CHAPTER 2

INTRODUCTION

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1. Neurodegenerative Diseases: a global disease emergency

Neurodegenerative Diseases (NDs) are considered by the World Health Organization to be part of the large group of neurological disorders affecting the brain and can be described as a degeneration of a specific population of neurons in the human nervous system. NDs selectively target specific population of neurons and neuronal circuits leading to the progressive failure of defined brain systems. In NDs patients, the degeneration of such neurons and circuits determines a different spectrum of symptoms, which reflect the loss of that particular population of neurons and their normal function in the central nervous system. Loss of neurons cause distinctive symptoms like motor disturbances such as chorea in Huntington’s disease (HD) or bradykinesia in Parkinson’s disease (PD), or loss of cognitive function such as dementia in Alzheimer’s disease (AD). The disease typically initiates in specific brain areas, but progressively affects other regions of the brain with manifestations of new symptoms or the worsening of early ones.

NDs are generally a chronic condition, characterized by a slow progression over time, which can extend for decades. Aging is strongly associated with the decline of brain functionality and the risk of developing sporadic forms of NDs, like AD and PD, rises sharply with age after 60, although some genetic forms of NDs, like HD, may have an early-age onset (Wolfe, 2018; section 2.3).

Given the increasing age of the world population and the absence of therapies against these chronic, highly debilitating and care-costing diseases, the World Health Organization considers brain disorders, AD and PD in particular, as leading contributors to the global disease burden. For instance, Alzheimer’s Disease International organization estimates that 50 million people worldwide are living with dementia in 2018 (AD accounts for an estimated 60-80 percent of these cases) (Alzheimer’s Disease International, 2018). This number is projected to increase to about 150 million by 2050, as the population ages (Figure 1).

![Figure 1: Number of people with dementia in low and middle income countries compared to high income countries. The World Alzheimer Report 2015 indicates 9.9 million new cases of dementia each year worldwide (one new case each 3.2 seconds). In the future, as the health care will improve, the consequent global demographic ageing will cause a progressive increase of dementia cases, especially in low- and middle-income countries (Source of the data from World Alzheimer Report 2015, https://www.alz.co.uk/research/statistics)](https://www.alz.co.uk/research/statistics)

Current treatments for all NDs are only symptomatic (e.g. Levodopa to control motor symptoms in PD or anti-cholinesterase agents against dementia in AD) (Wolfe, 2018). A better understanding of pathogenic mechanisms of NDs is essential to develop effective therapeutic strategies. Although
each ND shows a different biology, focusing on the common features of these diseases may provide important information about the key factors of neuronal degeneration.

2. Protein aggregation as common clue in neurodegenerative diseases

Although these pathologies have different characteristics and causes, the main common hallmark in the post-mortem brains of patients affected by different NDs is the presence of protein aggregates in the degenerated tissue. This provided an important clue to neurobiologists: the process of protein aggregation might be toxic for cells and somehow causative for NDs (Ross et al., 2004; Eisele et al., 2015; Wolfe, 2018).

Yet, there is an active discussion related to the type of aggregates responsible for (neuro) toxicity, with some investigators even arguing that aggregates may not be toxic at all and just a by-product of the degenerative process (Eisele et al., 2015).

Here I want to argue that the (process of) formation of aggregates is driving the disease because:

1. In all heritable, early onset forms of these diseases, the mutated genes encode proteins with reduced stability and high aggregation probability (section 2.3).
2. Mutations in molecular chaperones (chaperonopathies) and other protein quality control components (UPS or autophagy components) with a physiological role to prevent protein aggregation (see next sections), also lead to neuro and (cardio) muscular diseases associated with protein aggregation (sections 2.2 and 2.3).
3. In several studies using experimental models of these diseases, modulation of components of the protein quality control network were found to delay disease onset and progression (section 2.1).
4. Addition of in vitro generated protein fibrils can induce degeneration in several experimental models (section 2.6).

Therefore, an extensive part of the research in the field focused on understanding why and how proteins form aggregates and by which mechanisms aggregates may contribute to cell degeneration and death. In the neurodegenerative process, degeneration and death of neurons appear to primarily occur during the late stage of disease, preceded by functional (e.g. electrophysiological deficits, change in gene expression) and morphological (e.g. loss of synaptic connections and axon retraction) alterations (section 2.4).

Protein aggregation diseases (even the genetic forms) are typically late-onset. This suggests that in young individuals, the protein quality control (PQC) systems of the cells - the ensemble of systems that control protein folding and refolding, protein transport, protein complex (re)modelling and protein degradation via the ubiquitin-proteasome system (UPS) or the autophagy pathways - is capable to maintain protein homeostasis and hence inhibits the initiation of the toxic aggregation process. We here define protein homeostasis as the physiological balance between protein production and protein quality control. Protein aggregation in diseases may be initiated as a result of an age-related increases in the burden of unfolded or misfolded proteins and/or and age-related
decline in the functioning of the PQC network, (i.e. when protein homeostasis is perturbed). Aggregate-associated NDs can be then defined as age-related proteinopathies, in which protein aggregation is the main cell autonomous insult capable to trigger the neuronal degeneration (section 2.2).

In this Chapter, I will first discuss the PQC and the role of the heat shock proteins (HSPs), the key regulators of the cellular PQC and main contributors to the protein homeostasis: HSPs represent the first line of defense against protein aggregation, primarily acting in a cell-autonomous manner. Next, I will discuss NDs-associated aggregates and their toxicity in cells and the factors that trigger aggregation and overwhelm the cell autonomous protective strategies.

### 2.1: Protein quality control: HSPs and cellular strategies against aggregation

A protein is translated on ribosomes as a chain of amino acids, which must fold in a specific three-dimensional structure called “native state” to perform its biological functions. Although proteins may reach the native state guided by the primacy of their amino acid sequence (as postulated by Anfinsen’s dogma) (Anfinsen, 1973), protein folding in cells requires assistance not least because protein aggregation must be prevented. To prevent that native polypeptides, but also unfolded or misfolded protein species aggregate, all living systems have a PQC network in which HSPs (also called molecular chaperones) mediate multiple key processes to maintain the protein homeostasis in the normal cellular environment (Hartl et al., 2011). The human genome encodes more than 100 different HSPs which are grouped in different families: HSPH (HSP110), HSPC (HSP90), HSPA (HSP70), DNAJ (HSP40), HSPB (small HSPs) and the chaperonins HSPD-E (HSP60-HSP10) and CCT (TRiC). Several regulatory co-factors, such as the members of the BAG proteins family, can also be included in this network (Kampinga et al., 2009).

In a recent review, Kakkar and colleagues (Kakkar et al., 2014) summarized the literature on findings showing that HSPs can prevent protein aggregation in different NDs. This analysis also revealed that for each of the different types of proteins in the different neurodegenerative disease, different members of the HSPs family seemed required, implying that although aggregation drives these diseases, the types of aggregates or pathways of aggregation may substantially differ for the various disease-associated proteins.

However, how do HSPs generally contribute to PQC and aggregate prevention in cells? HSPs guide all the proteins in the cells from production to degradation, without being directly involved in their biological functionality, and show a great diversity in transcriptional regulation and functional capacity, which both depend on the “client” that they are processing and the conditions which the cells are coping with (Morimoto et al., 2008; Hartl et al., 2011). Despite their name, many HSPs are constitutively expressed in normal growing conditions and are essential for the cell viability (Morimoto et al., 2008). The constitutive HSPs participate in the de novo folding of proteins, in protein transport in the cellular compartments, in the (dis)assembly of protein complexes, in the protein degradation, and generally act as a buffer to counterbalance the natural tendency of proteins to unfold, misfold or aggregate (Hartl et al., 2011).
During co-translational folding, ribosome-associated HSPs (e.g. specialized HSPAs/HSP70 and DNAJs/HSP40) provide initial folding assistance to the nascent amino acidic chain (Kampinga et al., 2010; Hartl et al., 2011). Subsequently, the client is folded by the classical HSPA/HSP70 machinery (Kampinga et al., 2010). Here, partners of HSPA/HSP70 are DNAJs/HSP40 and nucleotide-exchange factors (NEFs), which regulate the interaction between the client and HSPA, by affecting the HSPA affinity for adenine nucleotides (ATP or ADP) (Kampinga et al., 2010).

A canonical HSPA machinery acts through the following steps (Kampinga et al., 2010; Dekker et al., 2015; Zuiderweg et al., 2017; Figure 2):

1) The J protein binds the non-native client protein and interacts with HSPA-ATP through its J domain. By this initial binding, the J protein prevents the possible aggregation of the non-native client (Dekker et al., 2015). Also, small HSPs (sHSPs) can act as an entry step of clients to the Hsp70 machine (Boncoraglio et al., 2012; Garrido et al., 2012). These sHSPs bind unfolded or misfolded protein to form chaperone-client complexes that prevent a (more) irreversible aggregate formation, keep substrate competent for processing by the HSPA/HSP70 machinery(Boncoraglio et al., 2012; Garrido et al., 2012; Carra et al., 2017; Haslbeck et al., 2019).

2) The client interacts with the peptide binding site of HSPA and, together with the J domain, stimulates HSPA to hydrolyze the ATP. This drives a conformational change in HSPA that stabilizes its interaction with the client by closing the peptide binding domain and causes DNAJ to leave the complex.

3) The NEFs (such as HSPSH/HSP110, HSPBP1 and BAG family proteins) bind HSPA-ADP and mediate the ADP-ATP exchange, reverting HSPAs to their “open” conformation and leading to the client release (Kampinga et al., 2010; Rampelt and Bukau, 2011; Bracher et al., 2015). In this phase, the client is released and may have reached its native, functional conformation. If folding is not completed after the release, the client will re-enter the cycle and may get to their final state by reiterative cycles of binding and release. Clients that cannot be completely folded by the HSPA/HSP70 machinery are transferred to or handled independently by the HSPC/HSP90 system, via the HSP-organizing protein (HOP) mediation (Young et al., 2001), or by the chaperonins (GroEL/GroES and TRiC) (Spiess et al., 2004). Clients that cannot be refolded at all, can be transferred to degradation machines (see below).

Acute proteotoxic stress conditions such as heat shock, oxidizing agents (e.g. reactive oxygen species (ROS)) and any other environmental factor can cause many proteins to become unfolded or misfolded and thus imbalance the protein homeostasis of the cells, with the risk of aggregate formation (sections 2.2.-2.3). Although the constitutively expressed HSPs might still assist in the refolding of these proteins, the cell also activates several stress responses pathways that upregulate selected HSPs via the induction of transcriptional programs in different cell compartments to rebalance protein homeostasis. Yet, only some HSPs genes are constitutively expressed and upregulated under stress or are expressed only under stress conditions; in fact most are not upregulated by proteotoxic stress and likely serve in other aspects of ensuring protein homeostasis.
Figure 2: Canonical model of action of HSPA / HSP70 machinery in protein folding. (Based on the model from: Kampinga et al., 2010 - The HSP70 chaperone machinery: J proteins as drivers of functional specificity).

The cytosolic stress response is mainly controlled by the heat shock transcription factor 1 (HSF-1) (Morimoto, 2011), which up-regulates several HSPs genes including members of small HSPs like HSPB1 (HSP27) and HSPB5 (αB crystallin), DNAJ proteins like DNAJB1 (HSP40) and HSPA members like HSPA1A, HSPA6 and HSPA8 (Kampinga et al., 2009; Hageman et al., 2010). The HSF-1 dependent cytosolic cell response is interconnected with the unfolded protein response (UPR) pathways occurring in the endoplasmic reticulum (UPRER) (Walter et al., 2011) and in the mitochondria (UPRMt) (Haynes et al., 2010). The accumulation of stress-denatured proteins in the lumen of these organelles is one of the main activation factors for both UPRER and UPRMt. From the ER, folded proteins are transported to the Golgi apparatus for further processing, whereas improperly folded proteins are degraded via proteasomes after retro-translocation in the cytosol, in a process called ER-associated degradation (ERAD). Under proteotoxic stress conditions, stress-denatured proteins are accumulated in the ER and are sensed by three different signal transducers (ATF6, PERK and IRE1) that activate the UPRER pathway (Walter et al., 2011). The UPRER positively regulates the expression of numerous genes, which down-tune the overall protein translation and encode for HSPs that increase the ER protein-folding capacity and the protein degradation via ERAD, Ubiquitin-Proteasome System (UPS) and lysosome-mediated pathways (Walter et al., 2011). In mitochondria, proteotoxic stress conditions, in particular aging-related factors (e.g. respiratory chain dysfunction

(Morimoto et al., 2008; Hageman et al., 2009; Mahat et al., 2016; Solis et al., 2016; Neueder et al., 2017).
and increased ROS in senescent mitochondria), concur to the accumulation of stress-denatured proteins in the mitochondrial matrix or intermembrane space. The consequent UPR\textsuperscript{MT} activation regulates a broad transcriptional program which includes genes encoding for mitochondrial chaperones that assist the organelle in protein refolding and quality control during the proteotoxic stress (Haynes et al., 2010).

**Figure 3: Protein degradation pathways in cells.** (Based on the model in Chiecanover et al., 2015 - Degradation of misfolded proteins in neurodegenerative diseases: therapeutic targets and strategies).

As stated above in case of failure of protein folding, the chaperone machineries can re-route the client for its degradation via: 1) the ubiquitin proteasome system (UPS) (Ciechanover et al., 2015) 2) the macroautophagy pathway (Tyedmers et al., 2010; Feng et al., 2014; Ciechanover et al., 2015) or 3) the chaperone-mediated autophagy (CMA) (Chiecanover et al., 2015; Kaushik and Cuervo 2018).

As in the folding process, the pathways involved in protein degradation depend on the client and the cellular conditions (e.g. starvation and amino acidic need, accumulation of unfolded or misfolded proteins during proteotoxic stress) (Tyedmers et al., 2010; Ciechanover et al., 2015) (Figure 3).

The prime pathway for most unfolded or misfolded proteins generated in the various cell compartments is the UPS, a selective proteolytic system for soluble single proteins, in which the conjugation of the client to ubiquitin (Ub) determines its degradation by the proteasome (Herskho et al., 1998). The process of ubiquitination is mediated by an enzymatic cascade composed by three main types of enzymes: the E1 Ub-activating enzymes, the E2 Ub-conjugating enzymes and, the E3
Ub-ligases that selectively recognize and (poly) ubiquitinate the client (Herskho et al., 1998). The properly ubiquitinated client is delivered to the proteasome. Here it is de-ubiquitinated, unfolded, inserted into the “chamber” of the proteasome and progressively cleaved into shorter small peptides. HSPs assist the UPS in the recognition of unfolded and misfolded proteins, in their ubiquitination by E3 ligases and finally in their delivery into the proteasome for the cleavage into small peptides, that can be further processes into single amino acids via aminopeptidases (Ravid et al., 2008; Cheicanover et al., 2015).

The interplay between HSPs and the proteasomal degradation is, however, far from understood: co-chaperones of the HSPA/HSP70 machinery such as DNAJB2 (Gao et al., 2011) and BAG1 (Luders et al., 2000) can interact with subunits of the proteasome and some E3 ligases can affect the function of the HSPA/HSP70 cycle (Rosser et al., 2007). This suggests that certain HSPs are part of the mechanism that targets proteins to proteasomal degradation, but how triaging between refolding and degradation occurs is still unknown.

In macroautophagy, the client intended for the degradation (e.g. a portion of the cytoplasm, unfolded and misfolded proteins, protein aggregates or even entire organelles) is collected into the autophagosome, a double-membraned vesicle, which subsequently fuses with a lysosome, causing the final degradation of the cargo by the lysosomal proteases (Nakatogawa et al., 2009). Each step of macroautophagy, such as the formation of the autophagosome and its fusion with the lysosome, is narrowly controlled by and involves several protein adaptors and regulators (Nakatogawa et al., 2009). HSPs can participate to the recognition of the cargo and its delivery into the autophagosome: a well-studied example of this is a process called “BAG-instructed proteasomal to autophagosomal switch and sorting” (BIPASS), a pathway that involves the co-chaperone BAG3 in promoting the degradation of the client to the autophagic pathway (Carra et al., 2008; Gamerdinger et al., 2011; Minoia et al., 2014).

CMA is a specific form of autophagy in which specific misfolded proteins that expose the amino acidic motif KFERQ (a motif found in about 30% of cytosolic proteins and normally buried in the native state), are selectively recognized by HSPA8/HSC70 and other co-chaperones. The client is delivered on the lysosomal membrane where it is unfolded and translocated into the lumen and degraded by lysosomal proteases into amino acids (Kaushik et al., 2018).

HSPs represent the first line of defense against protein aggregation and, coupled with the described mechanisms of protein degradation, contribute to maintain the protein homeostasis in the cell. However, if aggregation could not be prevented, cells have additional means to counteract aggregate toxicity by sequestering the misfolded or aggregated proteins into inclusions to prevent their toxicity. Such regulated deposition of endangered protein species in specific cellular deposit sites is a key strategy of defense against protein aggregation observed throughout the evolutionary trees (i.e. from bacteria to yeasts and mammalian cells) (Tyedmers et al., 2010). The type of deposition may differ depending on the stress conditions, the type of aggregating proteins and the cellular compartment. Particularly, several regulated and membrane-free deposition sites of aggregates have been observed in yeasts (Kaganovich et al., 2008; Miller et al., 2015a; Miller et al., 2015b; Rabouille and Alberti; 2017), including:
1) The juxtanuclear JunQs (Kaganovich et al., 2008) and intranuclear (INQs) (Miller et al., 2015b) quality-control compartments, in which ubiquitylated proteins are transiently stored when the degradative capacity of the UPS is saturated.

