CHAPTER 5

Astrocytic expression of the chaperone DNAJB6 results in non-cell autonomous protection in Huntington’s disease

ABSTRACT

Several neurodegenerative diseases like Huntington’s, a polyglutamine (PolyQ) disease, are initiated by protein aggregation in neurons. Furthermore, these diseases are also associated with a multitude of responses in non-neuronal cells in the brain, in particular glial cells, like astrocytes. These non-neuronal responses have repeatedly been suggested to play a disease-modulating role, but how these may be exploited to delay the progression of neurodegeneration has remained unclear. Interestingly, one of the molecular changes that astrocytes undergo includes the upregulation of certain Heat Shock Proteins (HSPs) that are classically considered to maintain protein homeostasis, thus resulting in cell autonomous protection. Previously, we discovered DNAJB6, a member of the human DNAJ family, as potent cell autonomous suppressor of PolyQ aggregation and related neurodegeneration. In the Chapter 4, we confirmed that the cell autonomous protective function of human DNAJB6 against PolyQ aggregation was also maintained in D. melanogaster. In this Chapter, using the same cell type specific expression systems in D. melanogaster, we show that exclusive expression of DNAJB6 in astrocytes (that do not express PolyQ protein) can delay neurodegeneration and expands lifespan when the PolyQ protein is exclusively expressed in neurons (that do not co-express DNAJB6 themselves). This provides direct evidence for a non-cell autonomous protective role of astrocytes in PolyQ diseases.

KEYWORDS

Neurodegeneration - Polyglutamine - Aggregation - Astrocytes - Chaperones - DNAJB6- prion-like aggregate spreading
1. Introduction

Several neurodegenerative diseases (NDs), including Huntington’s disease (HD), are characterized by protein aggregation in brain cells and this is thought to initiate or drive their pathology and degeneration (Kakkar et al., 2014; Kampinga and Bergink, 2016). Heat Shock Proteins (HSPs), which are fundamental regulators of the Protein Quality Control (PQC) system (Hartl et al., 2011), are considered to be protective against the aggregation of disease-related proteins and thereby capable to delay the onset of NDs (Hageman et al., 2011; Vos et al., 2010; Hageman et al., 2010; Kakkar et al., 2016a). In previous studies from our group, we identified DNAJB6, a human HSP of the DNAJ family and HSP70 co-chaperone, as a very potent and cell autonomous inhibitor of protein aggregation in different in vitro and in vivo models of Polyglutamine (PolyQ) diseases (Hageman et al., 2011; Kakkar et al., 2016b), including Huntington’s disease (HD, OMIM:#143100), which is characterized by the aggregation of mutant PolyQ Huntingtin (Htt).

In the study described in the Chapter 4, we showed that human DNAJB6 provides cell autonomous protection against the toxicity of PolyQ proteins (PolyQ-HTT for HD, and PolyQ-ATXN3 for SCA3) in D. melanogaster. Notably, the HD model used in this study (and described in the previous Chapters) exclusively express PolyQ-HTT and DNAJB6 in the same neurons in the fly brain. In line with our previous findings, we confirmed that the DNAJB6 cell autonomous protection is associated with a reduction in the PolyQ-HTT aggregate load in fly brains.

In addition to protein aggregation, a common hallmark of nearly all aggregation-related NDs is the reactivity of astrocytes, a specific type of glial cells that normally contributes to the brain homeostasis and supports the neuronal functions (Sofroniew and Vinters, 2010). The astrocytic response during aggregation-related NDs is found in degenerating areas of the brain and is characterized by a spectrum of progressive molecular, cellular and functional changes (Sofroniew and Vinters, 2010; Ben Haim et al., 2015), which are most likely a response triggered by the neuronal damage. On one hand, the (early) astrocytic response has repeatedly been suggested to serve in a protective manner to counteract the progression of neurodegeneration. On the other hand, (chronic) astrocytic reactivity has also been suggested to be a maladaptive response that leads to disease aggravation (Sofroniew, 2009).

Interestingly, one of the molecular changes detected in astrocytes in human brains affected by neurodegeneration includes the up-regulation of certain HSPs (Durrenberger et al., 2009; Seidel et al., 2012; Wilhelmus et al., 2006; Dabir et al., 2004 and Chapter 2), including DNAJB6 (Durrenberger et al., 2009). However, the functional implications of HSPs-up-regulations for the progression of neuronal degeneration have not yet been established.

To fully explore whether and how the expression of HSPs in astrocytes contributes to neuroprotection in NDs, we generated D.melanogaster lines that exclusively express PolyQ-HTT in neurons whilst co-expressing human DNAJB6 in astrocytes to study non-cell autonomous effects. As explained in the previous Chapters, to generate the D.melanogaster lines for this study, we used a
specific combination of parental lines carrying cell-specific promoters and transgenes that allow the selective expression of PolyQ-HTT and DNAJB6 respectively in neurons and glial cells/astrocytes in the fly brain (Chapter 3). Different combination of these parental lines have been used to also generate the experimental flies used in the study presented in the previous Chapter.

