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Is the ubiquitin-proteasome system impaired in Huntington's disease?

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Abstract. Ubiquitylated inclusion bodies (IBs) found in Huntington's disease (HD) postulate an impaired ubiquitin-proteasome system. However, this hypothesis remains controversial. *In vitro*-generated polyglutamine aggregates failed to inhibit purified proteasomes, while filamentous huntingtin aggregates isolated from mice resulted in inhibition. However, similarly isolated IBs did not, thus suggesting that IB formation is protective by sequestering smaller inhibitory aggregates. Accordingly, proteasome-activity assays in IB-containing mouse brain homogenates did not show decreased activity. On the contrary, some of the endoproteolytic proteasome activities increased, probably due to altered subunit composition. However, activity was found decreased in post-mortem human HD tissue. Finally, evidence supporting the hypothesis was found in HD cell models expressing fluorescent ubiquitin-proteasome system reporters but not in retina of SCA-7 mice with similar reporters. In summary, it seems that mutant huntingtin, probably in intermediate aggregate forms, has the potential to inhibit proteasome activity, but the global status of the system in HD brain tissue is not yet fully elucidated.

Keywords. Huntington′s disease (HD), polyglutamine (PolyQ), inclusion body (IB), ubiquitin-proteasome system (UPS), degron-fluorescent proteins, *in vitro* ezymatic activity, cell models, mouse models.

Huntington's disease

Huntington′s disease (HD) is a genetic autosomal dominant neurodegenerative disease caused by the expansion of a CAG repeat in the huntingtin (*htt*) gene [1]. This triplet expansion encodes a polyglutamine stretch (polyQ) in the N′ terminus of the high molecular weight (348-kDa) and ubiquitously expressed protein huntingtin (htt) [2, 3]. Normal individuals have between 6 and 35 CAG triplets, while expansions longer than 40 repeats lead to HD [1, 4]. The onset and severity of the disease depend directly on the length of the polyQ tract. The longer the polyQ is, the earlier the disease begins and the more severe the symptoms are [4, 5]. The disease is characterized by motor dysfunction, cognitive decline and psychological dysfunction, and there is currently no effective treatment to prevent or delay disease progression [6]. Emotional and cognitive changes often precede motor signs by several years (about 3 years). Motor symptoms include chorea (movements performed continuously for several hours that cannot be suppressed voluntarily), rigidity, dystonia and oculomotor dysfunction, among others [7]. Cognitive dysfunction involves subcortical dementia, including affective and personality changes, and problems in acquiring new knowledge. Depression and suicide, as well as mania and psychotic symptoms are frequent in patients with HD [8, 9].

The neuropathology of HD involves atrophy and gliosis in the caudate and putamen, with specific loss of the GABAergic Medium Sized Spiny Neurons (MSSNs) that project to the substantia nigra (SN) and globus pallidus (GP) [10]. To a lesser degree, pyramidal neurons in layers III, V and VI of the cerebral cortex

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are affected too [11, 12]. As the disease progresses, neuronal loss extends to a broad range of brain regions, such as the GP, thalamus, subthalamic nuclei, SN, hippocampus, spinal cord, and others [6, 12].

Another histopathological mark of the disease is the presence of neuronal inclusion bodies (IBs) that can be detected with anti-N-terminal htt antibodies and that show a fibrillar ultrastructure [13, 14]. Regarding the subcellular localization of the IBs, they are mainly located in neuronal projections, and accordingly, they are most commonly detected as neuropil inclusions and, to some extent, also as dystrophic neurites. Rarely, they can also be detected in perikarya and nuclei [14]. Although the striatum is the most affected brain region in HD, IBs are quite rare in this structure even when overt neuronal loss is detectable [14]. In contrast, IBs are most frequent in the cortex, especially in the insular, cingulated and dorsolateral prefrontal areas. Cortical neurons bearing IBs are preferentially located in layers V and VI and, at a much lower density, also in layer III. These cortical IBs are present even at a stage during which cortical degeneration has not been described [14]. Rare aggregates at a similar frequency to those in the striatum can also be observed in other brain regions, including the SN, hypothalamus, thalamus, and brain stem [14].

Apart from HD, there are eight additional inherited neurodegenerative diseases caused by CAG trinucleotide repeat expansions in their respective genes. These are the spinocerebellar ataxias (SCAs) -1, -2, -3, -6, -7, and -17; dentatorubro-pallidoluysian atrophy (DRPLA) and the spinobulbar muscular atrophy (SBMA) [15, 16]. In all these diseases the triplet expansions are within the coding sequence of the gene. Moreover, they are always translated in the reading frame that produces a polyQ sequence, and the threshold for the expansion to become pathogenic is around 40 repeats in most of these diseases [15]. The fact that all these diseases developed similar neurological syndromes despite the different nature and identity of the mutant proteins and the fact that they are dominantly inherited strongly suggest that the expanded polyQ tract confers a toxic gain of function to the mutant protein.

All expanded polyQ disorders also have in common the presence of neuronal IBs containing the polyQ-harbouring protein [17, 18]. Interestingly, polyQs were found to aggregate *in vitro* and the threshold length for *in vitro* aggregation correlates with the pathogenic repeat length threshold [19]. This is the reason why, at first, aggregates were thought to be pathogenic and, accordingly, aggregation the most obvious toxic property conferred by the mutation.