2) The insoluble protein deposits (IPoDs) (Kaganovich et al., 2008), in which aggregating proteins are accumulated in a seemingly more permanent manner (i.e., not destined to further processing or degradation via UPS).

In addition, a transient form of regulated aggregate deposition, called aggresomes, has been observed in mammalian cells (Johnston et al., 1998; Kopito et al., 2000; Garcia-Mata et al., 2002): aggregates formed at the periphery of the cell are moved along microtubules to the perinuclear site of the microtubule-organizing center (MTOC) (Garcia-Mata et al., 1999). This movement is mediated by the activity of the motor protein dynein and adaptor proteins, like histone deacetylase 6 (HDAC6), which are capable to recognize the ubiquitylated cargos (Kawaguchi et al., 2003). In how far aggresomes can be directly related to the IPoDs and/or JunQs is yet unclear. The INQs found in yeasts may be comparable to nucleolar sequestration of misfolded proteins in mammalian cells for nuclear proteins (Nollen et al., 2001; Park et al., 2013) that may also serve to transiently store misfolded cytosolic proteins, for further refolding (Nollen et al., 2001) or degradation (Park et al., 2013).

Interestingly, evidence has also shown that a process of disaggregation may occur that is conserved from bacteria to human, albeit that the players involved seem to differ (Mogk et al., 2018). Disaggregation is essentially characterized by the recognition of the aggregate by sets of HSPs that actively participate to the one-by-one extraction of misfolded polypeptides. The extracted proteins are then likely destined to subsequent HSPs-regulated processes of refolding or degradation. Disaggregation in yeasts is mediated by the yeast-specific chaperone HSP104, a member of the AAA+ ATPases associated with diverse cellular activities, in cooperation with the yeast HSP70 chaperone systems (Sanchez and Lindquist, 1990; Parsell et al., 1994; Lindquist et al., 1996; Glover and Lindquist, 1998). Importantly HSP104 homologs are absent from metazoan (with the exception of mitochondria); disaggregation in mammalian cells, however, does occurs and it is mainly mediated by HSPA/HSP70 (i.e. HSPA8 and HSPA1A in humans) which is assisted by specific set of co-chaperones that empowers HSPA/HSP70 to exhibit a potent, standalone disaggregation activity. These co-chaperones include members of the HSP110 family (HSPH1 in humans) and DNAJs (e.g. DNAJA2 and DNAJB1 in humans) (Nillegoda et al., 2015; Mogk et al., 2018). A proposed mechanism to describe the process of disaggregation is the “pulling model”, in which HSPA uses the energy of DNAJ-stimulated ATP hydrolysis to lock the substrate at the aggregate surface and apply force to “extract” it. sHSPs are thought to facilitate this “extraction” process if present during the formation of the aggregate: during this process, they bind to the aggregating substrates hereby changing the structure of the aggregates such that they remain in a (more) disaggregation-competent form (Nillegoda et al., 2015; Mogk et al., 2018).

Finally, another important strategy against protein aggregation, which is observed in bacteria (Lindner et al., 2008; Winkler et al., 2010), yeasts (Aguilaniu et al., 2003) and mammalian cells (Rujano et al., 2006; Fuentealba et al., 2008), is the asymmetric partitioning of aggregates during cell division. It has been shown that the mother cell in yeast and non-stem cells in metazoan may
retain most of the aggregate species during mitosis, benefiting the daughter cell with a lower aggregate load ("dilution effect"). Importantly, the almost total absence of mitosis in adult human neurons imply that these post-mitotic cells are not capable of this form of aggregates clearance, partially explaining the intrinsic vulnerability of neurons to protein aggregation and toxicity (section 2.5).

The presence of protein aggregates in the brain of NDs patients indicates that the previously described PQC pathways and strategies have failed to maintain the protein homeostasis. What are then the main contribution factors of protein aggregation? What are the main types of protein aggregates found in the brain of NDs patients? Which aggregates are characteristic for each specific NDs?

### 2.2. Factors leading to age-related protein aggregation

Aging is the main factor that enhances the probability of protein aggregation (Koga et al., 2011; Higuchi-Sanabria and Dillin, 2018). With age, a general decline in the capacity of the PQC and degradation pathways have been reported (Figure 4 - green line; Higuchi-Sanabria and Dillin, 2018). Inversely, the protein damage burden may increase due to many factors including accumulated oxidative damage, molecular errors during protein translation with mis-incorporation of amino acids or during the assembly of protein complex, and accumulation of somatic genetic alteration. As a consequence, protein homeostasis collapses when the burden exceeds the PQC capacity (Balch et al., 2008; Bhreme et al., 2014; Kampinga and Bergink, 2016;).

As illustrated in the hypothetical model in Figure 4, this would lead to the onset of sporadic forms of NDs, such as AD or PD (Figure 4 - red line). Support for such a model is furthermore provided by the genetic forms of NDs of AD (e.g. due to mutations in amyloid precursor protein or tau; Bird et al., 2012) or PD (due to mutations in α-syn) that lead to an elevated aggregation propensity of the affected proteins. Such evidence is even better illustrated by purely genetic NDs, like PolyQ diseases, where patients express an aggregation-prone protein from birth, yet are generally unaffected till mid-life (Figure 4 - blue line) due to an early collapse in protein homeostasis (Zuccato et al., 2010). Additional evidence for this model comes from diseases due to mutations in chaperones (so-called chaperonopathies; Macario et al., 2002, Kakkar et al., 2014) or other components of the PQC system (e.g. autophagy; Ciechanover, 2015), that are also often associated with protein aggregation (Figure 4-yellow line, and sections 2.3 and 2.4).
**Figure 4: Model for protein homeostasis collapse in age-related and aggregation diseases.** PQC declines with aging (green line); such decline is worsened by chaperonopathies (yellow line). The protein damage burden and risk of aggregation increases with age (red line) and is increased by factors such as pro-aggregation genetic mutations (blue line) and, likely, defects in the response to DNA damage (purple line). (Figure adapted from Kampinga and Bergink, 2016 - Heat shock proteins as potential targets for protective strategies in neurodegeneration).

Finally, an accelerated increase in the protein damage burden may underlay the early onset of NDs in patients with mutations that lead to defects in the response to DNA damage (Figure 4 - purple line, DDR deficiency) (Madabhushi et al., 2014), although there is yet no direct evidence supporting this.
2.3. The protein aggregation process: similarities and difference between different aggregation diseases

Protein aggregates are defined here as an incorrect non-functional association of polypeptide proteins. Hereby, we want to distinguish them from large, sometimes also detergent insoluble, functional oligomeric protein complexes or from the regulated sequestration of proteins into membrane-less structures in the cell (e.g. liquid droplets or phase separations) (Banani et al., 2017).

Aggregation can be a chaotic process, as often observed under acute protein-unfolding conditions (e.g. heat shock) and when based on hydrophobic interactions. In other cases, aggregates may instead be formed during a more ordered process, as driven for example by hydrogen bonding, which usually involves the same type of protein. However, other proteins may be trapped in disordered or ordered aggregates and hereby lose their normal functionality (which is considered to be one of the mechanisms of toxicity mediated by protein aggregation) (Ross et al., 2004; Iadanza et al., 2018).

Depending on the type of aggregation-prone proteins involved in the process and on the type of intermolecular interactions that drive the incorrect protein association (e.g. hydrophobic interactions or hydrogen bonds), cellular aggregates can be distinguished in amyloid aggregates and amorphous aggregates. Mutant proteins such those containing expanded polyglutamine in PolyQ diseases, α-synuclein in PD, or amyloid precursor protein in AD can form amyloid fibrils, which are thermodynamically stable, structurally organized, highly insoluble, filamentous protein aggregates composed by repeating units of β-sheets aligned perpendicularly to the axis of the fiber and therefore with the highest level of β-sheet organization. Differently, mutant superoxide dismutase 1 (SOD-1) in Amyotrophic Lateral Sclerosis (ALS) can form amorphous aggregates, which have a low degree of β-sheet organization and are not characterized by amyloid fibrils. Importantly, the cellular conditions that triggered the aggregation process (e.g. type of proteotoxic stress, protein modifications) may determine the type of intermediates formed during the process and the aggregate morphology, finally influencing the aggregate overall cellular toxicity (Ross et al., 2004; Iadanza et al., 2018).

Below, I will focus on a selected set of NDs in which proteinaceous aggregates are found in the brain of patients: Polyglutamine (PolyQ) diseases, PD, AD and ALS. Most forms of these NDs are largely sporadic, such as AD and PD, but some have clear genetic basis (familiar forms), such as PolyQ diseases that are entirely due to a single mutation. Although age- and environmental-related factors are important contributors of the pathological process, genetic NDs are mainly driven by a specific mutation in a single gene (monogenetic). The identification of the mutation in the genetic forms has provided a useful research tool to investigate the general pathological mechanisms of the NDs. Importantly, sporadic and familiar forms of the same ND, such as in the case of PD with α-synuclein aggregates (Lewy Body), show comparable histopathological features, such as type of aggregates and disease-involved brain area, making likely that both forms share a final common pathway.

Some genetic forms of NDs are recessive and the pathology is caused by the loss of normal function in the mutated protein. Notably, the most common mutations in PD have loss-of-function (LOF)
effects and occur in Parkin (PARK2), PINK1 (PARK6), DJ-1 (PARK7) and ATP13A2 (PARK9) genes, encoding for proteins involved in the UPS (e.g. Parkin is an E3 ubiquitin ligase) and mitochondrial turnover by autophagy (e.g. Parkin and PINK1) (Lesage et al., 2009; Schapira et al., 2010; Martin et al., 2011; Klein and Westenberger, 2012). Yet, also this LOF subsequently leads to protein aggregation that than (further) drives the disease.

Other genetic NDs are dominantly inherited and are characterized by a mutation in a gene that causes the encoded polypeptides to be aggregation-prone and to form aggregates either intracellularly (e.g. PolyQ huntingtin in HD, or α-synuclein in PD or superoxide dismutase-1 in ALS) or extracellularly (e.g. plaques of β-amyloid in AD) with gain-of-toxic function (Imarisio et al., 2008; Kiernan et al., 2011; Guerreiro et al., 2012; Wong et al., 2017). Although PolyQ diseases, PD, AD and ALS are all pathologically associated with protein aggregates, they are not always initiated by a protein that is intrinsically misfolded: whereas this is the case of mutant SOD-1 in ALS, mutant PolyQ proteins, for example, are not primarily misfolded, but instead need an additional processing to initiate their aggregation (Kampinga and Bergink, 2016).

Below, I will focus on genetic NDs characterized by gain-of-function mutations:

- **PolyQ diseases:**
  The PolyQ diseases are a heterologous group of trinucleotide (CAG) repeat disorders affecting proteins with entirely different function, yet leading to very similar disease phenotypes. This already indicates that LOF mechanism may not be the dominant mechanism driving these diseases. Huntington’s disease (HD) is the most prevalent form of CAG repeat disorders. It is an autosomal dominant ND with the CAG repeat expansion residing in the huntingtin (HTT) gene, leading to the expression of a mutant HTT protein with an expanded PolyQ tract. HD is associated with severe motor symptoms (“chorea”) and cognitive decline mainly caused by the degeneration of medium spiny neurons in the area of putamen and caudate nucleus (striatum of the basal ganglia) and various cortical regions with motor, visual and sensory functions. Intracellular aggregates of mutant PolyQ HTT are found in neurons in affected brain area of HD patients and these aggregates are considered to cause neurodegeneration via several toxic gain-of-function mechanisms (Zuccato et al., 2010; section 2.4). However, the full-length PolyQ HTT is unlikely misfolded, as it does not initiate a HSPs response (Hageman et al., 2010; Seidel et al., 2012; Seidel et al., 2016) and it does not seem a target for protein degradation (Cheichanover et al., 2015) as its steady state levels are indifferent from those of the wildtype protein (Zijlstra et al., 2010). Fragmentation of the full-length protein by cell proteases (caspases and calpains) or alternative mRNA splicing are considered key steps to initiate the formation of intracellular amyloid aggregates (Gafni et al., 2004; Haacke et al., 2007; Cowan et al., 2008). Spinocerebellar Ataxias (SCAs) are a set different and heterogeneous group of heritable PolyQ diseases, also characterized by the aggregation of proteins with expanded PolyQ and severe motor symptoms. This group, amongst others, includes the autosomal dominant Machado-Joseph disease (MJD or SCA3), in which intracellular amyloid aggregates of PolyQ ataxin-3 (ATXN3) are mainly found in brainstem and cerebellum (Costa et al., 2012). The role of proteolytic cleavage of PolyQ ATXN3 in SCA3 pathology is established (Berke et al., 2004; Haacke et al., 2007;
Koch et al., 2011) and ATXN3 cleavage products have been found in cellular and animal MJD models and in post-mortem brain tissues of MJD patients (Silva et al., 2018). Dentatorubral-pallidoluysian atrophy (DRPLA) and Spinal and bulbar muscular atrophy (SBMA) are two other notable examples of PolyQ diseases. DRPLA is autosomal and dominant, associated with the mutation of the atrophin-1 gene, presence of PolyQ aggregates, and characterized by a severe and diffuse degeneration of various regions of the brain (including cortical and subcortical areas), with cognitive and motor dysfunctions observed in patients (Katsuno et al., 2008). SBMA is due to the CAG repeat expansion in the Androgen Receptor (AR) gene, is inherited in an X-linked recessive manner, and is characterized by motor dysfunction and muscle weakness due to degeneration of motor neurons (Banno et al., 2012). Although SBMA is the only recessive form within the group of PolyQ diseases, it is probably the best example of pure gain-of-function protein aggregation disease. Male (XY) individuals suffering of complete androgen insensitivity do not show neurological symptoms meaning that such symptoms observed in SBMA patients are directly associated with the PolyQ expansion in the mutant AR. Interestingly, PolyQ-AR toxicity requires post-translational modifications: similarly to the normal AR, the binding with the androgen hormones induces an interdomain interaction in the mutant AR that seems associated to the propensity of the protein to aggregate and to the consequent toxicity (Zboray et al., 2015).

- **Parkinson's disease (PD):**
  PD is mainly characterized by motor symptoms due to the degeneration of dopaminergic neurons in substantia nigra pars compacta of brain basal ganglia, followed by dementia in the late phase of disease (Przedborski et al., 2017). About 5-10% cases of PD are genetic and caused by toxic gain-of-function or by the above-described loss-of-function mutations. Dominantly inherited forms of PD are those caused by mutations in or multiplication of the genes SNCA (PARK1, PARK4) (Lesage et al., 2009; Schapira et al., 2010; Martin et al., 2011; Klein et al., 2012). The mutations (e.g. A30P and A53T), as well as multiplications of SNCA gene, lead to aggregation of the encoded protein α-synuclein (α-syn) which is the main component of intracellular Lewy bodies and neurites, the characteristic inclusions found in PD neurons (respectively in the cell body and processes) (Wong et al., 2017). These amyloid aggregates/inclusions are also present in sporadic PD and in those caused by the LOF mutants, pointing to a central role of α-syn aggregation in PD pathogenesis. A-syn is an intrinsically disordered protein (Allison et al., 2014) that normally interacts with and binds to cellular membranes; such interaction has been suggested to be an important factor in the stability and aggregation of α-syn in PD (Zhu and Fink; 2003; Uversky and Eliezer, 2009). Abnormal covalent oxidative modifications of the protein itself may contribute to its aggregation (Schildknecht et al., 2013).

- **Alzheimer's disease (AD):**
  AD, the most common form of dementia, is mostly sporadic (although some genetic forms are also known) (Guerreiro et al., 2012) and characterized by the progressive loss of cognitive functions in patients. Common symptoms in AD are memory loss and general decline in thinking, language and learning capacity, reflecting the initial degeneration of neurons of the hippocampus region and progressive damage of other brain area. Extracellular plaques of β-amyloid and
intracellular tangles of hyper-phosphorylated tau are present in both sporadic and monogenic AD (Wolfe, 2018).

Genetic forms of AD show autosomal dominant mutations in the amyloid precursor protein (APP) gene. Mutant APP is normally folded and located at the plasma membrane where it requires cleavage by gamma secretase complex to generate aggregation-initiating amyloid β peptides, like amyloid β_{1-42} and amyloid β_{1-43}. Through this amyloidogenic pathways, Aβ peptides form toxic extracellular plaques of β-amyloid (Guerreiro et al., 2012). Autosomal dominant mutations in presenilin 1 and 2 (PSEN1 or PSEN2), important components of the gamma secretase complex, are also associated with the formation of Aβ peptides (Gotz et al., 2011; Guerreiro et al., 2012). The role of β-amyloid plaques in idiopathic AD has been recently heavily challenged (Morris et al., 2014), but their elevated presence in the brain of patients with genetic AD do support the idea that can contribute in AD pathology. In fact, Aβ amyloids may exist in different strains (i.e. forming different types of aggregates), that - dependent on the patient (and maybe its PQC capacity) - may be more or less modulated or detoxified.

Sporadic and genetic forms of AD are also characterized by intracellular aggregates of tau, a protein normally involved in the stabilization of microtubules in the axons of the neurons and therefore predominantly expressed in the central and peripheral nervous systems (Wolfe, 2018). Different isoforms of tau are expressed in human brain from the alternative splicing of mRNA from the gene MAPT. Several mutations in the MAPT gene have been found in patients affected by different NDs (Ballatore et al., 2007). Tau is subject to several post-translational modifications, and phosphorylation can reduce its ability to interact with the microtubules. Whether the phosphorylation of tau is a trigger for its aggregation still needs to be proven: aggregates of tau are always phosphorylated, but not all phosphorylated tau is aggregated; moreover there is not clear evidence that the activity of tau kinases or phosphatase is changed in AD (Iqbal et al., 2016; Wolfe, 2018). Expression in animal models of tau protein carrying disease-causing mutations, such as P301L and P301S, reproduces the typical molecular and cellular consequence observed in human disease including the formation of intracellular aggregates and neurodegeneration (Lewis et al., 2001; Allen et al., 2002).

