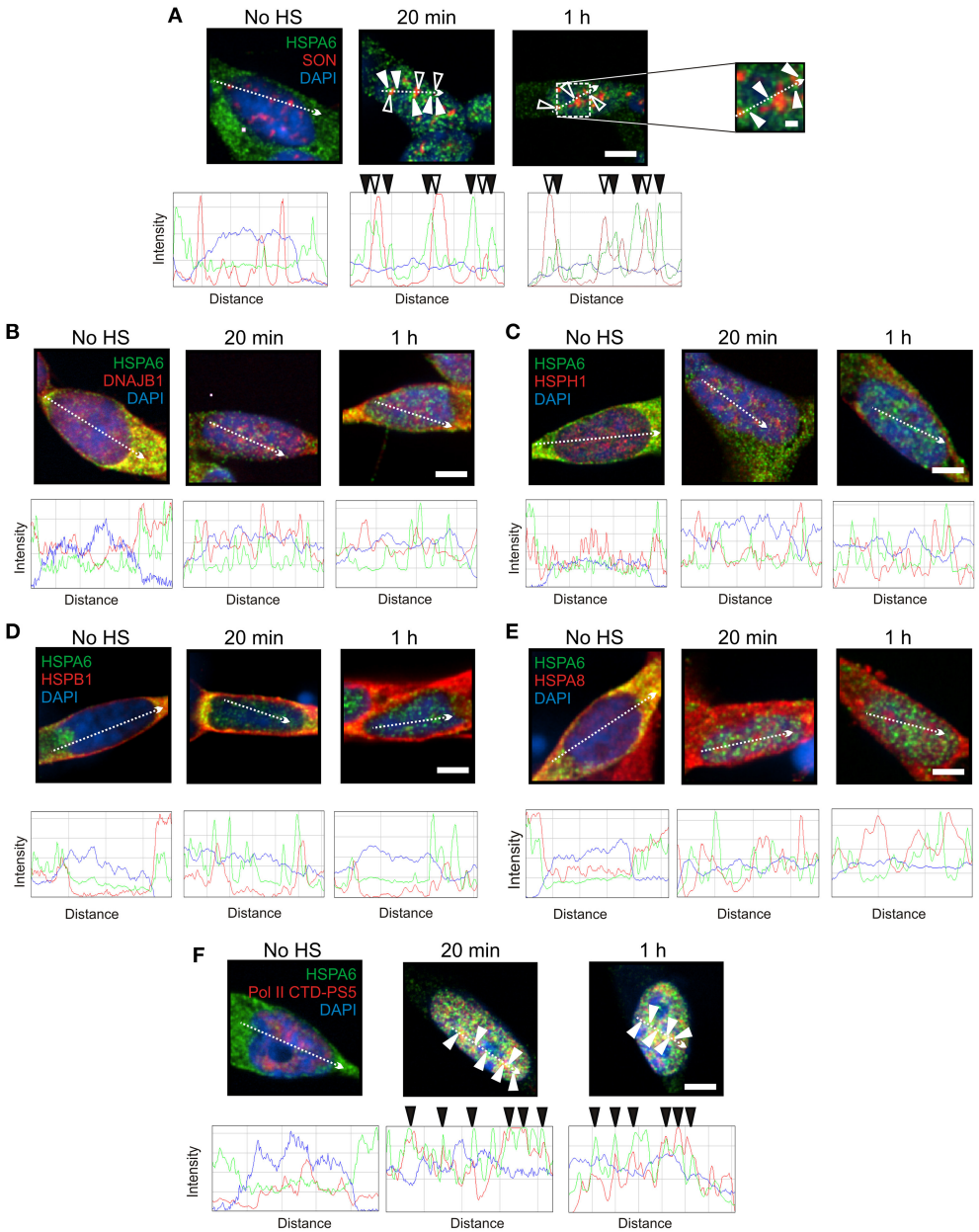

FIGURE 1 | HSPA6 was targeted to the periphery of nuclear speckles (perispeckles) following heat shock in differentiated human neuronal cells. (A) Prior to heat shock, HSPA6 (green) was distributed in the neuronal cytoplasm. After heat shock, HSPA6 localized to foci at the periphery of nuclear speckles (closed arrowheads) identified by the marker protein SON (red, open arrowheads). DAPI (blue) was used to identify neuronal nuclei. ImageJ line scans demonstrated that HSPA6 fluorescent peaks were offset from SON peaks. ImageJ line scans confirm that **(B)** DNAJB1, **(C)** HSPH1, **(D)** HSPB1, and **(E)** HSPA8 did not co-localize with HSPA6. **(F)** HSPA6-positive foci co-localized with the perispeckle marker RNA polymerase II (closed arrowheads). Scale bar represents 5 μm **(A–F)**. Inset scale bar in **(A)** represents 0.5 μm.

Excitation lasers: 405, 491, 561, and 644 nm. Emission filters (nm/bandpass): 460/50, 525/50, and 593/40.

Image Processing and Analysis

Image processing utilized Volocity 3D image analysis software (PerkinElmer, Waltham, MA, USA). ImageJ software (http://imagej.nih.gov/ij/) was employed for co-localization analysis using TIFF images exported from Volocity. Background subtracted images were used to generate intensity profile plots representing the fluorescence signal intensities for the indicated channels in a defined linear region using the RGB (red-green-blue) Profiler plugin. Images representative of 3 individual experiments are shown in which 25 cells were analyzed in coverslips harvested from each well of 6-well culture plates.

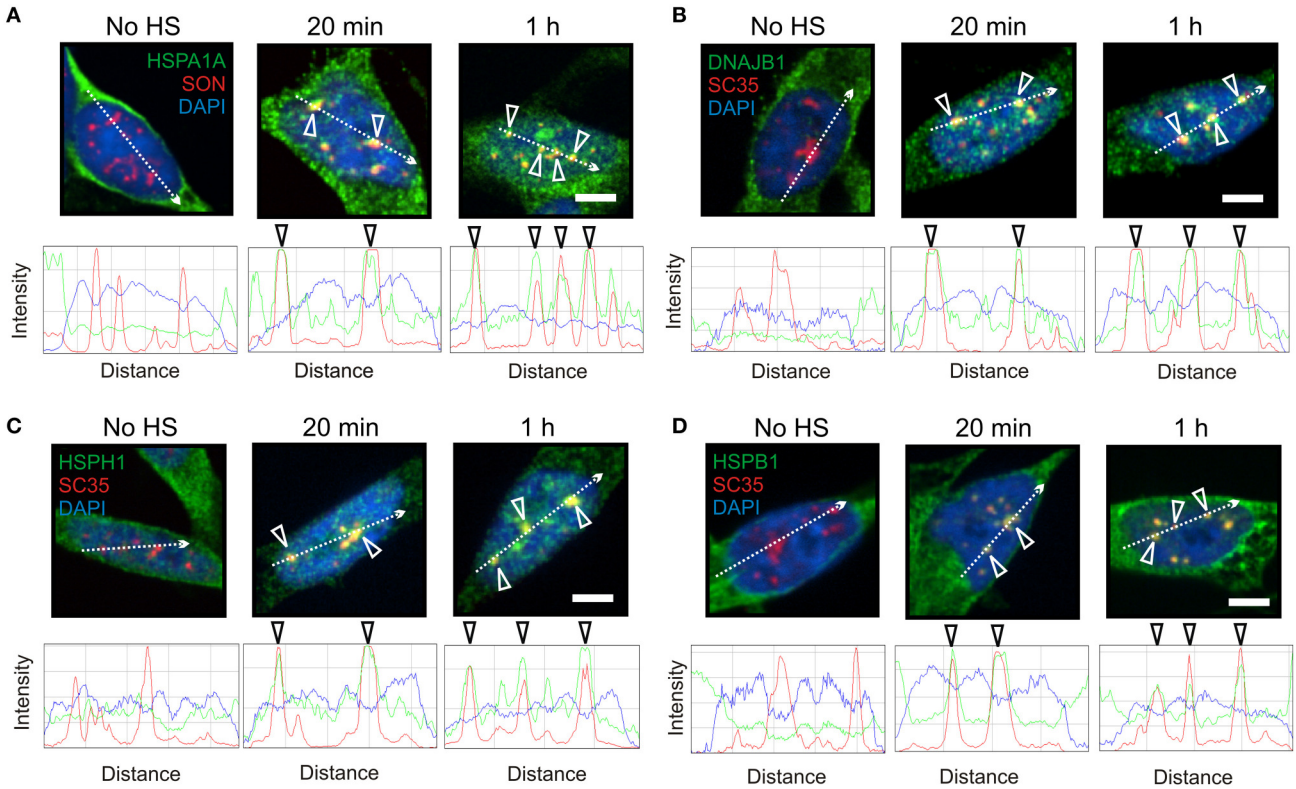

FIGURE 2 | HSPA1A co-localized at nuclear speckles with components of a mammalian protein disaggregation/refolding machine after thermal stress. **(A)** Prior to heat shock, HSPA1A (green) was distributed in the neuronal cytoplasm. After heat shock, HSPA1A co-localized with the nuclear speckle marker protein SON (red, open arrowheads) at 20 min and 1 h, confirmed by ImageJ line scans. DAPI (blue) was used to identify neuronal nuclei. Components of a mammalian protein disaggregation/refolding machine including **(B)** DNAJB1, **(C)** HSPH1, and **(D)** HSPB1, also targeted nuclear speckles (open arrowheads) after heat shock at 20 min and 1 h, as determined by co-localization with nuclear speckle markers SON (HSPA1A) and SC35 (HSPH1, DNAJB1, and HSPB1). Scale bar represents 5 μm.

RESULTS

Differential Targeting of HSPA6 and HSPA1A in Human Neuronal Cells following Thermal Stress

To induce Hsps, including HSPA6 (Hsp70B') and HSPA1A (Hsp70-1), differentiated human neuronal SH-SY5Y cells were treated with celastrol and arimoclomol as previously described (Deane and Brown, 2016). HSPA6 and HSPA1A were distributed in the neuronal cytoplasm prior to heat shock (**Figures 1A**, **2A**, No HS panels). At 20 min and 1 h after heat shock, HSPA6 localized to perispeckles (**Figure 1A**, closed arrowheads) around the periphera of nuclear speckles (open arrowheads) which were identified with the SON marker protein (Sharma et al., 2010; Sytnikova et al., 2011; Khalouei et al., 2014b). As shown in **Figures 1B–E**, components of the mammalian disaggregation/refolding machine, namely DNAJB1 (Hsp40-1), and the "disaggregase" HSPH1 (Hsp105α), and also HSPB1 (Hsp27) and HSPA8 (Hsc70), did not co-localize after heat shock with the HSPA6-positive perispeckles, confirmed by ImageJ line scans located below the immunocytochemistry panels. HSPA6 co-localized with the perispeckle marker RNA polymerase II (**Figure 1F**) that is associated with transcription sites (Ghamari et al., 2012).

