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Review

Preventing α-synuclein aggregation: The role of the small heat-shock molecular chaperone proteins

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ABSTRACT

Protein homeostasis, or proteostasis, is the process of maintaining the conformational and functional integrity of the proteome. The failure of proteostasis can result in the accumulation of non-native proteins leading to their aggregation and deposition in cells and in tissues. The amyloid fibrillar aggregation of the protein α-synuclein into Lewy bodies and Lewy neuritis is associated with neurodegenerative diseases classified as α-synucleinopathies, which include Parkinson's disease and dementia with Lewy bodies. The small heat-shock proteins (sHsps) are molecular chaperones that are one of the cell's first lines of defence against protein aggregation. They act to stabilise partially folded protein intermediates, in an ATP-independent manner, to maintain cellular proteostasis under stress conditions. Thus, the sHsps appear ideally suited to protect against α-synuclein aggregation, yet these fail to do so in the context of the α-synucleinopathies. This review discusses how sHsps interact with α-synuclein to prevent its aggregation and, in doing so, highlights the multi-faceted nature of the mechanisms used by sHsps to prevent the fibrillar aggregation of proteins. It also examines what factors may contribute to α-synuclein escaping the sHsp chaperones in the context of the α-synucleinopathies.

1. Introduction

Protein homeostasis (proteostasis) is the maintenance of proteins at appropriate levels and in the correct (functional) conformation [1]. Proteostasis involves a complex network of integrated systems, including molecular chaperone proteins, which ensures the conformational (and hence functional) integrity of synthesised proteins and the degradation of proteins which are no longer functional. When proteostasis mechanisms falter, proteins can misfold, aggregate and accumulate, often resulting in disease [2]. One example of this is the α-synucleinopathies, a group of neurodegenerative diseases that include Parkinson's disease (PD), multiple system atrophy and dementia with Lewy bodies [3], in which the protein α-synuclein (α-syn) aggregates into insoluble amyloid fibrils and forms deposits (termed Lewy bodies and Lewy neurites) inside cells. Whilst the aetiology of the α-synucleinopathies is multi-faceted, this review will focus specifically on the links of these diseases with the fibrillar aggregation of α-syn (for a more comprehensive review of the pathology and genetics of the α-synucleinopathies, see [4–8]).

Heat-shock proteins (Hsps) are a broad family of molecular chaperones that play important roles in proteostasis. They function under normal physiological conditions and when cells are under stress, e.g. when they are exposed to elevated temperatures or reactive oxygen species (ROS) [9]. One of the most up-regulated classes of Hsps induced by stress is the small heat-shock proteins (sHsps) which have been implicated as important components of the cellular response to the onset of many protein aggregation disorders, including the α-synucleinopathies.

The sHsps act as the cell's first line of defence against protein aggregation. Their ability to prevent protein aggregation *in vitro* has been well characterised using a variety of pathogenic and non-pathogenic proteins [10–13], including α-syn [11,14,15]. Yet since the α-synucleinopathies occur, it is evident that there are some circumstances whereby sHsps (and other classes of molecular chaperones) fail to prevent the aggregation of α-syn *in vivo*. This review outlines the role of sHsps in cellular proteostasis, with a particular focus on αB-crystallin (HSPB5, αB-c) and Hsp27 (HSPB1), as these have been the most studied sHsps, including in the context of the α-synucleinopathies (for recent reviews that focus on the association and impact of other major chaperone classes, e.g. Hsp70 and Hsp90, on the α-synucleinopathies see [16–19]). In doing so, this review provides insight into how these sHsps normally interact with α-syn to prevent its toxic fibrillar aggregation, and what factors may account for the chaperone activity of sHsps being ineffective and/or overwhelmed in the α-synucleinopathies.

Abbreviations: αA-c, αA-crystallin; αB-c, αB-crystallin; apoC-II, apolipoprotein C-II; α-syn, α-synuclein; AFM, atomic force microscopy; ALS, amyotrophic lateral sclerosis; DA, dopamine; EGFP, enhanced green fluorescent protein; Hsp, heat-shock protein; PD, Parkinson's disease; ROS, reactive oxygen species; sHsp, small heat-shock protein; TEM, transmission electron microscopy

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2. Proteostasis

Proteostasis operates both inside and outside the cell (for recent comprehensive reviews see [20,21]) and is dependent on numerous integrated pathways that control the lifecycle and fate of a protein (Fig. 1). These pathways include those involved in gene transcription, mRNA translation and protein synthesis on the ribosome, through to protein trafficking and compartmentalisation in cellular organelles, and finally to protein degradation by the proteasome [22]. As such, the macromolecular elements comprising the cellular proteostasis network include transcription factors, RNA processing and translocation factors, folding enzymes, trafficking components, molecular chaperones and degradation components. The capacity to maintain proteostasis varies between cell types [23], and is thought to be reflective of the composition and concentration of components of the network that arise as a result of differences during cellular differentiation and development [24]. Whilst this capacity is finite at any point in time, it can be spatially and temporally altered through varying the amount and/or activity of individual components. Thus, an appropriate analogy for proteostasis is a seesaw; there is an intricate balance required in the use of energy and resources to maintain the functional integrity of the proteome and thus avoid protein aggregation and disease.

2.1. Molecular chaperones

The biological function of (most globular) proteins is inextricably linked to them obtaining the correct (native) fold [25]. However, many proteins are intrinsically disordered and do not fold; instead they remain unfolded with some acquiring structure once they bind to other proteins or membranes [26–28]. Unfolding is important in the life cycle of many proteins; it is required in various biological processes including protein trafficking, secretion and translocation across membranes, as well as regulation of the cell cycle [29]. In all cases, attaining the correct native state, whether folded or disordered, is essential for protein function and often relies on the presence of molecular chaperones. Molecular chaperones protect and stabilise non-native regions of proteins, or assist in proteins acquiring their native state, without contributing conformational information or forming part of the final native structure [32,33]. Chaperones achieve this by interacting with (and stabilising) partially folded and unfolded protein intermediates in order to prevent improper associations that could otherwise lead to misfolding and aggregation [34]. In addition, chaperones can also facilitate the folding of multi-domain proteins, through transient sequestration of the folding intermediates [24].

Due to their role in assisting proteins to acquire and maintain their native conformation, molecular chaperones are key components of the proteostasis network. They participate in protein folding, complex assembly, protein trafficking, protein stabilisation and protein degradation. As their name implies, Hsps are molecular chaperones that are most commonly expressed as part of the cellular response to stress, although some members are constitutively expressed and play important roles under non-stress conditions [35]. The Hsps have been classified into groups based on the mass of their monomeric subunits. They include Hsp100, Hsp90, Hsp70, Hsp60 and the sHsps [36]. Chaperones can also be generally classified as having either a 'foldase' or 'holdase' type action. For example, Hsp70 and Hsp60 are classified as 'foldase' type chaperones as they actively facilitate the folding of protein intermediates to their native folded state. In doing so, they often act in tandem, with Hsp70 acting first on the polypeptide chain upon its exit from the ribosome. Their mechanism of action is dependent on ATP hydrolysis, which results in cycles of high- to low-affinity target protein binding, which promotes folding of the target protein [37,38]. Foldase chaperones also encompass the so-called 'unfoldase' action attributed to some chaperones, including Hsp70, in which ATP hydrolysis is used to unfold or disaggregate misfolded or aggregated proteins to provide folding-competent intermediates [39,40]. In contrast, so-called 'holdase'

