Extracellular Chaperones in Neuronal Proteinopathies: Protecting and Facilitating Neuronal Function

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ABSTRACT: 'Proteinopathy' refers to a class of diseases where the aggregation of misfolded or non-native proteins/inclusion bodies is identified to be the underlying cause. Most neurodegenerative diseases feature such non-native protein aggregates or inclusion bodies. Over 4 decades of research on Alzheimer disease and various other neurodegenerative diseases has highlighted the importance of an articulate and precise proteostasis machinery in protein quality control. This proteostasis machinery exists both within and outside the cells. A great number of studies have elucidated the role of intracellular chaperones in guiding the process of folding and clearance of native and non-native proteins. However, our idea about the mechanism and processes involved in regulation of proteostasis by extracellular chaperones is still largely unknown. In addition, the process of proteostasis differs within the cell types; for example, different populations of neuronal cells are known for selective vulnerability to physiological stress. This review focusses on the extracellular chaperones, their role in protein quality control, and their effect on neuronal structure, function, and survivability.

KEYWORDS: Clusterin, alpha-2-macroglobulin, haptoglobin, chaperones, extracellular space, neurodegeneration, amyloidosis

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Introduction

The abundance and diversity of proteins have propelled our scientific curiosity for understanding their biological function. Proteins undergo a series of modification after their biogenesis, which influences their structure, function, cellular location, and half-lives.[1] Attainment of non-native and misfolded protein confirmations is manifested in complex pathological conditions.[2] Regulation of protein structural dynamics, complex formation, trafficking, and degradation, both within and outside the cells are some of the processes that regulate the protein quality control machinery otherwise known as proteostasis machinery.[1] A fine-tuned balance and coordination of the production and disposal of proteins forms the basis of proteostasis.[3] An organism's response to internal and external stress depends on its level of multicellularity and systemic complexity.[2] The concept of 'proteostasis' broadly encompasses biochemical processes that intricately put-together ensure that cells maintain a steady expression (levels), structure, and function of the proteins for their physiological functioning.[1,4] This ultimately reflects in an organism's ability to maintain homeostasis and effectively reciprocate during cellular stress or damage.[5] Failure of these proteostasis pathways results in compromised health status and is often associated with ageing and reduced longevity.[6] Proteotoxic stress results in cytotoxicity arising from soluble or insoluble misfolded protein species (Figure 1).[4] An abnormal change in the structure and folding pattern of proteins leads to diseases associated with accumulation of misfolded or intrinsically disordered proteins, otherwise referred to as proteinopathies.[7] Thus, understanding the proteostasis machinery both within and outside the cells is of importance. A detailed role of heat shock proteins (HSPs), ubiquilin-1,[8] and other intracellular chaperones in proteostasis has been reviewed in detail elsewhere.[9] Here, in this review article, the focus is on the protein quality control at the extracellular space and the abundance of the extracellular chaperones (ECs) in this space on exposure to different stressors such as temperature, pH, oxidised microenvironment, and the shear stress arising from the flow of plasma throughout the body under certain pressure. This review focusses on adenosine triphosphate (ATP)–independent ECs and their role in functioning of neuronal cells, ie, chaperones secreted into the extracellular space on cellular stress and how these can be compared with the much well-studied HSPs in stressed cellular conditions.[10]

Chaperones and Protein Homeostasis

A retrospective look on the structure and confirmation of proteins highlights the crucial role of protein quality control mechanism that involves regulated modes of protein folding and trafficking. The discovery of chaperones in 1970 has opened doors for understanding the role of these proteins in guiding the folding of 'client' proteins and facilitating the trafficking of these proteins both within and outside the cell.[11] Chaperon proteins have been largely appreciated as 'guardians of the cell' for their unique and conserved role in maintaining proteomic integrity.[12] Chaperones are constitutively expressed in eukaryotes along with co-chaperones (proteins that facilitate the function of chaperones in protein folding but are not able to do so by themselves only).[8] These chaperones undergo various modifications during cellular stress such as glycosylation, phosphorylation, and

