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Heat shock proteins act at the crossroad between protein and lipid homeostasis: Implications for Parkinson's disease

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Abstract

Loss of protein homeostasis is associated with the accumulation of misfolded, condensed and aggregated forms of proteins, as well as with a wide range of neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases. Cells evolved a sophisticated protein quality control system to maintain protein homeostasis that includes molecular chaperones and degradation systems. Heat shock proteins (HSPs) are a class of molecular chaperones: they survey protein folding, prevent protein aggregation, and target damaged proteins to degradation. Several stimuli, beyond heat shock, can upregulate HSPs expression, which participate not only in the cell stress response, but exert also housekeeping functions, and may even regulate development and differentiation. Tremendous advances have been made in the last decades in understanding how HSPs are transcriptionally regulated. However, how stress signals are perceived by the cells and are integrated to activate the heat shock response is still only partly understood.

Changes in the physical state and lipid composition of cell membranes can activate the expression of HSPs, through yet unclear mechanisms. HSPs, in turn, can directly interact with lipids, thereby modulating the physical state of lipid membranes. Intriguingly, changes of the lipidome can elicit protein condensation and aggregation, and dysfunction in lipid homeostasis is a known risk factor of many neurodegenerative diseases.

Taken together, these data highlight the existence of a cross-talk between lipid and protein homeostasis. Based on their ability to interact both with proteins and lipids, one may envisage that HSPs represent key players acting at the crossroad between lipid and protein homeostasis. In this PhD thesis, we tackled this hypothesis using in vitro systems and cellular models. We used as a model system α-synuclein, a protein whose aggregation can be triggered by interaction with small lipid unilamellar vesicles (SUVs). We investigated whether recombinant small HSPs displace α-synuclein from lipids, reducing its aggregation (in collaboration with Prof. Vendruscolo, Cambridge University, UK). Our data demonstrate that, besides acting at the protein level and blocking α-synuclein aggregation by direct protein-protein interactions, small HSPs can interact with lipids, interfering with both lipid and protein dynamics and aggregation.

Next, we investigated whether changes in the physical state of the lipid membranes can induce the expression of HSPs. To do so, we used the aminosterol trodusquemine as a model system. Trodusquemine interacts with lipid membranes and changes their fluidity. It also prevents α-synuclein lipid-induced aggregation. Using cellular systems, we could show that trodusquemine acts as a co-inducer of the heat shock response, potentiating the expression of key HSPs. These, in turn, can synergize with trodusquemine to block protein misfolding and aggregation and help restoring lipid homeostasis.

In conclusion, this PhD work contributes to deciphering how cells regulate the cross-talk between lipid and protein homeostasis, and provides an example of how these processes may be pharmacologically exploited to combat protein aggregation.

1. Introduction

Protein homeostasis maintenance is fundamental for life

The protein homeostasis (or proteostasis) refers to the dynamic balance between processes of protein synthesis, folding, trafficking and degradation, and it is essential for cell function and viability (Hipp et al. 2019; Balch et al. 2008). Mammalian cells generally express more than 10.000 species of different proteins, which are involved in a wide range of crucial biological function. To perform their activity, most proteins need to fold into a well-defined three-dimensional structure and be transported in a specific cellular localization (Hartl et al. 2011; Clausen et al. 2019; Klaips et al. 2018). In addition, cells need to be able to restore basal homeostasis in response to stress environmental conditions, which constantly challenge the stability of their proteome (Balch et al. 2008). Cells evolved a sophisticated protein quality control (PQC) system to maintain proteostasis and ensure both cell and organism health. The PQC is an interconnected system of multiple components, including molecular chaperones, proteolytic machineries, and their regulators, and it is responsible for the cellular quality control by monitoring proteins from their synthesis through their degradation (Clausen et al. 2019; Dubnikov et al. 2017). Molecular chaperones predominantly guide de novo folding and assist in protein refolding, while degradation systems mediate the elimination of irreversibly misfolded and aggregated proteins, with the help of co-factors, adaptor proteins and proteolytic enzymes (Figure 1) (Hipp et al. 2019; Sin and Nollen 2015).

Understanding the organization of this extensive network in face of acute and chronic stresses is of fundamental relevance in biology and medicine, as its dysregulation is associated with several human pathologies, including metabolic and cardiovascular diseases, cancer, and neurodegenerative disorders (Hipp et al. 2019). Aging, oxidative stress, and protein dysfunction are common risk factors shared by these pathologies and all of them can perturb the proteostasis, underling that mechanisms supporting cellular homeostasis may be implicated in the pathogenesis of these disorders (Labbadia and Morimoto 2015; Taylor and Dillin 2011).

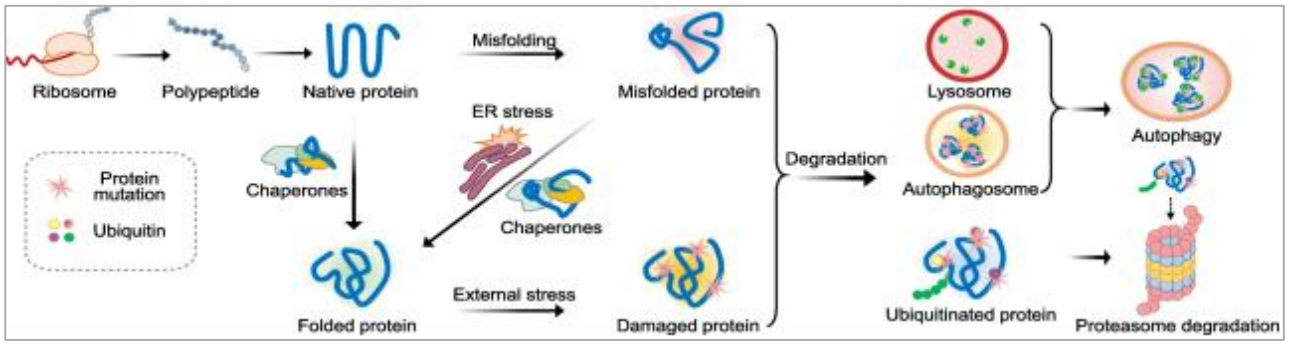


Figure 1: The Protein Quality Control System. Chaperones assist the folding of polypeptides into the native conformation. When misfolded proteins accumulate, cells activate the unfolded protein response (UPR) to re-establish proteostasis. Misfolded and damaged proteins are degraded via the ubiquitin-proteasome system (UPS) or the autophagy system. From: Yan et al., PMID: 33179756.

Proteostasis in Neurodegenerative diseases

Protein misfolding and aggregation are common hallmarks of several neurodegenerative disorders including Alzheimer's disease (AD) and Parkinson's disease (PD). The accumulation of aberrant or damaged proteins results in insoluble inclusions with potential cytotoxic effects, leading to axonal transport defects, synaptic dysfunction, and neuronal death. Besides intracellular aggregates, which can accumulate both in the cytoplasm and nucleus, extracellular aggregates have also been reported in neurodegenerative diseases (Chiti and Dobson 2006; Fusco et al. 2017). In this context, the proteostasis network is central in preventing non-native conformational state, acting as first line of cell defence against protein misfolding and aggregation. Maintaining proteostasis represents a constant challenge for the cell and the presence of protein inclusions supports the notion that proteostais network has collapsed in these diseases (Hipp et al. 2014).

Misfolding protein originate from multiple sources, including genetic mutations, amino acid misincorporation, premature translation termination and surplus of unassembled component of oligomeric complexes; in addition, conformational stress conditions, such as high temperature, oxidative stress, and viral infections, promote protein unfolding and misfolding (Sheikh et al. 2013; Dobson 2003). Chronic stress constantly challenges the cellular quality control system, promoting proteostasis imbalances during ageing (Labbadia and Morimoto 2015; Taylor and Dillin 2011). Recent findings have highlighted a decline of the proteostasis system during ageing (Hipp et al. 2014). Thus, alterations at the level of the physiologic “chaperones machineries” may trigger a toxic cascade of events that culminate in protein aggregation and promotes the development of neurodegenerative diseases-associated with protein aggregation (Dimant et al. 2012; Tittelmeier et al. 2020; Yoo et al. 2001). In addition, other age-related cellular changes can affect the chaperoning activity. As an example, the reduction of energy metabolic pathways decreases the bioavailability of ATP in senescent cells, resulting in a reduction of the activity of the ATP-dependent chaperones. Moreover, proteins can be altered by unwanted non-enzymatic modifications during ageing, which can interfere with chaperone’s ability to recognize their targets (Wiley and Campisi 2021; Amorim et al. 2022; Taylor and Dillin 2011).

When protein aggregation takes place, a cascade of events further compromises the protein quality control system. For example, chaperones are sequestered in the protein aggregates, which limits their availability and function (Hipp et al. 2019). The co-localization of different class of molecular chaperones with inclusions has been found in the brains of patients and in mouse models of several protein misfolding neurodegenerative diseases. On the one hand these colocalization studies suggest that chaperones play an active role in blocking the process of aggregation but, in turn, they also highlight that the deposition of chaperones in the inclusion itself can contribute to deplete them, ultimately leading to the loss of chaperone function (Kopito 2000; Hipp et al. 2014).

The progressive decline of the protein quality control system with age is accompanied by an increase in the rate of biosynthetic errors, in the accumulation of mutations due to genomic instability, by defects in the nucleocytoplasmic shuttling, which all together concur to further decrease cells’ ability to deal with proteotoxic insults. Thus, ageing is regarded as the major driver for the loss of

proteome integrity and widespread protein aggregation, promoting the pathogenesis of many age-related neurodegenerative diseases, including AD and PD (López-Otín et al. 2013; Labbadia and Morimoto 2015; Cuervo et al. 2005). Given the crucial role played by the protein qualuty control system in maintaining cellular functionality and viability and its implication in the development and progression of age-related neurodegenerative diseases, we will here describe in detail its major players.

The Protein Quality Control (PQC) system

Several intracellular and extracellular stresses, including heat shock, oxidative stress, mutations, and aging-related modifications can impair the proteome integrity, resulting in deleterious consequences for cell viability. To maintain the proteostasis, cells have evolved a sophisticated protein quality control (PQC) system, which is comprised of a multitude of molecular chaperones that cooperate with degradation systems through interconnected strategies aimed at identifying, refolding or degrading misfolded proteins and preventing irreversible protein aggregation (Macario and Conway de Macario 2007a; B. Chen et al. 2011; McClellan et al. 2005).

Molecular chaperones

A molecular chaperone can be defined as any protein that assists other proteins in folding or assembling into a functional conformation, without participating in the final structure (F. Ulrich Hartl and Hayer-Hartl 2002). By assuring the proper folding of a wide range of proteins – also known as clients or substrates - molecular chaperones are involved in many processes, such as regulation of cell cycle, differentiation, cell tolerance to stress and apoptosis. Many chaperones are ubiquitous (although tissue-specific chaperones also exist and play critical tissue-specific functions), and are expressed in different compartments of the eukaryotic cells, including the nucleus, cytosol and several types of organelles (Hartl 1996; Ellis 1993; Jeng et al. 2015).

There are numerous classes of structurally unrelated chaperones, and many of them belong to the superfamily of heat-shock proteins (HSPs) or stress proteins, as many of them are upregulated in response to stress conditions. Importantly, many HSPs are constitutively expressed in all cell types. HSPs are implicated in of the maintenance of the proteome, comprising de novo folding, refolding of denatured proteins to prevent their irreversible aggregation, protein maturation and targeting to their final destination; when misfolding occurs, HSPs can also cooperate with co-chaperones and adaptors to target the bound substrate to the degradation systems. HSPs are classified into six major families according to their molecular weight: Hsp100 (100-110 kDa), Hsp90 (83-90 kDa), Hsp70 (66-78 kDa), Hsp60 (60 kDa), Hsp40 (40 kDa) and small heat shock proteins (12-43 kDa) (Kampinga et al. 2009). Here, we list the main HSP chaperones and their functions.

Hsp70 family: Hsp70 proteins represent the major chaperone family, including eight homologous chaperone proteins, which mainly participate in the folding of newly synthesized protein chains, protein tracking across membranes, prevention of protein aggregation, and conformational maintenance. In eukaryotic cells, the stress-induced members are called Hsp70s and those that are constitutively expressed are called Hsc70s. For a detailed description of Hsp70 structure, which is characterized by three major functional domains, see the following articles: (Auluck et al. 2002; Kampinga and Craig 2010). Briefly, the conserved amino-terminal ATPase domain of Hsp70s binds to a molecule of ATP, whose hydrolyzation is required for their chaperone function. Hsp70s cycles between a low affinity-ATP bound state and a high affinity-ADP bound state. In the low affinity-ATP bound state, peptides can associate or disassociate quickly; instead, in the high affinity-ADP bound state, the peptide is tightly bound to the chaperone (Mayer and Bukau 2005; Langer et al. 1992; Hartl et al. 2011). The ATP-dependent binding and release cycles of Hsp70 is controlled by co-chaperones of the Hsp40 family, which accelerate the ATP hydrolysis and recruit Hsp70s to the protein substrates, and by nucleotide exchange factors (NEFs), which control the life span of the Hsp70-substrate complex (Mayer and Bukau 2005; Bascos and Landry 2019). Thus, Hsp70s cooperate with other chaperones and co-factors to create functional multicomponent molecular complexes.

Hsp40 family: Hsp40s are a heterogenous class of chaperones, also known as DnaJ family, which comprises more than 50 members. DNAjs/Hsp40s are implicated in folding, trafficking and degradation processes. DnaJ proteins can directly interact with non-native polypeptides and then recruit Hsp70s to the different substrates and cellular localizations (Qiu et al. 2006; Hipp et al. 2019).

Hsp90 family: the members belonging to Hsp90 family are highly conserved. While Hsc70 and Hsp70 are mainly involved in the folding of newly synthesized proteins, Hsp90 are mainly implicated in the correct folding and maturation of more than 60% of the cellular kinase proteins. More than 200 clients have been identified, including besides kinases also steroid receptors, and other signal-transduction molecules. Thus, Hsp90s participate in a plethora of biological processes ranging from cell-cycle regulation to apoptosis and hormone signalling, as well as innate immunity. In addition, Hsp90s participate in the cell stress response and together with Hsp70s, Hsp90s can be considered as the master chaperones in cells, due to their implication in essential cellular processes. (Taipale et al. 2010; Rutherford and Lindquist 1998; McClellan et al. 2007).Like Hsp70s, Hsp90s are ATP-dependent chaperones and switch conformational state owing to ATP binding and hydrolysis (Bascos and Landry 2019; Wandinger, Richter et al. 2008; McClellan et al. 2007). Hsp90s cooperate with numerous regulators and co-chaperones; many of them possess a tetracopeptide repeat (TRP) domain to dock onto the Hsp90. In particular, the TRP protein HOP binds concurrently to Hsp70 and to Hsp90 to promote a direct client transfer from Hsp70 to Hsp90 (Scheufler et al. 2000). Thus, the two main chaperone systems in the cells, Hsp70s and Hsp90s can cooperate to some extent.

Chaperonins: Chaperonins are large double-ring oligomers that encapsulate the nascent unfolded polypeptides to provide a protective compartment inside which they can reach their native conformation (36). There are group I and group II chaperonins. Group I chaperonins – also known as Hsp60s in eukaryotes – consists of seven-membered rings in mitochondria, and functionally

cooperates with a small co-factor - Hsp10. Group II chaperonins – also called TriC in eukaryotes – have two octameric ring-structure in cytosol and they are independent of Hsp10 protein (Hartl et al. 2011). The group I chaperonins is engaged in the folding of a set of mitochondrial proteins following their import from the cytosol. The process is regulated by ATP binding (Ranson et al. 1998; Xu et al. 1997; Horwich and Fenton 2009). The group II chaperonins consists of eight homologous subunits per ring, arranged back-to-back, similar to group I chaperonins in structure and in protein-encapsulation cycle. A typical example of group II chaperonins in eukaryotes is the chaperonin TRiC/CCTg. Group II chaperonins monitors roughly 10% of newly synthetised cytoplasmatic peptides, comprising actin and tubulin. TRiC is an essential chaperonin because the folding of many substrates of TRiC cannot be achieved with any other chaperone. TriC/CCT has also been shown to interfere with aggregation of the huntingtin protein, whose mutation is associated with the Huntington's disease, another example of neurodegenerative diseases characterized by protein aggregation (Lopez et al. 2015; Douglas et al. 2011).

Small heat shock proteins: Small Heat Shock Proteins (sHSPs) are found in all kingdoms of life, from archaea to humans. sHSPs are characterized by their low molecular weight (12–43 kDa) and they can assemble in dynamic oligomeric complexes of variable size (Vendredy et al. 2020; Carra et al. 2019). The human genome contains 10 different genes coding for 10 sHSPs- named HSPB1 to HSPB10 – and categorized as Class I or Class II, depending on their expression profile. Class I includes HSB1, HSPB5, HSPB6 and HSPB8. They are largely distributed in various tissues and are principally heat-inducible. This Class is implicated in cell sustainability in the face of stress factors. HSPB2-B4, HSPB7, HSPB9 and HSP10 are Class II chaperones; their expression profile is restricted to one or a few cell types and they are thought to play a major role in tissue-specific functions, such as development and differentiation (Bakthisaran et al. 2015; Janowska et al. 2019). A comprehensive characterization of Class II human HSPBs is still lacking and most of our knowledge is based on experimental data obtained using sHSPs from other organisms or human Class I HSPBs. Thus, caution should be taken when generalizing the properties and functions of sHSPs, because they display differences in expression, activation, and substrate specificity, while of course sharing some structural and functional homologies.

The essence of sHSPs/HSPBs relies on their ability to interact with themselves and other members of the family, forming an ensemble of homo and hetero-oligomeric complexes that are highly dynamic. Their primary structure presents a highly conserved α-crystallin domain (ACD), which is preceded by an N-terminal region (NTR) of variable length and followed by a short C-terminal region (CTR). The ACD is a sequence of 90–100 amino acids and is organized in two β-sheets forming an immunoglobulin-like β-sandwich structure. In contrast, both the C- and N-terminal domains are intrinsically disordered, resulting in the lack of well-defined three-dimensional structures in the native conformation of these proteins. The C-terminal is less longer than 20 amino acids and only marginally conserved across species. Also, it contains the characteristic three-residue I*X*(I/V) motif, which is fundamental for the oligomerization process and for client identification. The N-terminal is

highly variable in sequence and length, and it seems to be implicated in the subunit exchange (Haslbeck et al. 2019; Van Montfort et al. 2001; Rajagopal et al. 2015; Sudnitsyna et al. 2012).

The ability to assemble in inter-converting oligomers and the presence of disordered domains provide a huge flexibility to the structure of sHSPs/HSPBs. Indeed, they enable to acquire different functional conformations related to the interaction with diverse substrates, allowing the binding to a huge range of targets and performing pleiotropic functions. For these reasons, they are considered as the first line of defence in response to exposure to stress conditions (Haslbeck et al. 2019; Carra et al. 2019).

Originally, sHSPs were defined as "holdases" according to their ability to prevent the formation of insoluble and irreversible protein aggregates; this term was used in opposition to "foldases", which are involved in (re)-folding of unfolded proteins, including the Hsp70 and Hsp90 chaperones (Carra et al. 2019). Indeed, sHSPs are ATP-independent chaperones and they do not display enzymatic function or ability to refold clients. In vitro, sHSPs interact directly with aggregation-prone misfolded proteins and form multimeric complexes. When associated to sHSPs, the substrate can be refolded with the cooperation of ATP-driven chaperones, such as the Hsp70 system, and co-factors. Thus, sHSPs can reversely sequester non-native and aggregate-prone intermediates into a state that is competent for refolding or for targeting to proteolytic systems, both proteasomes and autophagosomes (Niforou et al. 2014; Clare and Saibil 2013; Mogk and Bukau 2017). Interestingly, sHSPs have been recently found to be incorporated in proteinaceous inclusions (Muchowski and Wacker 2005); here, they exhibit their function in facilitating the disaggregation and packing the peptide into inclusions. According to these findings, sHSPs act not only as "holdase" chaperones, but they could exert an active role in the aggregation process, promoting the formation of aggregated species that are reversible and that the cells can dissociate or clear during the stress recovery phase, following exposure to e.g. oxidative stress and changes in temperature and pH. This activity is referred to as "sequestrase" activity and is evolutionarily conserved, from Escherchia coli to yeast, C. elegans and humans. Thus, according to the current model sHSPs confers cellular protection acting as holdases and sequestrases (Shrivastava et al. 2022; Carra et al. 2019).

In this PhD thesis we will focus on this class of chaperones. Although different groups demonstrated their efficacy in blocking the aggregation of misfolded species associated with neurodegenerative diseases, including e.g. α-synuclein that is associated with PD, we still do not fully understand their mechanism of action. On the one hand, HSPBs can potently reduce the α-synuclein fibril elongation, a specific step in its aggregation process (see result section, *chapter 4.1* for details) (Cox et al. 2018; Bruinsma et al. 2011), can prevent the amyloid deposition and mitigate α-synuclein mediated-toxicity in neuronal cells and *Drosophila* PD model (Cox and Ecroyd 2017; Lu et al. 2019; Tue et al. 2012); moreover, their expression level is upregulated in tissue brain of AD and PD patients, as well as in animal models of protein misfolding diseases (Iwaki et al. 1992; Renkawek et al. 1999; Webster et al. 2019; Seidel et al. 2012). On the other hand, mutations in the sequence of several HSPBs have been associated to neuromuscular diseases, suggesting that the loss of their function (and/or the

toxic gain of function of their mutated variants) has a direct negative impact on neuron viability (Vendredy et al. 2020).

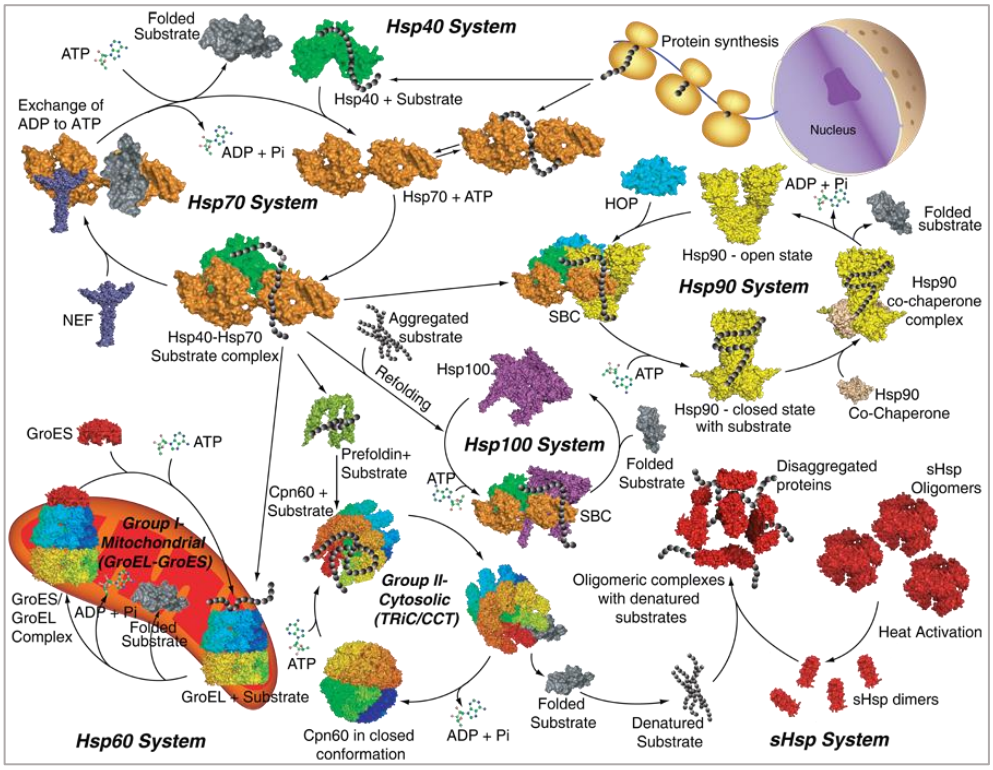


Figure 2: A functional HSP family network required for the maintenance of cellular protein quality control. From http://pdslab.biochem.iisc.ernet.in/hspir/chaperone.php.

All the molecular chaperones described above contribute to maintain proteostasis. Most of the newly synthesized polypeptides needs to fold into a defined three-dimensional structure to be functionally active, excepting for a remarkable fraction (~30%) of proteins with intrinsically unstructured regions that may acquire ordered structure only upon interfacing with partner substrates. Usually, the native protein conformations are thermodynamically stable, and the linear amino-acid sequence encodes all the required information to fold properly (Demarest et al. 2002; Dunker et al. 2008). However, due to the challenging environment, including the macromolecular crowding, the concentration of each given protein (which can be close to its solubility threshold) and the complex process of folding, proteins may adopt partially folded or misfolded conformations (Hipp et al. 2014). These partially folded or misfolded conformations have the great tendency to collapse into compact globular conformations, forming toxic aggregates that may be more thermodynamically stable than the native structure. In addition, misfolding-prone proteins can form β-sheet (amyloid) aggregates that can accumulate and also propagate from one cell to another (Figure 3) (Stefani and Dobson 2003).

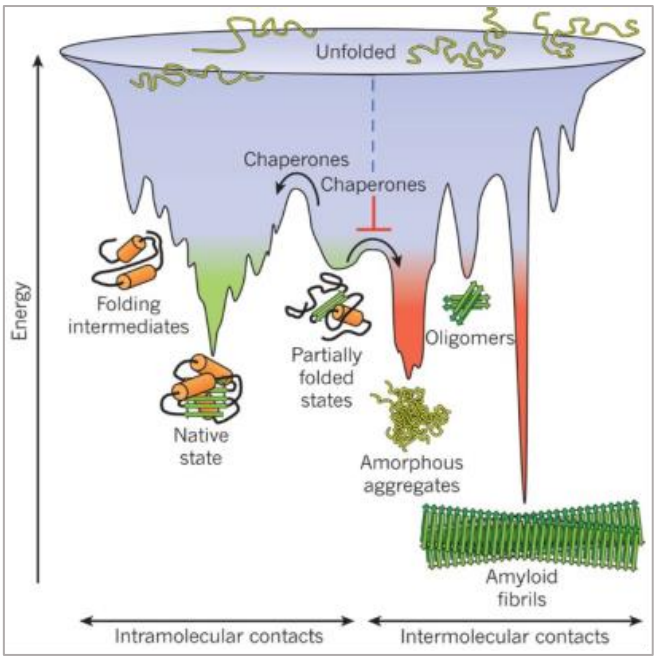


Figure 3: Scheme of the funnel-shaped free-energy surface that proteins explore as they move towards the native state (green) by forming intramolecular contacts. The ruggedness of the free-energy landscape results in the accumulation of kinetically trapped conformations that need to traverse free-energy barriers to reach a favourable downhill path. In vivo, these steps may be accelerated by chaperones. When several molecules fold simultaneously in the same compartment, the free-energy surface of folding may overlap with that of intermolecular aggregation, resulting in the formation of amorphous aggregates, toxic oligomers or ordered amyloid fibrils (red). Fibrillar aggregation typically occurs by nucleation-dependent polymerization. It may initiate from intermediates populated during de novo folding or after destabilization of the native state (partially folded states) and is normally prevented by molecular chaperones. From: PMID: 30733602.

Although it was originally believed that proteins can reach their native conformation spontaneously, it is now well established that they need molecular chaperones to fold efficiently and at the right biological timetable (Kim et al. 2013; Hartl 2017).Chaperone binding not only prevents or reverses intramolecular misfolding, but it may also block intermolecular aggregation. It has been suggested that chaperones can neutralize the aggregation process through different mechanisms (Hipp et al. 2019). Chaperones can interfere directly with amyloid-like formation at different kinetic steps by inhibiting completely the process or by changing the equilibrium between different aggregate species (Figure 3). These chaperones mediate their function by binding to hydrophobic sequences that are responsible to support aggregation. The HSP70-HSP40 system has been reported to block fibril elongation of a-synuclein, an aggregation-prone protein that is associated to Parkinson's disease; the TRiC chaperonin has been shown to promote the formation of larger and less toxic oligomers composed by the Huntington's disease associated protein huntingtin with an expanded polyglutamine tract (Kitamura et al. 2006; Tam et al. 2006; Behrends et al. 2006). Other chaperones are able to dissociate aggregates by ATP-dependent mechanism and they are referred to as "disaggregase" chaperones. In eukaryotic cells, the disaggregase activity is achieved through a sophisticated cooperation between Hsp70 and the nucleotide exchange factor HSP110. This Hsp70-Hsp110 system is coupled to degradation systems, since potentially toxic oligomers may be

generated from the disaggregation process (Mogk, Bukau, and Kampinga 2018; Nillegoda et al. 2015).

In order to get rid of the damaged species that cannot be refolded, cells need to selectively target them for degradation (Hipp et al. 2019). This is achieved through a complex cooperation between chaperones, adaptor proteins, cofactors and the degradation machineries. Chaperones cooperate with the ubiquitin–proteasome system, both in the cytoplasm and nucleus and with E3 ubiquitin ligases, to mediate the ubiquitination tagging of the bound substrate, an essential step for the degradation of the client (Arndt et al. 2007; Tekirdag and Cuervo 2018). Several chaperones can also directly inject the damaged proteins inside the lysosome, via the LAMP1 receptor. This process is referred to as chaperone-mediated autophagy (CMA), see below (Cuervo and Wong 2014; Bandyopadhyay et al. 2010).

Chaperones can also cooperate with the autophagy pathway, to promote the autophagy-mediated clearance of both substrates, aggregated proteins and damaged organelles (Klionsky 2007).

In conclusion, molecular chaperones not only assist in the generation of protein molecules, but they also contribute to the elimination of proteins when they are potentially dangerous or no longer required by the cell (Clausen et al. 2019).

Degradation machineries

As briefly mentioned in the previous section, degradation machineries play a fundamental role in the cellular mechanisms for protein quality control and homeostasis. In cooperation with chaperone molecules, they maintain a stable and functional proteome through the elimination of altered proteins that cannot be refolded or rescued. The main intracellular protein degradation pathways include the ubiquitin-proteasome system (UPS) and the autophagy system. The UPS predominately degrades short-lived proteins and small soluble misfolded proteins, whereas autophagy preferentially degrades large insoluble protein aggregates and even whole organelles (e.g., mitochondria, ribosomes and even portions of the nucleus) (Li et al. 2022; Finley 2009; Kriegenburg et al. 2012).

Ubiquitin–proteasome system (UPS)

Ubiquitin is a small molecule that is required for both proteolytic and nonproteolytic functions in the cells. It functions as a tag that labels a given substrate protein for different fates: proteasome-mediated degradation (typically polyubiquitin chains), autophagosome-mediate degradation, but also e.g. DNA repair and surface receptor re-uptake for recycling or destruction (Grumati and Dikic 2018; Dikic et al. 2009).

The ubiquitin–proteasome system (UPS) is the major proteolytic mechanism that regulates protein degradation, and it is implicated in various cellular processes, such as protein quality control, stress

response, DNA repair and apoptosis. The UPS comprises more than 800 elements, including specific enzymes for marking abnormal substrates by conjugation to a chain of ubiquitin, and 26S proteasomes responsible for proteolysis of ubiquitin-tagged substrates. This conjugation is a multistep process involving the sequential actions of three different enzymes. Firstly, ubiquitin-activating enzyme (E1) hydrolyses ATP to generate a thioester-linked bond between its catalytic site and the C-terminal glycine residues of ubiquitin; then, the ubiquitin-conjugating enzyme (E2) receives the activated ubiquitin from the E1 enzyme and creates a new similar thioester intermediate with ubiquitin; finally, a specific ubiquitin ligase (E3) cooperates with the E2 enzyme to transfer the ubiquitin to the substrate. Repetition of this process allows to elongate the ubiquitin chain by attaching additional molecules to the first ubiquitin via internal ubiquitin lysine residues (Grumati and Dikic 2018; Hochstrasser 2009; Grimm et al. 2012). Furthermore, E3 enzymes are responsible for the substrate specificity by recognizing sequences that are exposed by non-native proteins; however, how the complete mechanism is achieved is not fully clear yet. There has been evidence that some E3s interact directly with the substrate, others count on substrate recruitment via chaperones or other adaptor factors. For instance, the human protein CHIP docks on HSP70 and HSP90 through its tetratricopeptide domain, exploiting the molecular chaperones as recruiting elements for non-native proteins (Lackie et al. 2017; Clausen et al. 2019).

The 26S proteasome is an ATP-dependent multi-complex, made up of one 20S core particle (20S CP) and one or two 19S regulatory particles (19S RP). The 20S CP is the functional core of the complex and contains four stacked heptameric rings with a central cavity, where proteolysis takes place. The outer two noncatalytic α rings provide the binding to 19S RPs, the identification of the target substrate, and the binding to regulatory elements, while the inner two β rings present three subunits with proteolytic activity (Höhn et al. 2020; Kj 2001; Jung and Grune 2012). The 19S particle has at least 18 subunits with chaperone-like activity, and a lid that enables to detect polyubiquitin tags. After polyubiquitination, the protein is recruited by specific sensors of 19S particles and located in the central cavity, in which occurs its degradation in short peptides (Bedford et al. 2010). Finally, ubiquitin recycling enzymes called deubiquitinating enzymes (DUBs) remove ubiquitin from substrates and disassemble polyubiquitin chains, allowing to recycling of ubiquitin molecules in new reactions. Importantly, ubiquitin molecules are limiting in the cells. Therefore, the function of DUBs, which regulate UPS-mediated degradation in different cellular contexts, is fundamental to ensure proper ubiquitin-dependent functions (He et al. 2016; Snyder and Silva 2021) (Figure 4).

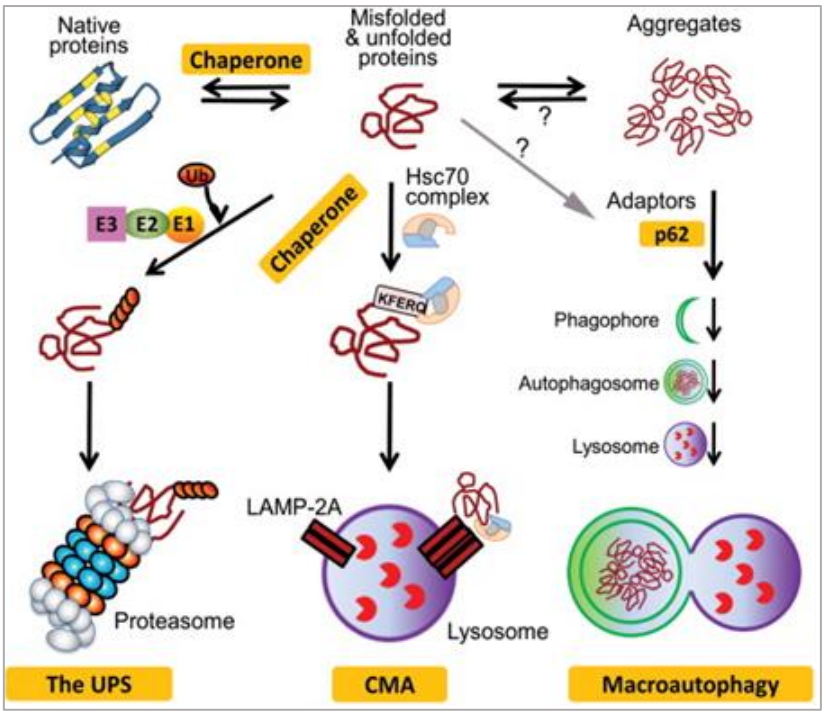


Figure 4: The protein quality control and the main degradation systems. Chaperones help fold nascent polypeptides, unfold misfolded proteins and refold them, and channel terminally misfolded proteins for degradation by the ubiquitin–proteasome system (UPS) or chaperone-mediated autophagy (CMA). When escaped from targeted degradation, misfolded proteins form aggregates via hydrophobic interactions. Aggregated proteins can be selectively targeted by macroautophagy to, and degraded by, the lysosome. From: PMID: 25092168.

Autophagy

Autophagy is a regulated degradative pathway in which cellular components are delivered to lysosomes for proteolytic degradation. Autophagy is a general term and different subtypes of autophagy have been identified. These include macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Klionsky 2007). Macroautophagy engulfs the cytoplasmic cargo within double-membrane vesicles, referred to as an autophagosomes, that fuse with lysosomes to originate an autolysosome, where the cargo is degraded through the action of acidic hydrolases.

Macroautophagy was initially considered to be a rather non-specific process. With time specific mechanisms that rely also on the cooperation between chaperones and adaptor proteins and enable the specific recognition of different cargoes have been identified, leading to the classification of different types of autophagy, based on the substrate and/or organelle engulfed; examples include aggrephagy, ribophagy, mitophagy, xenophagy that degrade, respectively aggregated proteins, ribosomes, damaged mitochondria and pathogens such as bacteria (Parzych and Klionsky 2014).

Microautophagy refers to a non-selective degradative process, where soluble intracellular substrates are enwrapped by autophagic tubes from the membrane. This mechanism can be classified by the morphology adopted to engulf the cargo (protrusion or invagination) and by location (the lysosome/vacuole or the endosome). Microautoghapgy is generally a non-selective process; however, under specific condition selective microautophagic pathways can be activated.

For instance, micropexophagy is a specific microautophagic pathway that engulfs damaged and/or superfluous peroxisomes (Oku and Sakai 2018; W. Li, Li, and Bao 2012).

Chaperone-mediated autophagy (CMA) is a process in which the cargo that contains a consensus CMA-targeting motif (KFERQ), is delivered across the lysosomal membrane. CMA requires the cooperation between a complex containing chaperones (such as Hsc-70), the co-chaperone Bag1, the CMA substrate and the lysosomal membrane receptor lysosomal-associated membrane protein 2A (LAMP-2A). During CMA, the chaperone bound substrate is unfolded in a Hsc70-dependent manner, injected inside the lysosome and degraded by the acidic proteases (Majeski and Dice 2004).

The biogenesis of autophagosomes is an intricate process that includes five key steps. (1) The formation of phagophore is triggered by the interaction of PIK3C3/VPS34 complex with endoplasmic reticulum (ER) or other membranes in response to differential stress signalling pathways. (2) The association between ATG5 and ATG9 ubiquitin-like proteins in a conjugation system (ATG12-ATG5-ATG16L1) is essential for phagophore membrane curvature and for engaging of the LC3 protein (microtubule-associated proteins 1A/1B Light Chain 3) and promoting its processing. (3) LC3 is firstly cleaved (LC3-I) and conjugated to phosphatidylethanolamine (PE) (LC3-II), then inserted in the extending phagophore membrane, where it plays a crucial role in both hemifusion of membranes and in selecting cargo for degradation. (4) The internalization of random or selected cargo completes the formation of the autophagosome. (5) Finally, the autophagosome fuses with the lysosome forming the 'autolysosome', where the proteolytic degradation of the cargo takes place by acidic lysosomal proteases (Glick et al. 2010; Chun and Kim 2018).

As briefly introduced, autophagy can selectively recognize specific cellular components, such as mitochondria, peroxisomes or lysosomes, or larger protein complexes, including proteasomes, or protein aggregates. The presence of adaptor proteins able to bind to LC3 through the LIR domain and with ubiquitinated proteins through UBDs, such as for example p62/SQSTM1, is essential in specifically targeting ubiquitinated proteins to autophagosomes (Figure 4 and 5) (Beese et al. 2020).

