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Licenciada

Modulation of α-synuclein aggregation and toxicity

Dissertação para obtenção do Grau de Mestre em Genética Molecular e Biomedicina

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Resumo

A α-sinucleína (aSin) é uma proteína amilodoigénica propensa à agregação. Esta proteína é encontrada em inclusões específicas denominadas corpos de Lewys em neurónios sobreviventes de pacientes com doença de Parkinson e outras sinucleinopatias. O processo de agregação é amplamente afectado por diferentes modificações pós-traducionais, como a fosforilação, acetilação e glicação. Recentemente foi demonstrado que as espécies oligoméricas de aSin são mais tóxicas do que os corpos de inclusão. As “heat shock proteins” (HSPs) são chaperonas moleculares com a capacidade de modular o “folding” e o “refolding” de proteínas. A sua sobre-expressão em modelos de doença de Parkinson reduz e previne a agregação de aSin. Como a redução da agregação da aSin pode levar a uma possível acumulação de espécies oligoméricas que podem causar danos celulares, o principal objectivo deste trabalho é melhor entender o papel das HSPs na formação de oligómeros de aSin, clarificando que espécies de aSin são formadas na presença de HSPs. Adicionalmente, como a glicação potencialmente acelera a deposição anormal de proteínas, investigou-se a forma como as HSPs interferem no processo de oligomerização de aSin glicada.

Neste estudo a Hsp70 parece induzir a agregação de aSin recombinante, gerando espécies com um elevado peso molecular mas sem toxicidade associada. A Hsp27 reduziu a oligomerização de aSin *in vitro*, possivelmente pela indução da formação de pequenos oligómeros não reactivos. A glicação por metilglioxal aumentou a agregação da proteína e a morte celular. Contudo, a sobre-expressão de Hsp27 reverteu a agregação de aSin glicada e diminuiu a sua toxicidade.

Estes resultados demonstram a importância da modulação das HSPs como alvo de possíveis terapêuticas da doença de Parkinson.

Palavras-chave: α-sinucleína, Hsp27, Hsp70, metilglioxal, glicação.

Abstract

It is widely known that α-synuclein (aSyn) is an amyloidogenic protein prone to aggregation. This protein is found in specific inclusions named Lewy bodies in the surviving neurons of Parkinsons's disease patients and other synucleinopathy brains. This aggregation process is greatly affected by different post-translational modifications, such as phosphorylation, acetylation, and glycation. Lately it was shown that aSyn oligomeric species are more toxic than the inclusion bodies. Heat shock proteins (HSPs) are molecular chaperones able to modulate the folding and refolding of proteins. Its overexpression in Parkinson's disease models reduces and prevents aSyn aggregation. As the reduction of aSyn aggregation can lead to an eventual accumulation of oligomeric species which may cause cell damage, the main goal of this work is to better understand the role of HSPs in aSyn oligomer formation, clarifying which are the aSyn resulting species formed in the presence of HSPs. Moreover, as glycation is suggested to accelerate abnormal protein deposition, we aimed to investigate how HSPs interfere with the oligomerization process of glycated aSyn.

In this study Hsp70 seemed to induce recombinant aSyn oligomerization, generating higher molecular weight species with no associated toxicity. On the other hand, Hsp27 reduced aSyn oligomerization *in vitro* possibly by inducing the formation of non-reactive small oligomers. MGO glycation increased protein aggregation and cell death. Interestingly, Hsp27 overexpression reversed glycated aSyn aggregation and its associated toxicity.

These results demonstrate the importance of HSPs modulation as a possible target of Parkinson's disease therapeutics.

Key words: α-synuclein, Hsp27, Hsp70, methylglyoxal, glycation.

Table of contents

Table of figures

Abbreviations

A30P	Alanine to proline substitution in residue 30 of α-synuclein
A53T	Alanine to threonine substitution in residue 53 of α-synuclein
Aβ	Amyloid β peptide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
aSyn	Alpha-synuclein
ATP	Adenosine triphosphate
Au	Arbitrary units
BCA assay	Bicinchoninic acid assay
Ctrl	Control
C-terminal	Carboxyl-terminal
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
E46K	Glutamic acid to lysine substitution in residue 46 of α-synuclein
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
HSF1	Heat shock factor 1
HSP	Heat shock protein
Hsp104	Heat shock protein 104
Hsp27	Heat shock protein 27
Hsp70	Heat shock protein 70
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertani
LDH	Lactate dehydrogenase
MGO	Methylglyoxal
NAC	Non-amyloid component of Alzheimer's disease
N-terminal	Amino-terminal
OD_{600}	Optical density at 600nm
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PD	Parkinson's disease
PLD2	Phospholipase D2
PrP	Prion protein
RNA	Ribonucleic acid
S129A	Serine to alanine substitution in residue 129 of alpha-synuclein
SDS	Sodium dodecil sulphate
SEC	Size exclusion chromatography
ThT	Thioflavin T

Tris	Trishydroxymethylaminomethane
UPS	Ubiquitin-proteasome system

1. Introduction

Parkinson's disease (PD) is a progressive, neurodegenerative disease affecting ~1% of the population above 60 years old (de Lau and Breteler, 2006). By 2030, the prediction is that it will affect between 8,7 to 9,3 million people throughout the world (Dorsey *et al.*, 2007). The cardinal motor symptoms of PD include resting tremor, bradykinesia, muscular rigidity and postural instability. However, non-motor symptoms like depression, dementia and psychosis are also present in PD (Weintraub *et al.*, 2008). Although several genetic alterations were shown to cause familial cases of disease (Pankratz and Foroud, 2007), ~90% of the cases are sporadic and highly linked to aging (Weintraub *et al.*, 2008).
Pathologically, PD is mainly characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta. This results in the depletion of dopamine in the striatum and other brainstem neurons, with the consequent disruption of the cerebral neuronal systems responsible for motor functions (Lotharius and Brundin, 2002). Other hallmark of the disease is the presence of protein inclusions, known as Lewy bodies, in the surviving neurons (Gibb, 1989; Greenamyre and Hastings, 2004). Among other components, Lewy bodies are composed by nearly 80 proteins (Licker *et al.*, 2009) being α-synuclein (aSyn) the main element of these structures (Spillantini *et al.*, 1997).

1.1aSyn

aSyn was originally identified in the neuromuscular junction of the Pacific electric eel *Torpedo Californica* (Maroteaux *et al.*, 1988), being highly conserved between vertebrates (George, 2002). Human aSyn is an abundant presynaptic protein with a perinuclear localization first identified as the precursor of the non-Aβ component (NAC) peptide, present in extracellular amyloid plaques in some forms of Alzheimer's disease (Iwai *et al.*, 1995). Recently this protein was also identified in bone marrow and peripheral erythrocytes (Nakai *et al.*, 2007).

1.1.1 aSyn sequence

aSyn is a natively unfolded, heat-resistant, acidic protein of 14kDa composed by 140 amino acid residues (Recchia *et al.*, 2004). Its sequence can be divided into three main regions: (i) the first 60 residues, containing five imperfect repeats (coding for amphipathic α-helices) with a conserved motif (KTKEGV); (ii) the residues 61–95, representing the hydrophobic and amyloidogenic NAC region, display three additional KTKEGV repeats; (iii) and the C-terminal region (residues 96-140) which is highly enriched in acidic residues and is therefore though to be responsible for the solubility of the protein. The first two regions include a membrane-binding domain, while the C-terminal is thought to be responsible for protein–protein and protein–small molecule interaction sites (Breydo *et al.*, 2012) (figure1.1). Missense mutations in the aSyn gene (*SNCA*) (Polymeropoulos, 1997; Krüger *et al.*, 1998; Zarranz *et al.*, 2004; Appel-Cresswell *et al.*, 2013; Kiely *et al.*, 2013) as well as gene multiplications (Singleton *et al.*, 2003) are associated with familial forms of the disease.

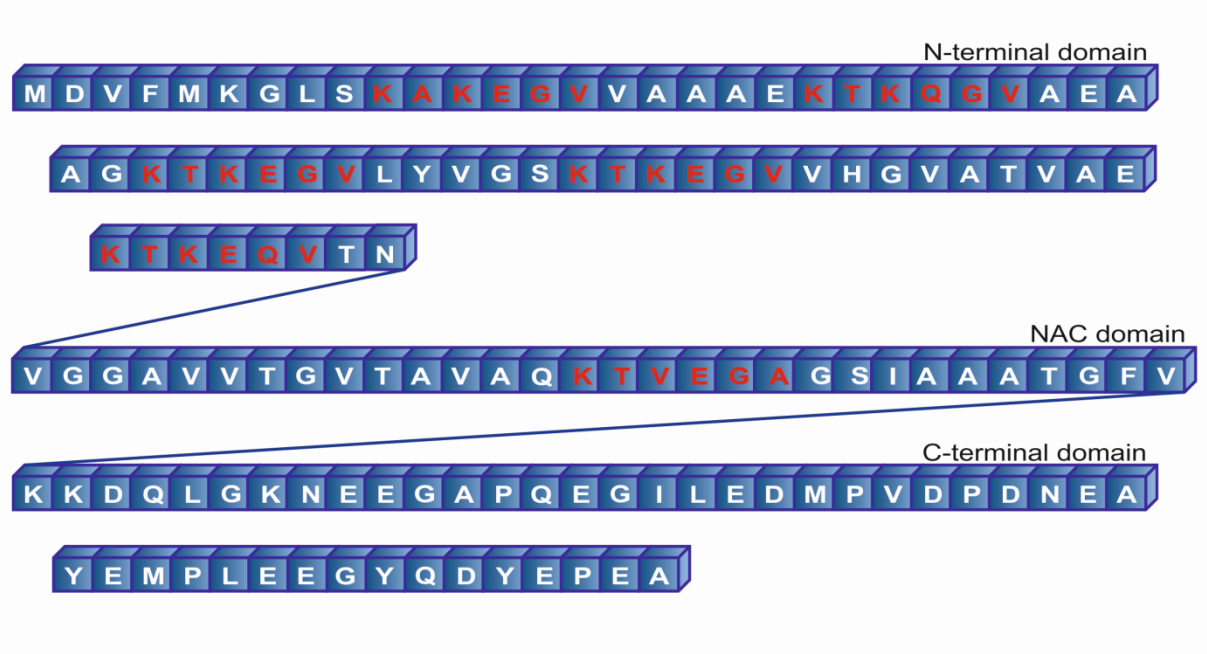

Figure 1.1 Schematic representation of the primary sequence of aSyn. The sequence can be subdivided into three domains: the N-terminal domain which includes six imperfect KTKEGV repeats (red sequences), the NAC (non-Aβ component of Alzheimer's disease) domain and the C-terminal domain. Both N-terminal and NAC domain comprise a membrane-binding region.

1.1.2 aSyn function

So far the function of aSyn remains largely unclear. However, several putative functions have been hypothesized. Since this protein is localized in the synaptic terminals and contain membrane-binding protein domains (Jenco *et al.*, 1998), it may be involved in neurotransmitter release. Interestingly, it was observed that membrane-bound aSyn inhibits phospholipase D2 (PLD2), suggesting aSyn may also participate in vesicle regulation (Jenco *et al.*, 1998; Lotharius and Brundin, 2002). PLD2 catalyzes the hydrolysis of phosphatidylcholine to generate phosphatidic acid, which is important in the regulation of vesicle transport and of changes in cell morphology (Lotharius and Brundin, 2002). aSyn may also display a role on dopamine transmission as it has an inhibitory activity on tyrosine hydroxylase, the rate-limiting enzyme involved in dopamine biosynthesis (Perez *et al.*, 2002), and also it may enable the increase of plasma membrane dopamine transporter molecules (Lee *et al.*, 2001). Recently, aSyn was shown to function as a non-classical chaperone, where its activity is essential for the maintenance of continuous presynaptic SNARE complex assembly and disassembly, which is crucial for the repeated release of neurotransmitters by the presynaptic nerve terminals (Burré *et al.*, 2010). aSyn is also believed to play an important role in nerve terminals protection against injury (Chandra *et al.*, 2005), although some studies show that aSyn increases the sensitivity of cells to the toxic effects of proteasome inhibition (Petrucelli *et al.*, 2002).