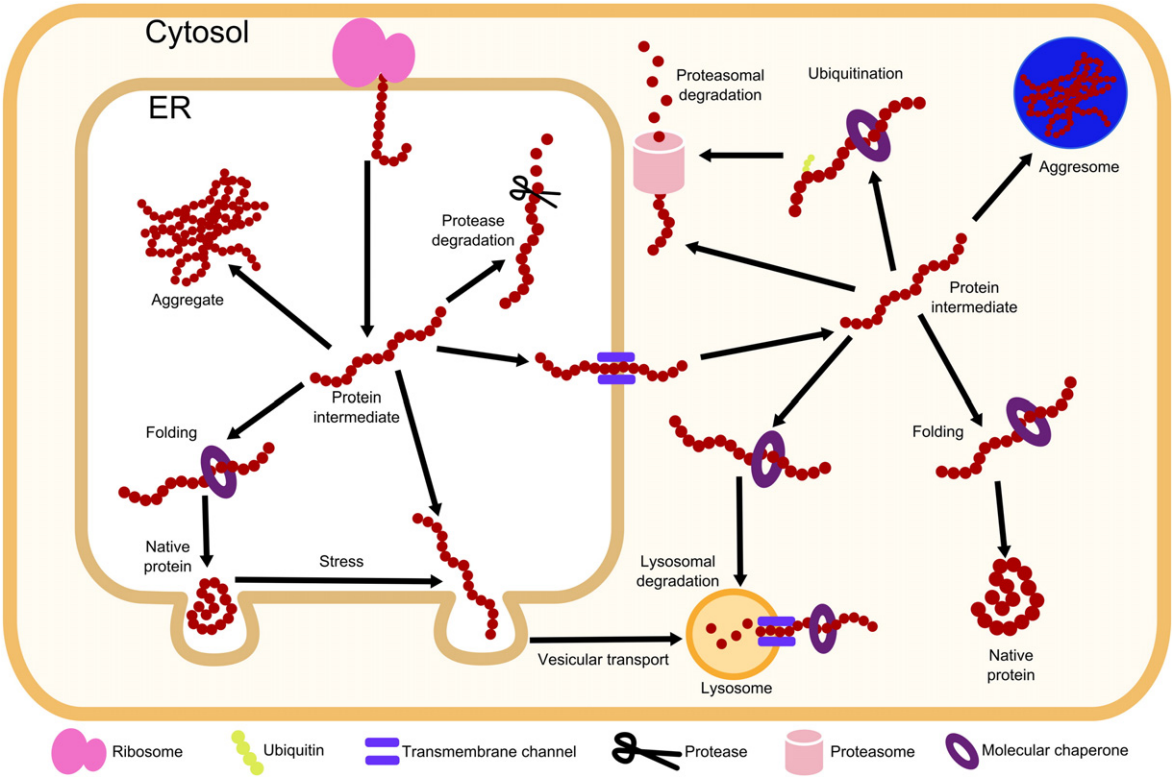

Fig. 1. Cellular proteostasis mechanisms. In the endoplasmic reticulum (ER), molecular chaperones assist newly synthesised protein intermediates to fold into their native conformation for transport into the cytosol. Persistent or misfolded protein intermediates can be proteolytically degraded within the ER or transported to the cytosol [30]. Once in the cytosol, protein intermediates are recognised by molecular chaperones and targeted for refolding or degradation via lysosomal or proteasomal pathways [31]. When these mechanisms fail to clear protein intermediates, insoluble aggregates can accumulate within the ER or cytosol as aggresomes. Adapted from [22].

chaperones, which include the sHsps, interact with the partially folded intermediate states of proteins to stabilise them and prevent their mutual association. The mechanism of action of holdase chaperones is ATP-independent since they do not have an active role in folding proteins and their association with target proteins is driven primarily through hydrophobic interactions. However, the ability of holdases to function in the ATP-depleted environment that occurs in the cell when it is under stress ensures their function is not compromised at the time cell viability is threatened. When energy levels permit, holdase chaperones can deliver target proteins to foldases for refolding, or to the cellular protein degradation systems such as the proteasome [37,38,41].

2.1.1. The role of molecular chaperones in protein degradation

When a protein can no longer maintain its correct conformation, the cell utilises degradation pathways such as the ubiquitin–proteasome machinery and autophagic–lysosomal trafficking systems to remove it. Misfolded proteins are recognised by the ubiquitin–proteasome system, which labels and degrades them through a highly regulated pathway. Using a series of ubiquitin ligase enzymes, ubiquitin polypeptide chains are covalently linked to misfolded proteins, marking them as substrates for selective degradation within the proteasome [42,43]. Lysosomal mechanisms, such as macroautophagy and microautophagy, are less selective; membrane-bound vesicles capture a selection of the cytosol, which is then targeted for degradation. By contrast, chaperone-mediated autophagy provides a level of targeted degradation as it relies on the recognition of a target motif in cytosolic proteins by specific chaperones, which then deliver the proteins to the membrane of the lysosome for internalisation and degradation [44]. The sHsps may play a role in all of these degradation pathways; for instance, αB-c stimulates ubiquitination of insoluble proteins which marks them for ubiquitin-dependent degradation [45], Hsp22 (HspB8), in cooperation with Bag3, promotes degradation of mutant Huntington protein through induction of macroautophagy [46] and Hsp27 targets misfolded cystic fibrosis transmembrane conductance regulator proteins for degradation in the proteasome [47]. Whether these activities are specific to individual sHsps or represent more generic traits of sHsp family members is yet to be determined, however it is clear from this work that as a chaperone class, sHsps function not only to bind to proteins to prevent their aggregation but can also facilitate their degradation when misfolded [48].

2.2. When proteostasis fails

A number of factors can influence the ability of a cell, tissue or organism to maintain proteostasis. Changes in cellular ATP levels, amino acid pools, metabolites, lipid homeostasis and ion balance can all disrupt the protein folding and degradation capabilities of the cell [24]. Signalling pathways can be exploited to control transcriptional, translational and post-translational mechanisms in the cell in order to regulate protein synthesis, folding, trafficking and degradation [24]. However, disruption of any element within this integrated network can result in proteostasis dysfunction. Aberrant protein folding and protein aggregation are now recognised as key factors in many diseases, collectively termed protein misfolding or conformational diseases [49]. These diseases, which include type II diabetes, cataract and neurological disorders such as Alzheimer's disease and PD [50], are associated with the aggregation and precipitation of misfolded protein into either amorphous or fibrillar aggregates. Their prevalence – and the prediction that this will increase dramatically over the next few decades as a consequence of the ageing population in many countries [51] – underlies the importance of understanding the network of pathways that maintain proteostasis.

3. Parkinson's disease is an aggregation disorder

PD is the second most prevalent neurological disorder, with its world-wide incidence projected to reach at least 8.7 million individuals over 50 years of age by 2030 [51–53]. PD is characterised by motor manifestations including tremor, rigidity, bradykinesia and postural instability, which can also be accompanied by non-motor symptoms such as sleep impairment, neuropsychiatric disorders and olfactory deficits [54,55]. All of these symptoms are linked to a gradual reduction in dopamine content associated with the progressive loss of dopaminergic neurons in the *substantia nigra pars compacta*, a region located in the midbrain.

The histological hallmark of PD is the presence of protein inclusions, known as Lewy bodies or Lewy neurites, localised to the cell body or cell processes respectively [56]. These distinctive spherical protein inclusions are found in the cytoplasm of nigral neurons and are characterised by a dense protein core surrounded by a halo of fibrils and auxiliary proteins [57]. The principal protein contained within these inclusions is α-syn [58]. The α-syn-rich proteinaceous deposits are not only found in PD but are also detected in other neurological disorders including dementia with Lewy bodies, some forms of Alzheimer's disease and multiple system atrophy [59–61].

Since α-syn is expressed in cell types other than neurons, often at higher levels (e.g. red blood cells [62,63]), the question arises: why are neurons so susceptible to forming α-syn inclusions? Neurons are thought to be particularly vulnerable to these protein accumulations as they are post-mitotic and therefore unable to dilute potentially toxic species through cell division [64]. Moreover, expression of some molecular chaperones, including the sHsps, appears to be lower in dopaminergic neurons compared to other cell types which may compromise their ability to prevent protein aggregation [23].