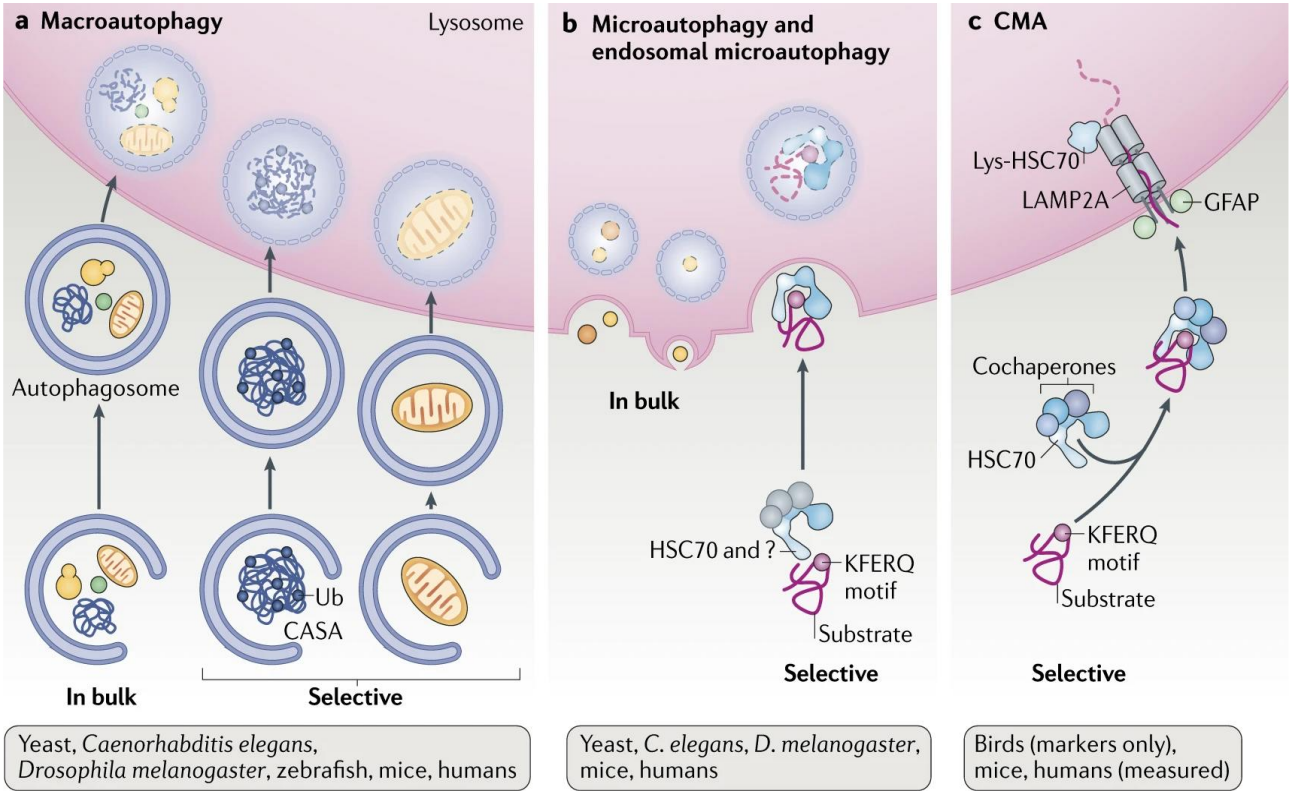


Figure 5: Autophagic pathways in mammals. a) Macroautophagy sequesters cytosolic cargo by a delimiting membrane that forms through conjugation of specific proteins among themselves and with lipids in a complex multistep process. The membrane then seals into an autophagosome that is trafficked by microtubules. Fusion of autophagosomes with lysosomes mediates degradation of the trapped cargo. Macroautophagy can be in bulk or selective depending on the cargo sequestered. b) Microautophagy entraps cytosolic cargo in small vesicles formed by invagination of the lysosomal membrane either in bulk or selectively via recognition and targeting by heat shock cognate 71 kDa protein (HSC70; also known as HSPA8) and cochaperones that are yet to be determined. c) Chaperone-mediated autophagy (CMA) involves the selective degradation of KFERQ-like motif-bearing proteins delivered to the lysosomes via chaperone HSC70 and cochaperones, such as carboxyl terminus of HSC70-interacting protein (CHIP), heat shock protein 40 (HSP40; also known as DNABJ1) and HSP70–HSP90 organizing protein (HOP), and their internalization in lysosomes via the receptor lysosome-associated membrane protein type 2A (LAMP2A). Bottom: evolutionary conservation of each autophagy pathway. CASA, chaperone-assisted selective autophagy; GFAP, glial fibrillary acidic protein; lys-HSC70, lysosomal HSC70; Ub, ubiquitin. From PMID: 29626215.

Heat Shock Proteins in Heat Shock Response

Heat shock proteins (HSPs) were originally described as a group of proteins expressed at high levels following organismal exposure to heat shock: their name derives from their serendipitous discovery by the Italian scientist Ferruccio Ritossa back in 1962 (Ritossa 1996) and was based on their molecular weight (Figure 2). It was later found that the expression of HSPs can be induced in response to a large variety of stress conditions, such as oxidative stress, cold, hypoxia, bacterial infections, exposure to heavy metals and toxins. HSP upregulation upon stress conditions was then shown to enhance the capacity of cells to recover from exposure to cytotoxic conditions (Richter et al. 2010; Piano et al. 2004). Later, it was shown that HSP can also be upregulated by physiological stimuli, and they also participate in processes linked to physiological cell function. Indeed, HSPs are not only expressed in response to external stimuli: HSPs account for 5-10% of total proteins in most cell types before stress condition occurs (Stetler et al. 2010). The induction of the expression of HSPs in response to multiple environmental and physiological stressors is generally referred to as the heat shock response (HSR). The HSR is evolutionarily conserved across diverse eukaryotes, due to its implication in physiological and developmental processes, as well as in the cellular stress response and underlying its crucial role for cell survival (Figure 6) (Feder and Hofmann 1999; Macario and Conway de Macario 2007b).

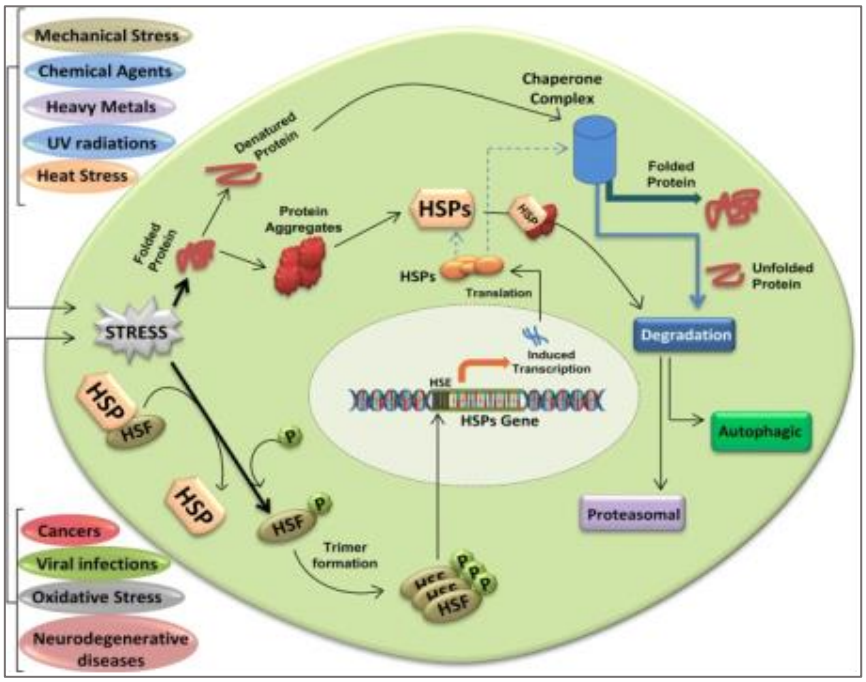


Figure 6: Molecular events and biological functions of different HSPs to maintain cellular homeostasis during stress conditions. Various kinds of stress elements induce denaturation of cellular proteins or formation of protein aggregates followed by transcription of different HSPs through activation of heat shock factors (HSFs). These HSPs form chaperone complex and refold the denatured proteins into the folded form or bind with protein aggregates/unfolded proteins and clear them through proteasomal or autophagic degradation. From: Kruthika Iyer et al., Cell Stress and Chaperones 2021. PMID: 34318439.

HSPs are classified as molecular chaperones and perform their chaperoning activities that range from peptide folding, assembling, and translocation, to guiding repairs, and the elimination of irreparable proteins (Miller and Fort 2018). Based on their expression, HSPs can be classified in two groups: constitutive or inducible, and ubiquitous or non-ubiquitous. Some HSPs are constitutively and ubiquitously expressed and act in housekeeping functions, such as e.g. Hsc70, or Hsp90β (HSP90 family), which is fundamental in the early embryonic development by modulating multiple pluripotency factors (Sreedhar et al. 2004). Other members are instead expressed only following specific stimuli and in a restricted number of cell types, such as e.g. testis-specific HSPB9 and HSPB10 (Kappé et al. 2003; Fontaine et al. 2003), and muscle-specific HSPB3, which is induced upon myoblast differentiation (Tiago et al. 2021; Sugiyama et al. 2000). HSPs can also act as cell cycle modulators in neuroectoderm induction and differentiation during neurogenesis (Miller and Fort 2018). For example, the heat shock 70 kDa protein 1, also termed Hsp72 or HSPA1A, is expressed under normal conditions and interacts with different transcriptional factors (HSF, c-Myc, pRb), nuclear receptors (steroid hormone receptors) and kinases (Raf, eIF2α-kinase, CyclinB1/Cdk1) to regulate their biological activity. Owing to the interaction with these clients, Hsp72 is central in several signal transduction pathways mediating cell homeostasis, cell cycle regulation and differentiation (Mayer and Bukau 2005). When endogenous or external stimuli occur, the expression of Hsp72 is induced to increase its levels and exert its cytoprotective functions as molecular chaperones against the stressors. Hsp72 acts in a coordinated manner in a complex with Hsp90, another molecular chaperones that can be found both in basal conditions and under stress conditions. Both Hsp70 and Hsp90 participate to housekeeping functions and play a critical role in the stress response (Rosenzweig et al. 2019). Taking the skeletal muscle system as an example, given the high expression levels of these chaperones in this tissue, we now recognize not only a function for Hsp70 and Hsp90 during muscle differentiation and development, but also during exercise, which leads to muscle damage, and during regeneration (Thompson et al. 2003; Senf 2013; Liu et al. 2006; Ubaida-Mohien et al. 2019; Dokladny et al. 2015). Thus, HSPs exert a plethora of functions and are involved during embryogenesis, tissue development, as well as tissue and organ maintenance in the adulthood (Figure 6).

Chaperones work in complex with other chaperones and co-chaperones; they also cooperate with enzymes or adaptor proteins that bridge the protein quality control with the degradation machineries, namely the ubiquitin-proteasome system and the autophagy system. Examples of such enzymes and adaptors are E3 ligases (e.g. CHIP) or p62/SQSTM1, which binds both to ubiquitinated proteins and to autophagosomes to facilitate the delivery of the chaperone-bound ubiquitinated substrate for clearance (McDonough and Patterson 2003; Yoon et al. 2021). Not only chaperones, but also co-chaperones can be expressed constitutively and can be inducible. The Bcl2-associated atherogenic 3 (BAG3) belongs to the family of BAG proteins (BAG1-BAG6) that are Hsc70/Hsp70 co-chaperones (Takayama et al. 1999). BAG3, as constitutive protein, is involved in tissue development and cytoskeleton organization, especially in resistance to mechanical stress in cardiomyocytes and skeletal muscle cells (Stürner and Behl 2017). It is instead induced in different cell types, such as leukocytes, epithelial and glial cells, after a wide range of stress factor, including oxidants, high temperature, serum deprivation, HIV-1 infection, electrophile stress, mediating cell adaptive responses through the promotion of selective autophagy (Rosati et al. 2011). It is also upregulated in neurodegenerative diseases characterized by protein aggregation, such as Alzheimer's and

Parkinson's disease, where it is thought to facilitate the autophagy-mediated clearance of aggregated and toxic species (Seidel et al. 2012).

The expression of HSPs, referred as the HSR, in both unstressed and stress conditions is tightly regulated to respond to specific cellular requirements. Tremendous progresses have been achieved in the last decades in understanding how HSPs are transcriptionally regulated, although some pivotal points of this complex system are still not well defined (Stetler et al. 2010).

Regulation of HSPs expression: the Heat Shock Factors

The heat shock factors (HSFs) are transcription factors that regulate the expression of the genes coding for the HSPs. HSFs bind to specific sequences called heat shock elements (HSEs) that are located in the promoter region of the target genes. In mammals, the HSF family comprises four members called HSF1, HSF2, HSF3 and HSF4, which exhibit both unique and overlapping functions. HSF1 and HSF3 are required for the induction of HSP upon stress conditions; in contrast, HSF2 and HSF4 regulate the expression of HSPs under resting and physiological conditions, as well as during cellular differentiation, development, or to maintain and enhance the innate immunity (Stephanou and Latchman 2011; Pirkkala et al. 2001). HSF4 seems to be highly expressed only in the brain and lung, and plays a well-characterized role in lens development, whereas HSF2 activity is crucial in the early embryogenesis stages of the heart, central nervous system, and testis (Tokunaga et al. 2022). The loss of HSF2 is linked with neurodegeneration, as exemplified by in vivo studies using the mouse model R6/2 of the Huntington's disease crossed with the HSF2-knockout mice. In particular, HSF2 deficiency shortens the lifespan of R6/2 Huntington's disease mice and enhances the accumulation of ubiquitylated misfolded proteins (Gomez-Pastor et al. 2018; Shinkawa et al. 2011). Concerning HSF3, it was originally described in avian species, and it was recently discovered in mouse, but not in humans. Finally, HSF1 is the most well studied member of the HSF family (Figure 7) (Stephanou and Latchman 2011; Neueder et al. 2017). We will thus describe more in depth the properties and activation of HSF1.

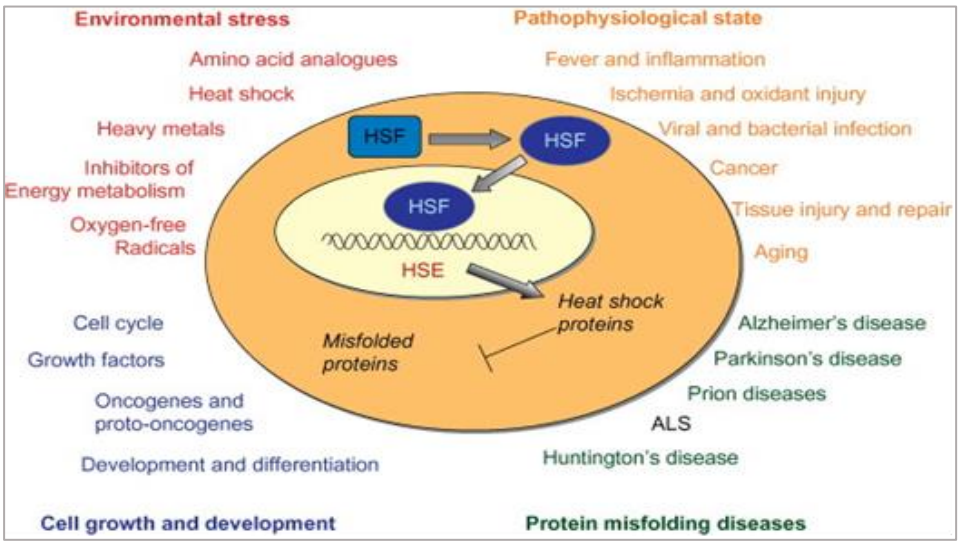


Figure 7: Conditions that activate the heat shock response. The expression of heat shock (stress responsive) genes is regulated by diverse exposures to environmental and physiological stress, conditions of cell growth, and development, in response to conditions of altered pathophysiological states, and protein aggregation diseases, that is, conditions that are associated with the appearance of misfolded proteins. The expression of heat shock proteins including molecular chaperones is regulated by the heat shock transcription factor HSF1. Molecular chaperones function to prevent and repair protein damage through interactions with the misfolded proteins. From: Morimoto et al., in Handbook of Cell Signaling (Second Edition), 2010. https://doi.org/10.1016/B978-0-12-374145-5.00268-0

Heat shock factor 1/HSF1

The HSF1 is the major transcriptional activator of HSPs in eukaryotes and it is constitutively expressed in most tissues and cell types. Knockdown or knockout of HSF1 in cells results in a deficiency of HSP expression after exposure to heat shock; this, in turn, correlates with increased vulnerability to apoptotic cell death after heat stress, highlighting the important protective role played by HSF1 upon stress (Morimoto et al. 1992).

Structurally, HSF1 is a multidomain protein. The N-terminal DNA-binding domain (DBD) is composed of three α-helices, a small two-stranded β-sheet, and a flexible loop or wing, that allows to bind to nGAAn motif of the heat shock element (HSE) in the promoter region of the target genes (Figure 8, panel a). The association of three HSF1 molecules into a trimer is required for high-affinity DNA binding and to stabilize the trimeric complex on the double helix of DNA (Figure 8, panel b) (Morimoto 2011; Neudegger et al. 2016). A flexible linker joins the DBD with the trimerization domain of two repeat regions (HR-A and HR-B), generating a coiled-coil triple leucine zipper that interacts with the same region of HSF1 protomers in the trimeric state. A heptad repeat region (HR-C) is thought to form a coiled coil with HR-A/B in the monomeric state, suppressing the HSF1 oligomerization. This region is followed by a highly flexible and disordered regulatory domain (RD) that is subject to PTMs and is mediating the activity of HSF1 (Figure 8, panel b). The C-terminal transactivation domain (TAD) contains two hydrophobic modules (AD1 and AD2) that are crucial for the responsiveness to stimuli. The flexible structure of both TAD and RD allows to interface with different target during activation and attenuation cycle of HSF1 (Åkerfelt et al. 2010; Morimoto 1998; Dayalan Naidu and Dinkova-Kostova 2017).

Several factors regulate the transcriptional activation of HSF1, such as its trimerization, its nuclear-cytosolic shuttling, multiple PTMs, phase transition and transient interaction with HSPs (chaperone titration) (Masser et al. 2020). In resting conditions, the mammalian HSF1 exists as inactive monomer in the cytoplasm, where its DNA-binding and transcriptional capacity are repressed at intramolecular and intermolecular levels (Figure 8, panel b). Both Hsp70 and Hsp90 bind to HSF1 monomers and act as repressors of HSF1, retaining it in the cytosol under unstressed conditions. Upon cellular insult, a quick cascade of events triggers the oligomerization of HSF1 to form DNA-binding active trimers (Figure 8, panel b). Upon proteotoxic stress conditions, the amount of non-native species increases. These non-native species compete with HSF1 for interaction with the chaperones. Hsp70 and Hsp90 dissociate from the complex with HSF1 to bind to the misfolded proteins, and, in turn, releasing HSF1 monomers to generate homotrimers (Kijima et al. 2018; Li et

al. 2017; Morimoto 1998). HSF1 contains a bipartite nuclear localisation signal and shuttles from the cytoplasm to the nucleus; instead, the active trimers are located in the nucleus, through mechanisms that are not completely clear. Two protein kinases, MEK and AMPK, have been hypothesized to promote or inhibit this process, respectively (Cargnello and Roux 2011). Numerous PTMs, including hyperphosphorylation, SUMOylation, acetylation, ubiquitination, and oxidation, are fundamental in regulating the transactivation capacity of HSF1 and drive the transcription of target genes by binding to their promoter. Such modifications are implicated in each step of the HSF1 activation cycle, providing both positive and negative effects. PTMs allow to guarantee an accurate and tight control of the HSR, instead of HSF1 being modulated by a simplistic on-off switch mechanism (Calderwood et al. 2010; Åkerfelt et al. 2010). The same PTM modification can have positive or negative impact on HSF1 activity, depending on the residue that is targeted. For example, hyperphosphorylation of HSF1 is required to enhance trimer stability and recruit the transcriptional machinery, thereby promoting transcription; yet, phosphorylation of specific residues such as S303 has been reported to inhibit trimerization (Masser et al. 2020) (Figure 8, panel b).

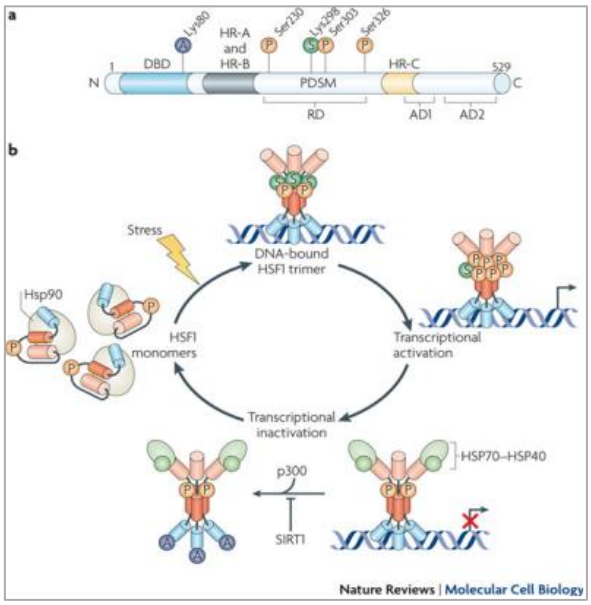


Figure 8: HSF1 undergoes multiple PTMs on activation. a) An overview of heat shock factor 1 (HSF1)-related post-translational modifications (PTMs). Some of the identified sites for acetylation (A), phosphorylation (P) and sumoylation (S) are indicated, as well as the phosphorylation-dependent sumoylation motif (PDSM). The DNA-binding domain (DBD) and the heptad repeats (HR-A and HR-B, and HR-C) are indicated as in, as well as the regulatory domain (RD) and activation domains (AD1 and AD2). b) The HSF1 activation and attenuation cycle, involving trimerization, multiple PTMs and feedback from heat shock proteins (HSPs). In the resting state, HSF1 is a monomer in both the cytoplasm and nucleus. Monomeric HSF1 is already a phosphoprotein under non-stress conditions, and it interacts with HSP90. On stress, HSF1 dissociates from the HSP90 complex, allowing HSF1 to trimerize and bind to the heat shock elements (HSEs) in HSP genes. Several PTMs, such as phosphorylation and sumoylation, are involved in regulating the transactivation capacity of HSF1. HSF1 acquires transcriptional activity, which is abrogated during the attenuation phase. Attenuation involves two regulatory steps: negative feedback from HSPs, which represses the transactivation of DNA-bound HSF1, and inhibition of DNA binding by the acetylation of Lys80 in the DBD of HSF1. The sirtuin SIRT1 regulates the attenuation phase of the heat shock response by preventing HSF1 acetylation42. From: Akerfelt et al., in Nat Rev Mol Cell Biol 11: 545-555. PMID: 20628411

Once the transcription of HSF1 target gene is activated, feedback inhibitory mechanisms are activated to fine tune the duration of the HSPs' induction and to attenuate the HSR, ensuring that the HSPs' induction is proportional to the stress signal. One factor that influences the duration of the HSG induction is represented by the number of HSEs in a given promoter region. In addition, another important factor that regulates the duration of the HSR is represented by the levels of chaperones; the high levels of chaperones, which accumulate as a result of the induction of the HSR, inhibit the HFS1 activity. By binding to its DNA-binding domain, chaperones release the HSF1 molecules from the promoter of HSP genes (Figure 9) (San Gil et al. 2017).

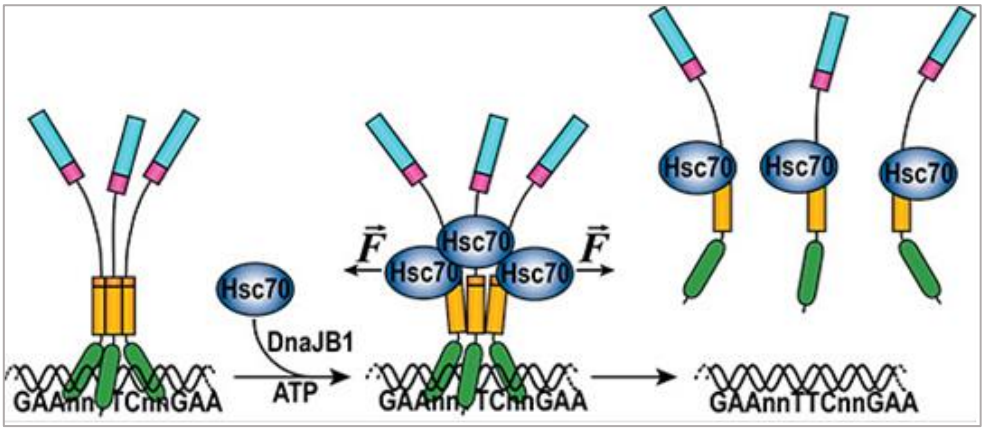


Figure 9: Hsp70 system attenuates the HSR and restores the resting state, by monomerizing trimeric Hsf1 and thus dissociating it from DNA. From: Kmiecik, Le Breton, Mayer, EMBO J 2020.

Another mechanism that can contribute to regulate HSF1 activity and turnover, thereby influencing the duration of the HSR is represented by post-translational modifications (PTMs). All known HSFs can be modulated through several PTMs such as e.g. phosphorylation, acetylation, ubiquitination and SUMOylation (Yan-Ming et al. 2012). For a detailed description of how PTMs regulate HSF activity please see Masser et al., 2020 (https://www.sciencedirect.com/science/article/pii/S001448272030495X)

Here we briefly summarize the mechanisms that regulate HSF1 activity. According to the traditional model, the common primary signal that activates the HSF1 is the increase in the amount of non-native proteins that accumulate upon stress and that bind to chaperones, leading to their dissociation from HSF1, which can undergo trimerization and nuclear translocation: this process is often referred to as chaperone titration(Prince et al. 2020). However, it seems that proteotoxicity is only associated with severe heat shock, whereas mild heat shock and other stimuli that can still induce the HSR do not appear to be coupled to protein denaturation, suggesting that cells perceive the stress through different mechanisms or "sensors" and have mechanisms in place that would not depend on chaperone availability to activate the HSR. Overexpression of SSA2, a member of the HSP70 family in Saccharomyces cerevisiae and a well-known protagonist in the feedback control of HSFs, does not prevent yeast from responding to heat shock and from HSPs induction, supporting the existence of multiple pathways and mechanisms leading to the activation of the HSFs and more generally regulating the HSG transcription (Nagy et al. 2007; Hjorth-Sørensen et al. 2001). Recent

experiments performed by Hentze et al. showed that upon heat shock the conformation of HSF1 changes. A domain of HSF1 itself unfolds, while the region involved in the formation of the active trimer becomes more stable. Therefore, the HSF1 protein itself can function as a temperature sensor that directly perceives changes in the temperature and responds to them, without the help of any chaperone (Hentze et al. 2016). Thus, changes in the conformation of HSF1 upon temperature increase seem to regulate its activity, along with PTMs and binding to chaperones, providing a very complicated scenario. It is likely that these different mechanisms coexist and can be interchangeably used by the cells or, rather can be used based on the duration and severity of the stress. In fact, as mentioned above, the expression of HSPs via HSFs can also be activated upon mild heat shock that does not induce massive protein misfolding (Zou et al. 1998; Kmiecik and Mayer 2022).

Role of cell membranes in the regulation of the HSR

Although intense research over the past decades allowed us to underpin the mechanisms regulating HSF activity, we still poorly understand how the cells perceive the change in the temperature, as well as other perturbations, and how the signal cascades are integrated to up-regulate HSPs via HSF1 activation.

Besides recent data showing that HSF1 itself can act as a temperature sensor (Hentze et al. 2016), increasing findings suggest that alterations in the fluidity of cell membranes may be one of the starting events that trigger specific signalling pathway/s and ultimately induce the expression of HSGs (Balogh et al. 2005). During the heat stress, the membrane quickly modifies its viscosity and architecture. Changes of the composition and physical properties of the membranes activate signalling pathways that modify gene expression to maintain lipid homeostasis; these signals could also activate the transcription of HSGs (Vigh et al. 1998; Sunshine and Iruela-Arispe 2017). Before discussing how changes in membrane fluidity may be perceived by the cells and may activate the expression of HSPs, we will briefly summarize key properties of the biological membranes.

Structure of the plasma membrane

The cell membrane provides the structural framework that separates and protects cells from the external environment; inside the eukaryotic cells, membranous organelles allow to compartmentalize the space, providing specialized microenvironments to fulfil distinct biological processes (along with the so-called membraneless organelles or biomolecular condensates that originate via liquid-liquid phase separation) (Banani et al. 2017). The plasma membrane consists of a lipid bilayer and proteins that can either cross entirely the lipid bilayer (integral membrane or transmembrane proteins) or interact with lipids along either side of the two leaflets (peripheral membrane proteins). Biological membranes are thus a fluid mosaic of components: lipids and proteins (Feigenson 2007; Manna et al. 2019). The lipid composition of membranes influences which types of proteins will associate with the lipids and regulates membrane receptor partitioning,

directly participating in and modulating signalling processes (Manna et al. 2019; Sunshine and Iruela-Arispe 2017).

Phospholipids, glycolipids, and sterols are the three main types of lipids found in biological membranes (Escribá et al. 2008). Phospholipids are the most abundant lipid type within the membranes: they contain a polar head group and two hydrophobic tails, consisting of fatty acids. One tail is usually unsaturated with one or more cis-double bonds, whereas the other tail is saturated. Variations in the length and saturation of the fatty acid tails have a significant impact on how tightly phospholipid molecules may pack against one another, thus influencing the fluidity of the membrane (Watson 2015). The amphipathic nature of phospholipids enables them to spontaneously form bilayers in aqueous environments. In this most favourable organization, the hydrophilic heads occupy the outer surface of each layer towards the aqueous environment, while the hydrophobic tails are sheltered from the water in the interior of the bilayer, facing each other in a lipid environment (Spector and Yorek 1985; Alberts et al. 2002).

Two general properties of phospholipid bilayers are essential to membrane function. Firstly, the structure of phospholipids provides the function as barrier between two aqueous compartments. Since the hydrophobic fatty acid chains reside in the inner side of the phospholipid bilayer, the membrane is impermeable to water-soluble molecules, such as ion and polar molecules, allowing to be selective permeable and control the molecular exchanges. Secondly, the double tails of fatty acids create a viscous structure, which allows them to move freely in the interior of the membrane and influence the physical state of the membrane. In addition, both phospholipids and proteins are able to diffuse laterally inside the membrane, which is a crucial feature for several membrane functions (van Meer et al. 2008; Cooper 2000; Singer and Nicolson 1972).

Phospholipid bilayers can adopt diverse phase of states depending on environmental factors, such as temperature, hydration, and pressure, and structural characteristics, including the lipid composition and the length of the hydrocarbon tails (Figure 10A). Usually, at low temperature the bilayer is in a solid phase – also known as “gel phase” – in which the hydrocarbon tails are highly ordered. The gel phase changes into a lamellar gel phase following heating (Kranenburg and Smit 2005). The hydrocarbon tails still exhibit a high degree of order, and the bilayer is more hydrated. At higher temperatures the gel phase transforms into the most disordered state, named liquid crystalline phase or fluid phase. In general, a lipid bilayer can exist in either a liquid or a solid phase at a certain temperature. Every lipid has a crucial temperature at which it converts from the gel to the liquid phase. In both phases, lipid molecules are confined to the two-dimensional plane of the membrane; however, the molecules are allowed to diffuse freely in the liquid phase bilayers. As a result, lipids can quickly move to different positions in a liquid-bilayer phase and migrate over the membrane through a process named random walk (Alberts et al. 2002; Casal and Mantsch 1984; Kranenburg and Smit 2005; Goñi 2014).

Besides phospholipids, the plasma membranes of mammalian cells also contain glycolipids and cholesterol. The glycolipids consist of two hydrocarbon chains linked to a polar head group

containing carbohydrates and they account only for 2% of the lipids of most plasma membranes. Cholesterol is a fundamental constituent of animal cells, and it is characterized by a hydrophilic region, a four-ring steroid central nucleus and a short hydrocarbon chain (Singer and Nicolson 1972). Owing to its rigid ring structure, cholesterol is crucial in determining the membrane structure, modulating both fluidity and permeability of the membrane. At high temperature, cholesterol partially immobilizes the fatty acid chains, providing lower permeability and less fluidity to the outer side of the membrane. However, cholesterol has opposite effects at low temperatures or at high concentration. By interfering with interactions between fatty acid chains, cholesterol prevents membranes from freezing and it inhibits possible phase transitions by preserving membrane fluidity (Krause and Regen 2014; Hac-Wydro and Wydro 2007).

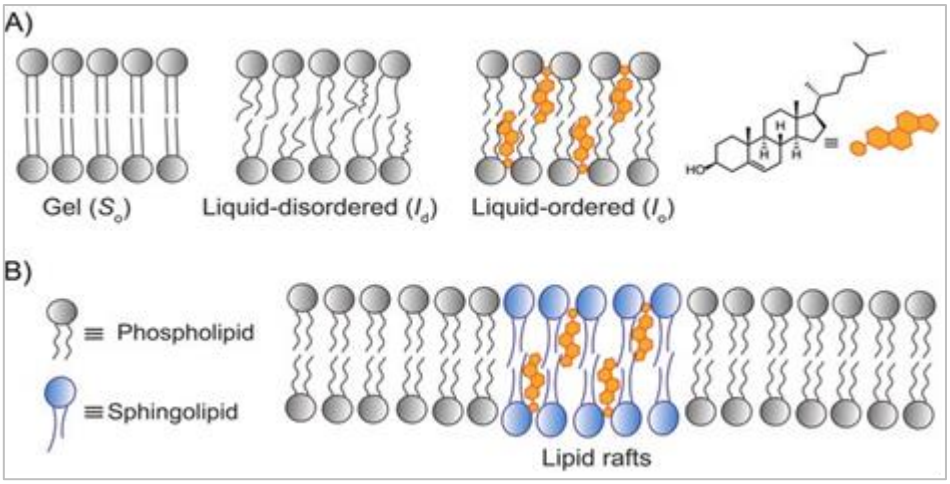


Figure 10. Schematic presentation of A) the gel phase, liquid-disordered phase, and liquid-ordered phase of phospholipid membranes, and B) membrane phase separation, leading to the formation of lipid rafts. From: Barba-Bon et al., in Chembiochem 2020. https://doi.org/10.1002/cbic.201900646

Membrane as platforms for cell signalling: lipid rafts

Membranes are characterized by heterogeneity in terms of their lipid and protein content. As mentioned before, a large variety of proteins are inserted inside the lipid bilayer and contain one or more membrane-spanning domains (referred to as transmembrane proteins); additional peripheral proteins interact with the outer or inner membrane layers. Transmembrane proteins include channels, receptors, and enzymes. Peripheral proteins are amphipathic proteins that can be directly anchored to the lipids; they are involved in various functions, from lipid transport to membrane signaling (Monje-Galvan and Klauda 2016).

Membrane composition is not uniform in terms of lipids and proteins and is organized in submicroscopic microdomains: the nature of lipids influences the types of proteins that can associate with a specific membrane microdomain (Laude and Prior 2004). Examples of these membrane submicroscopic microdomains are the lipid rafts, planar lipid rafts, also known as non-

caveolar, and caveolae (Figure 10B and 8). Lipid rafts (10–200 nm) are enriched in both sphingolipids and cholesterol. Planar rafts are continuous non-invaginated membrane domains without distinctive morphological characteristics, while caveolae are flask-shaped invaginated membrane structures generated by polymerization of caveolin proteins, which is a transmembrane palmitoylated protein with a hairpin-like structure that binds to cholesterol (Figure 11) (Pike et al. 2002; De Laurentiis et al. 2007). Lipid rafts and caveolae have received a lot of attention in recent years because of their implication in cell signalling, although their existence has long been controversial.

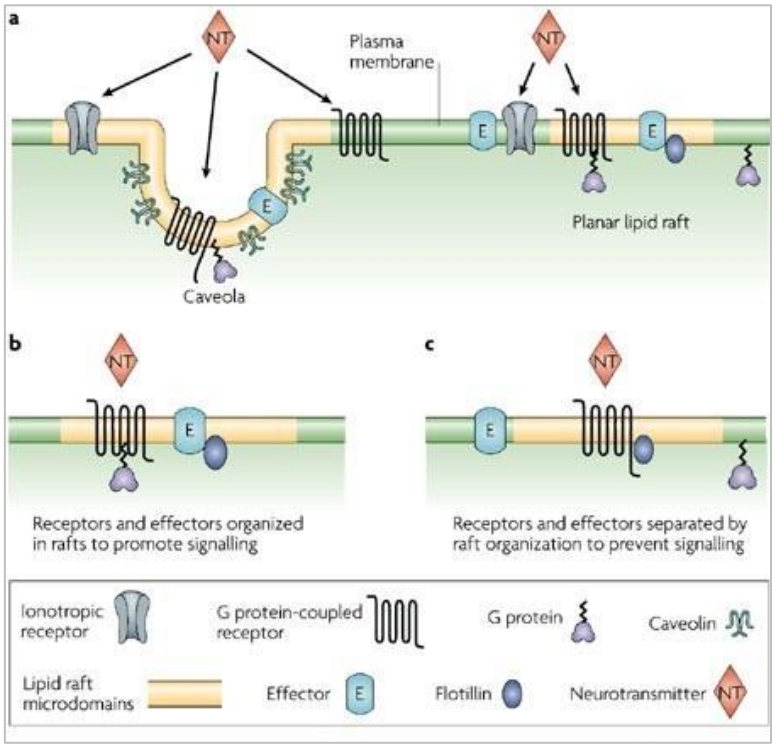


Figure 11: Planar lipid rafts and caveolae. Lipid rafts are cholesterol- and sphingolipid-enriched, highly dynamic, submicroscopic (25–100 nm diameter) assemblies, which float in the liquid-disordered lipid bilayer in cell membranes. a) There are two common raft domains in mammalian cells: planar lipid rafts and caveolae. Both possess a similar lipid composition. Planar rafts are essentially continuous with the plane of the plasma membrane and lack distinguishing morphological features. By contrast, caveolae are small, flask-shaped membrane invaginations of the plasma membrane that contain caveolins. Caveolin molecules can oligomerize and are thought to be essential in forming these invaginated membrane structures. Many neurotransmitter receptors (both ionotropic and G-protein-coupled), G proteins, and signalling effectors such as second-messenger-generating enzymes are found in lipid rafts. Neurotransmitters might activate receptors that are located both within and outside lipid rafts. b) The lipid raft signalling hypothesis proposes that these microdomains spatially organize signalling molecules at the membrane, perhaps in complexes, to promote kinetically favourable interactions that are necessary for signal transduction. c) Alternatively, lipid raft microdomains might inhibit interactions by separating signalling molecules, thereby dampening signalling responses. From: Allen et al., Nature Reviews Neuroscience volume 128–140 (2007). https://doi.org/10.1038/nrn2059

A large body of evidence confers to lipid rafts a crucial role in regulating the cell signalling, providing a distinct micro-environment for the functionality of receptors and intracellular molecules. In general, lipid rafts can selectively recruit to or exclude from the plasma membrane certain signalling proteins, kinases and phosphatases in response to ligand activation (Figure 11). In doing so, the lipid rafts serve as centralized platforms that can enhance or decrease the accessibility of the recruited proteins to effector molecules, facilitating or inhibiting the activation of a given signalling pathway. Additionally, lipid rafts can protect signalling molecules from the inhibitory effect of non-raft components (Simons and Ehehalt 2002; Li et al. 2021;). Although experimental evidence supports the notion that small changes in protein partitioning into a lipid raft can trigger signalling cascades, the dynamic features of initial lipid raft association is not fully understood (Simons and Toomre 2000; Varshney et al. 2016).

Considering how small changes in membrane microdomains can influence the activation of cell signalling pathways and the functionality of transmembrane proteins and because environmental stressors such as heat shock can change membrane fluidity, it is conceivable to think that changes in lipid microdomain properties, such as gel-liquid state transitions (Figure 10A), could be perceived by the cells and used as a signal to trigger the HSR. In other words, membranes could function as thermosensors.

Plasma membrane as a "cellular thermosensor"

Organisms are constantly exposed to dynamic changes of the environment and their survival depends on their ability to adapt rapidly to such changes. Cells need strategies to sense the fluctuations in temperature and respond properly by promoting physiological responses to maintain their functional integrity. The functions of the lipid bilayers are known to be strongly affected by their physical state, making them receptive to changes in e.g. the environmental temperature (Mansilla et al. 2004; Los and Murata 2004).