1.1.3 aSyn aggregation

Under conditions not currently fully understood, aSyn is able to misfold to assemble into fibrillar-like structures in a nucleation-dependent mechanism (Uversky *et al.*, 2001) (figure1.2). While initial studies suggested fibrils are the main toxic aSyn species inducing cell damage in PD pathology (de la Fuente-Fernandez *et al.*, 1998; Goedert, 2001), it is now widely accepted that the formation of these structures may be related with a protective cellular mechanism analogous to aggresome formation, in which the formation of fibrils serves to sequester potentially cytotoxic soluble aSyn species (McNaught *et al.*, 2002; Olanow *et al.*, 2004). In addition, a pathological examination of human brains revealed that dopaminergic neurons containing Lewy bodies appear morphological and biochemically "healthier" than the surrounding neurons (Tompkins and Hill, 1997). Interestingly, Lewy bodies are commonly observed at autopsy of aged individuals with no neurodegenerative disease symptoms (Bloch *et al.*, 2006). Thus it seems likely that protein aggregation intermediates, known as oligomers, rather than the larger intracellular inclusions, might be the pathogenic species in PD. Nevertheless, the neuronal death mechanisms remain unknown, possibly involving multiple molecular pathways (Conway, 2000; Outeiro *et al.*, 2008; Karpinar *et al.*, 2009; Winner *et al.*, 2011).

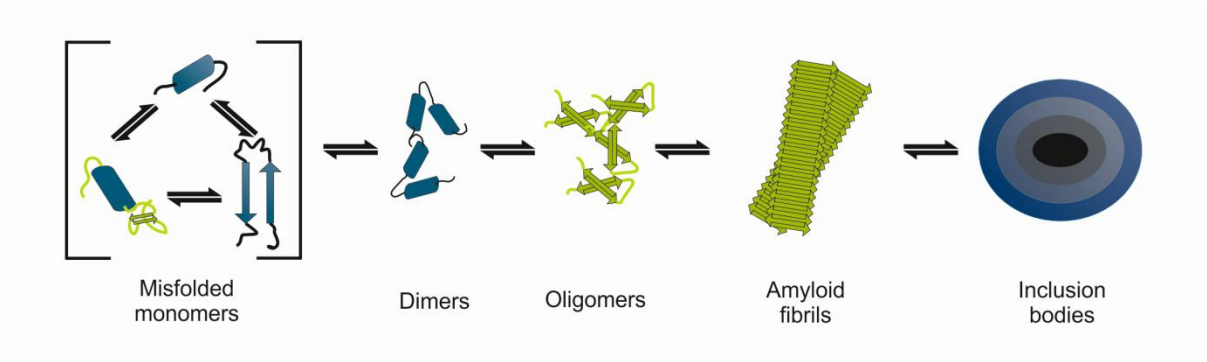

Figure 1.2 Scheme of aSyn oligomerization pathway. aSyn monomers may misfolded and aggregate into dimers and oligomers, ultimately being sequestered in proteinaceous inclusion bodies.

1.1.4 aSyn toxicity

Different studies showed that aSyn oligomers might promote the cell demise by affecting its proteostasis, namely by inhibiting the activity of the 20S and 26S proteasomes (Petrucelli *et al.*, 2002; Lindersson *et al.*, 2004; Xilouri *et al.*, 2013) and by repressing the protein refolding rate of the Hsp70 chaperone system (Hinault *et al.*, 2010). Impaired proteostasis can result in chronic endoplasmic reticulum (ER) stress that may culminate in neurodegeneration. aSyn oligomers can be formed and/or accumulated within ER, sensitizing neurons to ER stress (Colla *et al.*, 2012a, 2012b). Moreover, this accumulation impairs the ER-Golgi trafficking, disturbing endoplasmic reticulum associated degradation (Cooper *et al.*, 2006). Extracellularly, aSyn oligomers may be associated with different toxicity mechanisms according to their morphologic structure. aSyn oligomers with a globular morphology may act at synapses, altering the function of glutamatergic receptors, activating AMPA

receptor-mediated excitatory postsynaptic currents (Hüls *et al.*, 2011; Diógenes *et al.*, 2012). This may lead to dysfunctional synaptic signalling or excitotoxicity and neuronal death, and also to the impairment of hippocampal long-term potentiation (Diógenes *et al.*, 2012; Martin *et al.*, 2012). Either by decreasing the cell membrane lipid bilayer order (Stöckl *et al.*, 2013) or by pore-forming activity (Kayed *et al.*, 2009), aSyn oligomers with an annular structure are associated with the disruption of the cell membrane integrity, which might lead to an increase in intracellular calcium and transition metal dyshomeostasis that result in neuronal death (Pacheco *et al.*, 2012). Both globular and annular aSyn oligomers may promote neuronal death by a seeding phenomenon where misfolded soluble oligomers enter the cell and operate as seeding nuclei that trigger new aggregates formation (Danzer *et al.*, 2007) (figure1.3).

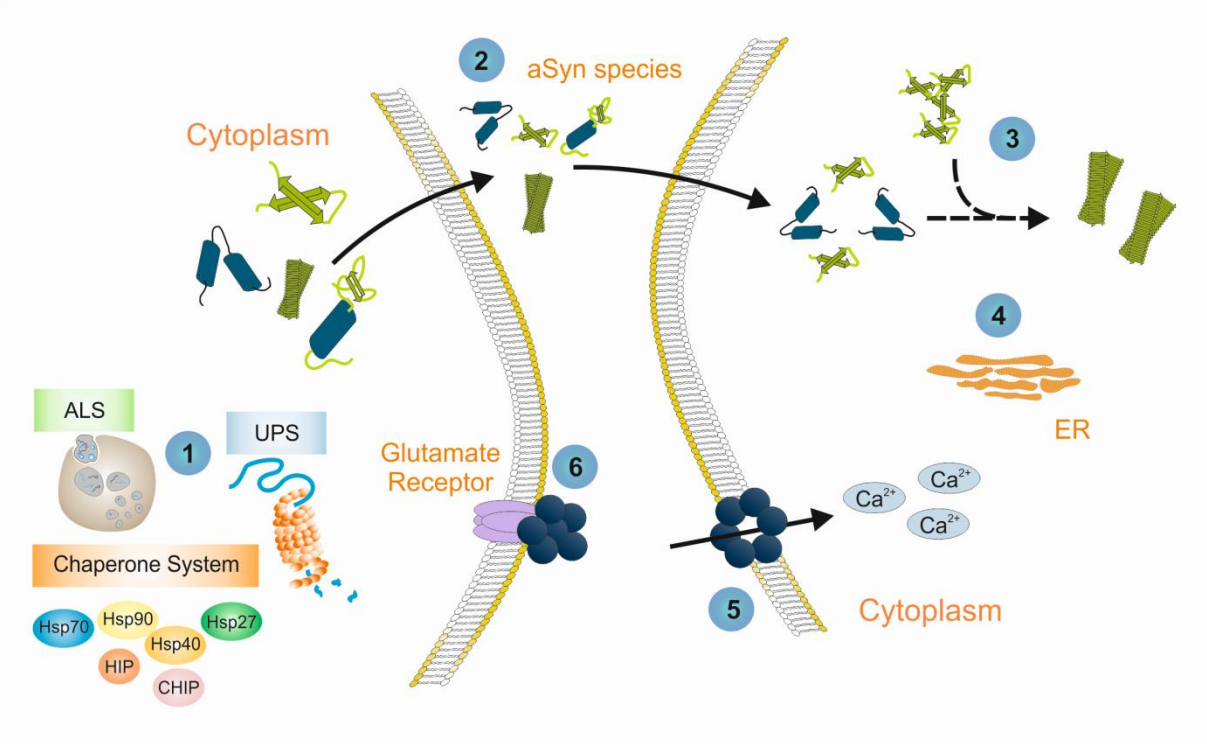

Figure 1.3 Oligomers are the main toxic aSyn specie in PD. aSyn oligomeric species are cytotoxic by mechanisms that include **1.** impairment of protein quality control systems, **2.** prion-like transmission, **3.** seeding effect, **4.** endoplasmic reticulum (ER) stress, (**5.**) membrane pore formation and **6.** glutamate receptor disorder, which may all be related with PD pathology (adapted from Kalia *et al.* 2012).

1.1.5 aSyn pathology transmission

Cell culture studies showed that aSyn oligomers are released either by passive release (like membrane disruption) or by active processes such as exocytosis or calcium-dependent exosomal mechanisms (Danzer *et al.*, 2012) (figure1.3). The uptake mechanism is thought to occur by endocytosis (Volpicelli-Daley *et al.*, 2011). This cell-to-cell transmission of aSyn was recently suggested upon observation of the widespread and acceleration of the synucleinopathy in central

nervous system upon injection of aSyn fibrils in mice neocortex and striatum (Luk *et al.*, 2012). This aSyn propagation process was also reported in cases where human PD patients grafts of fetal mesencephalic tissue (Li *et al.*, 2008) were also affected by the synucleinopathy. These results suggest a aSyn self-propagating mechanism such as those of infectious prion proteins (Goedert *et al.*, 2010).

1.1.6 Glycation of aSyn

aSyn is a target of several post-translational modifications including phosphorylation, ubiquitination, truncation, nitration and oxidation, which can modify the protein structure and influence its toxicity (Beyer and Ariza, 2013). Glycation is another aSyn putative post-translational modification in which several non-enzymatic reactions between carbonyl-containing groups and amino groups leads to the formation of advanced glycation end products (Vicente Miranda and Outeiro, 2010). It is suggested to increase aSyn proteolytic degradation resistance (Vicente Miranda and Outeiro, 2010). This type of modifications were first reported in the substancia nigra and locus coeruleus, revealing higher amounts near Lewy bodies in PD patients (Castellani *et al.*, 1996). Additionally, the levels of glycated proteins were shown higher in PD patients than in control cases (Dalfó *et al.*, 2005). *In vitro*, two glycation agents (methylglyoxal and glyoxal) were shown to induce aSyn oligomerization and to reduce its membrane-binding capacity (Lee *et al.*, 2009a).

1.2 Cell protein quality control systems

Cells possess powerful quality control systems that enable them to cope with protein misfolding. These maintain the proteome homeostasis by ensuring proteins correct folding or, whenever the folding process fails, by targetting these misfolded proteins for degradation, avoiding deleterious effects associated with protein aggregation. Molecular chaperones are part of the protein quality control system and contribute to the correct folding proteins (Buchberger *et al.*, 2010; Theodoraki and Caplan, 2012) (figure1.4). Whenever the refolding of misfolded proteins is not possible, proteins are targeted to elimination, a process that may occur by two major systems: (i) ubiquitin proteasome system, that is the primary route for the degradation of short-lived proteins and the mechanism that provide the control on the steady-state levels of many regulatory proteins; (ii) and the autophagy lysosome pathway, which is responsible for the degradation of long-lived proteins and other cell components (figure1.4). The elimination of misfolded proteins is controlled by both systems (Mittal and Ganesh, 2010; Hartl *et al.*, 2011).