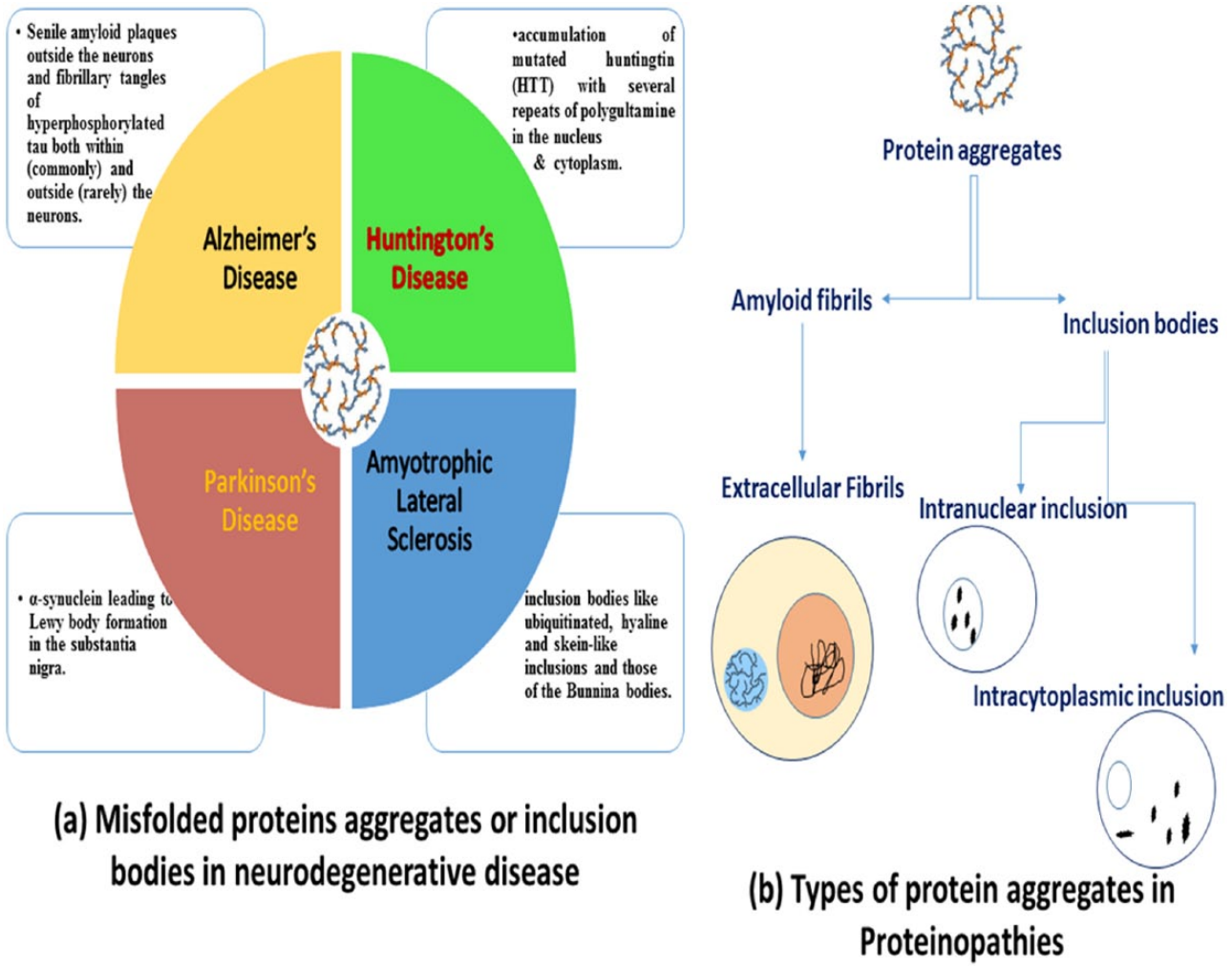

Figure 1. Pathological characteristics of amyloid-associated neuropathies and the protein aggregates in common proteinopathies. (a) All the major neurodegenerative diseases include amyloidosis and formation of amyloid fibrils/amorphous amyloid aggregates. For example, accumulation of senile amyloid plaques and fibrillary tangles of hyperphosphorylated tau in Alzheimer disease; α-synuclein leading to Lewy body formation in the substantia nigra in Parkinson disease; mutated huntingtin (HTT) with several repeats of polyglutamine in Huntington disease and ubiquitinated, hyaline, and skein-like inclusions and the Bunina bodies in amyotrophic lateral sclerosis. (b) The common protein aggregates causing proteinopathies include extracellular amyloid fibrils and intracellular inclusion bodies (of both intranuclear and intracytoplasmic subtypes).

biotinylation that regulate the binding of these chaperones to non-native client proteins.[13] Depending on their ability to refold client proteins, these chaperones have been classified into foldase and holdase chaperones.[14] Although the former binds to misfolded proteins and refold them to their correct 3-dimensional confirmation, the latter can only bind to misfolded proteins to trap them in an intermediate stable structure till they get refolded by foldase chaperones.[15] Precisely, chaperones can (1) facilitate proper folding of proteins within the endoplasmic reticulum (ER),[16,17] (2) form stable complexes or avoid formation of irreversible aggregates by selective binding to non-native proteins,[18] (3) compartmentalise misfolded proteins into aggresomes,[19,20] and (4) direct the non-repairable misfolded proteins for degradation by ubiquitin-proteosomal degradation (ubiquitin-proteasome system [UPS]),[21] autophagy[22,23] (chaperon-mediated autophagy), or ER-associated degradation (ERAD) pathways.[24] These chaperones are of significant interest in proteostasis, especially for neuronal cells as most of the neurodegenerative disorders have been linked to amyloid deposits (fibrillary proteinaceous aggregates) and the co-localisation of both intracellular and ECs.[18,20]

Most of the neurodegenerative disorders feature these amyloid deposits/inclusions within or in the space surrounding the neurons (Figures 2).[25] This has been specifically noted in the critical regions of the brain such as cortex and substantia nigra.[26] For example, accumulation of senile amyloid plaques outside the neurons and fibrillary tangles of hyperphosphorylated tau both within (commonly) and outside (rarely) the neurons are the associated pathological hallmarks in Alzheimer disease (AD).[27] Further details about the role of tau in AD progression have been discussed elsewhere.[28] Similarly, Parkinson disease (PD) is marked by the accumulation of α-synuclein leading to Lewy body formation in the substantia nigra,[29] and Huntington disease (HD) is characterised by

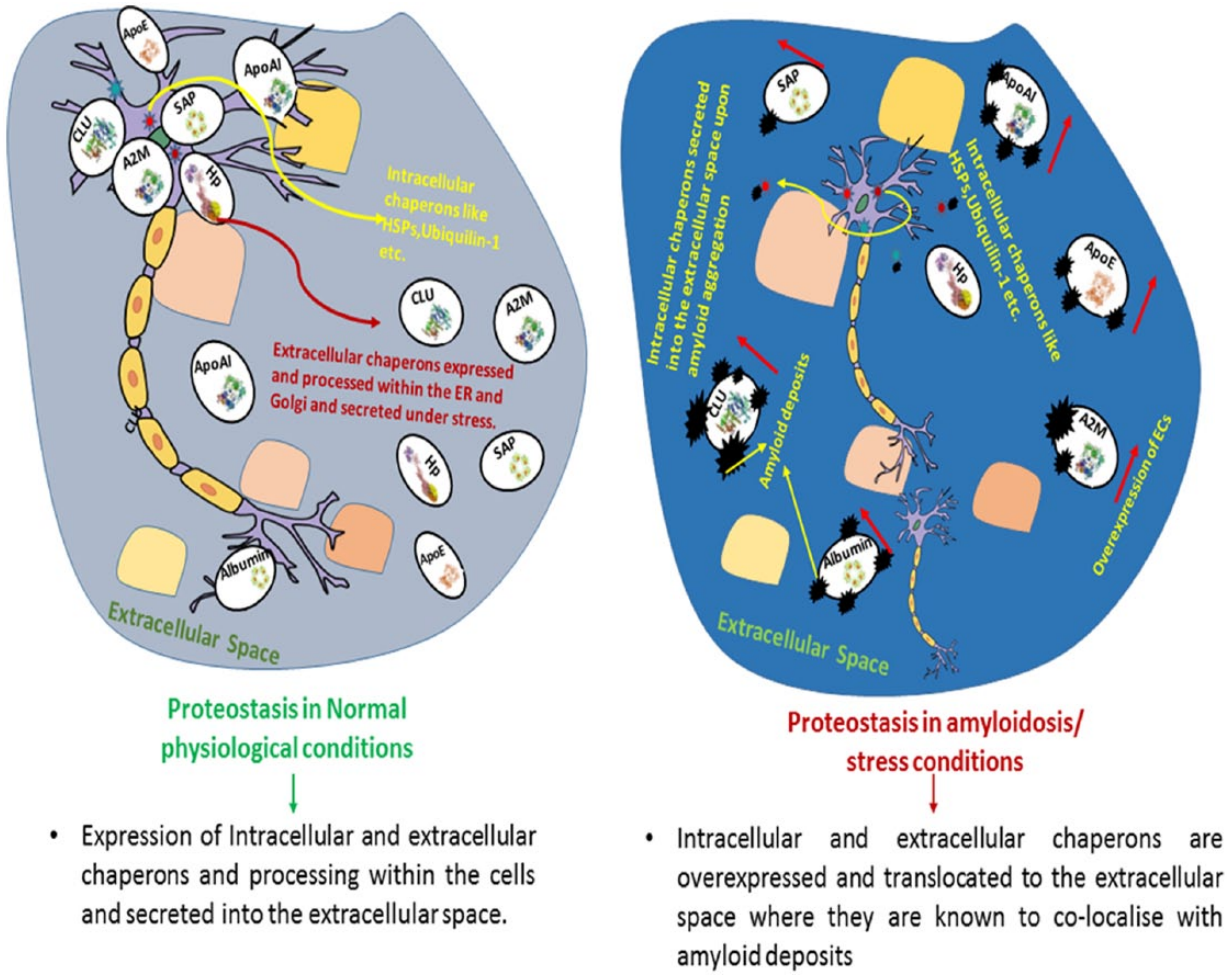

Figure 2. The proteostasis machinery in both normal and amyloid-associated neuropathy conditions. In normal physiological conditions, both the intracellular (marked as red and green dots) and extracellular chaperones (CLU, Hp, A2M, SAP, ApoE, and ApoAI) are expressed within the cells and undergo post-translational modifications within the endoplasmic reticulum prior to secretion. In amyloid-associated neuropathies, both the intracellular and extracellular chaperones are overexpressed and secreted into the extracellular space where they are known to interact with amyloid fibrils and intermediate by hydrophobic interactions. A2M indicates alpha-2-macroglobulin; ApoAI, apolipoprotein AI; ApoE, apolipoprotein E; CLU, clusterin; Hp, haptoglobin; SAP, serum amyloid P component.