- **Amyotrophic Lateral Sclerosis (ALS):**
ALS mainly affects the upper motor neurons (UMNs) in the motor cortex and lower motor neurons (LMNs) in the brain stem and spinal cord. LMNs communicate impulses from UMN s to muscles at the neuromuscular junctions, therefore ALS is typically characterized by a rapid and progressive loss of motor functions (i.e. control of the limbs, face muscles, jaws and tongue). Only 5-10 % of cases of ALS in humans are familiar and at least 16 genes with different dominant mutations are associated with genetic ALS (Andersen et al., 2011; Kiernan et al., 2011; Al Chalabi et al., 2012). In this Chapter, I will focus on the most frequent dominant heritable forms of monogenic ALS (about 5% of total ALS cases) associated to: 1) GGGGCC hexa-nucleotide repeat expansion in the c9orf72 gene; 2) mutations in the SOD1 gene (encoding for copper/zinc ion-binding superoxide dismutase 1 (SOD1)); 3) mutations in the TDP43 gene (encoding for TAR DNA-binding protein 43 (TDP43)).
An abnormal expansion of GGGGCC hexa-nucleotide repeat in c9orf72 gene has been recently discovered as the most common genetic autosomal dominant cause of familiar ALS. Although different hypotheses have been proposed to explain the relation between this expansion and ALS (e.g. loss of function of c9orf72 encoded protein, accumulation of toxic RNA foci), a non-exclusive mechanism is based on the evidence that repeat-associated non-ATG (RAN) translation of the c9orf72 gene generates toxic dipeptide repeat proteins (DPRs) that are highly prone to form intracellular amyloidogenic aggregates (Freibaum et al., 2017).

About 20% of familial ALS are due to dominant mutations in the SOD1 gene. Many mutations in SOD1 (e.g. A4V) have been identified and nearly all can cause protein misfolding and destabilize the functional SOD1 dimer, leading to an accumulation of the monomers. Due to their exposed hydrophobic surfaces, the monomers tend to form intracellular amorphous aggregates. Aggregates of SOD1 are also found in sporadic ALS, suggesting a common pathogenic pathway with the genetic forms (Luheshi and Dobson, 2009).

Also, mutations of TDP43 gene are associated with ALS and lead to the formation of intracellular aggregates. Notably, the aggregation of TDP43 seems one of the clearest “identifiers” of ALS and, interestingly, is also associated with the pathology of frontotemporal dementia, when degeneration occurs in the frontal and temporal lobes of the brain (Luheshi and Dobson, 2009; Andersen et al., 2011; Al-Chalabi et al., 2012).

An important aspect of the field is to unravel the mechanisms by which aggregates in NDs are toxic for the cell. In the next section, such mechanisms will be discussed with a particular attention to neurons, the brain cell type that shows a peculiar vulnerability to protein aggregation during the disease progression.
2.4. Mapping the toxicity of NDs-associated aggregates

Aggregates formed in all the above described NDs may exert their toxicity through several and potential different mechanisms, which mainly depend on the disease-associated proteins and on their cellular localization.

A detailed description of such mechanisms for each type of aggregate goes beyond the purpose of this Chapter. However, here I provide an overview of the cellular targets for toxicity of aggregates species (aggregating-prone proteins, intermediates and aggregates) associated with the above described NDs.

1) Gene transcription and histones modifications:
Genetic screens in cellular and in vivo disease models revealed that NDs, such as HD, are often associated with an altered gene transcription, although they did not always provide clear and reproducible outcomes and findings (Augood et al., 1996; Norris et al., 1996; Augood et al., 1997; Arzberger et al., 1997; Cha et al., 1998; Cha et al., 1999; Luthi-Carter et al., 2000; Chan et al., 2002; Fossale et al., 2002; Luthi-Carter et al., 2002a; Luthi-Carter et al., 2002b; Sipione et al., 2002; Hodges et al., 2006). A pleiotropic alteration in transcription could likely be a downstream consequence of protein aggregation in other cellular sites. Alternatively, but not mutually exclusive, aggregates, such as observed with HTT aggregates, are known to sequester specific transcription factors (TFs), which might finally contribute to cellular dysfunction and degeneration. The functional consequences of aggregate toxicity strongly depend on the type of TFs trapped (Boutell et al., 1999; Shimohata et al., 2000; Steffan et al., 2000; Holbert et al., 2001; Nucifora et al., 2001; Dunah et al., 2002; Zhai et al., 2005; Zuccato et al., 2007; Cui et al., 2006). Aggregates also trap chromatin regulators (i.e. histone-modification enzymes), hereby changing the epi-genetic landscape and leading to a more global change in gene expression profiles and hence neuronal functionality (Steffan et al., 2001; Sadri-Vakili et al., 2007).

2) Nucleocytoplasmic transport:
The nuclear pore complex (NPC) is a protein complex that controls the fundamental nucleocytoplasmic transport of RNA molecules and proteins. Recent data have revealed that dysfunction in NPC transport could be a very early effect in many different NDs aggregation diseases (Shur et al., 2001; Lee et al., 2006; Sheffield et al., 2006; Jovicic et al., 2015; Zhang et al., 2015; Freibaum et al., 2015; Zhang et al., 2016; Grima et al., 2017; ). Such early event will interfere and even disrupt the normal function of NPC and may actually have a high self-propagating nature as a disrupted nucleocytoplasmic transport will impede on nearly all metabolic and even many catabolic processes in the cell.

3) RNA metabolism:
RNA binding proteins (RBPs) are responsible for the mRNA maturation in the nucleus (in processes such as splicing, capping and nuclear export) and its translation in the cytoplasm. RBPs and transcripts transiently form different types of granules in nuclei and cytoplasm, which are essential for RNA metabolism. For example, processing bodies (P-bodies) are involved in mRNA silencing and degradation (Maziuk et al., 2017), whereas stress granules (SGs) are formed during cell stress (e.g.
heat shock, oxidative stress and ER stress) to silence non-essential transcripts and promote the translation of stress-response proteins such as HSPs (Maziuk et al., 2017). Transcripts are incorporated in SGs together with RBPs and other proteins that enable their interaction with the cytoskeleton and other organelles; importantly, RNA granules in neurons are also involved in mRNA transport along the axons for the final translation at the synapses (Maziuk et al., 2017). A typical RBP contains a RNA binding domain, nuclear import-export sequences and “low complexity” domains (LCDs), which mediates the formation of RNA granules (King et al., 2012). RNA granules are highly dynamic entities that readily disassociate to release the transcripts. A number of disease related proteins (in particular those causing ALS, such as TDP43) are normal constituents of these RNA granules. In disease, the presence of these mutated proteins reduces SG dynamics, likely because they now rapidly transform from a liquid to crystal phase (as highly stable amyloids). Such mechanism impedes on the physiological function of these granules and can be toxic to the cells (Mori et al., 2013; Liu-Yesucevitz et al., 2014; Kwon et al., 2014; Lee et al., 2016; Lin et al., 2016; Conicella et al., 2016).

4) HSPs:
As previously explained, HSPs play a central role in protein homeostasis and are the first-line of defense against protein aggregation. HSPs activity or inducibility declines with aging which may enhance the susceptibility to protein aggregation (Higuchi-Sanabria et al., 2018). Indeed, mutations in chaperones and hence impairment of protein quality control has been associated with neuro- and muscular degeneration associated with aggregation (Macario and Conway de Macario, 2002; Macario et al., 2005; Kakkar et al., 2016). Different HSPs are frequently found/recruited to NDs-associated aggregates. For example, HSP70 and HSP40 members are found in PolyQ inclusions (Wyttenbach et al., 2000; Suhr et al., 2001; Waelter et al., 2001). This might be due to the fact that HSPs recognize and interact with these aggregate species. However and inversely, aggregate species may sequester the HSPs in or at the aggregate (trapping) which may cause an impairment in the activity of HSPs further accelerating protein aggregation of the disease-relevant protein.

5) Ubiquitin-Proteasome System (UPS):
Similar to HSPs, the pathology of many NDs is associated with the reduced activity of the UPS capacity, which may occur during aging and result in a reduced capacity to degrade unfolded and misfolded proteins, perpetuating the formation of toxic aggregates. Several mutations in UPS components affect the ubiquitin-dependent processes and are linked to neurodegenerative processes and presence of protein aggregates: two examples are the ubiquitin-ligase Parkin, mutations in which cause an autosomal recessive form of PD (Kitada et al., 1998), and the de-ubiquitylating enzyme ATXN3, mutations of which are responsible of SCA3 (Evers et al., 2014). In these NDs, whereas the proteasome remains operative, the ubiquitination of the substrates is largely impaired, leading to reduce protein degradation and increased risk of protein aggregation. However, UPS can also be considered as a potential target of aggregate toxicity because aggregates species are capable to inhibit the activities of UPS components, therefore sustaining the pathological mechanism in a positive feedback loop (Ciechanover et al., 2015; Dantuma et al., 2014). There is evidence that aggregates (such as HTT and α-syn) may directly impede the proteasomal activity through direct interaction with its subunits (Stefanis et al., 2001; Snyder et al., 2003; Lindersson et al., 2004; Chen et al., 2006a; Diaz-Hernandez et al., 2006). Other studies suggest that
PolyQ proteins - which are poor substrates for the UPS - get stuck in proteasomes (clogging) (Homberg et al., 2004). However, more recent data rather suggest that such proteasomal impairment may be a late event in the diseases (Seidel et al., 2012; Seidel et al., 2016) and the result of an overall and complex imbalance of protein homeostasis, resulting in a proteasomal overload (Dantuma et al., 2014).

6) Autophagy:
Autophagy is an important pathway of removal of mis- or unfolded proteins, aggregates and damaged organelles such as mitochondria (via mitophagy). Notably, neurons retrogradely transport the cargo (e.g. aggregates and senescent mitochondria) from their axon and neuronal termini to their soma through a long and complex process, the efficiency of which is affected by aging and aggregates in NDs (Ciechanover et al., 2015; see also point 11). As in the case of HSPs and UPS, reduced autophagy is observed in aging and may contribute to NDs pathogenesis. Moreover, genetic impairment of autophagy has been shown to lead to neurodegeneration (Hara et al., 2006). Autophagy is often found to be impaired in NDs, as revealed by an abnormal accumulation of autophagosomes or reduced lysosomal activity in degenerating cells (Cuervo et al., 2004; Nixon et al., 2005; Morimoto et al., 2007; Boland et al., 2008; Martinez-Vicente et al., 2010; Wong et al., 2010; Nixon et al., 2011; Lee et al., 2012). Disease-associated aggregates have been shown to impair autophagy, through the direct interactions of the toxic species with the autophagic components, such as α-syn and HTT that have a high affinity with the autophagic protein complex LAMP-2A on the lysosomal membrane (Cuervo et al., 2004; Malkus et al., 2012; Qi et al., 2012). As result, the normal autophagic cycle may be blocked as suggested by the accumulation of autophagosomes and lysosomal impairment.

7) Ca\textsuperscript{2+} homeostasis:
In neurons, Ca\textsuperscript{2+} regulates the activity of several responsive proteins like Ca\textsuperscript{2+}-dependent enzymes (e.g. calpains and adenylate cyclases) and proteins involved in cellular signaling (e.g. calmodulin, kinases and phosphatases), gene transcription (e.g. calcineurin and cAMP response element binding protein (CREBP)), cytoskeleton dynamics (e.g. dynein) and synaptic functionality (e.g. synaptotagmins). Moreover, Ca\textsuperscript{2+} has a key role in neurotransmission and in the short- and long-term synaptic plasticity. Therefore, neurons control the intracellular levels of Ca\textsuperscript{2+} by carefully regulating the activity of different Ca\textsuperscript{2+} channels on the plasma membrane (e.g. NMDA and AMPA receptors, voltage-gated Ca\textsuperscript{2+} (VGCCs) and TRP channels, plasma membrane Ca\textsuperscript{2+} pump (PMCA) and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCE)) and on the membranes of Ca\textsuperscript{2+}-store-organelles like the ER and mitochondria (e.g. InsP3 and Ryan receptors, the sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) and the mitochondrial calcium uniporter (MCU)) (Bezprozvanny et al., 2009). A disturbance in Ca\textsuperscript{2+} homeostasis is observed in several NDs including HD, PD, AD and ALS (Bezprozvanny et al., 2009). Aggregate species associated with NDs, such as in the case of PolyQ-HTT and ATXN3 in HD and SCA3 respectively, disturbs the Ca\textsuperscript{2+} homeostasis by interfering with the normal activity of some of the channels located at the plasma and organelle membrane (Zeron et al., 2002; Tang et al., 2003; Swayne et al., 2005; Tang et al., 2005; Shehadeh et al., 2006; Fan et al., 2007; Kaltenbach et al., 2007; Chen et al., 2008; Zhang et al., 2008) or otherwise by forming transmembrane Ca\textsuperscript{2+} permeable pores in the bilayer (see point 13) (Buttrefield et al., 2010). This primarily determines a dysregulation of the levels of Ca\textsuperscript{2+} in the different cell compartments and consequently of the Ca\textsuperscript{2+}-dependent
pathways at the plasma membrane, ER (see point 8) (Hetz and Saxena, 2017; Remondelli and Renna, 2017), Golgi (see point 9) (Machamer et al., 2015), mitochondria (see point 10) (Lin and Beal, 2006; Federico et al., 2012) and synapses (see point 12). Slight changes in Ca^{2+} levels also further aggravates aggregation of proteins by modulating the activity of proteases like calpains and caspases that trigger the abnormal cutting of proteins in peptides with a high propensity to aggregate (e.g., huntingtin in HD) (Gafni et al., 2004; Cowan et al., 2008; Haacke et al., 2007).

8) ER:
The ER is a site of folding for at least one-third of the proteome. In NDs, the accumulation of protein aggregate species has been shown to cause chronic UPR^{ER} activation, impairing the normal functionality of the organelle in proteins maturation, trafficking and degradation. Like for several of the before mentioned effect of aggregation, ER impairment due to aggregate species may cause a forward vicious cycle of decline in protein homeostasis (Hetz and Saxena, 2017; Remondelli and Renna, 2017). Several mechanisms by which aggregate species can interfere with ER homeostasis have been identified. For example, aggregates disrupts the normal functionality of Ca^{2+} channels on the ER membrane (e.g. InsP3R) and consequently the overall Ca^{2+}-based regulation of ER and cytosolic proteins (see also point 7) (Tang et al., 2003; Higo et al., 2003; Belal et al., 2012; Selvaraj et al., 2012). Aggregates also have been shown to interact and interfere with the functions of ERAD components, therefore interfering with the normal degradation of proteins (Nishito et al., 2008; Yang et al., 2010; Abisambra et al., 2013). The ER-Golgi trafficking is altered by aggregate species through their interference with the normal functions of proteins involved in vesicles tethering, docking and fusion (e.g. Rab GTPases) (Cooper et al., 2006; Gitler et al., 2008), leading to a further toxic accumulation of proteins in the organelle. Finally, aggregate species also interfere with the UPR^{ER} pathway via its signal transducers (e.g. ATF6 in HD) (Fernandez-Fernandez et al., 2011; Naranjo et al., 2016).

9) Golgi apparatus (GA) and vesicular trafficking:
Through a finely regulated vesicular trafficking, proteins are transported from the ER to the GA where they are processed via post-translational modifications (e.g. glycosylation, proteolytic cleavage) and sorted to different compartments and membranes (including the plasma membrane, the extracellular space and the endo-lysosomal system). Notably, Golgi outposts are also present in neuronal axons and dendrites and may have an important role for the protein trafficking in these cellular sites. The GA structure, consisting of stacks of parallel cisternae, is primarily maintained by the microtubules of cytoskeleton, GRASPs and golgins and can be reversibly disassembled (fragmentation) during physiological cellular process (e.g. mitosis) (Machamer et al., 2015; Gonatas et al., 2006). Interestingly, GA fragmentation is also often observed before the degeneration of neurons in NDs and in concomitance with protein aggregation, although the link between the two has not been elucidated (Gonatas et al., 1992; Stieber et al., 1996; Mourelatos et al., 1996; Gosavi et al., 2002; Huse et al., 2002; Stieber et al., 2004; Gonatas et al., 2006; Gujita et al., 2008; Tong et al., 2012; Joshi et al., 2014; Baloyannis et al., 2014; Van Dis et al., 2014). Such irreversible fragmentation of the GA negatively impacts on the trafficking and processing of many essential proteins and membranes) and therefore can significantly contribute to NDs aetiology (Gonatas et al., 2006; Machamer et al., 2015). NDs-associated aggregate species perturb the homeostasis of many GA-specific proteins involved in vesicle trafficking or GA structure, leading to an abnormal
accumulation of proteins in GA and its final fragmentation (Stieber et al., 2004; Sundaramoorthy et al., 2013; Atkin et al., 2014; Soo et al., 2015; Sundaramoorthy et al., 2015).