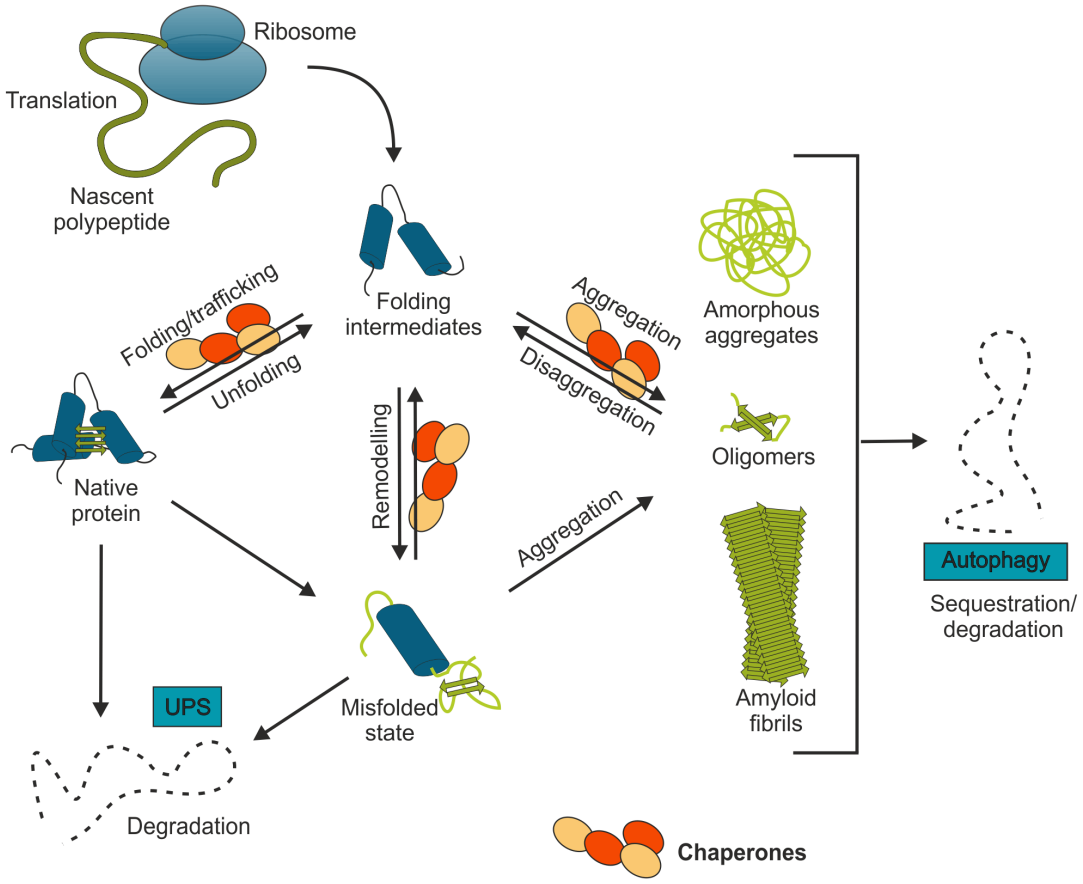

Figure 1.4 Protein quality control system. Chaperones are able to correctly assist the folding of the newly-synthetized proteins into their native conformation. When proteins reach a misfolded state, chaperones play a major role by facilitating the generation of intermediate folding states that can further aquire their correct folding. In some cases, chaperones may facilitate the aggregation of proteins that in a more soluble state would be noxious to the cell, or by oposition facilitate the dissociation of proteins to their active conformation state. Whenever the chaperone activity is not possible, proteins are targeted for degradation either by the ubiquitin proteasome system or by autophagy.

1.2.1 Heat Shock Protein 27

The heat shock protein 27 (Hsp27), a small molecular chaperone, is one of the main inducible HSPs, up-regulated in response to a variety of stress conditions including heat shock and oxidative environments (Landry *et al.*, 1989; Dimant *et al.*, 2012). This protein is mainly expressed in motor and sensory neurons in the brainstem, cranial nerve nuclei and cerebellum, having a limited expression in other post-mitotic neuronal types (Stetler *et al.*, 2010). The regulation of Hsp27 activity occurs through its oligomerization and phosphorylation status. This oligomerization process depends on the association of its C-terminal regions in higher molecular weight structures. After its N-terminal serine

residues phosphorylation, the high molecular weight targets dissociates into lower oligomeric structures, which are closely related with the promotion of cell survival under stress conditions (Stetler *et al.*, 2009).

Several studies investigated the role of Hsp27 in neurodegenerative disorders. In a mouse model of PD overexpressing aSyn from a viral vector, Hsp27 was upregulated, evidencing a clear correlation with the disease (St Martin *et al.*, 2007). Hsp27 significantly protected cultured cells against aSyn-induced stress, especially in A30P and A53T aSyn expressing cells (Zourlidou *et al.*, 2004), and to reduce aSyn aggregation and associated toxicity in cultured dopaminergic neurons (Outeiro *et al.*, 2006). Hsp27 is also able to reduce apoptosis in PC12 cells treated with oxidopamine (a cellular model of Parkinsonism), by delaying both cytochrome c release and caspase activation (Gorman *et al.*, 2005). Moreover, this chaperone also proved to play a role on other misfolding diseases. In Huntington's disease Hsp27 suppresses polyglutamine-mediated cell death in both Huntington's disease rodent and cell line models. This suppression probably occurs by limiting the production of reactive oxygen species and inhibiting caspase activation (Wyttenbach *et al.*, 2002; Perrin *et al.*, 2007). In Alzheimer's disease, β-amyloid peptide (1-42) fibril formation was inhibited by Hsp27 (Kudva *et al.*, 1997). This same molecular chaperone has a neuroprotective effect in cortical neurons exposed to β-amyloid peptide (King *et al.*, 2009). The level of hyperphosphorylated Tau, correlated with Alzheimer's disease, was found decreased by the presence of Hsp27, suppressing the Tau-mediated cell death (Shimura *et al.*, 2004).

1.2.2 Heat shock protein 70

Heat shock protein 70 (Hsp70) is present in several intracellular compartments, being essential in the cytosol, mitochondria, endoplasmic reticulum, lysosomes and extracellular compartments (Stetler *et al.*, 2010). Hsp70 is associated with different chaperone processes such as refolding of misfolded or aggregated proteins, prevention of protein aggregation, folding and assembly of newly synthetized peptides and promotion of the ubiquitination and consequent elimination of misfolded proteins by the proteasome system (Turturici *et al.*, 2011). Its activity is regulated by the interaction with and control of numerous co-chaperone families in a complex manner (Stetler *et al.*, 2010). The Hsp70 has a higher affinity to proteins in unfolded or partially folded states, interacting with an extended protein segment mainly hydrophobic, releasing them in an ATP-dependent manner (Bukau and Horwich, 1998).

Hsp70 reduces the formation of aSyn fibrils by the preferred interaction with prefibrillar species structural features (Dedmon *et al.*, 2005). However, it was shown that Hsp70 modulates aSyn fibril formation by inhibiting its elongation, reducing the quantity of mature fibrils available and therefore the risk of proteasome toxicity (Huang *et al.*, 2006; Luk, Mills, Trojanowski, and Lee, 2008). In addition to inhibiting aSyn aggregation in a mouse model of PD, Hsp70 also decreases aSyn toxicity in a cellular model (Klucken *et al.*, 2004a). Interestingly, in a *Drosophila* model, a reduction of aSyn toxicity was also observed. However, no effect on aggregation was detected (Auluck *et al.*, 2002). Geldanamycin is a chemical activator of the heat shock response that induces the expression of Hsp70 and other HSPs via inhibition of Hsp90 and subsequent activation of heat shock factor-1 (HSF1) (Zou *et al.*,

1998). This compound prevented aSyn aggregation and toxicity in a cell model (McLean *et al.*, 2004) and also prevented aSyn toxicity in mice (Shen *et al.*, 2005) and in flies (Auluck and Bonini, 2002; Auluck *et al.*, 2005). A recent study with carbenoxolone, a geldanamycin analogue, also reduced aSyn aggregation and associated toxicity in a cell model (Kilpatrick *et al.*, 2013). As previously mentioned aSyn is present extracellularly and is taken up by neighboring cells, having toxic consequences. Interestingly Hsp70 appears to act on extracellular aSyn preventing the formation of oligomeric species, rescuing the oligomer-induced toxicity (Danzer *et al.*, 2011).

1.2.3 Heat shock protein 104

The heat shock protein 104 (Hsp104) is only found in yeast and is essential for yeast survival at high ethanol concentrations. Moreover it also allows the stationary-phase yeast cells and spores to exhibit a naturally high thermo-tolerance (Parsell *et al.*, 1994). Hsp104 belongs to the AAA+ superfamily of ATPases, coupling energy from ATP hydrolysis to remodel an extensive variety of proteins, DNA and RNA (Shorter, 2008). It is mainly localized at the mitochondria, playing a role in the protein quality control by recognizing and solubilizing both structurally poorly defined aggregates and cross-β sheet amyloid fibrils to their native conformation (Stetler *et al.*, 2010; Murray and Kelly, 2012). The Hsp70 system is associated with this process and likely assists the protein refolding after the aggregates dissociation (Glover and Lindquist, 1998).

Interestingly, Hsp104 potently inhibited the fibrillization of wild type aSyn and also to the PD-linked mutant variants A30P, E46K and A53T; and also to serine 129 phospho-resistant mutant S129A and phospho-mimicking S129E. It also reduced the dopaminergic neurodegeneration in rat lentiviral model of PD (Lo Bianco *et al.*, 2008). In a *Caenorhabditis elegans* Huntington's disease model, Hsp104 was able to reverse the protein-folding homeostasis imbalance originated by the accumulation of polyglutamine aggregates (Satyal *et al.*, 2000). The reduction of polyglutamine aggregation and cell death by the action of Hsp104 was also observed in a mammalian cell line (Carmichael *et al.*, 2000), in a transgenic mouse (Vacher *et al.*, 2005) and in lentiviral-based rat models of Huntington's disease (Perrin *et al.*, 2007). Hsp104 likewise inhibited the fibril formation of human PrP106–126, disaggregated mature PrP106–126 (Liu *et al.*, 2011) and suppressed amyloid β protofibrils and fibrils growth and self-assembly (Arimon *et al.*, 2008).

1.2.4 Aim of the study

As some HSPs have the ability to inhibit aSyn aggregation and it was proposed that aSyn toxicity is tightly linked to oligomerization, the role of molecular chaperones as therapeutic targets for amyloidogenic neurodegenerative diseases needs to be further studied. With this study, we aim to better understand the role of HSPs on aSyn oligomerization, clarifying which aSyn species arise in the presence of HSPs. Moreover, being glycation suggested to accelerate abnormal protein deposition, we wanted to investigate how the HSPs interfere with the oligomerization process of glycated aSyn.

2. Materials and Methods

2.1 Proteins expression

2.1.1 Human recombinant aSyn expression and purification

Escherichia coli (*E. coli*) strain BL-21 (GE Healthcare, Buckinghamshire, United Kingdom) was transformed by heat shock with aSyn pT7-7 construct (a gift from Dr. Hilal Lashuel, Laboratory of Molecular and Chemical Biology of Neurodegeneration, Brain Mind Institute, Switzerland.). A transformed colony was transferred into 10 mL of LB medium supplemented with ampicillin (100 mg/L) and chloramphenicol (34 mg/L) and incubated overnight at 37º C with continuous shaking. 2.5 mL of the bacterial culture was added to a 2 L flask containing 500 mL of LB medium supplemented with antibiotics. The culture was incubated at 37º C under continuous shaking and the protein expression was induced with 0.3 mM isopropyl D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, Missouri, United States of America) at an OD_{600} of 0.6. After 3 h of expression cells were harvested by a 15 min centrifugation at 8000 g at 4º C and stored at -20º C until further use.

The cell pellet was resuspended in 20 mL of lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM ethylenediamine tetra-acetic acid, 1 mM phenylmethylsulfonyl fluoride) and lysed by freeze and thaw cycles with liquid nitrogen and five 30 sec sonication cycles (amplitude 10 microns) with 1 min incubation on ice between each sonication step (Soniprep 150 MSE). Taking advantage of aSyn thermostability, the cell extract was heated at 100º C for 20 min to precipitate non-thermostable proteins. After a 22000 g centrifugation for 30 min at 4º C, 30% (w/v) ammonium sulphate was added to the supernatant, allowing aSyn to precipitate by stirring for 30 min at 4º C. A new centrifugation was performed as previously described and the pellet resuspended in 5 mL of 30 mM Tris-HCl pH 7.4. The extract containing aSyn was centrifuged at 45000 g at 4º C for 30 min and the supernatant filtered with 0.22 µm filter and applied in a PD-10 gel filtration column for desalting (GE Healthcare, Buckinghamshire, United Kingdom).

