Cell-autonomous and non-cell autonomous protection of DNAJB6 in Huntington’s disease

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CHAPTER 6

DISCUSSION
1. Our findings and open questions

In this research, we generated and validated a *D. melanogaster* model that allows the independent and non-overlapping expression of two different transgenes of interest in neurons and astrocytes (or all glial cells) of the fly brain (Chapter 3). This model was developed for the *in vivo* investigation of the mutual interactions between neurons and astrocytes in the brain. In particular, we used this model to investigate the protective role of chaperones, and in particular both cell-autonomous and non-cell autonomous protective functions of the human chaperone DNAJB6 against Huntington’s disease (HD) (Chapters 4 and 5).

First, we showed that the previously observed cell-autonomous protective function of human DNAJB6 against PolyQ aggregation and toxicity (Hageman et al., 2010; Månsson et al., 2014, Kakkar et al., 2016) can be recapitulated in *D. melanogaster* models of HD (Chapter 4). We confirmed that DNAJB6 reduces PolyQ Huntingtin (Htt) aggregate formation and toxicity and improves neuronal fitness *in vivo*, consequently leading to a significant expansion of the lifespan of the flies expressing Htt in neurons. Most strikingly, we found that also the expression of DNAJB6 only in astrocytes leads to an expansion of the lifespan of flies expressing Htt in neurons, suggesting a non-cell autonomous protective activity of the chaperone in our *Drosophila* HD model (Chapter 5).

These main findings raises a number of new questions and perspectives, as described below:

1. Our data show that the non-cell autonomous protection of astrocytic DNAJB6 does not result in the same magnitude of lifespan extension as when DNAJB6 is directly co-expressed with PolyQ Htt in neurons, implying that direct cell-autonomous neuronal protection has the strongest effects. The relevance of these findings will be discussed in section 1.1 of this Chapter.

2. The neuronal PolyQ-HTT aggregates can trans-cellularly spread in the *D. melanogaster* brain in a prion-like manner and can end up in astrocytes. We hypothesised that the uptake of neuron-derived aggregates could imply that astrocytes act as a “reservoir” for these toxic prion-like species, thereby preventing their neuron-to-neuron spreading in the brain and hence delaying the progression of neurodegeneration. However, this prion-reservoir capacity is likely limited by the toxicity of captured aggregates. Possible mechanisms for PolyQ HTT prionoids entry in astrocytes will be discussed in section 1.2 of this Chapter.

3. Unlike for cell autonomous effects, the non-cell autonomous protection evoked by DNAJB6 expression in astrocytes is not associated with a reduction in the load of total PolyQ-HTT aggregation in the brain of our HD *Drosophila* model, implying that the DNAJB6 transmission from astrocytes to neurons (such as via exosomes) is unlikely. Although not supported by our data, the chaperone transmission from astrocytes to neurons still remains an intriguing mechanisms for non-cell autonomous protection in HD that might provide possible therapeutical options (section 1.3 of this Chapter).

4. In our HD *Drosophila* model, the frequency of astrocytes with inclusions is increased in case of DNAJB6 overexpression, which suggests that DNAJB6 may have enhanced the “reservoir”
capacity of astrocytes and their ability to prevent the spreading of prion-like species in the brain. How can DNAJB6 protect astrocytes from the toxicity mediated by PolyQ Htt prionoids (section 1.4 of this Chapter)?

5. Expression of DNAJB6 in all glial cells did not provide a longer extension of the lifespan of HD flies than DNAJB6 expression only in astrocytes, suggesting that astrocytes are the key players in the non-cell autonomous protection mediated by DNAJB6. Nonetheless our data does not exclude that other glial cells, such as microglia, might have an important role, together with astrocytes, in the non-cell autonomous protection against PolyQ Htt prionoids (section 1.5 of this Chapter).

1.1 - Cell-autonomous protection of DNAJB6 is more effective

Our data reveal that the cell-autonomous protection of neuronal DNAJB6 is more effective compared to the non-cell autonomous protection of astrocytic DNAJB6 in the HD Drosophila model expressing PolyQ Htt in neurons. This finding strongly supports the idea that PolyQ diseases are primarily neuronal diseases. Nonetheless, our data open to the possibility that the modulation of the chaperonome in astrocytes might be an important rearguard in providing protection. This is idea is also substantiated by the crucial role of astrocytes in protecting neurons (Section 3.1 and 3.2 of Chapter 2), and by their different capacity to handle toxic PolyQ aggregates (Sections 3.3 and 3.4 of Chapter 2).

1.2 - PolyQ Htt prionoids spreading and the role of astrocytes

Our data substantiate the interesting previous evidences showing that mutant PolyQ Htt aggregates might behave like prionoids (Scheckel et al., 2018) in line with several in vitro and in vivo studies and findings in patients based on the progression patterns of the polyQ diseases within the brains of poly Q patients (see Figure 6 of Chapter 2 and references in table 1 of Chapter 2, section 2.6). Being prionoids, the PolyQ Htt aggregates are capable of seeding, therefore of elongating by the recruitment of soluble polypeptide chains and of fragmenting, to generate additional elongation sites and amplify aggregation. Importantly, these aggregate species can recruit the normal Htt protein during the seeding process (Kazantsev et al. 1999, Busch et al. 2003; Ren et al. 2009; Trevino et al. 2012; Holmes et al. 2013; Tan et al. 2015; Ruiz-Alrandis et al. 2016).