As mentioned earlier, lipid bilayers exist in different phases: liquid, or liquid disordered, liquid crystalline and solid/gel phases (Figure 10A) (M'Baye et al. 2008). Temperature is one important factor that can change the transition of lipids from the liquid to the ordered gel phase and the temperature of transition changes based on lipid membrane composition. Lipid composition of course determines whether at a given temperature the membrane will exist as a liquid or a gel; cholesterol, for example, tends to inhibit possible phase transitions (Redondo-Morata et al. 2012).

How changes in the temperature affect membrane structure and the behaviour of lipid bilayers has been widely studied in different organisms, including bacteria, cyanobacteria, fungi, plants, and fish (Cossins et al. 1978; 1978; Horváth et al. 1998; Inaba et al. 2003). It has been shown that the membrane lipid bilayers need to be mostly fluid to perform proper cell functions, and the lipid bilayers of the majority of organisms are found completely or largely in a fluid state at physiological

temperature (Mendoza 2014). Membrane fluidity decreases upon reduction in temperature, leading to a “rigidification” of membranes that impairs their physiological functions. By contrast, high temperatures provoke the fluidification of membranes, disrupting the lipid bilayers (Fan and Evans 2015; Mansilla et al. 2004). Consequently, it is of crucial relevance to modulate the membrane fluidity in response to temperature shifts. This can be achieved by modifying the composition of the membrane bilayers, a process referred to as membrane remodelling. In particular, the temperature-induced remodelling of membranes is known as homeoviscous adaptation and is aimed at preserving their biophysical characteristics. Homeoviscous adaptation is an evolutionally conserved acclimatory response and it has been observed throughout several poikilothermic animals (e.g. fish, amphibians, reptiles and many invertebrates) exposed to different ambient temperatures. Homeoviscous adaptation is thus crucial for thermal regulation (Figure 12) (Ernst et al. 2016; Vigh et al. 2007). Although temperature is the best characterized environmental factor that modifies membrane viscosity, other physiochemical factors can perturb membrane fluidity, including hydrostatic pressure and various chemical effectors such as solute profile, ionic strength, and pH (Los and Murata 2004).

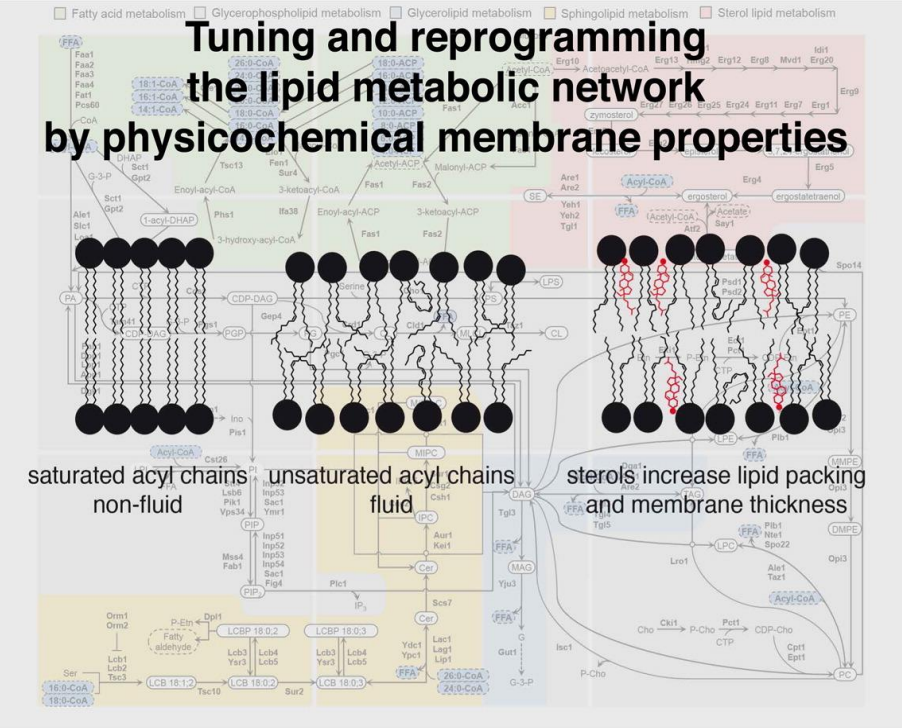


Figure 12: Homeoviscous adaptation and membrane homeostasis. Physical membrane properties and biosynthesis of membrane lipids are interdependent. Tailored production of lipid molecules ensures membrane homeostasis. From: Ernst R, et al. J Mol Biol. 2016. https://doi.org/10.1016/j.jmb.2016.08.013

Mass spectrometry-based lipidomics enabled to obtain accurate information on the lipid remodelling of living membranes during adaptive responses (Ernst et al. 2016). One of the most characterized responses to low temperature is the increase in the amount of unsaturated fatty acids in the cell membranes. Such adaptation is mediated by the induction of fatty acid desaturase enzymes, which are responsible to introduce *cis* double bonds at specific position into pre-existing

fatty acids, promoting the increase of membrane fluidity. As a result, the physical state of the membrane is restored to its original state. Cyanobacteria are especially well-suited for studying this adaptational mechanism because the amount of unsaturated bonds in their fatty acids can be modified easily by genetic manipulation. Indeed, it has been shown that the inactivation of *desA* and *desD* genes encoding for desaturase enzymes in *Synechocystis* sp. PCC 6803 leads to a drastic decrease in membrane fluidity, resulting in a loss of the ability to adapt to low temperatures. Additionally, when the *Synechocystis desA* gene encoding for Δ12 desaturase was inserted in the genome of *Synechococcus* sp. PCC 7942, a remarkable increase of unsaturated fatty acids was measured in the transformed organism, which was related to an increased tolerance at low temperature (Wada et al. 1990). In addition, the saturation of acyl chain lipids of the membrane by catalytic hydrogenation at isothermal conditions resulted in the activation of transcription of the *desA* gene, which normally is induced by cold stress. The catalytic saturation conditions affected only the plasma membrane, which acts as a thermosensor and influences the expression of low-temperature-inducible genes, such as genes coding for desaturases. However, it is still elusive how a perturbation in membrane fluidity is sensed and how the signal is transduced from the membrane to chromosomes to activate the expression of low-temperature-inducible genes (Vigh et al. 1993; Horváth et al. 1998).

Modifications in the lipid composition have been also associated with adaptational response to elevated temperatures, which increase the membrane fluidity and undermine the lipid bilayers integrity (Los and Murata 2004). At high temperature *Escherichia Coli* synthesizes an increased amount of saturated fatty acid to compensate for the hyperfluidization caused upon heat shock conditions. Such fluctuations in membrane fluidity are thought to be essential for triggering the regulatory responses that eventually result in acclimatization. The physical state of the membrane also affects the expression of HSGs induced by heat shock temperatures (Vigh et al., 1998). In fact, modification of the extent of saturated acyl chains in membrane of *Saccharomyces cerevisiae* influences the expression of the hsp70 and hsp82 genes (Carratù et al. 1996); this clearly identifies the existence of a link between membrane viscosity and the HSR.
Although the notion that cell membranes act as thermosensors is well-established, many questions remain to be answered regarding the mechanisms by which cells perceive the stimuli and transduce the signal to modulate the gene transcription. Because changes in the physical state of the membrane appears to be the primary signal upon temperature fluctuations, it is reasonable to assume that dedicated sensors are localized in the membrane to monitor its stability/viscosity. In this context, both integral membrane proteins and soluble proteins that can be recruited to specific cellular membranes have been identified as membrane sensors (Puth et al. 2015).

Integral proteins are embedded in the lipid bilayers through their transmembrane (TM) domains, which is ideally located in membrane to perceive alterations in the surrounding lipid bilayer. The TM domain consist of an α-helical secondary structure motif that is used for the integration within the membrane. The characteristic sequence of amino acids on one side of the TM helix (TMH) can confer a specific function to TM domains in oligomerization or lipid sensing (Lemmon and Schlessinger 2010). The most predominant motif is the GxxxG motif (G, glycine; x, any amino acid residue), which

is fundamental for the dimerization and allows for an intimate helix-helix association via non-polar van der Waals interactions further stabilized by backbone interactions (Senes et al. 2000). Although the mechanisms by which alterations in membrane properties affect the association of TMHs are still not fully determined, a well-known system used to study membrane sensing is represented by the DesK/DesR system in *Bacillus subtilis.* DesK functions as transmembrane membrane sensor, whereas DesR is a regulatory element to control the expression of lipid desaturase Δ5-Des. Upon low temperatures or increase of saturated fatty acids, the lipid packing of the membrane changes, leading to the alteration of the TM domain conformation of DesK, which switches from a kinase-active to a phosphatase-active state and regulates the lipid desaturase expression by modulating the activity of DesR (Figure 13) (Albanesi et al. 2004; Puth et al. 2015).

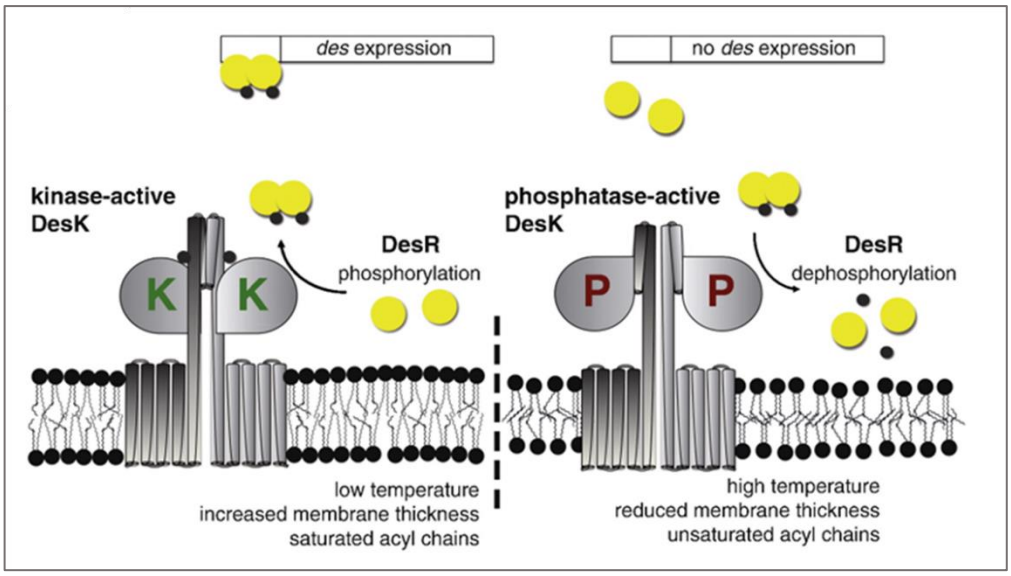


Figure 13. Sensing and regulating membrane viscosity. Schematic representation of the DesK/DesR system from B. subtilis regulating the expression of des encoding for a lipid desaturase. From: Ernst R, et al. J Mol Biol. 2016. https://doi.org/10.1016/j.jmb.2016.08.013

The class of soluble lipid sensors needs to be targeted to specific membranes to fulfil their functions. Thus, they can use different strategies aimed at recognizing the specific organelle to bind (Puth et al. 2015). Some lipid sensors can associate with species of lipids restricted to specific compartments, including phosphatidylinositol (PI) phosphates domain (Lemmon, 2008) or glycosylphosphatidylinositol (GPI) moieties (Paladino et al. 2004), while others can recognize lipid modifications, such as palmitoylation, that occur on the surface of membranes under certain conditions (Rocks et al. 2005). Another strategy consists of recognising the physicochemical features of membranes, comprising membrane curvature, charges density, or lipid packing (Hatzakis et al. 2009). Most of these sensors possess an amphipathic α-helix motif, which plays a crucial role in lipid and membrane sensing mechanisms (Magdeleine et al. 2016).

Eukaryotic cells contain different membranous organelles, each possessing membranes with distinct composition and properties, which confer them specific functions. This underlies the wide differences in the biophysical features of membranes and implies the existence of multiple sensing mechanisms and strategies (Figure 14).

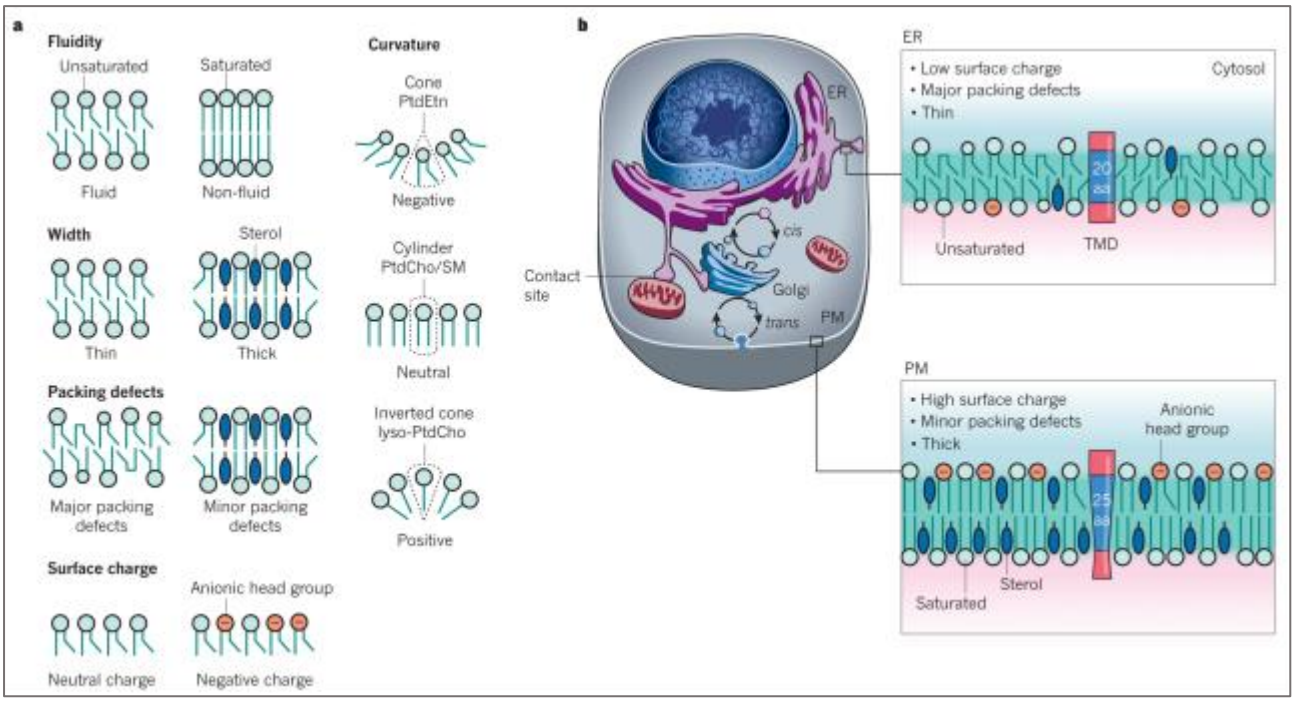


Figure 14: Differences between plasma membrane (PM) and endoplasmic reticulum (ER) membrane. TMD: transmembrane domain. a, Physical membrane properties are influenced by lipid composition. Fluidity is promoted by lipids with short, unsaturated fatty acids. The double bonds introduce kinks that lower the packing density of the acyl chains and inhibit transition of the membrane from a fluid to a solid gel phase. Phospholipids typically pair a saturated with an unsaturated fatty acid, which is an elegant means to prevent the two types of acyl chains from phase separating when the temperature drops. Thickness is promoted by acyl-chain length and sterols, which order and stretch the acyl chains. High levels of packing defects are found in lipids with unsaturated acyl chains and small head groups. Surface charge is determined by the presence of anionic lipids such as phosphatidylserine and phosphoinositides. Curvature is determined by lipid shape. Lipids with a small area ratio of polar head to acyl chain (creating a cone shape) induce negative curvature, lipids with an equal head to chain ratio (creating a cylinder shape) are neutral, and those with a much larger head compared with the acyl chain area (creating an inverted cone shape) induce a positive curvature. PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; SM, sphingomyelin. b, the endoplasmic reticulum (ER) has a thin bilayer, loose lipid packing and neutral cytoplasmic surface charge adapted for its biogenic function. The plasma membrane (PM) has a thick bilayer, tight lipid packing and negative cytoplasmic surface charge adapted for its barrier function. These contrasting physical properties are reflected in the length (20 aa and 25 aa, respectively) and geometry of transmembrane domains (TMDs) of ER and plasma-membrane-resident proteins. Membrane traffic between the ER and plasma membrane passes through the Golgi, a polarized and multi-cisternal organelle in which lipid sorting must occur to preserve the unique lipid compositions and, hence, specialized functions of the ER and plasma membrane. Note that lipid traffic can bypass membrane traffic at sites at which the ER contacts the trans-Golgi, plasma membrane or mitochondria. From: Holthuis JC, et al. Nature. 2014. PMID: 24899304

The endoplasmic reticulum (ER) is a cellular organelle implicated in fundamental biological processes, such as protein folding and secretion, calcium homeostasis and lipid metabolism. Several environmental and physiological insults can alter the ER homeostasis, resulting in the massive activation of the so-called ER-stress response and unfolded protein stress response (UPR) (Han and Kaufman 2016; Moncan et al. 2021). In mammalian cells the UPR is comprised of three signalling branches that are initiated by three ER transmembrane sensors, that include inositol-requiring enzyme 1 alpha (IRE1α), double-stranded RNA-dependent protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6). These sensors can perceive the accumulation of non-native proteins and, upon activation, they promote a complex transcriptional programme in order to restore the ER homeostasis (Figure 15) (Kono et al. 2017; Puth et al. 2015). In response to elevate amount of misfolding protein, the activation UPS ultimately leads to the augmentation of ER protein-folding ability by enhancing the expression levels of chaperones and foldases, the repression of protein translation, and the induction of ER-associated protein degradation (ERAD) of misfolded proteins (Hosoi and Ozawa 2009).

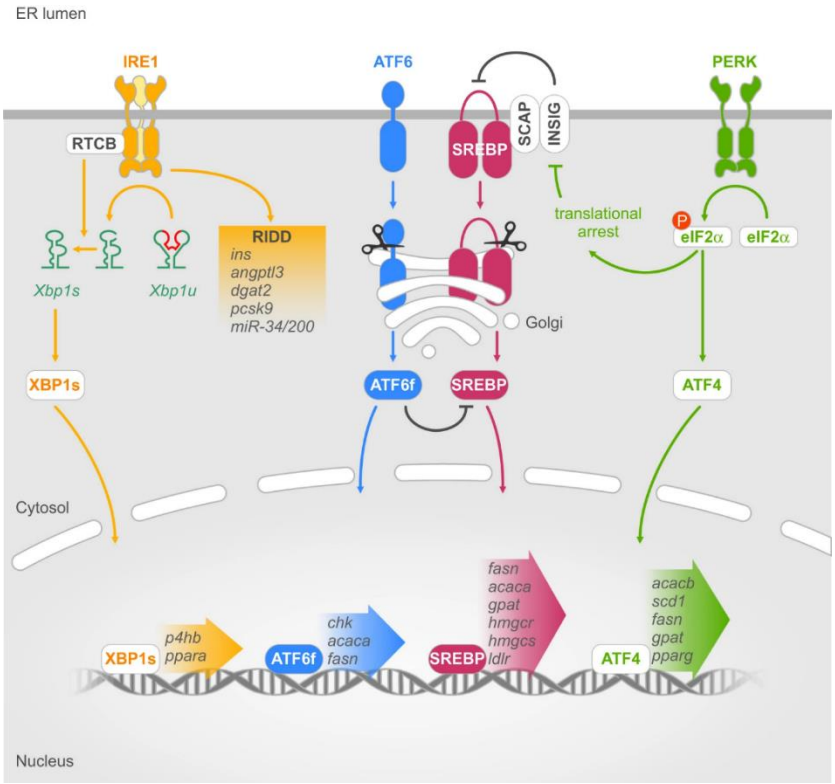


Figure 15. The Unfolded Protein Response (UPR) is controlled by three endoplasmic reticulum (ER) stress sensors: inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6) and PKR-like ER kinase (PERK). Upon activation, IRE1 splices x-box binding protein 1 (XBP1) mRNA, which is then ligated by RTCB and translated into XBP1s. IRE1 also cleaves cytosolic RNA in the process called regulated IRE1 dependent decay (RIDD), which reduces levels of target transcripts. Activated ATF6 is translocated to the Golgi, where its N-terminal fragment is released by proteases and translocated to the nucleus. PERK-mediated phosphorylation of eIF2α inhibits a global expression of genes, like SREBP activity-regulating Insig, and prompts selective translation of ATF4. The figure shows genes involved in lipid metabolism that are regulated by each of the three transcription factors, XBP1s, ATF6f and ATF4, produced by the UPR. From: Moncan et al., J Cell Mol Med. 2021 Feb; 25(3): 1359–1370. PMID: 33398919

Interestingly, although the UPR was originally described to survey the protein homeostasis in the ER, recent evidence has revealed that the ER stress/UPR activation is also crucial in maintaining the lipid homeostasis. Each of the three branches of the UPR were shown to affect the expression levels of key enzymes involved in lipid biogenesis. For instance, it has been found that IRE1α and its downstream mediator XBP1 promote the activation of genes implicated in fatty acid and cholesterol biosynthesis (Figure 16) (Basseri and Austin 2012; Moncan et al. 2021; Volmer and Ron 2015). Furthermore, ER-stress-dependent dysregulation of lipid metabolism was demonstrated to be implicated in various disorders, including dyslipidemia, insulin resistance, type 2 diabetes, and obesity. However, the exact mechanisms by which ER stress signalling pathways affect lipid homeostasis are not fully understood (Hosoi and Ozawa 2009; Engin and Hotamisligil 2010; Austin 2009; Kaplowitz et al. 2007). Next, the activating transcription factor 6 (ATF6), as well as the kinase PERK contribute to the regulation of lipid metabolism: ATF6 can inhibit the synthesis of cholesterol, while activates a lipid-specific transcriptional programme. PERK activates the lipogenesis through its downstream targets eIF2α, ATF4 and CHOP (Moncan et al. 2021).

There is a bidirectional relationship between ER stress and lipid metabolism. While activation of ER stress pathways can lead to lipogenesis and altered lipid homeostasis, lipids and abnormal lipid metabolism can also provoke ER stress. Indeed, UPR can be activated by signalling lipids, such as sphingolipids, and by potentially toxic excess of lipid classes including palmitate, stearate, and ceramides (Kono et al. 2017; Wei et al. 2006; Guo et al. 2007; Fu et al. 2011). Additionally, comparative analysis between proteomic and lipidomic studies of fractionated ER from normal and obese liver tissues have revealed an increase of enzymes implicated in lipid metabolism and a downregulation of protein biosynthesis genes in the obese ER proteome (Fu et al. 2011). This result suggests a crucial shift in the ER role, switching from representing the main site of protein synthesis to mostly fulfil lipid metabolism functions. Nevertheless, further investigations are required for a better comprehension of this intriguing interplay that support protein and lipid homeostasis in cells (Moncan et al. 2021; Basseri and Austin 2012; Han and Kaufman 2016).

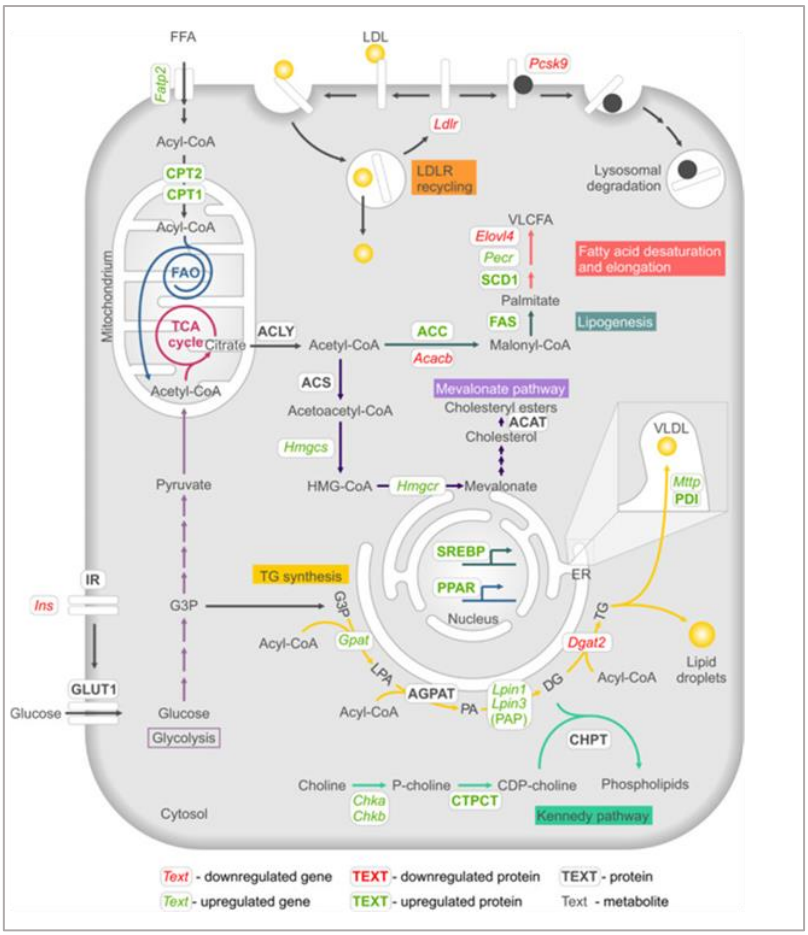


Figure 16. Overview of the major lipid metabolic pathways regulated by the UPR: Kennedy pathway, TG synthesis, lipogenesis, mevalonate pathway, fatty acid elongation and desaturation, fatty acid oxidation, tricarboxylic acid cycle, very low-density lipoprotein formation and low-density lipoprotein uptake. Only key nodes are shown. For further details, see the main text. Abbreviations: ACC, Acetyl-CoA carboxylase; ACACB, Acetyl-CoA carboxylase 2; ACAT, acyl-CoA:cholesterol acyltransferase; ACLY, ATP-citrate lyase; ACS, Acetyl-CoA synthetase; CoA, coenzyme A; AGPAT, Acylglycerol-P acyltransferase; CHKA, Choline kinase alpha; CHKB, Choline kinase beta; CHPT, Cholinephosphotransferase; CPT, Carnitine palmitoyltransferase; CTPCT, Phosphocholine cytidylyltransferase; DG, Diacylglycerol; DGAT2, DG acyltransferase; ELOVL4, Elongation of very long chain fatty acids-4; ER, Endoplasmic reticulum; FATP2, Fatty acid transport protein 2; FFA, Free fatty acid; FAO, Fatty acid oxidation; FAS, Fatty acid synthase; G3P, Glycerol-3 phosphate; GLUT1, Glucose transporter 1; GPAT, Glycerol-P acyltransferase; HMGCR, HMG-CoA reductase; HMGCS, HMG-CoA synthase; Ins, insulin; IR, insulin receptor; LDL, Low-density lipoprotein; LDLR, LDL receptor; LPIN, Lipin; LPA, Lysophosphatidic acid; MTTP, Microsomal TG-transfer protein complex; PA, phosphatidic acid; PAP, Phosphatidic acid phosphohydrolase; PCSK9, Proprotein convertase subtilisin/kexin type 9; PECR, Peroxisomal trans-2-enoyl-CoA reductase; PDI, Protein disulphide isomerase; PPAR, Peroxisome proliferator-activated receptor; SCD1, Stearoyl-CoA desaturase-1; SREBP, Sterol regulatory element-binding protein; TCA, tricarboxylic acid cycle; TG, Triglycerides; VLCFA, Very long chain fatty acids; VLDL, Very low-density lipoproteins. From: Moncan et al., J Cell Mol Med. 2021 Feb; 25(3): 1359–1370. PMID: 33398919

Plasma membrane as sites for the activation of the HSR.

Mild heat stress, as it occurs when a subject experiences fever, causes rearrangements of plasma membrane microdomains and also induces the expression of HSGs. Whether and how these events

are connected has been neglected for a long time. To address this question, researchers have postulated the so-called “membrane sensor” or “membrane thermosensor” hypothesis (Török et al. 2014; Vigh et al. 1993; Laszlo et al. 1998). The “membrane thermosensor” hypothesis postulates that the heat shock can lead to the reorganization of membrane structure and fluidity; it also assumes that specific sensors can perceive these changes and convert them into the activation of the HSR. In other words, the modification of the lipid composition and of the physical state of membranes are determining factors in the transduction of stress signals that induce the transcriptional activation of HSGs. In turn, the association between HSPs and specific membrane domains could allow to remodel the physical state of the membrane, restoring its fluidity and stability during the heat shock. As a result, HSPs would contribute to restore the biophysical properties of membranes, promoting the deactivation of the signal that perturbs the membrane, and repressing the induction of HSGs in a highly sensitive feedback loop (Török et al. 2001b; Maio and Hightower 2021).

In support of this hypothesis, mammalian cells exposed to certain membrane fluidizers or drugs that can interact with specific membrane lipids significantly activate the expression of HSGs (Balogh et al. 2005; Tóth et al. 2014; Török et al. 2003; Gombos et al. 2011). In particular, the two structurally unrelated compounds benzyl alcohol (BA) and heptanol (HE) can induce the HSR in K562 erythroleukemic cells treated at normal growth temperature, as demonstrated by the analysis of the increasing synthesis of Hsp70. The increased Hsp70 mRNA expression achieved at physiological temperature upon application of these membrane fluidizers was similar to the one obtained following exposure of the cells to heat stress at 42°C (Nagy et al. 2007; Balogh et al. 2010; 2005). Importantly, at doses that triggered the expression of HSPs, none of the two compounds caused measurable protein unfolding, suggesting that it is improbable that the activation of the HSR in treated K562 cells is triggered by protein denaturation events (Balogh et al. 2005) and strongly suggesting that membrane perturbations are sensed by the cells and can trigger the expression of HSGs.

Following the identification of these two compounds also the hydroxylamine derivative bimoclomol (BM) was identified as another membrane fluidizer that can activate the HSR. More precisely, BM cannot trigger the induction of HSGs without a concurrent stress; yet, in presence of BM, the stress-induced response is boosted owing to the synergic effect of the compound. Due to its properties, BM has been classified as a co-inducer of the HSR. Because BM does not influence protein denaturation in cells and its action is exerted by interacting with specific domains of the membrane, this implies that the membrane perturbation caused by BM is the initial signal sensed to trigger the expression of HSGs (Török et al. 2003). Taken together, these findings led to the formulation of the ‘‘membrane thermosensor’’ hypothesis, which predicts that one of the triggering stress signals that regulates the HSR is a change of the physical state of cell membranes (Tóth et al. 2014; Balogh et al. 2005; Török et al. 2014).

This mechanism of activation of the HSR could occur in parallel to the direct activation of the HSF1 via the chaperone titration mechanism and could actually be important upon mild stress conditions that do not lead to a massive production of unfolded/denatured proteins (when chaperones would

not be sequestered by misfolded proteins). One could speculate that small and local changes in membrane fluidity may be perceived by the cells and used to activate the expression of HSGs in order to boost specific functions of HSPs that go beyond protein refolding and anti-aggregation activities. This idea is supported by recent data showing that HSPs can directly associated with membranes via specific lipid interactions and regulate the properties of membrane lipid phase, comprising the fluidity and the permeability (see below) (Maio and Hightower 2021). Thus, one could envisage that membrane microdomain-associated HSPs could regulate the activity of specific raft-associated signalling platforms, participating to the cell stress response also acting at the level of the lipid bilayer. In turn, the transient binding of certain HSPs to membranes could restore the fluidity and bilayer stability and therefore recover the membrane functionality upon stress conditions.

HSP can interact with cell membranes.

HSPs are found in the cytoplasm, nucleoplasm and also in different membrane-bound organelles such as ER and mitochondria. In addition, HSPs can associate with lipids: morphological studies have shown the presence of HSPs on the surface and within the cellular membranes (Maio and Hightower 2021). Hsp17, a sHSP of bacterium *Escherichia coli*, was firstly found to sediment with membranes, and it was later observed to localize with the outer membrane, where it exhibits a protective action against heat and oxidative stress (Miyake et al. 1993; Laskowska et al. 2004). Studies employing immunocytochemistry have reported the localization of HspA in association with the thylakoid membranes in *Synechocystis* PCC; in particular HspA was shown to change its subcellular localization and translocated into the membrane in response to high temperature exposure, suggesting a protective function at the membrane level (Horváth et al. 1998). Additionally, the sHSP Lo18 of the bacterium *Oenococcus oeni* was upregulated and localized to the cellular membrane upon administration of benzyl alcohol (BA), the membrane fluidizer mentioned before in this thesis and was suggested to maintain membrane integrity during stress (Delmas et al. 2001).

Recent findings support the notion that sHSPs associate with membranes also in higher eukaryotes. In mammalian cells, many members of the sHSPs (HSPB) family have been reported to associate with membranes; for example, HSPB2 is associated with the outer mitochondrial membrane (Nakagawa et al. 2001), and HSPB5 interacts with the membrane of vertebrate lenses to ensure their transparency by inhibiting stress-induced protein aggregation (Cobb and Petrash 2002; Cobb and Petrash 2000) .

The mechanisms for sHSPs membrane insertion are complex and still elusive, especially considering that these proteins do not comprise major hydrophobic domains that could mediate their embedding into the lipids (Maio and Hightower 2021). Infrared spectroscopy studies indicate that sHSP-lipid interactions is mediated by the polar head group region (Tsvetkova et al. 2002; Horváth et al. 2008). Moreover, independent studies with various membrane models with different lipid composition and lipid:protein ratio have shown that sHSPs displayed lipid specificity in binding to membrane, suggesting that sHSPs may interact with specific microdomains, such as lipid rafts.

(Tsvetkova et al. 2002; Maio and Hightower 2021). The α-crystallin domain of sHSPs, which represents the signature of these proteins, might play a crucial role in their insertion within the membrane (Montfort et al. 2001). The β-sheet structures of this domain have been proposed to form oligomeric complexes that assist the incorporation into lipid membranes (Arispe et al. 1996, 1993; Mirzabekov et al. 1996; Rojas et al. 1992). In fact, the α-crystallin domain was found to be protected from proteinase K digestion after the incorporation of HSPB1 and HSPB5 into lipid vesicles, suggesting that this domain is inserted inside the lipid bilayer (De Maio et al. 2019).

The current studies support the view that the functional consequences of the association of sHSPs with membranes are related to the regulation of their physical state and the stability of lipid bilayer: in other words to maintain the integrity of membranes (Tsvetkova et al. 2002). Once bound to the lipids, HSPBs seem to stabilize the liquid-crystalline state and inhibit the formation of inverted hexagonal structures, regulating membrane fluidity (Tsvetkova et al. 2002). For example, HSB5 was shown to stabilize model membranes of synthetic and cyanobacterial lipids, by modulating their lipid phase behaviour (Maio and Hightower 2021; Tsvetkova et al. 2002). In addition, the association of Hsp17 to liposome increased the membrane order especially in the liquid-crystalline phase of the lipid bilayer, and neither interaction nor membrane ordering could be observed when the membrane lipids were restored to a gel state. A similar behaviour was found after addition of Hsp17 to lipid vesicles obtained from isolated *Synechocystis* membrane lipids, which do not form gel phase in the temperature range tested (Horváth et al. 1998; Török et al. 2001b; Tsvetkova et al. 2002). These data suggest that sHSPs can recognize membrane polymorphisms and, in particular Hsp17 preferentially binds to fluid phase membranes to increase the lipid bilayer physical order. Thus, considering the changes in membrane fluidity that occurs upon temperature shifts, we might deduce that the interaction between sHSPs and membranes could represent a general mechanism to safeguard membrane integrity during temperature fluctuations. sHSPs might behave as membrane-stabilizing factors and are able to antagonise the membrane hyper-fluidization caused by perturbating stimuli and concomitantly prevent the formation of a membrane-disrupting non-bilayer lipid phase (Horváth et al. 2008; Török et al. 2001a; Maio and Hightower 2021). In summary, sHSPs would play a dual role, acting as amphitropic proteins depending on their location: they might function as members of multichaperone protein-folding complex and/or bind reversibly to lipid membrane to restore its integrity after stress conditions and preserve its functionality (Török et al. 2001a).

The functional roles of sHSPs association with membranes is still understudied, especially in higher eukaryotes and mammalian cells. Using in vitro systems based on purified recombinant HSPBs and lipid vesicles of specific composition, it has been shown that HSPB1 and HSPB5 directly bind to the lipids and influence their viscosity (Maio and Hightower 2021; De Maio et al. 2019). Recent lipid interaction profile studies of HSPB1 have revealed that this protein not only interacts with membranes by lipid binding, but also exhibits a strong affinity to highly fluid membranes. HSPB1 plays a role in the regulation of the physical properties of the interacting membranes by altering rotational and lateral lipid mobility. In addition, the *in vivo* expression of HSPB1 strongly affected the phase behaviour of the plasma membrane under membrane fluidizing stress conditions.

Considering these current findings, a new function for HSPB1 as membrane chaperone has been hypothesized (De Maio et al. 2019; Csoboz et al. 2022). Also HSP70, the major stress-inducible member of the HSP70 family, can be localised within the membranes, including in endosomal/lysosomal compartment, where it plays a crucial role in maintaining the membrane integrity by inhibiting their permeabilization under stress, such as irradiation and oxidative stress (Nylandsted et al. 2004). HSP70 associates with the membranes of tumor cells, where it may repress the lysosomal permeabilization induced by multiple insults as cytokines, anticancer drugs, irradiation, thereby protecting these cancer cells. In line, the depletion of HSP70 from transformed cells causes the permeabilization of lysosomal membranes, resulting in the release of lysosomal components into the cytosol, and in the activation of cathepsin-mediated programmed cell death (Jäättelä 1999; Brunk et al. 2001; Leist and Jäättelä 2001). Additionally, HSP70 was shown to bind to different lipid components of the plasma membrane, especially to lipid rafts, where it has been supposed to play roles in maintaining the stability of lipid raft-associated signal transduction complexes under stress conditions (S. Chen et al. 2005; Broquet et al. 2003). Mechanistically, HSP70 interacts with negatively charged liposomes, but not with liposomes composed of neutral or positively charged lipids, underlining the lipid specificity of HSP70 (Arispe et al. 2004; Schilling et al. 2009; Armijo et al. 2014). Despite the insertion mechanism of HSP70 into the membrane remains to be clarified, the studies performed so far suggest the following model: HSP70 would incorporate into the membrane and expose a small region of the C-terminus end to the extracellular environment after the interaction with the lipid head group and subsequent change in peptide conformation (Armijo et al. 2014).

Because of the HSPs' ability to modulate the physical state of the membrane, one could speculate that their association with the membrane may restore the membrane perturbation caused by stress conditions and, ultimately, inactivate the expression of HSGs, serving as a negative feedback mechanism to regulate the duration of the activation of the HSR. As mentioned before, the association of HSPs to the membrane changes the order of the lipid bilayers, restoring the integrity of the membrane and, simultaneously, serving as a negative feedback loop. In summary, the interaction between HSPs and the lipid membranes would play a crucial role in the regulatory mechanisms of the HSR (Török et al. 2001b; Maio and Hightower 2021).

The effect of lipids on neurodegenerative diseases

Several neurodegenerative diseases are characterized by the accumulation of aberrantly folded (misfolded) proteins in form of aggregates (or inclusion bodies) in the brain. These neurodegenerative diseases, also known as protein misfolding diseases, comprise for example the Alzheimer's disease (AD), the Parkinson's disease (PD), the Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and transmissible spongiform encephalopathies. Despite the remarkable differences in clinical manifestation, a common hallmark of these disorders is the presence of inclusions of aberrant proteins in the cerebral tissue (and in the spinal cord in the case of ALS) (Sweeney et al. 2017; Fusco et al. 2017; Jellinger 2010). Compelling evidence supports the hypothesis that protein misfolding and aggregation result in synaptic dysfunction, neuronal death, and brain damages.

The conversion of polypeptides from a soluble into an insoluble state occurs through a multi-step process that includes the formation of misfolded states, their association into oligomeric species and can culminate with the formation of amyloid aggregates (Chiti and Dobson 2006; Dobson et al. 2020; Dobson 2003). These different states of misfolded proteins have been suggested to have different toxic properties, with small oligomeric species being considered far more toxic than large amyloid aggregates (Chiti and Dobson 2006; Dobson et al. 2020; Dobson 2003). Although substantial efforts have been done to understand at the molecular levels how protein aggregation occurs, the mechanism through which soluble proteins convert into aggregated states and the identification of the factors that influence the multi-step aggregation process are not fully determined.

