The role of the proteasome in Huntington's disease
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In this thesis, we examined the role of the proteasome in Huntington’s disease by addressing the following questions. Are intracellular polyQ fragments peptidase resistant and aggregation prone? Can we prevent their aggregation by chaperones? Are proteasomes sequestered and inactivated in HD? Can proteasomes degrade polyQ-expanded mHtt fragments, and if so, can we modify proteasome activity to improve mHtt degradation? These questions should resolve whether proteasomes are the good or the bad guys in HD, and whether manipulating proteasomal function can be used as a therapeutic strategy. To date, the proteasome has been considered as the bad guy in HD due to either the release of toxic polyQ fragments when degrading mHtt or due to its impairment.

Summary

In chapter 1 we give an short introduction to HD and proteasomes and their connection with each other in the cell, and we introduce the topics discussed in this thesis. In chapter 2 we review studies related to the role of proteasomes in HD to get a better insight in the current views and perspectives. The general idea is that proteasomes are impaired in HD and are unable to cleave polyQ sequences, although some studies show contradictory results. This may partly be due to the comparison of in vitro data to animal studies. Performing additional studies in living cells could give important answers that cannot be found in in vitro or in animal studies. In addition, we discuss the composition of the proteasome and how the activity of the proteasome can be modified by immunosubunits and proteasome activators.

The claim that proteasomes are unable to cleave inside polyQ sequences led to two possible scenarios. First, proteasomes could become clogged by the expanded polyQ sequences as they cannot be released by the proteasome. Second, polyQ peptides are released into the cellular environment where they rapidly initiate aggregation. In chapter 3 we show that when polyQ peptide release by the proteasome is mimicked, aggregation is initiated when the peptides exceed the disease-related threshold of approximately 37 glutamines. As has been observed in HD, other proteins with short polyQ tracts are recruited into polyQ peptide-initiated aggregates, as well as components of the UPS and chaperones, albeit during different stages of aggregation. Taken together, this indicates that when proteasomes release expanded polyQ peptides, it represents a model for aggregate formation that is common in all polyQ disorders.

In chapter 4, we examined whether the chaperones DNAJB6 and DNAJB8, which suppress aggregation and toxicity of polyQ-expanded proteins, also suppress aggregation induced by polyQ peptides. We show that both chaperones efficiently reduce aggregation of polyQ peptides, whereas the soluble levels of these peptides increase. Therefore, we conclude that these chaperones keep polyQ peptides in a soluble form, which allows degradation of these hazardous fragments before aggregation can occur. Furthermore, we also observed that these chaperones are recruited into polyQ peptide aggregates, which is a contra-productive process as the sequestered chaperones cannot affect peptide aggregation anymore. This data is in agreement with the findings of Månsson and colleagues, who showed that DNAJB6 efficiently prevents aggregation of polyQ peptides in vitro, but not of polyQ-expanded proteins.

While polyQ peptides were proven to be aggregation prone and initiate similar aggregation features as observed in HD, we next examined whether proteasomes are restricted in degrading polyQ-expanded mHtt fragments. In chapter 5, we studied whether proteasomes are capable of degrading mHtt entirely or whether expanded polyQ peptides are released. In order to circumvent degradation by the autophagic pathway, we added a degradation signal to mHtt for targeting towards proteasomal degradation. In a ubiquitination-dependent fashion and in agreement with the Nend rule, mHtt became a short-lived protein that was rapidly recognized and degraded by the proteasome. Surprisingly, no aggregation was observed when mHtt was targeted to the proteasome, and fragments containing the polyQ sequence were only temporarily observed in vitro. This indicates that proteasomes can efficiently degrade mHtt both in cells and in vitro, but we cannot exclude that the proteasome generates polyQ peptides while degrading the mHtt protein and that these polyQ peptides are subsequently degraded via additional rounds of 20S degradation in vitro or by other proteases or peptidases in vivo.