If the acceptor cells have a higher capability to cope against the PolyQ HTT toxicity, aggregation and seeding capacities, they might be capable to interfere with the prion-like spreading of toxic species and slow down the pathological processes.

In our model, astrocytes serve as acceptor cells: boosting their resistance against the up taken PolyQ Htt aggregates, through the overexpression of the protective chaperone DNAJB6, might slow down the pathological processes in HD.

An important open question is how PolyQ Htt prionoids can enter in astrocytes. Several mechanisms have been described for the spreading of PolyQ Htt prionoids in the brain:

1. Tunnelling nanotubes (TNTs)
2. Endocytosis and phagocytosis
3. Exosomes and exophers
4. Direct penetration of the plasma membrane
5. Transynaptic propagation

Although further investigations are needed, all these mechanisms might be involved in the PolyQ Htt spreading between cells that we also observe in our HD model. Here, we discuss each mechanism and the role of astrocytes herein as acceptor cells of the prionoids.

- **Tunnelling nanotubes**

Neurons, and other cell types including astrocytes (Wang et al., 2011), have the ability to produce temporary and retractable F-actin-based tubular protrusions, called tunnelling nanotubes (TNTs) that allow direct communication between cells (Abounit et al., 2012). TNTs are different from filopodia and are involved in normal physiological functions in the cells, such as the transmission of electrical signals in neurons (Smith et al., 2011). The finely regulated formation of TNTs requires SNARE proteins that allow the membrane curvature and fusion (Abounit et al., 2012). The active transport of vesicles and organelles via TNTs (Rustom et al., 2004; Wang et al., 2015) is mediated by molecular motors (Abounit et al., 2012). Brain cells can form TNTs under stress conditions (for example during starvation and during pathological processes). It has been proposed that TNTs are formed as a defence mechanism to allow cells to expel material that cannot be degraded, such as proteinaceous aggregates in NDs (Victoria et al., 2017). On the other hand, the transfer of these aggregates can contribute to the pathological process. In vitro experiments have shown that prions and prionoids can induce the formation of and hijack TNTs in donor cells. TNTs become channels for the spreading of toxic aggregates to acceptor cell (Gousset et al., 2009). A-syn, for example, have been found to be efficiently transferred from donor to acceptor neurons through TNTs inside endolysosomal vesicles (Abounit et al., 2016).

Data in in vitro experiments with co-cultured neurons have shown that TNTs serve as channels also for the prion-like spreading of PolyQ HTT (Costanzo et al., 2013). PolyQ HTT overexpressed in donor neurons induces the formation of TNTs. When the nanotubes get in contact with the acceptor cells transport of PolyQ aggregates is enable. Differently from α-syn, PolyQ HTT aggregates observed in these experiments are not embedded in vesicles but rather encaged by vimentin in aggresomes-like structures (Costanzo et al., 2013), suggesting that PolyQ HTT prionoids spread via TNTs without the involvement of endo-lysosomal pathways. PolyQ HTT aggregates induce TNTs formation (Costanzo et al., 2013), but the exact mechanisms that regulate the formation of TNTs during HD and the transport of the PolyQ HTT aggregates through TNTs still need to be elucidated. Moreover, data showing that PolyQ HTT transfer via TNTs also occurs in vivo are missing.

Astrocytes can also form TNTs under stress conditions (Wang et al., 2011). Moreover, recent data have shown that α-syn can be transferred via TNTs between co-cultured astrocytes (Rostami et al., 2017). Although further investigations are needed to unravel the in vivo functional significance of these findings, one can hypothesize that PolyQ HTT aggregates may be transferred from neurons to astrocytes via TNTs. Astrocytes might temporarily serve as reservoir of such aggregates released via TNTs from the neighbour neurons, providing non-cell autonomous protection. Nonetheless, after a certain threshold, astrocytes might initiate to protrude TNTs toward neighbouring cells to expel the
toxic not degradable aggregates. One possible interpretation of our data could be that by potentiating their resistance against aggregate toxicity via overexpression of protective chaperones, such as DNAJB6, the reservoir capacity of astrocytes might be enhanced.

- **Endocytosis and phagocytosis**

  Endocytosis mainly controls the internalization and recycling of plasma membrane components, receptors, and ligands and the uptake of extracellular macromolecules, nutrients and particles. The endocytic vesicles are formed at the plasma membrane and after internalization, they mature into early and late endosomes. These latter fuse with lysosomes for the degradation of the vesicle cargo.

  Data from *in vitro* and *in vivo* studies have suggested that NDs-associated aggregates, such as α-syn (Sung et al., 2001; Park et al., 2009; Hansen et al., 2011; Valpolicelli et al., 2011; Angot et al., 2012; Konno et al., 2012; Oh et al., 2016) and tau (Frost et al., 2009; Guo et al., 2011; Wu et al., 2012), can enter in cells in a prion-like manner through different mechanisms of endocytosis (Costanzo et al., 2013).