As shown in **Figure 2**, components of the disaggregation/refolding machine were targeted with HSPA1A to nuclear speckles, as determined by co-localization with nuclear speckle markers SON and SC35 (**Figure 2**, open arrowheads). The SC35 and SON antibodies used in the present study have been shown to co-localize at nuclear speckles that are enriched in RNA splicing factors in differentiated human neuronal SH-SY5Y cells (Lamond and Spector, 2003; Spector and Lamond, 2011; Khalouei et al., 2014b). ImageJ line scans confirmed the co-localization of HSPA1A at nuclear speckles with DNAJB1 (**Figure 2B**), HSPH1 (**Figure 2C**), and HSPB1 (**Figure 2D**). Hence, HSPA1A was targeted with components of a protein disaggregation/refolding machine to nuclear speckles after heat shock (**Figure 2**), while HSPA6 localized to perispeckles, where signal for these machine components was not detected (**Figure 1**).

Association of HSPA1A and HSPA6 with the Nucleolus in Neuronal Cells

At 1 h after heat shock, HSPA1A, but not HSPA6, was targeted to the granular component (GC) of the nucleolus (**Figure 3A**,

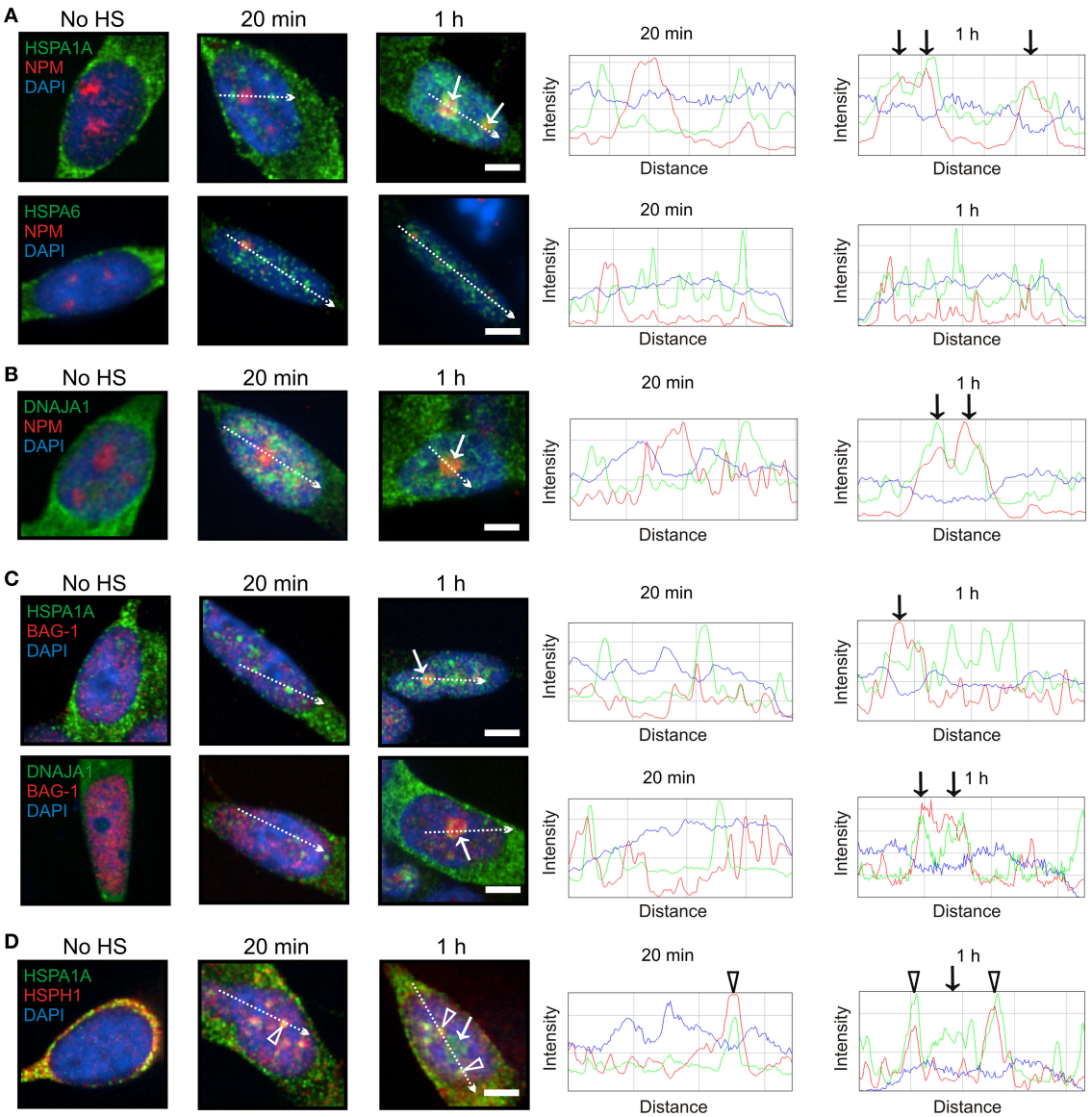

FIGURE 3 | HSPA1A, but not HSPA6, was targeted to the GC layer of the nucleolus at 1 h following heat shock. (A) At 1 h, HSPA1A (green, upper panel), but not HSPA6 (green, lower panel) co-localized with nucleophosmin (NPM) (red, arrow), a marker of the GC layer of the nucleolus. This localization was not observed at 20 min, confirmed by ImageJ line scans shown on the right. **(B)** DNAJA1 also co-localized with nucleophosmin at 1 h (arrows). **(C)** BAG-1 co-localized at 1 h with HSPA1A and DNAJA1 (arrows) that were shown to localize to the nucleolus in **(A,B)**, however the "disaggregase" HSPH1 did not (**D**, arrow). The open arrowheads represent HSPA1A and HSPH1 targeting to nuclear speckles, previously shown in **Figures 2A,C**. Scale bar represents 5 μm.

arrows), identified by nucleophosmin (NPM) marker protein (Hernandez-Verdun et al., 2010), which is the site of ribosomal RNA processing and ribosomal subunit assembly (Thiry and Lafontaine, 2005; Raska et al., 2006; Hernandez-Verdun et al., 2010). DNAJA1 also co-localized with nucleophosmin at the GC layer of the nucleolus (**Figure 3B**, arrows). As shown in **Figure 3C** (arrows), BAG-1 co-localized with HSPA1A and DNAJA1 which were targeted to the nucleolus at 1 h (**Figures 3A,B**, arrows), whereas the "disaggregase" HSPH1 did not (**Figure 3D**, arrow). The open arrowheads in **Figure 3D** represent the targeting of HSPA1A and HSPH1 to nuclear speckles, previously mentioned in **Figures 2A,C**.

Subsequently at 3 h after heat shock, HSPA6 (**Figure 4A**, arrow), but not HSPA1A (**Figure 4B**), co-localized at the GC layer of the nucleolus with components of a protein disaggregation/refolding machine, namely DNAJB1 and the "disaggregase" HSPH1 (**Figure 4C**, arrows), but, interestingly, not BAG-1 (**Figure 4D**). These results suggest differential targeting of HSPA6 and HSPA1A to nucleolar structures following thermal stress.

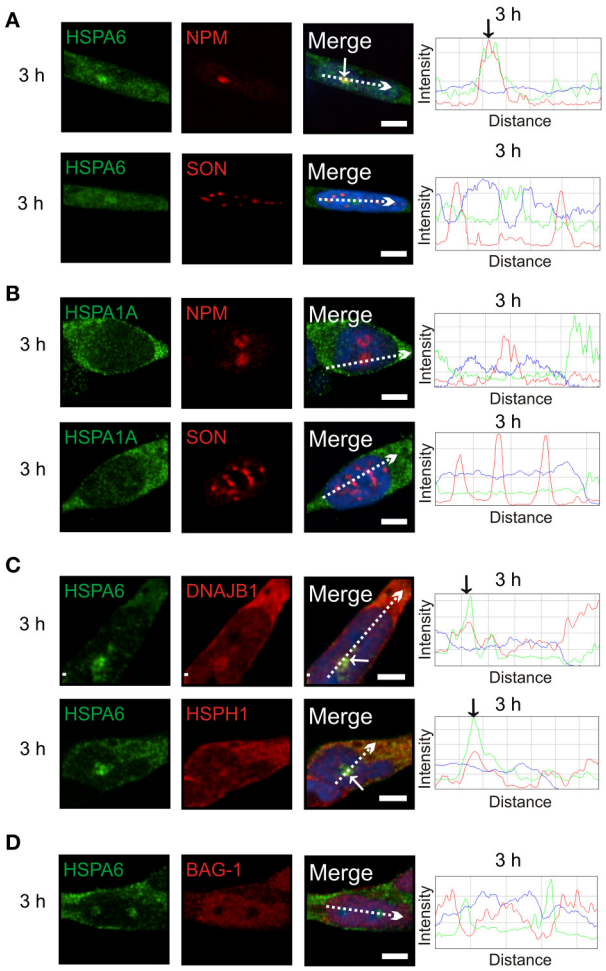

FIGURE 4 | At 3 h after thermal stress, HSPA6 co-localized with nucleophosmin, a marker of the granular component (GC) of the nucleolus. (A) HSPA6 co-localized at the 3 h time point with a marker of the GC layer of the nucleolus, nucleophosmin (arrow, upper panel), but not with the nuclear speckle marker SON (open arrowheads, lower panel). **(B)** HSPA1A did not co-localize with nucleophosmin (upper panel) or SON (lower panel) at 3 h. **(C)** Components of a mammalian disaggregation/refolding machine, including DNAJB1 and HSPH1, were also targeted to the GC layer of the nucleolus at 3 h (arrows), however **(D)** BAG-1 was not. DAPI (blue in merged panels and ImageJ line scans) was used to identify neuronal nuclei. Scale bar represents 5 μm.

Constitutively Expressed HSPA8 Exhibited Similar Heat Shock-Induced Targeting as HSPA1A, However HSPA6 Did Not

HSPA8 (Hsc70) is a constitutively expressed member of the HSPA family that is expressed at high levels in neurons compared to other cell types and has been proposed to provide pre-protection from neuronal stress (Manzerra et al., 1993, 1997; Chen and Brown, 2007a,b). Inducible HSPA members, particularly HSPA1A, have been more widely investigated in studies of protein misfolding and aggregation resulting from cellular stress. However, it has been recognized that constitutive Hsps, including HSPA8, also have stress-related functions (Manzerra et al., 1993; Vos et al., 2008; Stricher et al., 2013).

