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Review article

Extracellular heat shock proteins in neurodegenerative diseases: New perspectives

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ABSTRACT

One pathological hallmark of neurodegenerative diseases and CNS trauma is accumulation of insoluble, hydrophobic molecules and protein aggregations found both within and outside cells. These may be the consequences of an inadequate or overburdened cellular response to stresses resulting from potentially toxic changes in extra- and intracellular environments. The upregulated expression of heat shock proteins (HSPs) is one example of a highly conserved cellular response to both internal and external stress. Intracellularly these proteins act as chaperones, playing vital roles in the folding of nascent polypeptides, the translocation of proteins between subcellular locations, and the disaggregation of misfolded or aggregated proteins in an attempt to maintain cellular proteostasis during both homeostatic and stressful conditions. While the predominant study of the HSPs has focused on their intracellular chaperone functions, it remains unclear if all neuronal populations can mount a complete stress response. Alternately, it is now well established that some members of this family of proteins can be secreted by nearby, non-neuronal cells to act in the extracellular environment. This review addresses the current literature detailing the use of exogenous and extracellular HSPs in the treatment of cellular and animal models of neurodegenerative disease. These findings offer a new measure of therapeutic potential to the HSPs, but obstacles must be overcome before they can be efficiently used in a clinical setting.

1. Introduction

In 1962 Ferruccio Ritossa discovered an enlargement, or puffing, of chromosomes in the cells of *Drosophila* salivary glands when he subjected them to a prolonged thermal stimulus, otherwise known as "heat shock" [1,2]. Under the microscope Ritossa was looking at locally enhanced gene transcription in response to cellular stress and became the first person to identify and visualize the initiation of what we now know to be the heat shock response (HSR). This finding, which would later become recognized as a seminal discovery in the fields of genetics and molecular biology, shaped an entire focus of cell stress research and was the origin for the study of a family of macromolecules dubbed the heat shock proteins (HSPs) [3,4].

The HSR, characterized specifically by the upregulated expression of inducible members of the HSPs following cellular insult, is the single most evolutionarily conserved system across all cells and organisms, from yeast and bacteria to humans [5]. The HSPs are responsible for mediating intracellular homoeostasis by maintaining the correct folding behavior and translocation of proteins under both normal and stressful conditions [6]. Thus, the HSR is the cellular response to potentially toxic changes in the intra- and extracellular environment, such as alterations in blood flow, concentration of cytokines, metabolites, toxins, and increases in reactive oxygen and nitrogen species [6–9].

Much of our knowledge about the stress response and cell viability following cellular insult was shaped by the study of the HSPs, yet the large portion of research that has been dedicated to these proteins has focused on their intracellular role as a part of the protein quality control system. Surprisingly, differentiated adult neuronal populations have a weak or aberrant heat shock response, plausibly making them more susceptible to neurodegenerative pathology [10,11–15]. Interestingly, non-neuronal cell populations are able to secrete HSPs into the microenvironment that can be utilized by neighboring cells, including neurons [16–20]. As such, the delivery of exogenous HSPs and the increased secretion of HSPs by surrounding cells have been suggested as a therapeutic approach to treating neurodegenerative disease [10,20,21–26]. Herein we critically review extracellular HSPs in the context of neurodegenerative disease. We specifically focus on the known intracellular function of the molecular chaperones, the extracellular release of these proteins, and the unique roles that these HSPs may be playing in the extracellular space to promote neuronal viability

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Table 1
Subcellular localization and sequence homologies of the Hsp70, Hsp90, and Hsp110 families.

Family	HSP Isoform	Other Name	Subcellular Location	% Sequence Identity to Isoform 1	Reference # (If Studied Extracellularly in Neurodegenerative Disease)
Hsp110	HSPH1*	HSP105α	Cytosol	100%	N/A
	HSPH2	Apg-2	Cytosol	63.00%	N/A
	HSPH3	Apg-1	Cytosol	60.00%	N/A
	HSPH4	Grp170	Endoplasmic Reticulum	27.00%	N/A
Hsp90	HSPC1*	HSP90α	Cytosol	100%	[10]
	HSPC2	HSP90α A2 (Pseudogene)	Cytosol	96%	N/A
	HSPC3	HSP90β (Constitutive)	Cytosol	86%	N/A
	HSPC4	GRP94	Mitochondria	50%	N/A
	HSPC5	TRAP1	Endoplasmic Reticulum	34%	N/A
Hsp70	HSPA1A*	Hsp70-1	Nucleus/Cytosol	100%	[10,19,21–26,148,149,180]
	HSPA1B	Hsp70-2	Cytosol	100%	N/A
	HSPA1L	hum70t	Cytosol	89%	N/A
	HSPA2	Heat-shock 70 kD Protein-2	Cytosol	85%	N/A
	HSPA5	Bip, GRP78	Endoplasmic Reticulum	64%	N/A
	HSPA6	Hsp70-6	Cytosol	82.55%	N/A
	HSPA7	Hsp70-7	Cytosol	84%	N/A
	HSPA8	Hsc70 (Constitutive)	Cytosol	86%	10, 21
	HSPA9	GRP75	Mitochondria	52%	N/A
	HSPA12A	FLJ13874	Unknown	26%	N/A
	HSPA12B	RP23-32L15.1	Unknown	25%	N/A
	HSPA13	Stch	Microsomes	40%	N/A
	HSPA14	HSP70-4	Unknown	35%	N/A
Hsp40 Type I	DNAJA1*	DJ-2	Nucleus/ Mitochondria/ Endoplasmic Reticulum	100%	N/A
	DNAJA2	DNJ3	Membrane	54.17%	N/A
	DNAJA3	Tid-1	Cytosol/ Mitochondria/ Membrane	30.56%	N/A
	DNAJA4	DJ4	Membrane	73.37%	N/A
Hsp40 Type II	DNAJB1* vs DNAJA1**	Hsp40	Nucleus	100% 33.89%	10
	DNAJB2	HSJ1	Nucleus/Endoplasmic Reticulum	53.45%	N/A
	DNAJB3	Hsj3	Unknown	52.68%	N/A
	DNAJB4	Hsc40	Cytosol/Membrane	65.70%	N/A
	DNAJB5	Hsp40-3	Nucleus/ Cytosol	59.94%	N/A
	DNAJB6	Mrj	Nucleus	54.04%	N/A
	DNAJB7	Dj5	Unknown	46.40%	N/A
	DNAJB8	mDj6	Nucleus/ Cytosol	41.14%	N/A
	DNAJB9	Mdg1	Endoplasmic Reticulum	43.12%	N/A
	DNAJB11	Dj9	Endoplasmic Reticulum	31.78%	N/A
	DNAJB12	Dj10	Nucleus/ Endoplasmic Reticulum	47.11%	N/A
	DNAJB13	Tsarg6	Flagellum	48.52%	N/A
	DNAJB14	EGNR9427	Nucleus/ Endoplasmic Reticulum	40.16%	N/A
Hsp40 Type III	DNAJC1* vs DNAJA1** vs DNAJB1**	MTJ1	Nucleus/ Endoplasmic Reticulum	45.90% 41.94%	N/A
	DNAJC2	Zrf1	Nucleus/Cytosol	55.17%	N/A
	DNAJC3	p58	Endoplasmic Reticulum	41.79%	N/A
	DNAJC4	HSPf2	Unknown	34.29%	N/A
	DNAJC5	Csp	Membrane	41.27%	N/A
	DNAJC5B	CSP-beta	Membrane	41.94%	N/A
	DNAJC5G	gamma-CSP	Membrane	29.63%	N/A
	DNAJC6	auxilin	Cytosol	27.91%	N/A
	DNAJC7	Ttc2	Nucleus/ Cytoskeleton	36.76%	N/A
	DNAJC8	AL024084	Nucleus	32.29%	N/A
	DNAJC9	AU020082	Nucleus/ Membrane	38.30%	N/A
	DNAJC10	JPDI	Endoplasmic Reticulum	48.61%	N/A
	DNAJC11	FLJ10737	Mitochondria	39.39%	N/A
	DNAJC12	Jdp1	Cytosol	31.75%	N/A
	DNAJC13	Rme8	Endosome	35.85%	N/A
	DNAJC14	HDJ3	Endoplasmic Reticulum	39.71%	N/A
	DNAJC15	Dnajd1	Mitochondria	44.83%	N/A
	DNAJC16	mKIAA0962	Membrane	53.33%	N/A
	DNAJC17	C87112	Nucleus	40.00%	N/A
	DNAJC18	MGC29463	Membrane	35.85%	N/A
	DNAJC19	TIM14	Mitochondria	48.15%	N/A
	DNAJC20	JAC1	Mitochondria	No Homology	N/A
	DNAJC21	GS3	Nucleus	50.79%	N/A
	DNAJC22	FLJ13236	Membrane	39.62%	N/A
	DNAJC24	DPH4	Cytoskeleton	27.54%	N/A
	DNAJC25	DnaJ-like protein	Membrane	39.19%	N/A
	DNAJC26	GAK	Golgi Apparatus	24.39%	N/A