  Similarly, PolyQ HTT has been suggested to be internalized in cells through clathrin-dependent endocytosis (Ruiz-Alrandis 2016). PolyQ HTT exon-1 fibrils (HTTExon1Q44) added to the culture medium of neuronal-like cells were found in early endosomes in the first hours after exposure; at later times, the same aggregates were found in late endosomes and lysosomes, suggesting that the acceptor cells direct the endosomal cargo toward a degradation pathway (Ruiz-Alrandis et al., 2016). These findings indicate that endocytosis might be linked to prion-like spreading of PolyQ HTT, but also that fibrils that were internalized in fact may be detoxified by lysosomal degradation. Importantly, the results of this study indeed indicate that fractions of PolyQ HTT fibrils escape the endo-membranous compartment before being degraded and reach the cytosol, where they can initiate seeding. Although the authors do not provide data, it is suggested that PolyQ aggregates may escape the endosome through leakage or rupture, as observed for α-syn (Freeman et al., 2013) and amyloid beta peptide (Ji et al., 2002). In astrocytes, extracellular particles may not only enter via such endocytotic routes but also through phagocytosis as suggested by Pearce and colleagues (Pearce et al., 2015).

  Whilst uptake of aggregates by astrocytes could halt the speed of neuron-to-neuron transmission, a prerequisite would be that the astrocytes “survive” after having taken up this material. Internalized PolyQ HTT aggregates can escape the phago-lysosomal membrane and end up in the cytoplasm, where they induce co-aggregation of cytoplasmic normal HTT and co-localize with cytoplasmic chaperones such as HSP70/HSC70 and HSP90. If such escape occurs, aggregates might cause loss of astrocytes functionality and vitality with the consequent loss of their neuroprotective activity. As postulated before, and consistent with our findings, the clearance and reservoir capacities of these cells might be enhanced by potentiating their resistance against aggregate toxicity, via overexpression of protective chaperones, such as DNAJB6.
• **Exosomes and exophers**

Exosomes are extracellular vesicles (typically 40–100 nm) with a key role in intercellular communication and for the transmission of macromolecules between cells (including mRNA and proteins): they are formed by the fusion of the multivesicular body (an intermediate of endocytic compartment) with the plasma membrane, followed by the budding of the resulting vesicles, which are finally internalized by the acceptor cells (Edgar et al., 2016). Although the mechanisms underlying the selection and loading of the cargo, the budding, and the entrance in the acceptor cells still need to be completely elucidated, almost all mammalian cells with an endomembranes system are capable to release exosomes (Edgar et al., 2016).

It has been shown that NDs-associated aggregates can be also loaded in exosomes, secreted in the extracellular space and delivered to acceptor cells through these vesicles in a prion-like manner (Soria et al., 2017): similarly to TNTs, this might be a cellular strategy to expel non-degradable material that however contributes to the pathological spreading of toxic aggregates. The exact mechanisms by which aggregate species are loaded in exosomes and the destiny of the cargo, once internalized in the recipient cells, still need to be elucidated (Soria et al., 2017).

The subsequent uptake of exosomes by the recipient cell involves fusion with the plasma membrane and different cell-specific mechanism including endocytosis, phagocytosis (Feng et al., 2010, Fruhbeis et al., 2013; Abels and Breakefield, 2016) and macropinocytosis (Fitzner et al., 2011). Whether and how the acceptor cells select the exosomes with the cargo still need to be elucidated. Astrocytes can release and uptake exosomes to transmit many different types of cargos including mRNA, cytokines and peptides (Verkhratsky et al., 2016). Studies have shown that ND-associated aggregates, such as SOD-1, can be loaded and released as cargo in exosomes by astrocytes (Haidet et al., 2011; Basso et al., 2013). Investigations are needed to confirm whether PolyQ HTT aggregates are also loaded in astrocyte-derived exosomes.

Jeon and colleagues have shown that PolyQ HTT can be transferred between brain cells via exosomes *in vitro* and *in vivo* (Jeon et al., 2016). Interestingly this is the first study of human-to-mouse exosome-mediated transmission of toxic ND-associated aggregates. Co-culture of neurons derived from murine neural stem cells with human fibroblasts from an HD patient carrying a 143 CAG repeats mutation in *HTT* gene (HD143F) showed that mutant PolyQ HTT can spread in neurons in a prion-like manner, as cargo in exosomes from the donor fibroblasts. Also, the injection of exosomes derived from human HD143 fibroblasts in wild type mice triggered HD-like symptoms together with appearance of HTT aggregates in the animal brain. Data from this study also suggest that the release of PolyQ HTT in exosomes is not due to cell death and that the spreading of aggregates does not always require cell-to-cell contact (Jeon et al., 2016). Further investigations are needed to elucidate if the internalized Poly HTT aggregates are capable to escape from the endocytic pathway and to be vesicle-free in the cytosol.

More recently, Melentijevic and colleagues have shown that cellular stress and the presence of PolyQ HTT aggregates can induce in *C. elegans* the formation of vesicles named exophers (Melentijevic et al., 2017). They are capable to incorporate and extruding organelles and aggregates.
and are thought to be a cellular defence mechanism that however contributes to the cell-to-cell transmission of toxic aggregates (Melentijevic et al., 2017).

Due to their key role in vesicle trafficking and on the base of data from these studies, we can speculate that astrocytes may be capable to uptake exosomes (or exophers), loaded with PolyQ HTT aggregates released from neighbouring cells and that this prion-reservoir activity can contribute to protect the brain. The later release of exosomes by astrocytes might be an attempt to expel the aggregates. These unconventional secretory pathways might be only triggered once the cells are not further capable to cope with the presence of these toxic species (Basso et al., 2011).

- **Direct penetration of the plasma membrane**
Intracellular and extracellular aggregate species, including PolyQ HTT (Bäuerlein et al., 2017), directly interact with the phospholipid bilayer of the membranes. Aggregates can cross the phospholipid bilayer through the formation of transmembrane channels (Arispe et al., 1993; Jang et al., 2010), membrane deformation or disruption (Reynolds et al., 2011).