Another commonality of expanded polyQ diseases is the fact that they are all neurological disorders despite ubiquitous expression of the mutant protein, thus suggesting a selective vulnerability of neurons to expanded polyQ. Purkinje cells are affected in almost all the diseases caused by polyQ expansion, so this cell type seems to be especially susceptible and this might be the reason for the existence of so many ataxias among polyQ diseases [20]. However, there are also neuronal populations that are distinctly affected in some of these diseases, as is the case of the striatum in HD [10]. These specific patterns of neuropathology of the diseases could reflect the influence of the protein context harbouring the expanded polyQ on the execution of its toxic gain of function or that a partial loss of function may also participate in eliciting toxicity.

Normal functions of htt have not yet been fully elucidated (see [21] for a review). Htt has no overall sequence homology with other proteins and although it is ubiquitously expressed, the highest levels are found in central nervous system (CNS) neurons and in the testis [22, 23]. The presence of several HEAT domains and the reported interactions with many different proteins suggest that htt may participate in many physiological processes by acting as a scaffold for protein-protein interactions [24–26]. Regarding specific roles played by normal htt, it is proven that htt is essential for embryonic development because knockout mice lacking htt show an embryonic lethal phenotype [27, 28]. Interestingly, htt is known to participate in neuroprotective mechanisms in brain cells exposed to various apoptotic stimuli [29]. This anti-apoptotic activity seems to be particularly contained within the first 548 amino acids of the normal protein [29]. Htt is also involved in the cell machinery that controls synaptic transmission, and normal htt is known to interact with a number of cytoskeletal and synaptic vesicle proteins that are essential for exo- and endo-synaptic terminals [30]. Htt has also been shown to participate in brain-derived neurotrophic factor (BDNF) production and trafficking. Wild-type Htt, but not the mutant form, stimulates cortical BDNF production by acting at the level of Bdnf gene transcription [31, 32]. Normal Htt is known to sequester REST/NRSF in the cytoplasm, preventing it from forming the nucleus co-repressor complex at the RE1/NRSE nuclear site and allowing gene transcription [32]. Htt also enhances the vesicular transport of BDNF along microtubules [33], as it is involved in fast axonal trafficking in mammalian neurons [34]. Since BDNF levels are decreased in HD brains [31, 32] and BDNF is important for the survival of striatal neurons and the activity of cortico-striatal synapses [35–39], a loss of function of mutant htt in the above-

described effects on BDNF production and trafficking might contribute to striatal vulnerability in HD.

Although the partial loss of function of normal htt may contribute to some aspects of HD, we have to keep in mind that a toxic gain of function of expanded polyQ is the most likely determinant of the disease. As mentioned, the first suggested gain of toxic function was the aggregation capability. Currently there is a controversy concerning the role of aggregation in polyQ toxicity. Apart from the initial finding of the same threshold in polyQ expansion for *in vitro* aggregation and pathogenic mutation [19], it was also found that the aggregate formation precedes the onset of symptoms in a transgenic mouse model [40]. Studies using transfected cells further suggested that toxicity might be induced by aggregates [41]. On the other hand, more recent observations support that aggregates are not pathogenic or even that they might be protective [42–46]. Also, it was found that IBs are present in the cortex of HD brains before any sign of degeneration can be detected, and many MSSNs in the striatum lack IBs despite the presence of significant neuronal loss [14]. Similarly, an inverse correlation between aggregate distribution and brain region and cell type affectation was found [47]. Furthermore, there are also some transgenic mouse models of HD in which IBs appear only after symptoms begin [48]. Finally, it was found in transfected primary cultured neurons that the ability of a neuron to build an IB protects it from the toxicity elicited by mutant htt [44]. Regardless of its aggregation status, the toxic gain of function by which mutant htt causes disease is not known. There are some molecular pathways through which mutant htt might cause pathology (see [49] for a recent review). The proposed mechanisms, apart from conformational toxicity and protein sequestration, include transcriptional dysregulation [50], excitotoxicity and mitochondrial damage [51], transport disruption along axons [52], endocytosis and vesicle transport impairment [53], caspase and apoptosis activation [54], and impairment of the ubiquitin proteasome system [55], among others. This review will focus on the implication and possible impairment of the UPS in polyQ pathologies and more particularly in HD.

UPS

The UPS is responsible for the turnover of most soluble proteins present in the cytoplasm and the nucleus of the cell and plays an essential role in degrading key short-lived regulatory proteins and damaged or misfolded proteins [56]. The UPS comprises several elements that enable it to degrade the substrate proteins. These elements are 1) ubiquitin, a 76-amino acid protein universally distributed among eukaryotes and highly conserved that has to be added to the substrate to label it for degradation; 2) enzymes that transfer ubiquitin moieties to the substrate; and 3) a multicatalytic protease called 26S proteasome.