3.1. α-Synuclein in Parkinson's disease

α-Synuclein, a 140 amino acid protein, was initially identified as a neuron-specific protein localised within the nucleus and presynaptic terminals [65]. From a functional perspective, there is still no definitive evidence for the role α-syn plays in cells. It has been implicated in modulating synaptic activity through membrane processes, including neurotransmitter release, trafficking and biogenesis [66,67]. In addition to the full-length form of the protein, there are two shorter isoforms which are 126 and 112 amino acids in length and result from in-frame deletions of exon 3 and 5 respectively [68,69]. However the full length isoform is by far the most abundant in the brain [70]. Truncated versions, derived from proteolytic cleavage, have been identified as significant components of Lewy body inclusions [60,70,71]. Truncation fragments of α-syn also have a higher aggregation propensity than the full length form, both *in vitro* and *in vivo* [72–75].

As well as the association of α-syn with Lewy body pathology, there is a strong genetic association of α-syn with PD [76] which has further piqued interest into the role of this protein in disease. PD may be either early onset (before) or late onset (after 40 years of age). Although most commonly presenting as a late onset, sporadic disease of unknown aetiology, a familial form with an autosomal dominant pattern of inheritance among early onset patients was initially identified in two small kindred [77,78]. This led to the identification of three missense mutations in the α-syn gene which correlate to early-onset and aggressive PD: A53T, A30P and E46K [76,79,80]. Multiplication of the α-syn gene has also been associated with the development of α-syn inclusions [60,71]. Gene copy number is strongly correlated with the age of disease onset; duplication results in an average age of onset of 48.4 years, whilst triplication results in an average age of onset of 33.4 years [81].

3.1.1. α-Synuclein aggregation

Due to the presence of large amounts of aggregated α-syn in Lewy bodies and Lewy neurites, and the correlation between α-syn genetic abnormalities and early onset PD, the mechanism and kinetics by which α-syn aggregates to form amyloid fibrils *in vitro* have been examined in detail (Fig. 2). It has been generally accepted that α-syn exists as an unstructured monomeric protein in its native state in solution.

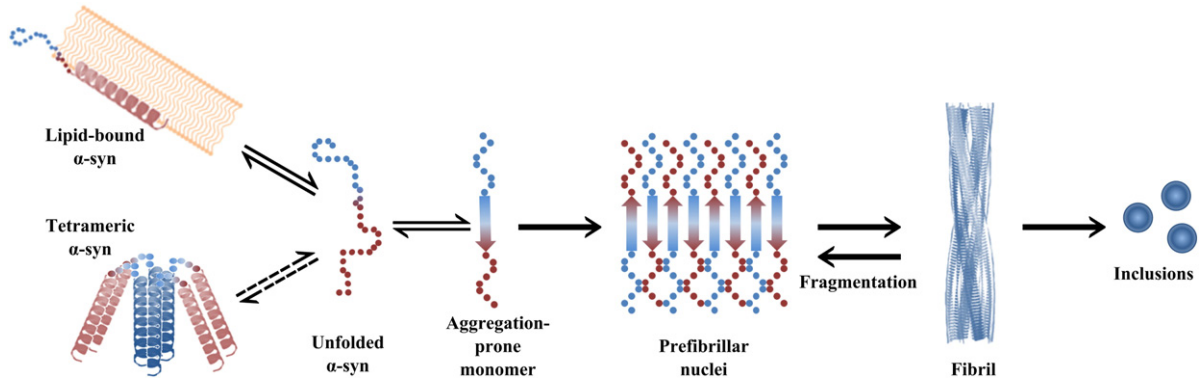

Fig. 2. Amyloid fibrillar aggregation of α-syn. Unfolded α-syn exists as a monomer that can interact with lipids, to form an α-helical structure [85]. There is some evidence that a tetrameric α-helical form of α-syn also exists in cells [84] and monomeric unfolded forms of α-syn may be in equilibrium with this tetramer. Unfolded α-syn aggregates through a nucleation-dependent mechanism, in which aggregation-prone α-syn monomers associate to form soluble prefibrillar oligomeric nuclei. This is followed by the elongation of these nuclei into mature amyloid fibrils. Fibrillar aggregates may then be sequestered into Lewy body or Lewy neurites. Alternatively, fragmentation of mature fibrils can generate additional oligomeric nuclei which seed secondary aggregation events.

However, when the N-terminus of monomeric α-syn interacts with lipids it attains an α-helical structure and recent evidence has suggested it may form a tetramer in some cell types [82–86]. Within inclusions, α-syn is assembled into highly-ordered, β-sheet rich amyloid fibrils. Similarly, under conditions of physiological pH and temperature *in vitro*, purified α-syn assembles into fibrils resembling those found in diseased brains [87–89]. Amyloid fibril formation occurs via a nucleation-dependent mechanism in which the formation of oligomeric nuclei is the rate limiting step, which is followed by rapid elongation and assembly into mature fibrils [90–92]. All three α-syn mutations associated with PD have been found to influence the early stages of aggregation, either nuclei formation or fibril growth, when their aggregation propensities have been assessed *in vitro* [93]. Each α-syn mutant exhibits distinct fibrillation kinetics and/or aggregate morphologies: A53T and E46K α-syn form fibrils more rapidly than the wild-type (WT) protein, and A30P α-syn forms mature fibrils more slowly, although smaller oligomeric species are formed more rapidly by this mutant [15,94,95]. The difference in fibrillation kinetics is generally attributed to variations in the rate of nucleation, as opposed to the rate of elongation [96,97]. As with gene multiplication, the aggregation propensity of each α-syn variant correlates well with disease onset and the severity of familial PD, with the A53T variant aggregating the fastest and being associated with the earliest and most aggressive disease phenotype [98].

In addition to the inherent aggregation propensity of α-syn, a number of factors have been shown to influence the rate at which the protein forms fibrils. For example, aggregation is promoted by the presence of metals, pesticides, lipids, membranes, and under conditions of low pH or molecular crowding [99–101]. In general, these factors increase the concentration of the prefibrillar intermediates crucial for the formation of nuclei during the rate-limiting step [57,99–101]. Conversely, aggregation is hindered by the presence of the related by non-aggregating proteins β- and γ-synuclein [102,103], as well as molecular chaperones such as sHsps [15,104]. Changes in the rate at which aggregation occurs (e.g. through the influence of the above mentioned factors), may therefore contribute to the failure of proteostasis elements to prevent α-syn aggregation in a disease context.

3.1.2. α-Synuclein pathogenicity

Although the exact physiological function of α-syn is yet to be defined, based on knowledge of its broad physiological role of modulating synaptic activity, some features of PD may be ascribed to toxicity associated with loss of function encountered when α-syn in the cell is sequestered and deposited following aggregation. The reduced availability of α-syn would impact on its ability to interact with cellular membranes [105]. Failure of α-syn to complete its physiological role within the cell could potentially cause neuronal damage, particularly in the synaptic terminal [106]. However, it is considered unlikely that this is the primary pathological effect of α-syn aggregation since α-syn knockout mice do not display any overt neuropathological or behavioural phenotypes, in contrast to mice that overexpress α-syn [107,108]. Instead, it is now generally accepted that the accumulation of α-syn into Lewy bodies and Lewy neurites leads to disease due to a toxic gain-of-function inherent in the protein when it exceeds a threshold concentration and adopts a fibrillar-type conformation [106]. The aggregation of α-syn, exacerbated by a decrease in the ability of the cell to dispose of damaged proteins, results in the accumulation of non-functional (potentially toxic) α-syn species, which may interfere with normal metabolic processes.

3.1.3. The debate surrounding the identity of the cytotoxic α-synuclein species

For some time, the toxic α-syn species responsible for neurodegeneration has been hotly debated. Initially, cytoplasmic inclusions were thought to be a characteristic feature of dead or dying neurons, with the deposition of mature amyloid fibrils into Lewy bodies identified as the neurotoxic event. This was primarily based on findings suggesting that inclusions may suppress organelle function, interfere with axonal transport, or induce energy failure via hyperubiquitination [109,110]. However, Lewy bodies are commonly found in living neurons, and are also present in up to 15% of healthy, aged individuals [111,112].