The sample was loaded into an ion-exchange chromatography Q Sepharose TM (GE Healthcare, Buckinghamshire, United Kingdom) fast flow column equilibrated with 30 mM Tris-HCl, pH 8.0. Proteins were eluted with a linear NaCl gradient (0.12– 0.5 M) at a flow rate of 1.5 mL/min and the elution monitored at 280 nm. Protein-containing fractions were collected and probed by immunoblot analysis using Syn-1 anti-aSyn antibody (BD Transduction Laboratories, catalogue number 610786, New Jersey, United State of America). Fractions containing aSyn were collected, concentrated by centrifugation using Amicon filters (Millipore, Massachusetts, United States of America), and applied to a gel filtration Superdex 75 column (GE Healthcare, Buckinghamshire, United Kingdom), equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl. Proteins were eluted with the same buffer at a flow rate of 1 mL/min. Fractions containing aSyn, probed by immunoblot, were collected, combined and applied in a 30 kDa Amicon filter (Millipore, Massachusetts, United States of America) to further purify monomeric aSyn. Final protein aliquots with ~100% purity were spectrophotometrically quantified in a nano-drop (using a molar extinction coefficient at 280 nm of 5960 M/cm) (Nanodrop

1000, Thermo scientific, Massachusetts, United States of America) and stored at -80º C until further use.

2.1.2 Hsp27 expression and purification

E. coli strain BL-21 was transformed by heat shock with Hsp27 PET16b construct (a kind gift from Dr. Paul Muchowski, Gladstone Institute of Neurological Disease, University of California San Francisco, United States of America). A transformed colony was transferred into 20 mL of LB medium supplemented with ampicillin (100 mg/L) and incubated overnight at 37º C with continuous shaking. 5 mL of the bacterial culture was added to a 2 L flask containing 500 mL of LB medium supplemented with antibiotic. The culture was incubated at 37º C under continuous shaking and the protein expression was induced with 0.3 IPTG (Sigma-Aldrich, Missouri, United States of America) at an OD_{600} of 0.5. After 3 h of expression cells were harvested by a 15 min centrifugation at 8000 g at 4º C and stored at -20º C until further use.

The cell pellet was resuspended in 15 mL of lysis buffer and 10 mg/mL of lysozyme (Sigma-Aldrich, Missouri, United States of America) was added. The cell suspension was then incubated on ice with constant stirring for 20 min. 0.33 mL/L benzonase (Sigma-Aldrich, Missouri, United States of America) was added to the cell suspension which was subsequently incubated at room temperature for 20 min with constant stirring. Insoluble cellular debris was removed by centrifugation at 36000g for 30min at 4º C. 53 mL/L of a 200 mM dithiothreitol (Sigma-Aldrich, Missouri, United States of America) solution was added to the soluble supernatant being incubated at room temperature with constant stirring for another 10 min. Insoluble contaminants were removed by centrifugation at 36000 g for 30 min at 4º C and the supernatant was filtered with a 0.22 µm filter.

The sample was loaded into an ion-exchange chromatography Q Sepharose TM fast flow column equilibrated with 20 mM Tris-HCl, pH 8.0. Proteins were eluted with a linear NaCl gradient (0 - 1.0 M) at a flow rate of 1.5 mL/min and the elution monitored at 280 nm. Protein-containing fractions were collected and probed by SDS-PAGE analysis using coomassie staining. Fractions containing the Hsp27 were collected, concentrated by centrifugation using Amicon filters and applied to a gel filtration Superdex 75 column, equilibrated with 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl. Proteins were eluted with the same buffer at a flow rate of 1 mL/min. Fractions containing Hsp27, as analyzed by SDS-PAGE, were collected, concentrated by centrifugation using Amicon filters and final protein aliquots with 95% purity were stored at -80º C until further use.

2.1.3 Hsp70 expression and purification

E. coli strain BL-21 was transformed by heat shock with Hsp70 pET28A construct with N-terminal His_6 tag (a kind gift from Richard Morimoto, Department of Molecular Biosciences, Rice Institute for Biomedical Research, Northwestern University, United States of America). A transformed colony was transferred into 10 mL of LB medium supplemented with ampicillin (100 mg/L) and kanamycin (50 mg/L) and incubated overnight at 37º C with continuous shaking. 5 mL of the bacterial culture was added to a 2 L flask containing 500 mL of LB medium supplemented with antibiotic. The culture was

incubated at 37º C under continuous shaking and the protein expression was induced with 0.5 mM IPTG when the culture reached an OD_{600} of 0.7. After another 3 h growth period, cells were harvested by a 15 min centrifugation at 8000 g at 4ºC and stored overnight at -20º C.

The cell pellet was resuspended in 20 mL of lysis buffer and lysed by three freeze and thaw cycles with liquid nitrogen and five times 30 sec sonication cycles (amplitude 10 microns) with 1 min incubation on ice between each sonication step. The Hsp70 containing extract was centrifuged at 15000 g at 4 ºC for 40 min and the supernatant was filtered with a 0.22 µm filter.

The sample was loaded into an immobilized Ni^{2+} affinity chromatography Histrap column (GE Healthcare, Buckinghamshire, United Kingdom) equilibrated with 20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4. Proteins were eluted with equilibration buffer containing 500 mM imidazole at a flow rate of 1 mL/min and the elution monitored at 280 nm. Protein-containing fractions were collected and probed by SDS-PAGE analysis using coomassie staining. Fractions containing Hsp70 were collected, concentrated by centrifugation using Amicon filter, and applied to a gel filtration Superdex 75 column, equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 150mM NaCl. Proteins were eluted with the same buffer at a flow rate of 1 mL/min. Fractions containing the 70 kDa protein were collected, concentrated by centrifugation using Amicon filters and applied in a PD-10 gel filtration column (GE Healthcare, Buckinghamshire, United Kingdom) to exchange for 30 mM Tris-HCl pH 7.4 buffer. Final protein aliquots with 75% purity were stored at -80º C until further use.

2.1.4 Hsp104 expression and purification

E. coli strain BL-21 was transformed by heat shock with Hsp104 pPROEX-Htb construct (a kind gift from Dr. James Shorter, Department of Biochemistry and Biophysics, Perelman School of Medicine at the University of Pennsylvania, United States of America) with N-terminal His_6 tag. A positive colony was transferred into 100 mL of LB medium supplemented with ampicillin (100 mg/L) and chloramphenicol (34 mg/L) and incubated overnight at 37º C with continuous shaking. 30 mL of the bacterial culture was added to a 2 L flask containing 500 mL of LB medium supplemented with antibiotics and incubated at 37º C under continuous shaking. When the culture reached an OD_{600} of 1 was placed at 18º C for about an hour. The protein expression was induced with 1 mM IPTG for 18 h at 18º C with continuous shaking. Cells were harvested by a 15 min centrifugation at 8000 g at 4º C and stored overnight at -20º C.

The cell pellet was resuspended in 20 mL of lysis buffer and lysed by three freeze and thaw cycles with liquid nitrogen and five times 30 sec sonication cycles (amplitude 10 microns) with 1 min incubation on ice between each sonication step. The Hsp104 containing extract was centrifuged at 15000 g at 4ºC for 40 min and the supernatant was filtered with a 0.22 µm filter.

The sample was loaded into an immobilized Ni^{2+} affinity chromatography Histrap column equilibrated with 20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4. Proteins were eluted with equilibration buffer containing 500 mM imidazole at a flow rate of 1 mL/min and the elution was monitored at 280 nm. Protein-containing fractions were collected and probed by SDS-PAGE analysis using coomassie staining. Fractions containing Hsp104 were collected, concentrated by centrifugation

using Amicon filters, and applied to a gel filtration Superdex 75 column, equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl. Proteins were eluted with the same buffer at a flow rate of 1 mL/min. Fractions containing Hsp104 were collected, concentrated by centrifugation using Amicon filters and eluted through a PD-10 gel filtration column to exchange for 30 mM Tris-HCl pH 7.4 buffer. Final protein aliquots were stored at -80º C until further use.

2.2 aSyn oligomerization

Purified monomeric aSyn was diluted at 140 μM in 30 mM Tris-HCl pH 7.4. Oligomerization was induced by continuous shaking for 4 days at 37° C in a Thermomixer (Eppendorf, Hamburg, Germany) at 800 rpm. aSyn oligomerization was established either with aSyn alone or in the presence of Hsp27 or Hsp70. Controls of the oligomerization of HSPs alone were performed. A glycation environment was also tested in the oligomerization process by adding 0.5 mM of methylglyoxal (MGO). Samples were taken at specific time points, diluted to an aSyn final concentration of 70 μM and stored at -20º C.

2.2.1 SDS-PAGE / Native PAGE Western blot analysis

The composition of different aSyn oligomerization species was evaluated by native or SDS-PAGE. 0.5 μg of each sample was resolved by native or SDS-PAGE using a Tetra Cell (Bio-Rad, California, United States of America) in a 12% polyacrylamide gel (Bio-Rad, California, United States of America) using standard procedures. Proteins were transferred to a nitrocellulose membrane (Bio-Rad, California, United States of America) using the Mini Tans-Blot system (Bio-Rad, California, United States of America). Prestained standard proteins were also loaded on the gel. Membrane was incubated for 1 h with constant shacking at room temperature with blocking solution (5% bovine serum albumin in 50 mM Tris, 150 mM NaCl, pH 7.4). The membrane was incubated overnight at 4° C with the primary antibody anti-aSyn (c-20) (BD Transduction Labs, New Jersey, United State of America) using a dilution of 1:1000 in blocking solution. Membrane was washed in PBS and incubated for 1 h at room temperature with anti-mouse-horseradish peroxide - conjugated secondary antibody (Invitrogen, California, United States of America) using a dilution of 1:10,000 in blocking solution. Detection procedures were performed according to ECL system (Millipore, Massachusetts, United States of America) with appropriate exposure time and films were scanned. Each immunoblot was repeated at least three times from independent experiments.

2.2.2 Thioflavin T binding assay

The formation of β-sheet enriched species was probed by Thioflavin T (ThT) binding assay (Nilsson, 2004). Briefly, ThT (Sigma-Aldrich, Missouri, United States of America) was incubated at a final concentration of 20 μM with 1.4 μM aSyn in 50 mM Tris-HCl, pH 7.4. Emission wavelength scan at 490 nm was performed with an excitation wavelength of 450 nm using a plate reader (Tecan Infinite 200, Männedorf, Switzerland).

2.2.3 Size exclusion chromatography (SEC)

aSyn samples (50 µg) were loaded on a SuperdexTM 200 10/300 column (GE Healthcare, Buckinghamshire, United Kingdom) using 30 mM Tris/HCl (pH 7.4, with 0.2 M NaCl) as an eluent at a flow rate of 0.5 mL/min and monitored at 220 nm.

2.3 Cell culture

2.3.1 Transfection of mammalian cells

Human H4 neuroglioma cells (gift from Dr. Bradley T. Hyman, Harvard Medical School) were maintained at 37° C in OPTI-MEM I (Gibco, Invitrogen, California, United States of America) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, California, United States of America) and seeded in 6 cm dishes (Corning, New York, United States of America) for triton-X 100 solubility assays or 35 mm imaging dishes (µ-Dish, Ibidi, Martinsried, Germany) for microscopy studies 24 h prior transfection. Cells were transfected with pcDNA3.1-SynT (a kind gift from Dr. Bradley T. Hyman), a plasmid encoding for aSyn fused with a truncated form of green fluorescent protein which forms *in vivo* inclusions (McLean *et al.*, 2001), and pcDNA3.1 (Invitrogen, California, United States of America) or pcDNA3.1-Hsp27 using FuGENE 6 (Roche diagnostics, Basel, Switzerland). 24 h after transfection the media was replaced by fresh media supplemented with either water or 0.5 mM MGO (for glycation experiments). Cells were collected 48 h after transfection.

