The role of the proteasome in Huntington's disease

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Citation for published version (APA):
Krom, S. (2013). The role of the proteasome in Huntington’s disease.
DYNAMIC RECRUITMENT OF ACTIVE PROTEASOMES INTO POLYGLUTAMINE INITIATED INCLUSION BODIES

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Revised version submitted to FEBS letters
**ABSTRACT**

Neurodegenerative disorders such as Huntington’s disease are hallmarked by neuronal intracellular inclusion body formation. A controversial topic in these protein misfolding disorders is the observed irreversible sequestration of proteasomes into inclusion bodies. These proteasomes may become clogged by the aggregated protein fragments, leading to impairment of the ubiquitin-proteasome system. Here, we show by fluorescence pulse-chase experiments in living cells that proteasomes are dynamically and reversibly recruited into inclusion bodies. As these recruited proteasomes remain catalytically active and accessible for substrates, our results challenge the concept of proteasome sequestration and impairment in Huntington’s disease, and support the reported absence of proteasome impairment in mouse models for Huntington’s disease.

**RESULTS**

Proteasomes directly interact with polyQ peptides when recruited into IBs

To examine the distribution patterns of intracellular proteasomes within polyQ IBs, we initiated aggregation using the N-terminal exon1 fragment of polyQ-expanded mHtt (mHtt(Q97)exon1), which represents the N-terminal cleavage product that is found in brain tissue of HD patients [1, 12]. In addition, pure polyQ peptides, representing products downstream of the proteasome, were used as initiators of aggregation, since it is considered that proteasomes are unable to degrade the polyQ tract [6, 7]. The Ub-Q99 constructs release Q99 peptides directly upon synthesis, as ubiquitin is cleaved off by ubiquitin C-terminal hydrolases [13]. The GFP-tagged proteasomal subunit PSMB4 (B7) was used to visualize proteasomes in living cells. This subunit is efficiently incorporated in both 20S and 26S proteasomes, as shown by complex separation on native gel and subsequent analysis of fluorescence (Supplementary Fig. S1). Recruitment of proteasomes into mHtt initiated IBs was observed when the PSMB4-GFP was co-expressed with tetracysteine-tagged mHtt (mHtt(Q97) exon1-C4). mHtt(Q97)exon1-C4 was labeled with the biosensit fluorophore ReAsH [14] and was found to be present in the core of IBs. However, dependent on IB size, proteasomes were differentially distributed (Fig. 1A). Proteasomes colocalized with mHtt(Q97)exon1-C4 in smaller IBs (Fig. 1A, upper panel), but were located in the outer layers of the larger IBs, forming a ring-like structure (Fig. 1A, lower panel). A similar redistribution of proteasomes was observed in cells stably expressing α7-GFP, this efficiently incorporated subunit was used as an alternative proteasomal subunit (data not shown). When C4-tagged polyQ peptides were used (Ub-Q99-C4), a similar proteasomal distribution pattern was observed (Fig. 1B). These findings suggest that proteasomes are not irreversibly trapped, but remain at the periphery of expanding IBs.

To determine whether these proteasomes interact directly with polyQ peptides, both in the core and also in the periphery, we used fluorescence lifetime imaging microscopy (FLIM) to study proteasome-polyQ interactions. Previously, we have shown by wide-field FRET-FLIM microscopy that GFP-tagged proteasomes, ubiquitin and the chaperone HSP70 were all recruited into polyQ aggregates but only GFP-tagged proteasomes directly interacted with polyQ fragments. Whereas GFP-ubiquitin and HSP70-GFP only showed moderate reductions in fluorescence lifetime [15]. By using confocal FRET-FLIM, we could distinguish between protein interactions in de core of the IB and at the periphery of IBs. Co-expression of ReAsH-labeled UbQ99-C4 and GFP-tagged PSMB4 showed a significant decrease in GFP donor lifetime compared to the negative control, both when proteasomes were present in the core or in the ring-like structures of IBs (Fig. 1C). This indicates that while chaperone HSP70 does not act on polyQ peptides in IBs, proteasomes continuously interact with polyQ fragments both at early stages of IB formation as well as during later stages.
Proteasomes are dynamically recruited into polyQ initiated IBs