Strikingly, the exclusive expression of DNAJB6 in astrocytes provides non-cell autonomous protection against progressive neuronal degeneration and prolongs organismal lifespan, although not accompanied by a reduction in the PolyQ-HTT aggregate load in the fly brains (therefore differently from what observed when the chaperone is expressed together with PolyQ-HTT in the same neurons). Rather, under these conditions, in flies that express DNAJB6 in astrocytes, a high fraction of astrocytes now contain neuronal derived PolyQ-HTT aggregates, in line with the suggestion that astrocytes might take up prion-like PolyQ-HTT aggregates species, a capacity that is enhanced by DNAJB6 expression (Brundin et al., 2010; Costanzo and Zurzolo, 2013; Ren et al., 2009; Babcock et al., 2015).

2. Materials and methods

Vectors

UAS/LexO vectors were obtained by cloning the sequences of HttQ100-mRFP (Prof. T. Littleton Group, MIT) or V5-DNAJB6 (isoform B) or eGFP (Clontech) in the multiple cloning site of pUAS attB or plExO attB (Prof. K. Basler Group, UZH). Driver (Promoter cell-specific expression) vectors were obtained starting from the backbone of plasmids pDPP-Gal4 attB or pDPP-LG attB or pDPP-LhG attB (Prof. K. Basler Group, UZH). DPP promoter was substituted with the sequence of promoter elav (pan-neuronal, from pElav-Casper vector, Prof. Liqun Luo, Stanford University), repo (pan-glial, from pENTRY-D-TOPO-Repo4.3 vector, Prof. C. Klämbt, University of Münster) or alm (astrocytic, from pAlrm-Casper vector, Prof. M. Freeman, UMASS). All obtained vectors were sequenced. See table T1 of Chapter 3 for vectors list.

Generation of new D. melanogaster lines

The D. melanogaster lines of table T1 were obtained by injection and transformation of embryos with the above mentioned attB vectors, based on attP-site specific PhiC31 integrase system, by Best Gene Inc. injection service (https://www.thebestgene.com/HomePage.do). D. melanogaster lines from Bloomington Drosophila Stock Center were also used: gmr-Gal4 (Line BDSC #1104 in Fig.3); alrm-Gal4 (Line BDSC #67031 in Fig.3); UAS-mCD8-GFP (Line BDSC #5130); UAS-mCD8-RFP (Line BDSC #27391); UAS-ATXN3-Q78 (Line BDSC #8150 in Fig.3); gmr-QF2 (Line BDSC #59283 in Fig.3 was a gift from C. Potter, Baltimore, MD, U.S.A.). gmr-QF2 and QUAS-ATXN3-Q78 are based on the Q expression system in D.melanogaster (Potter et al., 2010). All the lines were isogenised to remove background mutations by backcrossing each of them for 6 generations with the control stock w1118 line. See Chapter 3 for other details.
Genotypes

- Fig. 1A and 1B: w(-); UAS mCD8-RFP (or UAS HttQ100-mRFP) / alrm LhG; elav Gal4 / LexO eGFP.
- Fig. 3: for non-cell autonomous rescue: w(-), gmr-QF2; alrm-Gal4:QUAS ATXN3-Q78/ UAS DNAJB6 (or +); +/-.
- Fig. 5A and S3A: 1) control line (red): w(-); UAS HttQ100-mRFP / promoter (repo or alrm) LhG; elav Gal4 / LexO eGFP. 2) Rescued line (blue): w(-); UAS HttQ100-mRFP / promoter (repo or alrm) LhG; elav Gal4 / LexO DNAJB6.
- Fig. 7A and S4B: 1) Control (Fig. 7A, panel 1 and Fig. S4B, panels 1-3): w(-); UAS CD8-mRFP / alrm LhG; elav Gal4 / LexO eGFP. 2) Condition 1 (Fig. 7A, panel 2 and Fig. S4B, panels 4-6): w(-); UAS HttQ100-mRFP / alrm LhG; elav Gal4 / LexO eGFP. 3) Condition 2 (Fig. 7A, panel 3 and Fig. S4B, panels 7-9).
- Fig. 7B and Movie M1): w(-); UAS HttQ100-mRFP / alrm LhG; elav Gal4 / LexO DNAJB6.
- Fig. 8A and S5A: 1) Control line (red): w(-); UAS HttQ100-mRFP / alrm LhG; elav Gal4 / LexO eGFP. 2) Rescued line (blue): w(-); UAS HttQ100-mRFP / alrm LhG; elav Gal4 / LexO DNAJB6.

Antibodies and reagents

Antibodies (dilutions are indicated in brackets for western blots (WB) and immunofluorescence (IF)) against huntingtin (Chemicon, MAB2166, WB 1:5000), eGFP (Clontech-Living Colours, cat.No.632375, WB 1:5000), α-tubulin (Sigma Aldrich, clone DM1A, WB 1:2000), V5 epitope tag in DNAJB6b (Thermo Fisher Scientific, cat. No.R960-25, WB 1:2000, IF 1:50), NC-82 (DSHB, WB 1:5000) were used. DAPI for nuclei staining (cat.No.D1306) was from Thermo Fisher Scientific. 20% SDS Solution (cat.No.1610418) was from BioRad. PBS components (NaCl cat.No.S9888, KCl cat.No.P9541, Na2HPO4 cat.No.255793, KH2PO4 cat.No.V000225), Tween-20 (cat.No.P2287), Triton X-100 (cat.No.T8787), Bovine Serum Albumin (cat.No.A2058, BSA), glycerol (cat.No.G5516), 3.7% Formaldehyde (cat.No.11-0705 SAJ), Tris base (cat.No.T1503) and β-mercaptoethanol (cat.No.M6250) were from Sigma Aldrich.