10) **Mitochondria:**
Mitochondria provide energy through the production of ATP and are involved in metabolism and Ca\(^{2+}\) homeostasis (see also point 7). Importantly, they contain several antioxidant molecules and enzymes (e.g. glutathione, coenzyme Q10, catalase and glutathione peroxidase), which inhibit the toxicity of oxidant species produced during mitochondrial respiration (Lin et al., 2006b; Federico et al., 2012). Mitochondrial dysfunctions have been observed in most NDs and acknowledged to be able to trigger cell degeneration and such as may contribute to the onset of the disease (Jenkins et al., 1993; Gu et al., 1996; Kong et al., 1998; Nunomura et al., 2001; Pratico et al., 2001; Mattiazi et al., 2002; Damiano et al., 2006; Shapira et al., 2008). Importantly, mitochondrial dysfunctions can cause the accumulation of reactive oxygen species (ROS), causing oxidative stress, which could perpetuate and augment aggregate initiated damage by causing damage to the nuclear and mitochondrial DNA, to lipid membranes (e.g. through peroxidation, causing membrane breakage), to membrane proteins (causing leakage or impair mitochondrial import), and to soluble proteins (e.g. through fragmentation and oxidation, promoting their aggregation) (Lin and Beal 2006; Federico et al., 2012). NDs-associated aggregates species are known to alter the normal functions of the organelle (e.g. by interaction with its membranes, enzymes and membrane proteins) and, in some cases, interfere with its dynamics (e.g. fusion, fission, degradation via mitophagy and organelle movements) (Casley et al., 2002; Mattiazi et al., 2002; Anandatheerthavarada et al., 2003; Choo et al., 2004; Liu et al., 2004; Lustbader et al., 2004; Park et al., 2004; Pasinelli et al., 2004; Song et al., 2004; Crouch et al., 2005; Vijayvergiya et al., 2005; Manczak et al., 2006; Martin et al., 2006; Chinta et al., 2008; Orr et al., 2008; Song et al., 2011; Luth et al., 2014; Di Maio et al., 2016).

11) **Axonal transport and cytoskeleton:**
Neurons have a unique cellular morphology and rely on axonal transport for their maintenance and functions: several types of cargoes are transported along the microtubules of the axon cytoskeleton, with the direct participation of microtubule-associated proteins (e.g. Tau) and by several motor proteins like kinesins (for the anterograde “soma-synapse” transport) and dynein complexes (for the retrograde “synapse-soma” transport). Proteins, Golgi-derived vesicles, neurosecretory granules (containing molecules and proteins like neurotransmitters and neurotrophic factors), mRNA (to be translated at the synaptic terminal) and mitochondria are transported anterogradely; senescent mitochondria, autophagosomes (destined to degradation via autophagy in the soma, see also point 6) and endosomal recycling vesicles are instead transported retrogradely (Millecamps et al., 2013). Early events, observed in almost all NDs, are the defective axonal transport and accumulation of the cargoes in the soma, in the axon and in the synaptic terminal, which have been often linked to the presence of protein aggregates that physically may obstruct all these transport processes (Wagner et al., 1996; Patrick et al., 1999; Nguyen et al., 2001; Kamal et al., 2001; Saha et al., 2004; Gauthier et al., 2004; Ackerley et al., 2004; Dompierre et al., 2007; Morfini et al., 2009; Bosco et al., 2010). Interestingly, the morphological features of neurons in several aggregate-associated NDs - the so-called ballooned neurons - resembles the swollen neurons produced by neurotoxins that impairs the axonal transport.
12) **Synapses:**
Whilst several of the above-mentioned toxic effects of aggregates may indirectly affect the synaptic functionality (e.g. altered Ca$^{2+}$ homeostasis, transcription dysregulation, disruption of axonal transport, damage of the phospholipid bilayer), aggregates can also exert a direct toxicity on synapsis. For example, α-syn aggregates can interfere with the synaptic vesicle maturation and trafficking, with the normal function of synaptic proteins (e.g. SNAREs) and with the regulation of neurotransmitters release and re-uptake (Masliah et al., 2000; Chung et al., 2009; Garcia-Reitbo et al., 2010; Nemani et al., 2010; Lundblad et al., 2012; Scott et al., 2012; Choi et al., 2013; Janezic et al., 2013; Wang et al., 2014).

13) **Membranes:**
Intracellular and extracellular aggregate species can induce the disruption of the cellular membranes through their direct interactions with the phospholipid bilayer (Arispe et al., 1993; Jang et al., 2010; Reynolds et al., 2011; Bäuerlein et al., 2017). The main cytotoxic effects of such interactions are the modifications of chemical-physical properties (e.g. rigidity and conductance) and permeabilization of the membrane. Different mechanisms have been suggested to explain how aggregate species can destroy the bilayer: A) deformations and possible rupture of the membrane due to the interaction and physical impinge of the growing aggregate on the bilayer; B) detergent-like micellization, during which the monomers of toxic protein are adsorbed by the bilayer and, subsequently, the growing aggregate causes lipid extraction and membrane thinning; C) formation of transmembrane pores during which aggregates species are incorporated in the bilayer and form a channel which is permeable to molecules and ions (see also point 7). These mechanisms might involve both the plasma membrane and those of organelles, causing impairments in their organization and dynamics (Butterfield et al., 2010).
Figure 5: Mapping the toxicity of NDs-associated aggregates. The main sites/pathways of damage in neurons mediated by ND-associated aggregates are: 1) Gene transcription (including transcription factors) and histones; 2) Nucleocytoplasmic transport; 3) RNA metabolism; 4) HSPs; 5) Ubiquitin-Proteasome System (UPS); 6) Autophagy; 7) Ca\(^{2+}\) homeostasis; 8) Endoplasmic reticulum (ER); 9) Golgi apparatus (GA) and vesicular trafficking; 10) Mitochondria; 11) Axonal transport and cytoskeleton; 12) Synapses; 13) Membranes. Aggregate-mediated damage at these sites contributes to neuronal degeneration. The same toxic protein can form aggregates at different cellular sites and can affect multiple pathways. Dysfunctionalities in some of these pathways contribute to an alteration of protein homeostasis (e.g. HSPs, UPS, autophagy and Ca\(^{2+}\) homeostasis), therefore perpetuating the formation of toxic aggregates. NDs associated aggregates have also prion-like properties (14) (see section 2.6).

Given such a multitude of cellular damages that may result from aggregates in NDs (summarized in Figure 5), it is unlikely that strategies that focus to correct either one of these targets individually...
may produce long-lasting therapeutic effects in patients. For this reason, many research groups, including ours, have focused on the development of anti-aggregation therapeutic strategies that aim to prevent or delay the formation of these toxic species before they might initiate this cascade of these different toxic (and often self-perpetuating) processes.

### 2.5. Neuronal vulnerability in aggregate-related diseases

Neurons show a peculiar vulnerability to protein aggregation (Saxena et al., 2011). In some genetic NDs, although the disease-causing mutant protein is ubiquitously expressed in all cells of the body (such as in the case of PolyQ HTT in HD; Zuccato et al., 2010), neurons in particular show early onset pathology (neuronal vulnerability). Moreover, such vulnerability is often initially restricted to a specific sub-population of neurons (selective neuronal sensitivity). Even more peculiar, different PolyQ diseases, such as HD and SCA3, are both caused by a mutant protein with an expanded PolyQ stretch (which drives protein aggregation and disease), but whereas PolyQ HTT in HD primarily affects striatal neurons (Zuccato et al., 2010), PolyQ ATXN3 in SCA3 primarily affects cerebellar Purkinje cells (Evers et al., 2014).

The cause for these selective neuronal sensitivities is still not clear. Several research groups have characterized the transcriptome and proteome of different subgroups of neurons, aiming to identify specific candidates that could be linked to hypersensitivity of specific neurons to the pathogenic process. Differences have been found, but no clear marker explaining the vulnerability of a specific subset of neurons to a specific mutant protein has been identified so far (Mattson et al., 2006).

Nevertheless, the studies regarding the biology of neurons, the cellular PQC (see section 2.1) and the mechanisms by which aggregates can be toxic for cells (and particularly for neurons, see section 2.4) suggest that some of the following factors may contribute to the higher vulnerability of neurons to these diseases (independently from which neurons and which neuronal areas):

- **The distinct morphology**: neurons (and particularly the vulnerable subgroups) have very long axons that may connect many different CNS regions with each other (e.g. striatum of the basal ganglia and cortical regions in HD) or the CNS with the periphery (e.g. motor neurons in ALS) (Mattson et al., 2004; Zuccato et al., 2010; Kiernan et al., 2011). Such long axons require a high trophic support and imply a larger cell surface area exposed to toxic environmental conditions. The peculiar presence of an axon in the neuron also implies the presence of synaptic terminals which depend on an efficient transport of molecules and organelles from the very distant soma across a small space that easily may be affected by aggregates.

- **The metabolism with high energy requirements**: neurons are extremely energy-demanding cells (Belanger et al., 2011) and the high oxidative metabolism might contribute to a greater formation of ROS. For these reasons, the interlinked mechanisms of protein aggregation due to ROS-mediated modifications and aggregate toxicity on mitochondria (that increases levels of ROS) might be particularly exacerbated in neurons.
• Post mitotic: neurons are post-mitotic cells with no or extremely limited capacity of division and cell replacement (although few neuronal stem cells with such capacities are present in human CNS). Therefore, differently from other cells, neurons lack the protective strategy of asymmetric partitioning of aggregates during cell division and the possibility to replace damaged neurons.

• The responsiveness to neurotransmitters: the plasma membranes of neurons are rich of receptors for different neurotransmitters and, notably, the most vulnerable subgroups are particularly responsive to excitatory neurotransmitters such as glutamate and dopamine (e.g. striatal medium spiny neurons in the area of putamen and caudate nucleus in HD) (Mattson et al., 2006; Saxena et al., 2011). During excitotoxicity, post-synaptic receptors are over-activated by abnormal higher levels of glutamate, leading to increased influxes of Ca$^{2+}$ that may trigger a-specific activation of Ca$^{2+}$-dependent proteases to fragment disease-related proteins or otherwise trigger their unfolding (section 2.4, point 7).

• The activity of the neuronal PQC: the capacities of HSPs and protein degradation systems (UPS) might be intrinsically lower in neurons compared to other non-neuronal cells (section 3.4).

All together, these data indicate that the intrinsic cellular and molecular characteristics of neurons make them particularly vulnerable to protein aggregation in NDs.

2.6. Prion-like properties of NDs-Associated aggregates

In the previous section, we concentrated on how aggregates are formed in situ, in a compartment or organelle where they directly exert their toxicity (“cell-autonomous” effects). However, recent evidence in vitro and in vivo showed that NDs-associated aggregates may also have an ex situ origin (Brundin et al., 2010; Costanzo et al., 2013a).

Prion diseases (PrDs) are characterized by severe neurodegeneration and neuroinflammation and are caused by proteinaceous agents named “prions”, which consist of pathological aggregates (PrP$^{Sc}$) of the prion protein PrPC, a plasma membrane protein. The aggregation nucleus can entrap wildtype PrPC monomers, often referred to as conversion, and PrP$^{Sc}$ oligomers grow. Such oligomers can be fragmented, thus forming new nuclei, each of which can restart the nucleation and fragmentation cycle. The minimal self-replicating unit of aggregates is defined propagon and during the aggregate amplification process, normal proteins can be sequestered through co-aggregation. Prions show a great resistance to degradation by cellular proteases, heat and other denaturing agents. Human prion diseases typically affect the brain and neurons show a peculiar vulnerability. The most striking characteristic of prion diseases is the transmissibility of the proteinaceous agent between hosts: in the transmissible spongiform encephalopathies (TSEs), for example, transmission of the disease occurs when an individual is “infected” by prions mainly through ingestion of tissues or blood transfusion from another affected individual (Scheckel and Aguzzi, 2018). Prions are capable to reach the brain via the gut-brain axis where they can penetrate in neurons to start the aggregation cycle, co-sequestering the endogenous protein. Notably, experiments in animal models
confirmed that the normal protein is initially required for the prion disease to occur, suggesting that the prion must interact with the normal endogenous protein to initiate the vicious aggregation cycle.

As reviewed by Brundin, Melki and Kopito in 2010, an increasing amount of evidences suggests that also aggregates associated with the above-mentioned NDs, such as PD, HD and AD, might spread in a ‘prion-like manner’ and that disease progression is associated with the intercellular transfer of pathogenic proteins (Brundin et al., 2010).

This had already for long been suggested by the anatomical patterns of degeneration that are specific for each disease (Braak et al., 2003). Post-mortem analysis of brains from NDs patients showed that, whereas the disease starts in specific brain sites, protein aggregation and neuronal degeneration progresses over time to other brain regions, displaying a stereotypical anatomical pattern that is typical for each NDs and that follows specific networks of synaptic circuits as described below.

![Figure 6: how neuropathological changes in Parkinson’s, Alzheimer’s and Huntington’s diseases spread spatiotemporally during disease progression. The earlier the neuropathology develops in a given brain region, the darker the shading in the figure. (Image retrieved from Brundin et al., 2010 - Prion-like transmission of protein aggregates in neurodegenerative diseases)](image)

In HD, the area of putamen and caudate nucleus have been suggested to be the first to degenerate and show PolyQ HTT aggregates, although other studies indicate that motor and sensory cortical regions are also early affected by neurodegeneration (Zuccato et al., 2010). In MJD, PolyQ ATXN3 aggregation and neurodegeneration initially involve brainstem nuclei and cerebellum. In a later phase, these can be widespread and variable throughout many other region of CNS (Paulson et al., 2017).

In early-phase PD, Lewy bodies and neurites of α-synuclein appear and neurodegeneration occurs in the olfactory bulb, in the anterior olfactory nucleus and in the dorsal motor nucleus of the vagus nerve in the medulla oblongata (Brettschneider et al., 2015). Starting from the degeneration in these areas, other regions of the CNS (such as pons and midbrain) are affected in a later phase. Notably, the distinctive motor symptoms of PD are mainly associated with the degeneration of dopaminergic
neurons in the midbrain during the late phase. Interestingly, in early PD, α-synuclein aggregates and pathology have even been detected in peripheral neurons of the enteric nervous system, which are connected with the CNS through the brain-gut axis.

In AD, intracellular tangles of tau are first found in the hippocampus, in other regions of the temporal lobe and in the brainstem. Later, they also appear in the cortical regions (insular cortex and neocortex). The appearance of extracellular plaques of β-amyloid follow instead the opposite direction, earlier in the cortex and later in the brainstem. Although tau and β-amyloid are both connected with AD pathology and neurodegeneration, the reasons of this opposite pattern of spreading in the brain still need to be elucidate (Brettschneider et al., 2015).

Also in ALS, specific patterns of aggregation and degeneration of neurons have been recognized. However, it is still not clear whether ALS pathology starts in motor neurons of the cortex and brain stem/spinal cord (substantiating the “dying-forward” hypothesis) or in muscle cells (for the “dying-back” hypothesis) (Kiernan et al., 2011).

Different reasons for the domino-like degeneration of neuronal cells in such diseases have been proposed:

- Connected neurons normally inter-exchange molecules (including neurotransmitters), growth factors and anti-apoptotic signals for functionality, viability and support. Synaptic disruption leads to failure of this trophic support and “spreading” of degeneration along neuronal circuits. Loss of neurons may also dysregulate the pre- and post-synaptic transmission, triggering the degeneration of neighbor neurons (Mattson et al., 2006).

- Shared vulnerability of neuronal networks might be due to intrinsic cell autonomous factors (e.g. gene expression, neurotransmitters sensitivity, position in the circuit), that modulate a specific susceptibility (or resistance in no affected area) against the disease, although no clear markers have been identified so far (section 2.5.).

- Lastly and as discussed further below, protein aggregates associated with different NDs may spread between cells in a prion-like manner suggesting an intriguing explanation for this circuits-specific disease progression (this section).

Beside the observation of such anatomical patterns of neurodegeneration (Figure 6), experimental evidences for the “prion-like theory” were also initially provided by the injection of Aβ or tau or α-syn aggregates, obtained from the brain of AD or PD patients, into the brain of mice. This later caused the aggregation of the endogenous wild-type mouse protein in brain regions also really distant from the injection site (i.e. from one hemisphere to the contralateral one) (Claguavera et al., 2009; Eisele et al., 2010; Luk et al., 2012, Mougenot et al., 2012). Further, brain autopsies from PD patients, who received transplant of healthy embryonic neurons, showed that a subset of these engrafted cells displayed α-syn aggregates (Kordower et al., 2008a; Kordower et al., 2008 b; Li et al., 2008; Li et al., 2010). Similarly, Cicchetti and colleagues reported that neurons transplanted in HD patients showed disease-like degeneration (Cicchetti et al., 2009). Although they initially did not report HTT aggregates in these transplanted and degenerating neurons, they later re-examined the same cases using new techniques and they showed that the grafts do contain mutant HTT (Cicchetti
et al., 2014), providing evidence that also PolyQ HTT in HD shows prion-like properties similarly to α-syn in PD.

**In vitro** data have then shown that NDs-associated aggregates can be released from donor cells and enter in acceptor cells in which they are capable to initiate aggregation. “Initial” donors might be those neurons which are more vulnerable to protein aggregation (i.e. neurons in putamen and caudate nucleus in HD; neurons in hippocampus in AD), whereas acceptors might be neighbor cells (i.e. interneurons and glial cells) or neurons with synaptic contacts with the initial donor cells. In these *in vitro* experiments, two populations of cells are co-cultured using a specific culture system (i.e. “classic” co-culturing-, trans-well culturing- or conditioned media- experiments): one population (the donor cells) expresses the toxic protein usually tagged with a fluorescent protein (i.e. GFP) whereas the other co-cultured population (the acceptor cells) expresses the normal protein tagged with a different fluorescent protein (i.e. RFP). Transcellular spreading of the GFP-tagged aggregate species is evidenced by their appearance in the RFP fluorescent cells. Moreover, the presence of yellow fluorescent puncta suggests seeding and co-aggregation of the normal protein in the acceptor cells and ultimately the prionoids property of the ND-associate protein under study. In other experiments, prionoids are added to the cell medium, and their entering and seeding in the acceptor cells are verified after a certain time of incubation (Costanzo et al., 2013a).