Ren and colleagues have shown that synthetic PolyQ fibrils (PolyQ stretch flanked by lysine residues, K2Q44K2), added to the culture medium, enter in various type of acceptor mammalian cells (Cos-7, CHO, HEK, HeLa, and N2A) (Ren et al., 2009). This suggests that various brain cells, including astrocytes, might be recipients for these prionoids. K2Q44K2 fibrils directly spread into the cytoplasm of the recipient cells, where they co-aggregate with endogenous normal or PolyQ HTT and co-localize with cytosolic quality control components such as ubiquitin and HSP70. Using transmission electron microscopy, the authors showed the presence of fibrillar aggregates attached to the inner surface of the plasma membrane on a “bed” of cortical actin with no evidence that such aggregates were surrounded by endomembranous structures or clathrin (Ren et al., 2009). These data suggest that PolyQ species may directly penetrate into the plasma membrane and enter the cytosol. However, further research is needed to confirm this in co-culturing systems and in vivo experiments, and to unravel the mechanisms of membrane penetration. Importantly, data from the same group showed that PolyQ fibrils, different from those previously used (PolyQ HTT exon-1 fibrils, HTTExon1Q44), enter the cells through endocytosis (Ruiz-Alrandis et al., 2016).

- **Transynaptic propagation**
PolyQ HTT aggregates have been found in axonal terminals and associated with synaptic vesicles (Li et al., 2003). In a study by Pecho-Vrieseling and colleagues, a possible pathological significance for this has been investigated using ex vivo neural network models, in which neurons from R6/2 HD mice establish synaptic contacts with neurons from wild type animals (or alternatively, neurons differentiated from human embryonic stem cells) (Pecho-Vrieseling et al., 2014). Spreading of PolyQ HTT from the R6/2 neurons to the wild type neurons was observed. PolyQ HTT was located between and in close proximity to the marker synaptophysin in the pre-synapses, and to PSD-95 marker in the post-synapses. Additionally, up-taken aggregates by recipient neurons were initially located in the cytoplasm, but subsequently in the nucleus. Finally, the PolyQ HTT spreading was blocked by botulinum neurotoxins, which are well known to block synaptic vesicle fusion and neurotransmission in the pre-synapses by targeting different SNARE proteins (Pecho-Vrieseling et
Taken together, these data suggest a synaptic mechanism underlying the transneuronal propagation of PolyQ HTT aggregates. However further investigations are needed, for example to elucidate how PolyQ HTT in the presynaptic neuron can reach the terminal axon.

Each synapse in the brain is monitored by astrocytes and it is estimated that the processes of one astrocyte can contact over 100 000 synapses. Astrocytes exert key functions at the multi-partite synapse: they have a fundamental role in the vesicles trafficking in the synaptic cleft, respond to synaptic neural activity, regulate the synaptic transmission and keep the homeostasis of fluid, ions, pH and neurotransmitters (Araque et al., 1999; Halassa et al., 2007; Perea et al., 2009). We can therefore hypothesize that astrocytes can also play an important role in the transynaptic propagation of PolyQ HTT aggregates. Promoting their capacity to uptake PolyQ-loaded vesicles from the synaptic cleft and boosting their resistance to PolyQ HTT toxicity could greatly contribute to slow down the neuron-to-neuron spreading.

### 1.3 - Intercellular transmission of chaperones from astrocytes to neurons and implications in Huntington’s

Although our data do not support it, a possible intriguing mechanism - that we have taken in consideration to explain the non-cell autonomous DNAJB6 protection in our HD model - is the intercellular transmission of the chaperone from astrocytes to neurons.

A recent study from Takeuchi and colleagues showed that chaperones of the HSP40/DNAJ, HSP70/HSPA and HSP90/HSPC families can be transported between cells via exosome release and uptake. Notably, they found that HSP40/DNAJ-containing exosomes added to the culture medium of acceptor cells expressing PolyQ HTT can efficiently reduce aggregate formation (Takeuchi et al., 2015). Interestingly DNAJA1, DNAJA2, and DNAJB6a (a longer isoform of DNAJB6, different from the shorter DNAJB6b used in our study) (Hageman et al., 2010, Hanai et al., 2003), have been found to be secreted in exosomes, whereas DNAJ proteins localized in organelles (such as DNAJA3 in mitochondria, Hageman et al., 2010) have not. Exosome secretion of HSP40 resulted to be dependent by the J domain in the chaperone (Takeuchi et al., 2015).

The cell-to-cell exchange of HSPs using exosomes has been proposed by Takeuchi and colleagues as a physiological mechanism to cope with the variable capacity of different cells to express chaperones under stress conditions: in this proposed model, cells with an intrinsic limited capacity to respond to proteotoxic stress receive HSPs-loaded exosomes from the more responsive cells (Takeuchi et al., 2015).