Some features of the proteinaceous aggregates found in neurodegenerative diseases can be generalized. Although the main protein component is different in each disease, the protein deposits share similar morphological and structural properties (Figure 17, panel a). Their common core structure is characterized by cross-β fold, in which β-strands form long ribbon-like sheets that pack against each other to create long protofilaments that assemble together into fibrils, as a result of the protein misfolding and aggregation (Fitzpatrick and Saibil 2019). Exceptions of course can exist. For example, a quantitative biology approach supports the idea that the largest cytoplasmic inclusions formed by TDP-43, an RNA-binding protein associated with ALS, are more toxic than the smaller oligomers (Cascella et al. 2022), in sharp contrast with what observed for other types of aggregates associated with neurodegenerative diseases. Of note, TDP-43 can form both amorphous aggregates and more structured aggregates having amyloid properties (Capitini et al. 2021), further highlighting the complexity of the aggregation process and the different degree of toxicity of the various aggregated species formed by a given protein.

Interestingly, the development of sophisticated electron microscopy analyses, such as Cryo-electron tomography, enabled to study in detail the composition and organization of the proteinaceous aggregates associated with neurodegenerative diseases. It is now clear that the amyloid formations possess a highly heterogenous composition, indicating that they are not, as originally believed, comprised of a single type of protein fibril, but they contain various components, such as lipids, membranous organelles, and a wide variety of other proteins (Figure 17, panel b) (Lashuel 2021;

Creekmore et al. 2021). This finding supports the view that the protein aggregation can be influenced (or even driven) by multiple interactions with the misfolding-prone species and different cellular components, including organelles; such interactions may have an impact on the disease progression, making the understanding of this process extremely challenging (Soto and Pritzkow 2018). Here we briefly mention evidence for heterogeneous composition of the proteinaceous aggregates associated with PD, AD, HD and ALS.

Concerning PD, α-synuclein fibrils are the major component of intracytoplasmic inclusions, called Lewy bodies (LB), in Parkinson's disease. More than 300 proteins have been found in LBs; these proteins include players of the protein degradation systems and molecular chaperones. Nonproteinaceous components, such as metals, lipids and membranous organelles are also significantly enriched inside LBs (Lashuel 2021; Mahul-Mellier et al. 2020). As a consequence of this heterogenous composition, fibrils can arrange to form different protofilaments, affecting the final amyloid structure, and this in turn may be associated to different degrees of cytotoxicity. For example, six different α-synuclein fibrils in complex with lipids with differential quaternary arrangements have been identified through cryo-EM studies, exemplifying the degree of complexity in understanding protein aggregation and identifying the toxic species (Frieg et al. 2022).

Concerning AD, two main types of deposits are found in the brain of patients; intracellular neurofibrillary tangles, principally composed of the hyperphosphorylated aggregated tau protein, and extracellular plaques comprised of the β-amyloid protein (Aβ) (Selkoe 2002). Post-mortem characterization of Aβ amyloid fibrils from AD patients has shown that also amyloid plaques are heterogeneous in nature, with different fibril morphologies potentially involved in different steps of disease progression. Aβ plaques also contain a large variety of non-proteinaceous components, which can interact with amyloid fibrils in numerous ways and modulate the aggregation mechanism such as for example metal ions, cholesterol and multifunctional apolipoprotein clusterin (ApoJ). In particular, Zn^{2+} was suggested to enhance Aβ aggregation; cholesterol may facilitate Aβ fibril nucleation, while ApoJ can form slowly sedimenting Aβ complexes that cause oxidative stress, enhancing neurotoxicity (Tycko 2015; Grao-Cruces et al. 2023; Alexandrescu 2005; Stewart and Radford 2017).

HD is caused by an expanded poly-glutamine (polyQ) sequence in the mutant huntingtin (mHTT) protein that accumulate in intracellular aggregates called inclusion bodies (IBs). Using cryo-electron tomography and subtomogram averaging, it was shown that mHTT exon 1 (mEx1) and the polyQ tract only are structurally heterogenous and form filamentous structures; both they shared a subpopulation of filaments that exhibits a characteristic lumpy slab conformation, supportive of the polyQ core model. A morphological model reveals that polyQ strands might form a β-sheet structure, packed together by hydrogen bonds, in which the adjacent domains in mEx1 are mostly exposed at the filament surface, allowing them to modulate inter-filament aggregation. The length of the polyQ is the most relevant factor that influences the IB formation. In addition, other intrinsic factors can contribute to the aggregation process, including polyQ-flanking sequences and the specific conformation of mutant Htt. In fact, multiple monomeric conformations of mutant Htt may coexist, and some might show a higher propensity to aggregate, modulating both the rate and the

mechanism of aggregation (Sun et al. 2015; Fitzpatrick and Saibil 2019; Galaz-Montoya et al. 2021; Arrasate and Finkbeiner 2012). Also Htt aggregates are heterogeneous and many essential proteins, such as transcription factors, proteasomes or other ubiquitin proteases are sequestered in the IBs and their depletion has been proposed to contribute to cellular toxicity (Arrasate and Finkbeiner 2012).

Finally, we mention the poly-Gly-Ala (poly-GA) aggregates that result from the non-canonical RAN translation of an expanded GGGGCC repeat in the *C9orf72* gene, which represents the most frequent genetic cause of ALS and frontotemporal dementia (Edbauer and Haass 2016). Cryo-electron tomography studies highlighted that rather than filaments, poly-GA is characterized by densely packed twisted ribbons, each of which is comprised of a different number of laterally stacked protofilaments. Additionally, poly-GA aggregates contain and sequester 26s proteosome complexes, a lower number of ribosomes and the TriC/CCT chaperonins; these findings suggest that their toxicity may be, at least in part due, to the potential impairment of a large fraction of the cellular proteasomes, one of the most important degradative machinery presents in the cytoplasm and nucleus of mammalian cells. Other macromolecules are mainly excluded from the poly-GA aggregate composition, indicating some kind of “specificity” (Q. Guo et al. 2018; Y.-J. Zhang et al. 2016).

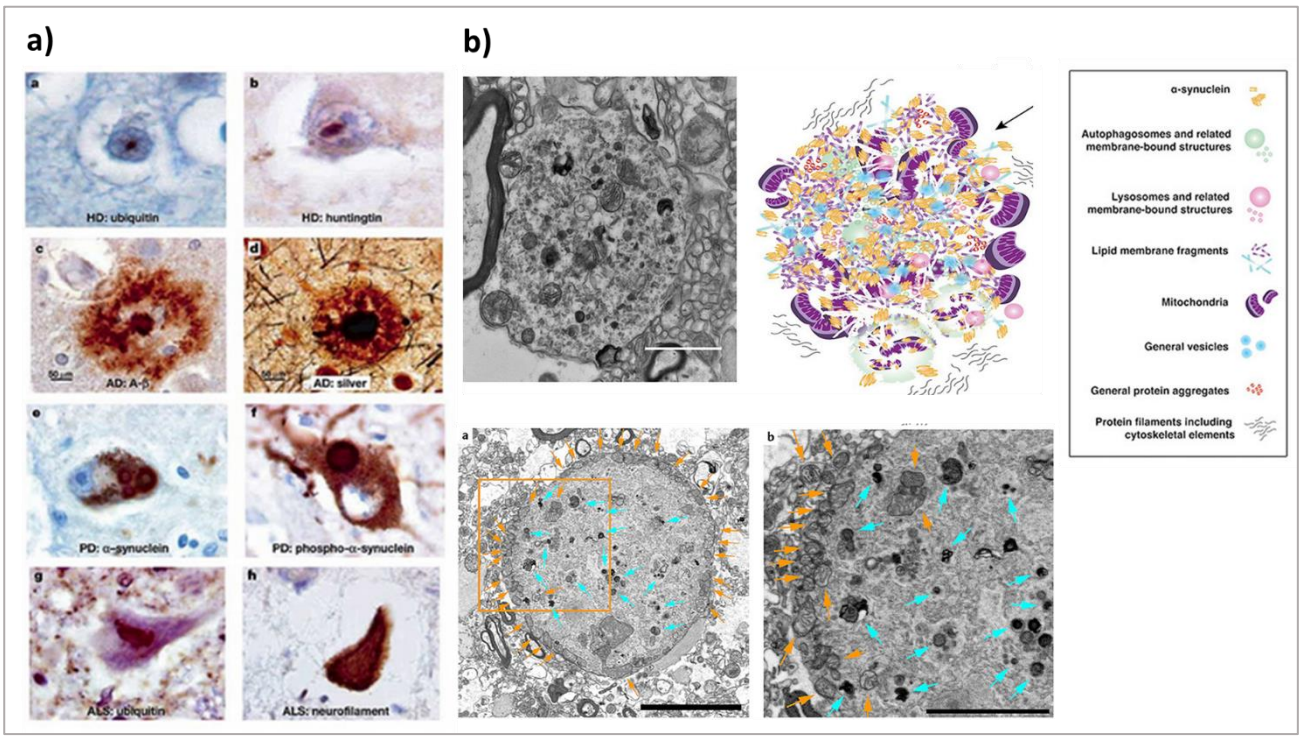


Figure 17. Unmasking the complexity of pathological aggregates in brain proteinopathies. (**a**) Images of protein aggregates found in the brain and that represent the pathological hallmarks of Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), and Amyotrophic lateral sclerosis (ALS) (**b**) Recent Electron microscopy (EM) images of LBs illustrating the diversity of their ultrastructure properties and the complexity of their composition, including the presence of proteins, vesicles, and membranous organelles. From: Lashuel, Current Opinion in Chemical Biology Volume 64, October 2021, Pages 67-75. PMID: 34174698.

Finding that the inclusion body are intimately associated with other intracellular elements and lipid vesicles reinforced the idea that multiple factors can influence the aggregation process, which for a long time has been studied using very simple test-tube assays, where the behaviour of the purified misfolding prone protein associated with disease was analysed in a minimal context, changing for example salt concentration and/or pH (Fitzpatrick and Saibil 2019). This knowledge inspired (and is inspiring) researchers in using more complex systems to understand which cellular components may influence or trigger protein aggregation. Interaction with lipids is emerging as an important player that can modify protein aggregation. In the next paragraphs we explore the effect of lipids on protein misfolding and amyloid aggregation focusing on Alzheimer's and Parkinson's diseases, the two main neurodegenerative diseases.

Lipids and protein aggregation diseases

Lipids are crucial for the normal development and function of the central nervous system (CNS). They are the major constituent of the brain and they are implicated in structural functions as well as in vital cell processes, including neurogenesis, synaptic transmission, neural communication, and modulation of gene expression (Mesa-Herrera et al. 2019; Estes et al. 2021). As mentioned in the previous chapters, lipids are important constituent of the lipid membranes and thanks to their heterogeneous composition, they can form microenvironments in which the proteins can be either inserted or excluded, regulating protein functionality. Lipids influence thus cell signalling and cell stress response, as well as cells' ability to adapt to environmental changes (Hotamisligil and Davis 2016). It is thus not surprising that lipid dysfunction is increasingly implicated in human disease, including neurodegenerative diseases. Genetic studies associated a large number of genes involved in lipid metabolism to the development of AD, PD, and other neurodegenerative disorders. For instance, the *Apolipoprotein epsilon4 allele*, which is essential for cholesterol transport into the brain, is the most common genetic risk factor associated with AD. In addition, mutations in the *GBA* gene, which encodes for a critical lysosomal enzyme of lipid catabolism, are associated with PD and have been suggested to promote the formation of toxic α-synuclein oligomers. These genetic studies have been corroborated by lipidomic studies that reinforced the concept of lipid metabolism imbalance as a common feature of neurodegenerative diseases (Fais et al. 2021; Cedazo-Mínguez 2007). For example, lipidomic analysis of tissues from AD patient and AD animal models have demonstrated changes in the amount of distinct lipid species, such as fatty acids, glycerophospholipids, sphingolipids, and cholesterol, supporting the idea that an unbalanced lipidome could actively influence the onset of neurodegenerative disorders (Estes et al. 2021).

Role of lipids in α-synuclein aggregation in Parkinson's Diseases

PD is a progressive neurological disorder and is mainly characterized by severe motor symptoms that include rigidity, hypokinesia and resting tremor (Balestrino and Schapira 2020) The most pronounced neuropathological feature is the loss of dopaminergic neurons in the *substantia nigra*, together with the presence of aberrant inclusions knows as Lewy bodies (LB), composed of the synaptic protein α-synuclein, as well as lipids (Spillantini et al. 1997)(see Figure 17b). Misfolding and

aggregation of α-synuclein is associated with a range of diseases that are called together synucleinophaties, which comprise PD, dementia with Lewy bodies (DLB), multiple system atrophy (MSA) and some variants of AD (Wong and Krainc 2017). Although the mechanisms underlying α-synuclein aggregation and toxicity are not fully understood, there is a general consensus that the formation of aggregation-prone oligomeric species of α-synuclein is a key event involved in neural dysfunction (Stefanis 2012).

α-synuclein is a 140-residues protein and is highly expressed in the brain, mainly localized in presynaptic terminals. From a structural point of view, this protein contains three domains : - the N-terminal domain (residues 1 to 60), which provides the ability to interact with lipids, especially with membranes and lipid micelles; - the central hydrophobic region (residues 61 to 95) called NAC (non-amyloid β component), which is essential for the conformational change of α-synuclein from random coil to β-sheets, and is involved in α-synuclein aggregation; - the C-terminal region (residues 96 to 140), which is enriched in acidic amino acids (Figure 18) (Mori et al. 2020; Sarchione et al. 2021). The N-terminal domain contains an amphipathic motif of 11 amino acids, similar to that one present in the apolipoprotein domain (Bussell and Eliezer 2003). This domain is important for interaction with lipids. The N-terminal domain is also the region where the PD disease-linked missense mutations A53T, A30P, A53E, and E46K are found. The C-terminal region represents the main site of post-translational modifications, such as phosphorylation and nitration. These modifications alter α-synuclein structure and provoke changes in hydrophobicity and protein-protein and protein-lipid interactions, thereby playing an important role in protein aggregation and disease pathogenesis (Zhang et al. 2019; Hassanzadeh et al. 2023).

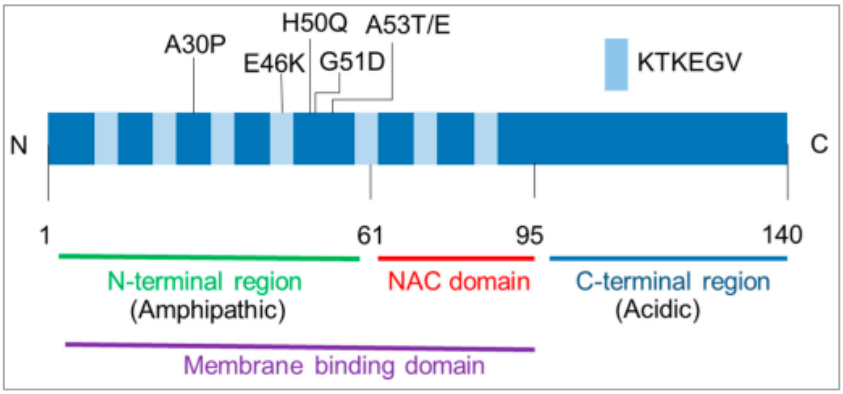


Figure 18. Alpha-synuclein protein structure. The N-terminal region and the non-amyloid-component (NAC) domain have seven imperfect KTKEGV repeats that contribute to membrane binding. All missense mutations linked to the familial Parkinson's disease (A30P, E46K, H50Q, G51D, A53T, and A53E) are located in the N-terminal region. From: Mori et al., IJMS 2020, https://www.mdpi.com/1422-0067/21/9/3301.

The structure of α-synuclein is considered largely flexible. α-synuclein is an intrinsically disordered monomeric protein in its cytosolic state, but in the presence of lipid surface the protein can adopt a helical structure that is ideally suited for lipid interaction. Thus, α-synuclein exists in a dynamic equilibrium between a soluble and a membrane-bound state (Galvagnion 2017). The helical

conformation is thought to be important for its physiological functions, including control of synaptic membrane processes, modulation of neurotransmitter release and synaptic plasticity. Upon binding to membranes, a segment composed of the first 95 residues of α-synuclein undergoes a structural transformation from random coil to α-helix; the fraction of the sequence implicated in this conformation change depends on the relative ratio between proteins and lipids and on the nature of the lipids species that reside in the membrane (Figure 19) (Chandra et al. 2003; Fusco et al. 2017; Bodner et al. 2009). Notably, the six familiar point mutations of α-synuclein, which reside in the N-terminal membrane-binding domain, show different lipid membrane interaction properties; this suggests that the lipid affinity of α-synuclein is playing a crucial role in the modulation of the α-synuclein aggregation process (Mori et al. 2020).

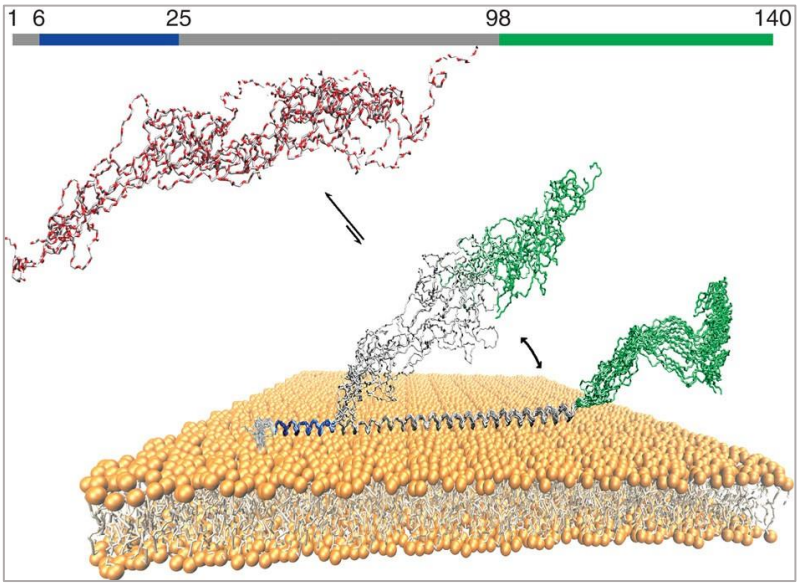


Figure 19. Identification of three different regimes of protein dynamics and membrane affinity by using a combination of solution and solid-state NMR spectroscopy. The N-terminal region (blue) is visible in DARR experiments, indicating that it is rigidly bound and anchored to the membrane. The central region (grey), showing intermediate dynamics and therefore being invisible in both CP and INEPT experiments, is suggested to play a key role in modulating the affinity of αS for membranes. Finally, a C-terminal fragment (green) maintains its unstructured nature and remains essentially uncorrelated with the membrane surface, despite showing weak and transient contacts in PRE experiments. From: Fusco et al., Direct observation of the three regions in α-synuclein that determine its membrane-bound behaviour. Nat Commun 5, 3827 (2014). https://doi.org/10.1038/ncomms4827.

Several binding assays of α-synuclein to lipid membranes have been performed using synthetic liposomes; these studies revealed that the physical, chemical and thermotropic properties of the membrane affect the strength of the interaction of α-synuclein. It has been demonstrated that α-synuclein preferably interacts with membranes composed of negatively charged lipids and/or with membranes that exist in a fluid state (Pirc and Ulrih 2015; Galvagnion et al. 2016; Galvagnion 2017). As a consequence, modifications of these properties can alter the interaction between α-synuclein and lipids, affecting the protein behaviour and promoting pathological mechanisms, such as its propensity to aggregate (Burré et al. 2015). For instance, the formation of α-synuclein oligomers has

been reported to be directly mediated by fatty acid levels in mesencephalic neuronal cells. Specifically, increasing the amount of polyunsaturated fatty acids can enhance the α-synuclein oligomerization, while the opposite effect is observed for saturated fatty acids (Sharon et al. 2003b), supporting the emerging view that impairment of lipid homeostasis can influence α-synuclein aggregation.

In parallel, many studies have focused on the kinetics of α-synuclein aggregation, in which lipids have shown to act as potent modulators of amyloid formation. The amyloid formation occurs through a complex series of microscopic processes that include primary nucleation, conversion, elongation and secondary nucleation (Buell et al. 2014; Knowles et al. 2014). *In vitro* studies with small unilamellar lipid vesicles (SUVs) have demonstrated that the rate of the initial step of α-synuclein aggregation process is greater than that occurring in bulk solution, underlining the promoter role of lipid in triggering α-synuclein aggregation on the membrane surface (Figure 20) (see also result section). The magnitude through which lipid systems mediate the process of amyloid formation was reported to correlate with the affinity of monomeric α-synuclein for membrane lipids (Buell et al. 2014; Cremades et al. 2012; Galvagnion et al. 2015; 2016).

More recently, it has been observed that a-synuclein aggregation can also occur via a liquid-liquid phase separation step, referred to as the condensation pathway (Ray et al. 2020). In this pathway, α-synuclein undergoes liquid-liquid phase separation by generating a liquid droplet state, which can convert into an amyloid-rich hydrogel. Such process can be enhanced by the presence of lipids, which may provide a scaffold for α-synuclein aggregation and remain integrated in the deposits, suggesting a potential explanation for the formation of Lewy bodies and their heterogenous composition (Figure 20) (Mahul-Mellier et al. 2020; Trinkaus et al. 2021; Hardenberg et al. 2021; Vendruscolo 2022).

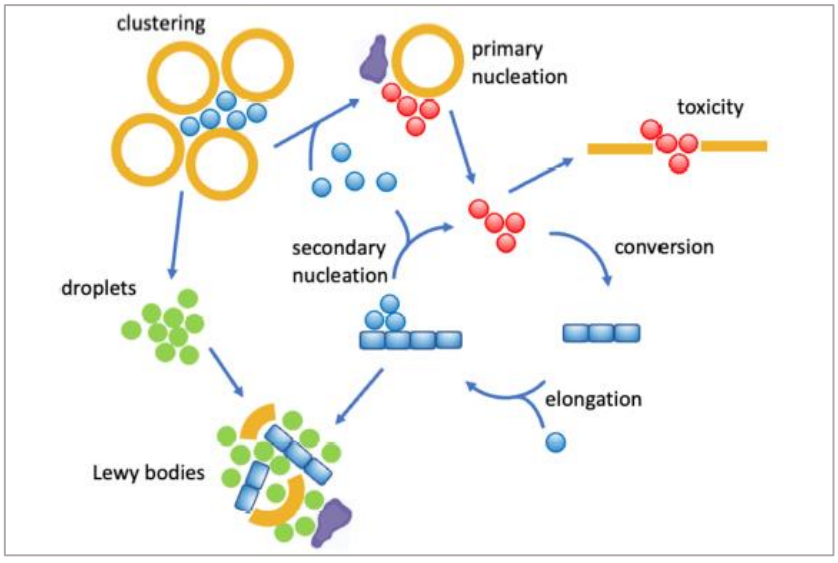


Figure 20. Mechanisms by which lipid dysregulation can affect α-synuclein aggregation in Parkinson's disease. One of the proposed physiological functions of α-synuclein (blue) is to bind reversibly synaptic vesicles (orange) and to promote their clustering (Fusco et al., 2016). However, when multiple α-synuclein molecules bind synaptic vesicles or other lipid assemblies of altered compositions, lipid droplets (Soste et al., 2019), they can self-assemble into disordered oligomers (red), which can be cytotoxic by disrupting lipid membranes (Danzer et al., 2007; Fusco et al., 2017), or progress to form amyloid fibrils and Lewy bodies. All these steps can be promoted by lipid membranes of specific compositions (Buell et al., 2014; Galvagnion et al., 2015, 2016) and regulated by interactions with molecular chaperones (purple). The clustering of synaptic vesicles can also open up the condensation pathway by concentrating α-synuclein molecules (Ray et al., 2020; Hardenberg et al., 2021), which may end up in the formation of Lewy bodies through the formation of α-synuclein droplets (Hardenberg et al., 2021) (green). From: Vendruscolo M. Lipid Homeostasis and Its Links With Protein Misfolding Diseases. Front Mol Neurosci. 2022 Mar 25;15:829291.PMID: 35401104

Because of the close relationship between lipid membranes and α-synuclein aggregation, researchers focussed on the understanding of the interplay between lipid and protein aggregation using biological material from PD patients and PD models. Lipidomic analysis of tissues from PD patients and PD animal models have revealed that several lipid species are dysregulated, such as fatty acids, glycerophospholipids, sphingolipids, and cholesterol, resulting in a perturbation of α-synuclein homeostasis (Estes et al. 2021; Mesa-Herrera et al. 2019). As examples, unsaturated fatty acids deficiencies, such as the level of α-linolenic acid oleic acid, were observed in CSF of PD patients and this decrease has been associated with increased formation of α-synuclein oligomers and α-synuclein cytotoxicity. Mechanistically, these changes were proposed to alter the equilibrium of membrane bound to soluble α-synuclein, culminating in the exacerbation of protein aggregation and toxicity. In addition, lower amounts of sphingolipids, in particular of ceramide, have been found in the brain tissue from PD patients and have been correlated to the acceleration of α-synuclein aggregation (Sharon et al. 2003a; Lee et al. 2011; Mesa-Herrera et al. 2019; Fanning et al. 2019).

Moreover, genome-wide association studies (GWAS) associated a large variety of enzymes involved in the regulation of lipid metabolism to α-synuclein dysregulation. Here we briefly describe a few of these identified genes.

GBA1 gene: Mutations in the *GBA1* gene strongly increase the risk of PD (Fais et al. 2021). This gene encodes a lysosomal enzyme, glucocerebrosidase (GCase), that hydrolyses glucosylceramide (GluCer) into glucose and ceramide in the lysosomes, allowing ceramide recycling to the plasma membrane and regulating its properties. The reduced activity of GCase results in the accumulation of defective lysosomes, impairing the chaperone-mediated autophagy process, which normally degrades α-synuclein and leading to its accumulation. Next, GCase deficiency in PD animal models and in PD patients is associated with the accumulation of α-synuclein (Mazzulli et al. 2011; Yang et al. 2017; Bonam et al. 2019). Two main mechanisms for α-syn aggregation induced by a GCase defective activity have been hypothesized. First, the abnormal levels of α-synuclein caused by the lysosomal dysfunction may promote the amyloid formation; second, the acceleration of α-syn aggregation may be induced by the accumulated substrate of GCase, that are GluCer and glucosylsphingosine, consistent with the observations that the composition of lipid membranes

controls the initial steps of a-synuclein aggregation (Mori et al. 2020; Vendruscolo 2022; Taguchi et al. 2017; Suzuki et al. 2015).

PLA2G6 gene: this is the causative gene for an autosomal recessive form of PD (PARK14) and it encodes for a phospholipase that regulates the membrane homeostasis by catalysing phospholipid hydrolysis to produce free fatty acid and lysophospholipids. It appears that PLA2G6-loss results in acyl-chain shortening in phospholipids, which affects ER homeostasis, neurotransmission and a-synuclein stability, promoting its aggregation (Fais et al. 2021; Lin et al. 2018; Mori et al. 2019). Further studies are required to elucidate the mechanism through which PLA2G6 cause PD and promotes a-synuclein aggregation.

Role of lipids in Aβ aggregation in Alzheimer's Diseases

AD is the most common neurodegenerative disorder and afflicts over 50 million people worldwide (Chiti and Dobson 2006). AD is clinically characterized by progressive memory loss, cognitive dysfunctions and aberrant behaviour (Rudolph and Lars 2005; Haass and Selkoe 2007). The main molecular signatures of AD is the accumulation of amyloid plaques due to impairment of β-amyloid (Aβ) peptides homeostasis, resulting in synaptic loss and neuronal death (Paolo and Kim 2011). In only 1–2% of AD cases, the aggregation of Aβ is enhanced by familial mutations (familiar Alzheimer's disease - FAD), causing the early onset of the disease and a faster rate of progression. All dominant FAD mutations are found either in the substrate (amyloid precursor protein) or in the protease presenilin that generates Aβ (Castellani et al. 2010; Bertram and Tanzi 2008; Walter and van Echten-Deckert 2013). However, the development of the disease is most frequently associated with environmental factors and age-related decline, which perturb Aβ processing, trafficking and degradation; these factors contribute to the onset and development of the most frequent sporadic Alzheimer's disease - SAD (Balch et al. 2008; Selkoe and Hardy 2016). Similar to PD, lipidomic analysis revealed alterations in the lipidome of AD patients, especially at the level of cholesterol, phospholipids and triglycerides (Paolo and Kim 2011).

Mechanistically, lipids are implicated in the regulation of Aβ production. The Aβ peptides are generated from the proteolytic cleavage of the amyloid precursor protein (APP), an integral membrane protein mainly expressed in the synapses of neurons. APP can be processed along two alternative pathways. The non-amyloidogenic pathway includes processing of APP through α-secretase cleavage at the plasma membrane, releasing soluble APPα (sAPPα) fragments into the extracellular space (Y. Zhang et al. 2011; Chew et al. 2020). Conversely, the amyloidogenic pathway involves cleavage of APP by β-secretase (BACE1) in early endosomes, which are further processed by the γ-secretase complex, generating as final products 40 or 42 amino acid peptides (Aβ-40 and Aβ-42), which are prone to aggregation (Figure 21) (Chew et al. 2020; Taseski 2004; Chen et al. 2017). The surrounding lipid bilayer has a significant impact on APP-processing. Because secretases are membrane-bound enzymes, the composition and organization of membrane lipids can affect their functionality. For example, the increased levels of cholesterol and ceramide enhance the β-secretase activity, whereas cholesterol and sphingolipids promote γ-secretase activity, influencing the production of aggregation prone peptides (Crivelli et al. 2020; Paolo and Kim 2011).

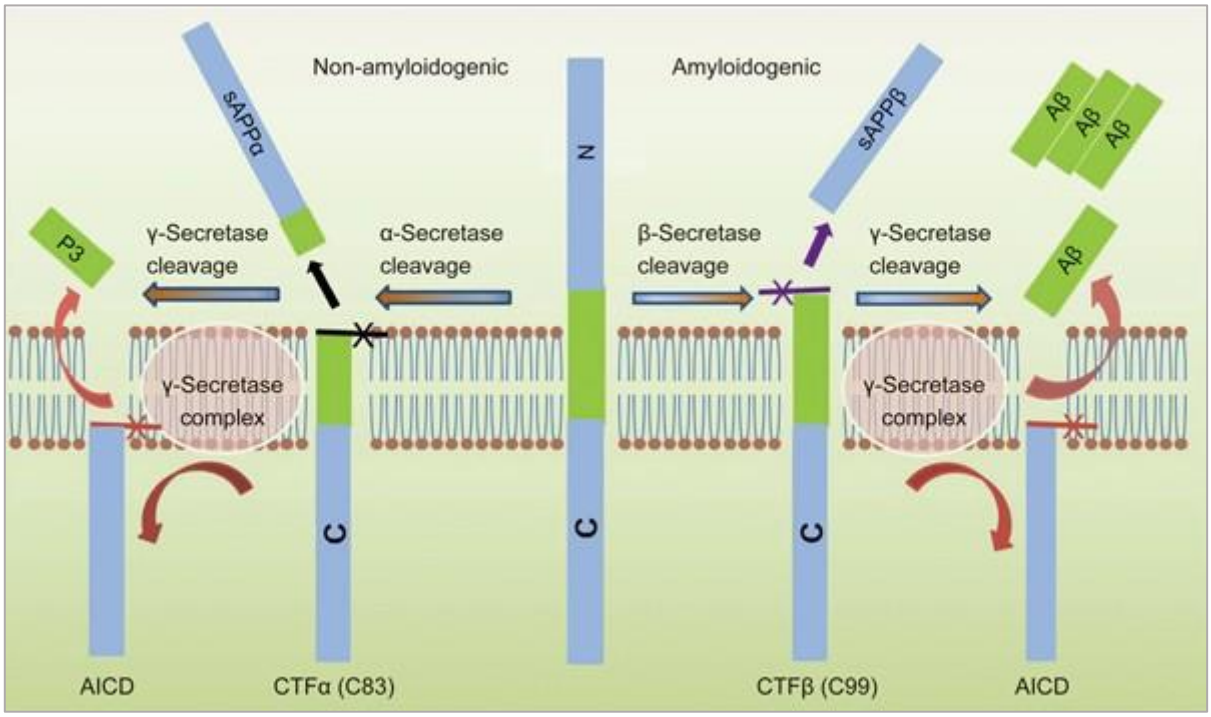


Figure 21. **Human APP proteolytic pathways**. Human APP proteolysis in the non-amyloidogenic pathway and amyloidogenic pathway. Non-amyloidogenic processing of APP refers to the sequential processing of APP by membrane bound α-secretases, which cleave within the Aβ domain to generate the membrane-tethered α-C terminal fragment CTFα (C83) and the N-terminal fragment sAPPα. CTFα is then cleaved by γ-secretases to generate extracellular P3 and the APP intracellular domain (AICD). Amyloidogenic processing of APP is carried out by the sequential action of membrane bound β- and γ-secretases. β-Secretase cleaves APP into the membrane-tethered C-terminal fragments β (CTFβ or C99) and N-terminal sAPPβ. CTFβ is subsequently cleaved by γ-secretases into the extracellular Aβ and APP intracellular domain (AICD) (https://doi.org/10.1038/aps.2017.28).

Moreover, lipids can also act as modulators of the Aβ aggregation. Briefly, the Aβ aggregation process involves the a primary nucleation step that generates disordered oligomers, which then grow into amyloid fibrils through fibril elongation, and secondary nucleation events (Knowles et al. 2014; Michaels et al. 2020). The spontaneous Aβ aggregation is a rare event (Knowles et al. 2014); however the presence of lipids, in particular cholesterol (Habchi et al. 2018) and gangliosides (Matsuzaki 2020) can trigger the primary nucleation of Aβ, accelerating the kinetic of this initial step (Figure 22) (Habchi et al. 2018). Importantly, the interaction with gangliosides, such as GM1, changes the conformation of Aβ from random coils to more ordered structures with are enriched in β-sheet content and are associated with neurotoxicity (Paolo and Kim 2011; Rangachari et al. 2018). Of note, some FAD mutations within the Aβ affect ganglioside-binding specificity (Paolo and Kim 2011). Also cholesterol-rich environments promotes Aβ aggregation, suggesting that it might take place preferentially in membrane raft domains (Matsuzaki et al. 2010; Ariga et al. 2008).

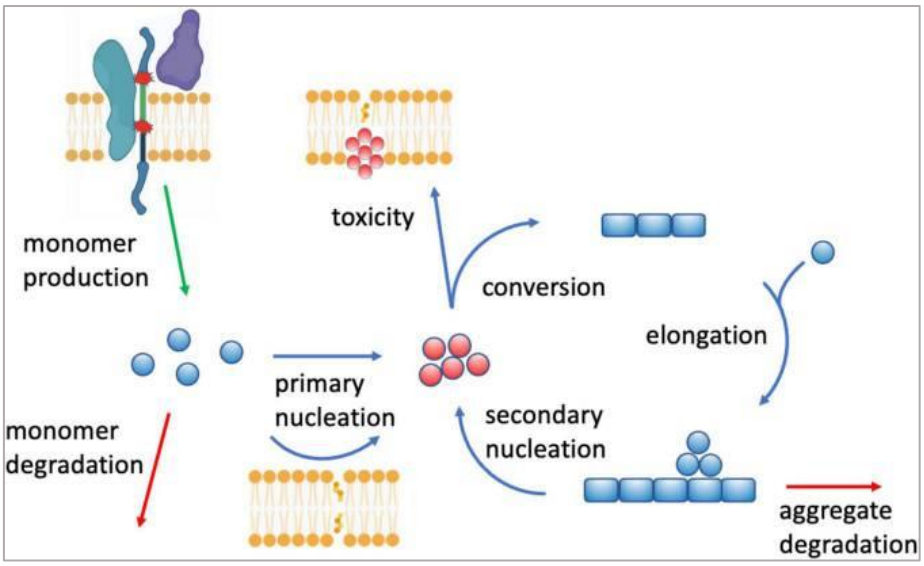


Figure 22. Crosstalk between perturbed lipid homeostasis and Aβ aggregation in Alzheimer's disease. There are at least three aspects of this crosstalk: (1) lipids affect Aβ homeostasis in a number of ways, for example by modulating the concentration Aβ monomers by regulating the activity of the secretases (top left) involved in the proteolytic cleavage of Aβ from APP (Di Paolo and Kim, 2011; Haass et al., 2012; Crivelli et al., 2020) ("monomer production"), of the endopeptidases ("monomer degradation") that degrade Aβ monomers (Yao et al., 2008), and of the endolysosomal system that removes Aβ aggregates (Morel et al., 2013) ("aggregate degradation"), (2) lipid membranes of altered composition cholesterol (Habchi et al., 2018) and gangliosides (Matsuzaki, 2020) can trigger Aβ aggregation by promoting the formation of disordered oligomers (in red) "primary nucleation"), which then grow into amyloid fibrils (Michaels et al., 2020) ("conversion," "elongation," and "secondary nucleation"), and (3) Aβ aggregates, including oligomers, can in turn compromise lipid homeostasis, including by perturbing lipid membranes by binding to them ("toxicity"), or by disrupting lipid signaling by binding membrane receptors (Haass and Selkoe, 2007). From: Vendruscolo M. Lipid Homeostasis and Its Links With Protein Misfolding Diseases. Front Mol Neurosci. 2022 Mar 25;15:829291.PMID: 35401104

In turn, lipid homeostasis can be perturbed by amyloid formation. Oligomers and protofibrils can impair the membrane integrity and signalling properties (Figure 22) (Christian Haass and Dennis J Selkoe 2007; Di Paolo and Kim 2011). The abnormal interaction of aggregating intermediate species with membrane receptors can trigger unwanted signalling pathways correlated to inflammatory responses (Christian Haass and Dennis J Selkoe 2007). In addition Aβ peptides can impair calcium homeostasis, subsequently leading to the dysregulation of calcium-sensitive phospholipid-metabolizing enzymes (Green and LaFerla 2008).

Similar to what reported for PD, GWAS studies identified as AD risk factors various genes encoding for proteins implicated in lipid metabolism. Here we briefly mention the *APOE, CLU* and *TREM2* genes. The ε4 allele of the *APOE* gene is the most common risk factor for SAD. ApoE is a lipoprotein involved in the transport of cholesterol and other lipids in the central nervous system (CNS), affecting the composition of physical properties of membranes (Grao-Cruces et al. 2023; Yamazaki et al. 2019; Silva et al. 2019). The *CLU* gene encodes a molecular chaperone, namely clusterin, which is implicated in Aβ clearance and in cholesterol metabolism and trafficking. The *TREM2* gene encodes for a receptor that is mainly expressed in microglia cells. TREM2 acts as a lipid sensor that

detects damage-associated lipid patterns and directly binds to Aβ oligomers with nanomolar affinity to promote its degradation (Y. Wang et al. 2015; Nugent et al. 2020; Lewcock et al. 2020).

Can we exploit changes in membrane fluidity to activate the HSR and prevent protein aggregation?

As already described in the previous chapters, protein aggregation is a complex multi-step process that can be affected by several environmental factors, including lipid membranes that can trigger the aggregation of e.g. α-synuclein and the Aβ peptide (Galvagnion et al. 2015; 2016).

More generally, the formation of toxic lipid species due to the dysregulation of lipid homeostasis can in turn promote the generation of toxic protein states.