D. melanogaster stocks maintenance

All stocks and experimental flies were kept in polystyrene vials 25x95 mm filled with 8 ml/vial of solidified media (17 g/l Agar; 26 g/l Yeast; 54 g/l Sugar; 1.3 mg/l Nipagin). All experimental flies were maintained in a humidified and temperature controlled incubator at 25 °C on a 12 hours’ light and 12 hours’ dark cycle (Premium ICH Insect Chamber, Snijders Labs). Experimental flies, anesthetized on a CO2 pad, were selected according to their gender and phenotype by light microscope visualization.

Lifespan curves

Parental flies (5-6 females and 5-6 males) were kept in vial for 3 days and then removed. Offspring virgin flies were collected in the same 24 hours. For each analysed group, ≈100 flies of specific
gender and phenotype were collected and kept in new vials (10 flies/vial). Flies were transferred to new vials containing fresh medium every 2 days and deaths were scored daily. Statistical significance of curves differences analysed with Log rank (Mantel-Cox) test (test 1) and Gehan-Breslow-Wilcoxon test (test 2) using Graph Pad Prism Software Version 5.00. All curves comparisons were made from flies analysed in the same experiment. T50 was defined as the time point at which 50% of the initial population has died.

Western Blotting *D. melanogaster* total head lysates preparation

30-40 *D. melanogaster* adults with specific phenotype, gender, age (days after pupal eclosion) and condition were collected; after freezing in liquid nitrogen and vortexing of entire flies, separated heads were collected, counted and lysed in SDS-rich buffer (SDS 1.45%, Glycerol 20%; Tris Base 0.2 M. 2.5 µl of buffer/head) using sonication (3 pulses of 50 Watt for 5 seconds). Homogenized lysate was then centrifuged at 1000 x g for 3 seconds to separate cuticle debris from supernatant. Proteins in supernatant were collected and quantified using spectrophotometry (Implant NanoPhotometer UV/Vis). Protein content was equalized. Samples, supplied with β-mercaptoethanol 5% and bromophenol blue, were boiled at 99 °C for 5 minutes. Equal amounts of volume were resolved on SDS-PAGE. Flies of the same line were collected from different vials and the entire experiment was repeated at least 2 times.

Western Blotting and Blot quantification

Following the preparation of samples, proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane and processed for Western Blotting. Primary antibodies (at concentrations mentioned above) were prepared in 3% BSA/PBS-Tween 20 0.1%, secondary antibodies at concentration 1:5000 (Invitrogen, horse peroxidase conjugated to IGG or IGM) in 5% milk/PBS-Tween 20 0.1%. For visualization membranes were incubated with Pierce ECL Western Blotting substrate (cat. No. 32106) for 2 minutes and visualized using ChemiDoc Touch Imaging System (BioRad). Blots have been quantified using Image Lab Version 5.2.1 software (BioRad).

Analysis of eye degeneration at light microscope

For each condition, at least 80 fly eyes were checked. The fraction of the eyes that showed degeneration phenotype were calculated as previously (Vos et al., 2010). For each analysed line, eyes of at least 40 flies were scored. The results were average of at least three independent experiments.

*D. melanogaster* Immunofluorescence (IF), Imaging and Punctae Quantification

Brains of experimental *D. melanogaster* adults with specific phenotype, gender, age (days after pupal eclosion) and condition were dissected by light microscope visualization, in 0.1% Triton X-100 PBS 1x buffer and then fixed in 3.7% Formaldehyde, 0.1% Triton X-100 PBS 1x buffer for 20 minutes at room temperature. Staining of brains was performed by blocking not specific sites using 2% BSA,
0.1% Triton X-100 PBS 1x buffer for 30 minutes at room temperature, then incubating with primary antibody (dilution 1:50) in blocking buffer for 2 days at 4°C and finally with secondary Alexa-conjugated dyes antibody (Invitrogen, Alexa Fluor 488 goat-anti-mouse; dilution 1:500) and DAPI (final concentration 0.2 µg/µl) in blocking buffer for 2 days at 4°C, to respectively visualize primary antibodies and nuclei. Brains were mounted between glass slide and coverslip, embedded in mountant solution (CitiFluor, Agar Scientific) and visualized by confocal microscopy within 24 hours. IF images of D.melanogaster brains were captured using confocal laser scanning microscope (Leica TCS SP8). Z-stack images were obtained to check for the punctae in cells at different Z-planes. Quantification of the punctae in cells was carried out manually. Photoshop, and Image J software were used for image processing and to generate the 3D reconstruction (Movie M1). Statistical significance of values differences analysed with 1-way ANOVA test using Graph Pad Prism Software Version 5.00.