As shown in **Figure 5**, HSPA8 localized to SON-positive nuclear speckles at 20 min and 1 h after heat shock (**Figure 5A**, open arrowheads), and to the nucleophosmin-positive GC layer of the nucleolus at 1 h (**Figure 5B**, arrows), before returning to the cytoplasm at 3 h. This pattern of heat-induced targeting to neuronal sites was similar to that of HSPA1A (**Figures 5A,B**), but not HSPA6 (**Figures 5C,D**). These results indicate that HSPA8 exhibits similar targeting after thermal stress as inducible HSPA1A. In contrast, HSPA6 exhibits features that are not observed for HSPA1A and HSPA8.

DISCUSSION

HSPA6 (Hsp70B') and HSPA1A (Hsp70-1) are inducible members of the HSPA (Hsp70) family (Chow and Brown, 2007; Noonan et al., 2007, 2008; Deane and Brown, 2016). We have previously shown that these proteins are not detectable in differentiated human neuronal SH-SY5Y cells but are induced by low dose co-application of celastrol and arimoclomol at concentrations that do not affect cell viability (Deane and Brown, 2016). Dividing human tissue culture cell lines, such as unstressed HeLa cells, express high basal levels of HSPA1A (Finka and Goloubinoff, 2013). However, this is not observed in unstressed, differentiated human neuronal SH-SY5Y cells which are non-dividing (Deane and Brown, 2016). The HSPA6 gene is present in the human genome, and in the marmoset monkey (NCBI gene ID: 100411854), camel (Elrobh et al., 2011) and goat (Banerjee et al., 2014) but is not found in the genomes of mouse and rat (Parsian et al., 2000), hence it is absent in current animal models of neurodegenerative diseases (Chow and Brown, 2007; Deane and Brown, 2016, 2017).

In order to advance knowledge of the little studied HSPA6, the present study investigated whether it is targeted to stress-sensitive neuronal sites with components of a mammalian disaggregation/refolding machine. Following thermal stress, HSPA1A, but not HSPA6, rapidly co-localized to nuclear speckles with DNAJB1 and HSPH1 components of a disaggregation/refolding machine. Nuclear speckles are rich in RNA splicing factors and splicing is inhibited by heat shock (Lamond and Spector, 2003; Spector and Lamond, 2011). In contrast, HSPA6, but not HSPA1A, was rapidly targeted by heat shock to perispeckles located at the periphera of nuclear speckles that are rich in RNA polymerase II and poly(A+)-containing RNA (Bregman et al., 1995; Mortillaro et al., 1996; Hall et al., 2006; Khalouei et al., 2014b) and have been characterized as "transcription factories" (Brown et al., 2008; Rieder et al., 2012, 2014). Interestingly, components of the disaggregation/refolding machine, namely DNAJB1, and the "disaggregase" HSPH1 (Hsp105α), did not co-localize with HSPA6 at perispeckles. This suggests a role for HSPA6 at perispeckles that does not require the elements of the disaggregation/refolding machine. It has been reported that HSPA6 is capable of refolding heat-denatured p53 in the absence of DNAJ proteins (Hageman

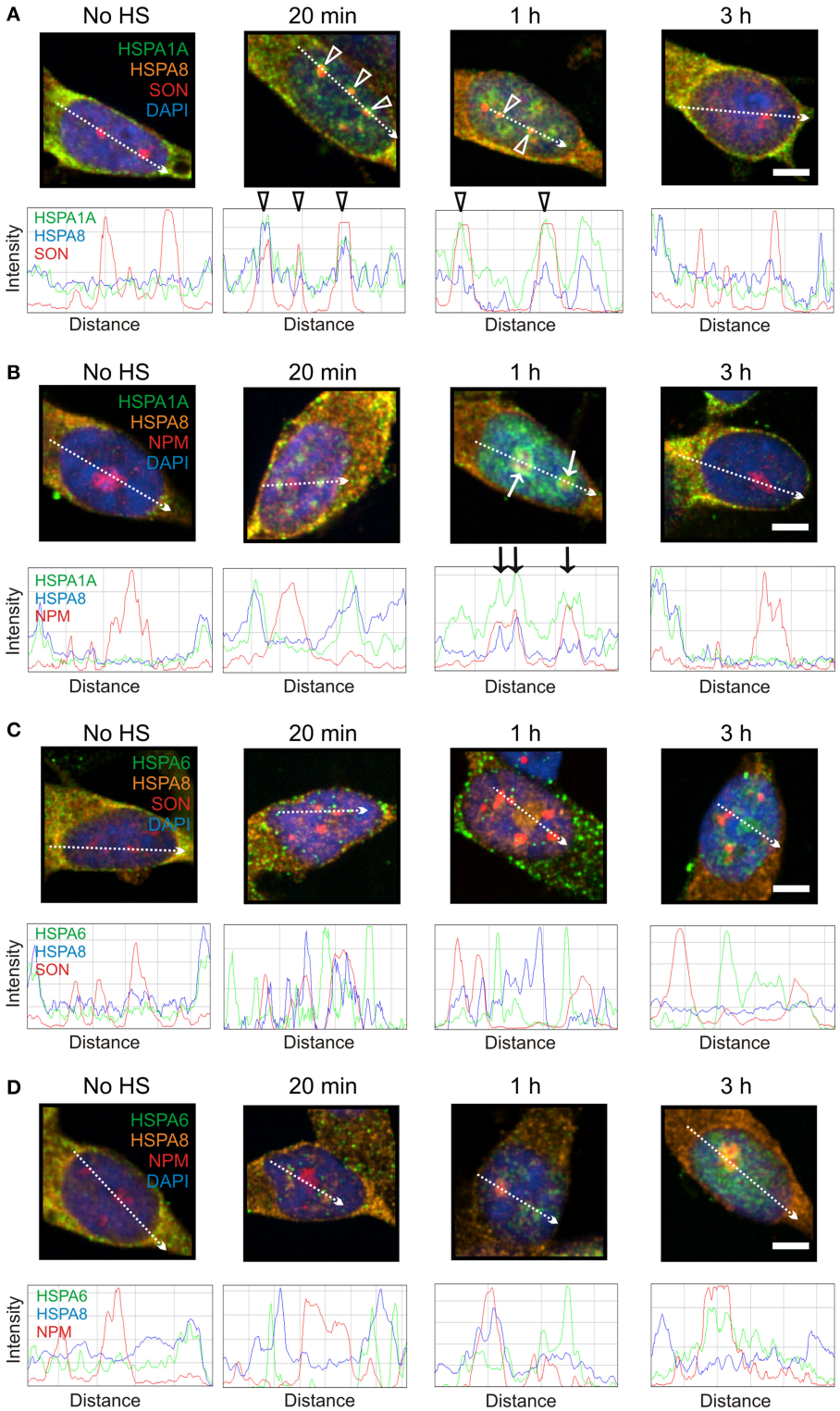

FIGURE 5 | Constitutively expressed HSPA8 exhibited similar heat shock-induced targeting as HSPA1A, however HSPA6 did not. (A) HSPA8 targeted SON-positive nuclear speckles at 20 min and 1 h after heat shock. **(B)** HSPA8 also targeted the GC layer of the nucleolus (identified by the marker protein nucleophosmin) at 1 h and co-localized with HSPA1A. **(C)** HSPA8 did not co-localize with HSPA6 at 20 min and 1 h at perispeckles or **(D)** at the GC layer of the nucleolus at 3 h. DAPI (blue) was used to identify neuronal nuclei. Scale bar represents 5 μm.

et al., 2011). Small heat shock proteins (sHsps) have been reported to enhance recovery from heat-induced nuclear protein aggregation (Kampinga et al., 1994; Stege et al., 1995) likely by maintaining denatured proteins in a folding competent state (Ehrnsperger et al., 1997; Lee et al., 1997; Deunnwald et al., 2012; Rampelt et al., 2012). The present results indicate that HSPB1 (Hsp27) co-localized at nuclear speckles after heat shock with disaggregation/refolding machine components including HSPA1A, DNAJB1, and HSPH1.

Later in the time course after heat shock, HSPA6 and HSPA1A are differentially targeted to the GC layer of the nucleolus which is involved in ribosomal RNA processing and ribosomal subunit assembly (Thiry and Lafontaine, 2005; Raska et al., 2006; Hernandez-Verdun et al., 2010). At the 1 h recovery time point, HSPA1A, but not HSPA6, co-localized at the GC layer of the nucleolus with DNAJA1 and BAG-1, but not with HSPH1 (Hsp105α). BAG-1 targets Hsp70 substrates to the proteasome to facilitate their degradation (Bracher and Verghese, 2015a,b) and does not promote the dissociation of protein aggregates in the presence of other members of the disaggregation/refolding machine (Rampelt et al., 2012). This suggests a possible role for HSPA1A in BAG-1-directed targeting of heat damaged nucleolar proteins to the proteasome for degradation, which is not observed for HSPA6. Subsequently at the 3 h recovery time point, HSPA6, but not HSPA1A, is targeted to the GC layer of the nucleolus with components of the disaggregation/refolding machine comprised of DNAJB1, and the 'disaggregase' HSPH1.

HSPA8 (Hsc70) is a constitutively expressed member of the HSPA (Hsp70) family that is present at high levels in neurons in the mammalian brain (Manzerra et al., 1997). It has been proposed that HSPA8 may pre-protect neurons from stress (Chen and Brown, 2007a,b). The present results indicate that following thermal stress, constitutively expressed HSPA8 is targeted to nuclear speckles with components of the disaggregation/refolding machine. This suggests that neurons may have the capacity to rapidly form a protein disaggregation/refolding machine without the time lag needed to induce HSPA1A. Enhancing levels of HSPA8 could represent an additional strategy to combat protein misfolding and aggregation. The current studies reveal that HSPA8 exhibits targeting features that are similar to HSPA1A and different from HSPA6, that is, (i) co-localization at nuclear speckles with machine components and (ii) targeting to the GC layer of the nucleolus with BAG-1.

Therapies for neurodegenerative diseases that showed promise in current animal models have failed to translate effectively in human clinical trials suggesting deficiencies in these animal models (Nestler and Hyman, 2010; Lang, 2010; Dunkel et al., 2012; t Hart et al., 2012; Sheikh et al., 2013; McGonigle and Ruggeri, 2014; Sasaki, 2015). The present results suggest that elements of the cellular stress response, involving targeting of HSPA6 to perispeckles and later to the GC layer of the nucleolus at 3 h, that are present in differentiated human neuronal SH-SY5Y cells, are absent in current mouse and rat models of neurodegenerative diseases that lack the HSPA6 gene. Primate models are currently being developed using the common marmoset, an animal that possesses the HSPA6 gene (NCBI gene ID: 100411854) (Lang, 2010; t Hart et al., 2012; McGonigle and Ruggeri, 2014; Sasaki, 2015).

AUTHOR CONTRIBUTIONS

CD and IB carried out the design of the work, data acquisition and data analysis. CD and IB also contributed to the writing of the manuscript, gave final approval of the version to be published, and agreement to be accountable for all aspects of the work.