Such studies have been first conducted using non-neuronal cells, like the human cell line HEK293 to investigate the prionoids properties of a certain aggregate; later the same studies have been repeated in neuronal cell lines from humans and rodents, finally demonstrating that α-syn, tau, SOD1, TDP-43, DPRs (from c9orf72 mutated gene), HTT and ATXN can enter in the cells in a prion-like manner (see table 1).

Five basic requirements have been identified to fulfill the criteria of a prion-like mechanism (Brundin et al., 2010; Costanzo et al., 2013a):

1. The protein aggregate must be capable of elongating by the recruitment of soluble polypeptide chains and of fragmenting to generate additional elongation sites and amplify aggregation.
2. The aggregates must be released from the donor cells. Several mechanisms have been proposed and are currently under investigation. These include: a) passive release of aggregates by membrane breakage during or after the donor cell degeneration; b) active release of aggregates via exocytosis, or as cargo in exosomes or as cellular material in exophers (Melentijevic et al., 2017).
3. The aggregates must be able to enter in the acceptor cells. Several mechanisms for the entering of NDs-aggregates have been proposed such as: a) passive uptake (i.e. via the disruption of the plasma membrane); b) active mechanisms of endocytosis and phagocytosis; c) movement of the aggregates via cell-to-cell membrane nanotubes. Importantly the internalized aggregate must be able to escape the endosomal system in order to interact with the endogenous protein and cause seeding and aggregation.
4. Acceptor cells, which are receiving the aggregates, must express the non-aggregated form for the “infectious” aggregate species to initiate the aggregate amplification process (aggregate *seeding*). Notably, the differential expression of the protein in different cells might partially
explain the specific vulnerability/resistance against aggregation: cells with lower expression of the protein might be more resistant to the seeding.

5. The aggregates must be resistant to degradation, especially during their cell-to-cell movement in the extracellular space (i.e. in the synaptic cleft).
Table 1: Pathogenic proteins in NDs are prionoids. In vitro and in vivo studies showing that NDs-associated aggregates in HD (HTT), AD (A-Beta, tau), PD (α-syn) and ALS (DPR proteins, TDP43 and SOD1) have prion-like characteristics.

<table>
<thead>
<tr>
<th>Neurodeg. disease</th>
<th>In vitro, in vivo studies and findings in patients</th>
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</table>
| Alzheimer’s (Aβ)  | **IN VITRO (cellular models):**  
|                   | • Nath S, et al. 2012 - Spreading of neurodegenerative pathology via neuron-to-neuron transmission of β-amyloid.  
|                   | • Sardar et al., 2018 - Alzheimer’s disease pathology propagation by exosomes containing toxic amyloid-beta oligomers.  
|                   | **IN VIVO:**  
|                   | • Meyer-Luehmann et al., 2006 - Exogenous induction of cerebral β-amyloidogenesis is governed by agent and host.  
|                   | • Eisele et al., 2010 - Peripherally applied Aβ-containing inoculates induce cerebral β-amyloidosis.  
|                   | • Harris et al., 2010 - Transsynaptic progression of amyloid-β-induced neuronal dysfunction within the entorhinal-hippocampal network.  
|                   | • Langer et al., 2011 - Soluble Aβ seeds are potent inducers of cerebral β-amyloid deposition.  
|                   | • Heilbronner et al., 2013 - Seeded strain-like transmission of β-amyloid morphotypes in APP transgenic mice.  
|                   | • Ruiz-Riquelme et al., 2018 - Prion-like propagation of β-amyloid aggregates in the absence of APP overexpression.  
|                   | • Ye et al., 2017 - Aβ seeding potency peaks in the early stages of cerebral β-amyloidosis.  
| Alzheimer’s (Tau) | **IN VITRO (cellular models):**  
|                   | • Frost et al., 2009 - Propagation of tau misfolding from the outside to the inside of a cell.  
|                   | • Kfoury et al., 2012 - Trans-cellular propagation of Tau aggregation by fibrillar species.  
|                   | • Michel et al., 2014 - Extracellular monomeric tau protein is sufficient to initiate the spread of tau protein pathology.  
|                   | • Woereman et al., 2016 - Tau prions from Alzheimer’s disease and chronic traumatic encephalopathy patients propagate in cultured cells.  
|                   | • Wu et al., 2016 - Neuronal activity enhances tau propagation and tau pathology in vivo.  
|                   | • Reilly et al., 2017 - Novel human neuronal tau model exhibiting neurofibrillary tangles and transcellular propagation.  
|                   | • Evans et al., 2018 - Extracellular Monomeric and Aggregated Tau Efficiently Enter Human Neurons through Overlapping but Distinct Pathways.  
|                   | **IN VIVO:**  
|                   | • Clavaguera et al., 2009 - Transmission and spreading of tauopathy in transgenic mouse brain.  
|                   | • de Calignon et al., 2012 - Propagation of tau pathology in a model of early Alzheimer’s disease.  
|                   | • Clavaguera et al., 2013 - Brain homogenates from human tauopathies induce tau inclusions in mouse brain.  
|                   | • Iba et al., 2013 - Synthetic tau fibrils mediate transmission of neurofibrillary tangles in a transgenic mouse model of Alzheimer’s-like tauopathy.  
|                   | • Dujardin et al., 2014 - Neuron-to-neuron wild-type Tau protein transfer through a trans-synaptic mechanism: relevance to sporadic tauopathies.  
|                   | • Holmes et al., 2014 - Proteopathic tau seeding predicts tauopathy in vivo. 2014.  
|                   | • Peeraer et al., 2015 - Intracerebral injection of preformed synthetic tau fibrils initiates widespread tauopathy and neuronal loss in the brains of tau transgenic mice.  
|                   | • Stancu et al., 2015 - Templated misfolding of Tau by prion-like seeding along neuronal connections impairs neuronal network function and associated behavioral outcomes in Tau transgenic mice.  
|                   | • Fá et al., 2016 - Extracellular Tau Oligomers Produce An Immediate Impairment of LTP and Memory.  
|                   | • Guo et al., 2016 - Unique pathological tau conformers from Alzheimer’s brains transmit tau pathology in nontransgenic mice.  
|                   | • Jackson et al., 2016 - Short Fibrils Constitute the Major Species of Seed-Competent Tau in the Brains of Mice Transgenic for Human P301S Tau.  
|                   | • Pickett et al., 2017 - Spread of tau down neural circuits precedes synapse and neuronal loss in the rTgTauEC mouse model of early Alzheimer’s disease. |
### Table 1: continued

<table>
<thead>
<tr>
<th>Neurodeg. disease</th>
<th>In vitro, in vivo studies and findings in patients</th>
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</table>
| **Parkinson’s (α-syn)** | **IN VITRO (cellular models):**  
  - Lee et al., 2005 - Intravesicular localization and exocytosis of α-synuclein and its aggregates.  
  - Desplats et al., 2009 - Inclusion formation and neuronal cell death through neuron-to-neuron transmission of α-synuclein.  
  - Nonaka et al., 2010 - Seeded aggregation and toxicity of α-synuclein and tau: cellular models of neurodegenerative diseases.  
  - Volpicelli-Daley et al., 2011 - Exogenous α-synuclein fibrils induce Lewy body pathology leading to synaptic dysfunction and neuron death.  
  - Freundt et al., 2012 - Neuron-to-neuron transmission of α-synuclein fibrils through axonal transport.  
  - Abounit et al., 2016 - Tunneling nanotubes spread fibrillar α-synuclein by intercellular trafficking of lysosomes.  
  - Domert et al., 2016 - Aggregated Alpha-Synuclein Transfer Efficiently between Cultured Human Neuron-Like Cells and Localize to Lysosomes.  
  **IN VIVO:**  
  - Luk et al., 2012a - Intracerebral inoculation of pathological α-synuclein initiates a rapidly progressive neurodegenerative α-synucleinopathy in mice.  
  - Luk et al., 2012b - Pathological α-synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice.  
  - Mougenot et al., 2012 - Prion-like acceleration of a synucleinopathy in a transgenic mouse model.  
  - Masuda-Suzukake et al., 2013 - Prion-like spreading of pathological α-synuclein in brain.  
  - Sacino et al., 2014 - Intramuscular injection of α-synuclein induces CNS α-synuclein pathology and a rapid-onset motor phenotype in transgenic mice.  
  - Bernis et al., 2015 - Prion-like propagation of human brain-derived alpha-synuclein in transgenic mice expressing human wild-type alpha-synuclein.  
  - Paumier et al., 2015 - Intrastriatal injection of pre-formed mouse α-synuclein fibrils into rats triggers α-synuclein pathology and bilateral nigrostriatal degeneration.  
  - Abdelmotilib et al., 2017 - α-Synuclein fibril-induced inclusion spread in rats and mice correlates with dopaminergic Neuredegeneration.  
  - Karampetsou et al., 2017 - Phosphorylated exogenous alpha-synuclein fibrils exacerbate pathology and induce neuronal dysfunction in mice.  
  - Shimozawa et al., 2017 - Propagation of pathological α-synuclein in marmoset brain.  
  - Thakur et al., 2017 - Modeling Parkinson’s disease pathology by combination of fibril seeds and α-synuclein overexpression in the rat brain.  
  **PATIENTS:**  
  - Li et al., 2008 - J. Y. et al. Lewy bodies in grafted neurons in subjects with Parkinson’s disease suggest host-to-graft disease propagation.  
  - Kordower et al., 2008b - Transplanted dopaminergic neurons develop PD pathologic changes: a second case report.  
  - Li et al., 2010 - Characterization of Lewy body pathology in 12- and 16-year old intrastriatal mesencephalic grafts surviving in a patient with Parkinson’s disease. |
<table>
<thead>
<tr>
<th>Neurodegenerative Disease</th>
<th>In vitro, in vivo studies and findings in patients</th>
</tr>
</thead>
</table>
| Huntington’s disease (Huntingtin) | **IN VITRO (cellular models):**
  - Ren et al., 2009 - Cytoplasmic penetration and persistent infection of mammalian cells by polyglutamine aggregates.
  - Herrera et al., 2011 - Visualization of cell-to-cell transmission of mutant huntingtin oligomers.
  - Costanzo et al., 2013b - Transfer of polyglutamine aggregates in neuronal cells occurs in tunneling nanotubes.
  - Pecho-Vriesling et al., 2014 - Transneuronal propagation of mutant huntingtin contributes to non-cell autonomous pathology in neurons.
  - Ruiz-Arlandis et al., 2016 - Binding, internalization and fate of Huntingtin Exon1 fibrillar assemblies in mitotic and non-mitotic neuroblastoma cells.

**IN VIVO:**
  - Babcock et al., 2015 - Transcellular spreading of huntingtin aggregates in the Drosophila brain.
  - Pearce et al., 2015 - Prion-like transmission of neuronal huntingtin aggregates to phagocytic glia in the Drosophila brain.
  - Tan et al., 2015 - Huntington’s disease cerebrospinal fluid seeds aggregation of mutant huntingtin.
  - Jeon et al., 2016 - Human-to-mouse prion-like propagation of mutant huntingtin protein.

**PATIENTS:**
  - Cicchetti et al., 2014 - Mutant huntingtin is present in neuronal grafts in Huntington disease patients.

Amyotrophic lateral sclerosis DPR-proteins (C9orf72) | **IN VITRO (cellular models):**
  - Westergard et al., 2016 - Cell-to-Cell Transmission of Dipeptide Repeat Proteins Linked to C9orf72-ALS/FTD.

Amyotrophic lateral sclerosis DPR-proteins (TDP-43) | **IN VITRO (cellular models):**
  - Nonaka et al., 2013 - Prion-like properties of pathological TDP-43 aggregates from diseased brains.
  - Feiler et al., 2015 - TDP-43 is intercellularly transmitted across axon terminals.
  - Smethurst et al., 2016 - In vitro prion-like behaviour of TDP-43 in ALS.
  - Pokrishevsky et al., 2016 - TDP-43 or FUS-induced misfolded human wild-type SOD1 can propagate intercellularly in a prion-like fashion.
  - Ishii et al., 2017 - Formation and spreading of TDP-43 aggregates in cultured neuronal and glial cells demonstrated by time-lapse imaging.

**IN VIVO:**
  - Porta et al., 2018 - Patient-derived frontotemporal lobar degeneration brain extracts induce formation and spreading of TDP-43 pathology in vivo.

Amyotrophic lateral sclerosis DPR-proteins (SOD-1) | **IN VITRO (cellular models):**
  - Munch et al., 2011 - Prion-like propagation of mutant superoxide dismutase-1 misfolding in neuronal cells.
  - Basso et al., 2013 - Mutant copper-zinc superoxide dismutase (SOD1) induces protein secretion pathway alterations and exosome release in astrocytes: implications for disease spreading and motor neuron pathology in amyotrophic lateral sclerosis.
  - Pokrishevsky et al., 2017 - Spinal cord homogenates from SOD1 familial amyotrophic lateral sclerosis induce SOD1 aggregation in living cells.

**IN VIVO:**
  - Ayers 2016 - Prion-like propagation of mutant SOD1 misfolding and motor neuron disease spread along neuroanatomical pathways.
Using the above-mentioned criteria, it can be concluded that the protein aggregates of HTT, Aβ, tau, α-syn, DPR-proteins (C9orf72), TDP-43 and SOD-1 have prion-like properties. However, except for α-syn, these proteins differ from the PrDs for one fundamental characteristic: infectability and transmissibility of the aggregates species between different individuals (i.e. for simple ingestion, blood transfusion and not through complex and artificial experiments *in vivo*) have not been fully demonstrated yet. Therefore, they cannot yet be referred to as prions and are further referred here as *prionoids* (as was proposed by Scheckel and Aguzzi, 2018).

In PD, evidence is accumulating that α-syn aggregates can actually be prions that can spread from the gastrointestinal tract to the brain. The initial idea was grounded by Braak and colleagues by observing that α-syn aggregates appeared in both the brain and the enteric nervous system (ENS) in post-mortem samples of PD patients (Braak et al., 2006). Experimental studies now indeed showed that direct injection of α-syn in ENS of animals can induce pathological features in the brain (including aggregation). The active transport of α-syn occurs through the vagal nerve. Other studies showed similar findings when animal’s gut received microbiota from PD donors (Holmqvist et al., 2014; Sampson et al., 2016; Uemura et al., 2018; Manfredsson et al., 2018). These data implies that α-syn, differently from other NDs-associated aggregates, show infectability and transmissibility properties similar to the prion protein, albeit cross-species transmissibility has not yet been shown to occur.

### 3. Glial cells and brain pathology

Several cell autonomous factors concur to determine neuronal vulnerability (section 2.5). However, our current understanding of brain pathology, which includes the evidence regarding the prion-like properties of NDs-associated aggregates (section 2.6), implies that also non-cell autonomous factors are important contributors of neuronal degeneration.

For a better understanding of NDs and how aggregates drive cell degeneration in NDs, it is therefore impelling to not exclusively focus on neurons and on the cell-autonomous activities of HSPs and PQC in these cells.

The human adult brain contains around 86 billion neurons, but also a similar number of non-neuronal cells that play a key role in the biology of the nervous tissue (Azevedo et al., 2009). Together with the vascular cells, which form the vessels for blood circulation, the main non-neuronal component of the brain is represented by glial cells. Neurons establish several non-cell autonomous interactions with glial cells and such interactions play a key role in the healthy and diseased brain. Our understanding of the nervous system evolved, throughout the years, from a “neuron-centric” view (that considered glia just as the “glue of the brain” with a passive role of structural support) to a more integrated picture, showing a complex network of electrically excitable neurons and electrically non-excitable glial cells with a high level of specialization and cooperativity. Whilst neurons fire action potentials through axons and are responsible for synaptic transmissions, glia have several mutual functional interactions with neurons, mainly aimed to maintain neuronal viability and functionality. Neurons die without glia: glial cells are therefore essential for the homeostasis of the brain (Barres et al., 2008).
Astrocytes, microglia and oligodendrocytes are the three main types of highly specialized glial cells in the mature human central nervous system (CNS) (Barres et al., 2008). In this Thesis, I will focus on astrocytes, the larger glial population in the human brain. An increasing body of evidence shows that astrocytes exert a number of fundamental functions in the healthy CNS, but also suggests that they also play a key role during pathogenesis in NDs (Sofroniew et al., 2010). Activation of astrocytes is a common hallmark of all NDs, including those associated with the presence of protein aggregates (Phatnani and Maniatis, 2015). This state of reactivity is called neuroinflammation and involves not only astrocytes, but also microglia which are CNS-resident macrophage-like cells, primarily deputed to remove cellular debris from the site of brain insult. It is believed that the initial damage-associated reactivity of astrocytes is aimed to positively counteract the disease progression. Nevertheless, a prolonged chronic state of neuroinflammation, which leads to the alteration or loss of the normal functions of astrocytes and gain of new detrimental activities, may rather contribute to the progression of neuronal degeneration (Sofroniew et al., 2010). This is notably observed in Alexander disease, a genetic neurodegenerative disorder that primarily affects astrocytes by a dominant gain-of-function mutation of the GFAP gene (Olabarria et al., 2017). Together, these observations suggest that the biology of NDs may not be exclusively ascribed to protein aggregation and PQC anti-aggregation activities in neurons (cell autonomous components of pathogenesis), but is also influenced by the positive and/or negative, molecular and functional interactions of neurons with the tissue environment and particularly with the other brain cells like astrocytes (non-cell autonomous components of pathogenesis). In the next sections, I will discuss the capacity of astrocytes to protect neurons against NDs-associated aggregates in a non-cell autonomous manner. In particular, I will focus on the observations that the molecular changes detected in reactive astrocytes during NDs include the up-regulation of certain HSPs. The functional implications of such HSPs up-regulation for the progression of neuronal degeneration have not yet been investigated. However, the peculiar role of astrocytes in the brain and the established key role of HSPs against protein aggregation and aggregate toxicity suggest that the HSPs astrocytic response in astrocytes may have a functional protective significance in these diseases.