3. Results

Astrocytes are generally considered to be more resistant to PolyQ-HTT aggregation and toxicity than neurons (Jansen et al., 2014; Jansen et al., 2016) and hence PolyQ diseases are primarily thought to be due to neuronal dysfunction and death. Moreover, although chronic astrocyte reactivity is considered to be detrimental, the initial astrocytic responses have been suggested to actually counteract the progression of neurodegeneration. However, direct evidence to substantiate this hypothesis is lacking. Importantly, altered expression of several HSPs in astrocytes are amongst such molecular changes (Durrenberger et al., 2009; Seidel et al., 2012; Wilhelmus et al., 2006; Dabir et al., 2004, and Chapter 2), the relevance of which has not been demonstrated so far.

In order to test whether and how the expression of HSPs in astrocytes might be relevant for the non-cell autonomous neuroprotection in PolyQ diseases, we used D.melanogaster as in vivo model of HD, and expressed DNAJB6 in glia/astrocytes (the same short nuclear and cytosolic isoform B used in the experiments of the previous Chapter; Hanai et al., 2003) to verify its protective activity against the toxicity mediated by the expression of HttQ100-mRFP in neurons (the same human PolyQ-HTT exons 1-12 construct used in the previous Chapter; Weiss et al., 2012). To do so, we used, also for these experiments, the GAL4-UAS (Brand and Perrimon, 1993) and LexA-LexO (Yagi et al., 2010) expression systems that can drive expression of transgenes in a completely independent and non-overlapping manner.

Astrocytes have been suggested to play a key role in many aggregate-related NDs, including HD (Sofroniew and Vinters, 2010; Ben Haim et al., 2015). On the one hand, it has been suggested that the neuronal damage leads to astrocyte reactivity, which compromises their functions in nourishing and protecting neurons. On the other hand, data from rodent HD models and indirect evidence derived from human HD brains (Jansen et al., 2016) suggested that PolyQ-HTT may also be directly toxic to astrocytes, thus contributing to their chronic reactivity and to a neurodegenerative phenotype. In line with these findings and those from rodent models expressing PolyQ-HTT in astrocytes (Shin et al., 2005; Bradford et al., 2009), we observed that the selective expression of HttQ100-mRFP in the astrocytes of D. melanogaster (using the validated and characterized
astrocyte-specific \textit{alrm}-Gal4 promoter; Doherty et al., 2009) reduced the lifespan of the cohort (Fig. S1A-B).

In the previous Chapter, we established that the neuronal cell-autonomous protection of human DNAJB6 is recapitulated in the \textit{D. melanogaster} system. Here we addressed whether the up-regulation of chaperones, like DNAJB6, in astrocytes might non-cell autonomously protect neurons against PolyQ mediated degeneration. To test this hypothesis, we selectively expressed DNAJB6 in astrocytes (using the specific promoter \textit{alrm}-LexA; Doherty et al., 2009), or in all glial cells (using the pan-glial promoter \textit{repo}-LexA; Xiong et al., 1994), in the flies co-expressing HttQ100-mRFP in neurons (using the validated and well-characterized pan-neuronal promoter \textit{elav}-Gal4 (Yao et al., 1993). Fig. 1 and Chapter 3).

Whereas PolyQ-HTT is ubiquitously expressed in HD human brains and rodent models of HD like R6/2 mice, in our fly model the PolyQ protein, HttQ100-mRFP, is exclusively expressed in neurons, but not in astrocytes or glial cells (Fig. 1): this enabled us to exclusively investigate whether astrocytic/glial DNAJB6 expression might exert non-cell autonomous protective effects against the toxicity mediated by the PolyQ proteins expressed in neurons. The tissue specific-expression via each of these promoters was confirmed by confocal microscopic analysis: control brains with mCD8-RFP in neurons and eGFP in astrocytes show non-overlapping staining as evidenced by diffuse mCD8-RFP fluorescence in neuronal lobes (e.g. antennal lobes, mushroom bodies), surrounded by a network of eGFP-positive astrocytes with ramified processes (Fig. 1A). Staining of brains of flies expressing neuronal HttQ100-mRFP was slightly lower. HttQ100-mRFP foci reminiscent of aggregate formation, were mainly detected in the neurons (Fig. 1B).
Figure 1: Co-expression of different transgenes in neurons and astrocytes of D. melanogaster brain. A,B) Representative confocal images of brains from 15-day-old adult male flies co-expressing transgenes in different cell types using the two independent expression systems Gal4-UAS and LexA-LexO: mCD8-RFP (Fig. 1A) or HttQ100-mRFP (Fig. 1B) in all neurons and eGFP in astrocytes. Neuronal lobes and regions rich of HttQ100-mRFP punctae are shown. In the detail of the merged image of Fig. 1A, astrocytes (closed circles) with their processes (arrows) are indicated. In the detail of the merged image of Fig. 1B, neurons bodies (dashed circles) with HttQ100-mRFP punctae (arrows) and astrocytes (closed circles) are indicated. Scale bar: 100 µm. Magnification: 20x. Genotypes in Materials and Methods.