Degradation of proteins via UPS involves tagging the substrate by covalent attachment of multiple ubiquitin molecules to synthesize the polyubiquitin chain signal that is recognized by the 26S proteasome. Attachment of a single ubiquitin domain to a target protein serves to modulate its activity or subcellular location [57–59], while attachment of a polyubiquitin chain results in targeting of the protein to the proteasome and subsequent proteolysis [56, 60]. Conjugation of ubiquitin to the substrate is a three-step cascade. In the first step, the C-terminal Gly residue of ubiquitin is activated by adenylation and subsequent rearrangement to form an intermolecular thiol ester with the ubiquitin-activating enzyme (E1) in an ATP-requiring reaction. Activated ubiquitin is then transferred to a Cys residue in the active site of a ubiquitin-conjugating enzyme (E2). In the third step, which is catalyzed by a ubiquitin-protein ligase (E3), ubiquitin is linked by its C-terminus through an amide isopeptide linkage to an ε-amino group of a Lys from the substrate protein. The E3 enzymes serve as the specific substrate recognition factors of the system through specific degradation signals termed degrons [60]. These degradation signals can consist in special protein domains, small motifs or unfolded, misfolded or other abnormal domains in the proteins [61]. In the case of some substrates, a ubiquitin elongation factor (E4) is required for achieving efficient polyubiquitylation [62, 63]. The process of ubiquitin addition is repeated until the polyubiquitin chain is synthesized [56].

The 26S proteasome is an energy-dependent multicatalytic protease localized both in the nucleus and the cytoplasm that degrades polyubiquitylated proteins to small peptides. It is composed by two complexes: the 20S proteasome, or core particle, refers to the barrel-shaped multisubunit proteolytic complex of proteasomes. In the inner part of the 20S complex are located the three types of catalytic subunits that execute the corresponding catalytic activities of the proteasome [trypsin-like, chymotrypsin-like and peptidyl-glutamyl preferring hydrolytic (PGPH) activity]. The 19S, or regulatory particles, are located on the sides of the 20S complexes and, in an ATP-dependent process, recognize the proteins labelled for degradation, open the outer ring of 20S particles and unfold the proteins to allow them to get into the proteolytic chamber inside the 20S complex. Upon recognition of the polyubiquitylated substrates, 19S complexes release polyubi-

quitin chains. Then, deubiquitylating enzymes (DUBs) disassemble them into ubiquitin monomers so they can be reused [64].

The hypothesis of impairment of the UPS in HD and in most nuerodegenerative diseases

In HD and other CAG repeat disorders, the fact that the IBs that are present in brains of patients were stained with anti-ubiquitin and anti-proteasome antibodies [13, 65] was the first evidence that supported the hypothesis of an impairment of the UPS. Similar findings were also obtained in brains of mouse models of HD [40]. Another initial observation supporting this hypothesis was the increase in the incidence of aggregates in cellular models of HD upon pharmacological inhibition of proteasome activity [41]. Similarly, the reversal of aggregates that takes place in primary neurons from the HD inducible mouse model upon shutdown of mutant htt expression no longer takes place in the presence of proteasome inhibitors [66]. Since the initial formulation of the hypothesis, many experiments have been performed to try to validate it. Here we will summarize them and will analyze the controversies and conclusions that arise from these studies.

Interestingly, aberrant proteinaceous deposits inside affected neurons are a common theme in most neurodegenerative diseases, and similar evidence supporting impairment of the UPS was also observed in many of these diseases. In AD, the characteristic intraneuronal deposits are the neurofibrillary tangles that consist in paired helical filaments made of hyperphosphorylated tau protein [67]. In PD, the Lewy bodies are present in brain stem and cortical areas, and their principal component is α-synuclein (α-syn) [68]. In Amyotrophic Lateral Sclerosis (ALS), Bunina bodies are mainly distributed in the lower motor neurons and formed by intermediate filament proteins [69]. In all these diseases, the intraneuronal inclusions can be detected with anti-ubiquitin antibodies [70, 71], and many experiments have also been conducted to specifically address the hypothesis of an impairment of the UPS in these diseases [72].

Although it is not the focus of this review, due to its soundness, we will briefly analyze the genetic evidence supporting UPS dysfunction as a key pathogenic mediator in familial cases of PD. In this disease, most of the patients develop the disease in a sporadic manner, but there is a number of patients that inherit it in an autosomal dominant or recesive manner (familial PD; FPD). So far, at least 11 genes (SNCA, PARK2, 3, 4, 5, 6, 7, 8, 9, 10, and NR4A2) causative for FPD have been identified [73–84]. The autosomal recessive form of parkinsonism (AR-JP), one of the most common familial forms of PD, is caused by point mutations or deletions in the parkin gene (PARK2) [75]. Parkin is a ubiquitin-protein ligase that acts along with the DUB enzymes [62, 85] and associates with the RPN10 (S5a) subunit of the 26S proteasome [86]. Many of the mutations found in parkin in AR-JP patients inactivate its ubiquitin ligating activity or possible association with its partners, thus causing the pathology. Furthermore, a mutation in the gene coding for the ubiquitin carboxy-terminal hydrolase UCH-L1 (PARK5) is also the cause of a form of FPD [76]. UCH-L1 has different activities depending on whether it is in monomeric or in dimeric form. The monomeric form of UCH-L1 catalyzes deubiquitination, while the dimmers display a ubiquitin ligase activity that generates ubiquitin-K63 bonds [87]. Mutation of UCH-L1 does not lead to complete inactivation, but to decreased activity and to a shortage in free ubiquitin that should have been recycled from conjugates. This then results in general impairment of the function of the UPS. Together, identification of parkin and UCH-L1 mutations as causative for FPD strongly suggests that UPS impairment may be at the root of neurodegenerative disorders.