In cell-based models and *in vivo*, neurotoxicity correlates best with the appearance of soluble α-syn oligomers as opposed to inclusions [113]. Toxicity is usually observed in the absence of mature α-syn fibrils or detectable deposition into inclusions in cell models [113,114]. Moreover, surviving dopaminergic neurons demonstrate equivalent viability irrespective of the presence or absence of Lewy bodies [115]. Furthermore, transgenic mice exhibit neurodegeneration outside the *substantia nigra* in the absence of fibrillary inclusions, and α-syn fibril-containing inclusions in *Drosophila* are observed in the absence of neurodegeneration [116–118].

A recent study utilised a rat lentivirus system to examine the cellular toxicity of E35K and E37K α-syn, which were specifically designed to form small oligomers, compared to an α-syn variant encompassing residues 30–110, which forms fibrils [119]. Following injection into the *substantia nigra*, cell loss was assessed based on a reduction in tyrosine hydroxylase-positive neurons in this region. Higher toxicity was observed in dopaminergic neurons of animals exposed to oligomer-forming variants of α-syn compared to those which formed fibrils [119]. Finally, dopamine and its metabolites inhibit the conversion of protofibrils to mature amyloid both *in vitro* and *in vivo* [120–122], providing a potential rationalisation for the vulnerability of dopaminergic neurons since they would therefore promote protofibril formation.

Alternatively, dopamine may act as a source of ROS in a process potentiated by α-syn, thereby promoting apoptosis [114].

In light of this evidence, the predominant consensus is now that soluble prefibrillar oligomeric species of α-syn are the most toxic entity, and fibrils are the less toxic end-product of the aggregation process [123,124]. Yet, mature fibrils can be a source of cytotoxic oligomeric species due to their fragmentation [125]. Thus, it has been proposed that the formation of protein inclusions may represent an additional protective mechanism employed by the cell [61,123,126]. The formation of inclusion bodies within cells may therefore represent a protection strategy used by the cell to sequester toxic oligomeric forms that may arise due to fibril fragmentation. In this way, potentially toxic species can be isolated in specific compartments in the cell to protect it from their harmful effects [127].

The generic toxicity of prefibrillar (i.e. small soluble oligomeric) species is also evident in non-disease-associated proteins capable of forming amyloid. For example, using two unrelated and non-disease-associated protein domains, the SH3 domain from bovine phosphatidylinositol-3'-kinase and the amino terminal of HypF from *E. coli*, Bucciantini and colleagues [128] demonstrated that oligomeric forms of these proteins generated during the early stages of aggregation were inherently cytotoxic when added to cells in culture. As well, early prefibrillar aggregates of apomyoglobin are toxic to cultured fibroblasts via their ability to alter membrane permeability [129]. Finally, soluble amyloidogenic oligomers of equine lysozyme are toxic to both primary and cultured neuronal cells; cytotoxicity is correlated with the size of the oligomers within the sample with larger species being less cytotoxic [130]. Thus, it is concluded that prefibrillar aggregates are the most toxic entity formed during amyloid fibril formation.

The toxicity of aggregates formed from a variety of pathogenic and non-pathogenic proteins correlates with the level of exposed hydrophobicity at the aggregate surface [131]. Initially, there is an abundance of soluble oligomeric aggregates with a high surface-to-volume ratio and a high degree of exposed hydrophobicity [132]. As the aggregate increases in size over time, there is a decrease in the surface-to-volume ratio and amount of exposed hydrophobicity [131–133]. The higher proportion of hydrophobic residues exposed on the smaller oligomeric species enables them to participate in inappropriate interactions that can ultimately lead to cell death [49]. It is important to note, however, that although this mechanism appears generally applicable to pathogenic and non-pathogenic proteins alike, ultimately the amino acid sequence defines the kinetics by which a protein aggregates and the specific properties of any aggregate formed. Thus, the specific toxicity of a protein is influenced by its relative ability to form oligomers, the rate these oligomers are then converted to mature fibrils and the amount of hydrophobicity exposed throughout this process [131].

The relative hydrophobicity of prefibrillar aggregates appears to endow them with a large potential to cause cellular damage. Many mechanisms have been proposed for α-syn toxicity (summarised in Fig. 3). When considering the mechanism of toxicity of these species, it is important to also take into account their physical properties. For example, α-syn is capable of lipid-binding [134,135] and the oligomers formed from α-syn can be small, flexible spheroids, characteristics which favour their association with membranes and pore formation [136]. Pore formation may occur on any of the cellular membranes, both inter- and intra-cellular, providing several targets for α-syn mediated toxicity [137]. For instance, pore formation within the plasma membrane may allow the abnormal flow of ions causing cellular dysfunction and leading to apoptosis [134,138]. This is indirectly supported by work showing that cells expressing α-syn have increased cation permeability [139]. Through interaction with lysosomal membranes, α-syn aggregates may inhibit chaperone-mediated autophagy, leading to an accumulation of substrates and proteasome inhibition [140]. Alternatively, prefibrillar oligomers can impair axonal transport via hyperphosphorylation of Tau, a protein normally responsible for stabilising and regulating the microtubule assembly and interacting with membranous cargo [141]. By disrupting transport from the endoplasmic reticulum (ER) to Golgi, α-syn can cause ER stress and Golgi fragmentation [113,140]. Energy production can also be impaired due to effects of oligomeric α-syn on mitochondria [142]. In addition, α-syn is implicated in reducing dopamine release and its subsequent reuptake in the synaptic terminal [143]. The active secretion or passive release (following death) of aggregated forms of α-syn can also result in cell-to-cell transfer of toxic intermediates that alter synaptic protein expression and excitability [146]. Moreover, these extracellular species of α-syn can activate surrounding astrocytes and glia, resulting in the production of ROS and proinflammatory cytokines, which in turn can be toxic to surrounding neurons [145]. Finally, although inclusion bodies may sequester potentially harmful aggregation intermediates, this process may also result in the depletion of proteins that become associated with the inclusions from the cytoplasm, leading to a loss in their biological activity [142]. Together, these factors may lead to compromised cell viability (and cell death). However, any one of these events (or combination thereof) may be sufficient given the delicate balance required to maintain proteostasis.

3.2. Lewy bodies and Lewy neurites: α-synuclein is not alone

Whilst α-syn is the main constituent of Lewy bodies and Lewy neurites, it is not the only protein found in these insoluble inclusions: a range of other proteins have been identified including synuclein-binding proteins, protein kinases, proteins implicated in the ubiquitin-proteasome system and proteins associated with the cellular stress response (e.g. molecular chaperones) [56]. Immunostaining of post-mortem brain tissue from PD patients indicates that Hsp90, Hsp70, Hsp40 and the sHsps are associated with Lewy bodies and Lewy neurites [118,147,148]. With regard to the sHsps, diffuse Hsp27 was identified throughout Lewy bodies and Lewy neurites in the *substantia nigra* [147,149] and αB-c also colocalises with α-syn in Lewy bodies and Lewy neurites [149–152]. In addition, post-translationally modified forms of αB-c are a major component of oligodendral cytoplasmic inclusions isolated from clinically confirmed cases of multiple system atrophy [59]. It remains to be established whether these chaperones associate with these protein inclusions before or after they form in the cell. In any case, it appears that although these chaperone proteins are available to inhibit α-syn aggregation in cells, under certain circumstances they are unable to prevent its deposition and instead become part of the inclusions that are the hallmarks of the α-synucleinopathies.