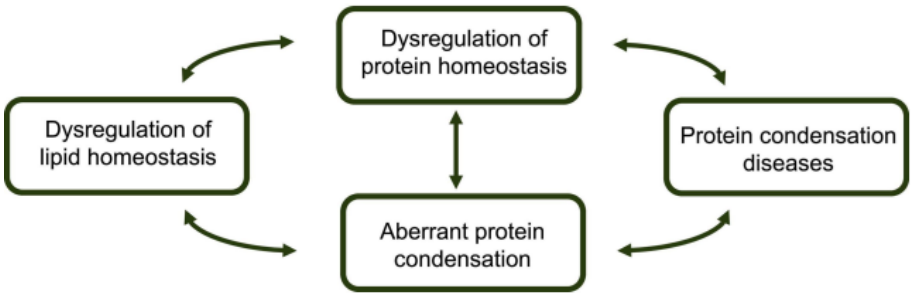


Figure 23. Lipid homeostasis dysregulation, toxic lipid species, and their links to protein misfolding diseases. Adapted from: Lipid Homeostasis and Its Links With Protein Misfolding Diseases. Michele Vendruscolo. PMID: 35401104

Cells sense dysregulation of both protein and lipid homeostasis and activate specific pathways to restore them. The sensors that are activated by protein and lipid imbalance and that regulate these pathways are overlapping, further supporting the intimate connection between these two systems. The typical example is represented by the unfolded protein response (UPR) that is activated when the levels of aberrantly folded proteins increase and modulates the expression of genes involved in the restoration of protein homeostasis, but also of lipid metabolism (see chapter 1 - Plasma membrane as a "cellular thermosensor"). One of these sensors, IRE1α , is involved in the regulation of fatty acid (FA) elongation and desaturation, triacylglycerol synthesis, lipolysis and lipogenesis (Moncan et al. 2021). IRE1α activates also the expression of genes coding for molecular chaperones, including HSPs.

In addition, the previously described "membrane thermosensor" hypothesis assumes that modifications of the lipid composition and the physical state of membranes are determining factors in the transduction of stress signals that ultimately induce the expression of HSGs (Török et al. 2014; Balogh et al. 2005). In turn, the association between HSPs and specific membrane domains would

allow the remodeling of their physical state, restoring their fluidity and stability under stress conditions (Horváth et al. 2008). Thus, changing the membrane fluidity could be exploited as a method to activate the HSR and induce the expression of HSPs, using compounds such as bimoclomol (BM) and other related molecules (Gombos et al. 2011; Török et al. 2003).

Recently, a group of aminosterols has aroused a great interest for the treatment of several human diseases. Aminosterols were firstly isolated from the gastrointestinal tract of dogfish shark *Squalus acanthias*, and they comprise the promoter squalamine and other related compounds, including trodusquemine (Lymbocker et al. 2022). These natural compounds were initially investigated for their potential antibacterial activity, and then they have been extensively studied for their therapeutic value against obesity (Rao et al. 2000; Ahima et al. 2002; Lantz et al. 2010), anxiety disorders (Krishnan and Tonks 2015), cancer (Krishnan et al. 2014) and, more recently, neurodegenerative diseases characterized by protein aggregation, including AD and PD.

From a structural point of view, trodusquemine - also known as MSI-1436 – belongs to the class of cationic amphipathic aminosterols and it shares the same steroid cholestane-like moiety as squalamine, but with a spermine moiety in place of the spermidine on the side chain, conferring a higher net positive charge (Figure 24) (Lymbocker et al. 2022).

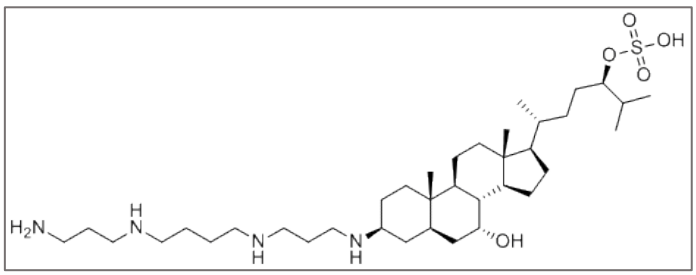


Figure 24. Chemical structures of trodusquemine. Trodusquemine is formed by a sulfate group (right), a sterol (centre) and a polyamine tail (left), that is a spermine**.** Adapted from: https://doi.org/10.1039/d1np00042j.

Trodusquemine is effective in inhibiting amyloid formation and reducing the toxicity related to oligomeric protein species (Ryan Lymbocker et al. 2022). In particular, *in vitro* studies have demonstrated that trodusquemine is able to prevent both the lipid-induced primary nucleation and secondary nucleation of α-synuclein. The inhibition of both steps would significantly decrease the amount of potential neurotoxic aggregates, providing protection both in cellular models and in *C. elegans* models of PD (Perni et al. 2018). Similar to squalamine (Perni et al. 2017), trodusquemine appears to exert its inhibitory action by displacing α-synuclein and its intermediate species from the membrane and preserving the integrity of membranes (Perni et al. 2018).

In contrast to its action on the aggregation of α-synuclein, trosdquemine was found to enhance, rather than inhibit, the rate of $A\beta_{42}$ aggregation, predominantly by stimulating the secondary nucleation microscopic step. Nevertheless, the treatment with this compound significantly decreases the $A\beta_{42}$-induced toxicity in neuroblastoma cells by inhibiting $A\beta_{42}$ association with the

cellular membranes (Limbocker et al. 2019). Additionally, the suppression of toxicity could result in increased rate of conversion of oligomers to less toxic fibrils, as demonstrated in *C. elengans* model of AD (Limbocker et al. 2019).

Recent studies with confocal microscopy, FRET and NMR performed by the laboratories directed by Prof. Fabrizio Chiti and Michele Vendruscolo have revealed that trodusquemine can be partially inserted within the membrane through its steroidal portion, affecting the physicochemical properties of the lipid bilayer and ultimately enhancing the resistance of membranes to the deleterious effects of misfolded proteins (Figure 25) (Errico et al. 2020; Perni et al. 2018; Limbocker et al. 2020). Hence, trodusquemine is a promising candidate therapeutic molecule for the treatment of these neurodegenerative diseases (Lymbocker et al. 2022; Errico et al. 2020).

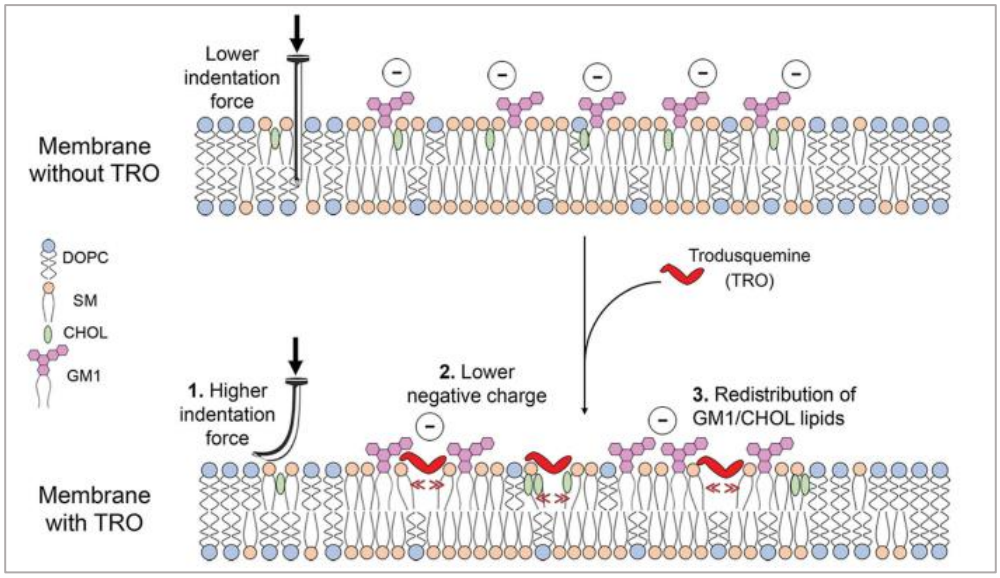


Figure 25. Effect of trodusquemine on physicochemical properties of cell membranes linked with the toxicity of misfolded protein oligomers. Schematic representation of the three major perturbations induced by the insertion of trodusquemine in a cell membrane and thought to protect it from the disruptive action of the oligomers. From: Errico et al., Nanoscale, 2020. DOI: 10.1039/d0nr05285j

Based on these findings, we asked whether part of the protective effect of trodusquemine in cell and animal models may be linked to the induction of the HSR. In other words, by modulating membrane fluidity, trodusquemine may potentiate the activation of the HSR in cells subjected to stress conditions. The increase in the levels of HSPs could then help the cells to restore both protein and lipid homeostasis (Figure 26). This hypothesis has been experimentally addressed in this PhD thesis (for a detailed description of the working hypothesis see Aim of the PhD Thesis and result sections).

2. Aim of the study

Dysfunctions in protein homeostasis (proteostasis) is associated with the development of a wide range of neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) that are characterized by the accumulation of misfolded and aggregated proteins (Hipp et al. 2019). The proteostasis is maintained by the coordinated action of the protein quality control (PQC) system that consists of molecular chaperones, which recognize and bind to aberrantly folded proteins limiting their aggregation, and degradation systems, which clear the damaged and mutated proteins. An important family of molecular chaperones is represented by the heat-shock proteins (HSPs), whose name derives from the finding that their expression can be upregulated upon several stress conditions, including heat shock. Although we gained detailed knowledge on the transcription factors that activate the expression of the heat shock genes (HSGs), we still do not fully understand how cells sense stressful conditions and transduce the stress signals into the activation of specific genes, including the HSGs (Dubnikov et al. 2017). Emerging findings suggest that changes in the physical state of the cell membranes can activate the expression of HSPs, through yet unclear mechanisms. In turn, thanks to their ability to bind to lipids, the major constitutes of the cellular membrane, HSPs would in turn restore the physical properties of the membranes, maintaining their integrity and functionality (Balogh et al. 2005; Török et al. 2014).

Interestingly, alterations in lipid homeostasis are associated with neurodegenerative diseases, such as AD and PD, with mutations of gene involved in lipid metabolism are main risk factors of many neurodegenerative disorders; moreover, perturbation of lipid membrane composition can influence protein aggregation and membranes can serve as platforms triggering protein aggregation (Vendruscolo 2022). Moreover, protein aggregation can perturb membrane integrity and functionality. Together these results underline the existence of a complex cross-talk between protein and lipid homeostasis.

Based on the ability of HSPs to interact both with aggregation-prone proteins and lipids, in this PhD thesis we investigated the hypothesis whether HSPs may act at the crossroad between protein and lipid homeostasis, functioning as lipid chaperones in addition to protein chaperones. Firstly, we used as a model system α-synuclein, a protein that is associated with PD. The reason for this choice is based on the finding that: 1) α-synuclein aggregation can be triggered by interaction with small lipid unilamellar vesicles (SUVs) (Galvagnion 2017); 2) small HSPs (HSPBs) are able to inhibit α-synuclein aggregation in vitro. Using in vitro assays developed in the laboratory directed by our collaborator Prof. Michele Vendruscolo (Cambridge, UK), we studied whether the anti-aggregation properties of several human HSPBs are due to interaction with α-synuclein (protein chaperone) or with lipids (lipid chaperone).

Next, we explored whether changes in the physical state of the lipid membranes can induce the expression of HSPs in human cells. To tackle this question, we used different approaches, including cell treatment with the aminosterol trodusquemine, which interacts with lipid membranes, modulating their fluidity and prevents α-synuclein lipid-induced aggregation by displacing toxic

oligomers species from the membranes. In particular, we tested the hypothesis that the protective effect of trodusquemine may be at least in part due to the induction of the HSGs.

Finally, we performed preliminary studies to translate our findings in vivo and we tested the potential therapeutic effects of trodusquemine in an AD mouse model characterized by severe AD-like pathology (5XFAD) focusing on Aβ aggregation and evaluating behavioural alterations and histological parameters.

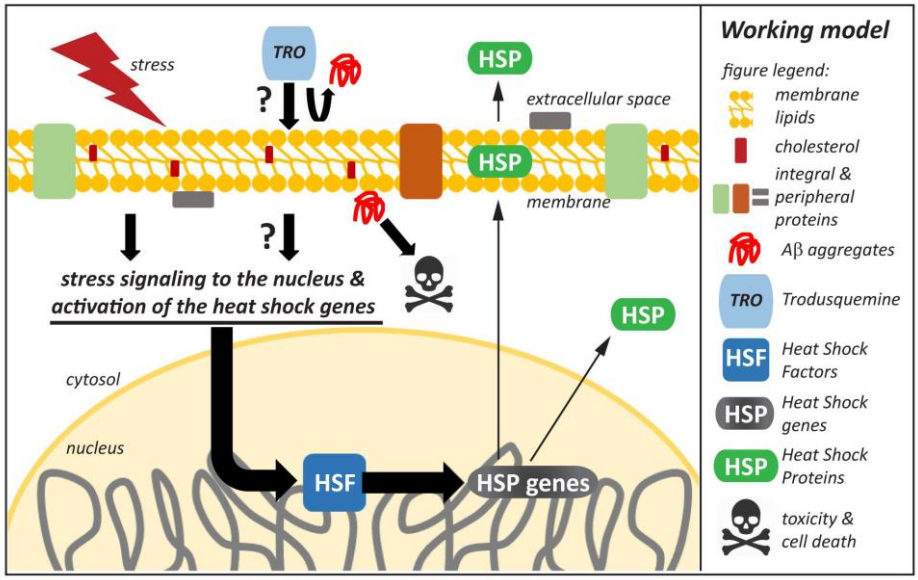


Figure 26. Schematic representation of the working hypothesis studied in this PhD thesis. Membranes are not only a physical barrier for the cell, but are involved in the cellular response to stimuli, including stress, and in the modulation of the intracellular signaling; one of the most important intracellular signaling pathway for cell stress response, adaptation and survival is represented by the Heat Shock Response that includes the Heat Shock Factors and the Heat Shock genes coding for Heat Shock Proteins. The physical state of the membranes can control the expression of Heat Shock genes; once synthesized, HSPs can block protein aggregation intracellularly, can interact with membranes and can be released in the extracellular space. Membranes can be damaged upon environmental stress and as a result of pathological state; in particular, aggregated proteins such as α-synuclein and Aβ peptides interact with lipid membranes (intracellularly but also extracellularly) and damage them, promoting toxicity and inflammation. Moreover, lipids facilitate α-synuclein and Aβ aggregation. Trodusquemine (TRO) displaces α-synuclein and Aβ from membranes and protects them from their toxic effects. Drugs that boost the HSR acting as co-inducers protect against protein aggregation-mediated toxicity.
Based on these data we hypothesize that TRO provides protection by acting on lipids and inducing the HSR and the synthesis of HSPs. small HSPs interact with lipids and reduce the aggregation or Aβ and α-synuclein acting directly on the peptides but also at the level of lipid-induced protein aggregation. We hypothesize that small HSPs displace α-synuclein from lipids, providing protection. Thus, co-treatment with TRO and HSP co-inducers may have synergistic protective effects.

3. Materials & Methods

Protein production

α-synuclein production

α-synuclein was expressed and purified as described previously (Galvagnion et al. 2015; 2016). Briefly, Escherichia coli strain BL21 competent cells were transformed with pT7-7 plasmid coding wild type human α-synuclein. Protein expression was performed by addition of 1 mM isopropylβ-thiogalactopyranoside (IPTG) at 30°C for 4h. Following purification, the purity of the aliquots was evaluated by SDS–PAGE and we used an extinction coefficient of 5600 M^{-1} cm^{-1} at 275 nm for protein concentration measurements. The protein solutions were aliquoted, flash frozen in liquid nitrogen and stored at -80°C, until required for use. Protein purification was performed in the laboratory directed by Prof. Michele Vendruscolo (University of Cambridge, UK).

HSPB3 and HSPB7 production

HSPB3 and HSPB7 were expressed and purified in Escherichia coli strain BL21 using pET21a containing the human HSPB3 coding sequence, or pET23b containing HSPB7 (kindly provided by Prof. Johannes Buchner, Technische Universität München). After the transformation performed with heat shock method, cell growth was carried out at 37°C with specific antibiotic under constant shaking at 250 rpm to an OD_{600} of 0.6. Subsequently, the protein expression was induced with 1mM IPTG at 30°C for 4h and cells were then harvested by centrifugation at 7,000rpm for 10min at room temperature. Cell pellet was lysed in Buffer A (20 mM phosphate buffer at pH = 7.40, with added 100 mM NaCl and 2 mM DTT) supplemented with 1 tablet of cOmplete Protease Inhibitor Cocktail and Benzonase 250 U/mL for 40min on ice, followed by sonication: 2x5min 60% amplitude with pulse 2sec ON/ 2sec OFF. After centrifugation at 13,500rpm for 1h, the inclusion bodies containing the protein were washed twice with Wash Buffer (20 mM phosphate buffer, pH 7.4; 100 mM NaCl; 0.05 % Triton X-100) using 4 to 6mL per g of wet cell weight. Then, to solubilize the inclusion bodies were incubated at 30°C for 15min in buffer A containing 6 M urea. After centrifugation at 14,000rpm for 1h, the solution was filtered through 0.22 µm filter. The sample in 6M was first diluted twofold with buffer A (to 3 M urea) and then fivefold from the sample in 3 M urea (to 0.6 M urea) to allow the refolding of the protein. Finally, the dialysis in phosphate buffer (pH 7.4) was performed overnight. The purity of the aliquots after each step was analysed by SDS–PAGE and the protein concentration was measured from the absorbance at 275nm using correspondent extinction coefficient. The protein solutions were aliquoted, flash frozen in liquid N2 and stored at -80°C, until required for use. Protein purification was performed in the laboratory directed by Prof. Michele Vendruscolo (University of Cambridge, UK).

HSPB5 production

HSPB5 was expressed and purified as described previously (Peschek et al. 2013). Escherichia coli strain BL21 was used to transform plasmid pET28b+ containing the human HSPB5 coding sequence (kindly provided by Prof. Johannes Buchner, Technische Universität München). Escherichia coli was cultured at 37°C with ampicillin (150 ug/mL) under constant shaking at 250 rpm to an OD_{600} of 0.6 and protein expression was induced by 1 mM IPTG for 4hr at 30°. After centrifugation, cell pellet was lysed in Buffer A (50 mM Tris/HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA; 2 mM DTT) supplemented with 1 tablet of cOmplete Protease Inhibitor Cocktail and Benzonase 250U/mL, followed by sonication: 2x5min 60% amplitude with pulse 2sec ON/ 2sec OFF. The lysate was filtered through 0.22 µm filter and was loaded on a Hi-Trap column equilibrated with TE buffer (50mM Tris-HCl; 2mM EDTA; pH 9) and then developed by a linear (20mM–1M) gradient of NaCl. Fractions containing the target protein were pooled and applied onto a Superdex 200-pg column equilibrated in TE buffer and eluted with linear (20mM–1M) NaCl gradient, followed by dialysis in phosphate buffer (pH 7.4). The purity of the aliquots after each step was analysed by SDS–PAGE and the protein concentration was measured from the absorbance at 275nm using protein extinction coefficient. The protein solutions were aliquoted, flash frozen in liquid N2 and stored at -80°C, until required for use. Protein purification was performed in the laboratory directed by Prof. Michele Vendruscolo (University of Cambridge, UK).

HSPB8 production

HSPB8 was expressed and purified as described previously (Carra et al. 2005). Escherichia coli strain BL21 was used to express pGEX-4T encoding the GST-tagged human HSPB8 coding sequence (Carra et al., 2005). Cultures of *Escherichia coli* were grown at 37°C with ampicillin (150 ug/mL) with shaking. Protein expression was induced OD_{600} 0.6 with 1 mM of IPTG and 10mL/L arabinose at 30°C for 4h. PBS buffer (supplemented with 1mM DTT, 1 tablet of cOmplete Protease Inhibitor Cocktail and Benzonase 250U/mL) was used to lysate the bacteria pellet, followed by sonication: 2x5min 60% amplitude with pulse 2sec ON/ 2sec OFF. The lysate obtained was loaded on Protino GST/4B Column and then eluted with buffer containing GsH (PBS pH7.4, 1mM EDTA, 1mM DTT, 20mM GsH). Dialysis was performed overnight at 4°C with rotation in PBS buffer and DTT, adding with 1 table of cOmplete and Benzonase 250U/mL to remove GsH and other elution buffer components. Solution was reloaded into Protino GST/4B Column and developed by a linear KCl gradient (20mM - 1M). SDS-PAGE gel was run to select the fractions containing HSPB8, which were applied on a Hi-Trap column equilibrated with low salt buffer (40mM Hepes pH 7.4, 20mM KCl, 1mM DTT, 1mM EDTA). A linear KCl gradient (20mM - 1M) was developed to eluate the protein, followed by dialysis in (pH 7.4). The purity of the aliquots after each step was analysed by SDS–PAGE and the protein concentration was measured from the absorbance at 275nm using protein extinction coefficient. The protein solutions were aliquoted, flash frozen in liquid N2 and stored at -80°C, until required for use. Protein purification was performed in the laboratory directed by Prof. Michele Vendruscolo (University of Cambridge, UK).

HSPB6 production

HSPB6 was expressed and purified as reported previously (Bukach, Sb, and Nb 2004). Plasmid pUBS520 containing the human HSPB6 coding sequence (kindly provided by Prof. Johannes Buchner, Technische Universität München) was transformed into Escherichia coli strain BL21. E. coli was cultured at 37°C with ampicillin (150 ug/mL) under constant shaking at 250 rpm to an OD_{600} of 0.6. 1mM IPGT was added for the protein expression and bacterial culture was continued for further 4 h at 30°C. Cells were then harvested by centrifugation and the pellet was lysed in Buffer A (Tris/HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA; 2 mM DTT) supplemented with 1 tablet of cOmplete Protease Inhibitor Cocktail and Benzonase 250 U/mL followed by sonication: 2x5min 60% amplitude with pulse 2sec ON/ 2sec OFF. The extract of HSPB6 was fractionated with (NH4)2SO4 (40% saturation – following the method from Hyman's lab) and stirred for 30min at 4°C. Then, centrifugate at 16,000rpm for 1hr and pellet was resuspended in Buffer B (20 mM Tris/acetate, pH 7.6; 10 mM NaCl; 0.1 mM EDTA; 0.1 mM PMSF). Ion-exchange chromatography was performed on a High-Trap Q column equilibrated with Buffer B and a linear (10 – 410 mM) gradient of NaCl was developed. Fractions containing the protein were gel filtrated on a Sephacryl S300 High-Prep 16/60 column, followed by dialysis in phosphate buffer (pH 7.4). The purity of the aliquots after each step was analysed by SDS–PAGE and the protein concentration was measured from the absorbance at 275nm using protein extinction coefficient. The protein solutions were aliquoted, flash frozen in liquid N2 and stored at -80°C, until required for use. A similar protocol was followed for the purification of the three HSB6 mutants. Protein purification was performed in the laboratory directed by Prof. Michele Vendruscolo (University of Cambridge, UK).

Site-Directed Mutagenesis

QuickChange II Site-Directed Mutagenesis Kit was used to produce the three mutant variants of HSPB6 (serine-16 was replaced with cysteine or aspartic acidc or alanine). The PCR protocol was followed from the Kit Manual and the sample reaction was prepared as follows: 5 µl of 10× reaction buffer, X µl (5{50 ng) of dsDNA template, X µl (125 ng) of oligonucleotide primer 1, X µl (125 ng) of oligonucleotide primer 2, 1 µl of dNTP mix, ddH2O to a final volume of 50 µl. Then add 1 µl of PfuUltra HF DNA Polymerase (2:5U=µl). X was calculated based on the molecular weight of the primers used (Sigma Aldrich). Site-directed mutagenesis was performed in the laboratory directed by Prof. Michele Vendruscolo (University of Cambridge, UK).

DNA Sequencing

DNA was sequenced using Sanger DNA Sequencing and analysis was carried out GeneStudio Professional Edition. 10 µl of plasmid DNA was sequenced.

Creation of Phosphorylation Mimetics

Using a semi-synthetic approach for the creation of phosphorylation mimetics, the cysteine was chemically transformed into a dehydroalanine (Dha) residue. To do so, the HSP6 cysteine mutants

were in 20mM sodium phosphate buffer pH 7.4. 200 µM of 5 protein aliquots were reacted with 5 molar equivalents of methyl 2,5-dibromopentanoate 20 (Sigma Alrich) for 48-72 hrs at 37 °C and shaking at 500 rpm. Excess methyl 2,5-dibromopentanoate was removed by running the reactions through 7k MWCO Zeba (ThermoFisher) spin desalting columns and then conversion to Dha was verified via LC-MS. To add the phosphate group, 100 µL aliquots of 50 µM of SXDha in 20 nM sodium phosphate pH 7.4 were reacted batchwise (5 min intervals) with 30,000 molar equivalents of sodium thiophosphate (pH 8.0, 690 mg/mL suspension, 5x5000 equivalents), where X = 9, 42, 87 or 129. The mixtures were left to react for 24 hours at 37 °C and shaking at 500 rpm. Excess sodium thiophosphate was removed via two 7k MWCO Zeba (ThermoFisher) spin desalting columns and reaction was verified via LC-MS. These experiments were performed in the laboratory directed by Prof. Michele Vendruscolo (University of Cambridge, UK).

Seed fibril preparation

Seed fibrils were produced as described previously (Kim et al. 2014). 500 µL samples of α-synuclein at concentrations from 500-800 µM were incubated in 20 mM phosphate buffer pH 6.5 at ca. 40◦C for 48-72hr and stirred at 1,500 rpm with a Teflon bar on an RCT Basic Heat Plate (IKA, Staufen, Germany). Fibrils were diluted to a monomer equivalent concentration of 200 µM. The aliquots were flash frozen in liquid N2 and stored at -80◦C. For assay at pH 6.5 the 200 µM fibril stock was sonicated for 1 min using a probe sonicator (Bandelin, Sonopuls HD 2070, Berlin, Germany), using 10% maximum power and a 50% cycle; while the 200 µM stock was diluted to 10 µM in water, sonicated 3 times for 5 s using 10% maximum power and 50% cycles using the probe sonicator for assay at low pH with nM fibril concentrations. Seed fibrils were produced in the laboratory directed by Prof. Michele Vendruscolo (University of Cambridge, UK).

Lipid vesicle preparation

Lipid vesicles were prepared as previously described using sonication (Galvagnion et al. 2016). DMPS lipid powder was dissolved in 20 mM phosphate buffer (NaH_2PO_4/NaH_2PO_4 - pH 6.5), and stirred at 50°C for 2hr. The solution was subjected to 5 cycles of freezing and thawing using dry ice and a water bath at 50°C. Lipid vesicles were obtained by sonication (Bandelin, Sonopuls HD 2070, 3 x 5min, 50% cycle, 10% maximum power) and centrifuged at 15,000 rpm for 20min at room temperature. After centrifugation, the sizes of the lipid vesicles were measured by dynamic light scattering (Zetasizer Nano ZSP, Malvern Instruments, Malvern, UK) and show to consist of a distribution centred at 20 nm diameter. Lipid vescicles were produced in the laboratory directed by Prof. Michele Vendruscolo (University of Cambridge, UK).

Measurements of aggregation kinetics (ThT assay)

α-synuclein monomers were incubated at the concentration 20μM, in the presence or absence of HSPBs at the concentrations indicated, and of 50 μM ThT and either preformed fibrils or DMPS vesicles at 37◦C or 30◦C, respectively (Flagmeier et al. 2016). The change in the ThT fluorescence signal was detected using a Fluostar Optima or Polarstar Omega fluorescence plate reader (BMG Labtech, Aylesbury, UK) in bottom reading mode under quiescent conditions. Corning 96 well plates with half-area (3881, polystyrene, black with clear bottom) non-binding surfaces with metal sealing tape were used for each ThT assay. At the end of each aggregation experiment the concentrations of monomeric and fibrillar states of the protein were determined as described previously (Flagmeier et al., 2016). ThT assay were performed in the laboratory directed by Prof. Michele Vendruscolo (University of Cambridge, UK).

Analysis of the aggregation kinetics

Determination of the lipid-induced aggregation rate

The change in mass concentration of fibrils with time M(t) was fitted using the model described previously (Galvagnion 2015; Flagmeier et al., 2016). Using AmyloFit (Meisl et al. 2016), we next estimated the rate of aggregation of each variant on lipid vesicles by fitting the kinetics to the model described above.

Derivation of the approach used to analyse highly seeded aggregation data

The change in mass concentration of fibrils for aggregation experiments at high seed concentrations (μM) was fitted using the model described previously (Flagmeier et al., 2016). To compare the effective rate constants when different HSPBs are introduced, we then calculated the ratio of the obtained k+ value for each chaperone to that of the control experiment with α-synuclein alone. All the formula required for the determination of aggregation kinetics and the lipid-induced aggregation rate were provided by the laboratory directed by Prof. Michele Vendruscolo (University of Cambridge, UK).

Cell lines

SH-SY5Y, U2OS wild type (WT) and U2OS HSF1-KO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (1 g/L d-glucose, l-glutamine, pyruvate) and supplemented with 10% Fetal Bovine Serum (FBS) and 100 U/mL penicillin/streptomycin and (FBS) in a humidified atmosphere at 37°C incubator with 5% CO2. Cells were routinely tested for mycoplasma contamination using the MycoAlert kit. U2OS WT and USOS HSF1-KO cells were kindly provided by prof. Valerie Mezger (CNRS, Université Paris Diderot, France). We kindly acknowledge prof. Valerie Mezger for sharing these cell lines with us. Cell culturing was performed in Prof. Carra lab.

Treatments in cells

Trodusquemine was kindly provided by Prof. Fabrizio Chiti (University of Florence). Trodusquemine aliquot was dissolved in sterile water and stored at +4°C (compound stable for 21 days). Protein Tyrosine Phosphatase 1B Inhibitor BML (#765317-72-4) and HSF1 inhibitor KNK (#15395-10) were bought from Cayman chemical, SREBP inhibitor PF-429242 dihydrochloride (#947303-87-9) was bought from Sigma-Aldrich. Cells were seeded 24hr before the treatment and were treated at 70-80% confluency. Treatment were performed with 5µM or 10µM trodusquemine at 37°C for 1hr ; with 20µM BML at 37°C for 1hr; with 20µM PF-429292 at 37°C for 1hr as indicated in each experiment. Cell treatments were performed in Prof. Carra lab.

Fluidity membrane assay

Membrane fluidity kit ab189819 (abcam) was used to perform the fluidity membrane assay. To evaluate the changes in membrane fluidity was used a lipophilic pyrene probe, pyrenedecanoic acid (PDA), that undergoes dimerization upon spatial interaction. When interacting with cell membrane, the probe diffuses through the lipid bilayers and it forms homodimers, or “excimers”, with an amount of excimer formation proportional to the membrane fluidity. The formation of excimers allows the change of emission spectrum of the probe, and by measuring the ratio of excimer (Em ~470nm) to monomer (Em max. ~400nm) fluorescence, a quantitative monitoring of the membrane fluidity can be obtained. The samples were prepared following the protocol provided in the Kit Manual. Labelling solution was prepared with 5 µM Fluorescent and and Pluronic F127 at a final concentration of .08%. Cells were previously seeded in 96-multiwell plate (ThermoFisher) and then were incubated with Fluorescent Lipid Reagent/Pluronic F127 solution for 1 hr at 25°C in the dark. After incubation, unincorporated PDA was removed by washing cells twice with perfusion buffer and then cells were resuspended in cell media. Fluorescence was red at both 400nm and 450-470nm using the appropriate filter for excitation at 350nm. For the analysis, the blanks from each sample were subtracted, following by the average the readings of duplicates, and calculate the normalized excimer to monomer fluorescence ratio. We acknowledge Prof. Livio Casarini for sharing with us the equipment required for measuring the fluidity membrane.

RNA extraction and RT-qPCR

For gene expression analysis on cell lines, total RNA extraction was performed using ReliaPrep RNA Cell Miniprep system following the protocol provided by the Kit Manual. Maxima cDNA Synthesis Kit was used to carry out the Retrotrascription. For each reaction 1µg of total RNA was used. The samples were incubated at 25°C for 10 minutes, then at 50°C for 15 minutes, and the last step was performed at 85°C for 5 minutes. The cDNA can be stored at -20°C. CFX96 Touch Thermal cycler (Bio-Rad, Hercules, CA, USA) was used to analyse the sample cDNA through qPCR. 25ng of cDNA was used for each reaction, supplemented with TAQ SYBR Green qPCR SYBR (Sigma-Aldrich) and primers of the gene of interest. The real-time PCR protocol was as follows: one cycle of denaturation (95°C for 3 minutes) followed by 40 cycles of amplification (95°C for 10 seconds, 60°C for 30 seconds). DNA amounts were quantified using

the ΔΔCt method, samples were normalized over housekeeping gene and calibrated over the untreated condition (control). A list of all primers used is provided in Supplementary Table below. RNA extraction and RT-qPCR were performed in Prof. Carra lab.

5XFAD mouse model

In this study were used heterozygous 5XFAD mice, which co-overexpress a triple-mutant human APP (Swedish mutation: K670N, M671L; Florida mutation: I716V; London mutation: V717I) and a double-mutant human PS1 (M146L and L286V mutations), and C57Bl6-SJL mice, as wild type (Wt) control. All mice were kept in conditioned rooms with stable temperature (21°C) and humidity (60%), on a light/dark cycle of 12 hours (h). Food and water were available ad libitum and body weight was recorded throughout the entire observation period. All animal procedures were approved by the Committee on Animal Health and Care of the University of Modena and Reggio Emilia and Italian Ministry of Health (protocol number: n°669/2021-PR) and conducted in accordance with National Institutes of Health guidelines.

Trodusquemine treatment *in vivo*

Trodusquemine was kindly provided by Prof. Fabrizio Chiti (University of Florence). Fresh trodusquemine solution was before the injection, dissolved in saline solution and protected from light exposition. The drug was administered intraperitoneally 1 time per mouse every week over a period of 9 week at a dose of 5 mg/kg body weight starting at 2 months of age. Vehicle-treated controls received sterile saline (0.9% NaCl in water).

Behavioural tests

Novel object place recognition (OPR)

The novel object recognition trials were conducted to determine memory acquisition based on the tendency for mice to explore unfamiliar objects. The task was adapted to the age of the animals (Dyets et al. 2019). Briefly, for two consecutive days mice were placed in a 50 cm squared box with gray floor and black walls with 4 visual cues differing for both shape and colour. During these 15 min trials mice were allowed to habituate to the empty apparatus. On the third day, 2 identical and parallel objects, distant 10 cm from the wall, were presented to the animal for 10 min. On the fourth day mice were recorded for 5 min while exploring objects placed as on the previous day for 5 min in the so called pre-test. Two h after the pre-test the position of one object was changed by placing it in front of a different wall (new placed object) and the time spent by the animal near the new placed object with respect to the familiar one was recorded in a 5 min trial. 6 and 24 hours after the pre-test the new placed object of the previous test remained in the same position and was used as familiar one, while the familiar object was moved in a new location and became the new placed object. On both trials, of 5 min duration, the time spent near the new and old-located objects were

recorded. Positive exploration was defined as touching the object with the nose or directing the nose to the object within a 10 cm long elliptical area surrounding the object. The time of exploration was recorded and analysed using ANY-maze video tracking system (Stoelting). The discrimination index was calculated as % of time exploring the new placed object minus the % of time on familiar object divided for the % of time exploring both objects ((% time new - % time familiar)/(% time new + familiar).
All data are shown as mean ± standard error of the mean (SEM) and were analysed by, two-way ANOVA followed by Bonferroni multiple comparisons using Graphpad Prism statistical software. Differences were considered significant with $p < 0.05$ while $0.10 > p > 0.05$ was considered as trend for a significant difference.

Morris Water Maze (MWM)

After 9 weeks of treatment, mice were subjected to Morris Water Maze (MWM) procedure to assess spatial learning and memory as previously described (Daini et al., 2022; Daini et al., 2021). Briefly, animals were placed in a circular white pool with a diameter of 120 cm filled with water at 23± 2°C made opaque by adding white non-toxic paint and allowed to swim for 90 seconds (sec) or until they found the location of a hidden circular platform with 11 cm diameter (escape latency). During this *learning phase* mice were trained with four consecutive trials per day (starting from a different quadrant with a different visual cue at each trial) for five days and escape latency was recorded and averaged across the daily trial session. To assess memory retention, the platform was removed on day 6 and the animals were allowed to swim for 60 sec (p*robe test*), and the latency to reach the platform area, total entries in the platform area and the % of time spent in target quadrant were evaluated.

Behavioural test was performed by an operator unaware of the treatment to avoid bias. Animal behaviour was conducted in a sound-proof room, recorded and automatically analyzed with ANY-maze Video Tracking system (Stoelting). Target search strategy was manually scored, independently, by two operators unaware of the treatment.

All data are shown as mean ± standard error of the mean (SEM) and were analysed by one-way analysis of variance (ANOVA), two-way repeated measures ANOVA followed as appropriate using the statistical GraphPad. Differences were considered significant with $p < 0.05$ as the level for a significant difference and $0.10 > p > 0.05$ as trend for a significant difference.

Sample preparation and histology

Twenty-four hours after the last behavioural test (9^{th} week of the study), 4 mo 5XFAD mice were anesthetized with isoflurane and sacrificed for histological analysis according to established protocols. Briefly, intracardial perfusion was performed with cold 4% paraformaldehyde (70 mL/7 min) preceded by an infusion of 50 ml of saline containing heparin sodium (5000U/L); brain

was dissected out and postfixed in the same solution for 12 h, rinsed in a 30% sucrose-PBS solution for two days. The brains were then frozen using dry ice, and coronal 20 µm thick sections series were cut at a cryostat, washed three times in cold PBS and stored at -20°C in a glycerol-PBS solution until use.

Immunohistochemistry of brain sections

Brain slices were processed according to established protocols to test AD-linked alterations; the following antibodies (Abs) were used: mouse anti-human Aβ/APP 6E10 (epitope human Aβ1-16, 1:500, Signet, #9300-10), mouse anti-human Aβ 11A1 (epitope synthetic peptide of E22P-Aβ 10-35 part, 1:500, Tecan, cat. n. 10379), rabbit anti-ionized calcium-binding adapter molecule 1 (IBA1, 1:1000, Wako, #019-19741).

A pre-treatment with formic acid was performed for anti-Aβ 6E10. Vectastain ABC-HRP (#PK-4002; #PK-4001) kit was used for peroxidase diaminobenzidine stainings; slices were placed on gelatinized glass slides, dehydrated and mounted with Eukitt® mounting medium.

Image and statistical analyses

Immunolabeled brain slices from wild type (Wt) and 5XFAD mice were photographed using a Nikon Eclipse CiL microscope (10x objective) through a Nikon DS-Fi3 camera under constant light conditions. To avoid any bias, mice were encoded by numbers and analyzed by an operator unaware of the treatment or the genotype.

For 6E10, 11A1 and IBA1 analysis, at least three images/mouse from cortex (CTX) (Bregma between -0.5 and -0.9), hippocampal dentate gyrus (DG), cornu Ammonis (CA) 1 (Bregma between -1.46 and -1.58 mm from Paxinos and Franklin 2019) were obtained using Nikon Eclipse Ci microscope and plaques/cells were manually counted in a $1mm^2$ area. IBA1 images were also converted to 32 bit grayscale and binarized applying a specific threshold (shift from the background mean grey value, -20) . Then, % of positive area, i.e., the area covered by pixels with gray value darker than the selected threshold, was automatically recorded through ImageJ software.

Data are expressed as mean ± SEM and were analysed by means of student t test or Mann-Whitney as appropriate. Statistical analyses were performed through GraphPad PRISM software and $p < 0.05$ was considered for a significant difference.

4. Results

4.1 HSPBs act on the α-synuclein aggregation process through multiple mechanisms

Small Heat Shock Proteins (sHSPs) are a ubiquitous class of molecular chaperones, found in all kingdoms of life from archaea to humans. The human genome contains 10 different genes coding for sHSPs, which are named according to the new nomenclature HSPB1 to HSP10 (Kampinga et al. 2009). While some HSPBs are expressed in many different cell types and tissues, such as HSPB1 and HSPB5, others are expressed only in a few cell types and are tissue-specific, such as HSPB3, HSPB9 and HSPB10 (Boncoraglio et al. 2012). In addition, HSPBs have been found intracellularly, in the extracellular space and can even be released by cells via extracellular vesicles, broadening their sites of action (Batulan et al. 2016; Van den Broek et al., 2021).