Returning to the UPS impairment hypothesis in the context of HD and other expanded PolyQ disorders, we might speculate several levels of the UPS at which impairment might be generated. It is possible that expanded polyQ could interfere directly with the 19S, blocking recognition of the substrate or its unfolding and the opening of the outer rings of the 20S catalytic core. It could also interfere directly within the 20S by clogging it so it cannot process additional substrate molecules. In both cases, proteins will not be degraded and will accumulate in the cell, increasing the possibility of aggregate formation. But, as in the described genetic forms of PD, the UPS impairment does not necessarily have to be at the proteasome level; it could take place at any level of the system such as ubiquitin availability (Ub, DUBs) or ligation process (E1, E2, E3). Any of these hypothetical pathogenic interactions might be exerted by soluble monomeric mutant htt or by aggregated forms of htt. However, since it has been reported that IBs recruit other proteins, including components of the UPS such as Ub and 20S or 19S subunits [13, 40, 43, 65], it has been hypothesized that this sequestration driven by aggregated htt can cause depletion of the cellular pool of these UPS components, thus leading to UPS impairment.

We will now summarize the different studies that have been conducted to determine whether impairment of the UPS does take place in HD. These studies employ different experimental approaches each of which has its particular advantages and limitations. We will

discuss the different findings according to the technique employed, starting from the most controlled *in vitro* studies to the most integrated *in vivo* approaches.

In vitro activity assays with purified proteasomes

The three endoproteolytic activities of the proteasome (trypsin-like, chymotrypsin-like and PGPH), cut the peptide bonds after basic, hydrophobic or acid residues, respectively [88]; Glutamine does not really fit in any of these categories, so a very basic question to answer is whether the proteasome is able to cut within a polyQ sequence or not. In order to elucidate this aspect, Venkatraman and co-workers performed experiments with peptides containing 10–30 residues of Gln, flanked by 2 Lys residues to enhance their solubility, and incubated them with purified proteasomes [89]. They observed no digestion of the polyQ sequence by the proteasome. The only cleavage made was cuts within the flanking basic residues. This failure to digest the polyQ sequence was reproduced with all forms of eukaryotic proteasomes tested, including mammalian 26S and 20S particles. In contrast, proteasomes from *Thermoplasma acidophilum* digested polyQ peptides completely. In order to further validate the inability of eukaryotic proteasomes to digest PolyQ sequences, a myoglobin Q(35) fusion protein was used as substrate. The eukaryotic proteasomes digested the protein but spared the polyQ sequence. This result is very important mechanistically because it shows that eukaryotic proteasomes, when degrading expanded polyQ-containing proteins, might be generating the most toxic and aggregation-prone fragments; i.e., the expanded polyQ with little or no flanking protein sequence. Moreover, such polyQ sequences (38–300Qs) exceed the lengths of normal proteasome products (2–25 residues) and a failure of these fragments to exit the proteasome may interfere with later proteasome function. In summary, these experiments that were designed to answer the very basic question of whether expanded polyQ can be degraded by proteasomes also had implications for potential pathogenic actions. One possible action would take place at the proteasome (that might be inhibited by clogging), while the other could take place either inside or outside the UPS but the proteasome would be the one generating the most toxic fragments.

In vitro proteasome activity assays have also been performed to test the possibility of direct inhibition of proteasomes by polyQ-containing proteins or aggregates. Bennet and co-workers tested the effect of *in vitro*-generated polyQ aggregates on the degradation of ubiquitin-dependent and ubiquitin-independent substrates by purified 26S proteasomes [45]. A ^{125}I-labelled fragment of cyclin B was ubiquitylated *in vitro* (Ub-cyclin N100) and used as a ubiquitin-dependent 26S proteasome substrate [90], while the small fluorogenic peptide Suc-LLVY-AMC was used as a ubiquitin-independent substrate of the chymotrypsin-like activity of 26S proteasomes. The polyQ-containing proteins consisted of N-terminus htt (exon 1 encoded) containing either 18 or 51 Gln residues fused to glutathione S-transferase (GST), with an intervening tobacco etch virus (TEV) protease cleavage site [45]. The presence of the GST tag maintains both Q18 and Q51 in soluble form [19]. Upon cleavage of the GST moiety with TEV protease, the Q51, but not the Q18, fragment rapidly formed high molecular weight aggregates. Neither the soluble htt fragments nor the aggregated ones produced a reduction in proteasome activity measured as the degradation of Ub_n cyclin. To further validate these results, the effect of small soluble oligomeric aggregates of synthetic polyQ as well as highly aggregated fibrillar species [90, 91] was also tested on the chymotrypsin-like activity of 26S proteasomes. Similarly, no differences in proteasome activity were observed [45]. In summary, these experiments strongly argued against the notion that a direct interaction between 26S proteasomes and monomers or aggregates of expanded polyQ could result in decreased proteasome activity.