4. Small heat-shock proteins as molecular chaperones

There are ten human sHsps (HSPB1–HSPB10) and of these the most well characterised are Hsp27, αA-crystallin (HSPB4, αA-c), αB-c and Hsp20 (HSPB6) [153,154]. Whilst sHsps are often described as 'holdase' chaperones, this term does not fully describe their chaperone activity [155] and not all sHsps have been shown to be capable of suppressing target protein aggregation (e.g. HSPB9 and HSPB10). Structural aspects of sHsps have been considered in detail elsewhere [156–159] and therefore the salient features are only summarised here. The sHsps are defined by their relatively small (compared to other Hsps) monomeric masses (12–43 kDa), and the presence of a conserved central region referred to as the α-crystallin domain. The α-crystallin domain is ~90 residues in length and contains up to nine anti-parallel β-strands organised into β-sheets in an immunoglobulin-like fold [160,161]. It is flanked by N- and C-terminal regions of variable length and sequence that predominantly lack structure [162]. In mammalian sHsps, the extreme C-terminus is a short, mobile and flexible extension which is typically polar in nature and contributes to stabilisation of the protein (and complexes it forms with target proteins) during chaperone action [163,164]. The N-terminal region contains regions of significant hydrophobicity and has been suggested to mediate the interaction between the chaperone and

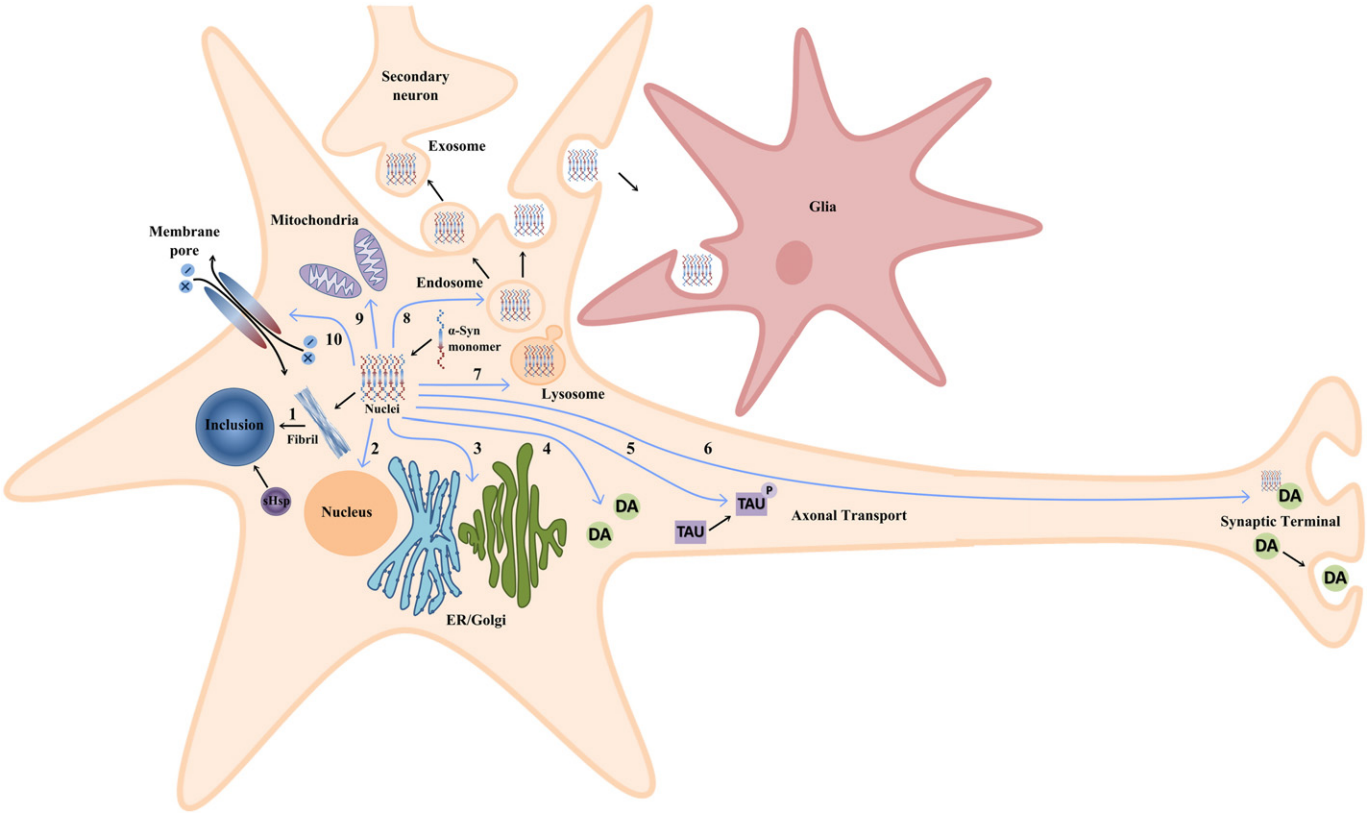

Fig. 3. A schematic model for the potential mechanisms by which α-syn aggregation is toxic to neuronal cells. Aggregation-prone monomeric α-syn associates to form soluble oligomeric nuclei leading to the formation of mature fibrils. **(1) Inclusion body formation:** Fibrillar α-syn is sequestered into protein inclusions, which also contain various other cellular proteins, including sHsps, potentially depleting the cell of these essential components **(2) Initiation of the heat-shock response:** An accumulation of toxic α-syn species within the cell activates the heat-shock response pathway, initiating changes in transcription of stress response genes. **(3) Blocking protein trafficking from the ER to Golgi:** Aggregation of toxic forms of α-syn in the cell can block protein transport from the ER to Golgi, inducing Golgi fragmentation. **(4) Oxidative stress:** Aggregation of α-syn induces dopamine (DA)-dependent ROS production, resulting in oxidative stress. **(5) Defects in axonal transport:** Aggregated α-syn induces hyperphosphorylation of tau in the axon, which causes defects in axonal transport through restricting the ability of tau to modulate microtubule assembly. This impairs essential cellular transport as well as resulting in the accumulation of aggregated material in the cell body. **(6) Altered synaptic terminal excitability and protein expression:** The presence of aggregated α-syn in the synaptic compartment alters the distribution of synaptic terminal proteins, diminishing synaptic vesicle release and leading to changes in synaptic terminal protein expression and excitability. **(7) Impaired autophagy:** Binding of α-syn to lysosomal membranes impairs chaperone-mediated autophagic function, resulting in substrate accumulation and proteasomal impairment in the cell body. **(8) Release of toxic α-syn species into extracellular space:** Aggregated α-syn may be actively secreted (e.g. via exosomes) or passively released by dying neurons and be subsequently taken up by neighbouring neurons, resulting in seeded aggregation and altered synaptic terminal activity. Uptake by surrounding glia can induce proinflammatory activity including ROS production, which is toxic to surrounding neurons. **(9) Impaired energy production and increased ROS production:** Localisation of α-syn to mitochondrial membranes may impair energy production or increase ROS formation. **(10) Membrane pore formation:** Ring-like oligomeric α-syn species may infiltrate cellular membranes forming pores within the membrane and altering ion permeability. Figure compiled using information from [113,114,134,138,140–145].

its target protein [165–168], although recent work demonstrates that the N-terminal region is not essential for chaperone function [169–171]. The formation of oligomeric assemblies is another defining feature of the sHsps. Whilst in some species sHsps form well-defined homogenous multimers (e.g. wheat Hsp16.9), many mammalian sHsps members (e.g. Hsp27, αB-c and αA-c) form large polydisperse oligomers which undergo rapid subunit exchange [169,172–178]. The popular model of sHsp chaperone action is that dissociated species (predominantly depicted as dimers) are the most chaperone-active form; the rationale being that these species have a higher degree of exposed hydrophobicity compared to the larger oligomeric forms, thereby facilitating their interaction with partially folded target proteins that expose significant hydrophobicity to solution [173,179]. In this model, the rate of subunit exchange of sHsps, which is highly dependent on solution conditions (e.g. temperature) but independent of sHsp concentration [178,180], dictates how fast chaperone active subunits can be liberated from large oligomers in order to interact with and prevent the aggregation of target proteins. Our previous work has shown that αB-c is most effective (on a mole: mole basis) at preventing the aggregation of slowly aggregating target proteins compared to those aggregating more quickly [10,181], presumably because of the requirement for active subunits to dissociate from larger oligomers as part of the chaperone action of this sHsp. Thus, when protein aggregation occurs very fast it may exceed the rate at which active sHsp subunits can dissociate, therefore leading to a decrease in the chaperone efficacy of the sHsp.