(*continued on next page*)

Table 1 (*continued*)

Family	HSP Isoform	Other Name	Subcellular Location	% Sequence Identity to Isoform 1	Reference # (If Studied Extracellularly in Neurodegenerative Disease)
	DNAJC27	auxilin-2	Nucleus	43.18%	N/A
	DNAJC28	RabJ	Golgi Apparatus	25.00%	N/A
	DNAJC29	SACS	Cytosol	40.00%	N/A
	DNAJC30	WBSCR18	Mitochondria	29.65%	N/A
Hsp27	HSPB1*	HSP27	Nucleus/ Cytosol/ Cytoskeleton	100%	10
	HSPB2	MKBP	Nucleus/Cytosol	40.99%	N/A
	HSPB3	HSPL27	Nucleus/Cytosol	40.24%	N/A
	HSPB4	crystallin alpha A	Nucleus/Cytosol	41.18%	N/A
	HSPB5	crystallin alpha B	Nucleus/Cytosol	45.03%	N/A
	HSPB6	HSP20	Nucleus/Cytosol	40.29%	N/A
	HSPB7	cvHSP	Nucleus/Cytosol	32.14%	N/A
	HSPB8	HSP22	Nucleus/Cytosol	39.56%	N/A
	HSPB9	FLJ27437	Nucleus/Cytosol	28.74%	N/A
	HSPB10	ODF1	Nucleus/Cytoskeleton	31.17%	N/A
	HSPB11	HSP16.2	Cilium	42.86%	N/A

* Indicates isoform used to compare sequence homology via NCBI BLAST protein-protein alignment.

** Indicates sequence homology comparison between the first members of the different types (I, II, or III) of DNAJ/Hsp40 proteins (eg. DNAJA1 vs DNAJB1).

in response to stress and pathology.

2. The molecular chaperones

The heat shock proteins are classified based on their molecular weight and intracellular functions [27–29]. The most prevalent of the HSPs are grouped in broadly conserved families: the Hsp100s, Hsp90s, Hsp70s, Hsp40s, and small HSPs. Within each family, individual protein isoforms vary by amino acid sequence homology, level of constitutive expression (e.g. constitutive Hsc70 vs stress-inducible Hsp70), and cellular location (see Table 1). In general, chaperone function is consistent within each group and the families can be architecturally defined by the presence of their two terminal domains – the nucleotide binding domain (NBD) and the substrate binding domain (SBD) (Fig. 1). The HSPs use these domains in conjunction to aid in the folding of nascent polypeptides, the subcellular translocation of proteins, and the degradation of unwanted proteins or complexes [30–35]. All of the molecular chaperones indiscriminately interact with a variety of unfolded proteins, which allows them to be present and active in all parts of a protein life cycle – from synthesis to degradation.

During de novo protein synthesis, there is a perpetual necessity for chaperone assistance in the folding and stabilization of nonnative proteins. Newly synthesized amino-acid sequences typically fold in an organized pattern, from a linear polypeptide chain to a three dimensional native state via structural intermediates that provide increasing kinetic favorability and architectural stability [29,36]. When a cell begins to undergo stress the molecular chaperone model predicts that this process is significantly perturbed, resulting in an increase in cytosolic protein species incapable of achieving their native folded state and the undesirable surface expression of large sequences of hydrophobic amino acids. In short, the surrounding environment traps the peptide in a less kinetically favorable and more reactive intermediate structure [37]. The molecular chaperones are responsible for accelerating the folding of the peptide's structure by reducing the activation energy required to move to the next kinetically favored intermediate [38,39].

In general, the HSPs are recruited to proteins that express large hydrophobic stretches, which act as a marker for partially or globally unfolded, misfolded, or aggregated proteins. The SBD of the HSPs binds to these hydrophobic patches, allowing the chaperones to reduce the potential for further damaging molecular interactions between nonnative peptides and for the subsequent refolding of the peptide back to a stabilized quaternary structure [39]. Part of what makes the HSPs so efficient in this process is their component structure. The SBD of each member of the molecular chaperones is comprised of a hydrophobic pocket that is capable of promiscuously binding to any and all peptides that present an attractive and available sequence, while the NBD is externally available for interaction with cochaperones which accelerate the turnover of adenosine-triphosphate (ATP) [40,41]. Additionally, this structural framework allows the HSPs to contribute to the refolding of the client peptide without providing any structural modifications to the protein sequence [29,38]. The functional binding and release of proteins by the HSPs is highly regulated and usually requires an ATP-dependent conformational change that is directed by a tightly coupled cochaperone network [35].

3. Hsp110

Hsp110 is a highly-conserved molecular chaperone that is able to directly associate with Hsp70 and J-domain proteins to form the major mammalian cytosolic disaggregase machinery [42]. As shown in Table 1, there are four, human Hsp110 isoforms [43,44]. The Hsp110s have the same general structure as that of the Hsp70 proteins, sharing the same N-terminal NBD and C-terminal SBD architecture (Fig. 1). The main function of the Hsp110s seem to be less associated with the refolding of nascent polypeptides, and instead appear to assist predominantly with nucleotide exchange for Hsp70 [43–46], although one recent report suggests that human Hsp105α can act as an ATP-dependent foldase capable of refolding misfolded polypeptides back to a native state [47]. *In vitro*, all three of the cytosolic Hsp110s appear to support nucleotide exchange, and thus protein disaggregation in an equal manner, however, *in vivo* there appears to be a hierarchy among Hsp110 family members as genetic knockout of Hsp105α in mice results in a severe deficit in the reactivation of aggregated proteins following heat shock [48,49]. As the major cellular mechanism for mammalian protein quality control, it seems logical that the individual components required to make the disaggregase machinery are abundantly available in the cytosol of the cell, yet the Hsp110 machinery is expressed at much lower concentrations compared to the constitutive Hsp70 (Hsc70) indicating that the availability of Hsp110 may be a necessary and crucial factor in using the HSPs as intracellular therapeutic modulators for neurodegenerative disease [48,50,51].