2.3.2 Cytotoxicity assay

Human H4 neuroglioma cells were maintained in Opti-MEM I supplemented with 10% FBS at 37° C. Cells were plated onto 24-well plates (1.9 cm^2) (TPP, Trasadingen, Switzerland), at a density of 25,000 cells/cm^2. Samples collected from aSyn oligomerization experiments were incubated at final concentration of 1 µM for 24 h. Media was collected and lactate dehydrogenase activity (LDH) (Clontech, California, United States of America) measured in a plate reader (Tecan Infinite 200, Männedorf, Switzerland), according to the manufacturer's protocol.

2.3.3 Triton-X 100 solubility assay

48h after transfection, cells were washed twice with PBS, placed at 4º C and lysed in 100 µL of lysis buffer (PBS supplemented with protease inhibitor and phosphatase inhibitor cocktail tablets). Samples were sonicated three times for 30 s (amplitude 10 microns) with 1 min incubation on ice between each sonication step. Total protein concentration was measured using bicinchoninic acid (BCA) assay (Pierce, Thermo scientific, Massachusetts, United States of America) and 1% of Triton-X 100 (Sigma-Aldrich, Missouri, United States of America) was added to 200 µg of protein extracts. Samples were incubated at 4º C for 30 min and the triton-insoluble fractions separated by centrifugation at 15000 g for 1 h at 4ºC. The triton-insoluble fractions were ressuspended with 40 µL of lysis buffer containing

2% of SDS and sonicated twice for 30 s (amplitude 10 microns) with 1 min incubation on ice between each sonication step.

2.3.4 Immunocytochemistry

48 h after transfection, cells in 35 mm imaging dishes were washed twice with PBS. 100% ice-cold methanol was added and dishes were incubated at -20º C for 10 min. Cells were washed three times with PBS and incubated with blocking solution (1.5% Normal Goat Serum in PBS) for 1 h at room temperature. Cells were incubated with the primary antibody anti-aSyn (Cell Signaling Technology, catalogue number 2642, Massachusetts, United States of America) using a dilution of 1:75 in blocking solution, overnight at 4º C. Cells were washed with PBS and incubated for 4 h at room temperature with Alexa Fluor® 488 Goat Anti-Rabbit conjugated secondary antibody (Invitrogen, catalogue number A11008, California, United States of America) using a dilution of 1:1000 in blocking solution.
A widefield fluorescent microscope Zeiss Axiovert 200M (Carl Zeiss MicroImaging, Jena, Germany) was used to visualize aSyn inclusions and at least 100 cells per condition were counted.

3. Results

3.1 Protein expression an purification

To investigate the effect of selected HSPs on the oligomerization process of aSyn, Hsp27, Hsp70, Hsp104 and aSyn were expressed in *E. coli*.

For aSyn, we first performed a thermal enrichment step, as previously described (Jakes *et al.*, 1994; Vicente Miranda *et al.*, 2013). By heating the bacterial protein extract, all non-thermo-resistant proteins precipitate, whereas aSyn remained in the soluble fraction (thermo-resistant proteins). After a second ionic exchange purification step, the fractions containing the 14 kDa aSyn (confirmed by Western-blot) were combined and further purified by SEC (figure 3.1A). aSyn positive fractions were again combined, buffer exchanged and the protein concentrated. aSyn was then quantified by spectroscopy and stored at -80º C until further use.

In the case of Hsp27, Hsp70 and Hsp104, the proteins were first purified by immunoaffinity. Next, the proteins were purified by SEC (figure 3.1B, C and D) and the positive fractions (confirmed either by Western-blot or by coomassie staining) combined, buffer exchanged, concentrated and quantified by spectroscopy, prior to storage at -80º C. Even though the expression of Hsp104 was successful after purification by immunoaffinity, the protein was unstable and precipitated irreversibly.

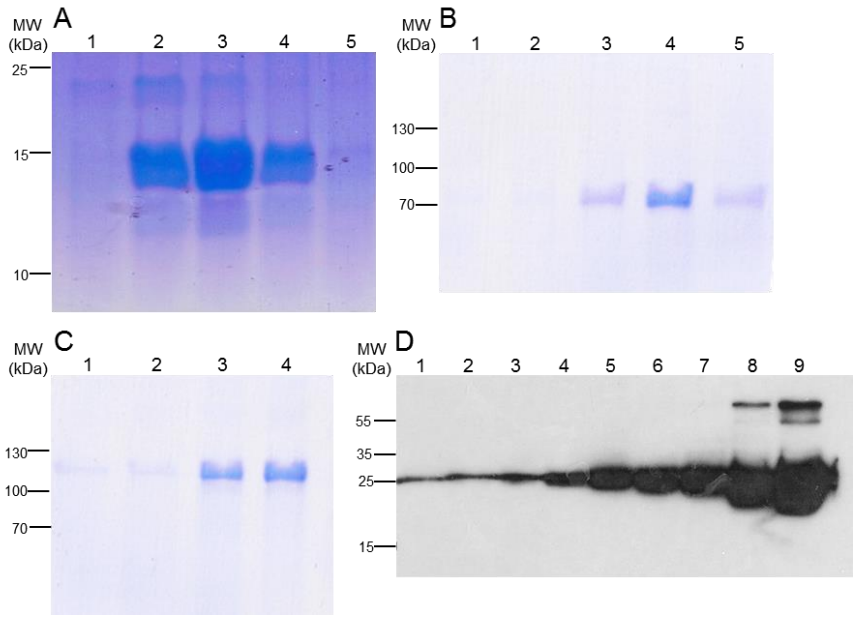

Figure 3.1 Protein expression in *E. coli* BL21+. Coomassie staining of an SDS-PAGE of eluted protein fractions after SEC: **A.** Fractions 2 to 4 are mainly composed by a ~14 kDa protein corresponding to aSyn **B.** Fractions 3 to 5 are mainly composed by a ~70 kDa protein corresponding to Hsp70 **C.** Fractions 3 and 4 are mainly composed by a ~104 kDa protein corresponding to Hsp104. **D.** Anti-Hsp27 Western-blotting of 12% SDS-PAGE: Fractions 5 to 9 are mainly composed by a ~27 kDa protein corresponding to Hsp27.

3.2 Effect of Hsp27 and Hsp70 on aSyn oligomerization *in vitro*

To determine the effects of the different chaperones on aSyn oligomerization, we studied aSyn oligomerization either alone or in the presence of each of the recombinant chaperones selected for our study. For this, we used different techniques including gel-based techniques such as native and SDS-PAGE, fluorimetric methods based on ThT reactivity, and SEC.

In native-PAGE, the protein structure and interactions remain unaltered and the proteins are resolved according to both their charge and molecular weight. In the case of aSyn the monomer will be the species with highest migration, and the fibrils will not enter the resolving gel. In SDS-PAGE, only the most stable associations will remain unaltered and the proteins will migrate according to their molecular weight.

In the ThT assay, the biding is proportional to the β-sheet structure content of the protein, a typical characteristic of amyloid fibrils.

Finally, for the SEC, the species populations are resolved according to their molecular weight.

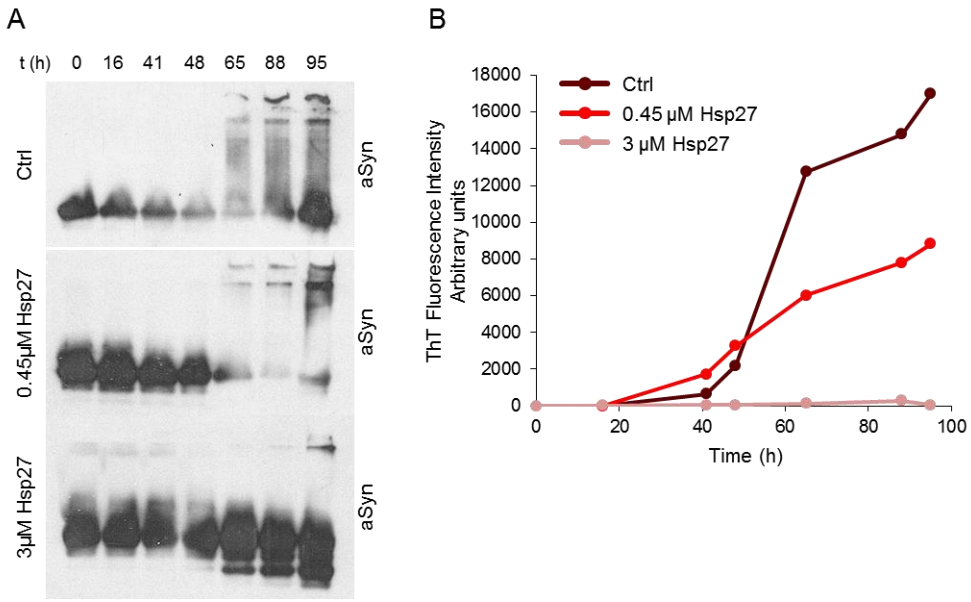

Figure 3.2 Hsp27 decreases aSyn oligomerization in a dose dependent manner. **A.** Anti-aSyn Western-blotting of 12% native-PAGE. aSyn oligomerization was performed with 140 μM aSyn in 50 mM Tris-HCl pH 7.4 in the absence or the presence of 0.45 and 3 μM of Hsp27, at 37º C, shaking at 800 rpm. **B.** Analysis of aSyn β-sheet formation by ThT fluorescence at aSyn final concentrations of 1.4 μM.

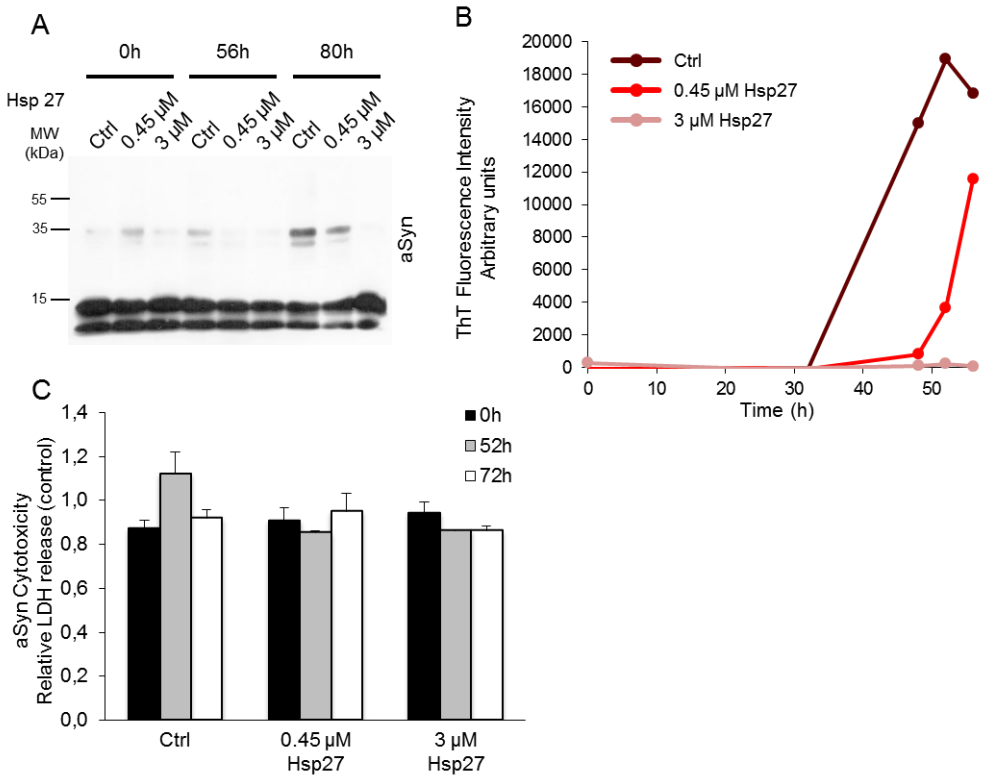

Figure 3.3 Hsp27 decreases aSyn oligomerization over-time in a dose dependent response. A. Anti-aSyn Western-blotting of 12% SDS-PAGE. aSyn oligomerization was performed with 140 µM aSyn in 50 mM Tris-HCl pH 7.4 in the absence or the presence of 0.45 and 3 µM of Hsp27, at 37º C, shaking at 800 rpm. **B.** Analysis of aSyn β-sheet formation by ThT fluorescence at aSyn final concentrations of 1.4 µM. **C.** Cytotoxicity of aSyn species population in H4 cells. LDH activity of H4 cells challenged to different oligomerization time-points. LDH release was expressed as relative levels of non-treated samples. Data are expressed as mean ± SD.