In the previously described experiments, the polyQ-containing fragments and peptides were generated by recombinant technology in bacteria or synthesized *in vitro* and, therefore, had not undergone ubiquitylation. If ubiquitylation of the monomers or aggregates of expanded polyQ-containing proteins were important for its potential inhibitory interaction with 26S proteasomes, the above-described experiments would not have been able to detect it. To overcome this limitation, similar experiments were performed with polyQ-containing IBs and filaments isolated from the brain of the Tet/HD94 inducible mouse model or from post-morten HD human brain tissue [92]. For this study we established a magnetic sorting-based protocol for isolation of N-terminal htt-containing IBs, and as expected, these were also immunopositive for ubiquitin [92]. We had also previously reported that the N-terminal htt- and ubiquitin-positive filaments that form the IBs can be isolated by high salt and non-ionic detergent extraction followed by sucrose gradient fractionation [93]. The potential inhibition by N-terminal htt- and ubiquitin-immunopositive IBs and filaments on 20s and 26S proteasome activity was analyzed [92]. The N-terminal htt- and ubiquitin-immunopositive filaments isolated from Tet/HD94 mice selectively inhibited the ATP-dependent endoproteolytic activities of the 26S proteasome (tested with small fluorogenic substrates for each of the activities: Suc-LLVY-AMC for the chymotrypsin-like

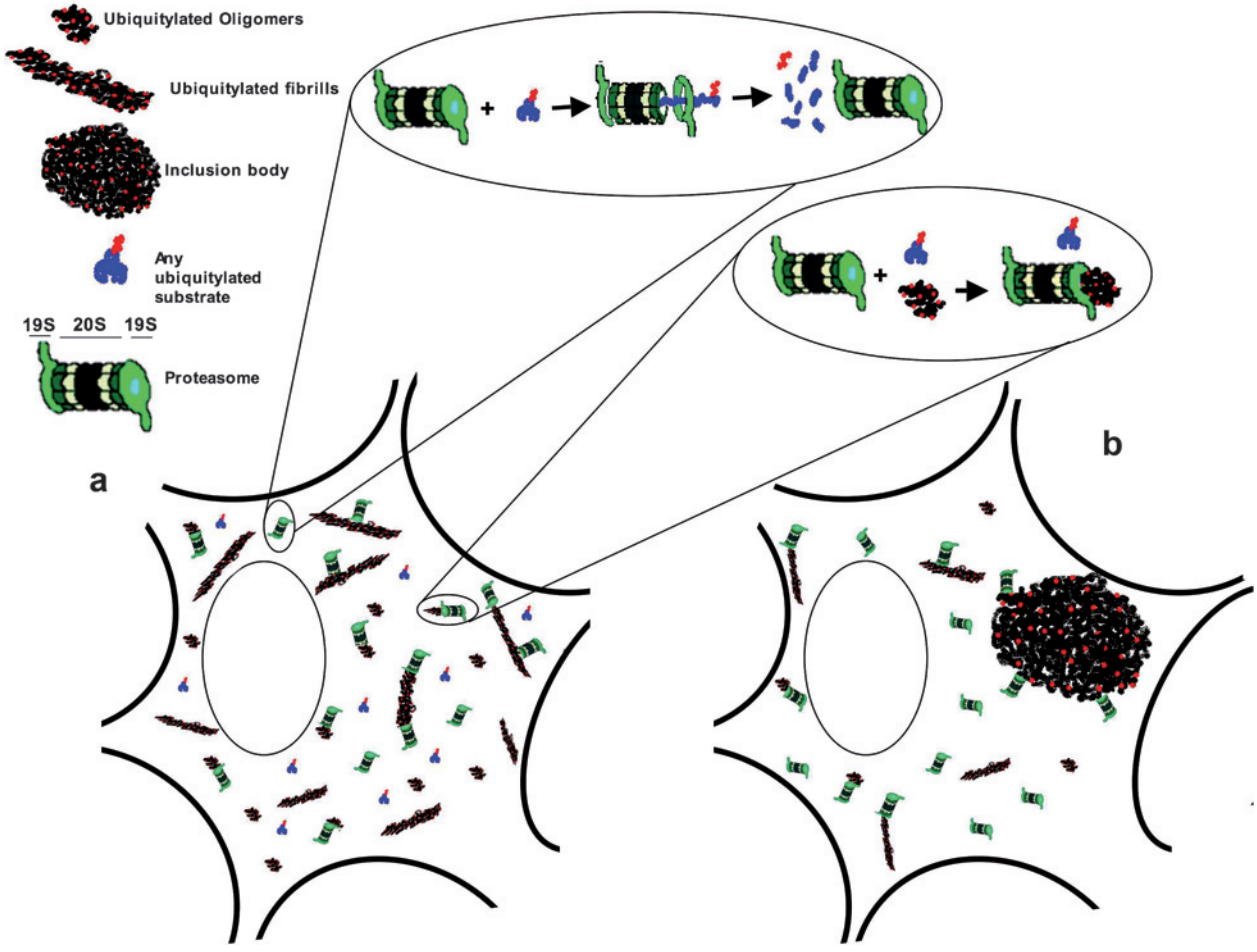

Figure 1. Postulated protective role of IB formation against UPS impairment. The 19S subunits of the proteasomes recognize ubiquitylated substrates, unfold them and open the 20S subunit to allow the substrates to get into it to be proteolyzed. (*a*) Neurons with oligomeric and/or fibrillary polyQ aggregates but that have not yet built an IB may have a higher concentration of these intermediate aggregated species. This would increase the chances for an inhibitory interaction of the ubiquitylated aggregates with the 19S caps of the 26S proteasomes. This interaction would prevent degradation of the rest of ubiquitylated cellular substrates that would then accumulate in the neuron. (*b*) In neurons harbouring IBs, the concentration of ubiquitylated intermediate aggregates of polyQ is lower, thus allowing the UPS to perform normally.