accumulation of mutated huntingtin (HTT)[30] with several repeats of polyglutamine in the nucleus and cytoplasm.[28] Furthermore, prion diseases (rare neurodegenerative diseases) are characterised by the aggregation of pathogenic prion protein (PrP^c).[24] However, in case of amyotrophic lateral sclerosis (ALS) instead of fibrillary amyloid deposits, the pathological characteristic includes different inclusion bodies such as ubiquitinated, hyaline, and skein-like inclusions and those of the Bunina bodies[24] (Figure 1). An overall understanding of the formation of these proteotoxic species in neurodegenerative diseases has led to the over-arching need for exploring 'the derailment of the proteostasis pathways in cases of neurodegeneration' or in other words understanding the 'difference in the proteostasis pathway between healthy and degenerative neurons'. Furthermore, it is essential to ask a multi-disciplinary question as to 'what determines the biological outcome of these non-native proteins'. Several dimensions to be evaluated include the loss of biological function, the gain of cytotoxic functions, tissue damages due to misfolded protein accumulation, and loss of other native proteins (proteosomal subunits) which are captured with the misfolded proteins due to extensive hydrophobic interactions.

This review tries to summarise our current knowledge of the ECs during stress or cell damage and look for barriers to chaperone function arising from (1) ineffective localisation, (2) inappropriate of random post-translational modifications, (3) error in trafficking within and outside the cells, and (4) mutations in the genes coding for these chaperones. Over the past few decades, significant knowledge has been gathered about the age-related progressive decline in the intracellular chaperones. However, the case of ECs remains less known. It is reasonable enough to hypothesise that with ageing or in diseased patients there is a decline in the levels of these ECs and/or a loss-of-function mutation in the coding region of the ECs that can lead to the onset of neurodegeneration. Therefore, understanding the role of ECs in protein quality control or proteostasis in the

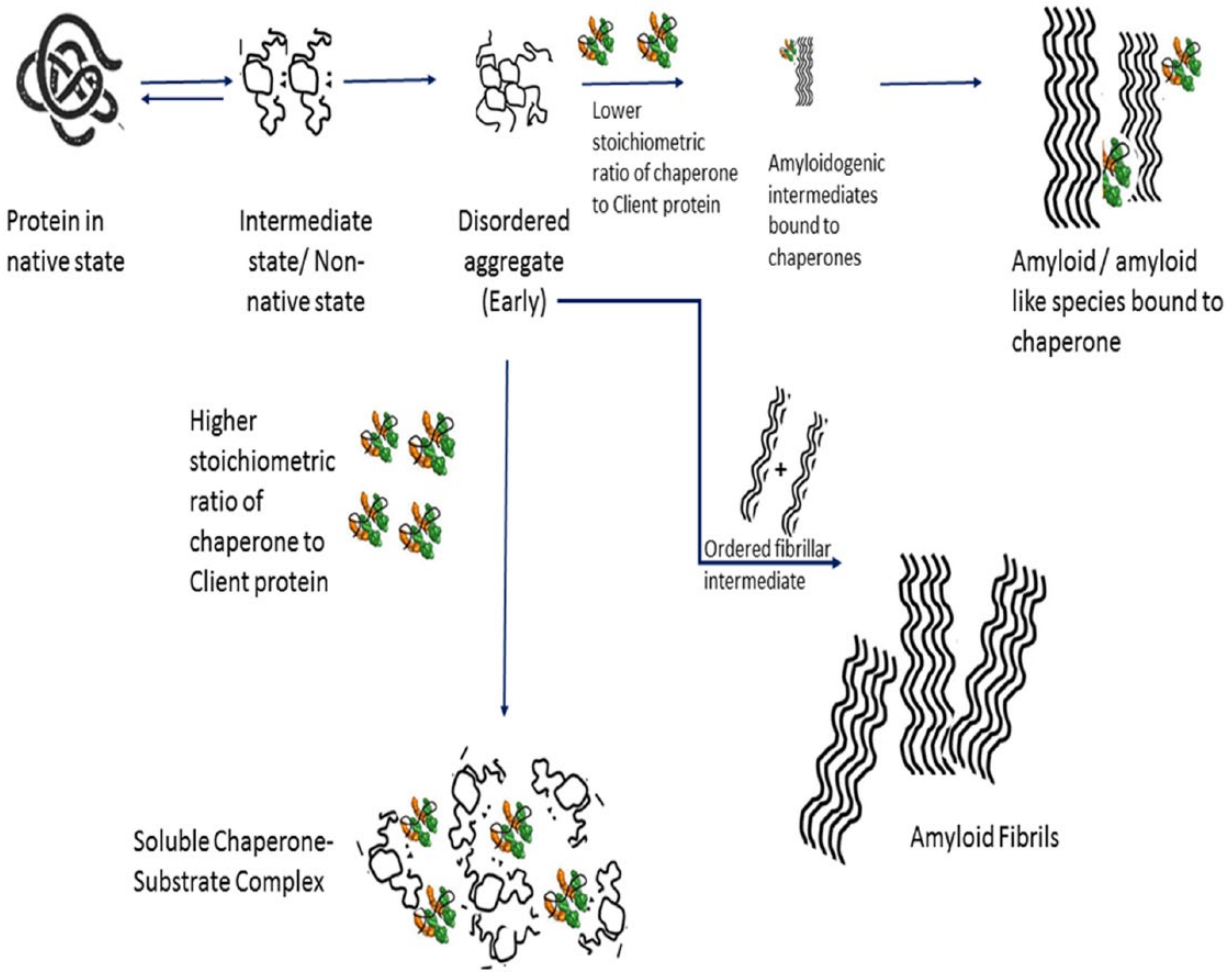

Figure 3. Effect of extracellular chaperones on amyloid fibrils. When a native protein encounters a denaturing stress, it enters into an intermediate non-native state which on continuous denaturing stress enters irreversibly into early disordered aggregates. On encountering higher levels of chaperones, the early disordered aggregates form soluble chaperone-substrate complex, whereas in low levels of chaperones, there is a chance of formation of either amyloid fibrils only or amyloid fibrils with chaperones bound to it.

extracellular space holds importance for dissecting the underpinning reasons for neuronal homeostatic functioning.