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Endoplasmic Reticulum Malfunction in the Nervous System

Joanna Jung[1], *Marek Michalak*[1]* *and Luis B. Agellon*[2]*

[1] Department of Biochemistry, University of Alberta, Edmonton, AB, Canada, [2] School of Dietetics and Human Nutrition, McGill University, Ste. Anne de Bellevue, QC, Canada

Neurodegenerative diseases often have multifactorial causes and are progressive diseases. Some are inherited while others are acquired, and both vary greatly in onset and severity. Impaired endoplasmic reticulum (ER) proteostasis, involving Ca^{2+} signaling, protein synthesis, processing, trafficking, and degradation, is now recognized as a key risk factor in the pathogenesis of neurological disorders. Lipidostasis involves lipid synthesis, quality control, membrane assembly as well as sequestration of excess lipids or degradation of damaged lipids. Proteostasis and lipidostasis are maintained by interconnected pathways within the cellular reticular network, which includes the ER and Ca^{2+} signaling. Importantly, lipidostasis is important in the maintenance of membranes and luminal environment that enable optimal protein processing. Accumulating evidence suggest that the loss of coordinate regulation of proteostasis and lipidostasis has a direct and negative impact on the health of the nervous system.

Keywords: calnexin, proteostasis, endoplasmic reticulum, lipidostasis, neurological disorders

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Robert Petersen, Case Western Reserve University, USA
Gaiti Hasan, National Centre for Biological Sciences, India

***Correspondence:**
Luis B. Agellon
luis.agellon@mcgill.ca
Marek Michalak
marek.michalak@ualberta.ca

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INTRODUCTION

Neurodegenerative disorders are diseases of the nervous system, often chronic, and progressive in nature, affecting many people worldwide and increasing in incidence each year[1]. They account for about 1% of deaths worldwide and pose one of the largest health, economic, and social capital burden. Environmental factors such as lifestyle, diet, and stress are high risk factors for developing neurological disorders (Migliore and Coppedè, 2009; Ochoa-Repáraz and Kasper, 2014; Perry and Holmes, 2014; Rothhammer and Quintana, 2016). Impaired cellular homeostasis is a hallmark of neurodegenerative diseases (Hetz and Mollereau, 2014). The maintenance of cell homeostasis is a complex and dynamic process relying on coordinated functions of the cellular reticular network, the interconnected network of membranes within the cell that includes the endoplasmic reticulum (ER). The ER is a dynamic membrane system and a multifunctional organelle. It is a major site of protein and lipid synthesis (Hebert and Molinari, 2007; Schwarz and Blower, 2016), and the major intracellular store of Ca^{2+} that is used by Ca^{2+} signaling processes (Krebs et al., 2015). The purpose of this article is to discuss the dynamic events coordinated by the ER, namely synthesis, quality control, and degradation of proteins and lipids, sensing of cellular lipid status as well as maintenance of the ER Ca^{2+} in the cellular signaling network that influence cellular proteostasis and lipidostasis, in the context of the pathogenesis of the diseases of the nervous system.

[1]http://www.who.int/mental_health/publications/neurological_disorders_ph_challenges/en/

CELLULAR STRESS RESPONSES IN THE NERVOUS SYSTEM

Cells, including neuronal cells (neurons, glial cells), are exposed to a wide variety of internal and external factors that induce cellular stress. These factors include gene variations that alter protein structure and function, inducers of oxidative stress, viral infection, environmental toxins, drugs, extremes in temperature, extremes in pH, inflammatory cytokines, lipotoxicity, Ca^{2+} depletion, aging, and other factors that cause loss of nutrient or energy homeostasis. Neurons are particularly susceptible to cellular stress, and disrupted cellular proteostasis or lipidostasis, due to their unique architecture and functional specialization (connectivity and excitability). In response to cellular stress, cells most frequently turn to the coping mechanisms such as the unfolded protein response (UPR; Groenendyk et al., 2013) and genome damage response (GDR; Dicks et al., 2015; **Figure 1**). The UPR works to restore protein homeostasis in the ER (Groenendyk et al., 2013; Hetz and Mollereau, 2014) whereas the GDR functions to repair DNA or chromatin damage (Dicks et al., 2015). Several recent review articles discuss these topics in greater depth (Cao and Kaufman, 2013; Groenendyk et al., 2013; Hetz and Mollereau, 2014; Wang and Kaufman, 2014; Dicks et al., 2015; Hetz et al., 2015). Disrupted proteostasis has been identified as an underlying cause of many neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Huntington disease, amyotrophic lateral sclerosis, prion related diseases, all of which have been referred to as diseases of protein folding (Hetz and Mollereau, 2014). These examples illustrate that long term alteration of cellular function in response to chronic disruption of proteostasis in the nervous system eventually lead to the pathogenesis of neurodegenerative disorders.

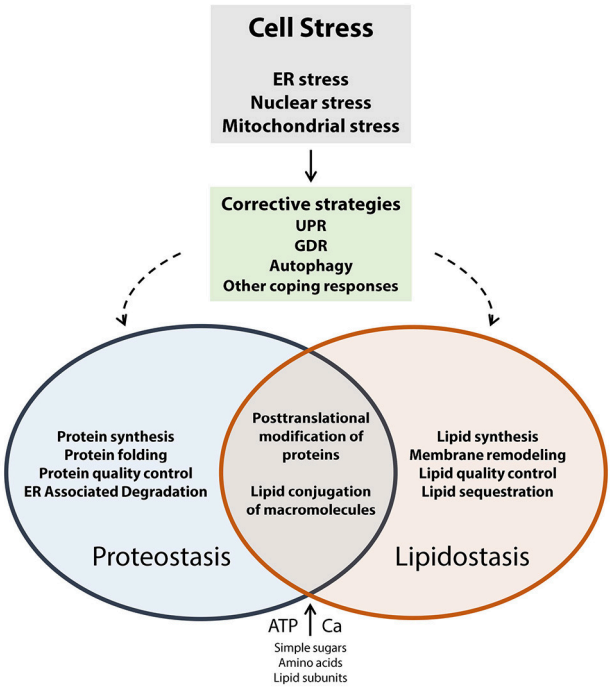

FIGURE 1 | Cell stress coping responses and the interplay between proteostasis and lipidostasis. Proteostasis refers to optimal protein biosynthesis and trafficking whereas lipidostasis pertains to optimal lipid biosynthesis, trafficking, and membrane assembly. Both of these processes rely on the availability of energy (ATP), and nutrients (such as Ca^{2+}, sugars, amino acids, lipid subunits, nucleotides, other essential cofactors). When cells experience external or internal insults that result in the loss of control of nutrient and energy metabolism corrective strategies (UPR, GDR, autophagy, other coping responses) are activated to counteract and eliminate cell stress. The regulatory and metabolic pathways that operate to recover proteostasis and lipidostasis are interconnected, and support each other in preserving global cellular homeostasis.

LIPID HOMEOSTASIS AND NEURODEGENERATIVE DISEASES

The ER is a critical organelle for maintenance of cellular lipid homeostasis (van Meer et al., 2008). It is the site of synthesis of the bulk of structural phospholipids, sterols, and storage lipids such as triacylglycerols and sterol esters (Higgins, 1974; Ikonen, 2008; Fagone and Jackowski, 2009; Chauhan et al., 2016). This organelle also supplies lipids to other cellular organelles, and is the driver of cellular lipid homeostasis. The brain is the most cholesterol enriched organ in the body (Dietschy and Turley, 2001; Zhang and Liu, 2015). Cholesterol in brain cells is derived primarily from *de novo* synthesis since lipoproteins are unable to cross the blood-brain barrier (BBB; Valdez et al., 2010; Zhang and Liu, 2015; Mistry et al., 2017). The majority of the cholesterol in the brain is found in the myelin sheaths that surround axons.

Impaired metabolism and transport of lipids in the brain has been linked to many neurodegenerative diseases such as Alzheimer's disease, Huntington disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, including inherited neurological diseases such as Niemann-Pick C disease, Smith-Lemli-Opitz syndrome, and Gaucher's disease (Cutler et al., 2002; Vanier, 2010; Wu G. et al., 2011; Don et al., 2014; Petrov et al., 2016; Schultz et al., 2016; Abdel-Khalik et al., 2017; Kim et al., 2017; Mistry et al., 2017; Schuchman and Desnick, 2017). In the case of amyotrophic lateral sclerosis, accumulation of ceramides, and cholesteryl esters which cause death of motor neurons (Cutler et al., 2002) is associated with defects in the metabolism of sterols (Cutler et al., 2002; Abdel-Khalik et al., 2017). Lipids may also affect the function of certain proteins; for example, the degree of membrane insertion of huntingtin, the brain protein involved in Huntington disease, is influenced by the amount of membrane cholesterol (Gao et al., 2016).

It is probable that inappropriate remodeling of membranes potentiates the loss of proteostasis by causing the malfunction of molecular chaperones and other membrane bound proteins (**Figure 1**). A recent study reported that long term feeding of mice with a diet enriched with saturated fats causes significant remodeling of the brain lipidome, particularly those lipids that make up the cell membrane (Giles et al., 2016). Considering the integral role of the ER in lipid synthesis, transport and degradation, we propose that lipidostasis is an emerging and

significant risk factor in the pathogenesis of neurodegenerative diseases.

PROTEOSTASIS AND CALNEXIN

The ER protein quality control system is comprised of many molecular chaperones and folding enzymes that closely monitor and facilitate the folding of proteins and their secretion in order to prevent formation and accumulation of toxic protein aggregates. Calnexin, calreticulin, and PDIA3 (a protein foldase that catalyzes the formation and correct isomerization of disulfide bonds and interacts with both calnexin and calreticulin), are the core components of the ER protein quality control system (Hebert and Molinari, 2007). Folding of most of non-glycosylated proteins is supported by BiP/GRP78, a protein that interacts with hydrophobic regions of newly synthesized proteins (Hebert and Molinari, 2007; Halperin et al., 2014). Other chaperons including GRP94, ERdj3, cyclophilin B, PDI, PDIA4, SDF2, and additional members of the PDI family proteins form large protein folding complexes that interact with misfolded and unfolded proteins (Hebert and Molinari, 2007; Halperin et al., 2014) to assist in their proper processing. Moreover, a class of small molecules, termed proteostasis promoters (Vega et al., 2016), have been identified.