To study the kinetics of aSyn aggregation, we incubated the protein under controlled temperature and shaking speed and collected different samples throughout time. We also incubated the different concentrations of HSPs alone. In these conditions, no ThT response was observed. By analyzing the resulting samples, we observed the formation of high molecular weight species along time (figure 3.2A and 3.3A), followed by an increase in the formation of β-sheet structures (figure 3.2B and 3.3B). This formation is most visible after 65 h of incubation where a smear of different-size aSyn species is observed in the native gels followed by a large increase in ThT fluorescence (figure 3.2).

The presence of Hsp27 inverted this tendency, decreasing the formation of high molecular weight species and also their β-sheet structure content (figure 3.2 and 3.3) in a concentration dependent manner. After 65 h the higher molecular weight species decreased in the presence of 0.45 µM of Hsp27, and were almost abolished in the presence of 3 µM of Hsp27 (Fig. 3.2A and 3.3A). In agreement, the ThT fluorescence emission also correlated with this observation (Fig 3.2B and 3.3B).

Interestingly, after 80 h of incubation, the presence of 3 μM of Hsp27 almost completely inhibited aSyn high molecular weight species and fibrils (Fig 3.2 and 3.3).

In contrast, the presence of Hsp70 tends to accelerate aSyn oligomerization and the formation of β-sheet structures (figure 3.4). As observed by SDS-PAGE analysis, at both 56 h and 80 h of incubation, at both concentrations of Hsp70, aSyn presents more higher molecular weight species than aSyn incubation alone (figure 3.4A). This data correlates with the increased ThT emission for both concentrations in a concentration dependent response (figure 3.4B).

The oligomerization pattern observed in both Native and SDS-PAGE for aSyn was not observed in SEC analysis (figure 3.5A). Although, an elution peak corresponding to aSyn oligomeric species was observed in the presence of Hsp27 (figure 3.5B, arrow), there were no significant changes in the SEC profile in the aSyn oligomerization in the presence of Hsp70 (figure 3.5C).

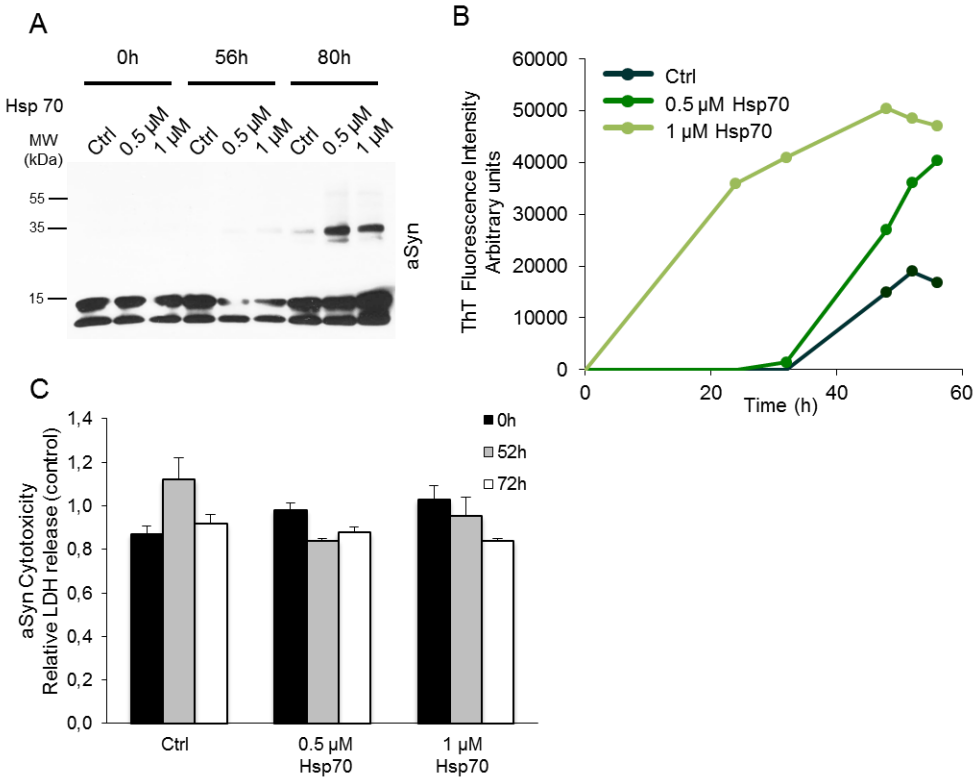

Figure 3.4. Hsp70 promotes aSyn fibrillization. A. Anti-aSyn Western-blotting of 12% SDS-PAGE. aSyn oligomerization was performed with 140 μM aSyn in 50 mM Tris-HCl pH 7.4 in the absence or the presence of 0.5 and 1 μM of Hsp70, at 37º C, shaking at 800 rpm. **B.** Analysis of aSyn β-sheet formation by ThT fluorescence at aSyn final concentrations of 1.4 μM. **C.** Cytotoxicity of aSyn species population in H4 cells. LDH activity of H4 cells challenged to different oligomerization time-points. LDH release was expressed as relative levels of non-treated samples. Data are expressed as mean ± SD.

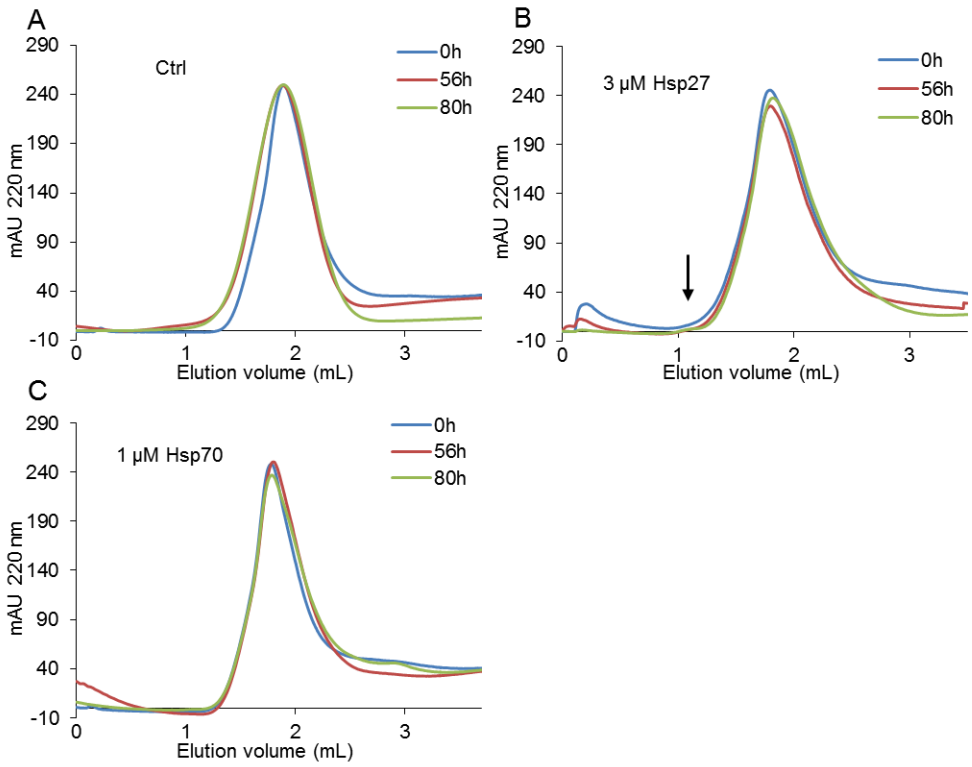

Figure 3.5 Hsp27 and Hsp70 do not alter the elution profiles of aSyn in SEC analysis. aSyn size exclusion chromatography elution profiles in Superdex-200 column. aSyn oligomerization in the absence **A.** or presence of 3 µM Hsp27 **B.** and 1 µM Hsp70 **C.**, 0, 56 and 80 h after oligomerization started. 50 µg of protein were injected. aSyn monomers display a retention time of 1.8 mL, while aSyn oligomers of 1 mL (arrow).

3.3 Cytotoxicity of aSyn species

To evaluate the cytotoxicity of the resulting aSyn species we performed LDH release measurements of cells challenged with aSyn species collected at different time-points, either in the presence or absence of the different chaperones. This method is based on the measurement of this enzyme's activity that correlates with the loss of membrane integrity and therefore the cytotoxicity.

We observed that the most cytotoxic species were those formed at 52 h of incubation (figure 3.3C). Interestingly, this toxicity was rescued in the samples where aSyn was oligomerized both in the presence of Hsp27 (figure 3.3C) or Hsp70 (figure 3.4C).

3.4 Effects of glycation on aSyn oligomerization

To evaluate the effects of glycation on aSyn oligomerization, we incubated aSyn with MGO and evaluated the oligomerization kinetics. Using SDS-PAGE and ThT analysis we observed that MGO increased both aSyn oligomerization and the formation of β-sheet structures (figure 3.6). However, in the presence of Hsp27, aSyn oligomerization was reduced and less β-sheet structures formed (figure 3.6A and 3.6B). Using SEC analysis, we observed MGO induced the formation of small oligomeric species along time (figure 3.7C, arrow). Interestingly, in the presence of Hsp27, an increase in the oligomeric species was also observed (figure 3.7D, arrow).

To evaluate the cytotoxicity of the glycated aSyn species, we performed LDH assays. Interestingly, treating cells with glycated aSyn alone resulted in higher cytotoxicity at time 0. However, in the presence of Hsp27, cytotoxicity was almost reduced to control levels in a concentration dependent manner (figure 3.6C).