Extracellular Chaperones

It is interesting to note that different proteins and their proteolytic fragments have different half-lives.[21,31] Similarly, half-lives of proteins in the cerebrospinal fluid (CSF) led to our earlier ideas of the presence of ECs in the body fluids.[32,33] These ECs ensure protein quality control, proteostatic balance, and normal functioning of proteins in the extracellular body fluids.[34] Extracellular chaperones facilitate proper stable complex formation via hydrophobic interactions,[36] folding of non-native proteins,[34] intracellular uptake and trafficking of these misfolded proteins,[34] and when required target these non-native proteins for degradation by UPS or autophagy.[35] Although several of the intracellular chaperones such as HSPs (HSP 101, HSP90, HS70/40, etc.)[35] can be overexpressed and translocated into the extracellular space during stress; however, these are present at low levels (extracellularly) and mostly require ATP for their functioning except for small HSPs (sHSPs).[36] Furthermore, ATP itself is 1000 times less concentrated in the extracellular space in comparison with that of the inside of a cell[37] and the common approach of most biosystems to expend less energy during stress, it is reasonable to have an abundance of ATP-independent chaperones predominantly in the extracellular space including blood plasma, CSFs, and interstitial fluids. Some of the well-known ECs include clusterin (CLU), haptoglobin (Hp), alpha-2-macroglobulin (α2M), and serum amyloid P component (SAP).[38] Interestingly, all ECs are ATP-independent,[38] and notably, the first 3 of these ECs even share sequence homology and functional characteristics with sHSPs.[39] These ECs are known to interact with non-native protein species via hydrophobic interactions.[30] It has been shown that these intermediate pre-fibrillar species poses higher cytotoxicity than that of their mature fibrillary structures[29] (Figure 3). Thus, interaction with intermediate states leads to stabilisation of non-native proteins and prevents the rise in cellular toxicity from further aggregation.[38] Although CLU, Hp, α2M, and SAP are some of the well-known ECs, CLU still remains the most well-studied among them:

Table 1. List of ECs.

LEVEL OF CHARACTERISATION OF EC	NAME OF EC IN HUMANS	STRUCTURAL DATA AVAILABLE (EG, X-RAY CRYSTALLOGRAPHY)	MOLECULAR WEIGHT, KDA	NO. OF DIMERS	INDUCED ON EXTERNAL STRESS
Characterised and moderate to well-studied EC	Clusterin	No	~63	Dimers of 1 α-chain and 1 β-chain (hetero)	Yes
	Alpha-2-macroglobulin	Yes (partial) NMR data – A (chain) position: 1337-1474 X-ray data – A/B (chain) position: 126-227 X-ray data – A/B/C/D (chain) position: 24-1474	720	Tetramer (homo)	Yes
	Haptoglobin	Yes (partial) X-ray data 2/C/H/M/R/W (chain) position: 92-406 X-ray data chain-C position: 148-406 X-ray data chain-C position: 148-406	~100 (Hp1-1)	Tetramer of 2 α-chains and 2 β-chains (hetero)	Yes
	Serum amyloid P component	Yes (full)		Pentameric (homo)	No
Less completely characterised putative ECs	ApoE	Not fully characterised	~36	Not fully characterised. Variable homo- and hetero-dimer have been reported for ApoE, albumin, etc.	Not well known
	Albumin		66.5		
	ApoAI		~30.7		
	Fibrinogen-420		~420		
	SPARC		~34.62		
	MIF		~46		
	Casein proteins		~19-25		

Abbreviations: ApoAI, apolipoprotein AI; ApoE, apolipoprotein E; ECs, extracellular chaperones; MIF, macrophage inhibitory factor; NMR, nuclear magnetic resonance; SPARC, secreted protein acidic and rich in cysteine.

- *Clusterin.* Clusterin is encoded by a single gene and is found in body fluids such as plasma, CSF, and seminal fluids.[29] The secreted form of CLU is the result of internal cleavage of cytoplasmic CLU resulting in approximately 63 kDa of CLU which is composed of anti-parallel strands of α-subunits and β-subunits followed by *N*-linked glycosylation (17%-27% carbohydrates by mass) within the ER.[29] However, on a sodium dodecyl sulphate polyacrylamide gel electrophoresis, CLU migrates at 75 to 80 kDa[40] largely because of its extensive glycosylation (Table 1). The abundance of CLU in the human body fluids is highest for seminal fluids (1000 μg/mL) followed by human plasma (100 μg/mL) and CSF (2 μg/mL).[41] In addition, CLU is known to share high level of amino acid sequence conservation between different species (approximately 70%-80%) also suggests for a potentially important role of CLU in extracellular protein quality control.[29] Under conditions of cellular stress (diseases, deficiency of growth factors, and exposure to noxious agents), CLU expression is hiked by the binding of transcriptional regulators such as heat shock factor 1 (HSF1) and several others as discussed by several others to its promoter region as discussed by Wilson and other authors.[42,43,44] This activated expression of CLU makes it viable for responding to overwhelming burden of both amorphous and amyloid protein aggregates.

Clusterin is known to have distinct intracellular and extracellular functions. For example, CLU interacts with the hydrophobic residues of misfolded proteins leading to the formation of high-molecular-weight soluble complexes and preventing their further aggregation.[29] However, CLU cannot refold the non-native proteins by itself.[29] Furthermore, an elevated expression of CLU has been confirmed in ageing humans and patient samples such as those suffering from nephritic toxicity, AD, preeclampsia, type II diabetes, Down syndrome, and systemic amyloidosis.[45]

In particular, Schrijver et al[46] have shown the increased expression of CLU in response to the progression and rise in the severity of AD. In this article, the authors hypothesise that

based on the results, it seems like increased CLU express confers neuroprotection from the misfolded protein deposits at the extracellular space.[45] In addition, depletion of CLU from human plasma increases the susceptibility of the plasma proteins to heat-induced aggregation and precipitation.[47] Interestingly, there are growing reports of intracellular function of CLU as a chaperone. Under oxidative stress conditions, the cellular levels of Cu-ATPases are regulated by elevated levels of CLU in the cytosol, which further targets these misfolded or non-native proteins (eg, Cu-ATPases) for lysosomal or proteosomal degradation.[48] For example, lysosomal degradation is observed for copper transport surface proteins such as ATP7A and ATP7B, whereas proteosomal degradation has been witnessed in case of COMMD1 (copper metabolism domain containing 1) and Iκ-β.[47]

Furthermore, tropical application of CLU has been shown to potently protect against dessicating stress at the ocular surface.[49] This was shown in a mouse model of dessicating ocular stress, where CLU has been suggested to protect the structural proteins on the surface of eye such as LGALS3 and OCLN.[50] In addition, CLU clears the misfolded protein species via autophagosomes. Interaction of CLU with LCIII aids in the formation of autophagosome membrane and thereby promotes clearance of misfolded protein species.[51] It is known that in prostate cancer cells, reduced expression of CLU blocks autophagy.[52,53]