Calnexin is a type I transmembrane molecular chaperone, and is of special interest as this protein is highly expressed during the development of the nervous system (Coe et al., 2008; Kraus et al., 2010). In mice, calnexin deficiency causes dysmyelination of peripheral and central nervous system (PNS; Kraus et al., 2010; Jung et al., 2011) as a result of misfolding of P0 and PMP22, two essential glycoproteins required for myelin formation (Jung et al., 2011). Calnexin has also been shown to interact with myelin oligodendrocyte glycoprotein (MOG; Jung and Michalak, 2011; Jung et al., 2015), a protein that is critically involved in the myelination of nerve cells in the central nervous system (CNS). Although MOG is only a minor component of CNS myelin it plays an important role in the pathology of multiple sclerosis (MS), a progressive neurological disorder caused by an autoimmune response against antigens of the CNS. Autoantibodies against MOG have been detected in the serum of MS patients (Reindl et al., 2013). Although deficiency in calnexin does not impact on the intracellular trafficking of MOG, the folding and stability of MOG are affected (Jung and Michalak, 2011; Jung et al., 2015). The discovery of a role for calnexin in maintaining myelin sheets (Kraus et al., 2010; Jung et al., 2011) and folding of MOG (Jung et al., 2015) provides new and unanticipated insights into the mechanisms responsible for myelin diseases of the PNS and CNS.

Calnexin interacts with the SH3-domain GRB2-like (endophilin) interacting protein 1 (SGIP1), a neuronal regulator of endocytosis, supporting a role for calnexin in the recycling of synaptic membrane proteins and maintaining synaptic homeostasis (Li et al., 2011). The balance between exocytosis and endocytosis is vital in maintaining the function of the brain cells (Lim and Yue, 2015). Endocytosis might also be a potential mechanism involved in cell-to-cell transmission of protein aggregates that underlie the pathogenesis of neurodegenerative diseases stemming from accumulation of protein aggregates (Lim and Yue, 2015). Synaptic transporters such as the serotonin transporter (Tate et al., 1999) and glycine transporter 2 which are expressed in the CNS (Arribas-González et al., 2013) are also calnexin substrates. The appearance of calnexin on the surface of hippocampal neurons has been reported (Itakura et al., 2013), further supporting the participation of calnexin in the integration of synaptic proteins to the plasma membrane as well as in the maintenance of synaptic proteostasis.

Global knockout of the PDIA3 gene in mice is embryonic lethal (Coe et al., 2010), however, targeted knockout of PDIA3 in the murine nervous system leads to severe motor dysfunction and growth retardation associated with a loss of neuromuscular synapses reminiscent of calnexin deficiency (Kraus et al., 2010), and more recently, of amyotrophic lateral sclerosis in humans (Woehlbier et al., 2016). Association between PDIA3 and the amyotrophic lateral sclerosis may not be surprising as PDIA3 expression is high in the brain during embryonic development (Coe et al., 2010). BiP, a key component of the UPR and essential regulator of ER proteostasis and Ca^{2+} homeostasis, has also been associated with neurodegenerative diseases (Hoozemans et al., 2005; Carnemolla et al., 2009; Wang et al., 2009; Gorbatyuk and Gorbatyuk, 2013). Global BiP gene knockout in mice is embryonic lethal (Luo et al., 2006). However, targeted deletion of BiP in developing Schwann cells manifests in a phenotype reminiscent of that seen in calnexin-deficient mice (Kraus et al., 2010), in particular PNS myelin abnormalities, diminished number of myelinating Schwann cells and hind limb paralysis (Hussien et al., 2015; Volpi et al., 2016). A class of small molecules, termed proteostasis promoters (Vega et al., 2016), have been described. For example, valproic acid, a drug that is currently used in the clinical management of mood disorders (Chiu et al., 2013), has been shown to induce the UPR coping mechanism and inhibit ER stress (Kakiuchi et al., 2003; Lee et al., 2014; Wang et al., 2015; Peng et al., 2016). Although the precise mechanism of action of specific compounds are not yet fully deciphered, proteostasis promoters have in common the ability of enhancing protein processing and relieving cellular stress, including in neuronal cells.

Disrupted autophagy has been linked with pathology of CNS disorders (Nikoletopoulou et al., 2015). Autophagy, a dynamic process promoting self-digestion, to help eliminate toxic aggregates through the lysosomal pathway (Yorimitsu et al., 2006) involves bulk degradation of proteins, lipids and organelles, including the ER (Kaur and Debnath, 2015). As neurons are post-mitotic cells, they rely on autophagy for removal of defective organelles, protection against protein aggregation and in preventing the accumulation of toxic proteins. Abnormal autophagy is involved in neurodegenerative disease pathology (Nikoletopoulou et al., 2015) as well as in acute brain injuries (Galluzzi et al., 2016). Calnexin is a component of the early autophagosomes (Gagnon et al., 2002) pointing to its potential role in an alternative mechanism for degradation of misfolded proteins and removal of organellar membranes in the nervous

system. The accumulating evidence from animal and clinical studies support a role for calnexin, and likely other ER molecular chaperones and folding enzymes, in maintaining neuronal proteostasis and perhaps also lipidostasis.

ER CALCIUM HOMEOSTASIS

The ER is the major Ca^{2+} storage depot of the cell. Ca^{2+} release from the ER impacts on the vast majority of cellular processes, including cell proliferation, transcription, exocytosis, apoptosis (Corbett and Michalak, 2000; Prins and Michalak, 2011; Krebs et al., 2015). Accordingly, maintenance of normal ER Ca^{2+} capacity is vital in supporting cellular stress coping responses in re-establishing proteostasis and lipidostasis (**Figure 1**), and therefore ER Ca^{2+} levels must be finely regulated. This can be accomplished by coordinating the function of multiple Ca^{2+} sensors, pumps, channels, exchangers, and Ca^{2+} binding proteins (Prins and Michalak, 2011; Brini et al., 2014; Krebs et al., 2015). Ca^{2+} in the lumen of the ER is frequently depleted by Ca^{2+} signaling events occurring within the ER and in other cellular compartments. Thus, in order to maintain Ca^{2+} signaling capacity, Ca^{2+} released from the ER lumen must be replenished. This process, which involves Ca^{2+} entry from the external environment of the cell into the ER, is referred to as store-operated Ca^{2+} entry (SOCE; Soboloff et al., 2012).

SOCE is initiated by Ca^{2+} release through inositol 1,4,5-triphosphate receptor (IP_3R) and/or ryanodine receptor Ca^{2+} channels and relies on ER luminal Ca^{2+} sensors (STIM proteins), a plasma membrane Ca^{2+} channel (ORAI), and sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA; Soboloff et al., 2012). Since ER chaperones and folding enzymes require Ca^{2+} to function, the sustained depletion of ER Ca^{2+} leads to the accumulation of misfolded proteins which subsequently activates UPR and other corrective strategies (Groenendyk et al., 2013). In neuronal tissue, Ca^{2+} signaling is especially important as it controls additional processes that do not occur in other tissues, such as synaptic signaling and neurotransmission. Neuronal Ca^{2+} signaling also plays an important role in learning, memory and neuronal plasticity (Brini et al., 2014). Not surprisingly, disturbance of ER Ca^{2+} homeostasis is commonly observed in severe neurodegenerative diseases (Mattson et al., 2000; Ong et al., 2010; Chen et al., 2011; Mekahli et al., 2011; Wu J. et al., 2011; Belal et al., 2012; Selvaraj et al., 2012; Bezprozvanny and Hiesinger, 2013; Popugaeva and Bezprozvanny, 2013; Zeiger et al., 2013; Koran et al., 2014). For example, mutations in the IP3R type 1 gene leads cerebellar neurodegeneration in mice and causes spinocerebellar ataxia type 15 (SCA15) leading to neurodegeneration in humans (van de Leemput et al., 2007; Sasaki et al., 2015; Tada et al., 2016). Mechanisms that ensure ER Ca^{2+} homeostasis might allow neuronal cells to effectively maintain both proteostasis and lipidostasis, and thereby prevent neuronal pathology. Overload of Ca^{2+} in the ER is also harmful hence ensuring constant supply without regulated release could lead to disease. Increased abundance of STIM1 and ORAI1 in HEK cells resulted in reduced formation and secretion of Aβ peptides (Zeiger et al., 2013). Furthermore, neuronal cell expressing mutant Huntingtin protein exhibit enhanced SOCE (Wu J. et al., 2011) and the loss of SOCE was observed in neuroblastoma cells treated with agent that mimics Parkinson's disease in mice (Selvaraj et al., 2012). Mechanisms that ensure the constant supply of Ca^{2+} in the ER might allow neuronal cells to effectively maintain both proteostasis and lipidostasis, and thereby prevent neuronal pathology.

BRAIN PERMEABILITY

The BBB is a physical structure that separates the CNS from the rest of the body, and selectively controls the flow of molecules in and out of the brain. Dysfunction of the brain endothelial cells, essential component of the BBB, is involved in the pathology of many CNS disorders (Deane et al., 2004; Cirrito et al., 2005; Zlokovic et al., 2005; Alvarez and Teale, 2006; Deane and Zlokovic, 2007; Tietz and Engelhardt, 2015), however the molecular mechanisms underlying its contribution are not fully understood. Abnormalities in BBB have been linked to pathogenesis of the Alzheimer disease (Cirrito et al., 2005; Zlokovic et al., 2005) involving defective clearance of β-amyloid (Deane et al., 2004; Deane and Zlokovic, 2007). Recent studies link ER stress coping responses and BBB disruption in the rat model of epilepsy (Ko et al., 2015). Brain endothelial cells are not only a physical barrier but also a dynamic interface involved in transport of the molecules and capable of response to inflammation on either side of the barrier. Brain endothelial cells are sensitive to proinflammatory factors, which affects the integrity and function of the BBB, originating from both sides of the BBB (Tietz and Engelhardt, 2015). The crossing of the auto-reactive lymphocytes across BBB accompanied by demyelination and neurodegeneration are hallmarks of MS pathology (Mahad et al., 2015). Experimental autoimmune encephalomyelitis (EAE), an animal model of MS allowed insights into a potential role of ER chaperones in initiation and progression of MS. ER quality control components including calnexin, calreticulin, BiP and PDIs likely play critical roles in facilitating the folding and trafficking of endothelial specific proteins such as ICAM, VCAM, and p-selectin in response to inflammation. Increased abundance of BiP has been seen in brain of MS patients (Mháille et al., 2008; Cunnea et al., 2011) and conditional knockout of the BiP gene and, consequently a disrupted proteostasis, exhibits exacerbated EAE symptoms that are not related to altered inflammatory response (Hussien et al., 2015). It is conceivable that other components of protein quality control, including PDIA3, calreticulin, and calnexin, may influence the function and integrity of the BBB. For example, calreticulin associates with MMP9 (Duellman et al., 2015) a matrix metalloproteinase that is critical for the integrity of BBB (Dubois et al., 1999; Rosenberg, 2009) and contributes to amyloid formation and clearance (Nalivaeva et al., 2008). Strategies allowing exogenous manipulation of the ER protein quality control system may offer a means to regain proteostasis as well as lipidostasis (**Figure 1**) in the

nervous system, and assist in the management of neurological disorders.