Although our data suggest that an exosome-secretion and uptake is not the dominant mechanism for the non-cell autonomous protection of astrocytic-DNAJB6 in our HD D. melanogaster model, the possible transport of protective chaperones between cells via exosomes still assigns a compelling physiological role of these vesicles in maintaining organismal protein homeostasis. In section 3.4.2 of Chapter 2, we discussed how brain cells have different capacities to induce the Heat Shock Response (HSR) (Sala et al., 2017; San Gil et al., 2017): astrocytes show a faster and stronger response than neurons (Nishimura et al., 1991) and whereas neurons in rodent in vivo models do
not induce HSPA/HSP70 expression after exposure to stress conditions, astrocytes do (Manzerra et al., 1992; Nishimura et al., 1996; Manzerra et al., 1997; Krueger et al., 1999; Oza et al., 2007; Pavlík et al., 2007; Yang et al., 2008). The lower intrinsic capacities to mount the HSR of neurons when compared to astrocytes might be functionally compensated by the intercellular astrocytes-to-neurons transmission of exosomes loaded with HSPs.

Takeuchi and colleagues also suggest that an eventual enhancement of the secretion of HSP-loaded exosomes might be a potential therapeutic strategy for HD and other neurodegenerative diseases (NDs) characterized by protein aggregation (Takeuchi et al., 2015). Although this indeed might be a potential strategy to promote the protein homeostasis in the brain affected by NDs (for example by stimulating astrocytes to express and release exosomes loaded of protective chaperones towards the surrounding neurons), this eventual therapy should aim to allow the exclusive loading of HSPs as cargo in the exosomes in the donor cells. This is a critical aspect in consideration of the fact that exosomes have been also found as vehicle of disease-associated prionoids such as α-syn (Emmanouilidou et al., 2010), Aβ (Rajendran et al., 2006), tau (Saman et al., 2012), SOD-1 (Gomes et al., 2007) and PolyQ-HTT (Jeon et al., 2016). The mechanisms underlying the selection of the exosome cargo during the pathology must be elucidated to develop potential therapies based on this strategy.

Exosomes are however ideal delivery vectors due to their low immunogenicity and toxicity and capacity to cross membranes (El Andaloussi et al., 2013). Recently, methods to produce engineered exosomes loaded with a specific protein have been developed. Exosomes engineered in laboratory have been successfully administered to the brain of mice, via the nasal route, proving their potential for the in vivo delivering of selected proteins (Sterzenbach et al., 2017). These findings open to future therapeutical approaches in which protective HSPs (such as DNAJB6) in exosomes are delivered to the brain for the treatment of aggregate-associated NDs.

1.4 - Protection by DNAJB6: cell autonomous and non-cell autonomous mechanisms

Our data showed that the cytosolic isoform DNAJB6b (Hanai et al., 2003) is an interesting candidate chaperone to enhance both the cell intrinsic resistance to polyQ aggregation (Chapter 4) as well as to boost the non-cell autonomous protection of astrocytes against HD pathogenesis (Chapter 5).

What is known about the cell-autonomous protective activity of DNAJB6 against PolyQ Htt aggregation and aggregate toxicity?

The formation of PolyQ Htt aggregates is mechanistically described as a process in different steps that starts with the formation of an initial “nucleus”, a kinetically unstable oligomer formed from interacting monomers (primary nucleation). Once the nucleus is assembled, it is thought to grow into large and highly stable β-sheet-rich structures with a fibrillar morphology. The growth of the fibrils proceeds through monomer addition on the existing β-sheet-rich aggregates (Morris et al., 2009; Cohen et al., 2011; Cohen et al., 2012). The aggregation process proceeds with secondary processes such as the fragmentation of the fibrils (Xue et al., 2008; Knowles et al., 2009), and the
nucleation at the surface and ends of the existing fibre (elongation sites) (Ferrone et al., 1985; Ruschak et al., 2007; Cohen et al., 2013) and fibril branching (secondary nucleation) (Andersen et al., 2009). A large amount of data indicates that the formation of PolyQ Htt aggregates occurs through these processes (Scherzinger et al., 1997; Scherzinger et al., 1999; Chen et al., 2002; Poirier et al., 2002; Dahlgren et al., 2005; Thakur et al., 2009; Crick et al., 2013; Wagner et al., 2018). The presence of the PolyQ expansion is thought to be the main driver of aggregation of PolyQ Htt, but other factors might influence its fibrillo-genesis, including the state of the PolyQ Htt monomers forming the aggregates (i.e. full length protein or truncated Htt fragments derived by proteases cleavage), the length of the PolyQ expansion, the protein concentration and the time.

DNAJB6 is a HSP70 co-chaperone that reduces the PolyQ-induced toxicity in vitro and in vivo (Hageman et al., 2010; Kakkar et al., 2016 and our data). The protective mechanism of action of the chaperone mainly relies in its capacity to form oligomeric complexes and to directly interact with the PolyQ client (Hageman et al., 2010). DNAJB6 strongly inhibits the primary nucleation step and perturbs the secondary nucleation in the aggregation process (Kakkar et al., 2016), by interaction of a serine/threonine (S/T)-rich region in its C-terminus domain with the substrate (Kakkar et al., 2016; Söderberg et al., 2018). The current model postulates that the hydroxyl groups in the side chains of the S/T region of DNAJB6 reduce the nucleation rate of the PolyQ species by competing with the hydrogen bonding necessary for formation of amyloid fibrils and beta-hairpins (Hoop et al., 2016).

However, little is now about the fate of the PolyQ Htt-DNAJB6 complex. Although not absolutely required for the anti-aggregation activity (Hageman et al., 2010), the J-domain of DNAJB6 allows the interaction with HSP70/HSPA that might direct the PolyQ species for further processing. Data concerning the physiological role of DNAJB6 (Watson 2007, Izawa 2000) or about chaperonopathies linked to mutations of the DNAJB6 gene (Harms et al., 2012; Sarparanta et al., 2012; Suarez-Cedeno et al., 2014) might provide insights about the possible processing of the PolyQ Htt-DNAJB6 complex.