Strikingly, the exclusive expression of DNAJB6 in all glial cells significantly expanded the lifespan of flies expressing pan-neuronal HttQ100-mRFP, increasing the T50 by 23% (Fig. 2A, Fig. S2A, E). In fact, the exclusive expression of DNAJB6 in astrocytes only resulted in a similar lifespan extension in flies expressing pan-neuronal HttQ100-mRFP (by 25%; Fig. 2B, Fig. S2B, E), indicating that the expression of DNAJB6 in this specific type of glial cells alone suffices to provide non-cell autonomous protection against PolyQ mediated toxicity. The lifespan extension in these lines is also not as pronounced as observed when DNAJB6 is expressed in neurons (in which T50 is increased by 43%; Fig. 4A, Fig. S2A, S2C of Chapter 4), suggesting that the pro-survival mechanisms mediated by DNAJB6 expression in different cell types might be mechanistically distinct and not completely equal in terms of effectiveness. Similar to what we found for exclusive DNAJB6 expression in neurons, DNAJB6 expression in all glial cells (Fig. S2C, E) or astrocytes (Fig. S2D, E) did not affect lifespan in control flies, indicating its specific effect on HttQ100-mRFP related toxicity.

We also found that both glial or astrocytic expression of DNAJB6 at lower levels than those in the previous experiments in Fig. 2, (using for DNAJB6, the weaker promoters repo-LG or alrm-LG
respectively, instead of repo-LHG or alrm-LHG; Yagi et al., 2010), did not lead to a non-cell autonomous protection in terms of lifespan in the pan-neuronal HttQ100-mRFP D. melanogaster model (Fig. S2F, S2G and S2J for glial DNAJB6; Fig. S2H, S2I and S2J for astrocytic DNAJB6). These data suggest that, similarly to what we observe for the cell autonomous protection by neuronal DNAJB6, also the DNAJB6-mediated non-cell autonomous protection depends on the level of the expression of the protective chaperone.

**Figure 2: Effects of glial or astrocytic DNAJB6 expression on lifespan in a pan-neuronal HttQ100-mRFP D. melanogaster model.** A, B) Lifespan of isogenised male flies co-expressing neuronal (N>) HttQ100-mRFP with either glial (G>) DNAJB6 or eGFP (Fig. 2A) or astrocytic (A>) DNAJB6 or eGFP (Fig. 2B). Lifespan of DNAJB6-expressing line (blue curve) is significantly expanded compared to the control line (red curve) both for Fig. 2A and Fig. 2B. Additional control lines, comparisons, statistics and genotypes are provided in Fig. S2A, B and E.

In a similar approach, we also found that DNAJB6 expression in astrocytes alleviated the level of eye degeneration caused by expression of ataxin-3 (ATXN3-Q78) in fly ommatidia (Fig. 3), in a different set of experiments. This truncated ATXN3 carries an expanded PolyQ (Q78) (Warrick et al., 1998), responsible for spinocerebellar ataxia type 3 (SCA-3; OMIM:#109150) (Costa and Paulson, 2012). Interestingly, whereas the neuronal expression of DNAJB6 leads to a near to complete eye protection in the same SCA-3 D. melanogaster model (Fig. 2, Chapter 4), the expression of DNAJB6 in astrocytes does not suffice to prevent depigmentation, but did attenuate progression into necrosis (Fig. 3A).
Figure 3: Non-cell autonomous protective activity of DNAJB6 against PolyQ-ATXN3 mediated degeneration in *D. melanogaster* ommatidia. A) Top panels show representative images of eyes of 1-day old adult female flies expressing the indicated transgene. Eye degeneration is quantified as the percentage of eyes showing either depigmentation (mild degeneration) or black necrotic spots (dotted line, arrowhead) (severe degeneration). Data are compared using an unpaired t test (SD; **: \( P \leq 0.01 \), ***: \( P \leq 0.001 \)). Genotypes in Materials and Methods.

In order to investigate the mechanism underlying this non-cell autonomous protection observed in the HD model, we first considered the possibility of intercellular transmission of DNAJB6 from glial cells to neurons (Takeuchi et al., 2015) (Fig. 4).
Figure 4: Schematic outline of possible intercellular transmission of DNAJB6 from glial cells/astrocytes to neurons in which the chaperone would reduce aggregation of PolyQ-HTT.

For this hypothesis to be true, such a transfer should lead to reduced aggregation of HttQ100-mRFP, similar to what we observed for the cell-autonomous protection via the expression of DNAJB6 in neurons (Fig. 4B, Fig. S2D of Chapter 4). However, DNAJB6 expression in all glial cells or astrocytes was not associated with a comparable dramatic reduction of HMW HttQ100-mRFP aggregates in total head lysates (Fig. 5A, Fig. S3A). These data confirm the specificity of the used expression systems. More importantly, these data imply that the intercellular glia-to-neurons transmission of DNAJB6 is unlikely the only or the dominant mechanism for the non-cell autonomous protective effects of DNAJB6 on the lifespan of neuronal HttQ100-mRFP flies.
In view of these results, we next investigated if other additional pro-survival and non-cell autonomous mechanisms would take place when DNAJB6 is expressed in astrocytes. It has been suggested that PolyQ-HTT aggregates may spread throughout the brain in a prion-like manner (Brundin et al., 2010; Costanzo and Zurzolo, 2013; Ren et al., 2009; Babcock et al., 2015) (Fig. 6) and that this progressive spread of disease-associated proteins contributes to the progression of the neurodegenerative process. In theory, astrocytes might restrict such spreading via actively taking up prion-like protein species, a capacity that might be limited by the progressive accumulation of toxic aggregates (Pearce et al., 2015) (Fig. 6).
Figure 6. Schematic outline of possible spreading of PolyQ-HTT prion-like species, role of astrocytes in uptake of seeds and the non-cell autonomous protection of neurons through expression of DNAJB6 in astrocytes.