One important property of sHSPs/HSPBs that influences their biological activity is represented by their ability to interact with themselves and other members of the family, forming an ensemble of homo and hetero-oligomeric complexes that are highly dynamic. This ability to assemble in inter-converting oligomers and the intrinsic flexibility of their structure allow to acquire different functional conformations; this, in turn, allow HSPBs to interact with many different substrates, performing pleiotropic functions. Key for the assembly of homo- and hetero-oligomers is the α-crystallin domain, which represents the signature of these proteins; yet, also the N- and C-terminal regions participate in the stabilization of the homo- and hetero-oligomers (Carra et al. 2019; Haslbeck et al. 2019).

One of the best characterized functions of HSPBs is their chaperone activity: HSPBs prevent protein misfolding and aggregation by recognizing and binding to exposed hydrophobic patches; since HSPBs do not possess ATP activity, the refolding of the substrate is mediated, whenever possible, by the HSP70 ATP-dependent system; instead, HSPBs can assist the targeting of damaged proteins to the proteasome and autophagy pathways for degradation, acting in concert with chaperones and co-factors (e.g. the HSPB8-BAG3-HSP70 complex targets misfolded species to autophagy) (Carra et al. 2019). Concerning the type of substrates recognized by HSPBs, they bind to non-native and aggregate-prone intermediates, preventing the deposition of insoluble amyloid formation (Carra et al. 2013; Mogk and Bukau 2017; Haslbeck et al. 2019) which is associated to the pathogenesis of several neurodegenerative disorders, including Parkinson's and Alzheimer's diseases.

In this PhD thesis, we studied the ability of five different members of the HSPB family - HSPB3 (Hsp17), HSPB5 (αB-crystallin), HSPB6 (Hsp20), HSPB7 (cardiovascular Hsp), and HSPB8 (Hsp22) – to inhibit the aggregation of α-synuclein, which was used as a protein model. In particular, we investigated whether, at the molecular level, HSPBs could bind to α-synuclein in its monomeric, oligomeric and fibrillar forms with different affinities, thus acting on specific steps of its aggregation process. Importantly, we addressed the question whether/how HSPBs, which can bind to lipid membranes, may also influence the lipid-induced aggregation process of α-synuclein, an aspect that has not been studied so far (Figure1).

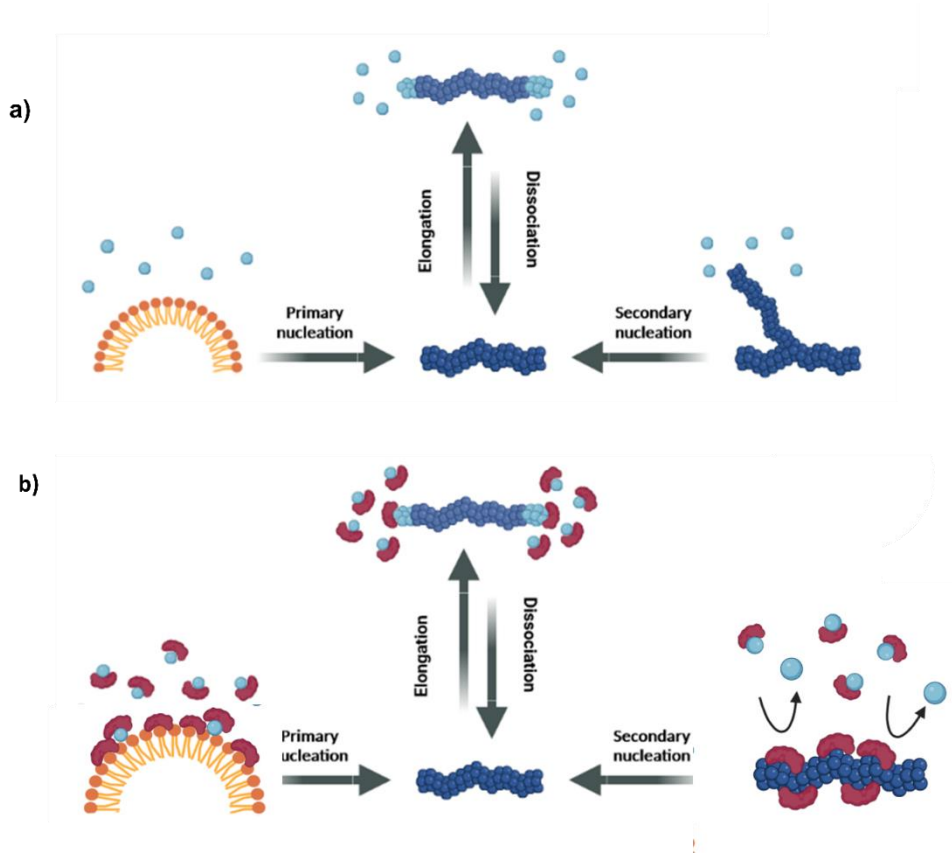


Figure 1. Schematic representation of the hypothetical mechanisms of action of HSPBs that would be involved in preventing α-synuclein aggregation. a) Schematic representation of the three main step of α-synuclein aggregation: primary nucleation, fibril elongation and secondary nucleation. b) HSPBs (purple) could bind to α-synuclein in its monomeric (light blue) and fibrillar forms (blue). HSPBs could also bind to lipid-associated forms of α-synuclein. Alternatively, but not mutually exclusive, HSPBs might compete with α-synuclein for binding to lipids and displace α-synuclein (see bended arrow).

We used a three-pronged approach, recently developed, which allows to disentangle the main steps of the aggregation process: primary nucleation, fibril elongation and secondary nucleation (Galvagnion et al., 2015).

Taking advantage from lipid interaction to induce primary nucleation, a lipid-induced primary nucleation assay has been developed, allowing to study α-synuclein aggregation at physiological concentrations, which are estimated to be lower than 30 - 60 µM (Figure 2a) (Flagmeier *et al*, 2016; Galvagnion *et al.*, 2015; Perni *et al*, 2018). Primary nucleation is then followed by secondary nucleation events that result in proliferation of exiting amyloid fibrils, such as elongation and surface-assisted nucleation (Buell et al., 2014). Upon formation of mature fibrils generated through primary nucleation, monomeric α-synuclein are inserted into the growing extremity of mature fibril to promote the fibril elongating. Hence, a second assay has been developed to characterize fibril elongation in the presence of preformed seed fibrils, where elongation of the added seeds is kinetically prevalent (Figure 2b). Next, a third assay has been developed to study fibril amplification, which occurs via secondary pathways, such as formation of surface-catalysed aggregates, under mildly acidic pH conditions (Figure 2c) (Flagmeier *et al.*, 2016). These mildly acidic environmental

conditions mimic the low pH (5.5) that is typical of dopaminergic neurons, which express the highest amount of α-synuclein (Sinning & Hubner, 2013).

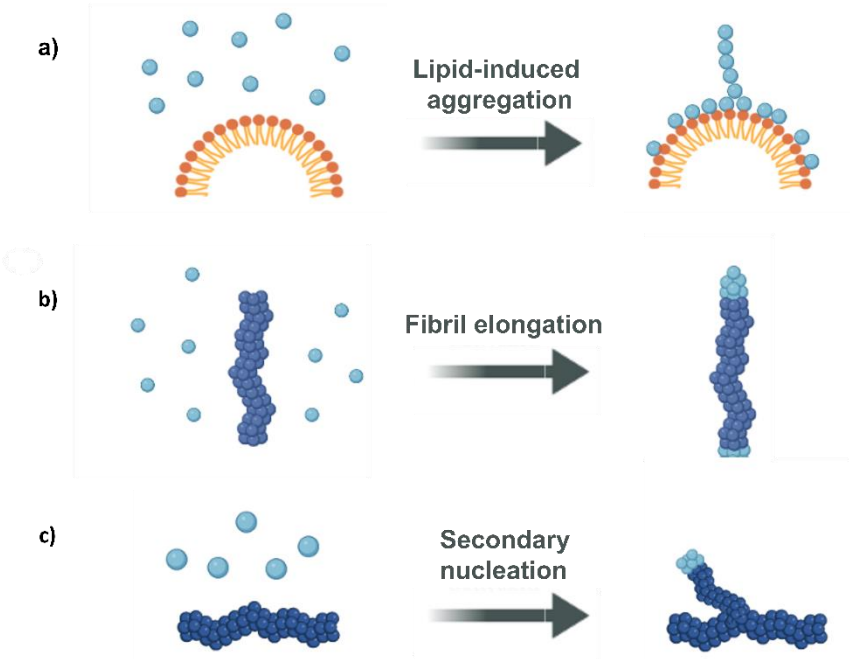


Figure 2. Three-pronged approach to study a-synuclein aggregation. a) Step 1: lipid-induced aggregation, where monomeric α-synuclein form growth-competent nuclei form due to the interaction with lipid surfaces. b) Step 2: elongation, where existing preformed fibrils at high concentrations assembly in longer fibrils in presence of monomeric α-synuclein. c) Step 3: secondary nucleation, where preformed fibrils at low concentrations along with monomeric α-synuclein under mildly acidic pH, lead to secondary pathways such as surface-catalyzed aggregates.

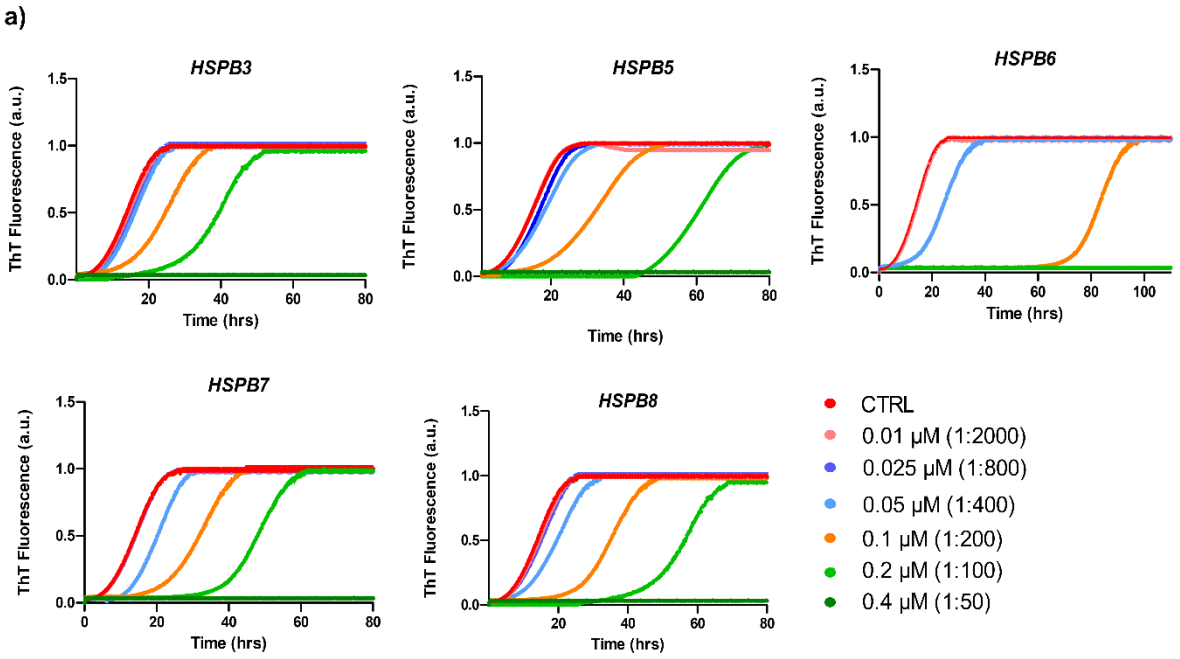
Step one: HSPBs inhibit α-synuclein lipid-induced aggregation with different degrees of efficacy

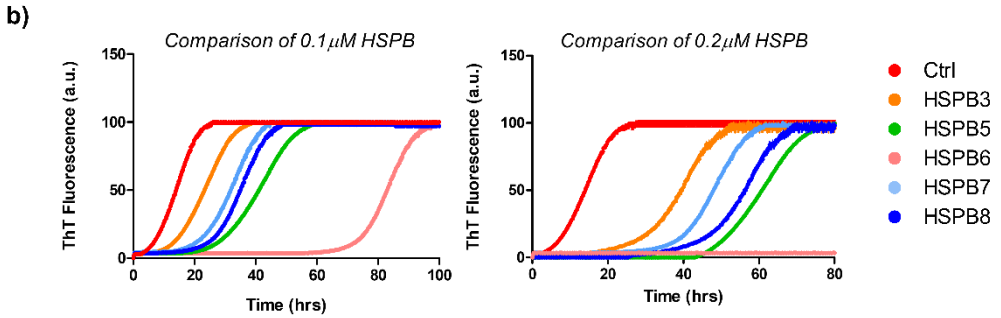
The primary nucleation event initiates the protein aggregation process with a slow and spontaneous formation of growth-competent nuclei from monomeric α-synuclein in solution. Recently, it has been shown that the primary nucleation is significantly prompted by the α-synuclein interaction with lipids, the main component of cellular membranes (Fusco *et al.*, 2017; Galvagnion *et al.*, 2016; Galvagnion *et al.*, 2015). In particular, small unilamellar vesicles (SUVs) prepared from model membrane lipid DMPS (1,2-dimyristoyl-sn-glycero-3-phospho-L-serin) were reported to enhance the α-synuclein aggregation rate in vitro (Figure 2a) (Galvagnion et al., 2015).

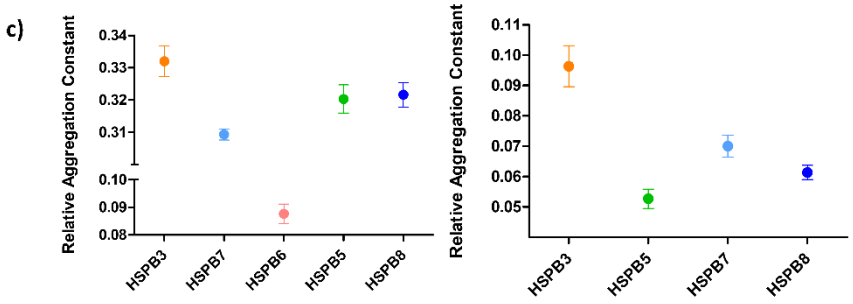
Focussing on five different HSPBs (HSPB3, HSPB5, HSPB6, HSPB7, and HSPB8), we investigated whether they influence the lipid-induced aggregation of α-synuclein in vitro, using artificial lipid vesicles made of specific types of lipids. The type of lipid vesicles used in this study are referred to as “small unilamellar vesicles” (SUVs) and they vary in composition and size, ranging from 20 to 100 nm. In particular, we employed SUVs composed of phospholipid 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (DMPS).

Using DMPS lipid vesicles mixed with α-synuclein and the Thioflavin T (ThT) fluorescence signal, we measured the amyloid fibril formation over time. ThT is a fluorescent dye used to quantify the formation and/or inhibition of amyloid fibrils owing to its ability to bind to β-sheet-rich structures, such as those found in amyloid aggregates, and display a characteristic shift of its emission spectrum. The aggregation of α-synuclein only in presence of DMPS was measured after 5 hrs of incubation; under these conditions we reproduced previously published data and we confirmed the lipid-induced aggregation of α-synuclein (Figure 3a) (Galvagnion et al. 2015). Next, we co-incubated the lipid vesicles with α-synuclein and each HSPB selected for our study, and we compared the aggregation rates. All the HSPBs tested delayed the lipid-induced aggregation on the timescale monitored; however, they displayed different strength of efficacy (Figure 3a,b). As reported in Figure 3a, using different concentrations we were able to show that all the HSPBs acted in a dose-dependent manner to inhibit α-synuclein lipid-induced aggregation. The amyloid formation was completely prevented at the highest concentration tested (0.4 μM) by HSPB6, while no effect was detected at the lowest concentration (0.01 μM) for all the HSPBs tested, since the aggregation curves were identical to that of α-synuclein and DMPS only (used as reference).

Comparing the behaviour of the different HSPBs at the same concentration (Figure 3b), we could extrapolate that HSPB3 showed the weakest inhibitory effect at both doses used, whereas HSPB6 exhibited the strongest effect, as it totally prevented α-synuclein lipid-induced aggregation (Figure 3b). The other HSPBs studied displayed intermediate effects (Figure 3b). Furthermore, we found that once the aggregation was triggered, it proceeded with similar kinetics regardless of the presence of HSPB5-8 (Figures 3b).


a)
HSPB3
HSPB5
HSPB6
HSPB7
HSPB8
ThT Fluorescence (a.u.)
Time (hrs)
CTRL
0.01 μM (1:2000)
0.025 μM (1:800)
0.05 μM (1:400)
0.1 μM (1:200)
0.2 μM (1:100)
0.4 μM (1:50)


b)
Comparison of 0.1μM HSPB
Comparison of 0.2μM HSPB
ThT Fluorescence (a.u.)
Time (hrs)
Ctrl
HSPB3
HSPB5
HSPB6
HSPB7
HSPB8


c)
Relative Aggregation Constant
HSPB3
HSPB7
HSPB6
HSPB5
HSPB8
HSPB3
HSPB5
HSPB7
HSPB8

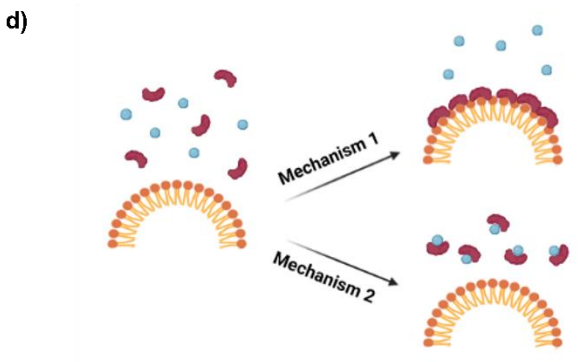

d)
Mechanism 1
Mechanism 2

Figure 3. HSPBs inhibit lipid-induced α-synuclein primary nucleation. (a) Changes in ThT fluorescence intensity when monomeric α-synuclein (20μM) is incubated with 100 μM DMPS vesicles at pH 6.5 and 30 °C and in the absence (red) or presence of each HSPB at increasing concentrations: 0.01 μM (pink), 0.025 μM (blue), 0.05 μM (light blue), 0.1 μM (orange), 0.2 μM (green), 0.4 μM (dark green). Data as absolute ThT fluorescence. a.u. arbitrary units. (b) Direct comparison of kinetic curves between all HSPBs tested at the same concentration: α-synuclein only (red), HSPB3 (orange), HSPB5 (green), HSPB6 (pink), HSPB7 (light blue) and HSPB8 (blue). (c) Relative rate of lipid-induced α-synuclein aggregation constant, determined by fitting a single-step nucleation model to the early time points of the reaction (Galvagnion *et al.*, 2015) in AmyloFit (Meisl et al., 2016), in the presence of HSPB3 (pink), HSPB5 (green), HSPB6 (pink), HSPB7 (blue), HSPB8 (purple), as per conditions in detailed in A. . n = 3; data shown as mean ± SEM. (d) Schematic representation of possible HSPBs effect on lipid-induced aggregation. α-synuclein monomers in light blue; HSPBs in purple.

To explore more in detail the aggregation kinetics, we applied a previously developed one-step nucleation model (Galvagnion et al., 2015), where the primary nucleation event is assumed to take place at the surface of the lipid vesicles and the lipid-bound α-synuclein represents a new growth-competent nucleus that facilitates the fibril formation (Figure 2a). The normalization of the elongation rate in the presence of each HSPBs over that of α-synuclein alone was performed in order to compare the impact of the different HSPBs tested. This comparative analysis showed that HSPB3 displayed the highest relative elongation rate for both the concentrations examined, implying the lowest inhibitor activity (Figure 3c); by contrast, HSPB5, HSPB7 and HSPB8 confirmed their intermediate efficacy in decreasing the lipid-induced aggregation (Figure3c). HSPB6 was excluded from this study as it fully inhibited α-synuclein primary nucleation at concentration of 0.2μM.

Based on these results, two possible mechanisms could be envisaged to explain how HSPBs may inhibit the lipid-induced primary nucleation (Figure 3d). In the first mechanism of action hypothesized, HSPBs compete with α-synuclein for the association with lipid vesicles, thus preventing the α-synuclein embedding into the membrane that would initiate the lipid-induced aggregation. In the second mechanism of action hypothesized, HSPBs interact directly with free α-synuclein monomers in solution, precluding their binding to the lipid surface and reducing the fibril formation (Figure 3b).

To discriminate between the two hypothesized mechanisms of action, we next tested whether HSPBs can directly associate with the lipid surface. To do so, we verified the impact of the various HSPBs on the fluidity of artificial membranes.

It is well known that when proteins interact with lipid membranes, they can influence their fluidity and melting temperature. In particular, it has been reported that when monomeric α-synuclein binds to vesicles composed of DMPS, its melting temperature decreases from 45 °C to 35 °C (Galvagnion *et al.*, 2015). Previous studies performed in collaboration with Prof. Michele Vendruscolo by a former PhD student working in the Carra lab addressed the impact of different HSPBs on membrane fluidity using DMPS lipid vesicles. These unpublished studies demonstrated that HSPB3, HSPB6 and HSPB7 decrease the melting temperature of DMPS lipid vesicles, suggesting

their association with the lipid surface; by contrast, HSPB5 and HSPB8 did not alter the membrane fluidity of DMPS vesicles, suggesting lack of direct association.

To further understand how HSPBs interact with lipids and may influence α-synuclein aggregation, we investigated the impact of HSPBs over time using DMPS as liposome model and carrying out the test at 30 °C. We found that the fluidity of DMPS did not change over time, although we detected changes in fluidity at the beginning of the measurement (Figure 4). According to previous observations (Galvagnion *et al.*, 2015), incubation of α-synuclein with DMPS lipid vesicles strongly enhanced the lipid fluidity, measured by DPH fluorescence polarization. Again, differential effects were observed on membrane fluidity depending on the type of HSPBs co-incubated with α-synuclein. HSPB3 and HSPB5 both affected lipid vesicle fluidity when incubated alone, and the loss of membrane fluidity was slightly rescued when α-synuclein was added (Figure 3b,d). These results may suggest that monomers of α-synuclein can still interact with the lipid membranes; yet, HSPB3 and HSPB5 would bind to the lipid-associated α-synuclein monomers to inhibit their subsequent incorporation, thus preventing α-synuclein lipid-induced aggregation (Figure 3f -Mechanism 1). Concerning HSPB7 and HSPB8 did not alter the fluidity per se (Figure 4a,c), but they could restore membrane fluidity in presence of α-synuclein (Figure 4a,c). We hypothesize that these HSPBs may associate with free α-synuclein monomers, hence preventing α-synuclein binding to lipids and maintaining the membrane fluidity (Figure 3f – Mechanism 2).
HSPB6 showed a different behaviour: when incubated alone with the lipid vesicles, HSPB6 increased their fluidity more than α-synuclein alone; intriguingly, when HSPB6 was co-incubated with α-synuclein and the SUVs, the membrane fluidity was not rescued but, instead, it was further increased (Figure 3e). Taken together, these data suggest that HSPB6 may compete with α-synuclein for binding to lipids and preventing its interaction with the membrane (Figure 3f -Mechanism 3).

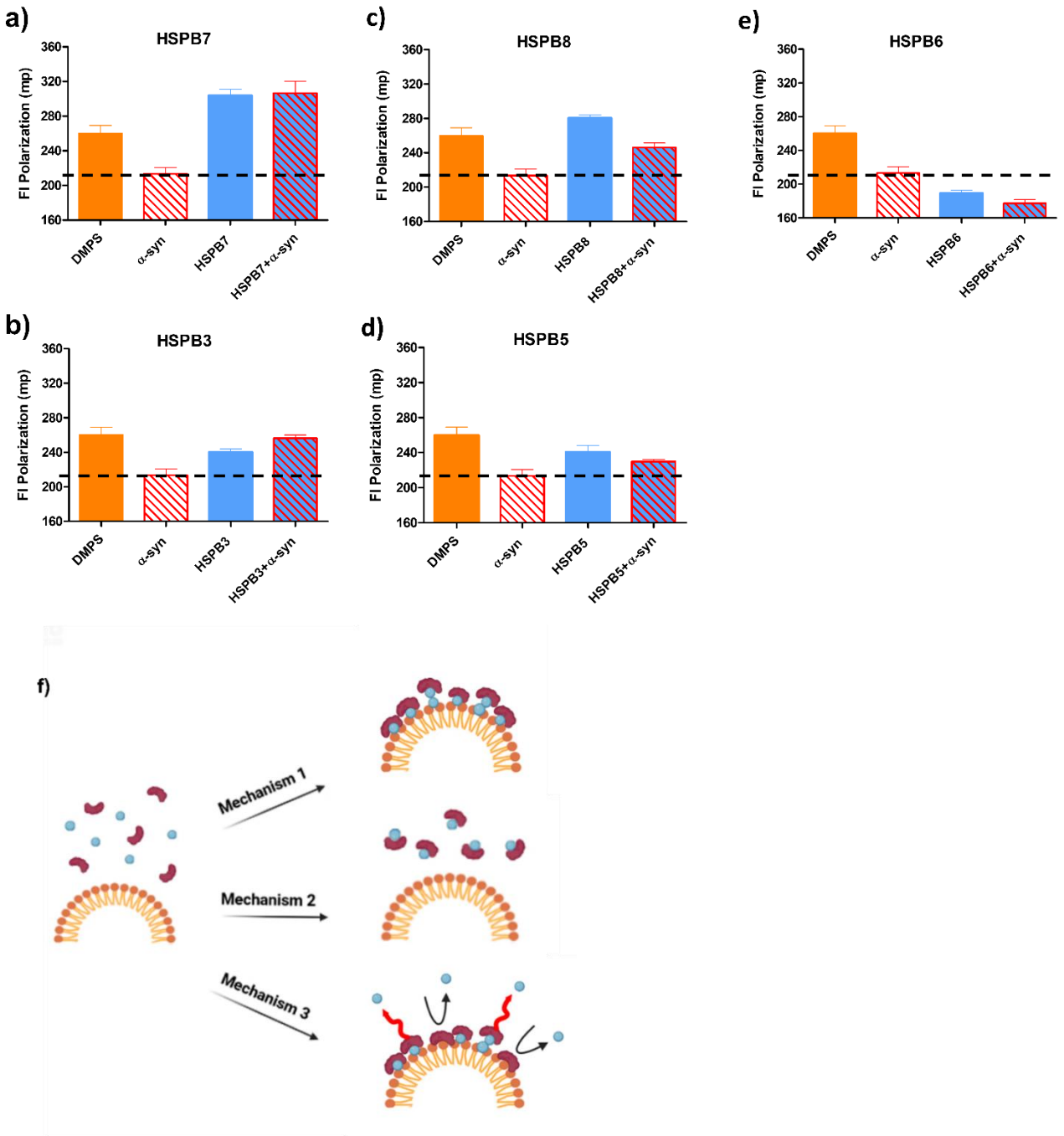


Figure 4. Impact of HSPBs on the fluidity of DMPS lipid vesicles and on α-synuclein lipid-induced aggregation. (a-e) Changes in DMPS (100 µM) membrane fluidity due to presence of α-synuclein (20 µM) or HSPBs (20 µM) detected through fluorescence polarisation of DPH at 30 °C for 4 h: (a) HSPB7, (b) HSPB3, (c) HSPB8, (d) HSPB5, (e) HSPB6. Data are shown as mean ± SEM, n=3. (f) Schematic representation of effect of HSPBs on the interaction of α-synuclein with lipid membranes. α-synuclein monomers in light blue, HSPBs in purple. In mechanism 1, HSPBs could bind to the lipid-associated α-synuclein monomers to inhibit their subsequent incorporation, thus preventing α-synuclein lipid-induced aggregation; in mechanism 2, HSPBs could associate with free α-synuclein monomers, hence preventing α-synuclein binding to lipids and maintaining the membrane fluidity; in mechanism 3, HSPB6 could prevent the embedding of α-synuclein by competing for binding to lipids (black arrow) and/or, one bound to α-synuclein embedded in the membrane, prevent the binding of new monomers of α-synuclein and therefore prevent the lipid-induced aggregation (red arrow).

Given the remarkable affinity of HSPB6 to lipids, we asked what the physiological meaning of this ability could be, and whether HSPB6 can represent a highly specialized chaperone to combat the protein aggregation induced by lipid. To date, it is known that HSPB6 is phosphorylated on serine 16 (Pser16-HSPB6) by PKA protein kinase A (PKA) and protein kinase G (PKG). HSPB6 phosphorylation has been suggested to play a role in muscle vasorelaxation and cardiac myocyte contractility, and mediate cardioprotection (Dreiza et al. 2010; Edwards et al. 2012). Based on these findings, we wondered whether phosphorylation could affect HSPB6 binding to lipids and its chaperone activity towards α-synuclein. To address this question, we generated phosphomimicking and non-phosphorylatable HSPB6 mutants. By site-directed mutagenesis we mutated the serine 16 for a cysteine residue (S16C), followed by a chemical addition of the phosphate (P) group to generate a phosphorylated HSPB6 mutant. Then, we replaced serine with aspartic acid (S16D) in HSPB6 WT to mimic the charge and size of the phosphorylated residue to create a phosphomimicking mutant; finally, we replaced serine 16 with alanine (S16A) to obtain a non-phosphorylatable mutant.

Using the lipid-induced aggregation assay, we investigated the efficacy of these three HSPB6 mutants in preventing α-synuclein aggregation. The phosphorylated mutant (S16C) delayed the α-synuclein lipid-induced aggregation more than the HSPB6 WT (Figure 5a). A similar effect was observed for the phosphomimicking mutant, which also displayed a stronger inhibitory effect compared to HSPB6 WT (Figure 5b). The non-phosphorylatable mutant S16A prevented the α-synuclein lipid-induced aggregation with an efficacy similar to the one of HSPB6 WT (Figure 5c). Together these data support the idea that phosphorylation enhances HSPB6 chaperone activity.

To obtain a detailed characterization of aggregation kinetics, we applied again the one-step nucleation model (Galvagnion *et al.*, 2015) previously described in Figure 2a. The normalization of the elongation rate in the presence of each HSPB6 mutant over that of α-synuclein alone was performed for direct comparison. This analysis confirmed that HSPB6 WT and the non-phosphorylatable mutant S16A behave in a similar way, while both S16D and S16C showed a stronger inhibitory activity. In particular the highest inhibitory effect was displayed by the phosphorylated mutant S16C at both concentrations considered (Figure d-e).

Thus, HSPB6 phosphorylation further enhances its affinity for lipids, resulting in a stronger inhibitory action as well as increased chaperone activity.

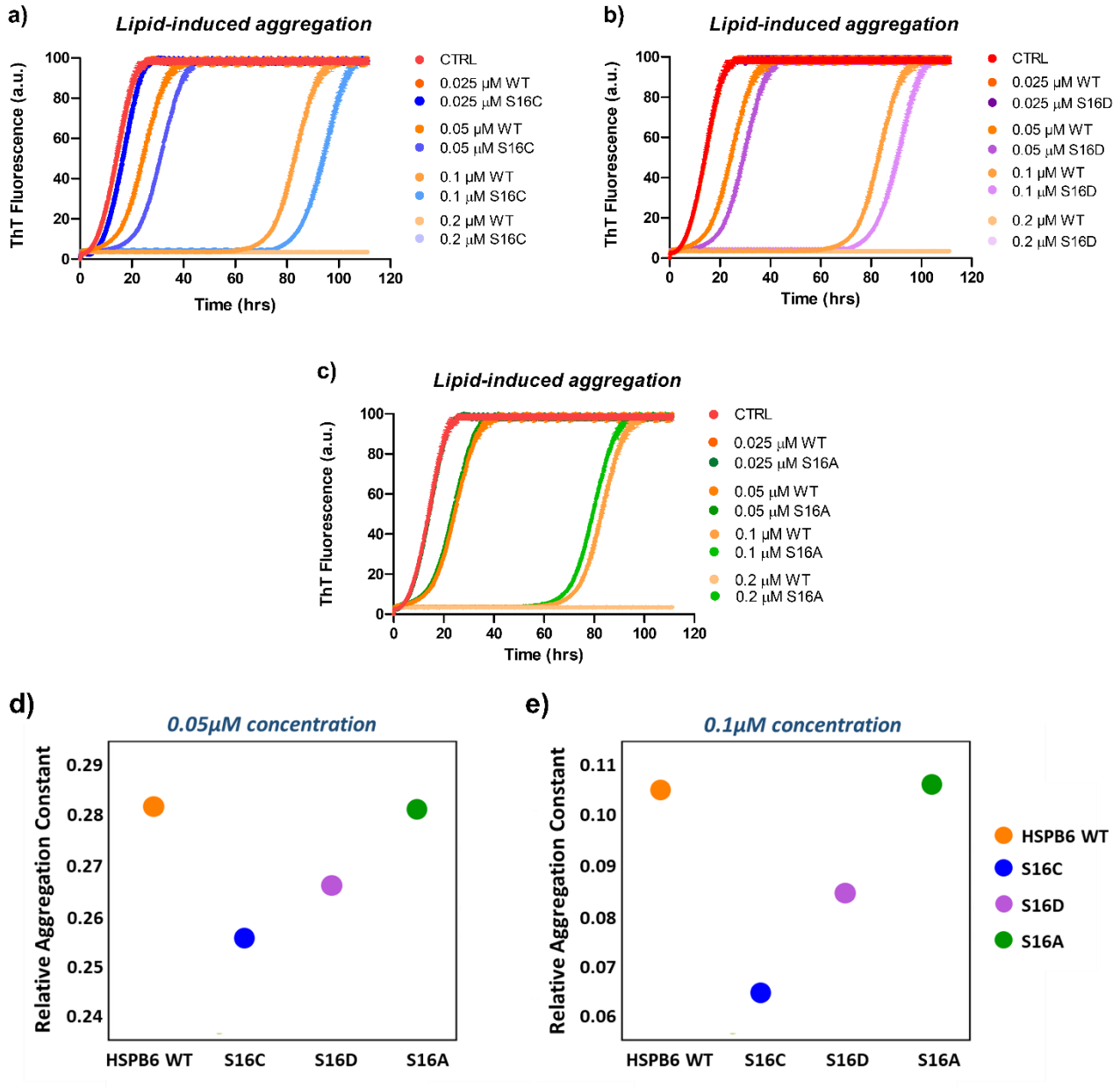


Figure 5. Phosphorilation enhances the chaperone activity of HSPB6. (a-c) Changes in ThT fluorescence intensity when monomeric α-synuclein (20μM) is incubated with 100 μM DMPS vesicles at pH 6.5 and 30 °C and in the absence (red) or presence of HSPB6 mutants at increasing concentrations (0.025 μM, 0.05 μM, 0.1 μM, 0.2 μM) . Data as absolute ThT fluorescence. a.u. arbitrary units. (d-e) Relative rate of lipid-induced α-synuclein aggregation constant, determined by fitting a single-step nucleation model to the early time points of the reaction (Galvagnion *et al.*, 2015) in AmyloFit (Meisl et al., 2016), in the presence of HSPB6 WT (orange), S16C (blue), S16D (purple), S16A (green) at 0.05 μM concentration in d) and at 0.1 μM concentration in e). n = 9; SEM = 2.2E-3 (HSPB6 WT); 3.64E-3 (S16C); 5.12E-3 (S16D) in d); SEM = 3.01E-3 (HSPB6 WT); 4.04E-3 (S16C); 3.82E-3 (S16D) in e).

Step two: HSPBs delay α-synuclein fibril elongation with different kinetics

As the initial formation of α-synuclein fibrils is followed by their elongation (Flagmeier *et al.*, 2016), we tested whether HSPBs could also affect α-synuclein fibril elongation using an assay previously developed (Flagmeier *et al.*, 2016; Galvagnion *et al.*, 2016; Galvagnion *et al.*, 2015). In this model, monomeric α-synuclein is inserted to the ends of fibrils preformed at neutral pH thereby causing fibril elongation and growth (Figure 2b). The elongation of the preformed fibrils was delayed by co-incubation with all the HSPBs studied here, albeit with different kinetics (Figure 6a). To directly compare the efficacy of the HSPBs analysed, we normalised the elongation rates over the one of α-synuclein alone (Figure 6b). HSPB3, HSPB5 and HSPB7 delayed the fibril growth with a relative fibril elongation ratio of approximately 0.1, regardless their concentration. Instead, HSPB8 delayed fibril elongation with a relative fibril elongation ratio ranging from of 0.2 to 0.4; when tested at lower concentrations, HSPB8 showed a slightly decreased inhibitory effect, (Figure 6a,b). HSPB6, which strongly suppressed lipid-induced aggregation of α-synuclein, could inhibit fibril elongation only when used at the highest concentration (20 μM). When HSPB6 concentration was 4-times lower (5 μM) than the one of monomeric α-synuclein (20 μM), the molecular chaperone lost all its inhibitory effect (Figure 6a,b).

In order to refine our characterization and better compare the efficacy on fibril elongation of the various HSPBs, we tested all HSPBs at lower concentrations, ranging from 0.1 μM to 1 μM (Figure 6d). We found that HSPB6 did not prevent the fibril elongation at none of the concentrations used, confirming the lowest inhibitory effect on α-synuclein fibril elongation showed already when performing the assay at higher concentrations. In contrast, all the other HSPBs maintained a strong effect even at the lower concentrations tested; especially, HSPB5 completely prevented fibril elongation at 1 μM concentration. Concerning HSPB8, its inhibitory activity on fibril elongation was reduced in a dose-dependent manner in the 0.1-1 μM concentration range (Figure 6d). Since we obtained similar results when using the 5-10 μM concentration range, our data together suggest that the effect of HSPB8 is dependent on the concentration of α-synuclein monomers.

To summarize our findings so far, the poor efficacy of HSPB6 in preventing fibril elongation, combined with its high binding affinity for lipids support the view that HSPB6 binds weakly to monomeric α-synuclein. Therefore, we speculate that the protective effect of HSPB6 is mostly due to the competition with α-synuclein for the binding to lipid membranes; this, in turn prevents the lipid-induced primary nucleation. This function is potentiated via phosphorylation of serine 16. For the other HSPBs studied, our results suggest two possible mechanisms of action. First, HSPBs might bind the extremities of α-synuclein fibrils, thus abrogating the subsequent incorporation of monomeric α-synuclein into the pre-existing fibrils. Second, HSPBs might interact directly with free α-synuclein monomers, preventing their addition to the fibril extremities. It is plausible that these two mechanisms are non-mutually exclusive and may co-exist (Figure 6c). Notably, the concentration of α-synuclein seeded fibrils (1 μM) used in this assay is significantly lower than the

monomer concentration (20 µM). Therefore, the kinetics found might be better explained by an affinity of HSPBs to intermediary species of α-synuclein, resulting in an inhibition of the earlier stages of the aggregation process. Our data suggest that HSPB8 might bind with higher affinity to α-synuclein monomers rather than to the fibril extremities, given that its inhibitory effect is concentration dependent. Hence, its ability to counteract α-synuclein monomers is influenced by the HSPB8:α-synuclein monomers ratio. Instead, the efficacy of HSPB3, HSPB5 and HSPB7 in preventing fibril elongation was not dependent on their concentration, implying that they might interact with higher affinity to the pre-formed fibrils, rather than to free α-synuclein monomers (Figure 6c).