To further explore the role of the proteasome in HD, we next focused on proteasomal functioning in cells expressing mHtt. The questions that we wanted to address were, if proteasomes are indeed irreversibly sequestered into aggregates, is the activity affected and can we manipulate proteasome activity to improve mHtt degradation? To answer these questions, we developed various methods to examine proteasome subunit incorporation, intracellular proteasome distribution and dynamics, proteasome activity and interaction of proteasomes with proteasomal activators. In chapter 6, we describe these methods and show that the efficiency of incorporation dramatically differs between various fluorescently-tagged proteasomal subunits, with β5i being the best candidate to be used for proteasome studies. We further explored and compared various methods to determine proteasome activity in cells and cell lysates, and after separation on SDS and native gels. Finally, we discuss how interactions between the 20S core and proteasome activators, such as the PA28αβ activating cap, can be measured by FRET-FLIM in living cells and which steps are needed to optimize interactions for FRET-FLIM measurements.

These techniques, we used to challenge the generally accepted concept of proteasome impairment in HD, which is mainly based on immunostainings of fixed cells and short-term FRAP experiments. As a consequence, it is assumed that proteasomes may become impaired due to an irreversible withdraw from the cell when recruited into aggregates, or proteasomes could become impaired due to clogging by the aggregating polyQ fragments. In chapter 7 we show by fluorescence pulse-chase experiments that proteasomes are not trapped in aggregates, but are dynamically recruited albeit with slow kinetics. When proteasomes that are recruited into aggregates, were visualized in cells with fluorescent activity based probes, we observed that these proteasomes are active and accessible for substrates, indicating that the UPS is not as severely affected by aggregate formation as assumed and that proteasomes remain functional in HD.
Since we show that proteasomes are able to degrade mHtt completely and its functioning is not as severely affected as initially suggested, we examined in chapter 8 whether modulation of the proteasomal activity improves mHtt clearance. In vitro degradation experiments revealed that activation of the 20S proteasome via opening of the α-ring accelerates mHtt degradation, although activation by the PA28αβ cap prevented folded mHtt from entering the catalytic cavity. When we modulated proteasome activity by exchanging constitutive catalytic subunits by immunosubunits, or by specific inhibition of the β5 activity, we observed improved mHtt degradation in vitro. Inhibition of β5 also resulted in reduced mHtt levels in cells, whereas exchanging the active sites in cells did not result in improved degradation. We conclude that modifying proteasomal activity does affect mHtt cleavage and improves its degradation but these results cannot simply be translated into a cellular system, most likely due to inefficient targeting of mHtt to the proteasome and competition by the autophagic pathway.

**Conclusion and future perspective**

In conclusion, we show in the present thesis that mHtt can be cleared by proteasomes in vitro and in cells, but apparently not efficiently enough to prevent protein accumulation in cells. By using microscopy techniques in living cells, we show that proteasomes remain functional when recruited into polyQ initiated IBs. In addition, modulation of proteasomal activity has proven to be an effective strategy for improving mHtt degradation, although targeting of mHtt for proteasomal degradation appears to be insufficient in cells. Our study contributes to a better understanding of proteasomal functioning in HD. Especially, since we give new perspectives on mHtt processing by proteasomes, which contradict the general assumptions. We propose that proteasomes may become a target for therapeutic intervention, particularly for clearance of nuclear mHtt fragments that cannot be cleared by autophagy. However, targeting of mHtt towards proteasomal degradation should be optimized to increase the effects of proteasomal modulation. Interestingly, we recently observed that nuclear mHtt fragments are ubiquitinated in a different way when compared to cytoplasmic mHtt, which may explain the low efficiency in mHtt turnover by proteasomes in cells. Whereas mHtt may be poly-ubiquitinated in the cytoplasm in order to facilitate degradation via the autophagic machinery, the limited levels of nuclear ubiquitinated mHtt may be due to the absence of a dedicated E3 ligase, thereby preventing mHtt degradation by the 26S proteasome.

*Månsson C, Kakkar V, Monsellier E, Sourigues Y, Härmmark J, Kampina HH, Melili R, Emanuelsson C, DNAJB6 is a peptide-binding chaperone which can suppress amyloid fibrillation of polyglutamine peptides at substoichiometric molar ratios, Cell Stress Chaperones, 2013*