In line with this idea, confocal microscopic analysis of flies co-expressing neuronal HttQ100-mRFP and astrocytic eGFP, revealed that approximately ~10% of eGFP-expressing astrocytes, contained mRFP punctae (Fig. 7A, C; Fig. S4A, S4B). These puncta are reminiscent of protein aggregation and under all conditions are qualitatively associated with the relative amounts of insoluble proteinaceous material detected in our western analyses. This phenomenon was not due to indiscriminate protein transfer, as shown by the absence of mRFP punctae in eGFP astrocytes of the control line expressing neuronal non-aggregating membrane-bound mCD8-RFP (Fig. 7A, C; Fig. S4A, S4B). These data support the hypothesis that PolyQ HTT aggregates can indeed be transferred from neurons to astrocytes. Importantly, the combined expression of neuronal HttQ100-mRFP with astrocytic DNAJB6 resulted in a significant increase in the frequency of astrocytes with RFP punctae to ~50% (Fig. 7A-C; Fig. S4A, S4B), suggesting that DNAJB6 expression positively influenced the capacity of astrocytes of taking up such prion-like species (Fig. 6). 3-D confocal analyses confirmed that HttQ100-mRFP aggregates are indeed inside astrocytes (Fig.7B, Movie M1).
Figure 7: Effect of glial or astrocytic DNAJB6 expression in the pan-neuronal HttQ100-mRFP D. melanogaster model on aggregate distribution within the brain and overall neuronal fitness. A) Representative confocal images of brains from 15-day-old adult male flies co-expressing neuronal mCD8-RFP or HttQ100-mRFP and astrocytic DNAJB6b or eGFP. For each indicated condition, a representative picture of the region in central brain indicated in Fig. S4A is shown. In panels 1-3, detailed images are provided showing astrocytes (closed circles) and HttQ100-mRFP punctae in astrocytes (arrows), used for the counting in Fig. 7C. Anti-V5 antibody and Alexa488 secondary antibody were used for (V5 tagged) DNAJB6 detection. Scale bar: 25 µm. Magnification: 40x. Genotypes in Materials and Methods. B) Frames from the representative Movie M1 (3D confocal image reconstruction) at different time points, showing astrocytes expressing DNAJB6b and containing HttQ100-mRFP puncta (arrows) from 15-day-old adult male flies expressing the indicated transgenes. Frames show the same cells from different angles. Anti-V5 antibody and Alexa488 secondary antibody were used for (V5 tagged) DNAJB6 detection. Magnification: 63x. Genotypes in Materials and Methods. C) Percentage of green fluorescent astrocytes containing HttQ100-mRFP puncta in a confocal
section of brains from flies of Fig. 7A and S4B (2 brains, 20 sections per condition). Statistical significance analysed with 1-way ANOVA test (SEM, ***, p<0.001).

Similarly to the experiments investigating the cell autonomous protection of DNAJB6 (in the previous Chapter), to also confirm that the expression of astrocytic DNAJB6 indeed resulted in the actual and specific non-cell autonomous protection of neurons, we analysed the level of the neuronal marker NC82 (Wagh et al., 2006) in total fly head lysates. As previously explained, the expression level of NC82 provides a direct measure of the functional fitness of the neuronal population. We found NC82 levels to be strongly decreased in 15-day-old flies solely expressing neuronal HttQ100-mRFP (Fig. S4A and S4B of Chapter 4). This NC82-decline was not only alleviated in flies co-expressing neuronal DNAJB6 (cell autonomous protection, Fig. 5A, B and S4C, D of Chapter 4), but also in flies co-expressing DNAJB6 in astrocytes (non-cell autonomous protection, Fig. 8A, B and Fig. S5A, B), implying an overall improvement of overall neuronal fitness in both cases.

Figure 8: Effect of glial or astrocytic DNAJB6 expression in the pan-neuronal HttQ100-mRFP D. melanogaster model on overall neuronal fitness. A) Western blots of NC82 from total head lysates of 5 and 15-day-old adult female flies co-expressing neuronal (Neur.) HttQ100-mRFP and astrocytic (Astr.) DNAJB6 or eGFP. Anti-NC82 antibody for NC82. Tubulin was used as loading control Number of fly heads per each lysate sample and genotypes in Materials and Methods. An independent repeat of the experiment is shown in Fig. S5A, B. B) Quantification of NC82 of data in Fig. 8A at day 15 (signal normalized on tubulin; a.u.: arbitrary units).

Our experimental findings further also support the conclusion that the effects of DNAJB6 driven by repo or alrm drivers are not due to cell-autonomous effects of “leaked” neuronal expression; indeed, under the latter condition, we should have seen a reduction of HTTQ100-mRFP aggregation, which is not the case (Fig.5A, Fig. S3). Additionally, as shown in Fig. S3 of Chapter 4, we found that neuronal co-expression of HTTQ100-mRFP with DNAJB6 at lower levels compared to those in the experiment of Fig. 4 of Chapter 4 (using for DNAJB6, the weaker promoter elav-LG instead of elav-LHG respectively; Yagi et al., 2010), did not lead to cell autonomous protection. In this case, we could even detect DNAJB6 in Western blots (Fig.S3D). This makes in extremely unlikely that an eventual leakage of repo and alrm drivers, causing non-detectable expression of DNAJB6 in neurons, can be held responsible for the 24% increase in obtained life span (Fig.2A, B).
4. Discussion

Our data are a direct demonstration of a long-assumed hypothesis that glial cells, and particular astrocytes, might play a modulating role in HD, which as in many other NDs, initially and primarily affects neurons.