4. Hsp90

The Hsp90s are the most unique, and arguably sophisticated, members of the molecular chaperones. In contrast with the Hsp70s, the Hsp90s appear to be much more selective in the client proteins with which they will associate [52,53]. Continually, these client proteins will more often than not be native-like proteins – those that are in the secondary and tertiary intermediate structures – rather than misfolded

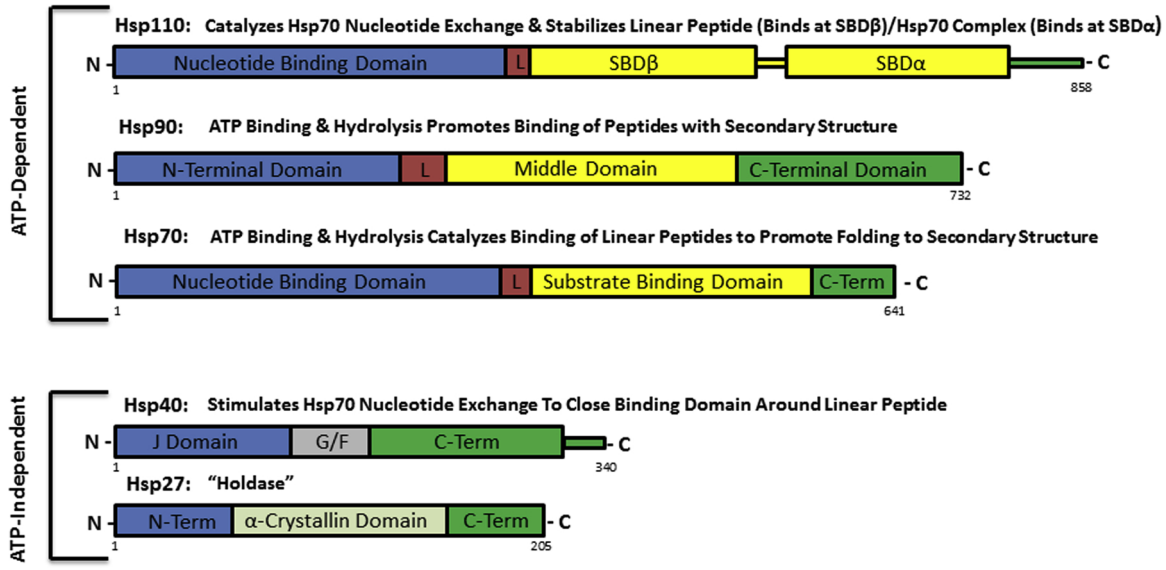

Fig. 1. Structural architecture and basic functions of human heat shock proteins. **Hsp110** (HSPH1; Hsp105α) is comprised of an N-terminal nucleotide binding domain (blue) and a C-terminal substrate binding domain (yellow) connected by a short linker region (orange). Hsp110 is responsible for stimulating nucleotide exchange for Hsp70 as well as helping to stabilize the unfolded peptide/Hsp70 complex by binding to Hsp70 with the α-binding domain and, if close enough, the linear peptide with the β-binding domain. **Hsp90** (HSPC1; Hsp90α) is comprised of three major domains – the N-terminal domain (blue) responsible for nucleotide exchange, the Middle domain (yellow) responsible for substrate folding, and the C-terminal domain (green). Hsp90 catalyzes the continued folding of polypeptides that have already achieved secondary structure. **Hsp70** (HSPA1A; Hsp70-1), much like Hsp110, is comprised of an N-terminal nucleotide exchange site (blue), and a c-terminal substrate binding domain (yellow) available for interaction with client proteins. Hsp70 typically functions with Hsp110 to stabilize a linear peptide chain and promote folding to the secondary structure. **Hsp40** (Type I J-domain containing proteins) are comprised of an N-terminal J-domain site (blue) that contains a His-Pro-Asp tripeptide motif responsible for stimulating Hsp70 nucleotide exchange, a short region of Gly-Phe (G/F) residues (grey) that allow the protein to have a strong level of flexibility, and a C-terminal domain (green). Hsp40 is responsible for stimulating ATP hydrolysis for Hsp70 so that it may close its binding domain around an unfolded peptide chain. **Hsp27** (HSPB1) is structurally comprised of an α-crystallin domain (light grey) that is approximately 100 amino acid residues in length, flanked by both an N-terminal domain (blue) and a C-terminal domain (green). Hsp27 is typically seen as an oligomeric "holdase", responsible for holding partially unfolded proteins and preventing their aggregation.

peptides. *In vitro* experiments using temperature-sensitive mutant protein strains to explore this relationship have demonstrated that Hsp90 preferentially interacts with nonnative polypeptides that have already attained a strong secondary structure [54–56]. This has led to the hypothesis that Hsp90's chaperone activity is responsible for preventing protein aggregation and providing the final acceleration in the formation of the mature protein [57,58]. Additionally, the Hsp90 machinery appears to have the most organized network of sequentially binding co-chaperones which work together in a defined order [59–61]. Interestingly, the Hsp90s appear to be more active in protein synthesis in homeostatic conditions than when the cell is under stress, thanks to a widely diminished co-chaperone network under heat shock. Experiments elucidating this in yeast demonstrated the upregulation of one major Hsp90 co-chaperone, Sti1, a non-competitive inhibitor of Hsp90 ATPase function. Upregulation of Sti1 forces Hsp90 into a "holdase" by preventing the necessary conformational changes required for release of the client peptide [62,63]. Thus, it appears that the state of the cellular environment mediates the co-chaperone networks that are required for the different functions of the molecular chaperones.

There are three main cytosolic mammalian isoforms of Hsp90: the stress inducible Hsp90α (HSPC1), its pseudogene (HSPC2), and the constitutively expressed Hsp90β (HSPC3) (Table 1). Similar to Hsc70 and Hsp70, these proteins are highly homologous [64,65]. Additionally, there is an endoplasmic reticulum isoform – GRP94 (HSPC4) that shares 50% homology with cytoplasmic Hsp90 – and a mitochondrial isoform – TRAP1 (HSPC5) that shares roughly 34% amino acid identity with its cytosolic counterparts [66,67].

5. Hsp70

Hsp70 is evolutionarily preserved across all prokaryotic and eukaryotic species. The mammalian protein shares roughly 60% amino acid similarity with its prokaryotic homolog, DnaK [68]. The human Hsp70 family is comprised of 13 unique isoforms encoded by 13 different genes, all of which differ from the next by amino acid sequence and expression level during homeostatic or stressful conditions [69] (Table 1). Two of these family members, HSPA5 and HSPA9 are organelle specific and remain confined to the endoplasmic reticulum and the mitochondria, respectively, while 7 different Hsp70 members are found in the cytosol or nucleus of the cell [69,70]. The high number of different Hsp70s is likely indicative of specialized functions (e.g. different binding affinities for co-chaperones or specific client peptides) for each individual member, however few, if any, studies have performed functional side-by-side comparisons with the individual, cytosolic isoforms. The 4 remaining Hsp70s – HSPA12A, HSPA12B, HSPA13, and HSPA14 – are more distantly related members, with little data available [69].