Clusterin expression is known to be correlated with tumour onset and progression. In a study by Shi et al,[54] the authors aimed to measure the correlation of CLU expression to that of several genes known to regulate cancer progression. For example, silencing or limited expression of CLU affects the expression of approximately 588 genes, ultimately upregulating around 17 pathways and downregulating 12 pathways.[53] Clusterin is known for its ability to promote cell survivability in cancer cells by inducing the phosphorylation of Akt, enhancing transforming growth factor β1-smad3 signalling and reducing the mesenchymal-epithelial transition via inhibition of Slug.[53] In several cancer models studied, CLU expression is elevated during chemotherapy which ultimately confers cytoprotection and limits the susceptibility to apoptosis.[55] For example, reduced expression of CLU via antisense CLU treatment in breast cancer cell lines showed an increased cell death after chemotherapy and tamoxifen treatment.[56] Apart from cytoprotection, CLU promotes cellular invasion and metastasis by upregulating Akt/MMP13 (matrix metalloproteinase 13)/EIF3I (eukaryotic translation initiation factor 3).[57]

Clusterin is trafficked within and outside the cell by a series of orchestrated events which regulates its secretion to the extracellular space on its maturation and further re-uptake of high-molecular-weight CLU-client protein complexes.[29] Furthermore, CLU is known to facilitate uptake and degradation of the protein aggregates by interacting with specific receptors, such as surface receptor megalin (LRP2), other lipoprotein receptors, such as LR8,[32] low-density lipoprotein receptors, such as ApoER2, and very low–density lipoprotein receptors,[32] plexin A4 (found in adult brain tissues of human and mice).[58] Within the cells, CLU is known to interact with other ER-resident chaperones such as GRP78/BiP (binding immunoglobulin protein) and protein disulphide isomerase to regulate misfolded proteins in the ER lumen.[29] Due to its interaction with ER-resident and membrane-bound chaperones (eg, BiP, calcitonin, and calreticulin) and localisation within the ER for its modification and packaging, it has been suggested that CLU may also influence the degradation of misfolded proteins via a mechanism similar to ERAD.[29] Furthermore, CLU has been shown to facilitate refolding of misfolded protein in the ER lumen and retro-translocates the folding incompetent misfolded proteins into the cytosol for lysosomal or proteosomal degradation.[33] There are several emerging evidence that suggest the retro-translocation of CLU (ie, movement of CLU from ER lumen into the cytosol) into the cytosol under conditions of cellular stress.[59] This has been discussed later in this review.

- *Alpha-2-macroglobulin.* Alpha-2-macroglobulin is composed of 4 identical subunits of 180 kDa each, giving rise to a high-molecular-weight blood glycoprotein of approximately 720 kDa.[60] The quaternary structure of α2M involves disulphide bonding of 2 subunits to form a dimer (Table 1), where the 2 dimers are then non-covalently bonded to ultimately form a tetrameric complex.[59] Unlike CLU, α2M has been accessed through X-ray crystallography studies, but the outcome has been limited and the major structural predictions are from its sequence homology with human complement system protein, ie, C3[61] (Table 1). The predicted domains include 8 fibronectin type 3 folded macroglobulin domains, 1 α-helical thiol ester containing domain, a receptor-binding domain, and a complement protein subcomponent–binding (CUB) domain.[34] The known complement subcomponents interacting at this CUB domain involve C1r/C1s, bone morphogenetic protein (BMP1), and urchin embryonic growth factor.[34] Alpha-2-macroglobulin is composed of approximately 10% carbohydrates by mass and is most abundant in the human plasma (1500-2000 μg/mL) followed by CSF (1-3.6 μg/mL).[62]

The levels of α2M in human body fluids are comparatively much higher than that of CLU, although it has been shown that CLU is more potent in inhibiting protein aggregation than α2M.[59] Alpha-2-macroglobulin, which is physiologically present in an inactive form, is known to interact proteases leading to a limited proteolysis of the tetramer.[34] This limited proteolysis results in the change of α2M confirmation which traps the protease within this bat region.[63] The activation of α2M is subject to reaction of the protease within α2M tetramer reacts

with (via methylamine) the thiol ester bond.[34] This ultimately results in exposing the distinct receptor recognition site for the binding of lipoprotein receptor–related protein (LRP).[36]

Alpha-2-macroglobulin is known to act as an ATP-independent molecular chaperone by binding indiscriminately to the hydrophobic residues of most of the non-native or misfolded proteins[34,36,59] and subsequent internalisation of the complex formed by LRP.[36] Interestingly, the internalised misfolded proteins are either subjected to cytosolic degradation machinery or re-presented on the cell surface, a function much similar to that of the HSPs. Therefore, α2M is one such EC which acts as a chaperone and also inhibits proteases thereby stabilising the misfolded proteins. Such a holdase action of α2M prevents further aggregation of misfolded proteins and directs these non-native proteins for degradation.[34] Similar to CLU, α2M depletion in the body fluids is known to raise the susceptibility of the plasma proteins for aggregation.[59] In addition, α2M has been shown to have role in immunomodulation and progression of cancers.[35]

- *Haptoglobin.* Haptoglobin is also known as 'acute-phase protein' because of its ability to be expressed at 8-fold higher level during stress.[64] Haptoglobin is an acidic glycoprotein produced in the liver and secreted into the extracellular space.[63] In normal physiological conditions, it is expressed at high levels in human plasma (300-200 μg/mL) followed by CSF (0.5-2 μg/mL).[37] Haptoglobin is coded by a single gene with 2 different alleles (Hp1 and Hp2) leading to a combination of 3 major phenotypes in humans (ie, Hp1-1, Hp 1-2, and Hp 2-2).[37,65] Hp1-1 is the simplest among all 3 with a single disulphide-linked $(\alpha 1)_2\beta_2$ resulting in approximately 100 kDa of final product (Table 1).[37] However, in Hp1-2 and Hp2-2, the presence of an additional cysteine residue in the α2-chain results in combination of disulphide-linked αβ polymers of different sizes ranging from 100 to 500 kDa.[38] There is no complete X-ray crystallography data available for Hp, and the prediction of the structure has been possible due to its sequence homology with chymotrypsinogen-like serine proteases.[38] Some of the partial X-ray crystallography data available have been listed in Table 1. Haptoglobin is known to bind with a wide range of receptors such as CD11b/CD18 integrin, CD22 B of the lymphocyte receptor, and iC3b fragment of complement.[66] One of the major biological functions of Hp involves its strong interaction with haemoglobin (Hb), and the resultant Hp-Hb complexes are known to bind to cell surfaces receptor CD163.[39] This promotes the formation of lipid peroxides and hydroxyl radical in the extracellular space in inflammed tissues.[39] It has been suggested that a large hydrophobic region next to the Hb-binding domain can putatively bind to hydrophobic residues on non-native or misfolded proteins.