PolyQ-HTT is expressed in glial cells in the brain of HD patients and animal models (Jansen et al., 2016). In line with previous data from rodent models (Shin et al., 2005; Bradford et al., 2009), we confirmed that PolyQ-Htt exclusively expressed in astrocytes reduces lifespan in D. melanogaster. These data thus indicate that astrocytic PolyQ-HTT may contribute to HD pathology and that astrocytic damage is generally detrimental to the health of the brain, as notably observed in Alexander disease (OMIM:#203450), a genetic neurodegenerative disorder that primarily affects astrocytes by a dominant gain-of-function mutation of the GFAP gene (Sofroniew and Vinters, 2010; Olabarria and Goldman, 2017).

However, our data revealing neuroprotection by expression of DNAJB6 in astrocytes differ from these latter studies (Jansen et al., 2016; Shin et al., 2005; Bradford et al., 2009), as astrocytes in our D. melanogaster model do not express PolyQ-HTT. Still, we found that DNAJB6-expressing astrocytes provide a non-cell autonomous protective effects against degeneration of neurons expressing PolyQ-HTT. Thus, these data for the first time reveal that astrocytes empowered with sufficient chaperone activity, provided by DNAJB6, can provide protection against degeneration of neurons in HD.

DNAJB6, the chaperone used in this study, has cell-autonomous protective effects as previously shown (Kakkar et al., 2016b, Chapter 4 of this Thesis), which are strongly associated with its ability to prevent the initiation of PolyQ aggregation by the core-polyQ fragment, irrespective of regions flanking the expansion (Hageman et al., 2010; Månsson et al., 2014), for which it is known that they can affect the aggregation propensity of the PolyQ-containing protein (Kuiper et al., 2017). Our findings for both in vivo PolyQ HTT and PolyQ ATXN3 fly models show that also not only the cell autonomous, but also the non-cell autonomous effects of DNAJB6 are generic for PolyQ proteins.

Interestingly, the non-cell autonomous protection evoked by DNAJB6 expression in astrocytes is not associated with a reduction in PolyQ-HTT aggregation. Although it has been suggested that DNAJs and other HSPs can be transmitted between cells via exosomes (Takeuchi et al., 2015), the absence of an effect on total aggregate formation in our study implies that additional mechanisms may underlie the DNAJB6-mediated non-cell autonomous protection.

Despite the use of well-established promoters and two independent expression systems, it could still be argued that low levels of neuronal DNAJB6, due to an eventual leakage of the repo-LexA and alrm-LexA promoters and beyond detection limit of our microscopic analyses, could have been responsible for the observed protection evoked by DNAJB6 expression in astrocytes. However, using a weaker elav promoter to drive neuronal DNAJB6 expression than those used in the presented data (i.e. using the elav-LG instead of the elav-LhG promoter (Chapter 3 of this Thesis and Yagi et al., 2010)) we found no significant cell autonomous protection, whereas we could still detect neuronal
DNAJB6 expression (Fig. S3 of Chapter 4). This implies that it is highly unlikely that the observed protection by astrocytic DNAJB6 expression is due to leakage of the repo-LexA and alrm-LexA promoters.

This implies that the observed non-cell autonomous protection via astrocytic DNAJB6 expression is directly due to an activity of the chaperone in the astrocytes enhancing its fitness and function. We hypothesize such may delay the trans-cellular spreading of PolyQ HTT aggregates in the D.melanogaster brain. Cellular experiments have indeed demonstrated that PolyQ-HTT aggregates can enter cells where they can initiate intracellular seeding (Kakkar et al., 2016b; Ren et al., 2009) and can spread between neurons in the D.melanogaster brain (Babcock et al., 2015). Our data show that, in the same organismal system, these neuronal PolyQ-HTT aggregates can end up in astrocytes in agreement with observations by Pearce and colleagues (Pearce et al., 2015), using axotomised neurons. 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. The prion-reservoir capacity of astrocytes is likely limited by the toxicity of captured aggregates. However, the upregulation of chaperones like DNAJB6 may enhance this capacity, which is supported by our data showing that the frequency of astrocytes with inclusions is increased in case of DNAJB6 overexpression. This provokes the speculation that DNAJB6 can positively influence the prion-reservoir capacity of astrocytes and their ability to prevent the spreading of prion-like species in the brain, via its protective functions against PolyQ-HTT toxicity (Hageman et al., 2010). The increased prion-reservoir capacity of astrocytes ultimately results in an improvement of overall neuronal fitness and in an increased lifespan of PolyQ-HTT D.melanogaster model (Fig. 6).

An alteration in neuroinflammation could be an additional contributor to the enhanced neuroprotection due to the DNAJB6 expression in astrocytes, which hereby might promote a more neuroprotective or less neurotoxic pathway. Indeed, astrocytic activation has been claimed to have both positive and negative effects on the progression of PolyQ diseases (Sofroniew and Vinters, 2010).