In our proposed model (Chapter 5, Figure 6), we speculate that somehow the DNAJB6-expressing astrocytes become a more effective reservoir of PolyQ HTT prionoids (which might be up-taken through different mechanisms such as TNTs-mediated transmission, uptake of exosomes and transynaptic vesicles or by endocytosis) and slow down the pathological spreading, as described above.

But the unanswered question remains: how can DNAJB6 make astrocytes more resistant to the uptaken PolyQ Htt prionoids, therefore maintaining their capacity to act as “prion-reservoir” and counteract the cell-to-cell spreading?

As said before, PolyQ Htt prionoids, independently from the mechanisms of entry, can end up in the cytosol as vesicle-free aggregate species with strong seeding properties and capable to interact with endogenous proteins and organelles. Interestingly, the isoform DNAJB6b (Hanai et al., 2003) is cytosolic (and nuclear) allowing interaction with these PolyQ species. Hence, based on the knowledge on PolyQ Htt prionoids and DNAJB6, we can speculate the following possible (mutually non-exclusive) mechanisms underlying the protective activity of astrocytic DNAJB6:
1. **Shielding/capping of PolyQ Htt prionoids and slowing down of seeding processes**

DNAJB6 may bind to fiber ends, hereby such shielding/capping of the prionoids might slow down their growth into (more) toxic amyloid fibrils. Even though DNAJB6 was found to be most effective in preventing primary nucleations, DNAJB6 has been shown to be still quite effective in suppressing secondary nucleations (Kakkar et al., 2016). Yet, data from our lab showed that the seeding of endogenously expressed soluble non-expanded polyQ proteins by extracellular addition of polyQ fibrils (Ren et al., 2009) was only marginally affected by DNAJB6 (Kakkar et al., 2016). PolyQ Htt prionoids provide surfaces for events of seeding with cellular proteins (including normal Htt) (Kazantsev et al. 1999, Busch et al. 2003; Ren et al. 2009; Trevino et al. 2012; Holmes et al. 2013; Tan et al. 2015; Ruiz-Alrandis et al. 2016), and - as well as endogenous aggregates - they can interact with organelles and cytoskeleton (Chapter 2, section 2.4). The DNAJB6 shielding/capping might impede the seeding and the interaction of prionoids with these cellular elements in the astrocytes. By slowing down all the cytotoxic events derived by seeding and interaction, DNAJB6 might provide a protective effect against the PolyQ Htt prionoids in the astrocytes. Alternatively, DNAJB6 binding of the fibrils may lead to active detoxification mechanisms as described below.

2. **(De-)acetylation of PolyQ prionoids for processing**

DNAJB6 can interact with several histone deacetylases (HDAC), including HDAC4 and HDAC6 (Hageman et al., 2010). HDAC4 is required for the full anti-aggregation activity of DNAJB6 and inhibition of HDAC4 results in a loss of function of DNAJB6 (Hageman et al., 2010). The acetylation state of DNAJB6 and PolyQ Htt might therefore be crucial in the mechanism. Hence, we can speculate that PolyQ Htt prionoids in astrocytes shielded by DNAJB6 are subjected to HDAC-mediated (de)acetylation. Interestingly, previous studies showed that HDACs are involved in protein quality control (Pandey et al., 2007; Jeong et al., 2009). The acetylation mediated by DNAJB6 and HDAC4 in astrocytes might direct the prionoids towards some of the pathways described below, or promote their compartmentalisation in specific cell sites. In other words, the acetylation of the prionoids mediated by DNAJB6/HDAC recognition might be the starting point for detoxification processing as described below.

3. **Promoting the sequestration in cellular deposit sites**

Cells can sequester misfolded or aggregated proteins into specific cellular deposit sites to prevent their toxicity as a key strategy of defense against protein aggregation (Tyedmers et al., 2010). Aggresomes in mammalian cells are a transient form of regulated aggregate deposition (Johnston et al., 1998; Kopito et al., 2000; Garcia-Mata et al., 2002): their formation and movement along the microtubules of the cytoskeleton (Garcia-Mata et al., 1999) is mediated by the activity of the motor protein dynein and adaptor proteins, like histone deacetylase 6 (HDAC6) (Kawaguchi et al., 2003). Such sequestration may serve to transiently store the cargo, for further refolding (Nollen et al., 2001) or degradation (Park et al., 2013). Hageman and colleagues found that DNAJB6 can interact with HDAC6 (Hageman et al., 2010). We might therefore speculate that DNAJB6 in astrocytes recognizes and binds the PolyQ Htt prionoids and through its interaction with HDAC6, direct the prionoids to these cellular deposition sites. In support of this, recent data have shown that DNAJB6, together with HSP70, is involved in protein sequestration in yeast (Kumar et al., 2018). The sequestration of PolyQ prionoids in a cellular deposit site, mediated by DNAJB6, might be an effective...
protective strategy in astrocytes also because, differently from post-mitotic neurons, astrocytes can divide. As last resort, astrocytes might asymmetrically partitionate the sequestered prionoids and benefit one of the daughter cells with a lower aggregate load (“dilution effect”; Rujano et al., 2006; Fuentealba et al., 2008). The survived astrocytes might then continue to support the neuronal fitness and continuing in the uptake of other spreading prionoids.