Under stress conditions, Hp is known to inhibit precipitation and aggregation of a wide range of proteins in the extracellular space.[63] This action of Hp has been noticed for all 3 phenotypes of human Hp with some preliminary reports suggesting Hp1-1 as the most effective among them.[38] From whole human serum in vitro experiments, it has been shown that Hp binds to misfolded proteins to form stable and larger molecular weight complexes.[38] Furthermore, Hp shows lowered hydrophobic interactions under acidic conditions.[39] A comparative analysis of the chaperone activity of Hp in stabilising non-native proteins has revealed that under normal physiological conditions, Hp is less effective than CLU and more effective than sHSPs.[29] In addition, Hp is known to be a potent immune-regulator and pro-angiogenic factor. For example, Hp binds to and is uptaken by the neutrophils to be stored in their cytoplasmic granules.[67] This facilitates secretion of Hp from neutrophils to the local extracellular environment during pro-inflammatory conditions.[63] Furthermore, this binding of Hp to neutrophils inhibits respiratory burst activity.[40] Although the binding of Hp to neutrophils has been studied in few cases, yet is not clearly known how this binding is regulated and also whether the binding sites predicted on the neutrophils and mast cells (using sequence homology) are functional or not.

- *Serum amyloid P component.* Serum amyloid P component (approximately 125 kDa) is composed of 5 non-covalently interacting identical monomers (approximately 25 kDa each) which finally results in pentameric disc-like structures (Table 1).[68] Although the pentameric quaternary structure of SAP is known (by X-ray crystallography), it is still debatable whether SAP can also be purified from the body fluid as a decamer (only on purification).[69] Furthermore, it has been shown that SAP circulates in decameric form in the body fluids (2 pentameric discs posed face-to-face with non-covalent interactions).[70] The SAP belongs to the penatraxin family of proteins and is rich in anti-parallel β-strands containing 204 amino acids, single intra-chain disulphide bond, and *N*-linked biantennary oligosaccharides.[42] Furthermore, it harbours a protease-resistant β-plated sheet structure, which inhibits SAP proteolysis.[69] The SAP with 8% carbohydrates by mass is synthesised by the liver and is expressed at high levels in human plasma (40 μg/mL) followed by CSF (98.4 μg/mL).[41] It has been shown that SAP is localised to specific regions in the cells due to interaction with specific and corresponding ligands expressed in the locations.[71] For example, SAP localises to elastic microfibrils, arterioles, bronchioles, glomerular and alveolar basement membrane, cardiac and smooth muscles, and almost all forms of amyloid.[68,69] The specific binding of ligands in all these cellular locations involves Ca^{2+}-dependent binding to oligosaccharides,

glycosaminoglycans, C-reactive proteins, aggregated IgG, fibronectin, c1q, complement C4-binding protein, chromatin, histones, and phosphoethanolamine-containing compounds such as phosphatidylethanolamine.[72] Interestingly, both Hp and SAP have been identified as acute-phase proteins in mice. However, in humans, SAP is not elevated on cellular stress.[42]

A major limitation is the lack of experimental studies to validate its activity as a chaperone. It is hypothesised that SAP has limited effectivity as refolding chaperone because of its role in recovery of only 25% enzymatic activity of denatured lactate dehydrogenase (LDH) when added along with refolding buffer.[73] This reaction is Ca^{2+} independent and observed at a supra-stoichiometric ratio of SAP pentamer to the LDH of 10:1.[72] Thus, functionally, SAP is known to be ATP-independent EC with much lesser known effects.[48]

Interestingly, still much is to be explored about the biology of ECs. It is not yet clear how these ECs are constitutively produced, modified, packaged, and translocated both within and outside the cells. The turnover of the ECs and their expression levels during cellular stress still remains largely unknown. In a study by Wyatt et al,[74] it has been shown that hepatocytes isolated from rats exhibited a 4- to 5-fold higher internalisation of CLU-client complexes in comparison with client proteins only. These misfolded proteins on internalisation in rats are known to be sequestered via lysosomal degradation.[49] Also, as discussed earlier, these ECs deploy a variety of degradation pathways into action such as ubiquitin-proteasome pathway, autophagy, and ERAD.[29] Very little is known about the receptors that are specific to each of these ECs, and thereby, exploring the mechanism of receptor-mediated endocytosis for the EC-client protein complexes remains poorly understood. Several preliminary studies have identified few of these receptors as scavenger receptors and lipoprotein receptors and megalin, which has been discussed in detailed earlier.[29] Another aspect of ECs yet to be studied in detail is their de facto structure-function relationship. For example, ECs such as CLU is poorly understood in terms of the correlation of its structure and function. Clusterin being a high intrinsically disordered protein with variable glycosylation makes it difficult for X-ray crystallography–based structure determination.[29] Few of the studies based on sequence homology analysis have predicted different structural domains of CLU such as amphipathic and coiled-coil α-helices, β-sheets, and cysteine-rich disulphide bonds in the core of the α and β monomers.[40] Furthermore, it is of interest to know how stressed or pathological conditions induce change in the confirmation of the ECs leading to different functionalities. For example, in physiological pH, CLU exists in different oligomeric forms in the aqueous solution which is known to dissociate under acidic conditions.[75]

Other Less-Studied Putative ECs – The Unsung Heroes

Many other secreted proteins have been reported for their ability to act as 'chaperones'. However, most of them have not been well studied, and thus, very little is known to support these proteins as ECs. Apolipoprotein E (ApoE), fibronectin-420, apolipoprotein-associated protein (apolipoprotein AI [ApoAI]), albumin, casein proteins, secreted protein acidic and rich in cysteine (SPARC), and macrophage inhibitory factor (MIF) are some of those secreted proteins known for their ability to influence amyloidosis (Table 1), ie, influence the process of amyloid generation in the cells from the amyloid precursor protein (APP).[29] Apolipoprotein E is designated as 'pathogenic chaperone' as it induces amyloid formation.[29] However, few other studies have shown that ApoE interacts with amyloid-forming proteins in vitro and can influence the amyloid clearance from cells in vivo.[29] Apolipoprotein AI has also been shown to be prevent amyloid aggregation, although its role as a general molecular chaperone is yet to be properly understood. One common feature of all those apolipoproteins is their ability to interact with most hydrophobic molecules and this can be due to the amphipathic α-helices.[29]

Fibrinogen-420, a subclass of fibrinogen family of blood protein, has been shown to possess chaperone activity in conditions of heat-induced protein aggregation.[76] The action of fibrinogen-420 was studied on thermally denatured citrate synthetase (CS) at equimolar concentrations of both fibrinogen-420 and CS.[51] Albumin with established roles in inhibition of amyloid synthesis is another such secreted chaperone but less characterised as a molecular chaperone.[29] However, from the preliminary data, it indicates that albumin cannot be regarded as an efficient chaperone due to its lower activity in correlation to its high molar requirement.[75] Finally, SPARC and MIF have been tested for their role in inhibiting amorphous protein aggregation with verified role of SPARC in amyloid fibril formation.[77,78]

The Impact of Amyloidosis on Neurons and Central Nervous System

In most neuropathies that feature amyloid deposits, the clinical manifestation of the disease depends on the region of brain affected and the downstream effects on sensory and motor functions.[79] Particularly, it has been shown that accumulation of amorphous misfolded proteins or amyloid fibrils leads to synaptic loss and neuronal death.[80] It has been reported that amyloid-associated neuropathies can be caused both due to environmental and genetic factors.[53] In addition, most of the known ECs are also known to be responsive to external stress conditions, ie, their expression is elevated on exposure to cellular and pathological stress.[53] However, this does not apply to SAP.[29] Some of the intracellular chaperones have been shown to regulate the formation and accumulation of amyloid deposits surrounding the neurons. For example, under the condition

of cellular stress, HSPs are transported to the axons and synapses to prevent amyloidosis and inhibit further aggregation.[82] In the CNS, non-neuronal cell lineages such as astrocytes have been reported to have an elevated level of HSPs to target misfolded protein degradation.[54] However, a major gap in the field is the lack of such reports about the ECs in the protection of neuronal structure and function.