The two members of the human Hsp70 family that share the highest amount of structural similarity are the stress-induced Hsp70 (encoded by the HSPA1 and HSPA2 genes) and the constitutively expressed Hsc70 (encoded by the HSPA8 gene), which share 86% amino acid identity. Under normal physiological conditions, the Hsp70s are generally involved in folding newly formed proteins and under stress they act to prevent aggregation and refold misfolded proteins [71]. There are a wide range of co-chaperones and nucleotide exchange factors that participate with Hsp70 in the protein refolding process under both homeostatic and stressful conditions. The major co-chaperones that associate with the Hsp70s are the Hsp40s/J-domain-containing proteins and Hsp110s, which are responsible for accelerating the catch and release of client proteins by stimulating nucleotide exchange [72,73].

6. Hsp40

The Hsp40 protein family constitutes the third piece of the major

mammalian cytosolic Hsp70-Hsp110-Hsp40 disaggregase machinery. Functionally, the Hsp40s are responsible for binding to misfolded client peptides and delivering them to Hsp70, while simultaneously stimulating the ATPase activity of Hsp70 to promote protein refolding [74,75]. All Hsp40 family members have a 70 amino acid residue stretch that comprises a J-domain (Fig. 1). Type I and II Hsp40s are classified by the presence of an N-terminal J-domain, where type III Hsp40's present with a J-domain in a separate region of the protein [72,73]. Structurally, J-domains consist of 4 α-helices with a highly conserved region between helices I and II called the His-Pro-Asp (HPD) tripeptide motif [73]. This HPD motif is essential for Hsp40s ability to stimulate ATP Hydrolysis for Hsp70 [72,76].

As shown in Table 1, there are over 50 members in the Hsp40/J-domain family of proteins that vary drastically in size and molecular weight [69]. Known mutations in 14 of these members contribute to a number of neurodegenerative pathologies including cerebellar ataxia, distal hereditary motor neuropathy, and Charcot Marie Tooth disease type II [77,78]. Intracellular upregulation of Hsp40 has been previously used as a therapeutic tool in models of a number of neurodegenerative diseases [79–83]; however a role for extracellular Hsp40 in the treatment of disease has yet to be established.

7. Hsp27

In terms of sequence homology, the small HSPs are the most variable members of the molecular chaperones (Table 1). Structurally these proteins are comprised of a 100 amino acid residue sequence called the α-crystallin domain, which is flanked by N- and C-terminals of varying size (Fig. 1) [84]. The small HSPs are typically found in large oligomeric complexes involving one or more family members, which have been suggested to provide cells with a large diversity of chaperone specificity [69,85–88]. Hsp27 – also known as HSPB1 – is, arguably, the most studied member of the 11 small HSPs [69,88]. Functionally, Hsp27 is an ATP-independent molecular chaperone involved in protein refolding, and serves a major role to trap and hold mis-folded polypeptides to prevent their aggregation and indirectly promote their refolding or proteolytic degradation under stress [89–91]. Additionally, Hsp27 is involved in cytoskeletal organization and has been shown to exhibit both antioxidant and anti-apoptotic properties [85,87,92–96].

Similar to many members of the HSPs, the upregulated expression of Hsp27 has been used as a tool to treat models of neurodegeneration [97–100], yet there are no established extracellular roles reported for this protein in the context of neurodegenerative disease, despite evidence of its secretion [101–105].

8. A role for extracellular HSPs

In 1986, Tytell and colleagues, while looking to extend the "Glia-Neuron Protein Transfer Hypothesis", were the first group to describe the presence of an extracellular "heat shock-like protein" that acted as a glia-axon transfer protein in the squid giant axon [16,106]. This was a 70–80 kDa polypeptide, that the group initially named Traversin. When the protein's expression and transfer was increased by exposure of the axon to heat, the investigators identified it as Hsp70 [16]. Shortly after, Hightower and Guidon demonstrated the rapid release of Hsp110, Hsp71, and Hsc70 from cultured rat embryo cells [18]. These two initial reports of the presence of extracellular HSPs (eHSPs) perpetuated two major questions: How do intracellular HSPs become eHSPs, and what function do these chaperones have in the extracellular space?

9. How do intracellular HSPs become extracellular?

Early experiments addressing the secretion of HSPs into the extracellular milieu suggested that these proteins are actively and selectively released by cells [18]. In Hightower and Guidon's initial work, they demonstrated that inhibition of the common secretory pathway with monensin or colchicine did not prevent the release of HSPs by stimulated cells. Importantly, they also demonstrated a strong association between Hsp70 and fatty acids, suggesting that if these proteins were being actively exported from the cell it was happening through a secondary, possibly lipid-derived mechanism [18]. A number of follow up studies exploring the active release of HSPs in different cell types have gone on to confirm the initial hypothesis that these proteins are purposefully exported by viable cells [10,20,107–109]. In one such example, Hsp70 release was identified by healthy peripheral blood mononuclear cells following heat shock [107]. The export of Hsp70 in this study was seen to be independent of classical secretory pathways and is once again suggestive of an alternative mechanism of secretion.

The accumulation of eHSPs following blockade of classical secretory pathways in stressed cells is an interesting observation. Hsp70, for example, lacks a classical N-terminal leader sequence supporting the finding that its release is not occurring through conventional exocytosis. This has led investigators in the field to pose independent hypotheses as to how these proteins escape the cytosol, many of which take aim at Hsp70's specific interaction with lipids and lipid membranes [108–110]. The proposed mechanism for the release of HSPs that has garnered the most support is through release by extracellular vesicles, most notably lipid rafts and exosomes [20,110–112]. Cells release extracellular vesicles in response to stress, and included in those vesicles are stress signals that allow neighboring cells to communicate. It's likely that, because of their abundance in the cytosol under stressful conditions, the HSPs either become trapped or are actively loaded into these extracellular vesicles and released into the extracellular space. A number of studies have detected members of HSP families within extracellular vesicles released by a variety of different cell types including reticulocytes, dendritic cells, B cells, hepatocytes, and astrocytes [20,113–116]. The presence of HSP containing vesicles in these cell types has driven this hypothesis to the forefront of the field [117,118].

10. Predominant extracellular sources of HSPs for neurons

Active release of HSPs may be a source of trophic support for neurons [10,13–15]. Many studies have established that astrocytes rapidly upregulate Hsp70 in response to stress *in vitro*, and that the upregulation of Hsp70 in astrocytes is crucial to the release of specific neurotrophic factors [10,20,119–124]. Previous work from our group demonstrated that astrocytes in culture readily release exosomes containing Hsp70 into the microenvironment in a signaling kinase dependent manner following thermal or oxidative stress. In this study it was determined that Hsp70 release was promoted by the increased activity of extracellular-signal-regulated kinase (ERK1/2) and phosphalidylinositol-3 kinase (PI3K), and tempered by activation of c-jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) [20]. Thus it would appear that both the upregulation and release of Hsp70 by astrocytes may be supportive to neurons.

Another predominant source of eHSPs for neurons is skeletal muscle. The dependence of motor neurons (MNs) on trophic support is well documented and the loss of trophic support has been presented as an underlying hypothesis for neurodegenerative diseases [21,125–127]. It has been well established that limb muscle extract (MEx) – a solution derived from embryonic hind limbs containing a combination of target derived neurotrophic factors – promotes MN survival *in vitro* better than any single trophic factor alone [125]. Previous work from our group has demonstrated that Hsp70 is a major and crucial component of MEx, and that depletion of Hsp70 from MEx potently reduced its survival promoting effect [10]. Further, we found that administration of either purified recombinant Hsp70, or the constitutively expressed isoform Hsc70, to MN cultures devoid of MEx ameliorated cell death, confirming that extracellular Hsp70 is a critical component for the survival of MNs. It is of note to mention that exogenously administered Hsp90 also ameliorated MN cell death, Hsp27 had no effect, and Hsp40 increased MN cell death in these experiments, suggesting that some, but

not all eHSPs are protective for neurons [10].