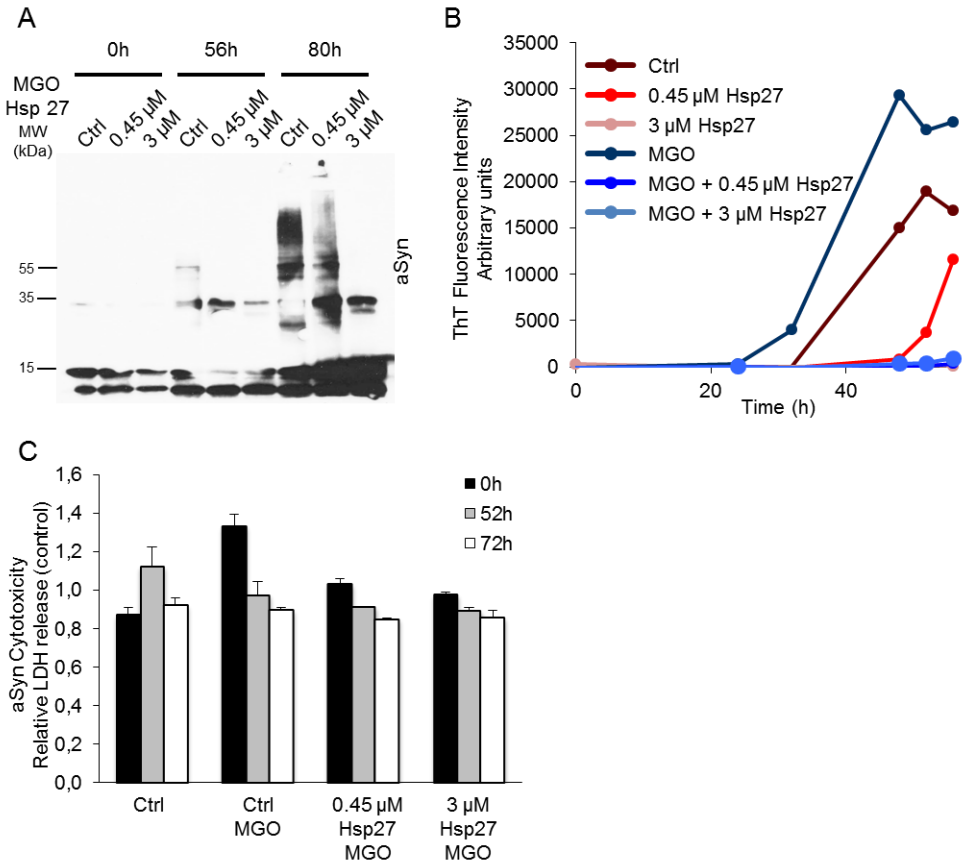

Figure 3.6 MGO induces and Hsp27 reverses aSyn fibrillization. A. Anti-aSyn Western-blotting of 12% SDS-PAGE. aSyn oligomerization was performed with 140 µM aSyn and 0,5 µM MGO in 50 mM Tris-HCl pH 7.4, in the absence or presence of 0.45 and 3 µM of Hsp27, at 37º C, shaking at 800 rpm. **B.** Analysis of aSyn β-sheet formation by ThT fluorescence at aSyn final concentrations of 1.4 µM. **C.** Cytotoxicity of resulting aSyn species population in H4 cells measured by LDH activity. LDH

release was expressed as relative levels to non-treated samples. Data are expressed as mean ± SD.

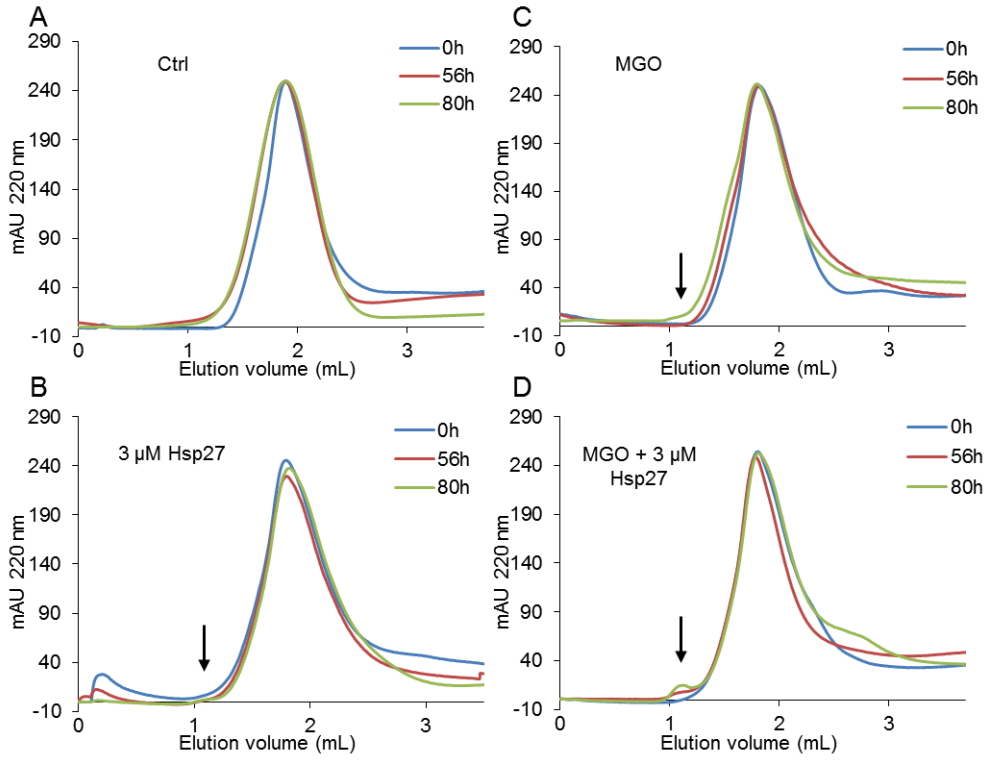

Figure 3.7. MGO induces oligomerization of glycated aSyn. aSyn size exclusion chromatography elution profiles in Superdex-200 column. aSyn oligomerization in the absence **A.** or presence of 3 µM Hsp27 **B.** and in glycation conditions **C.** and **D.**, 0, 56 and 80 h after oligomerization started. 50 µg of protein were injected. aSyn monomers display a retention time of 1.8 mL, while aSyn oligomers of 1 mL (arrow).

3.5 Effects of Hsp27 on aSyn oligomerization in human cells

To evaluate the oligomerization of aSyn in H4 cells in the presence of Hsp27, we used a well-established paradigm of aSyn aggregation comprising the expression of a C-terminally-modified version of aSyn (synT) together with a control vector or with an Hsp27 expressing vector.

To evaluate the oligomerization of aSyn we used two different techniques: (i) a triton X-100 solubility assay where the proteins are separated according to their solubility at 1% triton X-100; (ii) a microscopy analysis of aggregates formation by immunocytochemistry.

We observed no differences in aSyn triton X-100 solubility in the presence of Hsp27 (figure 3.8). Nevertheless, immunocytochemistry assay, showed a non-significant decrease of cells presenting aSyn aggregates (figure 3.9).

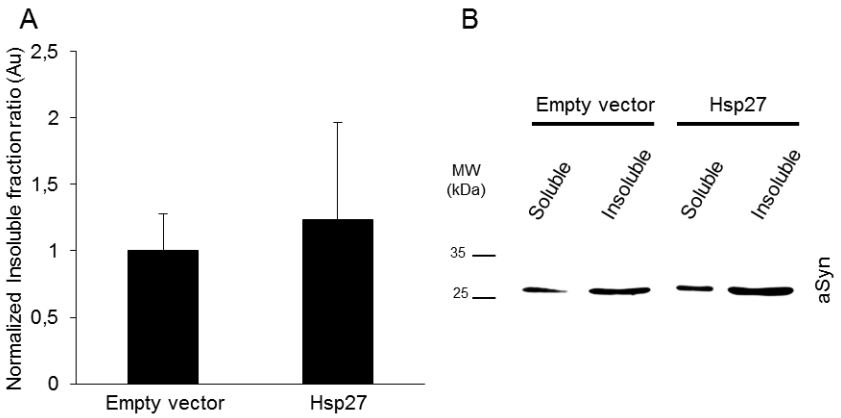

Figure 3.8 Hsp27 does not alter aSyn solubility. A. Triton-X normalized insoluble fraction ratio. Data are expressed as mean ± SD, n=3. **B.** Representative anti-aSyn Western-blot of 12% SDS-PAGE.

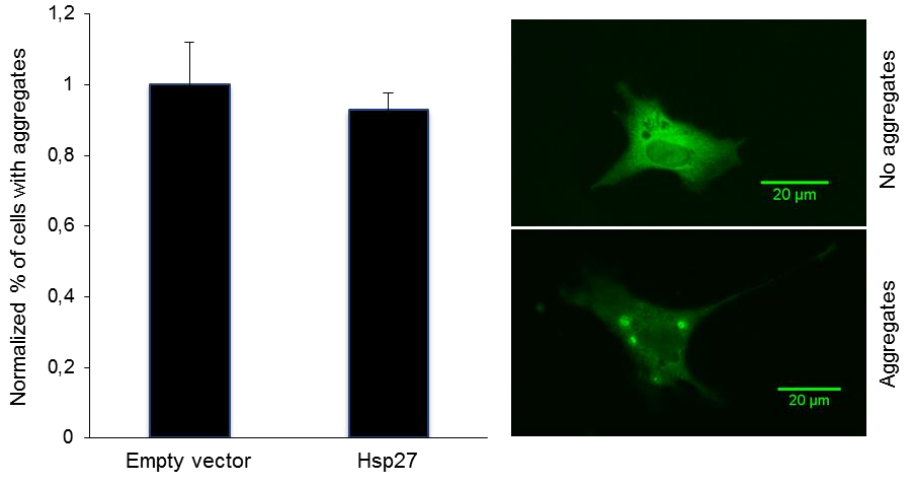

Figure 3.9 Hsp27 reduces aSyn aggregates in a H4 cell PD model. Normalized percentage of cells with aggregates. Data are expressed as mean ± SD, n=4.

Interestingly, the challenge of MGO to cells expressing synT alone increased the triton X-100 insoluble aSyn. However, the co-expression of Hsp27 reversed this increase on triton X-100 insoluble aSyn (figure 3.10), followed by a decrease in the number of cells displaying aggregates (figure 3.11).

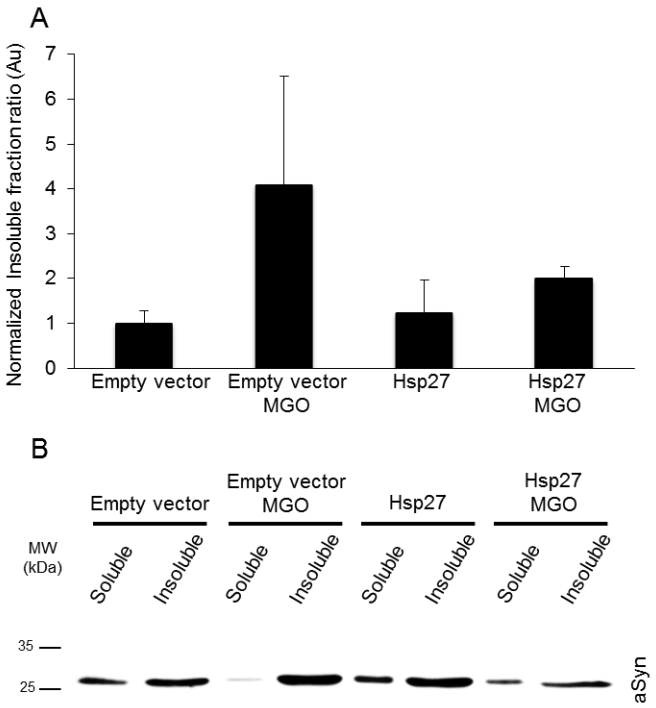

Figure 3.10 MGO induces aSyn insolubilization. A. Triton X-100 normalized insoluble fraction ratio. Data are expressed as mean ± SD, n=3. **B.** Representative anti-aSyn Western-blot of 12% SDS-PAGE.

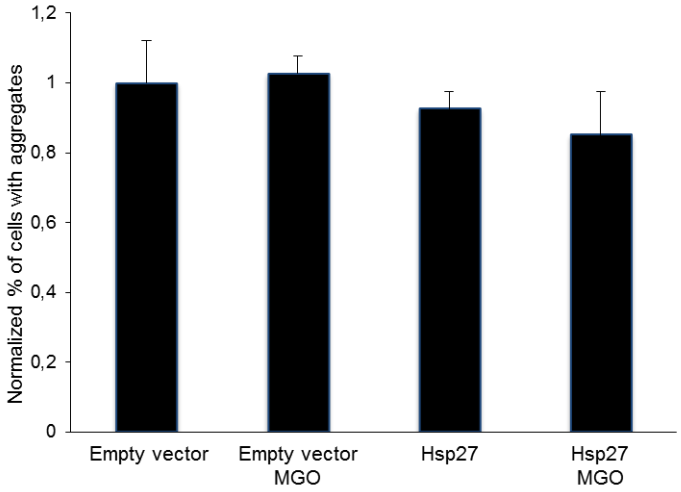

Figure 3.11 MGO increases aSyn aggregation in a H4 cell PD model. Normalized percentage of cells displaying aSyn aggregates. Data are expressed as mean ± SD, n=2.