Although not fully understood, several preliminary evidence have suggested that different cells have different susceptibility to proteotoxic stress. The unfolded protein response evoked by cells in response to proteotoxic stress involves an orchestrated functioning of the proteostasis pathways.[82] Neuronal cells and other cells of the CNS bear an increased risk of being burdened by misfolded proteins under stressed or diseased conditions.[83] For example, dopaminergic neurons in the substantia nigra in case of PD, motor neurons in the motor cortex and spinal cord in ALS, and cholinergic neurons in the hippocampus and entorhinal cortex in case of AD are those cells with greater susceptibility in comparison with other cells.[51] One way of looking at this is to explore the correlation of the expression of the proteostasis components in these cells under stressed conditions and thereby identify the level of pathological marker proteins such as amyloid-β, α-synuclein, Lewy body, HTT, and Bunina bodies.[51] A comparison of deficiencies or abnormal expression pattern of proteostasis components and non-native protein accumulation will help us to understand greater vulnerability of neuronal cells.

ECs in Ameliorating CNS Amyloidosis

Most cells in our body are bathed in enriched extracellular fluid that is rich in plasma, ions, proteins, enzymes, and other soluble compounds. Neurons have a hyper-oxidised extracellular space in comparison with their cytoplasm, which is subject to constant shear stress from the flow of plasma under pressure.[29] One hypothesis is that the presence of an effective blood-brain barrier (BBB) further limits the ability of lymphoid clearance of these amyloid deposits without being complexed with ECs. In the CNS, ECs are expressed in some of the neuronal cell populations including astrocytes and can also be shuttled across the BBB.[84] This indicates that the extracellular space of neurons can respond to cellular stress for the integrity of their structure and function.[83] Most of the experiments on cytoprotection conferred by ECs against amyloid have been performed in in vitro conditions.[29] Our current understanding of ECs explains the biological role of ECs in inhibiting non-native protein formation and also regulating the degradation and clearance of the misfolded proteins via ubiquitin-proteosomal degradation, autophagy, or ERAD (Figure 3).[27] All these intracellular protein degradations rely on an effective shuttling of the amyloid deposits in complex with ECs from the extracellular deposits into the cytoplasm and this can be regulated by the receptors on the cell surface.[29] Receptor-mediated endocytosis thus remains a major focus in understanding the clearance and degradation of amyloid deposits within the cells.[30] One question of interest is to identify different receptors for facilitating receptor-mediated endocytosis for different EC-client protein complexes. Furthermore, it is essential to identify any shared homology or structural similarity of the ligand-binding site of these receptors.

The receptors on the surface of neuronal cells include those on the axonal body (ie, on myelin sheath), dendritic nerve endings, and the pre-synaptic dendrites.[85] It has been shown that the abundant ECs cannot confer cytoprotection against amyloid deposits in the absence of a selective receptor-mediated endocytosis pathway.[84] In addition, those receptors on the astrocytes[86] and microglia[87] can also regulate the formation of misfolded proteins in the extracellular space of the neurons. It has been shown that the glial cells not only act as supportive cells or phagocytic cells but also have been implicated in the disease pathology of neurodegenerative diseases especially AD.[88,89]

Another fascinating aspect of ECs and extracellular proteostasis is that of an extracellular protease machinery. Wyatt et al[90] in several of their review articles have discussed about the role of an extracellular protease machinery in protein quality control mechanism at the extracellular space. In case of neurons, a major role of extracellular space involves the maintenance of a normal neuronal structure for effective signal generation and transduction along with coordinated synaptic transmission of the neuronal signals.[91] The idea of an existing extracellular proteolytic machinery relies on the expression of certain proteases that can be triggered by amyloid deposits. For example, it has been shown that Aβ can induce the activation of plasminogen system in the body fluid.[60] Plasmin, an active protease component of plasminogen system along with plasmin-α2-anti-plasmin system, is known to be triggered by stressed Aβ deposits and in systemic amyloidosis, respectively.[60] In addition, the necrosis of cells (cross-linking of proteins) due to sudden injury can also activate the plasminogen system.[60] However, when our search for an extracellular protease system is to counter the overwhelming level of non-native proteins in the extracellular space, it is also important to note that any such system causing proteolytic degradation can result in proteolytic fragment generation from these non-native proteins, which may or may not be cytotoxic. The reason that degradation of fibrin clots by plasmin results in cytotoxic proteolytic fragments stands indicative of a possible similar case for an extracellular protease system.

The formation of senile amyloid plaques and neurofibrillary tangles of hyperphosphorylated tau presents a complex and debilitating case for synaptic neuronal communication as well as causes loss of neuronal structure and function leading to neuronal death.[60] In some cases, such as PD, selective neurons, ie, dopaminergic neurons are significantly reduced in the substantia nigra.[27] Emerging fields of AD, PD, ALS, HD, and Lewy body motor neuron disorders[31] have also implicated the

role of amyloid plaques and inclusion bodies both within and outside the neurons and their effect on neuronal communication[31] (Figure 1).

Extracellular chaperones discussed below play specific roles in clearance of amyloid deposits from the extracellular space.

- Clusterin is known to be overexpressed in several aged healthy humans and in disease models such as AD, diabetes, and Down syndrome, and most of these feature a progressive amyloidosis.[29] This proves that ageing itself is detrimental in the induction of amyloidosis.[29] Furthermore, CLU is known to bind strongly to intermediate amyloid oligomers/pre-fibrils in comparison with that of native amyloidogenic proteins and the mature amyloid fibrils.[76] In addition, the intermediate amyloid oligomers and pre-fibrils are more cytotoxic in comparison with mature fibrils.[65] The role of CLU either in promoting amyloidogenesis or inhibiting amyloid aggregation has been much debated for.[61] In $CLU^{-/-}$ APP transgenic mice, it has been shown that the burden of Aβ fibrillary species is reduced.[29] In addition, CLU depletion renders proteins in the extracellular body fluid more susceptible to denaturing stress and amorphous aggregate formation.[61] Apart from neurodegenerative diseases, CLU is known to be overexpressed in cerebrovascular diseases such as cerebral amyloid angiopathy (CAA)[76] and cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL).[92] It has been shown that in CADASIL, CLU exhibits noted immunoreactivity in the glial cells and damaged axons due to white matter damage.[63,74]