4. **Disaggregation processing**
Disaggregation is characterized by the recognition of the aggregate by sets of HSPs that actively participate to the one-by-one extraction of misfolded polypeptides (Mogk et al., 2018). Disaggregation in mammalian cells is mainly mediated by HSPA/HSP70, assisted by specific set of co-chaperones - members of the HSP110 family and DNAJs (e.g. DNAJA2 and DNAJB1 in humans) (Nillegoda et al., 2015; Mogk et al., 2018) - that empowers HSPA/HSP70 to exhibit a potent, standalone disaggregation activity. Small HSPs (sHSPs) are thought to facilitate this “extraction” process by binding the aggregating substrates and changing the structure of the aggregates such that they remain in a (more) disaggregation-competent form (Nillegoda et al., 2015; Mogk et al., 2018). Although data from our lab showed that DNAJB6 is not per se capable to disaggregate PolyQ aggregates (Hageman et al., 2010), we might hypothesize that DNAJB6-capping of PolyQ Htt prionoids might still promote their recruitment in the disaggregation processing pathway. Similarly to sHPS, DNAJB6 might be capable to keep the PolyQ substrate in a disaggregation-competent form. DNAJB6 might then recruit HSPA via its J domain directing the prionoid toward a disaggregation processing.

5. **Re-engagement towards the autophagic flux or proteasomal degradation**
The J-domain allows to DNAJB6 to recruit HSPA, providing a link to direct the PolyQ Htt prionoids towards protein degradation pathways, such as autophagy and proteasome degradation. This might occur right after acetylation and disaggregation of the DNAJB6-capped PolyQ substrate. Previous data already suggest that DNAJB6 is involved in protein degradation and catabolic processes (Izawa et al., 2000; Watson et al., 2007, Kakkar et al., 2016). Although PolyQ Htt is known to be a poor substrate for these proteolytic pathways (Ciechanover et al., 2015), they might represent a line of defence against the toxicity of the prionoids in the astrocytes.

Future studies might explore how DNAJB6 is capable to protect astrocytes by starting from the above hypothesis. Data would provide further insights in the mechanisms of protection of DNAJB6 against PolyQ Htt, but also about the processing of toxic prionoids by astrocytes and how this influences the course of the pathology.

All the previous mechanisms relies on the idea that PolyQ Htt prionoids enter in astrocytes where they interact with DNAJB6. Would it be possible instead that DNAJB6 is first released by astrocytes and next uptaken by the suffering neurons expressing PolyQ Htt? In the next section, I will discuss this interesting option.
1.5 - Other glial cells might come into play

Our data showed that astrocytes are key players in the non-cell autonomous protection mediated by DNAJB6 against PolyQ Htt. Although our data suggest that other glial cells seem not crucial in the DNAJB6-related non-cell autonomous protection - emphasizing the pivotal role of astrocytes in the maintenance of brain homeostasis - still we cannot exclude the participation of other glial cell types, such as microglia in the protective mechanisms as described above. Microglia and astrocytes establish several functional interplays during brain disease, therefore an important line of research would be the investigation of how the modulation of the chaperonome in astrocytes influences such astrocyte-microglia interplay in HD.

Microglia are the resident macrophages in the central nervous system, they monitor the environment and respond to neural degeneration by switching to different activation states. Similarly to astrocytes (Section 3 of Chapter 2), microglial activation might play a dual role in HD either being neuroprotective or detrimental for neurons. They are fundamental actors in the mechanism of neuroinflammation because involved in the release of inflammatory cytokines and clearing of cell debris (Ywang et al., 2017). Microglial activation is a component of the pathogenesis of HD and it is believed to be triggered by PolyQ Htt-mediated cytotoxicity (Ywang et al., 2017). Accumulating evidences show that PolyQ Htt can trigger microglial activation (Crotti et al., 2015). Importantly, microglia hardly show PolyQ Htt aggregates in different HD rodent models and brains of HD patients (Jansen et al., 2017). Such less frequent presence of aggregates in microglia compared to neurons might be due to differences in protein quality control, and expression/activity of HSPs.

A cross-talk between astrocytes and microglia occurs during HD pathology, meaning that both the cell populations release molecules and other signals that are crucial for the regulation of their cellular activities (Crotti et al., 2015; Ywang et al., 2017). Such crosstalk is also important for the modulation of the processes of neuroinflammation (Crotti et al., 2015).

The interplay between astrocytes and microglia offers new perspectives to our findings. Astrocytes with potentiated chaperonome might be capable through the release of specific signals to modulate the microglia activation and neuroinflammation, maintaining the microglia state toward a neuroprotective path (e.g. control of cytokine release). The DNAJB6-potentiated astrocytes and microglia might also efficiently collaborate in limitating the spreading of PolyQ Htt prionoids.

One intriguing possibility would be to investigate the effects in HD pathology of the expression of protective chaperones in microglia. Given the crucial role of both glial cell types in maintaining neuronal homeostasis, a combined expression of protective chaperones in astrocytes and microglia might offer an additional layer of protection against PolyQ Htt aggregates.
2. Saving neurons is good, together with astrocytes is better: the “healthy-astrocytes” approach

The development of treatments of neurodegenerative diseases - a field that in the last decades showed several important advances in the understanding of the pathology, but with very limited success in finding a therapy - should be based on evidences that go beyond an exclusive neuron-centric view and that consider more wide aspects such as the strict functional interdependency between neurons and astrocytes (and other glial cells). Neurons are the “kingmakers” of the brain physiology and pathology, but an increasing amount of evidences is showing that NDs are diseases of neurons with a key co-participation of astrocytes and other glial cells: if neurons are sick, also astrocytes are, and vice versa.