activity, Boc-LSTR-MCA for the trypsin-like activity and Z-LLE-βNAP for the PGPH activity). However, when the same experiments were performed with the 20S proteasomes in the presence of SDS to facilitate the entrance of the substrate into the catalytic chamber, no inhibition was detected. Similarly, degradation of in vitro ubiquitylated IκBα (Ub-IκBα) by 26S proteasomes was also inhibited by the filament-containing fraction from Tet/HD94 mice. The selective inhibition of 26S proteasome but not of 20S proteasome activity suggested a direct interaction of the ubiquitylated filaments and the 19S ubiquitin-interacting regulatory caps of the 26S proteasome. Interestingly, this interaction was in fact confirmed by immunoelectron microscopy [92]. However, a preparation of IBs from the brain of Tet/HD94 mice (containing the same protein amount as the filament-containing fraction used in the previous experiments) did not modify proteasome activity. All together, these results suggest that fibrillar, and possibly also oligomeric, ubiquitylated PolyQ aggregates have the potential to interfere with 26S proteasomes but only when these aggregates are not recruited into IBs. These results therefore strengthen the notion that IB formation may be protective, in this case, by neutralizing the inhibitory action of dispersed ubiquitylated polyQ filaments (see Fig. 1).

Proteasome activity measurement in homogenates from cell or animal models and human tissue

A more integrated approach is to use cell or tissue lysates from cell or animal models of HD or post-morten human brain tissue to measure proteasome activity. In this way, all the compounds that are present

in the cell and that could interfere with the UPS will be present in the experiment, although they will be much more diluted than in the *in vivo* situation.

The simplest way to measure proteasome activity in these lysates is by incubating them with the above-mentioned small fluorogenic substrates that are specific for each of the proteolytic activities. Most studies explored only one or, at most, two of the activities with analyses measuring chymotrypsin-like activity being the most commonly used. These kinds of approaches have been performed on cell model lysates [94], mouse brain homogenates [46, 95, 96] and post-mortem HD human brain homogenates [97]. Regarding the experiments performed with cellular lysates, mouse neuro2a (N2a) cell lines stably transfected to express an inducible system of N-terminal htt with normal (16Q) or expanded polyQ (60Q and 150Q) were used [94]. In these cell lines, the presence of expanded polyQ caused proteasome redistribution from a diffuse pattern in both the nucleus and the cytoplasm to an aggregate-associated pattern. Homogenates from these cell lines were fractionated into a soluble and a precipitated fraction by centrifugation at 15 000g for 15 min. The chymotrypsin-like proteasome activity was then assayed in either fraction by incubation with Suc-LLVY-AMC. The expression of expanded 150Q N-terminal htt resulted in increased chymotrypsin-like activity in the precipitated fraction and in decreased activity in the soluble fraction. Since in these cells proteasomes redistribute to aggregates, and these are mainly represented in the precipitated fraction, this was the expected outcome of assaying activity in soluble and precipitated fractions separately. Therefore, it is not possible to conclude from these experiments whether total proteasome activity is decreased or not in those cells expressing 150Q N-terminal htt.

The first analysis of proteasome activity on HD mouse model brain extracts was performed on the Tet/HD94 inducible mouse model [95]. These mice express exon 1 htt with a 94 polyQ repeat in the forebrain in a tetracycline-regulated manner [98, 99]. In this study, the three endoproteolytic activities of the proteasome were tested, and no inhibition of any of them was detected [95]. On the contrary, the chymotrypsin- and trypsin-like activities increased selectively in mutant htt-expressing and IB-harbouring regions such as cortex and striatum but not in cerebellum that does not express the transgene. Interestingly, the overall level of 20S proteasome subunits was not altered in these mice. This, together with the fact that only two of the activities were changed, suggested a qualitative change in the subunit composition of proteasomes. This was confirmed to be the case since the interferon-inducible catalytic subunits of the immunoproteasome LMP2 and LMP7 were increased in Tet/HD94 mouse brain as well as in brain tissue from HD patients. In summary, since endoproteolytic proteasome activity was not decreased in proteasomes of homogenates of tissue containing soluble and aggregated mutant htt, these experiments argue against the postulated inhibition of proteasomes by clogging of the catalytic core. On the other hand, these experiments also provide evidence that proteasome subunit composition may be altered during the course of disease, and it may be as a consequence of neuroinflamatory processes [100].

A recent similar study was performed in R6/2 mice that also express exon 1 mutant htt but with a longer polyQ and a with broader tissue distribution. This study also revealed no inhibition of 20S proteasome activity but an increase of the chymotrypsin-like activity that is detectable after the age of 13 weeks [96]. Furthermore, no change was observed between wild-type and R6/2 mice in the 26S proteasome activity measured as degradation of radio-labelled ubiquitylated lysozyme.