To further determine proteasome dynamics in IBs, we performed a fluorescence pulse-chase experiment. Since FRAP studies did not show fluorescence recovery of proteasomes within IBs over a time span of minutes [5], we studied protein distribution over a time span of hours. Therefore, we first labeled the pre-existing pool of the C4-tagged proteins with red fluorescence using ReAsH. After a subsequent chase period of 8 or 20 hours, to allow the synthesis of new C4-tagged proteins, the cells were incubated with FlAsH to label the newly-synthesized proteins with green fluorescence. Pulse-chase labeling of mHtt(Q97)exon1-C4 (Fig. 2A) or Ub-Q99-C4 (Fig. 2E) showed a sequential sequestration of these polyQ fragments, as the newly synthesized mHtt(Q97) exon1-C4 or polyQ-C4 peptides were recruited to the outer layers of the IBs whereas the initial polyQ fragments were exclusively found in the core of the IB, both after an 8 and 20 hours chase period. The difference in distribution was not due to uneven penetration of FlAsH and ReAsH, as both dyes efficiently label the core of Q99-C4 aggregates [15]. In contrast, C4-tagged proteasomes (PSMB4-C4) showed a different distribution pattern in IBs that were initiated by co-expression of untagged mHtt(Q97)exon1 (Fig. 2C) or polyQ-peptides (Fig. 2F). After an 8 hours chase period, there was a partial overlap between the pre-existing and newly-synthesized proteasomes. After a 20 hours chase period, a complete overlap in localization of both proteasome pools was observed in ring-like structures. Indicating that the initial ReAsH-labeled pool exchanged with the newly-synthesized FlAsH-labeled pool of proteasomes. These findings indicate that proteasomes are dynamic within IBs. Fluorescence intensity plots of cross sections of IBs show the difference in localization of the initial pool (red) and the chased pool (green) of mHtt(Q97)exon1-C4 or proteasome subunit PSMB4-C4 in the IB (Fig. 2B and Fig. 2D, respectively). The difference in recruitment between proteasomes and mHtt was also shown biochemically. Proteasomes were present only in the SDS-soluble fraction of cells expressing mHtt(Q97)exon1, whereas polyQ proteins were present in both the SDS-soluble and SDS-insoluble fraction (Supplementary Fig. S2). Together, these findings indicate that proteasomes are not irreversibly trapped in mHtt IBs, but are dynamically recruited albeit with relatively low exchange rates.

Proteasomes recruited into IBs are still catalytically active and accessible

The dynamic recruitment of proteasomes into IBs and the direct interaction between proteasomes and polyQ fragments does not exclude that proteasomes may become catalytically impaired due to clogging by polyQ fragments, as suggested before [5, 6]. To examine whether proteasomes are active and accessible for substrates in IBs, we added activity based probe 1 (ABP1) (BodypiFL-to clogging by polyQ fragments, as suggested before [5, 6]. To examine whether proteasomes are still catalytically active, we performed in vitro zymography to identify only those proteasome complexes that are labeled by the activity probe (Fig. 3C). All proteasome complexes were labeled by ABP1 when cell lysates were incubated with the probe prior to native gel electrophoresis (left panel). However, uncapped 20S proteasomes were not labeled with ABP1 when the probe was added after separation of the different proteasome complexes by electrophoresis (right panel). This indicates that proteasome labeling with ABP1 only occurs when proteasomes are in complex with either 19S or PA28 activating caps. Since the probe was visualized via RFP-tagged PSMB4, indicating that there were no proteasomes present in the dense core that were not labeled and thus catalytically inactive (Fig. 3B). To further examine whether the activity-labeled proteasomes are also accessible to substrates, we preformed zymography to identify only those proteasome complexes that are labeled by the activity probe (Fig. 3C). All proteasome complexes were labeled by ABP1 when cell lysates were incubated with the probe prior to native gel electrophoresis (left panel). However, uncapped 20S proteasomes were not labeled with ABP1 when the probe was added after separation of the different proteasome complexes by electrophoresis (right panel). This indicates that proteasome labeling with ABP1 only occurs when proteasomes are in complex with either 19S or PA28 activating caps. Since the probe was visualized via RFP-tagged PSMB4, indicating that there were no proteasomes present in the dense core that were not labeled and thus catalytically inactive (Fig. 3B). To further examine whether the activity-labeled proteasomes are also accessible to substrates, we preformed zymography to identify only those proteasome complexes that are labeled by the activity probe (Fig. 3C). All proteasome complexes were labeled by ABP1 when cell lysates were incubated with the probe prior to native gel electrophoresis (left panel). However, uncapped 20S proteasomes were not labeled with ABP1 when the probe was added after separation of the different proteasome complexes by electrophoresis (right panel). This indicates that proteasome labeling with ABP1 only occurs when proteasomes are in complex with either 19S or PA28 activating caps. Since the probe was visualized via RFP-tagged PSMB4, indicating that there were no proteasomes present in the dense core that were not labeled and thus catalytically inactive (Fig. 3B). To further examine whether the activity-labeled proteasomes are also accessible to substrates, we preformed zymography to identify only those proteasome complexes that are labeled by the activity probe (Fig. 3C). All proteasome complexes were labeled by ABP1 when cell lysates were incubated with the probe prior to native gel electrophoresis (left panel). However, uncapped 20S proteasomes were not labeled with ABP1 when the probe was added after separation of the different proteasome complexes by electrophoresis (right panel). This indicates that proteasome labeling with ABP1 only occurs when proteasomes are in complex with either 19S or PA28 activating caps. Since the probe was visualized via RFP-tagged PSMB4, indicating that there were no proteasomes present in the dense core that were not labeled and thus catalytically inactive (Fig. 3B). To further examine whether the activity-labeled proteasomes are also accessible to substrates, we preformed zymography to identify only those proteasome complexes that are labeled by the activity probe (Fig. 3C). All proteasome complexes were labeled by ABP1 when cell lysates were incubated with the probe prior to native gel electrophoresis (left panel). However, uncapped 20S proteasomes were not labeled with ABP1 when the probe was added after separation of the different proteasome complexes by electrophoresis (right panel). This indicates that proteasome labeling with ABP1 only occurs when proteasomes are in complex with either 19S or PA28 activating caps.
needs access to the 20S core via these activator caps, activity labeling in IBs can therefore only occur when 20S proteasomes are activated and thus accessible for protein substrates, representing functional proteasomes. In addition, subsequent analysis by SDS gel electrophoresis under reducing conditions demonstrated that all three proteasomal catalytic subunits remained equally active in cells with mHtt(Q97)exon1-C4 or Ub-Q99-C4 initiated IBs (Supplementary Fig. S3).