4.1. Expression of sHsps in the brain

Of the ten identified human sHsps, some are ubiquitously expressed (e.g. Hsp27 and αB-c), whilst others are found only within specific tissues [182,183]. For example, αA-c is only present at appreciable levels within the eye lens, where, together with αB-c, it forms α-crystallin, the hetero-oligomeric lens protein which is responsible for maintaining lens transparency via its chaperone action and ordered arrangement [184,185]. Given the important role sHsps have in proteostasis, it is surprising that there has not been a systematic study of sHsp expression and localisation in the human brain. In relation to neurodegenerative conditions such as the α-synucleinopathies, five sHsps are expressed within the central nervous system. Myotonic dystrophy protein kinase binding protein (HSPB2) is expressed in smooth muscle of vessel walls of the brain [186], and compartment-specific expression of Hsp20, Hsp22, Hsp25 (the murine ortholog of human Hsp27) and αB-c has been demonstrated within other brain tissues of the mouse [187]. Hsp27 is expressed in motor and

sensory neurons in the brainstem and cranial nerve nuclei [188], and is also constitutively expressed, along with αB-c, in glial cells [186].

Although there is some evidence for the neuroprotective capabilities of Hsp20 and Hsp22 [182], Hsp27 and αB-c have attracted the most attention in relation to neurodegenerative disease. Expression of both αB-c and Hsp27 is highly induced in response to neurological stress [151,189]. Hsp27 and αB-c are expressed in reactive astrocytes adjacent to senile plaques in both normal aged brains and in neurodegenerative conditions, such as Alzheimer's disease [186], and their expression is increased in reactive astrocytes in the hippocampus of PD patients with dementia [190]. Moreover, Hsp27 is one of the most strongly induced proteins across several brain regions in PD patients [191] and its levels are 2.5-fold higher in pathologically confirmed cases of Dementia with Lewy bodies than age-matched controls. Chen and Brown [23] compared constitutive and inducible Hsp27 expression in several neuronal subtypes associated with neurodegenerative diseases, including PD and amyotrophic lateral sclerosis (ALS). Constitutive Hsp27 expression was found within motor neurons of the spinal cord (the degeneration of which is associated with ALS), but Hsp27 was not detected within dopaminergic neurons of the *substantia nigra* (the degeneration of which is associated with PD). Chen and Brown speculated that one reason ALS is approximately 33 times less frequent than PD is that motor neurons in the spinal cord are better equipped to manage misfolded proteins than dopaminergic neurons due to the protection provided by the levels of Hsp27 [23]. Thus, the low basal expression of Hsp27 in dopaminergic neurons [23,154] may facilitate the onset of α-syn aggregation in these cells. The increased expression of sHsps in the context of the α-synucleinopathies may be a consequence of the cellular stress conditions that accompanies the onset and progression of these diseases.

4.2. The effects of small heat-shock proteins on α-synuclein aggregation

The presence of sHsps within Lewy bodies and their up-regulation in the surrounding neuronal tissues associated with a number of α-synucleinopathies have led to an examination of how sHsps interact with, and influence the aggregation of, α-syn. These findings, based on studies using *in vitro* α-syn aggregation assays, and cell- and animal-based models of α-syn aggregation are summarised in Table 1.

4.2.1. In vitro α-synuclein aggregation assays

sHsps interact with multiple species formed along the α-syn off-folding aggregation pathway (Fig. 4). Various sHsps, including Hsp27 and αB-c, bind monomeric α-syn *in vitro*, with a dissociation rate constant (k_{off}) in the range of 10^{-3} s^{-1}, as determined by surface plasmon resonance measurements, suggesting a weak, transient interaction that nonetheless inhibits its aggregation [15]. The ability of dyes such as thioflavin T (ThT) to bind to β-sheet structures (such as those found in α-syn fibrils) has been used to monitor the aggregation kinetics of α-syn and provide a quantitative measure of fibril formation. Bruinsma et al. [15] assessed a panel of sHsps, including Hsp27, Hsp20, Hsp22, αB-c and hetero-oligomers of HSPB2 and HSPB3, for their ability to inhibit α-syn aggregation using both *in vitro* ThT assays and atomic force microscopy. They reported that Hsp27 is the most efficacious of these sHsps with regards to preventing fibril formation of WT α-syn and it does so by inhibiting both the lag and elongation phases of aggregation [15]. This leads to an overall reduction in the number and size of fibrils. Comparable effects were observed when Hsp27 was incubated with E46K and A30P α-syn, however, the presence of Hsp27 increased the aggregation of A53T α-syn compared to when no chaperone was present [15]. αB-crystallin also inhibits the aggregation of α-syn (and its disease-related mutant forms) *in vitro* and does so at sub-stoichiometric levels [11]. In addition, the *in vitro* fibrillar aggregation of A53T α-syn isolated from brain tissue extracts of transgenic mice is significantly reduced by the presence of αB-c [193]. As with Hsp27, the addition of αB-c not only increases the lag phase of α-syn aggregation, slowing the formation of prefibrillar intermediates, but also inhibits the elongation phase, indicating that it acts to stabilise monomeric and prefibrillar α-syn species [11,14]. Notably, the efficiency with which αB-c inhibits α-syn fibril formation correlates with the aggregation-propensity of the α-syn isoform [11,15]. Thus, at a given molar ratio, αB-c is more effective at inhibiting the aggregation of WT α-syn (which aggregates the slowest) and is less effective against A53T α-syn (which aggregates the fastest).

Most studies that have tested the *in vitro* chaperone action of the sHsps have involved addition of the chaperone to α-syn before aggregation has commenced, and therefore focussed on the interaction of the chaperone with species formed in the early stages of aggregation [11, 15,196]. However, in addition to interacting with monomeric and

Table 1
A summary of studies that have investigated interactions between sHsps and α-syn. Key findings regarding this interaction are categorised according to the method used.

Method used	α-Syn Isoform	sHsp(s)	Key Findings	Reference
***In vitro* aggregation assays and atomic force microscopy**	WT A53T A30P E46K	Hsp27 αB-c Hsp20 Hsp22 HspB2B3	• sHsps bind α-syn variants in a weak, transient but specific manner. • sHsps reduce the amount of fibrillar aggregation resulting in fibrils that are shorter, and have a clustered morphology.	[15]
***In vitro* aggregation assays and transmission electron microscopy (TEM)**	WT A53T A30P	αB-c	• αB-c inhibits α-syn fibril formation at substoichiometric ratios. • The number of fibrils is significantly reduced and amorphous-like aggregates are produced.	[11]
***In vitro* aggregation assays**	WT	αB-c	• The ability of αB-c to suppress α-syn aggregation increases with temperature. • αB-c inhibits further aggregation when introduced during the elongation phase of fibril growth.	[14]
***In vitro* assay, TEM**	A53T	αB-c	• The interaction of αB-c with α-syn monomers is weak and transient. αB-c binds along the face and ends of mature α-syn fibrils. • Fibril-bound αB-c inhibits further elongation.	[192]
***In vitro* aggregation assays**	A53T	αB-c	• αB-c significantly reduces the *in vitro* aggregation of α-syn extracted and purified from brain tissue of transgenic mice.	[193]
Cell-based model	WT A53T A30P	Hsp27	• Hsp27 expression protects stably transfected ND7 α-syn-expressing cells from cell death stimuli including serum withdrawal.	[194]
Cell-based model	WT A53T	Hsp27 αB-c	• Both Hsp27 and αB-c co-localise with α-syn inclusions in co-transfected H4 neuroglioma cells. • Hsp27 reduces inclusion formation, although both αB-c and Hsp27 reduce α-syn toxicity. • Hsp27 protects primary dopaminergic neurons from α-syn toxicity.	[149]
Cell-based model	WT	Hsp27	• Hsp27 does not colocalise with α-syn in inclusions.	[147]
***In vivo* murine**	A53T	Hsp25* αB-c	• Hsp25 levels are significantly up-regulated in both the soluble and insoluble fractions of spinal cord tissue of A53T α-syn. over-expressing transgenic mice. • αB-c levels are increased in the insoluble fraction of spinal cord tissue from these transgenic mice.	[193]
In vivo Drosophila	WT	αB-c	• αB-c reduces the 'rough eye' phenotype induced by α-synuclein expression and aggregation in *Drosophila*.	[195]

* Hsp25 is the murine ortholog of human Hsp27.