11. A role for extracellular HSPs in neurodegenerative diseases

A key hallmark in many neurodegenerative diseases is potentially neuro-toxic changes in the intra- and extracellular environment. Protein aggregation is another hallmark feature of neurodegenerative diseases. Whether cause or effect, a contributing factor to these environmental change is the misfolding and aggregation of a protein species, that results in a further perturbation of the protein quality control system. Indeed, the subsequent increase in misfolded and reactive cytosolic proteins in affected cells should initiate the HSR. However, differentiated neurons appear to have an aberrant or attenuated HSR without increased expression of Hsp70 [10–15]. Consequently, the increased synthesis of intracellular HSPs through pharmacological and genetic mechanisms has been investigated as a therapeutic approach in treating several neurodegenerative disorders [128–132]. Why neurons have a diminished capacity to specifically upregulate Hsp70 is unclear. Some have reported attenuated neuronal HSR is a result of inadequate transcription or activation of heat shock factor 1 (HSF1) – the master regulator of stress proteins [133,134]. This, however, cannot be the only reason for the deficit in Hsp70 expression in neurons since these cells are capable of upregulating other HSPs (e.g., Hsp90) that are synthesized under the transcription of HSF1 [10,21].

The failure to upregulate Hsp70 may further contribute to potential toxic changes in the cytosol of neurons that may be amplified at the synapse. In animal models of neurodegenerative diseases, damage to synapses and axons has been reported to occur long before clinical pathology and activation of cell death pathways that lead to cell degeneration [106,135–141]. Lack of readily accessible proteins distant to the site of production may generate further dysfunction in the distal axon or synapse. Thus, particularly under times of stress, supplementation of nutritive proteins, such as Hsp70, to axons and synapses far distal to their neuronal cell bodies may be a key factor in slowing neurodegenerative disease [23,142,143]. Several studies have begun to investigate whether administration of extracellular HSPs is effective for delaying or abrogating neuropathology in animal models of disease [22–26,144–149]. Below we review potential mechanisms that have been investigated in Alzheimer's disease and ALS where extracellular Hsp70s may exert a therapeutic effect.

12. Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disease characterized by difficulty remembering newly acquired information and subtle behavioral changes that progress to increased severity of dementia and disorientation [150]. These symptoms likely result from the dysfunction and degeneration of neuronal populations in brain structures including the hippocampal formation, entorhinal cortex, and neocortex [151]. The mechanisms precipitating pathology and clinical symptoms have been fiercely investigated and debated. Pathologically, there are two main hallmarks of AD: the presence of extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles of hyperphosphorylated tau (NFTs). The deposition of amyloid, generally, develops first in the neocortex before spreading to entorhinal and hippocampal areas, whereas the presence of NFTs seem to appear first in the subcortical structures before spreading to the cortex [151,152]. The formation of NFTs appears to be a downstream process from the amyloidosis of Aβ [153]. In addition to these two pathological protein products, the impairment of synaptic function and synapse loss are tightly coupled with diseae progression. Indeed, even at early stages in the Tg2576 mouse model of AD, detectable increases in Aβ levels are seen concomittant with synapse dysfunction, and it is well established that the presence of Aβ is detrimental to both long-term potentiation (LTP) and depression (LTD), both of which are key processes in the formation or extinguishing of memories [154–158]. Interestingly, knockout of endogenous Tau mitigates both Aβ-induced synapto-toxicity and cognitive dysfunction, suggesting that Tau is required for Aβ toxicity in neurons *in vivo* [158,159]. Continually, RNAi knockdown of Tau is capable of attenuating Aβ-induced destruction of microtubules and dendritic spines *in vitro* [160,161]. Together these studies suggest that both Aβ and Tau play roles to drive synapse impairment and contribute to AD pathogenesis.

The progressive buildup of amyloidogenic Aβ as the root cause for AD was initially proposed in the mid 1980's and has since become one of the dominant models of AD pathogenesis, despite a high-level of skepticism and scrutiny [153,162–167]. Aβ, a 42 kDa protein, is the product of improper cleavage of amyloid precursor protein (APP) by β- & γ-secretases – the latter of which is formed by a complex including the presenilin 1 & 2 proteins [168]. Self-assembly of Aβ results in the formation of distinct structures including dimers, oligomers, unstructured aggregates, and fibrils, with evidence suggesting that the oligomers are the most neurotoxic [169,170]. It is still largely unclear whether the accumulation of amyloid aggregates is a driving cause behind AD. One reason why the amyloid cascade hypothesis has been questioned is the presence of a lengthy phase in AD pathogenesis in which abnormal Aβ is generated in the absence of clinical evidence for the disease. The importance of this phase has only recently been acknowledged in the field, and its presence in the pathogenesis of the disease supports a role for either unrelated or related-but-downstream mechanisms in AD pathology [171]. It is most likely that AD, as in most neurodegenerative diseases, has a multitude of pathological mechanisms that accumulate overtime, until the cell is no longer able to compensate and reaches the point of dysfunction and failure. Indeed a number of other driving mechanisms, such as slow accumulation of DNA damage in the aging brain, aberrant re-entry of neurons into the cell cycle, myelin degradation, disruptions in de novo protein synthesis, disruptions in cellular metabolism, hippocampal hyperactivity, and abnormal *wnt* signaling have all been suggested as independent mechanisms capable of driving AD pathogenesis [167,172–174]. Considering the roles of HSPs in gene transcription, protein synthesis, folding, transport and degradation, potential consequence of neurons' inability to adequately mount an HSP response may contribute to pathology.

Intracellular overexpression of Hsp70 in neurons has cytoprotective roles in models of AD most notably through its chaperone functions [175–177]. Intranasal injection of recombinant human Hsp70 was shown to ameliorate spatial memory deficits as measured by latency to platform in the Morris water maze, and decreased levels of soluble Aβ in cortex and area CA1 of the hippocampus in two rodent AD models [25]. Intranasal administration of Hsp70 was subsequently shown to preserve neuronal morphology and revert gene expression of cortical and hippocampal neurons in transgenic AD model mice to a pattern similar to that seen in non-transgenic controls [148].

A strong binding affinity for Aβ by extracellular Hsp70 has been reported previously, and it has been hypothesized that extracellular Hsp70 can bind to soluble monomers, dimers, or trimers of Aβ, preventing them from (a) forming larger oligomeric species that are cytotoxic and (b) disturbing neuronal cell membranes [178,179]. While this is speculation, it seems likely that the hydrophobic surface residues on Aβ peptides would be the most attractive client for Hsp70 in the areas where the intranasally injected protein localized. One could further posit that the removal of extracellular Aβ by Hsp70 is then responsible for the amelioration of stress, and thus the appearance of the "normal" hippocampal and cortical spectrum of gene expression seen in the transgenic mice treated in the group's follow up study. Additionally we suggest that the removal of Aβ by extracellular Hsp70 would have a positive impact on synaptic transmission by decreasing the interaction between Aβ and synaptic membrane proteins. This hypothesis is further supported by a recent report demonstrating that the holdase activity of secreted Hsp70 is capable of masking Aβ in the extracellular space, suppressing neurotoxicity, and protecting structural integrity in adult neurons in *Drosophila* [180].

13. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by progressive and rapid muscle weakness, spasticity, and atrophy leading to the inevitable paralysis and death of patients within an average of 2–5 years following diagnosis [181,182]. All of these symptoms arise from the denervation of neuromuscular junctions (NMJs) and the dysfunction and death of spinal and cortical motor neurons (MNs). While the vast majority (~90%) of ALS cases are sporadic, approximately 20% of the genetic, or familial, cases have been associated with autosomal dominant mutations in the gene coding for the protein Cu/Zn superoxide dismutase-1 (SOD1) [183]. To date, over 100 different mutations in the SOD1 protein have been discovered, all of which promote varying degrees of SOD1 demetallation and a reduction of the stability of SOD1 monomers. These molecular changes result in a toxic gain of function, non-specific interaction with unnatural targets, and the insufficient degradation of superoxide radicals by the mutant protein [184–188]. Soon after the discovery of SOD1 mutations in a subset of familial ALS patients, the first mouse model for ALS was created. The $SOD1^{G93A}$ mouse model of ALS is the best characterized rodent model for the disease, and is still the most commonly used today.

A number of distinct, damaging mechanisms within MNs, including mitochondrial dysfunction, increased oxidative and nitrative stress, axonal transport dysfunction, and aberrant RNA and protein metabolism, coupled together with NMJ denervation and pathological activation of surrounding glial cells have been implicated in the pathogenesis of ALS [189–190]. Each of these proposed mechanisms independently contributes to cellular stress, but culminate to produce an environment that is highly detrimental to MN survival. Pathologically, early changes in the cell bodies of spinal cord MNs correlate with the initial signs of denervation at the NMJ, both of which long precede cell death [189,191,192]. While the mechanism of denervation is not yet clear, evidence from both human sporadic ALS patients and the $SOD1^{G93A}$ mouse model has led to the hypothesis that these early disturbances in motor neuron cell bodies propagate axonal and synaptic deficits that lead to a retraction of the motor axon from the neuromuscular synapse [192–196]. The combination of the early intracellular damage and loss of trophic support due to denervation lends support to exploring both central and peripheral regions of MNs as areas of potential therapeutic intervention early in disease pathology.

The diminished capacity for MNs to mount a routine stress response in the face of cellular insult is well documented [10,14,21]. As mentioned above, the exogenous administration of both stress-inducible and constitutively expressed isoforms of Hsp70 is capable of maintaining cell viability for MNs devoid of trophic support *in vitro* [10]. These initial experiments from our group suggest that the exogenous delivery of Hsp70 or the secretion of the protein from non-neuronal sources may be crucial to maintaining MN viability in disease [10,20,21]. Indeed, evidence from our follow up studies using systemic injection of recombinant human Hsp70 *in vivo* supports these hypotheses [22,23]. In these studies, we found that intraperitoneal injection of Hsp70 was capable of delaying disease onset, prolonging survival, and maintaining NMJ innervation in the $SOD1^{G93A}$ mouse model. An interesting observation from this work was the localization of recombinant protein to peripheral tissues, including skeletal muscle. The protein was not detected centrally. These results suggest that exogenous Hsp70 has a direct, survival promoting effect for MNs in disease through a mechanism at the neuromuscular synapse.

The failure of MNs to upregulate a “normal” stress response is an observation that has piqued the interest of other investigators as a potential therapeutic target for the treatment of MN disease. As such, a strong line of investigation using both genetic and pharmacological manipulation to restore a complete heat shock response in models of ALS pathology has been explored [50,197–202]. However these studies have provided ambivalent evidence towards the effectiveness of intracellular upregulation of HSPs in MNs.

Using a cross between two different SOD1 mouse strains and mice engineered to overexpress Hsp70 specifically in MNs, investigators determined that genetic upregulation of Hsp70 was incapable of ameliorating disease [199]. One explanation for the striking ineffectiveness of intraneuronal Hsp70 upregulation could be a deficiency in shuttling the synthesized protein down the axon. This study demonstrated the inclusion of Hsp70 in cytosolic aggregates, suggesting that it was not freely translocating down the axon to feasibly more disease-prone areas of the MN, such as the NMJ. Indeed, axonal transport deficits in MNs are early pathological events in SOD1 mice [203], thus it may not be sufficient to simply increase cytosolic protein expression in MN cell bodies. Instead, perhaps the local delivery of HSPs to the synapse may offer a more accessible and protective route.

The pharmacological approach to manipulation of the HSPs that has garnered the most interest is the hydroxylamine-derived compound Arimoclomol – a co-inducer of HSP expression. Indeed, treatment with Arimoclomol considerably increased the life span and motor function of $SOD1^{G93A}$ animals compared to untreated controls [198,204]. Mechanistically, Arimoclomol acts to amplify HSP expression by stabilizing active HSF1 in the cytosol and nucleus of cells that are undergoing stress [129]. Following treatment in these studies, SOD1 mice showed a significant upregulation of Hsp70 and Hsp90 in spinal cord MNs, however the non-cell autonomous nature of ALS pathology suggests that cells other than neurons are undergoing high levels of stress [205]. As a result, the amplification of HSPs through arimoclomol, and its survival promoting effects, must be considered in the context of surrounding cell types as well as the failure of genetic upregulation of Hsp70 in MNs to confer a survival promoting effect.

Arimoclomol has previously demonstrated profound effects in augmenting a stress response in astrocytes, generating a preferential upregulation of Hsp70 and Hsp90 following peripheral nerve injury [206]. The release of Hsp70 by astrocytes is well established [20]. It is possible that Arimoclomol exerts its protective effects, at least in part, by increasing the expression, and secretion, of astrocytic Hsp70, which may be capable of providing extracellular support to vulnerable neurons in ALS. Skeletal muscle cells also represent a non-neuronal target for the effects of Arimoclomol. It is well established that the denervation of NMJs happens far before the frank degeneration of MN cell bodies in ALS mouse models [189,192–196], suggesting that therapeutic strategies aimed at maintaining peripheral MN-muscle connections may have profound effects on slowing disease progression [22,23,138,199,207]. Additionally, muscle cells have a more robust HSR than MNs [14,208], and indeed Hsp70 is a critical trophic component of MEx [10]. Thus, just like with astrocytes, the upregulation and increased secretion of HSPs by muscles to maintain NMJ innervation may be a contributing factor to the survival promoting effects of Arimoclomol in ALS models. Excitingly, Arimoclomol was recently advanced to a Phase III clinical trial to assess the drug’s efficacy in slowing ALS disease progression after succeeding in a Phase II safety and tolerability trial at a dose of 200 mg tid [209].