SUMMARY

We propose that disrupted proteostasis and lipidostasis underlie many neurological disorders. Recent studies suggest that molecular chaperones are intimately involved in coordinating the cellular proteostasis and lipidostasis in the nervous system, including the cells that make up the BBB, by ensuring the quality of key proteins and lipid components of the membranes. Importantly, the activity of ER chaperones depends on ER Ca^{2+} homeostasis. A detailed knowledge of the regulatory and metabolic pathways involved in proteostasis and lipidostasis in cells that make up the nervous system, will provide better insights into the heterogeneity of neurological disorders and uncover new opportunities for therapeutic development.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Chaperone Proteins in the Central Nervous System and Peripheral Nervous System after Nerve Injury

Shalina S. Ousman[1], Ariana Frederick[1] and Erin-Mai F. Lim[2]*

[1] Departments of Clinical Neurosciences and Cell Biology & Anatomy, Hotchkiss Brain Institute, University of Calgary, Calgary, AB, Canada, [2] Department of Neuroscience, Hotchkiss Brain Institute, University of Calgary, Calgary, AB, Canada

Injury to axons of the central nervous system (CNS) and the peripheral nervous system (PNS) is accompanied by the upregulation and downregulation of numerous molecules that are involved in mediating nerve repair, or in augmentation of the original damage. Promoting the functions of beneficial factors while reducing the properties of injurious agents determines whether regeneration and functional recovery ensues. A number of chaperone proteins display reduced or increased expression following CNS and PNS damage (crush, transection, contusion) where their roles have generally been found to be protective. For example, chaperones are involved in mediating survival of damaged neurons, promoting axon regeneration and remyelination and, improving behavioral outcomes. We review here the various chaperone proteins that are involved after nervous system axonal damage, the functions that they impact in the CNS and PNS, and the possible mechanisms by which they act.

Keywords: peripheral nerve injury, spinal cord injury, axotomy, chaperones, chaperone proteins, regeneration, neuronal cell death, myelination

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****Correspondence:***
Shalina S. Ousman
sousman@ucalgary.ca

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PNS AND CNS NERVE DAMAGE

Following damage to the axons of central nervous system (CNS) and peripheral nervous system (PNS) neurons, a number of cellular and molecular processes are initiated to promote regeneration of damaged nerve fibers, remyelination and target reinnervation (Zochodne, 2008). In the injured PNS for example, re-expression of regeneration associated genes (RAGs) such as c-jun and growth associated protein-43, are involved in axon outgrowth (Hall, 2005; Zochodne, 2008) while inhibitory axonal and myelin debris is phagocytosed by Schwann cells and infiltrating hematogenously-derived macrophages (Waller, 1850; Gaudet et al., 2011). Schwann cells also (Liu H. M. et al., 1995) secrete neutrophic factors (Madduri and Gander, 2010) and adhesion molecules (Colognato et al., 2005; Nodari et al., 2008) that allow for survival and directional growth of regenerating axons. Because of these favorable conditions, axotomized PNS neurons generally regenerate robustly, although less so in humans. Compared to the PNS however, injured CNS neurons regrow poorly and this has been attributed to reduced and/or premature truncation of beneficial processes. For example, the damaged CNS displays inadequate RAG expression, poor immune responses and, death of oligodendrocytes (David and Ousman, 2002). As a consequence, many labs are interested in identifying the molecular factors that promote axon regeneration in the damaged CNS and PNS. Over the last three decades, it has become evident that chaperone proteins are involved in the PNS and CNS after nerve damage. This mini-review will focus on which chaperones have been found

to be modulated following axon damage (**Table 1**) and, the functions that they have been assigned (**Figure 1**).

CHAPERONE PROTEINS

There are thousands of original manuscripts and reviews on chaperone proteins and so we will only introduce them in general terms here. Proteins have to be in a specific conformation in order to perform their functions. When cells experience stresses such as high and low temperatures, altered pH, oxygen deprivation, or disease states, proteins have difficulty in forming and maintaining their proper structures. Also, misfolded proteins can cause correctly structured ones to unfold. If not corrected, misfolded proteins can form aggregates that could lead to cell death (Reddy et al., 2008). Chaperones assist in the correct non-covalent assembly of polypeptides. They have the ability to recognize unfolded or partially denatured proteins and prevent incorrect associations and aggregation of unfolded polypeptide chains (Derham and Harding, 1999). In addition to correcting protein misfolding, some chaperones such as heat shock protein (HSP)70 and alphaB-crystallin (αBC)/HSPB5 promote (Campisi and Fleshner, 2003) or suppress (Ousman et al., 2007) inflammatory responses, while others such as HSP27 and αBC are involved in cell survival (Parcellier et al., 2005). Chaperones therefore play an important role in maintaining cell homeostasis and survival.

EXPRESSION OF CHAPERONES AFTER PNS AND CNS NERVE INJURY

Hsps were one of the earliest chaperones discovered to have altered expression after PNS and CNS axonal damage. Cauley et al. (1986) found that expression of a 30 kDa HSP was augmented after optic nerve crush in goldfish. HSP70 was subsequently discovered to be induced after facial nerve axotomy in hamsters (New et al., 1989) and following crush damage to rat spinal cords (Gower et al., 1989), along with the ability to be retrogradely transported in damaged frog sciatic nerves (Edbladh et al., 1994). Other groups have also observed augmented expression of HSP70 in transected zebrafish optic nerves (Nagashima et al., 2011) and rat spinal cord (Keeler et al., 2012) as well as in macrophages and astrocytes after spinal cord contusion in rats (Mautes and Noble, 2000). After these first observations, increased expression of other hsps including HSP25 (Iijima et al., 2003) and HSP27 (Hirata et al., 2003) has been noted in the retina, optic tract and superior colliculus of transected rat optic nerves (Krueger-Naug et al., 2002; Hebb et al., 2006), in transected rat spinal cord (Keeler et al., 2012), and in inferior alveolar and sciatic nerves and their associated Schwann cells and dorsal root ganglia (DRG) (Costigan et al., 1998; Kim et al., 2001). Of interest, there may be some selectivity in the location of HSP27 and phosphorylated-HSP27 after cervical spinal cord injury since the hsp was found to be expressed only in subpopulations of injured neurons in the rostral ventral respiratory group, the dorsal part of the gigantocellularis (Gi), and vestibular nucleus, but seldom in the ventral Gi and raphe nucleus (Vinit et al., 2011). Work by Willis et al. (2005) expanded the list of HSPs found to be enhanced in the injured PNS to include HSP60, glucose-regulated protein (GRP)75 and αBC which they discovered were capable of being synthesized within damaged PNS axons. In the CNS, increases in the levels of HSP32, HSP72 and HSP90 were evident following transection of the spinal cord and even after constriction and transection of peripheral nerves (Klass et al., 2008; Sharma et al., 2015). The latter observation showed that PNS nerve damage could alter expression of hsps in the CNS.

Other chaperone proteins besides hsps have been associated with axonal damage in the PNS and CNS. In 1997, Bonnard et al. (1997) found that clusterin/ApoJ/sulfated glycoprotein-2 mRNA increased in rat sciatic nerve after crush damage with expression of the chaperone possibly being specific to sensory axons as observed in a mouse sciatic nerve transection paradigm (Wright et al., 2014). Since the Bonnard observation, an augmentation in clusterin mRNA and protein has also been seen in numerous nerve injury scenarios including the spinal cord dorsal horn and gracile nucleus following rat sciatic nerve transection (Liu L. et al., 1995), the rat hippocampus after entorhinal cortex lesioning (Lampert-Etchells et al., 1991), the mesodiencephalic after hemitransection (Zoli et al., 1993), and the hypoglossal nucleus after hypoglossal nerve transection (Svensson et al., 1995). Finally, GRP94 is another chaperone whose expression increases after contusion injury in the rat spinal cord (Xu et al., 2011) while Bcl-2-associated athanogene-1 (BAG1), a co-chaperone for HSP70/HSC70 expression, is enhanced in Schwann cells following sciatic nerve crush in rats (Wu et al., 2013).

Not all chaperones however are augmented after PNS and CNS injury. Klopstein et al. (2012) noted a reduction in the small hsp, αBC, after spinal cord contusion injury in mice, and we have also observed a decrease in this crystallin after sciatic nerve crush damage in mice (Lim et al., 2017). Further, sigma-1 receptor (σ1R), an endoplasmic reticulum chaperone protein which is present in both sensory neurons and satellite cells in rat DRGs, was found to be downregulated in neurons as well as in their accompanying satellite glial cells after sciatic nerve ligation (Bangaru et al., 2013). HSP60 is another chaperone that was decreased in the brains of rats with pain and motor deficits following sciatic nerve ligation (Mor et al., 2011) while HSP90ab1, HSPa4, and HSPe1 were reduced after contusion injury in rats (Zhou et al., 2014).

Altogether then, the expression of various chaperones and co-chaperones is altered after CNS and PNS axon damage in either an enhanced or reduced manner. The question is, do these proteins play an active role in the injury or repair processes.

FUNCTION OF CHAPERONE PROTEINS AFTER PNS AND CNS AXONAL DAMAGE

Neuronal Survival

Because of the increased expression of clusterin in axotomized motorneurons, Törnqvist et al. (1996) suggested that the chaperone may be involved in the death of damaged neurons. Other studies instead indicate that some chaperone proteins play

TABLE 1 | Expression of chaperone proteins after CNS and PNS axotomy.