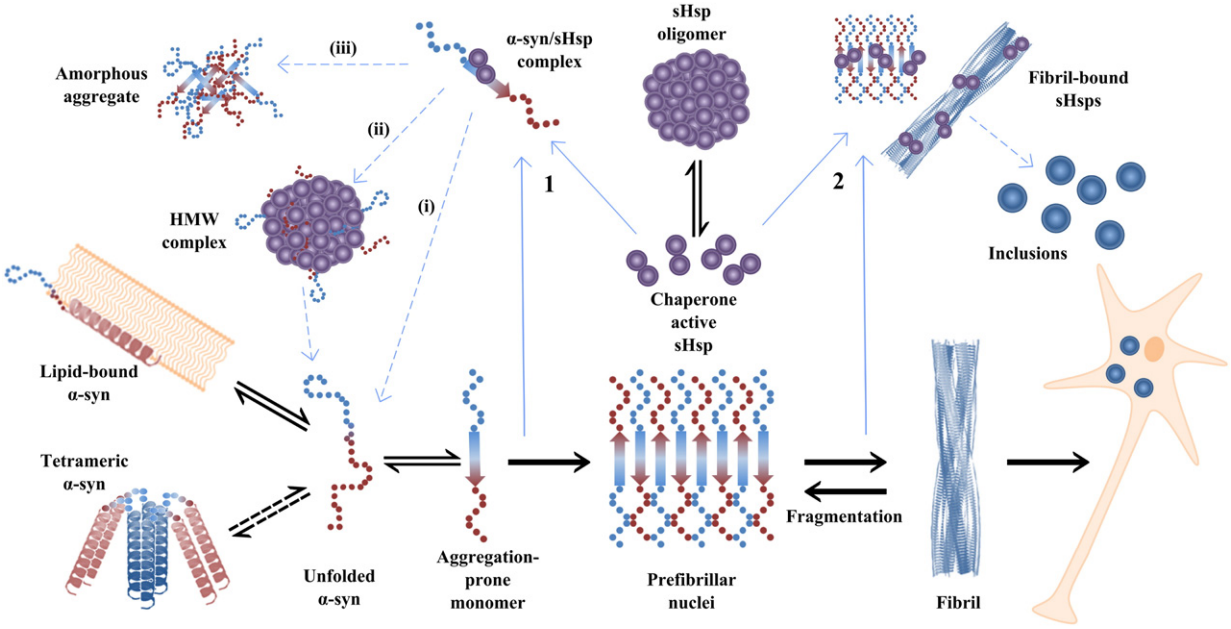

Fig. 4. sHsps interact with various species formed during the fibrillar aggregation of α-syn. Unfolded α-syn aggregates through a nucleation-dependent mechanism, and the resultant fibrillar deposits may then be sequestered into inclusion bodies. Fragmentation of mature fibrils can generate additional oligomeric nuclei, further perpetuating aggregation. **(1) The interaction of sHsps with monomeric aggregation-prone α-syn.** The mechanism of this interaction remains to be fully elucidated but may involve one or more of (i) weak transient interactions with α-syn which prevent it from associating into oligomeric nuclei, (ii) the formation of a stable complex between α-syn and the sHsps which, when cellular conditions permit, enable monomeric α-syn to be released, or (iii) α-syn being induced to form amorphous aggregates rather than fibrils. **(2) The interaction of sHsps with prefibrillar intermediates and mature α-syn fibrils.** sHsps can bind to oligomeric and fibrillar forms of α-syn with moderate (μM) affinity. In doing so, they inhibit further fibril growth and may lead to tangling of the fibrils into larger 'inclusion-like' deposits. Solid lines represent pathways that are well supported by the current literature, dotted lines represent proposed pathways in which the details are yet to be fully resolved.

pre-fibrillar α-syn species, recent work has shown that sHsps also bind to species formed further along the aggregation pathway including mature fibrils [14,192]. Thus, when introduced during the elongation phase of α-syn aggregation, αB-c prevents further fibril growth by binding along the length of mature fibrils [14,192]. Recent work, using apolipoprotein C-II (apoC-II) as a model fibril-forming protein has shown that, by binding to fibrils, αB-c stabilises them and prevents their dilution-induced fragmentation [12]. Moreover, binding of αB-c to apoC-II fibrils also causes them to associate (tangle) into larger species reminiscent of protein inclusions [12]. Thus, it appears that by binding to fibrils, sHsps inhibit their fragmentation and prevent secondary nucleation events, both of which contribute to fibril toxicity [197]. Moreover, whilst the presence of sHsps within inclusions had been considered a by-product of their failed attempt to mitigate aggregation [196], their ability to stabilise mature α-syn fibrils provides another rationale for their localisation in these deposits. Finally, recently αB-c was shown to also promote the dissociation of potentially toxic β_2-microglobulin oligomers into monomers, highlighting another role these chaperones may have in cells to protect them from α-syn oligomer-induced toxicity [198].

In summary, the interaction of sHsps with aggregation-prone α-syn is multi-faceted; it involves binding to monomeric, oligomeric, prefibrillar and fibrillar forms of the protein in order to prevent the toxicity associated with the aggregation process. However, as the chaperone action of sHsps is dependent on the supply of dissociated (chaperone-active) species from larger oligomers, the dynamic relationship between the rate at which α-syn aggregates and the rate of sHsp subunit exchange therefore appears to be an important factor with regards to whether or not sHsps can prevent α-syn deposition in the context of the α-synucleinopathies.

4.2.2. Cell-based models of α-synuclein aggregation

Whilst it is clear that sHsps can interact with α-syn to inhibit its aggregation *in vitro*, it remains to be conclusively established whether this occurs in the cellular environment. Most cell-based models of α-syn aggregation and associated toxicity are based on the exogenous application of aggregated α-syn to cells in culture. The susceptibility of dopaminergic neurons to exogenous α-syn fibrils was illustrated using preaggregated fragments of α-syn derived from a fibrillogenic region of the protein encompassing residues 61-95 [199]. Administration of these fibrillar α-syn peptides to rat primary mesenchephalic neurons results in a reduction of dopaminergic neurons and dopamine content [199]. Whilst exogenous application of α-syn fibrils may have relevance to cell-to-cell propagation of aggregated α-syn [200–203], the intracellular aggregation of α-syn into Lewy body-like deposits most likely recapitulates the earliest events in disease and therefore is considered the most relevant with regard to α-syn's interaction with sHsps.

Overexpression of α-syn (via transfection or viral induction) has been examined in both primary and immortalised cell lines. Overexpression of both WT and A53T α-syn in primary mesenchephalic neuronal cultures not only results in significant cell death, which is specific to dopaminergic neurons and does not impact the viability of the other cells in the culture, but also renders surviving dopaminergic neurons more susceptible to neurotoxic insults [204,205]. Overexpression of WT, A53T and A30P α-syn in human neuroblastoma cells (SH-SY5Y) is not sufficient to cause extensive inclusion formation. However, co-treatment with various agents that induce generation of ROS (e.g. rotenone, papaNONOate and $FeCl_2$) results in the formation of cytoplasmic protein inclusions in cells [206,207]. The inclusions contain both α-syn and ubiquitin and are therefore typical of Lewy bodies isolated from diseased tissue [206,207]. Thus, it appears that the presence of aggregation prone forms of α-syn *per se* is not sufficient to disrupt cellular proteostasis, however, a second insult that impacts on the proteostasis network can induce the formation of these protein inclusions.