The failure of intraneuronal upregulation of Hsp70 to confer protection to MNs in disease is a striking observation [199]. Another reason for the lack of the protective effect of Hsp70 could be a diminished cytosolic co-chaperone network. Indeed, the cytosolic expression of Hsp110 is far lower than that of Hsc70 in neurons [51]. If the primary effect is a result of co-upregulation of Hsp70 and Hsp90 in neurons, then treatment of SOD1 mice with arimoclomol is supportive of this hypothesis, as is a recent study from the Horwich group [50]. In this study $SOD1^{G85R}$ rats were crossed with rats genetically engineered to overexpress either Hsp110 or both Hsp110 and Hsp70. The overexpression of Hsp110 in the rat model of ALS was sufficient to increase lifespan, an effect that was exaggerated following co-overexpression of Hsp110 and Hsp70 [73]. Hsp110 works predominantly with the Hsp70s to catalyze a rate limiting step in Hsp70 nucleotide exchange [48], thus increasing Hsp70’s effectiveness. While this study provides exciting

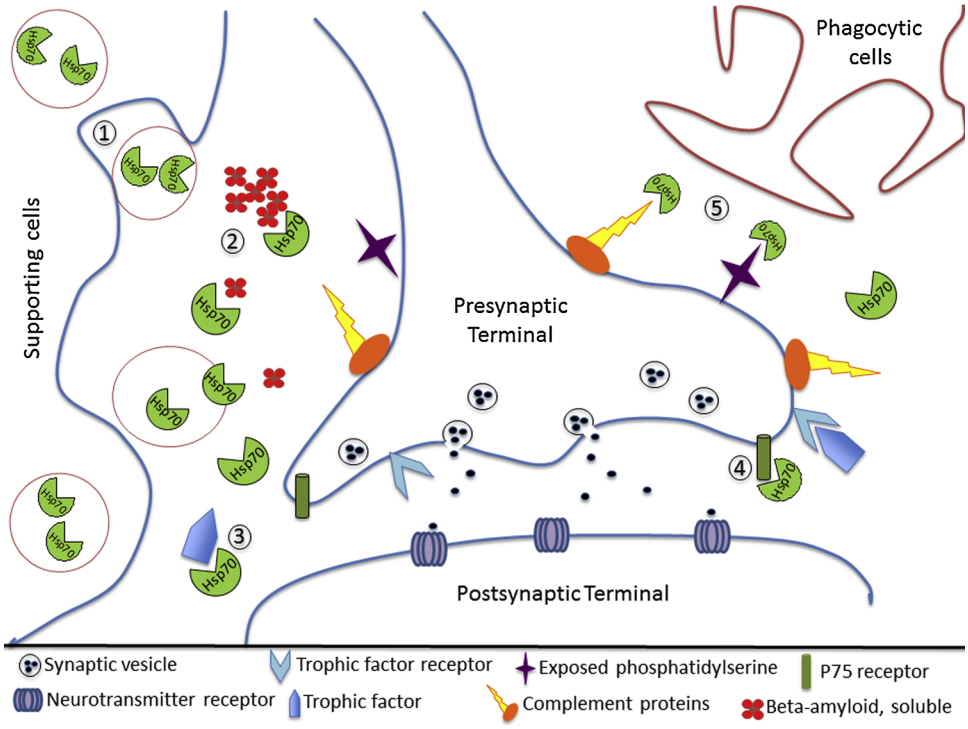

Fig. 2. Hypothesized neuroprotective roles for extracellular Hsp70. A key early pathological event in most neurodegenerative diseases is the dysfunction and removal of synapses in affected neuronal populations. Here we speculate that extracellular Hsp70 may play key roles in ameliorating some of the driving mechanisms of synaptic impairment and clearance. **(1)** Neuronal support cells such as astrocytes, skeletal muscle cells, and terminal Schwann cells release Hsp70 into the extracellular space in times of stress. **(2)** In the extracellular space, Hsp70 is capable of binding to toxic protein species that express large stretches of hydrophobic amino acid residues, such as aggregates or oligomers of Beta-amyloid, preventing them from interacting with synaptic proteins and disrupting synaptic transmission. **(3)** Additionally, Hsp70 may be capable of binding to and increasing trafficking of trophic factors from supporting cells to their target neurons, helping to strengthen synapses and maintain innervation. **(4)** Hsp70 may also be able to block apoptotic signaling to populations of cells, such as motor neurons, by masking the p75NTR at synapses, preventing its activation by potentially toxic signaling factors released by muscle. **(5)** Hsp70 has a strong binding affinity for the hydrophobic stretches present on membrane bound complement proteins and phosphatidylserine, and may prevent synaptic stripping by masking these "find me" and "eat me" signals from tissue-resident phagocytes.

evidence for the role of HSP upregulation as a treatment in ALS, a protective role for exogenous Hsp110 is still to be determined.

Similar to Hsp110, the genetic upregulation of Hsp27 as a therapeutic tool has been attempted in a mouse model of ALS. Interestingly, these experiments had ambivalent results. In one study, the overexpression of Hsp27 was capable of delaying symptom onset, but did not increase survival in the SOD1^{G93A} mouse [99]. In a second study, Krishnan and colleagues found that the over expression of Hsp27 was capable of protecting against spinal cord ischemia, but had no effect on SOD1^{G93A} mouse viability [100]. Together, these studies suggest that there might be a minimally protective role for the intracellular upregulation of Hsp27 early on in ALS disease progression; however a protective role for exogenous Hsp27 is still to be determined in disease.

14. Proposed mechanisms by which extracellular HSPs confer neuroprotective effects: areas for future research

While there is now substantial evidence supporting a mechanism of release for HSPs, the specific mechanism by which extracellular HSPs exert protective effects is less understood. The endocytosis of HSPs by neurons in culture systems has been readily described [10,16,19–21]. Indeed, extracellular Hsp70 has been shown to be taken up by neurons/axons following axotomy when added directly to the stump [210], a phenomenon that may be mediated by toll-like receptors [211], however there are no reports of systemically injected HSPs freely entering the nervous system at a detectable level. This raises an interesting question: Do extracellular HSPs need to be taken up by neurons in order to provide a survival promoting effect?

We have postulated that the protective effect of systemically administered Hsp70 in the SOD1 mouse model of ALS was due to a peripheral function at the neuromuscular junction, as muscle was the primary localization of the protein following treatment [22,23]. As discussed above, synaptic dysfunction is an early event in neurological disorders. Extracellular HSPs may exert beneficial effects through specific interactions with macromolecules that may promote this dysfunction. One hypothesis is that the protein localizes to the synapse and works to either remove or mask potentially toxic extracellular proteins or maintain a steady flow of trophic factors to the motor neurons (Fig. 2). Indeed a similar function could be taking place in the structures affected in Alzheimer's disease [25].

Another possibility is that extracellular HSPs may be playing a role in the protection of synapses by blocking expression of proteins involved in synaptic pruning or stripping. An exciting line of investigation implicating a role for the complement cascade in neurodegenerative diseases has recently gained traction as a novel mechanism for the selective pruning of synapses [212–214]. In the developing nervous system microglia selectively prune back synapses that express "eat me" signals such as complement components C1q and C3 [215,216], and seem to avoid synapses expressing "don't eat me" signals such as CD47 [217]. A similar exclusionary principle may also be regulating the microglial phagocytosis of synapses early in Alzheimer's disease [212,213]. One of the major "eat me" signals, phosphatidylserine (PS), is a small hydrophobic molecule that presents itself on the exterior surface of injured or dying cells, marking them for phagocytosis [218]. Hsp70 has a prominent binding affinity for hydrophobic [38,39] and negatively charged proteins, and indeed a strong attraction between Hsp70 and PS has been reported [219]. Hsp70's high propensity for binding these types of proteins may allow it to mask hydrophobic, membrane-bound "eat me" signals like PS and complement proteins extracellularly at synapses, thus preventing their recognition by microglia or peripheral tissue macrophages in neurodegenerative diseases [215,216] (Fig. 2). This is an open line of investigation that we are currently pursuing in a mouse model of ALS.