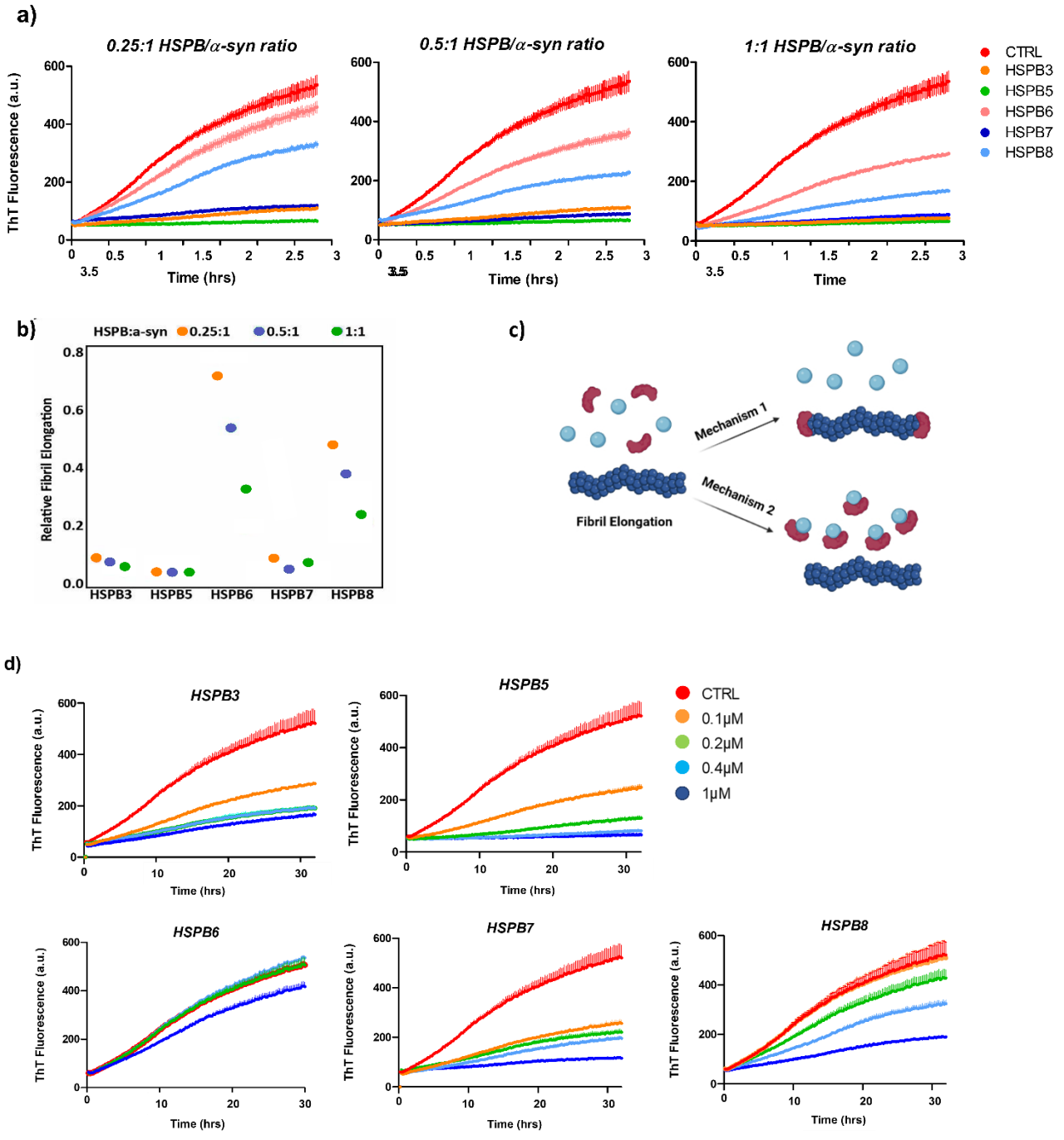


Figure 6. HSPBs delay α-synuclein fibril elongation. (a) Changes in ThT fluorescence intensity when monomeric α-synuclein (20 μM) is incubated with 1 μM preformed fibrils at pH 6.5 and 37 °C and in the absence (red) or presence of HSPBs: HSPB3 (orange), HSPB5 (green), HSPB6 (pink), HSPB7 (light blue), HSPB8 (blue), HSPBs at 5 μM, 10 μM or 20 μM. Data as absolute ThT fluorescence. a.u. arbitrary units. (b) Relative rate of α-synuclein fibril elongation, determined as per Flagmeier et al., 2016. n = 3; SEM = 6.08E-4 (HSPB3 5 μM); 4.20E-3 (HSPB 5 μM); 5.99E-3 (HSPB6 5 μM); 3.17E-4 (HSPB7 5 μM); 2.73E-3 (HSPB8 5 μM); 2.62E-3 (HSPB3 10 μM); 5.53E-3 (HSPB 10 μM); 1.04E-4 (HSPB6 10 μM); 2-09E-4 (HSPB7 10 μM); 1.08E-2 (HSPB8 10 μM); 3.18E-3 (HSPB3 20 μM); 4.13E-3 (HSPB 20 μM); 1.24E-1 (HSPB6 20 μM); 2.62E-2 (HSPB7 20 μM); 4.97E-3 (HSPB8 20 μM). (c) Schematic representation of the putative effect of HSPBs on fibril elongation. α-synuclein monomers in light blue, α-synuclein fibrils in blue, HSPBs in purple. (d) Changes in ThT fluorescence intensity when monomeric α-synuclein (20 μM) is incubated with 1 μM preformed fibrils at pH 6.5 and 37 °C and in the absence (red) or presence of HSPBs at 0.1 μM (orange), 0.2 μM (green), 0.4 μM (light blue), or 1 μM (blue).

Third step: HSPBs delay α-synuclein secondary nucleation with different kinetics

To further investigate how HSPBs interfere with the α-synuclein aggregation process, we then assessed their effect on α-synuclein fibril amplification, which occurs via secondary nucleation pathways including the formation of surface-catalysed aggregates, under conditions of mildly acidic pH (Buell *et al*, 2014b; Flagmeier *et al.*, 2016) (Figure 2c). Because this assay is performed at low pH, we firstly determined by circular dichroism (CD) whether the pH used might affect the folding and stability of the various HSPBs; this in turn may affect their chaperone activity. Only the CD spectra of HSPB5 was affected by decreasing the pH from 6.5 to 5.5; thus, the latter chaperone was excluded from this study to avoid data misinterpretation (data not shown). The other HSPBs tested could all delay α-synuclein secondary nucleation, although with different efficacies (Figure 7a). HSPB7 and HSPB3 strongly inhibited α-synuclein secondary nucleation at all concentrations used, while HSPB8 had a moderate effect with slight differences based on the dose tested. By contrast, HSPB6 only weakly prevented α-synuclein secondary nucleation compared to other HSPBs, confirming the lowest affinity for α-synuclein (and the higher affinity for lipids). However, HSPB6 still showed some chaperone activity (Figure 7a). Similar to the primary nucleation and fibril elongation assays, two possible mechanisms of action could explain our results. According to the first hypothesized mechanism, HSPBs might exert their effect by binding to α-synuclein fibril surfaces and prevent the addition of monomers to the pre-formed fibril. According to the second hypothesized mechanism, HSPBs might inhibit α-synuclein secondary nucleation by interacting with α-synuclein monomers and prevent them from binding to and amplifying the pre-existing fibrils (Figure 7b). Considering the results obtained with the three-pronged approach, one could envisage that HSPB8 prevents α-synuclein aggregation by binding to and neutralizing its monomeric forms, while HSPB3 and HSPB7 preferentially interact with α-synuclein oligomers and/or fibrils, blocking their further aggregation.

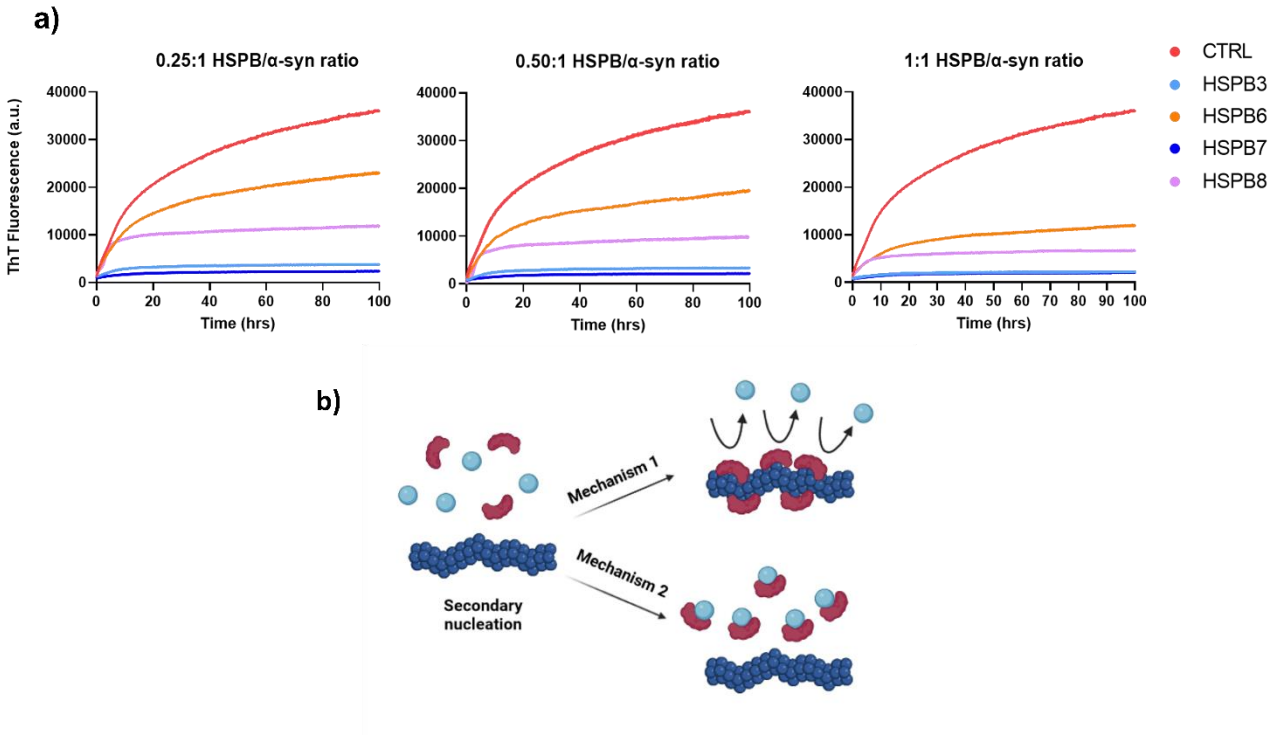


Figure 7. HSPBs delay α-synuclein secondary nucleation. (a) Changes in ThT fluorescence intensity when monomeric α-synuclein (20 µM) is incubated with 50 nM preformed fibrils at pH 5.5 and 37 °C and in the absence (red) or presence of HSPBs at 5µM (0.25:1 HSPBs/α-syn ratio), 10µM (0.50:1 HSPBs/α-syn ratio), 20µM (1:1 HSPBs/α-syn ratio): HSPB3 (light blue), HSPB6 (orange), HSPB7 (blue), HSPB8 (lilac). Data are shown as absolute ThT fluorescence. a.u. arbitrary units. (b) Schematic representation of possible HSPBs effect on secondary nucleation. α-synuclein monomers in light blue; α-synuclein fibril in blue; HSPBs in purple.

Discussion

In this chapter, we have shown that HSPB3, HSPB5, HSPB6, HSPB7 and HSPB8 can inhibit or delay α-synuclein aggregation with manifold mechanisms and with different strength. Previous studies have reported that HSPB1 and HSPB5 bind to monomeric and fibrillar α-synuclein and inhibit fibril elongation (Bendifallah *et al.*, 2020; Cox *et al.*, 2018; Waudby *et al*, 2010). Here we show that this activity is shared by the other members HSPB3, HSPB6, HSPB7 and HSPB8.Their interaction with monomeric and fibrillar α-synuclein may explain their presence inside the α-synuclein deposits(Cox & Ecroyd, 2017; Outeiro *et al*, 2006; Waudby *et al.*, 2010; Wilhelmus *et al*, 2006).

Most importantly, we validated initial findings showing that HSPB can interact with lipids (De Maio *et al.*, 2019) and we propose that HSPB may function as lipid chaperones that, on the one hand, can stabilize membranes maintaining their integrity upon stress conditions and, on the other hand, may render the membranes less vulnerable to protein misfolding, by decreasing the embedding and

aggregation of proteins within the lipid bilayer. To what extent the physiological protective functions of HSPBs is due to their association with membranes is still poorly understood.
Although all the HSPBs tested inhibited the lipid-induced primary nucleation, even at low concentrations, they displayed different degree of efficiency, with HSPB6 being the most active member that also showed high binding affinity to lipids and the lowest efficacy on fibril elongation and secondary nucleation assay, which are carried out in absence of lipid vesicles. HSPB6 was previously reported to bind to monomeric α-synuclein only transiently and weakly (Bruinsma *et al.*, 2011). This is in agreement with our findings. Since HSPB6 has a higher affinity for lipids than for α-synuclein, we propose that it acts as a lipid chaperone that outcompete α-synuclein and abrogate the lipid-induced aggregation. Our results open new questions for a better understanding of HSPB6 mechanism of action. Is this lipid chaperone activity due to a competition mechanism? Is HSPB6 displacing from lipids only a specific subset of aggregation-prone proteins? Or does this mechanism apply to other proteins whose aggregation is promoted by the interaction with lipids? Or rather, does HSPB6 embedding into the membrane change its local fluidity, reducing the binding affinity for aggregating species? Finally, to what extent does phosphorylation modulate the lipid chaperone activity of HSPB6? Future studies will need to address these questions in order to exploit HSPB6 properties for potential therapeutic application.

Similar to HSPB6, the other HSPBs studied delayed α-synuclein lipid-induced primary nucleation, yet with distinct mechanisms. Based on our results, we propose that HSPB5, which only mildly restored the changes induced at the level of membrane fluidity by α-synuclein would bind to α-synuclein inserted in the lipid surface; instead, HSPB3, HSPB7 and HSPB8, would interact with higher affinity with α-synuclein monomers, preventing their embedding within the lipid vesicles (Figure 5f). In agreement, HSPB8 and HSPB3 were reported to interact with monomeric α-synuclein with higher affinities compared to HSPB5 (Bruinsma et al., 2011).

In summary, our results demonstrate that HSPBs can modulate α-synuclein aggregation through multiple mechanisms that go beyond direct protein-protein interaction and suggest that HSPBs can act at the crossroad between the lipid and protein homeostasis.

4.2. Trodusquemine acts as co-inducer of the HSR

The aminosterol trodusquemine has recently emerged as a promising drug for the treatment of the neurodegenerative diseases AD and PD. Using in vitro systems, cellular and animal models of AD and PD it has been shown that trodusquemine can displace Aβ and α-synuclein, the main aggregating species associated with AD and PD, from membranes, thus preserving their integrity and reducing the toxicity mediated by Aβ and α-synuclein (Ryan Lymbocker et al. 2022). The protective effect exerted by trodusquemine would be at least in part due to its ability to directly interact with the cellular membrane and change its physical properties, enhancing their resistance against the deleterious interactions with misfolded proteins. Although enormous progress in the understanding of the mechanism of action of trodusquemine (and related aminosterols) has been done thanks to the work of independent laboratories (Ryan Lymbocker et al. 2022), the molecular targets of trodusquemine are still not fully determined.

Given its ability to interact with membranes, modulating their physical state, we postulated that trodusquemine may induce the expression of specific genes, including those coding for HSPs. Potentiation of the HSR, in turn, may contribute to the protective function of trodusquemine. Our hypothesis is based on the observations that compounds such as bimoclomol (BM) and arimoclomol interact with the membranes, change their fluidity and activate the expression of HSGs (Gombos et al. 2011; Török et al. 2003). These drugs are thought to activate specific sensors embedded and/or associated with the membranes, according to the so-called “membrane thermosensor” hypothesis that we described in the introduction (Török et al. 2014; Balogh et al. 2005).

Trodusquemine synergistically acts with heat shock to change the membrane fluidity

To test the hypothesis that trodusquemine may affect membrane fluidity, thereby activating or potentiating the HSR, we firstly tested whether changes in fluidity membrane occur upon heat shock (HS) or following to treatment with drugs such as benzyl alcohol (BA), used as positive control since it is a known membrane fluidizer (Balogh et al. 2005).

SH-SY5Y neuronal cells were treated with two different concentrations of BA at 37°C for 45 min; in parallel, SH-SY5Y neuronal cells were exposed to heat shock at 42°C for 2hr (HS). Changes in membrane fluidity were assessed using a lipophilic pyrene probe that, when interacting with membranes, exhibits changes in its spectral properties (membrane fluidity kit ab189819; see materials and methods section for a detailed description). A quantitative analysis of membrane fluidity (or viscosity) can be obtained by measuring the ratio of monomer (EM max. 372 nm) to excimer (EM 470 nm) fluorescence. As expected, the fluidity of cell membranes was altered upon HS and its effect was comparable to that of the highest concentration tested (45mM) of BA (Figure 1a).

Because trodusquemine is able to modify the physiochemical properties of the membrane (Errico et al. 2020), we then asked whether trodusquemine alone or in the presence of HS can affect the membrane fluidity. We observed that trodusquemine alone did not affect the overall membrane fluidity measured using this assay. However, when cells were pre-treated with trodusquemine, followed by temperature upshift, we observed a synergistic effect on membrane viscosity compared to cells exposed to HS alone (Figure 1b).

One of the best characterized mechanisms of action of trodusquemine is the inhibition of protein tyrosine phosphatase 1B (PTP1B) (Ryan Lymbocker et al. 2022). PTP1B an endosomal phosphatase that decreases the activation of signalling pathways such as leptin, insulin, growth factors and cytokines (Lantz et al. 2010). Trodusquemine significantly enhanced insulin-stimulated tyrosine phosphorylation of insulin receptor (IR) β and STAT3, direct targets of PTP1B, in HepG2 cells in vitro and/or hypothalamic tissue in vivo (Lantz et al. 2010). The inhibition of PTP1B by trodusquemine has been correlated with its pharmacological activity in disparate disease models ranging from metabolic syndromes (diabetes) to myocardial regeneration and memory impairments (Ryan Lymbocker et al. 2022). It is thus fundamental to understand to what extent the changes observed at the level of membrane viscosity are linked to the inhibition of PTP1B, especially considering that under our experimental conditions cells are treated with trodusquemine for a long time, which is sufficient to activate/inhibit specific cell signalling pathways. Thus, we tested the effect on lipid membrane viscosity of BML-267, a commercially available specific inhibitor of PTP1B. Cells were treated with BML alone or were subjected to BML pre-treatment, followed by exposure to HS. BML per se did not affect the membrane viscosity, similar to trodusquemine, using this assay. Importantly, we observed no changes in membrane fluidity when cells were pre-treated with BML followed by HS (Figure 1b). We conclude that the synergistic effect of trodusquemine and HS on membrane viscosity is not due to inhibition of PTP1B.

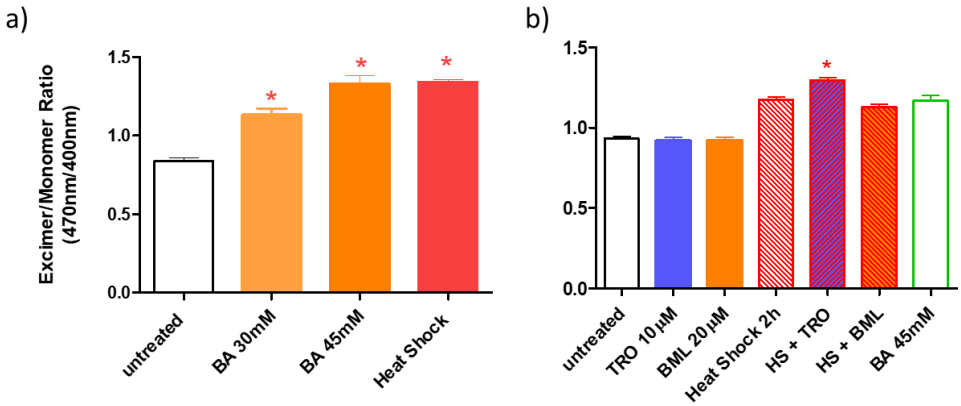


Figure 1. Quantitative analysis of membrane fluidity (or viscosity) upon cell treatment with trodusquemine, BML and heat shock. a) SH-SY5Y cells were treated with 30mM or 45mM benzyl alcohol (BA) for 45 min or were exposed at heat shock stress (42°C for 2h). b) SH-SY5Y cells were treated with 10µM trodusquemine (TRO) for 1h or with 20µM BML for 1h or with 45mM BA for 45min, followed by HS at 42°C for 2h, where reported. The membrane fluidity parameter was determined after 1h incubation with 5 µM

PDA and 0.08% pluronic F-127, by measuring the ratio of monomer (EM max. 372 nm) to excimer (EM 470 nm) fluorescence. n = 9 experimental replicates, ± SEM; One-way ANOVA, Tukey's post-test, *pValue < 0,05.

Trodusquemine potentiates the HSR

Once established that trodusquemine synergizes with HS to affect the membrane fluidity, and considering that this can affect the expression of HSGs, we then asked whether trodusquemine can act as a co-inducer of the HSR, similar to what previously found for arimoclomol (Dairin et al. 2004; Parfitt et al. 2014) and other compounds that change the physical state of membranes (Török et al. 2014; Balogh et al. 2005). To address this question, we evaluated the expression of selected HSGs in SH-SY5Y cells treated with trodusquemine alone, HS alone or in combination. We monitored both the expression level of selected HSGs after acute treatment and during the stress recovery time.

As mentioned in the introduction, some of the genes coding for HSPs can be constitutively expressed (e.g. Hsc70/HSPA8), while others can be induced by stress conditions, such as HS or developmental and differentiation stimuli. Here we focussed on the stress-inducible genes coding for HSP70/HSPA1A, DNAJB1, HSPB1 and we also included the genes coding for HSPB8 and its co-chaperone BAG3 that form a complex with known anti-aggregation activity and that is upregulated in the brain of AD and PD patients (Seidel et al. 2012). Treatment of SH-SY5Y cells with trodusquemine alone did not induce any transcriptional activation of the HSGs studied; however, the drug synergyzed with HS to enhance the transcriptional levels of these stress-inducible genes (HSPA1A, DNAJB1, HSPB1, HSPB8, and BAG3; Figure 2). The results obtained on the transcription of the HSGs are in agreement with the impact of trodusquemine alone or together with HS on membrane viscosity (Figure 1). These data together support the idea that trodusquemine functions as a co-inducer of the HSR and suggest that it would do so by modulating the physical states of membranes. We next evaluated the duration of the co-inducing effect observed upon exposure of neuronal cells to trodusquemine and HS. Also, during the recovery period, cells pre-treated with trodusquemine showed higher expression levels of the HSGs analyzed (Figure 2). Based on these observations we suggest that trodusquemine is not only a co-inducer of the HSR, but it also extends in time the HSR.

In light of these results, we speculate that trodusquemine may modulate the fluidity of the membrane to induce the expression of HSPs and potentiate the HSR in cells subjected a stress conditions, such as HS.

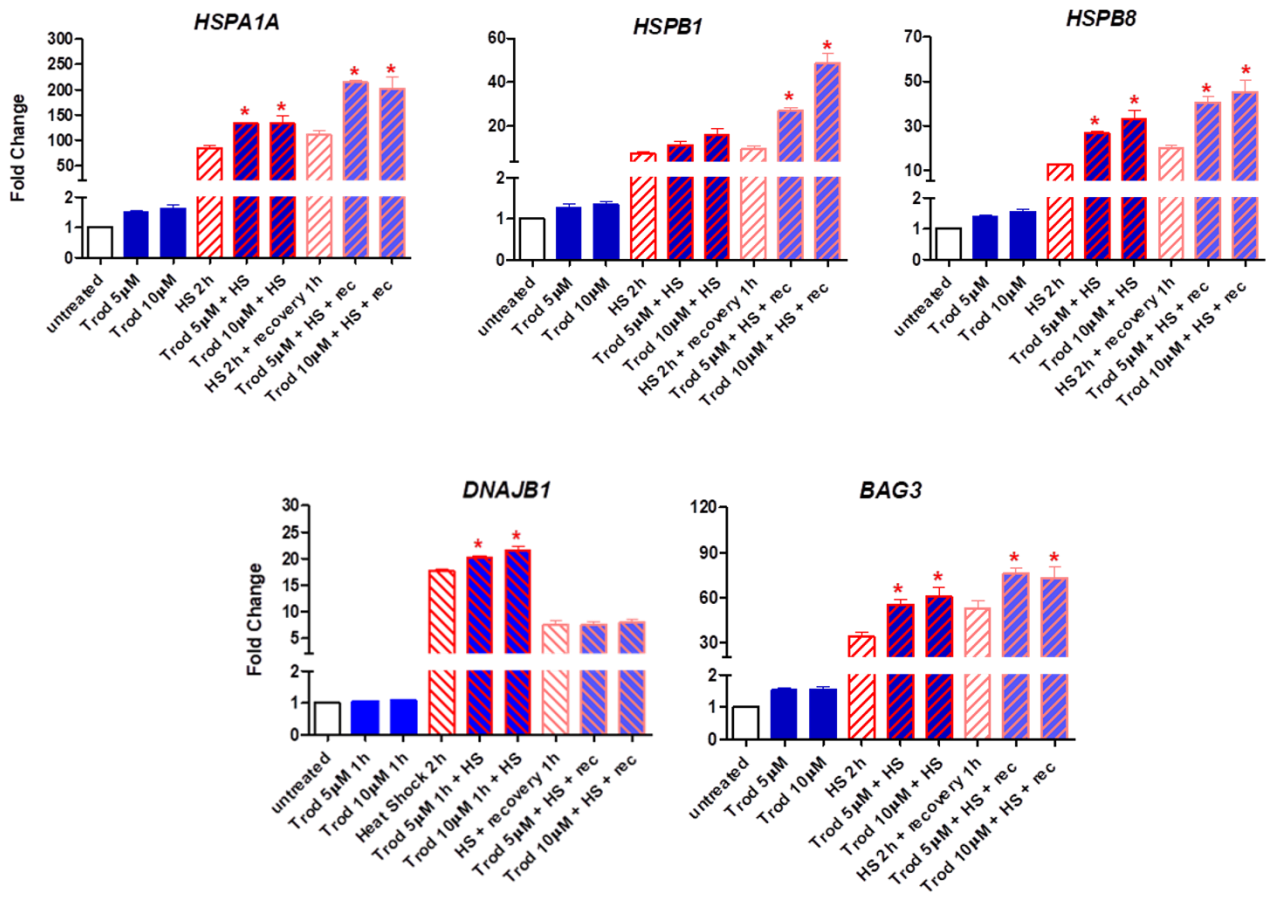


Figure 2. mRNA levels of stress-inducible HSGs upon treatment of neuronal cells with trodusquemine and HS. RT-qPCR analysis of molecular chaperones HSPA1A, HSPB1, HSPB8, DNAJB1 and co-chaperone BAG3 in SH-SY5Y cells treated as indicated in the graphics and using different concentrations of trodusquemine. Experimental conditions: untreated: total RNA was extracted after incubation of 1h at 37°C; Trod 1h: total RNA was extracted after exposure of the cells with the indicated concentration of trodusquemine for 1h at 37°C; Heat shock 2h: total RNA was extracted after exposure of the cells at 42°C for 2h; Trod + HS: total RNA was extracted after exposure of the cells with the indicated concentration of trod for 1h, followed by heat shock at 42°C for 2h; HS + recovery 1h: total RNA was extracted after exposure at 42°C for 2h and incubation at 37°C in drug-free for 1h; Trod + HS + rec: total RNA was extracted after exposure of the cells with the indicated concentration of trodusquemine for 1h, followed by heat shock at 42°C for 2h and by incubation at 37°C in drug-free for 1h. Abbreviations used: Trod: trodusquemine; HS: heat shock; rec: recovery. RPL0 was used as housekeeping control gene. Fold change normalized over RPL0 and calibrated over untreated. n = 9, ± SEM; One-way ANOVA, Tukey's post-test, *pValue < 0,05.

Trodusquemine potentiates the HSR acting via the Heat Shock factor 1

To achieve insights on how trodusquemine exerts its effect, we explored which pathway(s) may be involved in its mechanism of action. Because the heat shock factor 1 (HSF1) is the main transcriptional factor that induces the expression of HSGs in response to stress (Morimoto et al., 1992), we firstly asked whether trodusquemine function is mediated by HSF1. To do so, we used the commercially available compound KNK437, which is classified as a selective HSF1 inhibitor (Koishi

et al. 2001). Treatment of SH-SY5Y cells with KNK437 alone did not induce the expression of any of the HSGs analyzed (Figure 3a, orange bar KNK 1h) compared to untreated cells (Figure 3a, CTL). As expected, pre-treatment of SH-SY5Y cells with KNK437, followed by exposure to HS inhibited the induction of HSGs (Figure 3a, orange bar, KNK+HS), compared to cell treatment with HS alone (Figure 3a, white bar, HS). In agreement with what shown in Figure 2, cell pre-treatment with trodusquemine followed by HS exerted a synergistic effect on the expression of the HSGs analyzed (Figure 3a, green bar, TROD+HS). Co-incubation with KNK437 completely abolished the co-inducing effect of trodusquemine (Figure 3a, blue bar KNK+TROD+HS). KNK437 co-treatment also prevented the synergistic effect on the HSR mediated by trodusquemine during the stress recovery phase (Figure 3a, columns +rec). Taken together, these results further support the idea that trodusquemine acts as co-inducer of the HSR, acting via HSF1.

To further validate our findings, we next employed Crispr/Cas9 knock-out (KO) U2OS cells wild-type (WT) or lacking the HSF1 gene. U2OS cells are a cell line with epithelial morphology derived from a osteosarcoma patient. Trodusquemine could slightly, but significantly, increase the expression of HSPA1A in U2OS WT cells exposed to HS (Figure 3b). Importantly, this effect was abolished in U2OS HSF1-KO cells (Figure 3b), in support of the idea that HSF1 is a new, indirect, molecular target of trodusquemine. Comparative analysis between U2OS and neuronal SH-SY5Y cells revealed that U2OS WT cells showed a lower susceptibility to trodusquemine (Figure 3c). Thus, different cell types may respond differently to trodusquemine; alternatively, the pathway sensing changes in membrane fluidity may be differentially expressed and activated in these two different cell types. Although preliminary and between only two cell types (both immortalized), this observation highlights the need to further investigate how cells with different membrane compositions may display different sensitivity to changes in membrane fluidity, thereby activating to different degrees the HSR upon stress and responding differently to the same concentration of a given compound. This type of knowledge will be relevant for the tissue-specific function of compounds with therapeutical potential like trodusquemine.

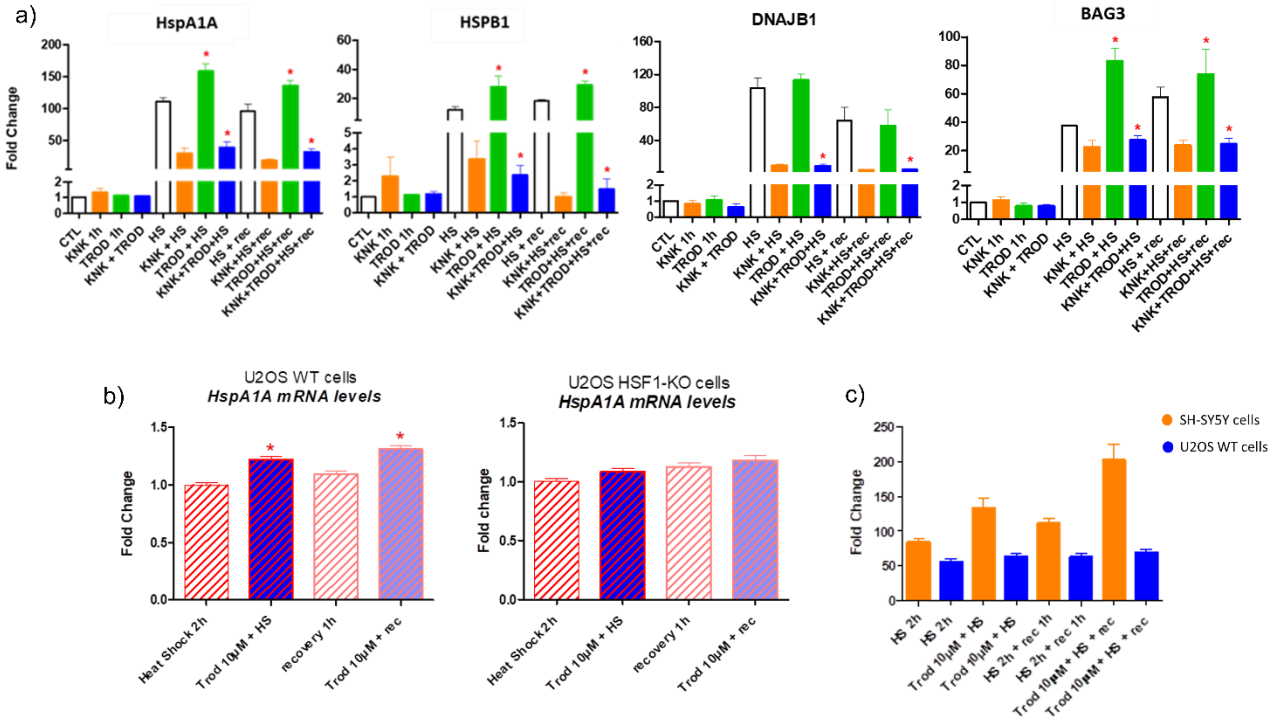


Figure 3. a) mRNA levels of stress-inducible HSGs in presence of the HSF1 inhibitor KNK437. RT-qPCR analysis of molecular chaperones HSPA1A, HSPB1, DNAJB1 and co-chaperone BAG3. Experimental conditions: CTL: total RNA was extracted after incubation of 1h at 37°C; KNK 1h: total RNA was extracted after exposure of the cells with 20µM of inhibitor KNK437 at 37°C for 1h; TROD 1h: total RNA was extracted after exposure of the cells with 10µM of trodusquemine for 1h at 37°C; KNK + TROD: total RNA was extracted after exposure of the cells with 20µM of inhibitor KNK437 and with 10µM of trodusquemine at 37°C for 1h; HS 2h: total RNA was extracted after exposure of the cells at 42°C for 2h; KNK + HS: total RNA was extracted after exposure of the cells with 20µM of inhibitor KNK437 at 37°C for 1h, followed by heat shock at 42°C for 2h; TROD + HS: total RNA was extracted after exposure of the cells with 10µM of trodusquemine at 37°C for 1h, followed by heat shock at 42°C for 2h; KNK+TROD+HS: total RNA was extracted after exposure of the cells with 20µM of inhibitor KNK437 and with 10µM of trodusquemine at 37°C for 1h, followed by heat shock at 42°C for 2h; HS + rec: total RNA was extracted after exposure at 42°C for 2h and incubation at 37°C in drug-free for 1h; KNK+HS+rec: total RNA was extracted after exposure of the cells with 20µM of inhibitor KNK437 at 37°C for 1h, followed by heat shock at 42°C for 2h and incubation at 37°C in drug-free for 1h; TROD+HS+rec: total RNA was extracted after exposure of the cells with 10µM of trodusquemine at 37°C for 1h, followed by heat shock at 42°C for 2h and incubation at 37°C in drug-free for 1h; KNK+TROD+HS+rec: total RNA was extracted after exposure of the cells with 20µM of inhibitor KNK437 and with 10µM of trodusquemine at 37°C for 1h, followed by heat shock at 42°C for 2h and incubation at 37°C in drug-free for 1h. Abbreviations used: CTL: control; KNK: inhibitor KNK437; TROD: trodusquemine; HS: heat shock; rec: recovery period. Rlp0 was used as housekeeping control gene. Fold change normalized over RPL0 and calibrated over untreated. n = 12, ± SEM; One-way ANOVA, Tukey's post-test, *pValue < 0,05. **b) Trodusquemine cannot induce the expression of HSPA1A mRNA in U2OS HSF1-KO cells exposed to HS.** Comparison between HSPA1A mRNA levels in U2OS WT cells vs U2OS HSF1-KO cells exposed to trodosquemine, followed by HS. RT-qPCR analysis of molecular chaperones HSPA1A. RPL0 was used as housekeeping control gene. Fold change normalized over RPL0 and calibrated over untreated cells. n = 9, ± SEM; One-way ANOVA, Tukey's post-test, *pValue < 0,05. **c)** Different cell types (SH-SY5Y cells and U2OS WT cells) respond differently to trodusquemine and HS and induce to different degrees the expression of HSPA1A mRNA.

Inhibition of Protein Tyrosine Phosphatase 1B (PTP1B) does not synergize with HS to coinduce the expression of stress-inducible HSGs

Cells integrate a multitude of signals and can use different receptors to activate a given signal transduction pathway; different signals can converge onto the same pathway or activate simultaneously different signaling pathways, which can then even influence each other. Although PTP1B did not synergize with HS to change membrane fluidity (Figure 1b), we cannot exclude the possibility that, in cells, inhibition of PTP1B upon trodusquemine treatment may mediate, at least in part, the induction of selected HSGs.

Thus, we next investigated whether PTP1B inhibition may synergize with HS to induce the expression of selected HSGs. SH-SY5Y cells were pre-treated with the selective inhibitor of PTP1B (BML), followed by HS. Similar to trodusquemine, treatment of SH-SY5Y cells with BML alone did not affect the expression of any of the HSGs analyzed compared to untreated (CTL) cells (Figure 4). In addition, upon cell pre-treatment with BML followed by HS, we found no increase in the expression levels of HSPA1A, DNAJB1 and BAG3, compared to HS (Figure 4). These data suggest that, in contrast to trodusquemine, PTP1B inhibition does not synergize with HS to induce the HSR. We conclude that, under our experimental conditions, the co-inducing effect of trodusquemine on stress-inducible HSGs is mediated by the HSF1 and not by inhibition of PTP1B.

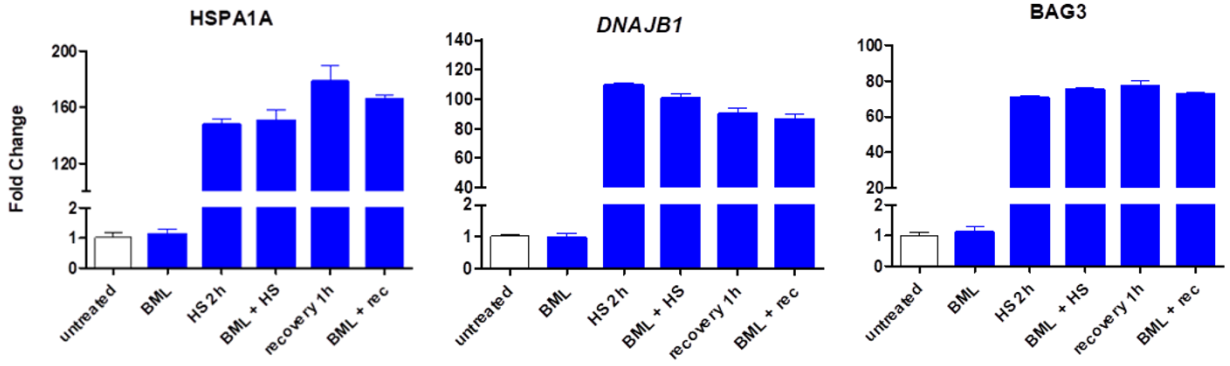


Figure 4. Inhibition of PTP1B does not synergize with HS to coinduce the expression of stress-inducible HSGs. RT-qPCR analysis of molecular chaperones HSPA1A, DNAJB1 and co-chaperone BAG3. Experimental conditions: untreated: total RNA was extracted after incubation of 1h at 37°C; BML: total RNA was extracted after exposure of the cells with 20µM of BML-267 for 1h at 37°C; Heat shock 2h: total RNA was extracted after exposure of the cells at 42°C for 2h; BML + HS: total RNA was extracted after exposure of the cells with 20µM of BML-267 for 1h at 37° for 1h, followed by heat shock at 42°C for 2h; recovery 1h: total RNA was extracted after exposure at 42°C for 2h and incubation at 37°C in drug-free for 1h; BML + rec: total RNA was extracted after exposure of the cells with 20µM of BML-267 for 1h at 37° for 1h, followed by heat shock at 42°C for 2h and incubation at 37°C in drug-free for 1h. Abbreviations used: HS: heat shock, rec: recovery period. RPL0 was used as housekeeping control gene. Fold change normalized over RPL0 and calibrated over untreated. n = 12, ± SEM; One-way ANOVA, Tukey's post-test, *pValue < 0,05.