In studies in which α-syn is over-expressed, the extent of α-syn aggregation is dependent on both the level and isoform of α-syn expressed: A53T α-syn had an increased tendency to form inclusions, consistent with its increased propensity to form fibrils *in vitro* and its genetic association with early onset PD [206]. McLean et al. [208] expressed an α-syn isoform which was C-terminally tagged with enhanced green

fluorescent protein (EGFP) in H4 neuroglioma cells and demonstrated that overexpression of this protein results in cytoplasmic inclusions in ~5% of transfected cells. Interestingly, cotransfection with synphilin-1, a protein which binds α-syn *in vivo*, increased the percentage of cells containing inclusions to ~55% [208,209]. Notably, although the α-syn was expressed with an EGFP fluorescent tag, inclusions were not fluorescent or reactive to anti-EGFP antibodies [208]. This was due to C-terminal truncation of the EGFP attached to α-syn in these cells, generating a non-fluorescent form of the protein which aggregated to form inclusions. Thus, these findings highlight the potential problems with tagging α-syn (and indeed other proteins) to monitor its aggregation in cells. Alternative methods for studying full-length α-syn aggregation in cells have also been developed. For example, using a tetra-cysteine tagged α-syn, which was either microinjected or transfected into cells and detected by labelling with a fluorogenic biarsenical compound, Bertoncinni et al. [210] demonstrated that the aggregation of α-syn into inclusions is significantly increased (by up to 10%) by co-treating the cells with $FeCl_2$ to induce oxidative stress. More recently, α-syn aggregation in cells was modelled by the addition of a 16 amino acid peptide (termed CL1) to the C-terminus of α-syn, and this was used to demonstrate a correlation between increasing numbers of α-syn aggregates in cells and a reduction in cell viability [211].

Much of what is known about the interaction between sHsps and α-syn in cells comes from cell-based models of α-syn like those described above. However, in general these cell models have only been used qualitatively to assess the effects of sHsps on α-syn aggregation. One issue with these cell culture models of α-syn aggregation (which has hampered their use in quantitative studies) is that often only a small proportion of the cells in culture develop inclusions when transfected [208]. This severely limits the types of biochemical analyses that can be undertaken with these models. Despite this, there is some evidence for sHsps inhibiting α-syn aggregation in cells. For example, using an α-syn overexpression system coupled with viral-mediated Hsp expression in ND7 cells, Zourlidou et al. [194] reported a reduction in α-syn-induced cellular toxicity following overexpression of Hsp27. Although this study focused on the inherent susceptibility of α-syn-expressing cells to external cell death stimuli, such as serum removal, without directly considering α-syn aggregation, it was concluded that Hsp27 is neuroprotective. Outeiro et al. [149] used overexpression of the truncated EGFP-tagged WT α-syn in H4 cells (a model discussed above) to examine the effect of sHsps on α-syn aggregate formation and reported that cotransfection with Hsp27 significantly reduced the percentage of cells containing α-syn inclusions. Both Hsp27 and αB-c reduced the inherent toxicity of α-syn expression to levels similar to those seen when Hsp70 is expressed in these cells [149]. The same effect was reproduced when A53T α-syn was overexpressed in primary midbrain cultures [149,212]. Furthermore, a reduction in the endogenous levels of Hsp27 by siRNA resulted in a concentration-dependent increase in α-syn toxicity in the transfected H4 model [149].

Apart from their ability to inhibit aggregation, sHsps may protect cells from the toxicity associated with α-syn aggregation in other ways. Both Hsp27 and αB-c increase the resistance of cells to oxidative stress [213–215] and can inhibit apoptosis via a range of interactions with partner proteins involved in cell death pathways [216]. For instance, αB-c inhibits autocatalytic maturation of caspase-3 [217], and Hsp27 binds to caspase-3 and cytochrome-c released from mitochondria causing inactivation of the caspase cascade [218,219]. Caspase-independent mechanisms have also been described, in which Hsp27 inhibits the ability of Daxx (death domain-associated protein 6) to interact with Ask1 (apoptosis signal-regulated kinase 1) and Fas (a cell death regulator) [220], whilst αB-c interacts with Bax and Bcl-2 to inhibit their translocation to the mitochondria [221]. These interactions have all been shown to provide sHsp-mediated protection from apoptosis in cells. Thus, sHsps may protect cells from the cytotoxic effects of α-syn aggregation via multiple mechanisms, not just by affecting its aggregation state. Together, these cell culture studies have provided promising glimpses of the role (and therapeutic potential) of sHsps in preventing toxicity associated with α-syn aggregation in cells. However, more robust and reliable cell culture models of α-syn aggregation are required so that the precise impact of sHsps on α-syn aggregation and its associated toxicity can be unequivocally determined.

4.2.3. Animal-based models of α-synuclein aggregation

Surprisingly little work has been conducted into the impact of sHsps on α-syn aggregation and its associated toxicity using animal models. Various transgenic mouse models have been developed to study the α-synucleinopathies (extensive reviews of this work are found in [222–224]). An example of one such model is that developed by Lee and colleagues [225], in which the transgene is introduced via embryonic pronuclear injection resulting in overexpression of human α-syn in glia and the neurons of the *substantia nigra pars compacta*. This model is characterised by pathological accumulation of α-syn in neuronal cell bodies and neurites in regions of the brain and spinal cord, in parallel with ubiquitin deposition, reminiscent of the proteinaceous inclusions characteristic of the α-synucleinopathies [225]. Using this model, immunohistochemical analysis of tissues from symptomatic transgenic A53T α-syn mice demonstrated that the expression of αB-c and Hsp25 increases in reactive astroglia and oligodendrocytes within affected regions of the central nervous system [193].

Transgenic α-syn models have also been developed in rats, *Drosophila melanogaster* and *Caenorhabditis elegans*, as well as alternative models in which preaggregated α-syn protein or fragments are injected directly into the *substantia nigra* [195,199,226–231]. These models have been used as experimental tools to study the toxicity associated with α-synuclein aggregation. However, the impact sHsps have on α-syn aggregation in these models is yet to be explored. Tue et al. [195] did demonstrate a reduction in the rough-eye phenotype of α-syn transgenic *Drosophila* in the presence of αB-c, however the inhibition of α-syn aggregation was not directly examined. Disease-relevant models such as these provide valuable tools to further examine the role (and therapeutic potential) of sHsps in preventing α-syn aggregation *in vivo* and also may shed further light on the manner by which the cellular defences against protein aggregation are overwhelmed in the α-synucleinopathies.

5. Conclusions

The aggregation of α-syn into protein inclusions (Lewy bodies and Lewy neuritis) underlies the onset and progression of the α-synucleinopathies and represents a failure of the proteostasis network in maintaining the protein in a biologically active, non-toxic form. Key components of the proteostasis network are molecular chaperones and of these the sHsps are the cell's first line of defence against protein aggregation. We need to better understand why sHsps fail to prevent α-syn aggregation in the context of the α-synucleinopathies. One of the main factors appears to be that the relatively low basal levels of αB-c and Hsp27 in dopaminergic neurons of the *substantia nigra* makes them more susceptible to protein aggregation. If this is the case, then boosting their expression may be a target for therapeutic intervention.

Whilst it has been generally considered that the role of sHsps in the chaperone network is to stabilise partially folded intermediate states of proteins to prevent their aggregation, recent work has demonstrated that their mechanism of action is much more multi-faceted. In relation to α-syn aggregation, sHsps interact with multiple species formed during the aggregation process, from monomeric partially folded intermediate states through to the mature fibrils themselves. The biological relevance of these interactions must now be further examined using more sophisticated disease-relevant cell and animal models of the α-synucleinopathies.

In addition, whilst many studies have primarily considered the ability of the sHsps to inhibit or prevent α-syn aggregation *in vitro*, the limited number of cellular and *in vivo* studies performed to date indicates

that they may not significantly alter the number of inclusions formed in cells. Instead, sHsps may afford protection against the toxicity associated with α-syn aggregation via their impacts on other cellular pathways, such as by inhibiting ROS formation or apoptosis. The common theme in all this work is that sHsps act, through their chaperone activity, to stabilise proteins and prevent their improper interactions in cells. In doing so, they maintain cell viability and therefore are key components of the proteostasis network. In the α-synucleinopathies, targeting their chaperone activity may open up new therapeutic avenues to treat these currently intractable and debilitating diseases.

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