3.1: The role of astrocytes in the healthy brain

For their homeostasis, neurons depend on astrocytes, with which they establish multiple functional interactions (Belanger et al., 2011). Astrocytes are the larger glial population in the brain and provide structural support, tiling the entire CNS. The most striking feature of astrocytes is represented by their many long processes, that give their typical star-shape and are required for contacting neurons in different cell regions including synapses (synaptic processes) (Sofroniew et al., 2010).

Like neurons, astrocytes can show a high level of heterogeneity in morphology and function. Based on their anatomical position in the brain tissue and on morphology, they are classified as protoplasmic, when found in the gray matter and showing finely branching processes, or fibrous when located in the white matter and showing long fiber-like processes (Cajal, 1909).

Interestingly, astrocytes are “territorial” cells: with its processes, a single astrocyte can contact many contiguous neurons and synapses, covering a “territory” (also defined as “domain”), with little
overlap between neighboring astrocytes (Nedergaard et al., 2003; Halassa et al., 2007b). These domains are functionally interconnected through connexins-gap junctions (Giaume et al., 2010), which permit to astrocytes to communicate through signaling waves of calcium ions and to exchange molecules each other. Such interconnection of several astrocytes forms a glial syncytium, a multicellular network that embraces the neuronal circuits, with which they have several interactions (Nedergard et al., 2003; Obhereim et al., 2006).

The functional interactions of astrocytes with neurons include the following:

1. **Function in the multi-partite synapse:**
   An example of the cellular specialization in CNS is the multi-partite synapse, a site of interplay between astrocytes with the pre- and post-synaptic neurons (Araque et al., 1999; Halassa et al., 2007a; Perea et al., 2009). Here, astrocytes exert essential functions by maintaining the fluid, ions, pH and transmitter homeostasis. Astrocytic processes are in fact rich in aquaporin water channels (e.g. AQP4) and transporters for ions (e.g. K⁺, Na⁺, H⁺ and bicarbonate) (Simard et al., 2004; Seifert et al., 2006; Obara et al., 2008) and neurotransmitters (e.g. glutamate, GABA and glycine) (Sattler et al., 2006; Seifert et al., 2006). The neurotransmitters released in the synaptic cleft by the pre-synaptic neuron can be taken up by astrocytes and are then enzymatically converted into precursors and recycled back to synapse for reconversion into active transmitters. A typical example of this neurotransmitter release and recycle between neurons and astrocytes is the glutamate-glutamine cycle (Bak et al., 2006; Mckenna et al., 2007). As also previously discussed, any imbalance in pH and levels of neurotransmitters and ions in the synaptic cleft are detrimental for neurons and may play an important role in many NDs (e.g. in the process of excitotoxicity). Therefore, the buffering capacity of astrocytes in the multi-partite synapse is crucial to maintain the neuronal fitness. This can impact on protein aggregation in the over-activated post-synaptic neurons because the resulting intracellular ionic imbalance (i.e. Ca²⁺) can trigger the unfolding of disease-associated proteins or cause their cleavage by proteases (section 2.4).

2. **Support to neuronal plasticity:**
   Astrocytes release different molecules into the extracellular space, some of which can activate neurons, such as glio-transmitters (e.g. glutamate, ATP, adenosine, GABA and D-serine) (Nedergaard et al., 2003; Volterra et al., 2005; Halassa et al., 2007a; Perea et al., 2009), growth factors (Banker et al., 1980; Sofroniew et al., 2010; Belanger et al., 2011), neuroactive steroids (e.g. estradiol, progesterone) (Garcia-Segura et al., 2006) and matrix-associated proteins (e.g. thrombospondins; Christopherson et al., 2005). The molecular exchanges with neurons are fundamental for the adaptive plasticity of CNS, implying a role of astrocytes in controlling dendritic and axonal arborization, synapses connections (synaptogenesis and synaptic pruning), receptors composition at synaptic cleft and neurotransmitters regulation (Sofroniew et al., 2010). Adaptive plasticity has an important role not only in developing CNS, but also during functional regeneration of neurons after a damage, therefore with implications also in NDs. As discussed before, NDs-aggregates in neurons may exert a toxic activity on synaptic functionality and structure (section 2.4), which could be eventually exacerbated by the loss of astrocytic functions.
3. Metabolic cooperation:
The CNS is high energy-consuming and neurons have a very high energy requirement, making them extremely dependent upon the supply of energy substrates from the blood circulation. Astrocytes strictly monitor the trafficking of energy metabolites (such as glucose and lactate), being in contact with both neurons and the blood vessels. Although neurons and astrocytes show a different metabolism, they are also highly cooperative (Belanger et al., 2011). Neurons have a high oxidative metabolism (Lebon et al., 2002; Itho et al., 2003; Boozier-Sore et al., 2006; Boumezbeur et al., 2010a) efficiently use lactate as primary source of energy substrate (Schurr et al., 1997; Bouzier et al., 2000; Qu et al., 2000; Itoh et al., 2003; Serres et al., 2005; Bouzier-Sore et al., 2006; Boumezbeur et al., 2010b) and are low glycolytic (Almeida et al., 2004; Herrero et al., 2009; Bolanos et al., 2010) (a specialization that protects them from oxidative stress). Differently, astrocytes have low oxidative metabolism, take up glucose, are high glycolytic and generate glycogen as energy reserve (Itho et al., 2003; Herrero et al., 2009; Bittner et al., 2010). Importantly, astrocytes transfer energy metabolites to neurons by responding to any increase of neuronal activity and energy demand. To sense this, astrocytes monitor the level of extracellular glutamate released by neurons and increase glycolysis and lactate production. The energy metabolites produced by astrocytes are then released into the extracellular space and taken up and used by neurons for ATP production (Belanger et al., 2011). Another important example of astrocytes-neurons cooperation concerns the cholesterol metabolism: brain cells are separated from the periphery due to the Blood-Brain-Barrier and they cannot receive the cholesterol from the blood. Therefore, astrocytes synthetize de novo the cholesterol pool in the brain and, similarly to what happens for energy metabolites, they transfer it to neurons, by which it is used for membranes generation and synthesis of derivative molecules (Priefeger et al., 2011). During NDs, metabolic processes for ATP production, that increase the production of ROS, might boost protein aggregation in neuronal and non-neuronal cells in the brain. Interestingly, it has been observed that in HD, mutant aggregate species of HTT are capable to reduce the cholesterol biosynthesis by interfering with the transcription regulatory pathway of SREBP (sterol responsive element-binding protein). This might have detrimental effects on the astrocytic capability to de novo produce the cholesterol pool in the brain, for being used by neurons to build up membranes and other important functions (Valenza et al., 2011).

4. Neuronal protection from oxidative damage:
ROS, generated with the cellular oxidative metabolism, can stress and damage neurons. As previously discussed, ROS can also directly cause covalent modifications in proteins, an important trigger in the process of aggregation (section 2.4). Astrocytes release anti-oxidant molecules and ROS-scavengers (e.g. glutathione) to neurons, providing a non-cell autonomous protection from oxidative damage (Chen et al., 2001; Shih et al., 2003). As explained before, a loss of astrocytic function in producing and releasing such scavengers might promote the accumulation on ROS and consequently protein aggregation in neurons (section 2.4).

5. Formation of the Blood-Brain-Barrier (BBB):
The vascular endfeet of astrocytes cover all the vascular surfaces in the brain. Together with the basal lamina, the capillary endothelial cells, and the perivascular pericytes, astrocytes form the BBB, an interface that selects and controls the movement of molecules and cells between blood and the CNS extracellular space, providing an essential protection to neurons, also from all those factors.
that can trigger protein aggregation. Through these contacts, astrocytes release molecular mediators (e.g. prostaglandins, nitric oxide and arachidonic acid) that control the CNS blood vessel diameter and blood flow. In response to increased neuronal activity, astrocytes can also control and increase the delivery of oxygen and nutrients from blood to the active brain region. These several functions of BBB might also have a great impact in NDs pathogenesis: a significant role of structural and functional changes of BBB observed in NDs suggests that BBB alteration might contribute to the aetiology of these disorders. However, as similar BBB changes are observed in many different NDs, it might be likely that the barrier dysfunctions are consequence of the neurodegeneration. BBB changes are an important component of neuroinflammation and they can alter BBB integrity, transport and regulatory functions (e.g. as signaling interface), enhancing the entering of immune cells from the periphery into the nervous tissue (Carvey et al., 2009).

Below, I will next describe whether or not neuroinflammation is detrimental or beneficial in NDs, in particular focusing on the role of astrocytes.

### 3.2: The role of astrocytes in the brain during disease and implications in NDs

Neuroinflammation used to be considered as an unregulated glial response to brain diseases that might cause neurotoxicity and exacerbate the pathological conditions. This had led to the idea that inhibition of neuroinflammation might be used as a therapeutic strategy against NDs. More recently, however, neuroinflammation is no longer considered an all-or-none uncontrolled response, but a fine-tuned regulated continuum of progressive changes in glial cells. These changes may be initially positive for neurons and aim to counteract the disease process, being part of the normal protective functions of glia. Nevertheless, a prolonged chronic state of neuroinflammation, which might occur in NDs, changes glial activities through alteration or loss of their normal functions and gain of detrimental functions which may finally contribute to degenerate neurons in a non-cell autonomous way (Sofroniew et al., 2009; Sofroniew et al., 2010).

Together with protein aggregation, neuroinflammation represents another common hallmark of NDs. In this state, reactive astrocytes and microglia, the major glial mediators of the immune response in CNS, respond to the pathological condition and to the tissue damage through a spectrum of molecular, functional and cellular changes during the disease progression, which mainly aim to restore the homeostasis of the tissue (Ben Haim et al., 2015; Heneka et al., 2014). Astrocytes, due to their several functional interactions with neurons during health and disease, have a fundamental role during neuroinflammation and in the pathogenesis of NDs associated with protein aggregates. In such context, two open questions are currently under investigation: 1 to) understand how astrocytes react to the degeneration of neurons and other brain cells during the disease; 2) to establish how the cellular and functional changes that characterize the astrocytic reactivity can influence the neuronal fitness during the pathology in a non-cell autonomous manner.
In NDs, reactive astrocytes are generally found in those CNS regions that are more vulnerable to the respective protein aggregation and neurodegeneration:

- In HD brains, reactive astrocytes are primarily observed in caudate and putamen during the early stage and later also in the motor cortex, globus pallidus, thalamus and hippocampus (Vonsattel et al., 1985; Faideau et al., 2010).
- In PD brains, they are found in substantia nigra (Forno et al., 1992; Damier et al., 1993).
- In AD patients, reactive astrocytes appear before clinical symptoms and are initially found in the hippocampus and entorhinal cortex and later in the temporal, frontal and parietal lobes. Notably, they are also found around the extracellular plaques of β-amyloid, although not in all cases (Simpson et al., 2010; Carter et al., 2012).
- Also in ALS, reactive astrocytes are observed in the vulnerable regions and notably before the manifestation of motor symptoms (Maragakis et al., 2006; Philips et al., 2011).

Importantly, the reactive astrocytes may already appear in the early pre-symptomatic stage of the disease (Halm et al., 2015), supporting the idea that the initial astrocytic response might be a protective process to counteract the progression of neurodegeneration.

In NDs, reactive astrocytes are hypertrophic, they overexpress GFAP, and reorganize and polarize their processes toward the site of neurodegeneration (Soﬁroniew et al., 2009; Ben Haim et al., 2015). Astrogliosis - the increased proliferation of astrocytes - is a component of neuroinflammation. Data from disease rodent models indicate that the magnitude of astrogliosis is very limited during the early phase of NDs, compared to the reaction observed after acute brain injury (Khamphuis et al., 2012; Sirko et al., 2013). This again supports the idea that the astrocytic reactivity is not always characterized by an intense cell division that ultimately aims to merely form a glial scar, but rather is a regulated set of progressive and functional changes that strictly depend by the type and extent of brain damage.

During neuroinflammation, astrocytes proceed through different levels of reactivity. In the healthy brain, astrocytes show non-overlapping domains and do not express high levels of GFAP. During a mild-moderate neuroinflammation, which may characterize the early phase of any brain disease, the structural organization of astrocytes is maintained and they incur molecular changes and hypertrophy that are potentially reversible if the origin of the damaged is resolved. Instead, if the insult continues and degeneration of neurons persists, such as in chronic and progressive brain diseases (e.g. NDs), the cellular and functional changes in astrocytes progress alongside with the brain damage and their structural domain-based organization is disrupted. The process may end with the formation of glial scars along the border of the damage site: this structure is mainly formed by astrocytes and other glial cells with high deposition of dense collagenous extracellular matrix. The state of glial scars is considered an irreversible condition of astrocytes and it is thought to primarily serve as a barrier between the healthy tissue and the area of damage and inflammation (Soﬁroniew et al., 2010).

Perturbations in the homeostasis of the brain are sensed by astrocytes through plasma membrane-receptors, which are responsive to molecules released by other cells in the damaged tissue. Such
signals may be released by all the cells involved in the pathological process, including affected neurons and the glia in the site of damage and degeneration. Astrocytes, together with microglia, are the main mediators of the immune response, and once activated they can signal to other cells, propagating the neuroinflammation state throughout the brain (Buffo et al., 2010; Burda et al., 2014; Kigerl et al., 2014). The spectrum of released molecules can vary, depending on the origin of the damage (e.g. NDs, infections, trauma, stroke) and can differ in case of acute injury (such as in a stroke) or chronic slow degeneration of cells (such as in NDs). Although the exact molecular triggers of astrocyte reactivity for each specific NDs are still under investigation, several of them have been identified. The spectrum of signals, which characterizes the neuroinflammation during NDs includes polypeptide growth factors (e.g. CNTF, BDNF, GDNF, NT3, EGF, bFGF, IGF1) (Nieto-Sampedro et al., 1990; Schar et al., 1993; Schwartz et al., 1994; Kahn et al., 1997; Hinks et al., 1999; Messermsmith et al., 2000; Smith et al., 2001; Chen et al., 2006b; Escartin et al., 2007) cytokines (e.g. interleukins, TNFα, INF-γ, TGFβ) (Yong et al., 1991; Chiang et al., 1994; Korder et al., 1996; Giulian et al., 1998; Campbell et al., 2001; Swartz et al., 2001; Lin et al., 2006), neurotransmitters (e.g. glutamate) (Bekar et al., 2008), ROS, nitric oxide (Swanson et al., 2004) and other molecules associated with unpaired metabolism (e.g. NH4+) (Norenberg et al., 2009).

Receptors for these signals are present on the plasma membrane of astrocytes and, once activated, they trigger intracellular signaling cascades (e.g. JAK/STAT-, NF-κB-, calcineurin- and MAPK-pathways) that initiate those molecular changes that lead to the cellular and functional changes in astrocytes during neuroinflammation (Buffo et al., 2010; Ben Haim et al., 2015). As previously said, astrocytes monitor each synapse in the brain, and it is estimated that the processes of one astrocyte can contact over 100,000 synapses (Araque et al., 1999; Halassa et al., 2007a; Perea et al., 2009). Astrocytes are therefore well positioned at the tripartite synapses to rapidly and efficiently detect, through these receptors, any abnormal neuronal activity (e.g. release of ROS and NH4+), during the pathological processes associated with NDs. The presence of such signals in the extracellular space may justify the presence of reactive astrocytes also in brain regions which are relatively distant from the site of neuronal degeneration.

In the early phase of NDs, the activity of reactive astrocytes may be beneficial to and protective for neurons (Silver et al., 2004). In addition to the previously mentioned functions, reactive astrocytes exert several new important activities that support neuronal viability and counteract the disease. Important examples of these are:

- The release of neuroprotective molecules such as cytokines and growth factors (e.g. CNTF, BDNF, NGF and FGF) (Nieto-Sampedro et al., 1990; Schar et al., 1993; Schwartz et al., 1994; Kahn et al., 1997; Hinks et al., 1999; Messermsmith et al., 2000; Smith et al., 2001; Chen et al., 2006b; Escartin et al., 2007; Vargas et al., 2008).
- The release of anti-oxidant molecules that block ROS toxicity (e.g. glutathione, ascorbic acid) (Chen et al., 2001; Shih et al., 2003).
- The re-uptake of excitatory neurotransmitters, like glutamate, from the synaptic cleft (therefore counteracting excitotoxicity) (Rothstein et al., 1996; Bush et al., 1999). The repair and maintenance of the BBB (Carvey et al., 2009).
• The restriction of inflammatory cells at the site of damage (via the formation of the glial scar) (Bush et al., 1999; Faulkner et al., 2004; Myer et al., 2006; Okada et al., 2006; Herrmann et al., 2008; Voskul et al., 2009).

It is important to note that the commonality of the changes in reactive astrocytes (i.e. release of the same neuroprotective molecules) that is observed among different NDs (i.e. different types of neurons, different protein aggregates, different aggregate location), strongly supports the hypothesis that such changes are a consequence and not a cause of the disease. Protein aggregation primarily in neurons and neuronal damage trigger astrocytic reactivity and neuroinflammation in the site of damage.