Protein	PNS/CNS, species	Tissue	Cell type	Change	Authors
alphaB-crystallin	CNS, mouse	Spinal cord	Astrocytes and oligodendrocytes	Decreased	Klopstein et al., 2012
	PNS, rat	DRG	Neurons	Increased	Willis et al., 2005
	PNS, mouse	Sciatic nerve	Schwann cells and neurons	Decreased	Lim et al., 2017
BAG1	PNS, rat	Sciatic nerve	Schwann cells	Increased	Wu et al., 2013
BiP	CNS, rat	Spinal cord	Neurons and glia	Unchanged	Penas et al., 2007
	CNS, rat	Spinal cord	Neurons and glia	Decreased	Penas et al., 2011a
Clusterin	CNS, rat	Hippocampus	Astrocytes and neurons	Increased	Lampert-Etchells et al., 1991
	CNS, rat	Brain	Non-neuronal	Increased	Zoli et al., 1993
	CNS, rat	Spinal cord	Astrocytes and oligodendrocytes	Increased	Liu et al., 1998
	CNS, rat	Spinal cord	Glia	Increased	Liu L. et al., 1995
	CNS, rat	Red nucleus	Neurons and glia	Increased	Liu et al., 1999
	CNS, rat	Spinal cord	Neurons and glia	Increased	Klimaschewski et al., 2001
	CNS, rat	Spinal cord and brain	Neurons and glia	Increased	Törnqvist et al., 1996
	PNS, rat	Sciatic nerve	Non-specific	Increased	Bonnard et al., 1997
	PNS, rat	Sciatic nerve	Schwann cells	Increased	Wright et al., 2014
	PNS, rat	Hypoglossal nerve	Neurons and glia	Increased	Törnqvist et al., 1996
	PNS, rat	Hypoglossal nerve	Neurons and glia	Increased	Svensson et al., 1995
GRP75	PNS, rat	DRG	Neurons	Increased	Willis et al., 2005
GRP94	CNS, rat	Spinal cord	Neurons and astrocytes	Increased	Xu et al., 2011
HSP25	CNS and PNS, mouse	Spinal cord and sciatic nerve	Neurons	Increased	Murashov et al., 2001
	PNS, rat	Inferior alveolar nerve	Schwann cells, neurons, and endothelial cells	Increased	Iijima et al., 2003
HSP27	CNS, hamster	Retina	Retinal ganglion cells	Increased	Liu et al., 2013
	CNS, mouse	Spinal cord	n/a	Increased	Yi et al., 2008
	CNS, rat	Spinal cord	n/a	Increased	Zhang et al., 2010
	CNS, rat	Spinal cord	n/a	Increased	Park et al., 2007
	CNS, rat	Spinal cord	Neurons	Increased	Keeler et al., 2012
	CNS, rat	Brainstem	Neurons	Increased	Vinit et al., 2011
	CNS, rat	Retina	Retinal ganglion cells	Increased	Hebb et al., 2006
	CNS, rat	Retina, optic tract, and superior colliculus	Retinal ganglion cells and astrocytes	Increased	Krueger-Naug et al., 2002
	CNS, rat	Medulla oblongata	Neurons	Increased	Hopkins et al., 1998
	CNS, rat	Retina, optic nerve, optic tract, lateral geniculate leaflet, visual cortex	Non-specific; astrocytes	Increased	Chidlow et al., 2014
	CNS and PNS, rat	Sciatic nerve, DRG, spinal cord	Neurons	Increased w/anterograde transport	Costigan et al., 1998
	PNS, rat	Sciatic nerve	Schwann cells	Increased	Hirata et al., 2003
	PNS, rat	DRG	Neurons	Increased	Benn et al., 2002
	PNS, rat	Sciatic nerve	Non-specific	Increased	Klass et al., 2008
	PNS, rat	Sciatic nerve	Non-specific	Increased	Kim et al., 2001
	PNS, rat	Sciatic nerve	n/a	Increased	Tsubouchi et al., 2009
HSP30	CNS, goldfish	Retina	n/a	Increased	Cauley et al., 1986

(Continued)

TABLE 1 | Continued

Protein	PNS/CNS, species	Tissue	Cell type	Change	Authors
HSP32	CNS, mouse	Spinal cord	n/a	Increased	Yi et al., 2008
	CNS, rat	Spinal cord	n/a	Increased	Park et al., 2007
HSP60	PNS, rat	DRG	Neurons	Increased	Willis et al., 2005
HSP70	CNS, zebrafish	Optic nerve	Retinal ganglion cells	Increased	Nagashima et al., 2011
	CNS, rabbit	Spinal cord	Neurons	Increased	Sakurai et al., 1997
	CNS, mouse	Spinal cord	n/a	Increased	Yi et al., 2008
	CNS, rat	Spinal cord	Neurons and glia	Increased	Gower et al., 1989
	CNS, rat	Spinal cord	Macrophages and glia	Increased	Mautes and Noble, 2000; Mautes et al., 2000
	CNS, rat	Spinal cord	n/a	Increased	Park et al., 2007
	CNS, rat	Spinal cord	n/a	Increased	Sengul et al., 2013
	CNS, rat	Spinal cord	n/a	Increased	Zhang et al., 2010
	CNS, rat	Spinal cord	Non-specific	Increased	Song et al., 2001
	CNS, rat	Spinal cord	Neurons	Increased	Keeler et al., 2012
	CNS, rat	Spinal cord	Non-specific	Increased	Kalmar et al., 2003
	CNS, rat	Retina, optic nerve, optic tract, lateral geniculate leaflet, visual cortex	Non-specific	No change	Chidlow et al., 2014
	CNS & PNS, dog	Spinal cord and DRG	Neurons and glia	Increased	Cízková et al., 2005
	PNS, hamster	Facial nerve	Non-specific	Increased	New et al., 1989
	PNS, frog	Sciatic nerve	Neurons	Retrograde transport	Edbladh et al., 1994
	PNS, rat	Sciatic nerve	Non-specific	Increased	Klass et al., 2008
HSP72	CNS, rat	Spinal cord	n/a	Increased	Sharma et al., 2015
	CNS, rat	Spinal cord	Neurons	Increased	Sharma et al., 2006
	CNS, rat	Spinal cord	Non-specific	Increased	Tachibana et al., 2002
	CNS, rat	Spinal cord	n/a	Increased	Chang et al., 2014
	PNS, rat	External carotid nerve ganglion	Schwann cells and neurons	Increased	Hou et al., 1998
HSP90	CNS, rat	Spinal cord	n/a	Increased nitration	Franco et al., 2013
	PNS, rat	Sciatic nerve	Non-specific	Increased	Klass et al., 2008
HSP90ab1	CNS, rat	Spinal cord	Non-specific	Decreased	Zhou et al., 2014
HSPa4	CNS, rat	Spinal cord	Non-specific	Decreased	Zhou et al., 2014
HSPe1	CNS, rat	Spinal cord	Non-specific	Decreased	Zhou et al., 2014
σ1R	PNS, rat	DRG	Neurons and satellite glia	Decreased	Bangaru et al., 2013

a role in maintaining survival of damaged neurons. For instance, Benn et al. (2002) found that upregulation and phosphorylation of HSP27 was required for the survival of sensory and motor neurons after sciatic nerve transection. The lab then extended this work both *in vivo* and *in vitro* to show that survival of PNS neurons from injured neonatal or adult animals following nerve growth factor (NGF) removal was related to whether DRG cells expressed HSP27. They also demonstrated that overexpression of human HSP27 in neonatal rat sensory and sympathetic neurons significantly increased survival after NGF withdrawal (Lewis et al., 1999). With respect to other chaperone proteins, HSP70 was observed to promote retinal ganglion cell survival and optic nerve regeneration in zebrafish since these processes were enhanced and reduced respectively if the hsp was inhibited (Nagashima et al., 2011). Moreover, exogenous application of HSP70 to the proximal end of transected rat sciatic nerves prevented the death of almost all sensory neurons (Houenou et al., 1996). Other evidence for a role of chaperones in promoting neuronal survival after CNS and PNS injury was demonstrated by Chang et al. (2014) who showed that HSP72 expression in neurons and astrocytes correlated with less apoptosis of these cell types in a rat spinal cord compression model. Also,

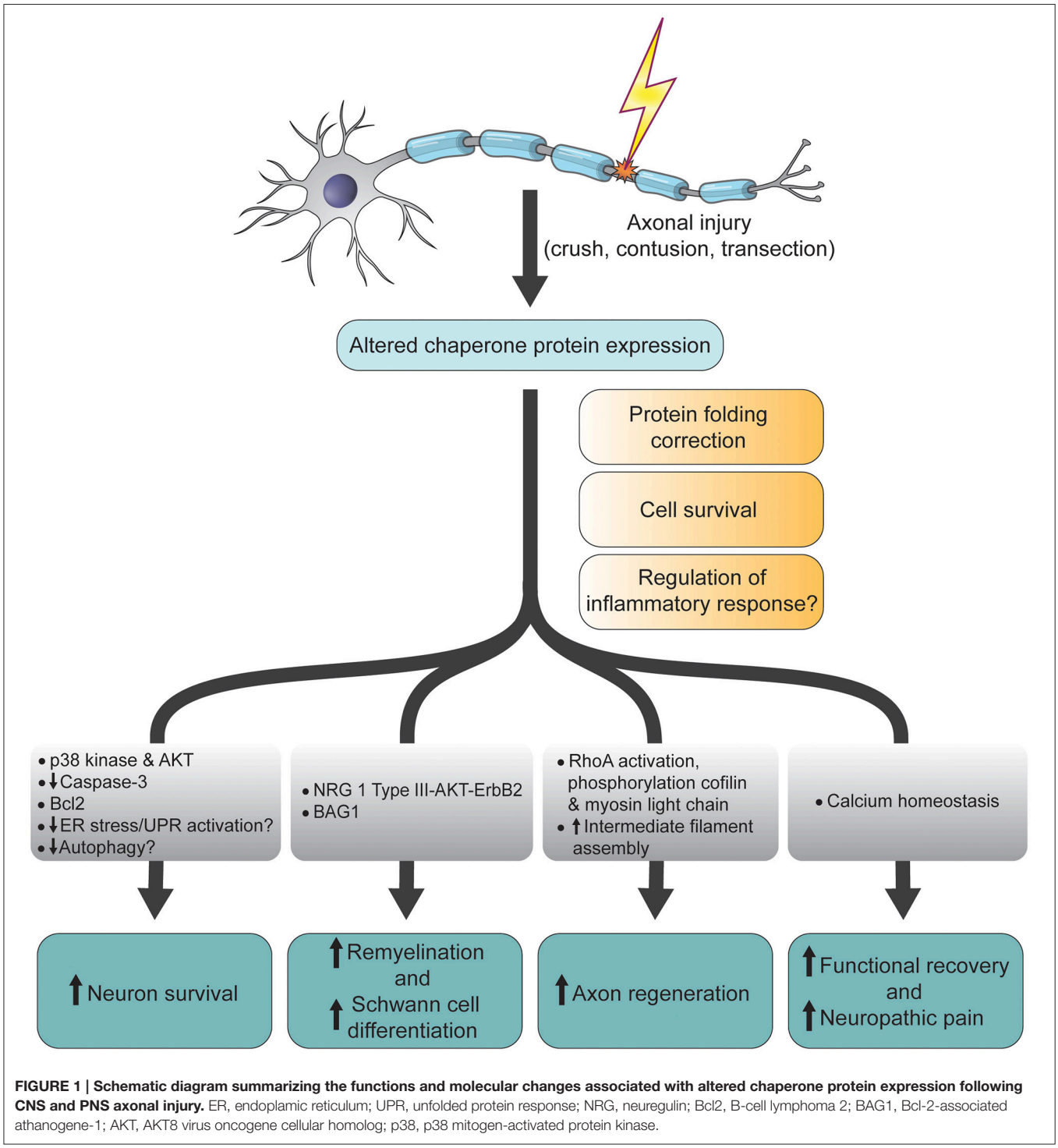

FIGURE 1 | Schematic diagram summarizing the functions and molecular changes associated with altered chaperone protein expression following CNS and PNS axonal injury. ER, endoplamic reticulum; UPR, unfolded protein response; NRG, neuregulin; Bcl2, B-cell lymphoma 2; BAG1, Bcl-2-associated athanogene-1; AKT, AKT8 virus oncogene cellular homolog; p38, p38 mitogen-activated protein kinase.

survival of retinal ganglion cells following optic nerve transection in hamsters correlated with HSP27 expression after remote ischemic pre-conditioning (Liu et al., 2013) while Penas et al. (2011a) showed that a decrease in binding immunoglobulin protein (BiP)/GRP78 correlated with retrograde degeneration of damage peripheral motor neurons. Interestingly, if chaperones themselves are altered by post-translational modification, this could negatively impact neuronal survival as shown by Franco et al. (2013) who found that the presence of nitrated HSP90 in contused rat spinal cord was linked to motor neuron death.