In search for sensors activated by trodusquemine at the level of membranes: Sterol regulatory element binding proteins (SREBPs)

Sterol regulatory element binding proteins (SREBPs) are a class of transcriptional factors that mediate the synthesis of sterols in the cells, whose levels influence membrane viscosity. Based on the intracellular sterol content, SREBPs can activate the promoter of genes involved in cholesterol biosynthesis and uptake by binding to specific DNA sequences called sterol regulatory elements (SREs) (Shimano and Sato 2017). Moreover, it has been reported that SREBPs can modulate the transcription of various chaperones, including e.g. DNAJB1, DNAJB5 and DNAJB9 (Rome et al. 2008). Hence, we asked whether inhibition of these specific transcription factors may reduce the co-inducing effect of trodusquemine upon HS. To address this specific question, we used the commercially available compound PF-429242, a reversible competitive inhibitor of SREBP site 1 protease. By preventing the proteolytic processing and nuclear translocation of SREBP, PF-42924 reduces the expression of SRE-containing genes. Similar to treatment with trodusuqmine or BML alone, treatment of SH-SY5Y cells with the inhibitor PF-429242 did not induce the expression of any of the HSGs analyzed, namely HSPA1A, HSPB1, HSPB8 and BAG3 (Figure 5). When cells were pre-treated with PF-429242 followed by exposure to HS, we did not observe any synergistic effect on the expression levels of the HSGs analyzed (Figure 5). Cells could induce the expression of HSGs as efficiently upon inhibition of the SREBP site 1 protease. Yet, when cells were co-incubated with trodusquemine and PF-429242, followed by temperature upshift, we observed no significant differences in the expression of the HSGs tested compared to cells exposed to HS alone (Figure 5). Based on these findings, we suggest that the proteolytic processing and nuclear translocation of SREBP may contribute to the induction of the expression of HSGs in cells exposed to trodusquemine and HS. Of note the pre-treatment with PF-429242 lasted 24 hrs, a typical time-point used in the literature to completely inhibit SREBP mediated transcription (T. Wang et al. 2021; Hawkins et al. 2008; Monnerie et al. 2017), while pre-treatment with the HSF1 inhibitor KNK437 and with the PTP1B inhibitor BML lasted only 1 hr. It is thus possible that pre-treatment with PF-429242 for 24 hrs leads to changes in the membrane composition and responsiveness to trodusquemine, a hypothesis that we cannot exclude. Future investigations are required to characterize this preliminary finding and understand to what extent SREBPs are implicated in the expression of HSGs and may affect trodusquemine activity, especially when used only for a short time treatment.

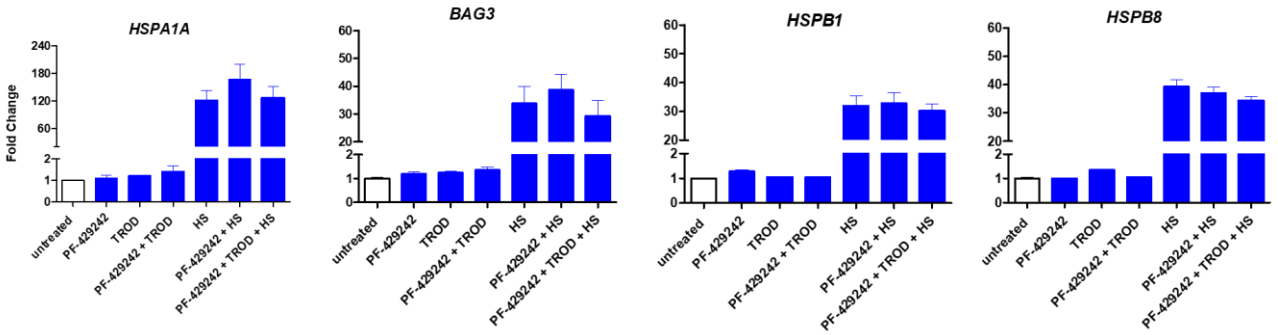


Figure 5. HSGs mRNA levels following inhibition of the SREBP site 1 protease. RT-qPCR analysis of molecular chaperones HSPA1A, HSPB1, HSPB8 and co-chaperone BAG3. Experimental conditions: untreated: total RNA was extracted after incubation of 1h at 37°C; PF-429242: total RNA was extracted after exposure of the cells with 20µM of PF-429242 for 16h at 37°C; TROD: total RNA was extracted after exposure of the cells with 10µM of trodusquemine for 1h at 37°C; HS: total RNA was extracted after exposure of the cells at 42°C for 2h; PF-429242 + HS: total RNA was extracted after exposure of the cells with 20µM of PF-429242 for 16h at 37°, followed by heat shock at 42°C for 2h; recovery 1h: total RNA was extracted after exposure at 42°C for 2h and incubation at 37°C in drug-free for 1h; PF-429242 + TROD + HS: total RNA was extracted after exposure of the cells with 20µM of PF-429242 for 16h at 37°, followed by treatment with 10µM of trodusquemine for 1h at 37°C and then followed by heat shock at 42°C for 2h. Abbreviations used: TROD: trodusquemine, HS: heat shock. RPL0 was used as housekeeping control gene. Fold change normalized over RPL0 and calibrated over untreated. n = 12, ± SEM; One-way ANOVA, Tukey's post-test, *pValue < 0,05.

Discussion

In this chapter we have shown that trodusquemine can induce the expression of genes coding for stress-inducible HSPs, potentiating in strength and duration the activation of the HSR in cells exposed to temperature upshift. Thus, similar to bimoclomol and arimoclomol, trodusquemine is emerging as a novel chaperone co-inducer that cannot activate the expression of HSPs *per se*, but can potentiate the HSR induction in combination with concomitant stresses (Gombos et al. 2011). Our data suggest that this compound can amplify the fluidity perturbation induced by HS, making the cells more responsive to stress signals that can affect the lipid states.

Amongst the HSGs whose expression was amplified by trodusquemine, we found the stress-inducible genes coding for HSP70/HSPA1A, a master chaperone involved in maintaining proteostasis upon several stress conditions, and the genes coding for its two partners HSPB8 and the co-chaperone BAG3. HSPB8 and BAG3 form a complex with Hsc70 and Hsp70/HSPA1A that promotes the targeting of misfolded and aggregated proteins to autophagy for degradation. Of note, HSPB8 and BAG3 are upregulated in the brain of patients affected by AD and PD. Drugs that are able to boost the expression of these key chaperones acting as co-inducers such as trodusquemine hold promise for thetreatment of these neurodegenerative diseases (Seidel et al. 2012). Whether

trodusquemine also potentiates the HSR in cells exposed to different types of stress conditions such as for example oxidative stress or proteasome inhibition is unknown and will deserve future investigations. Moreover, whether trodusquemine selectively potentiates the HSR or also may influence the activation of the so-called cold shock response, which is activated upon cell exposure to low temperatures, is unclear and also requires future investigations.

Next, we searched for the sensor and the transcription factor(s) that may mediate the transcriptional upregulation of HSGs in cells treated with trodusquemine. We provided evidence that the co-inducing effect of trodusquemine on stress-inducible HSGs is mediated by the HSF1, the main transcriptional factor activated in response to stress conditions (Morimoto et al., 1992). We also excluded that the inhibition of PTP1B, a well-known target of trodusquemine, is involved in the transcriptional co-induction of HSGs. Thus, trodusquemine acts as co-inducer of the HSR acting via HSF1.

Additionally, we considered the potential implication of other signalling pathways that are known to regulate lipid biosynthesis and can also modulate the expression of specific HSGs. We focused on the sterol regulatory element binding proteins (SREBP) pathway. This pathway can up-regulate the genes coding for the chaperones DNAJB1, DNAJB5 and DNAJB9, as demonstrated following overexpression of SREBP2 in adipocytes (Rome et al. 2008). Using the reversible competitive inhibitor of SREBP site 1 protease, we found no synergistic effect on the expression levels of the HSGs. Yet, long-term inhibition of proteolytic processing and nuclear translocation of SREBP reduced the ability of trodusquemine to act as co-inducer of the HSR. Based on our findings, we cannot presently conclude on the implication of this pathway in trodusquemine's mechanism of action. Future studies will need to rule out if the lack of activity of trodusquemine in cells exposed to PF-429242 is a consequence of changes in membrane composition that may, in turn, decrease the binding affinity of trodusquemine. It has been recently observed that trodusquemine binds preferentially to cholesterol and ganglioside GM1 (composed of glycosphingolipid and sialic acids) thanks to its positively charged spermine moiety (Errico et al. 2020); this supports the idea that local changes in lipid membrane composition may affect its sensitivity to trodusquemine.

In summary, the mechanism that we identified here is novel and interesting, considering the increasing evidence supporting a cross-talk between lipid and protein homeostasis (Vendruscolo 2022). Considering that HSPs, including HSPA1A and HSPBs (as shown in this thesis) can bind to membranes, by reducing the association of misfolded α-synuclein and its lipid-induced aggregation, it is tempting to speculate that part of the protective effect of trodusquemine in PD in vitro and cellular models might be due to the upregulation of HSGs. Once synthesized at higher rate thanks to the co-inducing effect of trodusquemine, these HSPs would act as protein chaperones, by binding to aggregating species and inhibiting e.g. fibril elongation, but they could also act as lipid chaperones, by decreasing the association of aggregated species with the membranes and facilitating the maintenance of membrane integrity upon stress. A combination of in vitro and cellular approaches will help in the future to elucidate the exact mechanism of action of trodusquemine and identify the membrane sensor/s that mediate its activity as co-inducer of the HSR.

4.3. Study of trodusquemine effect in 5XFAD mouse model.

As mentioned above, several protective effects of trodusquemine have been reported in literature, underlying its importance as a potential therapeutic strategy. Using cellular and animal models of neurogenerative diseases, it has been demonstrated that trodusquemine can reduce protein aggregation and their mediated toxicity (Lymbocker et al. 2022).

In this thesis, we employed a severe mouse model of AD-like pathology (5XFAD) to test whether trodusquemine may exert beneficial effects at early stage disease. The 5XFAD mice express human Amyloid Precursor Protein (APP) and presenilin 1 (PSEN1) transgenes with a total of five familial AD (FAD) mutations that show an additive effect in promoting $A\beta_{42}$ overproduction. As a consequence, 5XFAD mice develop quite rapidly AD-related phenotypes, achieving the full Aβ pathology within 7-10 months (mo) of life. In particular, they exhibit intraneuronal $A\beta_{42}$ aggregation at approximately 2 mo of age, which is followed by the progressive amyloid plaque deposition, neuroinflammation and neuron loss at about 6 mo of age in the brain region with the most pronounced amyloidosis. In addition, these 5XFAD mice exhibit memory loss and other cognitive deficits at 6 mo of age. Aβ aggregates and plaques are the main hallmark of this neurodegenerative disease, and there is a huge consensus on their cytotoxic effects, resulting in cellular dysfunction, synaptic loss, and neuronal death. Hence, finding new compounds able to combat and reduce the Aβ deposition holds promise for the treatment of AD.

Here we tested whether trodusquemine treatment may alleviate the intracellular Aβ (iAβ) accumulation, which in turn may prevent and/or delay extracellular Aβ (eAβ) deposition. In order to tackle this question, we performed weekly injection of saline solution (sal) or trodusquemine (Trod) 5mg/kg for 8 weeks in 2 mo wild type (wt) and transgenic (tg) 5XFAD mice. Of note, the choice of dosage and timing was based on studies previously performed (Zasloff et al. 2001; Ahima et al. 2002). Both behaviour tests and ex-vivo histological analyses were performed to evaluate the potential neuroprotective effects of trodusquemine, if any.

Behavioural tests

Firstly, we excluded any effect of chronic Trod treatment on metabolic parameters, such as body weight, with a potential influence on in vivo results. The body weight of experimental mice was monitored throughout the study and the data obtained are shown in (Figure 1a-b; two way repeated ANOVA: Genotype/treatment: $F(3, 53)=0.3667$, $p=0.7773$; days post injection: $F(7, 371)=65.66$, $p<0.0001$; genotype/treatment*days post injection: $F(21,371)=1.061$, $p=0.38$

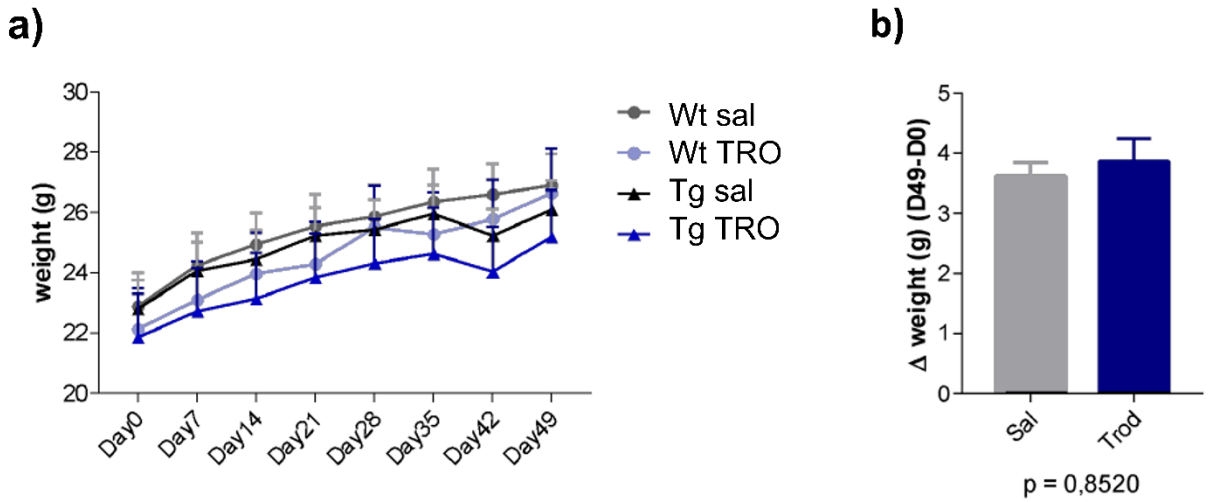


Figure 1. Body weight progression. (a-b) Chronic trodusquemine treatment did not affect the physiological weight gain under our experimental conditions. a) Body weight progression. Data are shown as mean ± SEM and analysed according to two-way repeated measures ANOVA. b) Comparison of body weight gain between sal and trod injected mice, expressed as the difference between the measure at D49 and D0 of treatment**.** Data are shown as mean ± SEM and analysed by one-way ANOVA, p=0,8520. Experimental groups: C57Bl6-SJL wt (black bar): sal n=17; TROD n=9; 5XFAD tg (red bar): sal n=20, TRO n=11.

Novel object place recognition

5XFAD mice are characterized by a series of behavioural deficits such as e.g. cognitive decline and motor deficiencies (Oblak et al. 2021). We thus verified whether trodusquemine influences the behaviour of 5XFAD mice. 5XFAD mice were subjected to the novel object place recognition (NOR) task after 7 weeks of chronic trodusquemine treatment, at 4 mo. NOR is based on the innate behaviour of mouse to spend more time in exploring a novel object/place than a familiar one and was performed with retention tests at varying intervals (2, 6 and 24h) to assess the retention capacity of recognition memory of 5XFAD mice.

A decreased spontaneous exploration of objects during pre-test session was detected in Tg mice, without any significant improvement observed after chronic exposure to trodusquemine (Figure 2a). Concerning memory performance, Tg mice exhibited a reduction in the time spent in exploring after training at each interval considered compared to WT mice and this was not improved in Tg mice treated with trodusquemine (Figure 2b). Trodusqumine did also not improve the ability to discriminate between old and new positions, as demonstrated by the lower discrimination index measured in Tg mice compared to that of Wt mice (Figure 2c).

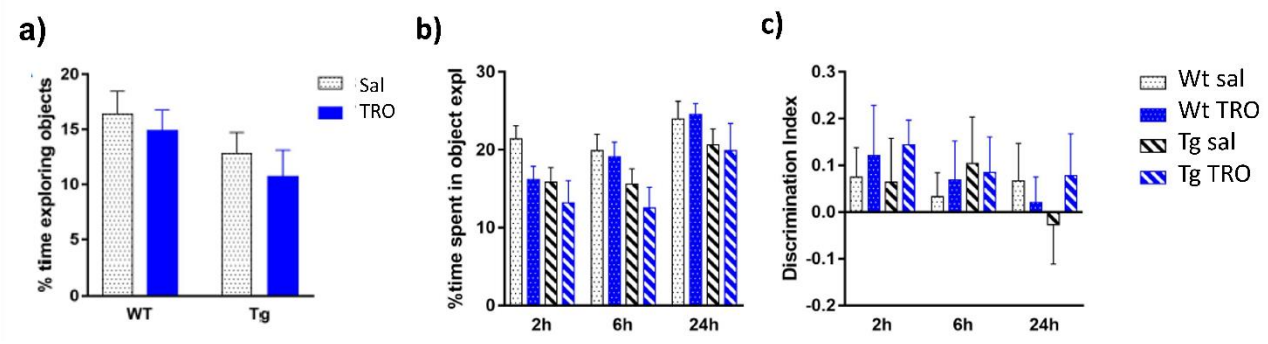


Figure 2. Trodusquemine treatment did not ameliorate the performance of 5XFAD mouse model in exploring behaviour and recognition memory test. a) Comparison of percentages of exploring time in object familiarization phase. b) Comparison of percentages of time spent with the object in the novel place. c) Comparison of discrimination index at different intervals (2h, 6h, 24h). Data are shown as mean ± SEM and analysed by Two-Way repeated measures ANOVA. Experimental groups: C57Bl6-SJL Wt: sal n=17; TROD n=9; 5XFAD Tg: sal n=20, TRO n=11.

Morris Water Maze

The spatial learning and memory were evaluated by the Morris water maze (MWM) task performed 9 weeks after the first trodusquemine injection. The learning index is measured by the escape latency to find the position of the hidden platform during five consecutive days. In our experimental conditions, the performance in a cue-guided training was not different between B6SJL (wt) and 5XFAD mice suggesting that spatial learning abilities are preserved in AD mice at this experimental age; of note, Trod treatment did not influence mouse behaviors (daily escape latency, two way repeated measures ANOVA; days of training $F(4,208)=13.00$, $p<0.0001$; genotype/treatment $F(3,52)=0.9668$, $p=0.4155$; days of training*genotype/treatment $F(12,208)=1.358$, $p=0.1885$) (Figure 3a). After 24 hours from the last training session, the probe test was performed to measure the task performance regardless of precise position of the hidden platform; the analysis of the performance was determined by measuring the percentage of time spent in the target quadrant. Our analysis demonstrated that all the experimental groups displayed a similar spatial memory (two way ANOVA, genotype $F(1,39)=1.182$, $p=0.2839$; treatment, $F(1,39)=0.03$, $p=0.8610$; genotype*treatment $F(1,19)=0.1799$, $p=0.6738$) (Figure 4b-c).

Based on these findings, we were not able to detect any substantial difference in both learning and memory between the Wt and Tg treated with trodusquemine at the experimental age investigated.

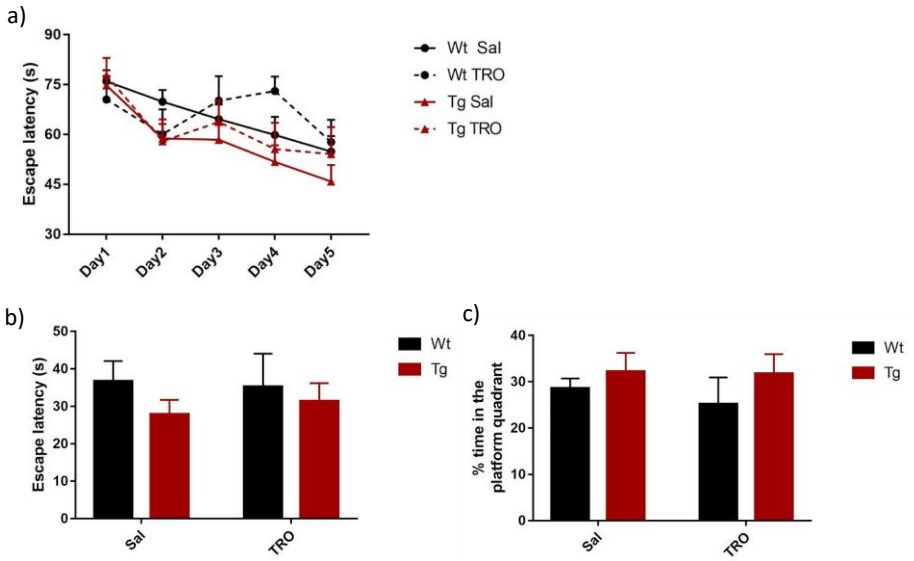


Figure 4. Trodusquemine treatment did not improve learning and memory in 5XFAD mouse model. a) In the acquisition training for the Morris water maze (MWM), no significant differences between all group mice were detected in escape latencies. Data are shown as mean ± SEM and analysed by means of two-way ANOVA. b) No differences in the preference for platform quadrant were shown among the mice group trained. Data are shown as mean ± SEM and analysed by means of one-way ANOVA. Experimental groups: C57Bl6-SJL wt (black bar): sal n=17; TROD n=9; 5XFAD tg (red bar): sal n=20, TRO n=11.

Immunohistological analysis

Amyloid plaques analysis

We investigated whether trodusquemine treatment can influence Aβ deposition during the early phase of AD progression in 5XFAD mice. Brain slices from 5XFAD mice treated with saline (control) or trodusquemine were prepared and processed through immunohistochemistry staining for plaque visualization with an anti-6E10 Ab, which detects both Aβ and APP, and an antibody specific for Aβ42 neurotoxic oligomers (anti-11A1 Aβ). The number of Ab-positive protein aggregates were compared in the saline treated and trodusquemine treated animals. The number of amyloid plaques was measured in the cortex (CTX), in the dentate gyrus (DG), and in the CA1 hippocampal region. Representative images showing cortical and hippocampal 6E10+ amyloid plaques and their relative quantifications are reported in Figure 5. We found that 5XFAD mice treated with trodusquemine displayed approximately the same number of 6E10+ amyloid plaque compared to control (sal) in all analysed brain regions (CTX (above fimbria): Tg sal vs Tg Trod p=0.7302; CTX (above DG): Tg sal vs Tg Trod p= 0.9353; DG: Tg sal vs Tg Trod p=0.8741; CA1: Tg sal vs Tg Trod p=0.9048) (Figure 5).

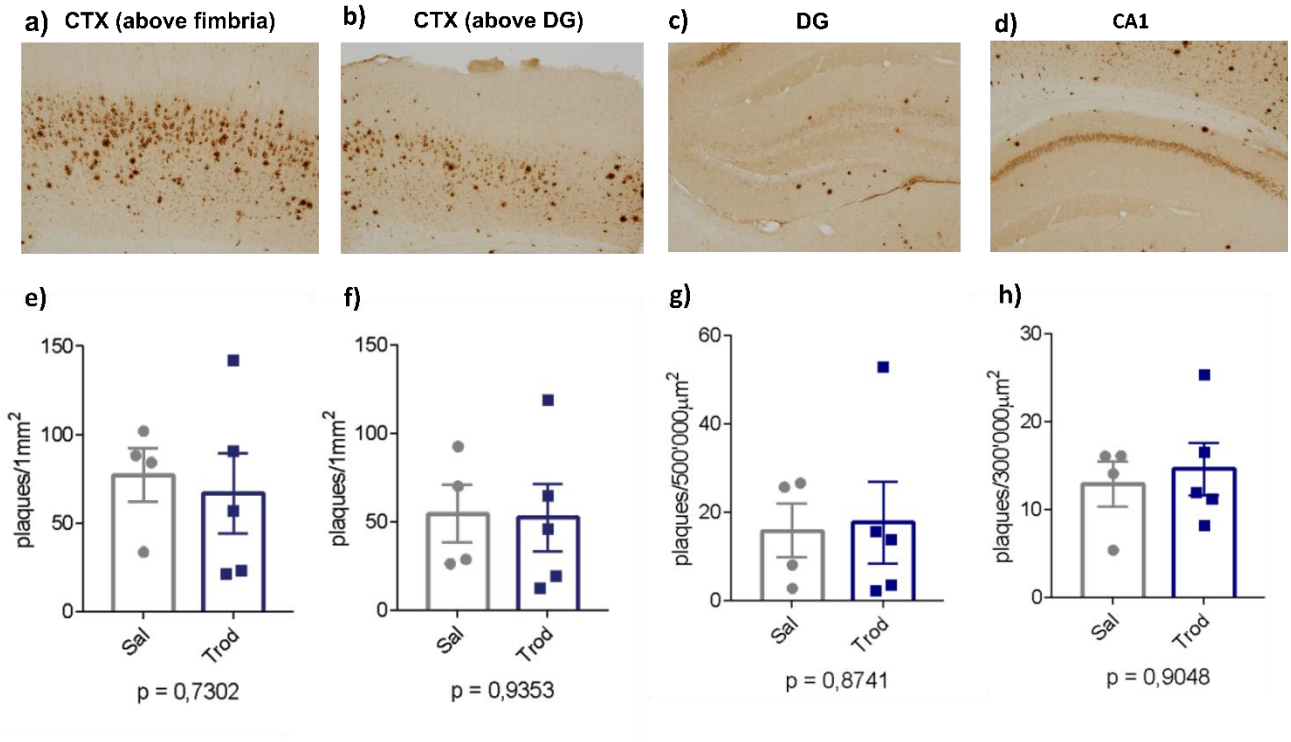


Figure 5. Trodusquemine treatment did not affect the amyloid deposition in 5XFAD mice at early stage of AD progression. (a-d) Representative images showing 6E10+ plaque in (a) CTX (above fimbria), (b) CTX (above DG), (c) DG and (d) CA1 of 4 mo 5XFAD mice treated with Sal (grey bar) or trodusquemine (blue bar). Data are shown as mean ± SEM and were analyzed according to Mann Whitney test. Experimental groups: 5XFAD: Sal n=20, Trod n=11. Abbreviations: CTX: cortex; DG: dentate gyrus; CA1: cornus Ammonis 1.

We further differentiated toxic and non-toxic conformer of Aβ, by means of IHC with mouse anti-human Aβ 11A1. Although the relationship between ternary structure of Aβ and the inherent toxic is still unclear, it has been recently reported that aggregates of $A\beta_{42}$ contain two main conformers with different toxicity: a major one with a turn at positions 25 and 26, and a minor one with a turn at positions 22 and 23 with a higher toxicity (Izuo et al. 2013; A. Morimoto et al. 2004). The monoclonal antibody 11A1 recognizes the turn at positions 22 and allows the discrimination between the two different Aβ conformers.

Representative images showing cortical and hippocampal 11A1+ amyloid plaques and their relative quantifications are reported in Figure 6. Similar to the previous analysis, the number or 11A1+ amyloid plaques were not differently affected by Trod treatment in young 5XFAD mice (CTX (above fimbria): Tg sal vs Tg Trod p=0,7302; CTX (above DG): Tg sal vs Tg Trod p= 0,7302; DG: Tg sal vs Tg Trod p> 0,9999; CA1: Tg sal vs Tg Trod p=0,4127).

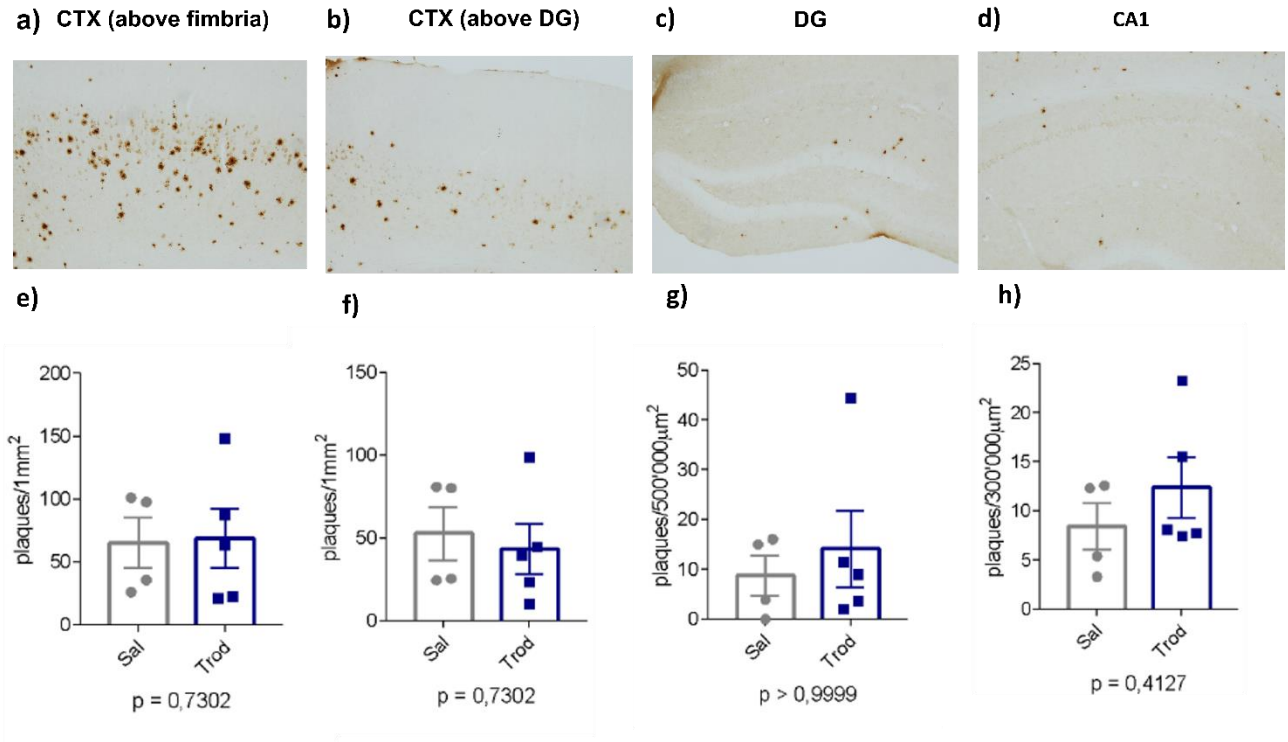


Figure 6. Trodusquemine treatment did not affect amyloid deposition expressing Aβ toxic conformer in 5XFAD mice in early stage of AD progression. (a-d) Representative images showing 11A1+ plaque in (a) CTX (above fimbria), (b) CTX (above DG), (c) DG and (d) CA1 of 4 mo 5XFAD mice treated with sal (grey bar) or trodusquemine (blue bar). Data are shown as mean ± SEM and were analyzed according to Mann Whitney test. Experimental groups: 5XFAD: Sal n=20, Trod n=11. Abbreviations: CTX: cortex; DG: dentate gyrus; CA1: cornus Ammonis 1.

Ionized calcium-binding adapter molecule 1 immunoreactivity

Inflammation is a common phenomenon that characterizes the AD pathology (Heneka et al. 2015) and 5XFAD mice are characterized by signs of neuroinflammation with microgliosis in the areas with amyloid deposition (Oblak et al. 2021; Daini et al. 2021). Thus, we used a marker of microglial cells, IBA1, to determine whether trodusquemine treatment can mitigate the microgliosis in the 5XFAD mouse model (Daini et al. 2021). Representative images showing cortical and hippocampal IBA1+ cells in different brain regions and their relative quantifications are reported in Figure 7. We found that trodusquemine did not influence the number and the area of $IBA1^+$ cells in any of the brain region considered, indicating that this compound did not reduce the microglia response at this stage of the pathology in 5XFAD mice (Figure 7a: count: CTX (above fimbria): Tg sal vs Tg Trod p=0.6470; CTX (above DG): Tg sal vs Tg Trod p>0.999; DG: Tg sal vs Tg Trod p=0.4127; CA1: Tg sal vs Tg Trod p=0.8341; Figure 7b: % area: CTX (above fibria): Tg sal vs Tg Trod p=0.8834; CTX (above DG): Tg sal vs Tg Trod p=6828; DG: Tg sal vs Tg Trod p=0.6211; CA1: Tg sal vs Tg Trod p=0.6262).

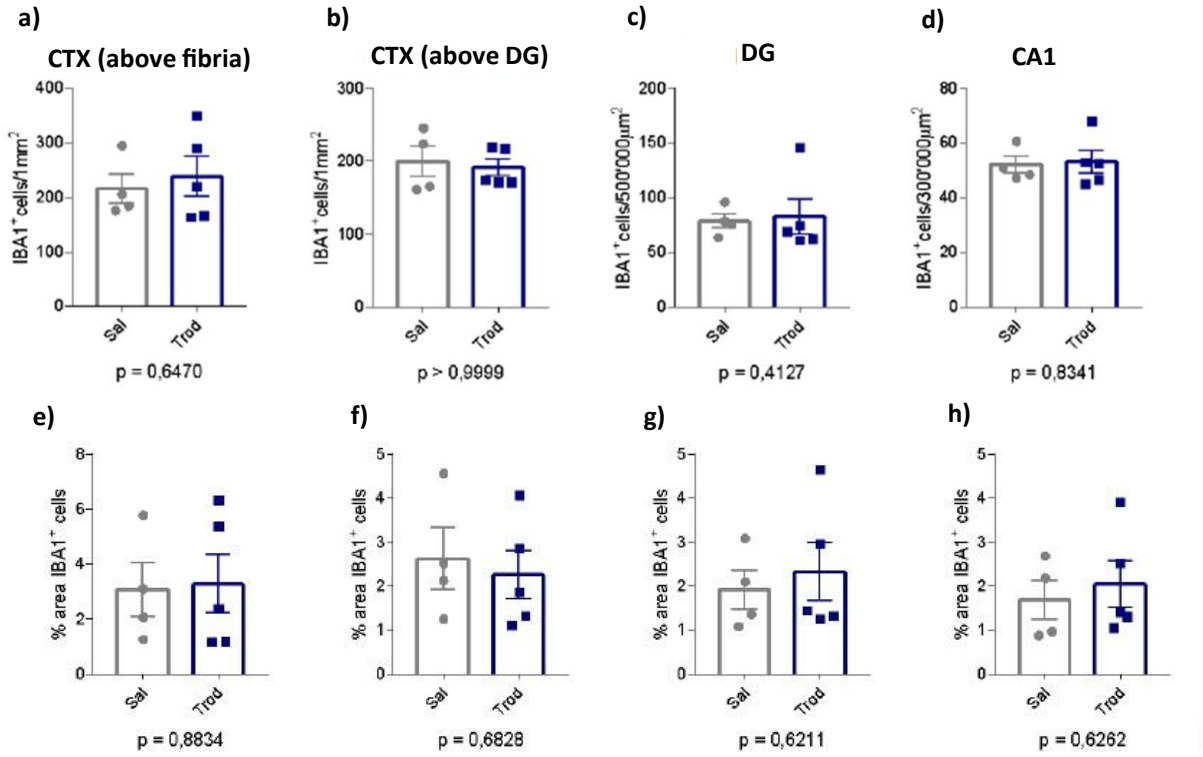


Figure 7. Trodusquemine treatment did not modulate microglial activity in 5XFAD mice. Analysis related to the number of IBA1+ cells expressed in (a) CTX (above fimbria), (b) CTX (above DG), (c) DG and (d) CA1 and the percentage of area coved by IBA1+ cells in (a) CTX (above fimbria), (b) CTX (above DG), (c) DG and (d) CA1 of 4 mo 5XFAD mice treated with saline (grey bar) or trodusquemine (blue bar). Data are shown as mean ± SEM and were analyzed according to one-way ANOVA. Experimental groups: 5XFAD: Sal n=20, Trod n=11. Abbreviations: CTX: cortex; DG: dentate gyrus; CA1: cornus Ammonis 1.

Discussion

In this chapter, we tested the neuroprotective function of trodusquemine in 5XFAD mice at early stage od AD progression, by evaluating both behavioural phenotype and histological parameters.

Concerning hippocampal-dependent cognitive functions, 5XFAD mice were subjected to two different behavioural tests to evaluate any influence of trodusquemine on memory and learning abilities. Recent data from literature demonstrated that chronic TRO treatment (5 mg/kg/5 d, i.p. for 5 weeks) ameliorated cognitive impairment and memory in 6 mo hAPP-J20 mice, another animal model of AD (Ricke et al. 2020), through PTP1B downregulation. hAPP-J20 mice develop a less sever AD pathology compared to the 5XFAD mouse model. Indeed, heterozygous hAPP-J20 mice express the human APP carrying only two familiar mutations (Swedish and Indiana familial mutations), while 5XFAD mouse model expresses a total of five familial AD in APP and PSEN1 transgenes, driving to an earlier onset of the disease with severe AD-related phenotypes. Here, using the more aggressive 5XFAD mouse model we could not identify any protective effect exerted by trodusquemine at the

dose and time point used. Several reasons may explain the lack of effect observed. These include low dose of treatment or improper time of administration or too aggressive mouse model phenotype. Future studies will need to address more carefully whether changing the trodusquemine dose and administration frequency may lead to a delay in disease progression in the 5XFAD mice. Finally, our study was carried out in the prodromal stages of AD, predominantly characterized by histological alterations and not by significant cognitive changes. It would be interesting to evaluate whether chronic treatment with trodusquemine in older 5XFAD mice may ameliorate disease progression.

5. Conclusion

This PhD thesis tackles two important questions: 1) understand the exact mechanism of action of the family of small HSPs, which are highly dynamic in structure and can associate with proteins and lipids; 2) understand how changes in membrane viscosity influence the cellular stress response and elucidate the interplay between lipid and protein homeostasis, which are both deregulated in protein misfolding diseases. A range of biochemical techniques to study lipid vesicle and proteins in vitro, along with molecular biology techniques to study gene expression in neuronal cell culture have been used to tackle these questions.

In the first section of this PhD thesis, we studied whether HSPBs can act as lipid chaperones, in addition to their well-documented protein chaperone activity. Our multidisciplinary approach enabled us to show that HSPBs can prevent or delay α-synuclein aggregation acting as lipid chaperones and as protein chaperones. HSPBs can interact directly with monomers or fibrils of α-synuclein and can compete with α-synuclein for binding to lipid membranes, decreasing its lipid-induced aggregation. Importantly, we highlight differences in the mechanism of action of the various members of the HSPB family studied. HSPB6 showed extraordinary affinity for lipids and is emerging thanks to our work as lipid chaperone. In general, the take home message from this experimental work is that the expression of the different HSPBs by a given cell type may help restoring the membrane integrity under stress conditions through direct binding to lipids, while also protecting the membranes from the deleterious interaction with misfolded proteins. This function can be achieved with different mechanisms: by buffering monomeric aggregation-prone species in solution, thus decreasing their embedding into the lipids; by buffering pre-existing fibrils and preventing their growth; by competing with misfolded proteins for interaction with lipids. Finally, we cannot exclude that once bound to the lipid membranes, HSPBs may locally change their viscosity, decreasing their affinity for misfolded species.

In the second section of this PhD thesis, we studied how mechanistically the aminosterol trodusquemine, a drug that was shown to prevent protein aggregation and that can modulate the membrane fluidity, protects neuronal cells against aberrant protein species. Our studies revealed that trodusquimine acts as a co-inducer of the HSR by modulating the fluidity membrane. This in turn lead to the potentiation of the HSR induced by a concomitant stress condition, temperature upshift. Mechanistically, trodusquemine potentiates the HSR acting via the HSF1; we excluded the implication of PTP1B, a known target of trodusquemine, in the induction of HSGs. The finding that exposure of neuronal cells to trodusquemine in absence of any additional stress cannot induce the HSR is important and suggest that this drug may selectively potentiates the HSR in those cell types that are experiencing a stress condition. This observation is important considering that trodusquemine has been tested in Phase 1 trials for the treatment of diabetes, obesity and cancer and is a promising compound for the treatment of neurodegenerative diseases such as PD (Lymbocker et al. 2022)

The chaperones that are upregulated by trodusquemine, e.g. HSPA1A and HSPB8, have been reported to bind to lipid. It is thus tempting to speculate that once synthesized at higher rates due to the co-inducing effect of trodusquemine, the HSPs would act as protein chaperones, by binding to aggregating species, but also as lipid chaperones, by decreasing the association of aggregated species with the membranes and helping the restoration of membrane integrity upon stress.

Altogether the data presented in this PhD thesis highlight the existence of an intricate cross-talk between lipid and protein homeostasis, with important implications in the cell stress response and in neurodegenerative diseases. Understanding how this cross-talk works and identifying the key players and mechanisms of action will provide novel targets for the repurposing of existing drugs and for the design of innovative therapeutic compounds.

6. References

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