In contrast to the results obtained on brain homogenates from HD mouse models, a similar study performed on human post-morten HD brain tissue has reported decreased activity [97]. Seo and coworkers analyzed the chymotrypsin and post-glutamyl activities of the 20S proteasome in post-mortem brain samples from HD patients and normal subjects. They found decreased chymotrypsin activity in the striatum of grade 0–1 and of grade 3–4 HD patients and also in the cerebellum of grade 0–1 HD patients. This activity did not change in cortex or SN of HD patients. The post-glutamyl activity was decreased in the striatum and cerebellum of grade 0–1 and of grade 3–4 HD patients, in the cortex of grade 0–1 HD patients and in the SN of grade 3–4 HD patients. These results would suggest that either there is a decrease in 20S proteasome content in HD brain or, if the same amount of 20S proteasomes is present that these are functionally impaired. Unfortunately, this study lacks measurement of total proteasome content in the analyzed brain samples. Moreover, the individual proteasome activity values from each post-mortem patient's brain samples were normalized using the atrophy index that results from the macroscopic evaluation of ventricular size of the same HD patient brains. In summary, with the limitations associated with analyzing enzymatic activities in post-mortem tissue, this study suggests differences in the UPS, but it is difficult to conclude whether proteasome activity is altered in human HD brain or not.

Degron-fluorescent proteins

The approaches mentioned in the previous sections have in common that they only monitor the status of the UPS at the level of proteasome activity. As we said before, impairment of the UPS does not necessarily have to be at the proteasome levels; it could be at any other level of the UPS pathway, including the ubiquitin-ligating process. In this case, all the experiments described above will not detect it. A more integrated analysis of UPS in HD models has been accomplished with the expression of degron-reporter proteins. These reporter proteins result from fusing a UPS degradation signal to a fluorescent protein that converts it into a reporter of impaired UPS function. These modified proteins have an extremely short half-life and will accumulate only if the UPS is not working efficiently. This is evidenced when cells expressing the reporters are exposed to proteasome inhibitors [101–103]. An additional advantage of the degron-fluorescent protein reporters is their cellular resolution. If the impairment is not synchronous in all the cells in a culture or mouse model, or if it only takes place in a small subset of cells, the biochemical methods will fail to detect impaired enzymatic activities. With degron-fluorescent protein-based approaches it is possible to detect those individual cells in which an impairment of the UPS is taking place. This approach, however, also has some limitations. Depending on the degradation signal attached to the fluorescent protein, the combination of E2 and E3 enzymes that will guide it to degradation via UPS will be different. This is especially relevant for E3 ligases because, as previously described, different E3s recognize different substrates depending on the type of degradation signal they carry. Therefore, if the UPS impairment takes place at the level of certain E2 or E3 enzymes, not all degron-fluorescent proteins will be able to detect it [104]. This type of reporter protein has been used both in cell and animal models of PolyQ diseases.

Regarding studies in cellular models, the exon 1 fragment of htt containing a pathogenic polyQ repeat (HttQ103) was transiently transfected into HEK 293 cells that stably expressed a reporter protein consisting of a CL1 degron fused to the COOH-terminus of green fluorescent protein [105]. The CL1 signal is a 16-amino acid sequence that easily destabilizes proteins by labelling them for ubiquitylation. In this study, only those cells that presented aggregates showed significantly higher GFP (green fluorescent protein) fluorescence compared to control cells. Thus, it was concluded that the presence of aggregates led to inhibition of the UPS in transfected cells expressing N-terminal mutant htt.

Similar experiments have recently been conducted by the same group but using nuclear and cytoplasmic compartment-specific UPS reporter proteins [45]. In this case, the reporter protein, apart from the CL1 degron, carried a nuclear export signal (NES) [106] or the SV40 nuclear localization signal (NLS) [107]. This approach makes it possible to explore whether nuclear or cytoplasmic-localized protein aggregation would result in UPS worsening only in the cis compartment or also in the trans compartment [45]. In cells expressing N-terminal HttQ103, where juxtanuclear cytoplasmic IBs were common, fluorescence was substantially elevated in the nucleus as well as in the cytoplasm. Surprisingly, contrary to previous observation, diffusely localized HttQ103 in the absence of detectable IBs also induced significantly elevated levels of the reporter in the nucleus and cytoplasm. Therefore, this study shows that expression of N-terminal mutant htt in transfected cells causes global impairment of the UPS that results from an intrinsic property of N-terminal mutant htt and not from its sequestration into inclusions [45].

The approach of degron-fluorescent protein as a reporter of UPS impairment has also been applied in whole animals. The first mouse model expressing one of these reporters for *in vivo* monitoring the status of the UPS was generated by Lindsten and co-workers [108]. The degron-fluorescent protein was GFP carrying a ubiquitin fusion degradation (UFD) signal. This is an N-terminal-linked ubiquitin molecule that, on one hand, has a G76V substitution that prevents removal of the ubiquitin by DUBs and, on the other hand, serves as acceptor for polyubiquitin chains. In these mice, the transgene is under control of the cytomegalovirus immediate-early enhancer and the chicken β-actin promoter, and accordingly the mice show ubiquitous expression of the Ub^{G76V}-GFP reporter. These mice, therefore, became a unique tool to explore *in vivo* the hypothesis of impairment of the UPS by breeding them with mouse models of HD and of neurodegenerative diseases in general [93, 109].