With respect to which molecular mechanism(s) may be involved in chaperone-mediated neuronal survival post-nerve damage, the activation of the p38 kinase was found to be required for induction of HSP25 expression after sciatic nerve axotomy.

Here, HSP25 formed a complex with AKT to prevent neuronal cell death (Murashov et al., 2001). Also, nitrated HSP90 induced neuronal death via P2X7 receptor-dependent activation of the Fas pathway (Franco et al., 2013) while HSP27's neuroprotective action was found to be downstream of cytochrome c release from mitochondria and upstream of caspase-3 activation (Benn et al., 2002). Another possible mechanism involved in cell survival post-axotomy may involve endoplasmic reticulum (ER) stress and the unfolded protein response (UPR), both of which are induced and activated after nerve damage (Penas et al., 2007; Ohri et al., 2011; Fan et al., 2015). If ER stress is activated, a number of chaperone proteins are upregulated such as GRP78/BiP (that co-chaperones with HSP70 or σ1R) and GRP94, an ER homolog of HSP90. These chaperones attempt to correct the stress by mediating proper protein folding and thus prevent cell death through induction of pro-survival factors such as Bcl2. If chaperones are unable to prevent accumulation of unfolded or misfolded proteins, the UPR is activated to minimize overloading by unfolded proteins. If however the stress is still unmanageable and homeostasis cannot be restored in a timely manner, apoptosis is induced (Fu and Gao, 2014). Enhancement of ER stress components such as CHOP, XBP1, and ATF6 has been observed after spinal cord contusion (Penas et al., 2007; Ohri et al., 2011), L5 spinal nerve ligation (Zhang et al., 2015) and sciatic nerve crush (Mantuano et al., 2011), with BiP enhancement observed in the latter two studies. The hypothesis is that the chaperones are attempting to correct the injurious processes and prevent cell death. However, very few studies have definitively linked chaperones with correcting ER stress and UPR activation after PNS and CNS axotomy. Penas et al. (2011b) have ventured into this area by noting that the balance between BiP and CHOP drives cell fate. As a result, they sought to modulate this ratio in favor of BiP using valproate (VPA). Although it did not augment BiP levels, high doses of VPA in a severe spinal cord contusion model reduced CHOP levels which correlated with reduced oligodendrocyte, myelin and axonal loss and better functional recovery. Of interest, the same lab has implicated chaperones in preventing another cell death process, autophagy, after axotomy. It was noted that autophagy markers such as BECLIN 1, LC3II, and LAMP-1 were enhanced in motor neurons after spinal root avulsion along with downregulation of BiP. The authors concluded that BiP decrease is a signature of the degenerating process, since its overexpression led to an increase in motor neuron survival (Penas et al., 2011b). However, this is correlative and more studies are needed to clearly clarify that the upregulation of chaperone proteins seen after PNS and CNS nerve damage is an attempt to correct ER stress or autophagy-induced cell death.

Axon Regeneration

In addition to cell survival, chaperones have been associated with regeneration of damaged PNS and CNS axons. For example, clusterin was found to be important for regrowth of sensory neurons after sciatic nerve transection and crush injury since this process was impaired in clusterin null mice (Wright et al., 2014). Further, exogenous application of the small heat shock protein, αBC, promoted neurite outgrowth of rat retinal cells (Wang et al., 2012). *In vivo*, the crystallin mediated regeneration of crushed rat optic nerve fibers through reduced activation of RhoA and phosphorylation of cofilin and myosin light chain. Other mechanisms by which chaperones mediate regeneration may involve modulation of axonal and Schwann cell cytoskeleton. Specifically, HSP27 was found to promote axonal outgrowth by possibly promoting assembly of intermediate filament proteins in Schwann cells (Hirata et al., 2003). In addition, Ma et al. (2011) noted that enhanced motor function recovery attributed to HSP27 was likely due to increased motor synapse reinnervation. To expand upon effects on functional recovery, exogenous application of αBC was discovered to promote locomoter recovery following spinal cord contusion injury in mice, with the improvement linked to reduced tissue damage and inflammation (Klopstein et al., 2012).

Myelination

We recently showed that αBC contributes to remyelination in the PNS after sciatic nerve crush in mice (Lim et al., 2017). Specifically, the small HSP appears to modulate the conversion of de-differentiated Schwann cells back to a myelinating phenotype and that this occurs through a neuregulin 1 Type III/AKT/ErbB2 mechanism. A hint that the crystallin may be involved in myelination was demonstrated earlier by D'Antonio et al. (2006) who found a correlation between the expression of the HSP and formation of myelinating Schwann cells during development. αBC is not the only chaperone found to be involved in Schwann cell function after PNS injury. BAG1, which is enhanced in Schwann cells after sciatic nerve crush, was shown to be important for differentiation of myelinating Schwann cells since knockdown of BAG1 with siRNA reduced the number of protein zero positive Schwann cells in culture (Wu et al., 2013).

Neuropathic Pain

All of the functions discussed above are beneficial, but chaperones may be involved in promoting injury-related neuropathic pain. The chaperone σ1R, was found to be associated with neuropathic pain after sciatic nerve ligation since use of an antagonist called 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl]morpholine) inhibited spinal sensitization and pain hypersensitivity (Romero et al., 2012). In corroboration, Pan et al. (2014) showed that σ1R was involved in pain sensitivity by sensory neurons in a rat sciatic nerve ligation model, since its antagonism with 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride or *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine dihydrobromide, reduced pain through restoration of calcium influx. Further, $\sigma 1R^{-/-}$ mice display reduced central sensitization and diminished hyperalgesic responses after sciatic nerve ligation (de la Puente et al., 2009). These results would suggest that the expression of σ1R would have to be increased or at least maintained from its non-injured level. However, Bangaru et al. (2013) showed that σ1R expression was reduced in DRG cells after sciatic nerve ligation. Whether there is differential expression in DRGs vs. axons after injury needs to be clarified especially knowing that local protein synthesis can occur within PNS axons (Court et al., 2011).

Inflammation

There is a large literature on chaperones and inflammation. However, very little research has been conducted on how these proteins affect the immune response after PNS and CNS nerve injury. Klopstein et al. (2012) showed that αBC reduced inflammation in the mouse spinal cord after contusion injury. On the other hand, Fan et al. (2015) linked GRP78/BiP with necroptosis and ER stress in macrophages/microglia after mouse spinal cord contusion. Considering the beneficial role that the robust immune response plays after PNS axon damage, there is an opportunity to understand what function(s) chaperone proteins play in the protective PNS immune response as opposed to the slow, limited and seemingly detrimental inflammation seen after CNS axotomy.

Altogether, a number of chaperones play various beneficial roles after PNS and CNS injury including promoting neuronal survival, axon regeneration, remyelination, Schwann cell differentiation, and functional recovery. However, others such as σ1R appear to mediate less desirable effects such as neuropathic pain.

THERAPEUTIC STRATEGIES THAT INVOLVE CHAPERONES

Because of the many reported beneficial functions of chaperone proteins after CNS and PNS nerve injury, some efforts have been made to harness their protective properties to enhance repair. Peroxisome proliferator-activated receptor-gamma (PPARγ) is a ligand-activated transcription factor of the nuclear hormone receptor superfamily whose agonist pioglotazone improved functional recovery and reduced motor neuron loss, astrogliosis, and microglial activation after rat spinal contusion injury. This improvement was attributed in part to the augmented expression of HSP27, HSP32, and HSP70 in the cord (McTigue et al., 2007). Using another PPARγ ligand, rosiglitazone, Yi et al. (2008) implicated chaperones in promoting survival of the damaged spinal neurons after brain contusion injury since neuronal survival correlated with enhanced expression of HSP27, HSP70 and HSP32. Along the same lines on neuronal survival, because σ1R agonists possess potent anti-apoptotic abilities, one of its agonists, 2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate, was studied in the context of spinal root avulsion in the rat. Here, a marked increase in motor neuron survival was evident, which correlated with a decrease in astrogliosis and an increase in the σ1R co-chaperone, BiP. However, considering the neuropathic pain-inducing effects of σ1R described in the previous section, one would have to decipher how to achieve the benefits of neuronal survival without developing pathological effects such as pain.

With respect to other therapies involving chaperones, improvement in functional recovery in rats with a contusion spinal cord injury following interferon-beta 1b treatment was found to be related to enhanced HSP70 expression in the cord along with reduced polymorphonuclear leucocyte infiltration, hemorrhage, oedema and necrosis (Sengul et al., 2013). An interesting therapeutic intervention in spinal cord injury experiments has been the use of exercise in injured animals. Keeler et al. (2012) showed that functional recovery improved in animals that had been previously subjected to an exercise regiment. This benefit was associated with increased expression of HSP27 and HSP70 in the spinal cord. In the PNS, a similar effect was seen after chronic constriction of the rat sciatic nerve where exercise training attenuated neuropathic pain and which correlated with reduced inflammation and enhanced HSP27 expression (Chen et al., 2012).

Another chaperone-inducing agent that has been used as a therapy after nerve transection has been BRX-220 (bimoclomal analog). BMX-220 is a hydroxylamine derivative that promotes cell survival through increased expression of HSP70 and HSP90 (Vígh et al., 1997). In the CNS, BRX-220 improved motor neuron survival while enhancing HSP70 and HSP90 in the damaged spinal cord (Kalmar et al., 2002, 2003). Follow up studies in the PNS, showed that BRX-220 reduced pain sensations that correlated with enhanced HSP70 expression in sensory DRG neurons (Kalmar et al., 2003).

Finally, immunophilins are a group of proteins that serve as receptors for the immunosuppressant drugs cyclosporin A and FK506. Systemic administration of FK506 dose-dependently increased the rate of axonal regeneration and functional recovery in rats following a sciatic nerve crush injury. This was attributed to binding of FK506 to the immunophilins FKBP-12. However, it is possible that the beneficial function was through chaperone proteins—FK506 can also bind FKBP-52/FKBP-59, which has been identified as a heat shock protein (HSP56) and shown to enhance expression of HSP70 (Gold, 1997; Gold et al., 1997).

CONCLUSION

Numerous studies over the past three decades have shown that the expression of chaperone proteins are not only altered following nerve damage to CNS and PNS neurons but that these proteins play an active role in the repair processes and in mediating some of the pathological events. Ongoing and future studies will have to consider how to harness the beneficial properties while reducing the injurious functions to enhance CNS and PNS recovery.

AUTHOR CONTRIBUTIONS

SO wrote the manuscript with content discussion, editing, and figures design by AF and EL.

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