An increasing amount of evidences, including our findings indicate that targeting the prion-like spreading of PolyQ HTT prionoids in order to slow down the pathological processes in HD might have a therapeutical value.

Although further investigations are needed to confirm whether and how the spreading occurs in vivo, the data from the above suggest the following key points:
1. The mechanisms of spreading of PolyQ HTT prionoids, which involve TNTs communication, exosomes / exophers release, and transsynaptic propagation, are not unregulated passive events due to the death of the cells. Rather, they are active and well-regulated processes that the donor cells use when they are not further capable to cope with the presence of these toxic species, used as mechanisms of defense in the early phase of the pathological process. Importantly, these mechanisms result to be less active and efficient when the disease is progressing and the cells are degenerating. If from one side, these mechanisms can temporarily protect the donor cell, from the other side, they can promote the spreading of the prionoids to the recipient cells.
2. Cell-to-cell contact seems an important factor to promote the spreading of PolyQ HTT, but it is not always required.
3. HTT prionoids can actively enter the cells embedded in vesicles or vesicles-free, and in a regulated manner (for example, by exosome uptake or by clathrin dependent endocytosis). However other mechanisms can also occur, such as passive membrane penetration or phagocytosis. The mechanism of uptake mainly depends by the types of acceptor cells, the aggregate species and disease stage.
4. Differently from other prionoids, PolyQ HTT prionoids, independently from the mechanisms of entry in the recipient cells, can end up in the cytosol. Here, these vesicle-free aggregate species have seeding properties and can interact with endogenous proteins (including components of the protein quality control, such as cytosolic HSPs). Further investigations are needed to understand if this occurs by escaping of the PolyQ HTT aggregates from the endocytic-lysosomal compartment.

Taking in consideration the above, one can speculate that by inhibiting the mechanisms involved in the spreading of PolyQ HTT prionoids, it would be possible to slow down the disease progression of HD. However, there is a number of limitations to consider about this approach:
• Some of these mechanisms such as the exosome release and the TNTs-mediated communication are used by the donor cells as defense strategies against the aggregate toxicity. A complete blockage of the spreading could result in an acceleration of the pathological process in the most vulnerable cells (likely neurons). Therefore, the magnitude by which the spreading should be inhibited and the specific cells to target are factors that must be taken in consideration to design an effective therapy.

• The inhibition of some of these mechanisms could have detrimental consequences for the cell functionality and viability. Some of them, such as transynaptic communication, endocytosis, and TNTs communication can be blocked by a pharmacological treatment (for example by using respectively botulinum neurotoxins, chlorpromazine or cytochalasin B) (Ruiz-Alrandis et al., 2016; Pecho-Vrieseling et al., 2014; Bukoreshtliev et al., 2009). However, we must consider that they also are key fundamental cellular processes that are important for the transport of molecules, nutrients, neurotransmitters and more, and do not involve only the spreading of PolyQ HTT prionoids. Moreover, many pharmacological inhibitors might not target the selected pathway in a specific manner, and therefore produce side-effects, due to off-target inhibitions.

• The blockage of the spreading of PolyQ HTT aggregates might likely not suffice to efficiently slow down the pathology as the formation of the aggregates still occurs in a cell-autonomous manner (Walsh et al., 2016).

Here we speculate that a “healthy astrocytes” approach against toxic PolyQ HTT prionoids based on the overexpression of the protective chaperone, such as DNAJB6, in the astrocytes (as investigated in our study), might slow down the pathological processes and overcome some of the previous described limitations and side effects.

The combined strategies to provide cell-autonomous and non-cell autonomous protection by expressing DNAJB6 in different types of brain cells - neurons or astrocytes respectively - might be therapeutically very effective. DNAJB6 is expressed ubiquitously (Hageman et al., 2010) and in vivo brain-specific overexpression of DNAJB6 is not associated with deleterious phenotypic effects (Kakkar et al., 2016): these findings support the idea that DNAJB6 might be a safe therapeutic target in HD and that its expression might be promoted in both neurons and astrocytes.

As shown by our findings, such approach also provides new insights about the functional role of chaperones in the brain during the pathology of PolyQ diseases (when these chaperones are expressed in the different cells of the nervous tissue). The non-cell autonomous protection via HSP expression in astrocytes highlights the unique role of these glial cells in supporting and protecting neurons and paves the way towards new therapeutic strategies against HD.

In more general terms, we can conclude that the potentiation of the brain cell chaperonome might have a great therapeutical value in neurodegenerative diseases. By overexpressing the chaperone in neurons and stopping the protein aggregation, we aim to directly protect these cells. However, this approach may be scaled-up and improved by also boosting the chaperonome of astrocytes: in this case, we do not only provide protection in a cell-autonomous manner, but also in a non-cell autonomous manner, promoting the capacity of astrocytes to keep the neuronal viability and fitness.
In conclusion, astrocytes can indeed be an important target of treatment in neurodegenerative diseases. Paraphrasing the words of Prof. Ben Barres in a 2008 perspective article, many drug trials exclusively target neurons, whereas the role of astrocytes and other glial cells in the pathological process still has received little attention. If the astrocytes that physiologically support neurons are killed, “how can the neurons be saved by just targeting the neurons?” (Barres, 2008).