Apolipoprotein E has also been shown to influence amyloidosis in a concentration-dependent manner and regulates clearance of Aβ deposits.[69] However, similar to CLU, ApoE can also be either pro- or anti-amyloidogenic depending on its molar ratio, and in $ApoE^{-/-}$ APP transgenic mice, it has been shown that the burden of Aβ fibrillary species is reduced.[93] A double-knockout of CLU and ApoE leads to significant increase in Aβ levels and amyloidosis.[94] In addition, both CLU and ApoE are known to harbour specific single-nucleotide polymorphisms (SNPs) which have been correlated to increased risk for AD.[69,95] In a study on CLU from diverse ethnic and racial background of population, it was found that the C-terminus of β-sheet (exon 7) possesses putative sites for SNPs that are associated with high risk for AD.[96] Several other SNPs have been reported in this study with low to mild risk factor.[95] Similarly, the ϵ4 allele of ApoE has been related to genetic predisposition for AD.[95] Several SNPs in ϵ4 allele have also been correlated to sporadic onset of AD.[97]

- Alpha-2-macroglobulin, a high-molecular-weight glycoprotein, is known to act as an ATP-independent EC. Alpha-2-macroglobulin forms complexes with Aβ deposits in the extracellular space and can lead to their disposal via receptor-mediated endocytosis (ie, LRP).[36] Furthermore, at sub-stoichiometric levels, α2M inhibits the aggregation of amyloid-forming proteins and confers protection against cytotoxic Aβ deposits.[29,98] Even as CLU, α2M interaction is specific to the intermediate pre-fibrillar species or oligomers and thus has limited or no effect on mature fibrillary Aβ species.[36] Furthermore, most of the genes associated with AD are located on chromosome 12, and α2M has a similar chromosomal location.[99] This indicates a linkage of α2M with AD onset and progression. Further polymorphisms in α2M and potential LRP can be related to the onset and progression of the neurodegenerative diseases.[100]
- Haptoglobin is known to bind to wide variety of amyloid-forming proteins, and the molar ratio for such a complex formation is much within sub-stoichiometric levels.[41] Similar to other ECs, Hp can also inhibit the formation of amyloid aggregate formation.[41] However, due to complexation with Hb, there is a noticeable reduction in this inhibitory effect of Hp on both amorphous and fibrillary Aβ aggregates.[101] The known polymorphism of Hp (Hp1-1, Hp 1-2, and Hp 2-2) does not influence the susceptibility for diseases related to amyloid deposits.[41]
- Serum amyloid P component has been found in almost all the amyloid deposits tested for, and it has been shown that the abundance of SAP in amyloid fibrils is extremely high in comparison with its low expression levels in human plasma.[42] This, along with the fact that the interaction of SAP with amyloid fibrils is highly selective, justifies a potential role of SAP in amyloidosis in neuronal cells.[42] This still remains largely unknown. Tennent et al. had reported that SAP can confer protection from amyloid deposits by specific binding to amyloid fibrils, masking these fibrils and inducing proteolytic degradation of amyloid deposits.[102,103]

Apolipoprotein AI is another potential secreted mammalian EC that is known to directly interact with APP and inhibit Aβ toxicity and aggregation.[104] Albumin is also known to inhibit amyloid formation by selectively binding to Aβ peptides.[105] Casein proteins have been related to interaction with amyloid deposits in mammary tissues, but no such evidence in neuronal tissues have been reported.[29] Little is known about MIF regarding amyloidosis, but it has been shown that affinity chromatography–based purification of Aβ peptides also resulted in purification of MIF both in normal rat brain and in AD brain.[29]

Our present understanding of cytotoxicity arising from amyloid deposits is based on the damage to cellular structure (neuronal structural damage and neuronal death). However, it

is not well understood as to how the hydrophobic domains of these amyloid deposits (the site for interaction with most of the ECs) regulate their cytotoxicity.

Therapeutic Relevance of ECs in Neurodegeneration

The search for an effective therapeutic regime that has low side effects and focusses on the underlying cause of the neurodegenerative diseases is still on. Most of the contemporary therapeutic approaches rely on targeting the symptoms and thus have been of limited efficacy in reversing the loss of cognitive and functional abilities. One major limitation in this direction has been the lack of a precise understanding of the disease biology. For example, AD has been the major amyloid disease affecting the nervous system, cognitive abilities, and loss of movement-based functions.[106] Some of the contemporary therapeutic regimes against AD rely on inhibiting the production and aggregation of $A\beta_{1\text{-}42}$.[107] Interestingly, it has been subsequently validated that level of $A\beta_{1\text{-}42}$ expression remains unchanged both in healthy controls and patients with AD.[81] A major key to designing an effective therapeutic regime is to focus on the molecular chaperones and look for methods to ensure cellular and extracellular clearance of amyloid deposits (uptake and proteosomal or lysosomal degradation). The range of ECs known till date poses potential for being therapeutic targets for amyloid-associated neurodegenerative diseases. A common therapeutic strategy may rely on the heat shock response (HSR) axis which relies on upregulating the EC expression by overexpression of HSF1.[108] There is a wide range of chemical compounds known for their ability to induce HSR, ie, celastrol, arimoclomol, withaferin A, geranylgeranylacetone, and 17-AAG.[107] These chemical compounds and other strategies of inducing the HSR axis are emerging therapeutic strategies for treating neurodegenerative disorders.

The use of systems biology approach and induced pluripotent stem cells are other dimensions for designing and development of personalised therapies against these neurodegenerative disorders. These have added to our efforts for small molecule–based drug designing for targeting allosteric activity, designing of antibody-based therapies that target these amyloid deposits (recent report of aducanumab),[109] and specific receptor targeting that can elevate the clearance of the misfolded proteins.[109] Current scientific technology has ensured that we can work for therapeutic development on 3 different platforms such as in silico, in vitro, and in vivo. However, the diversity of the misfolded proteins in terms of their size and structure along with their high abundance nullifies the role of relatively less abundant ECs as therapeutic targets.

Conclusions

An overall understanding of this review is that proteostasis is central to normal functioning of cells and system as a whole. Over the years, our focus has been limited to intracellular events of proteostasis, protein manufacturing, and quality control mechanisms. However, given that the cells are bathed in an extracellular space, it is important to look at the extracellular proteostasis events. Extracellular chaperones such as CLU, α2M, Hp, and SAP are some of the most important and abundant chaperones in the extracellular space with potential to influence amyloidosis, inhibit amorphous and fibrillar protein aggregates, and confer cytoprotection from these toxic proteins. A role of these ECs in effective neuronal communication can be viewed as their role in clearance of senile amyloid plaques, inclusion bodies, and neurofibrillary tangles (hyperphosphorylated tau). Although it seems indicative that proteostasis, ECs, neuronal damage, and death are all related, yet our current understanding is based on intermediate linkages and not solid understanding of their precise cross-talk. Therefore, this review tries to understand the role of ECs in normal neuronal functioning and also elucidate processes of their dysregulated expression and function in neurodegenerative diseases.

Author Contributions

SS conceived and designed the experiments, analysed the data, wrote the first draft of the manuscript, made critical revisions and approved final version, and reviewed and approved the final manuscript.

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