4. Discussion

The process of aSyn aggregation has been extensively studied *in vitro.* In the process that culminates with the formation of aSyn amyloid fibrils, which can be promoted with agitation of monomeric aSyn at 37º C, oligomeric species can be formed (Fink, 2006; Giehm *et al.*, 2011). In our study, we detected aSyn oligomerization using Native/SDS-PAGE (figures 3.2A and 3.3A) and the formation of β-sheet structures using ThT assays (figures 3.2B and 3.3B). Treating H4 cells with the resulting aSyn species populations demonstrated that the oligomeric species (intermediate time-point) were more toxic than the fibrils (later-time point) or monomers (earlier time-point), in agreement with other studies (Outeiro *et al.*, 2008; Winner *et al.*, 2011). In the presence of Hsp27, the formation of higher molecular aSyn weight species was decreased (figure 3.2 and 3.3 A and B). This result is again in agreement with other reports where Hsp27 reduced aSyn oligomerization (Bruinsma *et al.*, 2011; Aquilina *et al.*, 2013). The neuroprotective role of Hsp27, previously observed in neuronal cells either exposed to exogenous aSyn species (Zourlidou *et al.*, 2004) or co-expressing both proteins (Outeiro *et al.*, 2006), was also confirmed in our H4 cytotoxicity studies (figure 3.3 C). Hsp27 reduced the toxicity of aSyn species in a concentration dependent manner to levels lower than the control (with monomeric aSyn). In contrast, Hsp70 promoted aSyn aggregation while reducing aSyn-induced toxicity (figure 3.4). Although some studies reported that Hsp70 is able to reduce aSyn cytotoxicity and aggregation when co-expressed with aSyn (Klucken *et al.*, 2004b; McLean *et al.*, 2004; Outeiro *et al.*, 2008; Danzer *et al.*, 2011; Kilpatrick *et al.*, 2013), studies in *Drosophila* co-expressing Hsp70 (Auluck *et al.*, 2002) or treated with geldanamycin (Auluck and Bonini, 2002; Auluck *et al.*, 2005) showed protection against the aSyn toxicity without affecting aSyn aggregation, since aSyn inclusions were still present in the neurons. Thus, our observation is in line with the potential beneficial role of aSyn inclusion formation, where putative toxic soluble forms of aSyn are arrested, preventing the impairment of different cellular systems (Waxman and Giasson, 2009; Breydo *et al.*, 2012).

Upon glycation of aSyn we observed an increase in the formation of higher molecular weight species and β-sheet-rich structures (figure 3.6A and B). MGO is the most significant and highly reactive glycating agent in cells and is generated as a by-product of glycolysis (Richard, 1993). Incubation of aSyn with MGO was previously shown to induce the formation of aSyn oligomeric or globular structures, rather than fibrils (Lee *et al.*, 2009a; Padmaraju *et al.*, 2011). With Aβ peptide, relevant in the context of Alzheimer's disease, MGO induced the formation of oligomeric species and larger aggregates, with higher β-sheet content (Chen *et al.*, 2006). These observations are in agreement with our results with aSyn were we found an increase in ThT fluorescence in the presence of MGO, indicating an increase in β-sheet content.

Interestingly, glycated aSyn species displayed higher cytotoxicity when applied exogenously to cells (figure 3.6C). Previously, MGO was described to promoted apoptosis (Ghosh *et al.*, 2011; Antognelli *et al.*, 2013). Moreover, it was demonstrated that MGO turns a neuroglial cell line more vulnerable to

cytotoxic effects (Lee *et al.*, 2009b). Also, rat and hippocampal slices treated with MGO produced increased levels of reactive oxygen species (Heimfarth *et al.*, 2013).

We observed a substantial decrease in the formation of aSyn higher molecular weight species in both the presence of Hsp27 and MGO (figure 3.6A and B). Interestingly, MGO was shown to modify the α-crystallin structure of HSPs (namely, Hsp27) probably by exposing hydrophobic sites that otherwise would not be available for chaperone function. In addition, MGO might induce Hsp27 oligomerization, enhancing its activity (Nagaraj *et al.*, 2003; Oya-Ito *et al.*, 2006). This enhanced Hsp27 activity may explain the effect on reducing aSyn cytotoxicity (figure 3.6C). On one hand, the higher chaperone activity of Hsp27 may explain the reduced formation of aSyn oligomers. On the other hand, glycated Hsp27 was shown to have an extraordinary anti-apoptotic power, being associated with several cancers (Sakamoto *et al.*, 2002; van Heijst *et al.*, 2006; Oya-Ito *et al.*, 2011). To investigate this hypothesis, we aim to investigate whether MGO is able to directly modify Hsp27 and promote its oligomerization, therefore increasing its activity. To that purpose, we will first glycate Hsp27 and further compare aSyn oligomerization kinetics in the presence of unmodified or glycated Hsp27.

Although we observed Hsp27 to reduce aSyn oligomerization *in vitro*, it did not alter aSyn Triton X-100 solubility in cells (figure 3.8), nor did it significantly decrease the percentage of cells with aSyn inclusions (figure 3.9). Hsp27 has previously shown to reduce the aggregation of aSyn (Outeiro *et al.*, 2006) and of other proteins associated with other neurodegenerative disorders (Kudva *et al.*, 1997; Lee *et al.*, 2006; Wilhelmus *et al.*, 2006; Yerbury *et al.*, 2013) . In some Huntington's disease models, Hsp27 did not show a neuroprotective effect (Zourlidou *et al.*, 2007) whereas in others it rescued polyglutamine toxicity (Wyttenbach *et al.*, 2002; Perrin *et al.*, 2007).

Some Hsp27 homologues are known to bind misfolded proteins, co-aggregating with them. This process maintains the misfolded proteins in a conformation that allows other HSPs to disaggregate and possibly refold them, being an important stress-protection mechanism (Ehrnsperger *et al.*, 1997; Katoh *et al.*, 2004; Cashikar *et al.*, 2005; Duennwald *et al.*, 2012).

Treating H4 cells with MGO induced the formation of Triton X-100-insoluble aSyn species (figure 3.10). Interestingly, we also observed a trend towards an increase in the formation of aSyn aggregates (figure 3.11). The glycation process is known to increase the levels of intracellular and mitochondrial reactive oxygen species, inducing oxidative stress (Fukunaga *et al.*, 2005; Shangari and O'Brien, 2004; Wang, Liu, and Wu, 2009; Yan *et al.*, 1994). Glycation is also responsible for the impairment of several protein quality control systems, namely the ubiquitin proteasome system and chaperones (Bento *et al.*, 2010), inducing the accumulation of aggregated proteins (Lee *et al.*, 2009a; Oliveira *et al.*, 2011, 2013) that can be toxic (Li *et al.*, 2013), which explains the results observed.

Surprisingly, overexpression of Hsp27 dramatically reduced the presence of insoluble Triton X-100 aSyn in H4 cells treated with MGO (figure 3.10), which is followed by a reduction in the percentage of cells displaying aSyn inclusions (figure 3.11). In addition to the putative direct function of Hsp27 on glycated aSyn, the possible increased activation of Hsp27 by glycation previously mentioned may be responsible for the decrease in aSyn aggregation. Moreover Hsp27 has the ability to lower the levels of reactive oxygen species by increasing the intracellular glutathione and reducing the intracellular iron (Mehlen *et al.*, 1997; Arrigo, 2001; Arrigo *et al.*, 2005). Glutathione is an important cofactor of the major catabolic route of MGO, the glyoxalase system (Thornalley, 1993; Vicente Miranda and Outeiro, 2010). Therefore Hsp27 overexpression may also decrease the general levels of protein glycation in the cell, therefore resulting in decreased aSyn aggregation. Thus, it will be important to investigate the levels reactive oxygen species in the presence of Hsp27 in control and glycation conditions. This may be achieved using specific fluorescent dyes that bind specific types of ROS.

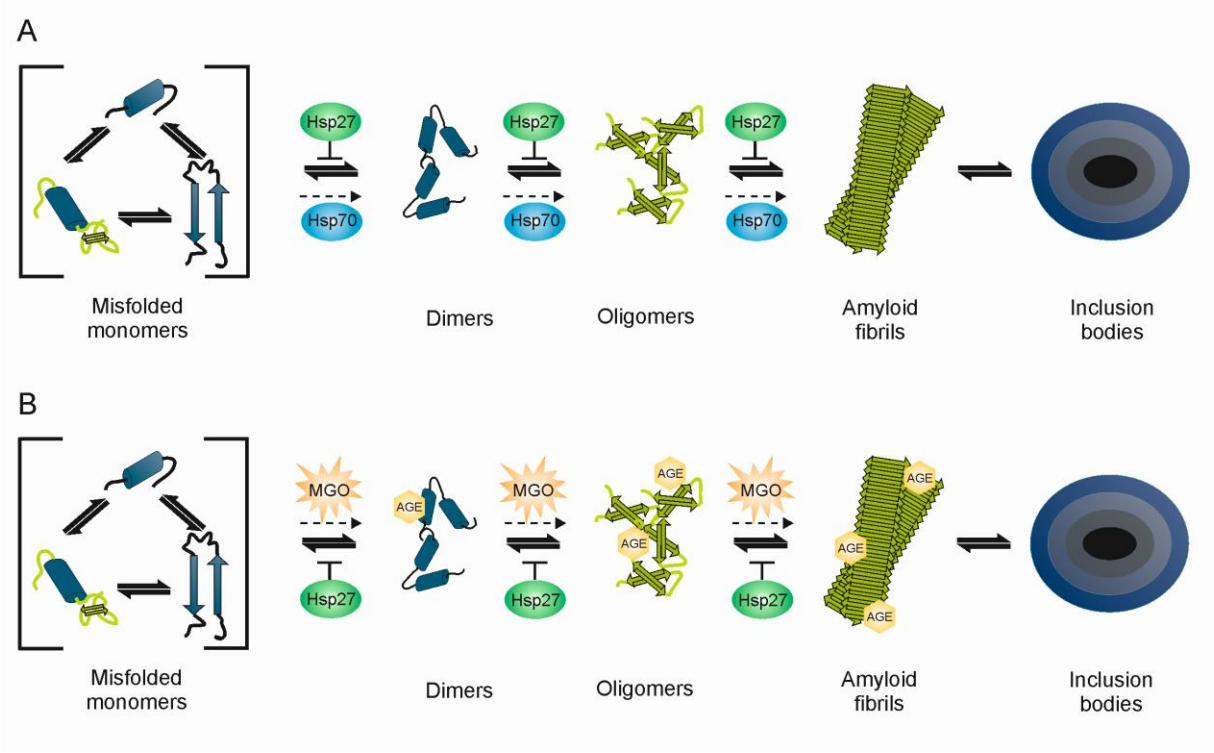

Figure 4.1 HSPs effect in aSyn oligomerization *in vitro*. A. Hsp27 reduced aSyn oligomerization, while Hsp70 appears to induce this process. **B.** In a glycation environment, aSyn is more prone to aggregation, however, when Hsp27 is present its oligomerization reduction activity is highly enhanced.

The co-localization of Hsp27 and Hsp70 in Lewy bodies suggests an important role for these molecular chaperones in PD (Auluck *et al.*, 2002; McLean *et al.*, 2002). Actually, a decrease in the activity of HSPs and of other protein quality control components is associated with ageing and neurodegeneration (Chen *et al.*, 2005; Baraibar and Friguet, 2012). Moreover, several familial PD-associated mutations impair the ubiquitin proteasome system, lysosomal activity, and mitochondrial function, and increase the aSyn aggregation, which leads to a general destabilization of the proteome (Leroy *et al.*, 1998; Ardley *et al.*, 2004; Bender *et al.*, 2006; Pankratz and Foroud, 2007; Pan *et al.*, 2008). The activity of molecular chaperones in stabilising protein conformations and assisting in their trafficking for degradation is of extraordinary importance in the cell. Thus, understanding the precise molecular mechanisms underpinning chaperone function is essential so that we can use them as targets for therapeutic intervention in PD and in other neurodegenerative disorders.

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