Due to the chronic nature of NDs and the progressive accumulation of the neuronal damage over time, (reactive) astrocytes may lose their normal functions of support and protection toward neurons (see previous section) and acquire new toxic and detrimental functions (Sofroniew et al., 2010; Ben Haim et al., 2015). In the final step, astrocytes may even die, further aggravating the pathology. In addition to the loss of their normal functions, chronic reactive astrocytes may produce and release abnormal cytotoxic levels of inflammation-associated signals (Brambilla et al., 2005; Brambilla et al., 2009), ROS (Hamby et al., 2006) and other toxic metabolites like glutamate (Takano et al., 2005) that can exacerbate the disease condition. Moreover, although fundamental to isolate the site of damage, the formation of the glial scar in the late phase is considered detrimental for brain regeneration, because it also inhibits the axon regeneration and the formation of new synaptic connections (Silver et al., 2004).

The key role of protein aggregates in NDs and the presence of reactive astrocytes in the brain of the patients suggest that aggregates might trigger astrocyte reactivity, as an important component of ND pathology. But inversely, does this reaction also serves to handle aggregates? This question is even more relevant in the context of the prion-like behavior of many aggregating ND proteins. So, what do we know about the presence of aggregates in astrocytes? And what is their sensitivity to aggregates. How do they handle them and may this handling play a role in the ethology of the disease? These questions are addressed in the next sections.

### 3.3: Astrocytes and NDs-associated aggregates

Astrocytes with intracellular aggregates are indeed observed in most NDs, mainly in the affected brain regions where protein aggregation in neurons also occurs. However, the frequency of astrocytes with aggregates is much lower compared to the frequency of neurons with intracellular aggregates (considering the same affected brain region) (Miller et al., 2004; Maragakis et al., 2006; Jansen et al., 2014; Phatnani et al., 2015). This is also observed in PolyQ diseases, including HD (Jansen et al., 2017).

The reason for this is unknown, but one could speculate the following one or more options:
- Astrocytes are mitotic cells and, differently from neurons, they can be regenerated in the adult brain; during division and differentiation, cells might then be capable of asymmetric partitioning of
aggregates, an important protective survival strategy conserved from bacteria to yeast and dividing (stem) cells in metazoan (section 2.1).

- Astrocytes are resistant to intrinsic triggers for cell autonomous aggregation. They are shown to have a low oxidative metabolism (Belanger et al., 2011) and higher levels of endogenous molecules (such as ROS-scavengers) that might protect them from protein damage (Sofroniew et al., 2010). Moreover, they might have better PQC to prevent or handle/degrade the aggregates (sections 3.3.1 and 3.4).

- Astrocytes do not take up aggregates and thus do not take part in the prion-like processes; inversely they do take up the prion-like aggregates, but have better capacity to handle them compared to neurons (see 3.3.2 and 3.4).

### 3.3.1 Aggregates intracellularly formed in astrocytes

Several aggregation-prone proteins responsible for different NDs are ubiquitously expressed in all the cells of the body. For example, the \( HTT \) human gene is regulated by a promoter with a widespread constitutive expression with little regulation. \( HTT \) is therefore ubiquitously expressed in neuronal and non-neuronal cells (Zuccato et al., 2010).

Due to the ubiquitous expression of toxic proteins in the brain of the patients, the specific effects of ND-associated protein aggregation in astrocytes cannot be easily discriminated and characterized through the post-mortem analysis of these samples. Such characterization is also not possible using \( in \) \( vivo \) disease models with ubiquitous promoters.

Only by using \( in \) \( vivo \) models in which the toxic protein is exclusively expressed in astrocytes, it is possible to evaluate its impact on the overall homeostasis of the brain. The studies summarized in Table 2 used the promoter of the \( GFAP \) gene for the selective astrocytic expression of transgenes in rodent models. The expression of different NDs-associated proteins in astrocytes was found to cause formation of intracellular aggregates, alteration of their neuronal-supporting functions (e.g. activity of glutamate EAAT transporters, BBB integrity), triggered astrocytic reactivity and induced neuroinflammation. Interestingly, another important and common finding in these models is the degeneration of neurons close to the site of reactive, aggregates-containing astrocytes. Phenotypically, animals show age-dependent neurological symptoms (e.g. motor impairments) and reduced lifespan, meaning that protein aggregation is detrimental for astrocyte fitness and/or functionality and can trigger their reactivity (cell-autonomously). The affected astrocytes next can lead to neurodegeneration, in a non-cell autonomous manner (Forman et al., 2005; Dabir et al., 2006; Bradford et al., 2009; Bradford et al., 2010; Gu et al., 2010; Tong et al., 2013).
Table 2: Expression of pathogenic proteins in astrocytes leads to protein aggregation and neurodegeneration in in vivo models of NDs. Studies are summarized that illustrate the phenotype of rodent models expressing different NDs-associated proteins exclusively in astrocytes. Type of toxic proteins, transgene sequence, promoter for expression and main findings are reported.

<table>
<thead>
<tr>
<th>Toxic Protein</th>
<th>Construct</th>
<th>Promoter</th>
<th>Method</th>
<th>Phenotype</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTT</td>
<td>N-terminal human HTT (1–208 amino acids) with 160Q</td>
<td>human GFAP promoter</td>
<td>Overexpression in Astrocytes</td>
<td>Reactive astrocytes (increased GFAP) and neuroinflammation, altered astrocytic functionality, PolyQ HTT aggregates in astrocytes, no clear sign of cellular degeneration, age-dependent neurological symptoms and reduced lifespan.</td>
<td>Bradford J et al., 2009.</td>
</tr>
<tr>
<td>HTT</td>
<td>N-terminal human HTT (1–171 amino acids) with 82Q</td>
<td>human GFAP promoter</td>
<td>Overexpression in Astrocytes</td>
<td></td>
<td>Bradford J et al., 2010.</td>
</tr>
<tr>
<td>α-syn</td>
<td>mutant human A53T α-syn</td>
<td>human GFAP promoter</td>
<td>Overexpression in Astrocytes</td>
<td>Reactive astrocytes (increased GFAP) and neuroinflammation, altered astrocytic functionality, A53T α-syn aggregates in brain, neuronal degeneration, age-dependent neurological symptoms and reduced lifespan.</td>
<td>Gu et al., 2010.</td>
</tr>
<tr>
<td>Tau</td>
<td>human Tau</td>
<td>human GFAP promoter</td>
<td>Overexpression in Astrocytes</td>
<td>Reactive astrocytes (increased GFAP) and neuroinflammation (with BBB disruption), altered astrocytic functionality, Tau aggregates in astrocytes, neuronal degeneration</td>
<td>Forman et al., 2005.</td>
</tr>
<tr>
<td>Tau</td>
<td>human Tau, mutation P301L (FTDP-17)</td>
<td>human GFAP promoter</td>
<td>Overexpression in Astrocytes</td>
<td>Reactive astrocytes (increased GFAP) and neuroinflammation, altered astrocytic functionality, Tau aggregates in astrocytes, motor impairments.</td>
<td>Dabir et al., 2006.</td>
</tr>
<tr>
<td>TDP43</td>
<td>Human TDP43, mutation M337V</td>
<td>human GFAP promoter</td>
<td>Overexpression in Astrocytes</td>
<td>Reactive astrocytes (increased GFAP), altered astrocytic functionality, ubiquitin inclusions in astrocytes, neuronal degeneration, motor impairments.</td>
<td>Tong et al., 2013.</td>
</tr>
</tbody>
</table>

All together, these studies show that astrocytes are intrinsically sensitive to aggregates and that this may lead to their functional impairment. These can subsequently lead to signs of neurodegeneration. Nonetheless, it is important to emphasize that these data do not sustain the idea that neurodegeneration is exclusively due to protein aggregation in astrocytes, as the selective expression of the same disease-causing proteins in neurons (and not in astrocytes) also leads to a phenotype characterized by protein aggregation and neurodegeneration.

3.3.2 Prionoids spreading into astrocytes

As mentioned above (section 2.6), NDs-associated aggregates in HD (HTT), AD (A-Beta, tau), PD (α-syn) and ALS (DPR proteins, TDP43 and SOD1) have prion-like characteristics. Besides neuron-to-neuron transmission, spreading may also involve neuron-to-astrocyte transmission. In fact, one
could hypothesize that a fraction of NDs-associated aggregates observed in astrocytes are not originated in situ, but they might be prionoids that have entered into the astrocytes in a prion-like manner. Even more so, by taking up aggregates, astrocytes may counteract spreading to neurons and hence could slow down the process of degeneration. Below, I summarize some speculations in support of such scenarios.

First, as much as any other cell types, astrocytes may be “vulnerable” to the entry of prionoids via passive uptake (i.e. by disruption of the plasma membrane), because this is an intrinsic property of the aggregates and does not seem to depend by the type of acceptor cells (Butterfield et al., 2010).

Second, it has been shown that the active entry of prionoids is mediated by mechanisms which are also observed in astrocytes: notable examples are receptor-mediated endocytosis or the transport via tunneling nanotubes (Davis et al., 2008 and Discussion of this Thesis).

Whereas most in vitro studies have used various human and rodent (neuronal) cell lines to investigate transmission of prionoids (table 1), some have done the same for astrocytes. Accumulating evidence comes from in vitro studies on PD that have shown that α-syn aggregate species released from neuronal cells can enter in astrocytes (via either endocytosis or the formation of tunneling nanotubes) (Lee et al., 2010; Braidy et al., 2013; Lindstrom et al., 2017; Rostami et al., 2017). Data in vitro (Danzer et al., 2007; Hansen et al., 2011) and in vivo (Angot et al., 2012) have shown that these α-syn prionoids have seeding properties in several types of recipient cells (although this still needs to be confirmed to happen also in astrocytes).

Similar data have been suggested for aggregates of dipeptide repeat proteins linked to the C9orf72 (Westergard et al., 2016).

Alongside with these in vitro studies, investigations conducted in rodent and insect models permitted to further substantiate the possibility of in vivo spreading of prionoids into astrocytes. Luk and colleagues (Luk et al., 2012) have shown that a single injection of synthetic α-syn fibrils in the brain of α-syn transgenic mouse can initiate cell-to-cell transmission and dramatically accelerate both the formation of PD-associated intracellular aggregates and the onset of disease; interestingly, they also found evidences that astrocytes -- among other cells in the injected mouse brain -- were acceptor for these aggregates.

In another study, exosomes containing α-syn, isolated from the frontal cortex of patients with Lewy bodies dementia and injected in the hippocampus of wild-type mice, caused α-syn aggregate formation localized in both neurons and astrocytes (Ngolab et al., 2017).

Similarly, the intracerebral inoculation in mice with brain homogenates from AD patients induced tau-like pathology, tau aggregation and spreading with astrocytes being acceptor cells for the tau protein (Clavaguera et al., 2013; Boluda et al., 2015).
Another approach to study in vivo the spreading of aggregates is the expression of the transgenic toxic protein in a specific population of brain cells (donors) and verify if such protein can be later found in another cell type (acceptors). Using such an approach, Pearce and colleagues have shown that HTT expressed in D. melanogaster neurons can spread to glial cells. They showed that glia requires the scavenger receptor draper (homolog of the human gene MEGF11) and uses a phagocytic engulfment machinery to accept the HTT prionoids (Pearce et al., 2015).

Similarly, de Calignon and colleagues observed that mice expressing mutant tau in a specific population of neurons of the entorhinal cortex found tau aggregates to appear in astrocytes at 24 months of age but not at earlier time points, indicating that tau is likely released from neurons and taken up by astrocytes, as the axons degenerate (de Calignon et al., 2012).

Inversely, some studies have shown that prionoids can be released from astrocytes and enter neurons. For example, astrocytes generated from ALS patients can release exosomes containing mutant SOD-1 that are toxic to co-cultured neurons (Haidet-Phillips et al., 2011; Basso et al., 2013). Similarly, stem cells-derived (rat) astrocytes overexpressing TDP43 can release TDP43 prionoids, which subsequently can be internalized in co-cultured neuronal cells (Ishii et al., 2017).

These in vitro and in vivo studies highlight that astrocytes could play different roles in the biology of ND as possible acceptor cells of prionoids, hereby functioning as a matrix-barrier in slowing down the spreading of toxic species to neurons. However, astrocytes may have a limited capability to do so, e.g. due to the intrinsic toxicity of the accumulated aggregates. Hereby, their loss may contribute to the disease, either by loss of their neuronal support functions and/or by now becoming donor cells of the prionoids. In such perspective, boosting the capacity of astrocytes to take up and sustain their viability by properly handling the toxic prionoids might have a therapeutical value.

In the next section, I will summarize the existing knowledge on how astrocytes react to the presence of the aggregates and which factors might make them more resistant to such aggregates.

### 3.4: Astrocyte response to NDs-associated aggregates: intrinsic resistance, protein quality control and expression of HSPs

The less frequent presence of aggregates in astrocytes compared to neurons (as described in section 3.3) may be due to differences in protein quality control, and expression/activity of HSPs. In the next sections I will explore such differences, highlighting some key aspects that may be crucial in the process of neurodegeneration.

#### 3.4.1- Differences between neurons and astrocytes in the UPS

Tydlacka and colleagues reported that UPS is less active in neurons in comparison to white matter glia, suggesting that the glial resistance to protein aggregation might be due to a higher activity of the UPS in sustaining protein degradation and turn-over (Tydlaka et al., 2008; Jansen et al., 2014). Interestingly, it has been observed that during ND-associated neuroinflammation, the immunoproteasome is induced in glia (particularly in astrocytes and microglia) and neurons. In fact, the immunoproteasome induction is a common hallmark in AD (tau) (Mishto et al., 2006; Orre et
al., 2013) and ALS (SOD-1) (Puttahaparty et al., 2007; Cheroni et al., 2009). Recent data have shown the same in PD (α-syn) (Ugras et al., 2018). In HD (HTT), the induction of the immunoproteasome is observed in neurons, but no data have shown the same in glia so far (Díaz-Hernández et al., 2003).

The expression of the immuno-subunits is induced by factors released by the cells during neuroinflammation (e.g. IFNγ). In the immunoproteasome, certain proteasomal subunits are substituted by specific immuno-subunits that change the cleavage capability of the proteasome (Jansen et al., 2014) Whether such substitutions improve the proteasomal activity in degrading ND-associated toxic proteins and, therefore, in counteracting aggregation is yet unclear. However, some recent data support such assumption: Ugras and colleagues (Ugras et al., 2018) have shown that immunoproteasome is capable of degrading α-syn fibrils in vitro in cell-free conditions. Next, they confirmed that α-syn fibrils are capable to enter in cells and induce a neuroinflammation response, as previously shown (Lee et al., 2010). Finally they showed that the inhibition of the immunoproteasome increases intracellular accumulation of the aggregates, suggesting a neuroprotective role of the immunoproteasome in response to α-syn aggregation.

3.4.2- Heat shock response in neurons and astrocytes and HSPs upregulation in astrocytes during disease

As previously explained (section 2.1), HSPs can be constitutively expressed or stress-induced.

In different tissues and in cells of the same tissue, the HSR can vary in speed of transcript generation and fold increase in HSPs levels (Sala et al., 2017). Importantly, different cells within the same tissue can have different capacities to induce the HSR. Interestingly, such difference has been also observed for cells in the brain (San Gil et al., 2017).

In one of the first in vitro studies where cultured cortical neurons and astrocytes were compared for induction of mRNA and protein levels of HSPA/HSP70 after heat shock, astrocytes showed a faster and stronger response than neurons (Nishimura et al., 1991).

In line, several in vivo studies (Manzerra et al., 1992; Nishimura et al., 1996; Manzerra et al., 1997; Krueger et al., 1999; Pavlik et al., 2007; Oza et al., 2007; Yang et al., 2008) have shown that in rodent models treated with hyperthermia or other stress conditions (i.e. induced ischemia), neurons do not induce HSPA/HSP70 expression after exposure to stress conditions, whereas surrounding astrocytes do.

These studies indicate that neurons under different conditions of stress have lower intrinsic capacities to mount the HSR as compared to astrocytes. As the HSR contributes to maintain protein homeostasis, this difference might be one possible factor (but not the unique) that explains why neurons are more affected by protein aggregation in neurodegenerative diseases and why astrocytes are more capable to cope protein aggregation.

However, several studies in vitro and in vivo revealed that several pathogenic proteins (i.e. PolyQ-HTT and SCA3, SOD-1 and TDP43) do not activate HSF-1, at least not before a massive aggregation has already occurred (Tagawa et al., 2007; Chafekar et al., 2012; Bersuker et al., 2013; Seidel et al.,
2016; San Gil et al., 2017), meaning that the capacity to activate the HSR may not be a prime factor responsible for the differential sensitivity of astrocytes and neurons to the initiation of aggregation of the ND-associated proteins.

In fact, HSPs-overexpression screens from our lab have indicated that not always the HSR-regulated HSPs are the best suppressors of aggregation, in particular not for PolyQ proteins (Kakkar et al., 2014). Importantly, it has been established that the human chaperonome consists of many other HSPs that are not regulated (or only marginally) by the HSR and HSF-1, but instead by “non-canonical” regulatory pathways that take place in chronic stress conditions (such as during NDs) (Hageman et al., 2009; Kakkar et al., 2014; Mahat et al., 2016; Solis et al., 2016; Neueder et al., 2017).

Some of these non-canonical and less investigated HSPs show protection in in vivo models of protein aggregation diseases. The protective activity of these HSPs differs between different disease-associated proteins, suggesting that each type of aggregate is biochemically distinct and requires specific HSPs to prevent its formation or target it for degradation. This also suggests that no generic explanation can be given in terms of HSP expression that would explain the relative resistance of astrocytes to aggregation.
### Table 3: section A (HSPAs, DNAJs, HSPCs and chaperonins) and section B (small HSPs): HSPs expression in astrocytes in the brain of NDs patients or in vivo animal disease model. Data are grouped per HSPs families and per each HSP member. Disease, type of analyzed sample, method of analysis, and main findings are reported. (Abbreviations: MSA: multiple system atrophy; α-synP: α-Synucleinopathy; tauP: sporadic and familiar tauopathies; FTLD: Frontotemporal lobar degeneration; IHC= Immunohistochemistry; MA=microarray analysis; RT qPCR= Real Time qPCR; ISH= In situ hybridization).