In conclusion, our findings support the hypothesis that increasing astrocyte fitness and functions by chaperones like DNAJB6 (tentatively by increasing their prion-reservoir capacity) has protective significance in HD and possibly other protein aggregation-related NDs which show a pathology associated with these prion-like species such as Alzheimer’s (OMIM:#104300) and Parkinson’s (OMIM:#168601) (Brundin et al., 2010; Costanzo and Zurzolo et al., 2013).
REFERENCES

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**SUPPLEMENTARY DATA**

**Figure S1. Lifespan analyses of D.melanogaster line expressing PolyQ HTT in astrocytes.** A) Lifespan of isogenised male expressing HttQ100-mRFP or control transgene (eGFP or only promoter) in all astrocytes. Detailed statistics, comparisons and genotypes are shown in Fig.S1B. B) Genotypes of lines, comparisons, and statistical analysis of lifespan curves of Fig.S1A. Statistical significance analysed using ≈100 flies/group with Log Rank Mantel-Cox test (Test 1) and Gehan-Breslow-Wilcoxon Test (Test 2).
Figure S2 (A–E). Lifespan analyses of control lines depicted in Fig. 2. A) Lifespan of isogenised male flies (additional control lines of Fig. 2A) co-expressing neuronal (N>) HttQ100-mRFP and glial (G>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S2E. B) Lifespan of isogenised male flies (additional control lines of Fig. 2B) co-expressing neuronal (N>) HttQ100-mRFP and astrocytic (A>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S2E. C) Lifespan of isogenised male flies expressing glial (G>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S2E. D) Lifespan of isogenised male flies expressing astrocytic (A>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S2E. E) Genotypes of lines, comparisons, and statistical analysis of lifespan curves of: Fig. 2A and S2A; Fig. 2B and S2B; Fig. S3C; Fig. S3D. Statistical significance analysed using ≈100 flies/group with Log Rank Mantel-Cox test (Test 1) and Gehan-Breslow-Wilcoxon Test (Test 2).
Figure S2 (F-J). Lifespan analyses of lines with moderate expression of glial or astrocytic DNAJB6 in the pan-neuronal HttQ100-mRFP D. melanogaster model. F) Lifespan of isogenized male flies co-expressing neuronal (N>) HttQ100-mRFP with glial (G>) DNAJB6 or eGFP. Data shown are for moderate expression of LexO-DNAJB6/eGFP using repo-LG promoter. Additional control lines, comparisons, statistics and genotypes are provided in Fig.S2G and J. G) Lifespan of isogenized male flies (additional control lines of Fig. S2F) co-expressing neuronal (N>) HttQ100-mRFP and glial (G>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig.S2J. H) Lifespan of isogenized male flies co-expressing neuronal (N>) HttQ100-mRFP with astrocytic (A>) DNAJB6 or eGFP. Data shown are for moderate expression of LexO-DNAJB6/eGFP using alrm-LG promoter. Additional control lines, comparisons, statistics and genotypes are provided in Fig.S2I and J. I) Lifespan of isogenized male flies (additional control lines of Fig. S2H) co-expressing neuronal (N>) HttQ100-mRFP and astrocytic (A>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S2J. J) Genotypes of lines, comparisons, and statistical analysis of lifespan curves of Fig. S2F-G and S2H-I. Statistical significance analyzed using =100 flies/group with Log rank Mantel-Cox test (Test 1) and Gehan-Breslow-Wilcoxon Test (Test 2).
**Figure S3.** Effect of glial or astrocytic DNAJB6 expression in the pan-neuronal HttQ100-mRFP D. melanogaster model on aggregates formation. A) Independent repeat of experiment shown in Fig. 5A.
Figure S4. Supplemental experiments to Fig.7. A) Representative confocal image of D. melanogaster brain (expressing pan-neuronal mCD8-RFP, via elav), indicating the different neuronal lobes and highlighting the central brain region analysed for data of Fig. 7A, S4B, 7B and 7C. B) Representative confocal images of a repeat of the experiment shown in Fig.7A. In panels 1-9, detailed images are provided showing neurons (dashed circles), astrocytes (closed circles) and HttQ100-mRFP punctae in astrocytes (arrows).
Figure S5. Supplemental experiments to Fig.8. A) Independent repeat of experiment shown in Fig. 8A. B. I) Quantification of NC82 for data of Fig. S5A at day 15 (signal normalized on tubulin; a.u.: arbitrary units).

**MOBILE M1**

M1) Representative 3D reconstruction from confocal microscopic imaging showing astrocytes expressing DNAJB6b (green fluorescence) with HttQ100-mRFP puncta. Sample from 15-day-old adult male flies expressing neuronal HttQ100-mRFP and astrocytic DNAJB6b. Anti-V5 antibody and Alexa488 secondary antibody were used for (V5 tagged) DNAJB6 detection. DAPI was used for nuclei detection. Magnification: 63x. Genotypes in Materials and Methods. Available here: [https://www.sciencedirect.com/science/article/pii/S0969996118304327?via%3Dihub#ec0005](https://www.sciencedirect.com/science/article/pii/S0969996118304327?via%3Dihub#ec0005)

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