Unfortunately, there are not yet reports of the level of the reporter in brain of Ub^{G76V}-GFP mice combined with mouse models of HD or other PolyQ diseases. There is only a reported study of the UPS status in retinal neurons of Ub^{G76V}-GFP mice combined with a SCA7 knockin mouse model [46]. In this disease, apart from the cerebellar and brainstem pathology, there is also marked retinal degeneration. Analysis of the UPS reporter level in retinas of 6-week-old animals (early stage of pathogenesis) revealed no significant difference between animals that express 266 polyQ stretch (Ub^{G76V}-GFP^{+} $Sca7^{266Q}$) and control animals (Ub^{G76V}-GFP^{+}). It suggests that, at least in initial stages, retinal dysfunction occurs in the presence of a functional UPS. By contrast, in animals 13 weeks of age (terminal stage of pathogenesis), there

was a significant increase in reporter levels in Ub^{G76V}-GFP^{+} $Sca7^{266Q}$ retinas. However, it is not clear whether this reflects a true UPS impairment or a transcriptional dysregulation in retinal neurons that would result in increased Ub^{G76V}-GFP mRNA levels. Additionally, Bowman and colleagues tested whether inclusion bodies may have a protective role against neuronal dysfunction as has been postulated by other investigators [42–45]. For this, they stained retinal sections for detection of ataxin-7 nuclear inclusions by immunofluorescence. These investigators observed a strong inverse correlation between Ub^{G76V}-GFP protein levels and the size and intensity of nuclear inclusions in sections from animals 9 (middle stage of pathogenesis) or 14 weeks of age. In summary, this work argues against significant impairment of the UPS as a necessary step for SCA-7 retinal degeneration and supports the hypothesis that IBs can have a protective effect.

A transgenic Caenorhabditis elegans with expression of a degron fluorescent protein and a mutant form of ataxin-3 has also recently been reported [110]. In this case, the reporter protein was ubiquitin-conjugated dsRed (Ub-dsRed). Therefore, the degron was also a UFD motif, although no G76V substitution or similar modification was introduced to reduce the effect of DUB enzymes. The expanded polyQ-containing protein expressed in this animal was truncated human ataxin-3 with a Q127 polyQ [110]. The constructs were expressed in GABA neurons of *Caenorhabditis elegans* under control of the unc-47 promoter. In animals expressing both dsRed and truncated 127Q ataxin, cells possessing polyQ aggregates had bright red fluorescence, whereas only a minority of cells without aggregates showed red fluorescence. Interestingly, the increase in the fluorescence was not due to enhancement of the Ub-dsRed transcription. Therefore, from this experiment it can be concluded that expression of truncated 127Q ataxin-3 in C. elegans neurons produces impairment of the UPS.

Future perspectives

The experiments described above do not offer a definite answer to the question of whether the UPS is impaired in HD. The measurement of the enzymatic activities of the proteasome have different limitations depending on the experimental approach used. In experiments performed *in vitro*, not all of the cellular components involved in the degrading process are present, and this could lead us to a deceitful result. In experiments performed with cellular or animal model homogenates, the substrates and the pathogenic species are much more diluted than in physiological conditions. Moreover, if the impairment of the proteasome takes place in a restricted number of neurons, this technique may not be sensitive enough to detect such small changes. Besides, the studies performed on human brain tissue have the additional limitations associated with the post-mortem interval and tissue preservation and, therefore, the comparability of the samples. Moreover, as previously described, the impairment of the UPS can take place at any level of the degradating pathway, not only at the proteasome level. However, until now, all the enzymatic assays performed to probe or discard the hypothesis of UPS impairment were based on assaying the proteolytic activity of the proteasome, and the enzymatic activities of E1, E2, E3 and DUB enzymes have not been yet explored.

The degron-fluorescent reporter proteins are a very promising tool because they provide a more integrated answer to the status of the UPS. Studies performed with transfected cells stably expressing these reporter proteins support the hypothesis of UPS impairment, but the high levels of pathogenic polyQ constructions obtained by transfection could give rise to positive results that do not reflect reality in the tissue of HD patients. In animal models, the concentrations of pathogenic polyQ proteins are compatible with normal embryonic development and acquisition of a disease phenotype in adult life. Results obtained in animal models are therefore expected to be less artefactual. Unfortunately, the brain phenotype of degron-fluorescent protein reporter mice combined with mouse models of HD still has to be explored. Although the Ub^{G76V}-GFP reporter mice have been combined with an SCA-7 model, they have been studied only in retina [46]. It is expected that the combination of degron-reporter mice with mouse models of HD may offer a comprehensive and definite answer to the question whether the UPS is impaired in HD brain. In this regard, in collaboration with Nico Dantuma's group, we are combining the Ub^{G76V}-GFP mice with various mouse models of HD, including the inducible one. Since biochemical and enzymatic assay data suggest that the potential inhibition of the UPS could be transient and take place in a nonsynchronous fashion in certain neurons within a given population, combining degron-fluorescent mice with the inducible model of HD could facilitate the study by inducing synchronous expression of mutant htt in all neurons. Interestingly, two transgenic mouse models with ubiquitous expression of a different degron fluorescent reporter that results from fusion of the consensus ubiquitylation signal CL1 have recently been reported [111, 112]. Since every reporter protein follows different combinations of the E2-E3 enzymes for their ubiquitylation, the more reporter proteins that are

tested in mouse models, the greater the possibility of detecting impairment.

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