<table>
<thead>
<tr>
<th>HSP</th>
<th>NDs</th>
<th>Samples and Meth.</th>
<th>HSPs in astrocytes: main findings</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HSPA (HSP70)</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>HSPA1A (HSP70-1; HSP72; HSPA1)</td>
<td>MSA, α-SynP (α-Syn)</td>
<td>In vivo, human IHC</td>
<td>Increased HSPA1A/HSP70 in MSA brain. Many reactive astrocytes in the area of neurodegeneration were positive for HSPA1A/HSP70. Co-localization of HSPA1A/HSP70 with glial cytoplasmic inclusions.</td>
<td>Kawamoto et al., 2007</td>
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<td>HSPA9 (GRP75; HSPA9B; MOT; MOT2; PBP74; mot-2; mortalin)</td>
<td>PD</td>
<td>In vivo, human IHC</td>
<td>HSPA9/mortalin, normally expressed in astrocytes, was reduced in the astrocytes of PD patients located in affected brain regions.</td>
<td>Cook et al., 2016</td>
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<td><strong>DNAJ (HSP40)</strong></td>
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<tr>
<td>DNAJB6 (Mrj; mDj4)</td>
<td>PD</td>
<td>In vivo, human RT-qPCR IHC ISH IHC</td>
<td>DNAJB6 is a component of Lewy bodies in both PD substantia nigra and PD cortex. DNAJB6 is strongly up-regulated in parkinsonian astrocytes, and located especially at the astrocytic endfeet.</td>
<td>Durrenberg et al., 2009</td>
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<td>Michael et al., 2011</td>
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<td><strong>HSPCs (HSP90)</strong></td>
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<td>HSPC1 (HSP90AA1; HSPN; LAP2; HSP86; HSPC1; HSPCA; HSP89; HSP90A; HSP90N; HSPCAL1; HSPCAL4; FLJ31884)</td>
<td>MSA (PD-like α-synP)</td>
<td>In vivo, human IHC</td>
<td>HSPC1/HSP90 is expressed in glial cells (including astrocytes) and co-localizes with glial α-synuclein inclusions</td>
<td>Chiba et al., 2012</td>
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<td></td>
<td>PD (and other α-synP)</td>
<td>In vivo, human IHC</td>
<td>HSPC1/HSP90 is expressed in glial cells (including astrocytes) and often co-localizes with glial α-synuclein inclusions • See also for HSPB5 same study</td>
<td>Uryu et al., 2006</td>
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<td><strong>Chaperonins</strong></td>
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<td>HSPD1 (HSP60; GroEL)</td>
<td>PD (and other α-synP)</td>
<td>In vivo, human IHC</td>
<td>HSPD1/HSP60 is expressed in glial cells (including astrocytes), but rarely co-localizes with glial α-synuclein inclusions</td>
<td>Uryu et al., 2006</td>
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<tr>
<td></td>
<td>PD AD</td>
<td>In vivo, human IHC</td>
<td>Astrocytes in all brain areas showed expression of HSPD1/HSP60, which is increased when the cells are reactive.</td>
<td>Martin et al., 1993</td>
</tr>
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### Table 3: continued

<table>
<thead>
<tr>
<th>HSP</th>
<th>NDs</th>
<th>Samples and Meth.</th>
<th>HSPs in astrocytes: main findings</th>
<th>Ref.</th>
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<tr>
<td><strong>HSPB (Small Heath Shock Proteins)</strong></td>
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<tr>
<td>HSPB1 (MT2F; HMN2B; HSP27; HSP28; HSP25; HS.76067; DKFZp586P1322)</td>
<td>AD</td>
<td>In vivo, human IHC</td>
<td>In AD brains, reactive astrocytes were present at the site of neuronal degeneration and amyloid aggregates formation. Reactive astrocytes showed increased expression of HSPB1/HSP27. HSPB1/HSP27 did not co-localize with A-Beta aggregates. ● See also for HSPB2, HSPB5, HSPB6 and HSPB8 (same study).</td>
<td>Bruinsma et al., 2011</td>
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<tr>
<td></td>
<td>AD</td>
<td>In vivo, human IHC</td>
<td>In control brains, HSPB1/HSP27 was occasionally observed in astrocytes. In AD brains, the reactive astrocytes were immunopositive for HSPB1/HSP27. The chaperone occasionally co-localized with the amyloid plaques (frequency &lt;35%). ● See also for HSPB5 and HSPB6 (same study).</td>
<td>Wilhelmus et al., 2006.</td>
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<tr>
<td></td>
<td>AD</td>
<td>In vivo, human IHC</td>
<td>In AD brains HSPB1/HSP27 is highly expressed in affected brain regions rich in senile plaques, and notably, in a large number of reactive astrocytes. Expression of HSPB1/HSP27 increased with the severity of AD-specific morphological changes, and with the duration of dementia. Similar patterns of immunoreactivity were present in PD brains although they showed less expression of HSPB1/HSP27 in reactive astrocytes compared to AD brains.</td>
<td>Renkawek et al., 1994</td>
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<td></td>
<td>AD (tau)</td>
<td>In vivo, rat model: truncated tau protein into the Thy-1 gene RT qPCR IHC</td>
<td>HSPB1/HSP27 is predominantly overexpressed in reactive astrocytes located in regions with tau pathology, rarely in neurons.</td>
<td>Filipcik et al., 2015</td>
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<tr>
<td></td>
<td>AD</td>
<td>In vivo (mouse model): P301S tau transgenic mice IHC</td>
<td>Reactive astrocytes in the transgenic mice upregulated HSPB1/HSP27. Neurons do not show the same upregulation. Tau aggregates and HSPB1/HSP27 do not co-localize.</td>
<td>Yata et al., 2011 -</td>
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<td>Table 3: continued</td>
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<p>| PD | In vivo, human | HSPB1/HSP27 was upregulated in reactive astrocytes in the brain of PD patients. Such increased expression was correlated with the neuronal degeneration rather with the amount of senile plaques. ● See also HSPB5 (same study) | Renkawek et al., 1999 |
| ALS (SOD-1) PD | In vivo, mouse model: - transgene regulated by mouse prion protein promoter. - SOD1G37R for ALS - α-SynA53T for PD | In symptomatic SOD1G37R mice, reactive astrocytes showed increase in HSPB1/HSP25 immunoreactivity in brain regions associated with SOD-1 pathology. Increased HSPB1/HSP25 was not observed in neurons of these regions. Inclusion-like deposit were present in some cells showing increased HSPB1/HSP25 expression and with astrocyte-like morphology. In symptomatic α-SynA53T mice, similar results are found. | Wang e al., 2008 |
| ALS (SOD-1) | In vivo, mouse model: Leu126delTT mutation in the Cu/Zn superoxide dismutase gene (SOD1) | HSPB1/HSP27 is upregulated in the transgenic SOD-1 mice. HSPB1/HSP27 is markedly increased in post-symptomatic transgenic mice compared to the same animals at disease onset. Moderate increased levels of HSPB1/HSP27 are found in glial cells of transgenic mice at disease onset (compared to a normal control). The expression of HSPB1/HSP27 was localized in the cytoplasm of GFAP-positive glial cells and stronger in reactive astrocytes than microglial cells. | Fukada et al., 2007 |
| ALS (SOD-1) | In vivo, mouse model: human gene for SOD1 with aG93A mutation | Motor neurons of SOD-1 mice showed very little or no immunostaining for HSPB1/HSP25 (whereas the same neurons in control mice showed robust expression of HSPB1/HSP25). In contrast, numerous reactive astrocytes of SOD-1 mice contained HSPB1/HSP25 (Figure 8, second and fourth or bottom panels). Such reactive astrocytes overexpressing HSPB1/HSP25 are diffuse throughout the white matter of all columns. HSPB1/HSP25 and SOD1 co-localized in the cytoplasm of neurons and in some astrocytes. The processes of many reactive astrocytes were immunolabeled with HSPB1/HSP25, but not with SOD1. | Strey et al., 2004 |</p>
<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Methodology</th>
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<tr>
<td>HSPB2</td>
<td>AD</td>
<td>In vivo, human IHC</td>
<td>In AD brains, reactive astrocytes were present at the site of neuronal degeneration and amyloid aggregates formation. Reactive astrocytes showed increased expression of HSPB2. HSPB2 co-localized with A-Beta aggregates. <strong>See also for HSPB2, HSPB5, HSPB6, and HSPB8 (same study).</strong></td>
<td>Bruinsma et al., 2011</td>
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<tr>
<td>HSPB5</td>
<td>AD</td>
<td>In vivo, human IHC</td>
<td>In AD brains, reactive astrocytes were present at the site of neuronal degeneration and amyloid aggregates formation. Reactive astrocytes showed increased expression of HSPB5/aB-crystallin. HSPB5/aB-crystallin did not co-localize with A-Beta aggregates. <strong>See also for HSPB1, HSPB2, HSPB6 and HSPB8 (same study).</strong></td>
<td>Bruinsma et al., 2011</td>
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<td>In control brains, HSPB5/aB-crystallin was occasionally observed in astrocytes. In AD brains, the reactive astrocytes were immunopositive for HSPB4/aB-crystallin. The chaperone did not co-localize with the amyloid plaques. <strong>See also for HSPB1 and HSPB6 (same study).</strong></td>
<td>Wilhelmus et al., 2006</td>
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<td></td>
<td>TauoP</td>
<td>In vivo, human IHC</td>
<td>Increased expression of HSPB5/alphaB-crystallin in glial cells (including astrocytes) in affected brain regions. Not all glial cells/astrocytes over-expressing HSPB5/alphaB-crystallin were also positive for tau aggregates. Not all astrocytes over-expressing HSPB5/alphaB-crystallin were reactive (increased expression of GFAP).</td>
<td>Dabir et al., 2004</td>
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<td></td>
<td>AD</td>
<td>In vivo, human IHC</td>
<td>Increased expression of HSPB5/alphaB-crystallin in reactive astrocytes in AD-affected brain regions</td>
<td>Tomimoto et al., 1997</td>
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<td></td>
<td>AD</td>
<td>In vivo, human IHC</td>
<td>Increased expression of HSPB5/alphaB-crystallin in reactive astrocytes in AD-affected brain regions. Immunoreactivity to HSPB5/alphaB-crystallin in astrocytes was found mainly restricted to areas with senile plaques and neurofibrillary tangles (tau).</td>
<td>Renkawek et al., 1994</td>
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<tr>
<td></td>
<td>AD</td>
<td>In vivo, human IHC</td>
<td>Increased expression of HSPB5/alphaB-crystallin in astrocytes in AD-affected brain regions</td>
<td>Shinohara et al., 1993</td>
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<tr>
<td>Disease</td>
<td>In vivo, human</td>
<td>IHC</td>
<td>Note</td>
<td>Reference</td>
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<tr>
<td>AD</td>
<td>Increased expression of HSPB5/alphaB-crystallin in reactive astrocytes in AD-affected brain regions</td>
<td>Iwaki et al., 1992</td>
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<tr>
<td>ALS</td>
<td>Increased expression of HSPB5/alphaB-crystallin in reactive astrocytes in AD-affected brain regions. AD aggregates do not co-localize with HSPB5/alphaB-crystallin.</td>
<td>Lowe et al., 1992</td>
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<td>PD</td>
<td>HSPB5/alphaB-crystallin is expressed in glial cells (including astrocytes) and often co-localizes with glial α-synuclein inclusions. See also for HSPC1 (same study)</td>
<td>Uryu et al., 2006</td>
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<td>HD</td>
<td>HSPB5/alphaB-crystallin immunoreactivity was up-regulated in reactive astrocytes of symptomatic mice; Glia cells, including astrocytes, accumulated mutant SOD1 immunoreactivity.</td>
<td>Wang et al., 2005</td>
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<td>FTLD (TDP-43)</td>
<td>TDP-43 aggregates were observed in the brain of the patients and they were variably positive for the HSPB5/alphaB-crystallin and less often GFAP. Bundles of astrocytic glial fibrils characteristic of reactive astrocytes were often found in proximity, but glial fibrils were negative for TDP-43. These processes might be astrocytic end-feet with abnormal TDP-43 fibrillary inclusions.</td>
<td>Lin et al., 2009</td>
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<td>PD (and other α-SynP)</td>
<td>HSPB5/alphaB-crystallin was upregulated in reactive astrocytes in the brain of PD patients. Such increased expression was correlated with the neuronal degeneration rather than with the amount of senile plaques. See also for HSPB1 (same study)</td>
<td>Renkawek et al., 1999</td>
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<tr>
<td>ALS (SOD-1)</td>
<td>HSPB5/alphaB-crystallin immunoreactivity was up-regulated in astrocytes of symptomatic mice; Glia cells, including astrocytes, accumulated mutant SOD1 immunoreactivity.</td>
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<td>HSPB6 (HSP20; FLJ32389)</td>
<td>SCA3</td>
<td>HSPB8 (AH11; HMN2; CMT2L; DHMN2; E2IG1; HMN2A; HSP22)</td>
<td>HSPB8 (AH11; HMN2; CMT2L; DHMN2; E2IG1; HMN2A; HSP22)</td>
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<tr>
<td><strong>AD</strong></td>
<td>In vivo, human IHC</td>
<td>mass intact and amyloid aggregates formation. Reactive astrocytes showed increased expression of HSPB6/HSP20. HSPB6/HSP20 did not co-localize with A-Beta aggregates. ● See also for HSPB1, HSPB2, HSPB5 and HSPB8 (same study).</td>
<td>Bruinsma et al., 2011</td>
<td></td>
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<tr>
<td><strong>AD</strong></td>
<td>In vivo, human IHC</td>
<td>In control brains, strong upregulation of HSPB8 and a moderate upregulation of BAG3 specifically in astrocytes in the cerebral areas affected by neuronal damage and degeneration, for all the investigated diseases. No significant change in the HSPB8-BAG3 expression levels was observed within neurones, irrespective of their localization or of the presence of proteinaceous aggregates.</td>
<td>Seidel et al., 2012b</td>
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<tr>
<td><strong>AD</strong></td>
<td>In vivo, human IHC</td>
<td>In AD brains, reactive astrocytes were present at the site of neuronal degeneration and amyloid aggregates formation. Reactive astrocytes showed increased expression of HSPB8. HSPB8 did not co-localize with A-Beta aggregates. ● See also for HSPB1, HSPB2, HSPB5 and HSPB6 (same study).</td>
<td>Bruinsma et al., 2011</td>
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</table>
In Table 3, I summarized the available data on responses of astrocytes in terms of HSPs expression as seen in patient brains or in in vivo animal disease models. Given the cellular work, these may thus not always and necessarily be due to a direct response of the cells to the presence of aggregation-prone proteins or aggregates. In fact, intra-astrocytic protein aggregates (e.g. tau in AD, α-syn in PD) do not always co-localize with the upregulated HSP in the GFAP-positive astrocytes (studies reported in Table 3). These data suggest that the HSP upregulation in astrocytes might not be exclusively a “stress” response to protein aggregation in that specific cell, but a more concerted intercellular response against the brain damage: astrocytes, in which aggregation is still not initiated, might receive signals (i.e. cytokines) from the neighbour degenerating neurons and overexpress specific protective HSPs in response.

It is, however, striking that astrocytes that show upregulation of HSPs are almost exclusively found in the corresponding region of neurodegeneration where the remaining neurons contain protein aggregates (studies reported in Table 3). In fact, the astrocytes are generally found to up-regulate the specific chaperone at a higher level compared to affected neurons in that area; moreover, also more astrocytes than neurons in a specific brain region are found to be positive to a certain HSP (studies reported in Table 3). On one hand, this might be explained by the neuronal vulnerability to aggregates (neurons degenerate and therefore less cells positive for a certain HSP may be identified); however, this might also substantiate the hypothesis that the HSPs upregulation is part of an astrocytic protective response.

So, the upregulation might fulfill a dual function. It may protect the astrocyte in a cell-autonomous manner, but also protect the neurons in a non-cell autonomous manner. In fact, HSP-boosted astrocytes, which are more resistant to protein aggregation and aggregate toxicity, might keep their functional activity to maintain neuronal fitness and viability. Moreover, the same astrocytes might be capable to release protective HSPs as cargo in exosomes towards neurons or uptake prionoids released by the neurons to impede their spreading in other brain regions (see section 1.2.7.b and Discussion of this Thesis). Nonetheless there is still a general lack of knowledge concerning such aspects.

### 3.5. Conclusion

The functional implications of the HSPs up-regulation in astrocytes for the progression of neuronal degeneration have not yet been established. The key protective role of the HSPs against protein aggregation and aggregate toxicity suggests that this upregulation might not be merely a stress marker or a compensatory effect, but rather be part of the protective response of astrocytes against diseases characterized by toxic aggregates species.

To get a better insight in the role of astrocytes in HD and whether and how the expression of a specific HSP expression in astrocytes may play a role in this disease, we generated a D. melanogaster model of HD that exclusively express the mutant toxic PolyQ HTT in neurons, whilst co-expressing a protective chaperone either in the same neurons or in astrocytes. The chaperone that we choose to investigated is DNAJB6, a member of the human DNAJ family that has potent cell autonomous effects against PolyQ-related neurodegeneration (Hageman et al., 2010; Månsson et al., 2014;
Kakkar et al., 2016) and that has been found to be upregulated in astrocytes in some NDs (Durrenberg et al., 2016).
REFERENCES


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