There is a long-standing argument in the field as to whether or not there is an immune-modulating role for extracellular Hsp70. Hsp70 has demonstrated profound effects associated with both immunostimulatory and immunosuppressive activities [220]. Additionally, extracellular Hsp70 has been reported to increase phagocytosis, modulate monocyte response to endotoxin, and increase chemotaxis of neutrophils, however the mechanisms by which Hsp70 affects these immune cells is still unknown [221–224]. Both inflammatory and immune responses have been hypothesized to play a

role in the pathogenesis of neurodegenerative diseases [225,226]. One possible immunosuppressive role for extracellular Hsp70 is to mask toxic species at the synapse, thus reducing synapse dysfunction and preventing neurons from signaling to and activating glial cells (Fig. 2). Another possibility is that extracellular Hsp70 is driving adaptive immune signaling by neurons. Indeed one recent report has demonstrated that intranasal infusion of Hsp70 in a rodent model of AD resulted in a significant increase in the neuronal expression of genes responsible for surface antigen presentation, specifically major histocompatibility complex I and II [144], suggesting that Hsp70 may be exerting a protective effect through an activation of adaptive immunity. While this study provides a compelling argument to continue the study of an immunomodulatory role for the HSPs, at present there is not enough evidence to definitively conclude that extracellular Hsp70 is exerting a protective effect through immunomodulation in the context of neurodegenerative diseases.

15. Considerations for treatment with exogenous HSPs

The treatment of neurodegenerative diseases with exogenous HSPs is in a stage of infancy. There is strong promise for the protection of neurons susceptible to pathological stressors in disease, but investigators must be wary of a few considerations. The first of these considerations is in the choice of chaperone isoform. Because of their structural similarities, the cytosolic members of the HSP families have long been considered functionally redundant; however, very few studies have explored the functional prowess of each of these members in specific side by side assays. Thus, it is imperative to consider that the small changes in sequence homology between these proteins may account for stronger functional differences than originally appreciated. Indeed, data from our group indicate that the small differences in homology between Hsc70 and Hsp70 may have a profound effect on the way that these proteins are metabolized following exogenous delivery.

In follow up experiments to our previous work treating $SOD1^{G93A}$ mice with recombinant human Hsp70 [22,23], we purified an endotoxin free, recombinant human Hsc70 (rhHsc70) with a 6x-Histadine tag on the substrate binding domain, and repeated the same systemic treatment paradigm we used previously (for purification methods, see [21]). Since there is uncontrollable variability in the recombinant protein production process, we aimed to normalize our doses based on the ATPase activity of the protein before treating the animals (1 unit of activity was equal to approximately 1 ug of protein). We treated age-, litter-, and sex-matched $SOD1^{G93A}$ mice with either rhHsc70 or an equivalent sized dose of bovine serum albumin (BSA), a protein of roughly the same molecular weight that we previously found to have no survival promoting effect. Surprisingly, unlike in the previous study [23] all doses of rhHsc70 were ineffective in delaying the denervation of NMJs in the tibialis anterior, a muscle comprised predominantly of type IIb muscle fibers – those that are most susceptible to denervation – compared to BSA treated controls (Data not shown). To determine if Hsc70 had a toxic effect, we next assessed NMJ denervation in the soleus, a muscle comprised predominantly of slow muscle fibers – those most resistant to denervation. We observed no significant difference in NMJ denervation in the soleus at any dose of rhHsc70 compared to BSA treated controls, suggesting that rhHsc70 was not promoting denervation. Using a nickel affinity chromatography approach, we precipitated out the rhHsc70 from a subset of treated animals and prepared the elutant for western blot to determine the final localization of injected protein. In the previous study full length Hsp70 was detected in liver and muscle of treated animals [22]. In the experiments using rhHsc70, lower molecular weight fragments were identified in liver as a 40 Kda protein band that had positive immunoreactivity with an antibody to 6x-Histidine, but no full length protein was able to be detected. This band appeared to be the correct molecular weight for the 6x histidine-tagged substrate binding domain of Hsc70. These results suggest that the subtle differences in amino acid sequence between Hsp70 and Hsc70 have profound effects on the way the protein is processed following exogenous delivery.

The second consideration for the treatment of neurodegenerative diseases with exogenous HSPs is the method of delivery. It would appear, at least in the case of models of AD that intranasal delivery of Hsp70 is efficient at allowing the protein to get into the CNS without much difficulty [25,148]. Conversely, our group has never detected Hsp70 or Hsc70 in the CNS following intraperitoneal injection. If extracellular HSPs are required for synaptic protection in some way, local administration of the protein to those synapses is likely the best method to pursue. In the case of neurodegenerative diseases that manifest in the CNS, intranasal administration or intracerebroventricular injections should be considered. In neurodegenerative diseases like ALS, where MNs synapse onto muscle in the periphery, intramuscular injection of the protein may offer a more targeted approach to the NMJ as compared to systemic administration. Intramuscular injection may also provide a route of administration where injected protein is less likely to be metabolised.

The final consideration for treatment with exogenous HSPs is an immune response. Extracellular HSPs are capable of interacting with membrane bound Toll-like receptors [211], which are present on antigen presenting cells (APCs). In the early 2000s, two studies demonstrated that recombinant Hsp70 was capable of stimulating increased activity in macrophages, suggesting that these extracellular proteins had a role in regulating the immune system [227,228]. These original studies characterizing the immunomodulatory role of Hsp70 were initially challenged due to early preparations of recombinant heat shock proteins being contaminated with lipopolysaccharide or other bacterial proteins [229,230], however subsequent investigation with endotoxin-free protein purification methods has confirmed that Hsp70 is capable of activating inflammatory cells [231–233]. Thus, while it is imperative to use endotoxin-free proteins, one must also account for an immune response to the protein itself. Indeed, our group has determined that antibodies are made to recombinant human Hsp70 following exogenous administration to mice [22], however mice also created antibodies to BSA in these experiments, suggesting that this was not a targeted response to the recombinant Hsp70. While an immune response needs to be considered, it should not deter investigators from exploring the use of exogenous HSPs to treat neurodegenerative diseases, as antibodies are made to other recombinant proteins (e.g. insulin, IFN, etc.) used in clinical treatment.

16. Conclusion

With the prominence of damaging, hydrophobic proteins implicated in a vast number of neurodegenerative pathologies (e.g. mSOD1 in familial amyotrophic lateral sclerosis, Aβ in Alzheimer's disease, α-synuclein in Parkinson's disease, etc.), it seems intuitive that biology would provide us with a protective macromolecule capable of masking toxic protein species before they can wreak havoc on vulnerable cells. Intracellularly, the HSPs perform a number of crucial and well-understood functions under homeostatic and stressful conditions in order to maintain proteostasis; however these internal functions do not protect cells from the effect that toxic extracellular species can exert. Excitingly, the findings that HSPs can be secreted into the extracellular environment provide investigators with new opportunities to target previously unmanageable sources of stress and mask extracellular signals that may mark susceptible cells for clearance. These findings offer a new measure of therapeutic potential to the HSPs, but obstacles must be overcome before they can be efficiently used in a clinical setting. Future exploration into the mechanism by which these proteins are released, the roles they play in the extracellular environment and the route by which they can be optimally delivered to aid vulnerable cells is imperative to unlocking their full therapeutic potential.

Declaration of Competing Interest

The authors confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

None of the authors has any conflict of interest to disclose.

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