**IBs in mHtt transgenic mice brains contain active proteasomes**

To translate our finding to an in vivo model, we incubated brain sections of R6/2 transgenic mice that express mHtt(Q200) [17] with ABP1 in order to confirm the presence of active proteasomes in neuronal IBs. Both the cerebral cortex and striatum in R6/2 mice have the most mHtt-induced IBs, and immunohistochemistry was used to identify the mHtt-containing IBs in neuronal cells in the cerebral cortex (Fig. 4). Co-localization of N18 (mHtt), NeuN (neuronal nuclei marker) and ABP1 confirmed the presence of active proteasomes within IBs in mouse brains, with 93 % (±6 %, SD) of the nuclear inclusions efficiently labeled with the proteasome activity probe, and 87 % (±5 %, SD) of the cytoplasmic inclusion bodies. To confirm the specificity of proteasome activity labeling in mouse brain tissue we pre-incubated brain slices with proteasome inhibitor epoxomycin, resulting in a decrease of fluorescence labeling (data not shown).

**DISCUSSION**

When we visualized the dynamics of proteasomes over a time span of hours, an exchange of proteasomes was observed (Fig. 2C). This is in contrast to earlier Fluorescence Recovery After Photo-bleaching (FRAP) experiments in living cells that did not show recovery of fluorescently-tagged proteasomes within a time span of minutes [5]. This indicates that proteasomes have a slow on-off rate into IBs, which was also suggested by the differences in distribution of fluorescently-tagged proteasomes in aggregates (Fig. 1A). Our data is in correspondence with the assumed protective role of IB formation, which was shown when aggregating GFP-tagged mHtt fragments in striatal neurons were imaged in time by automated fluorescence microscopy [18]. IB formation improved cell survival when compared to cells that showed only a diffuse mHtt distribution but no aggregates. Similarly, IB formation also coincided with less proteasomal impairment, which was detected using a short-lived UPS reporter [19]. A drop in proteasome activity was only observed just before IBs were formed, indicating that IB formation would indeed be a protective mechanism to sequester toxic mHtt species in the cell that may otherwise impair the UPS.

The observed recruitment of proteasomes into IBs appears to be independent of ubiquitination of the aggregated polyQ fragments. Aggregates induced by mHtt [20] but also by pure polyQ peptides [7] are initially devoid of ubiquitin, while proteasomes are directly recruited when small aggregates are formed. Although we show that the recruited proteasomes remain catalytically active and

![Figure 2](https://example.com/figure2.png)

**Fig. 2. Proteasomes are dynamically recruited into polyQ initiated IBs.**

(A) Fluorescence pulse-chase experiments show sequestration of mHtt polyQ fragments into IBs. Cells expressing mHtt(Q97) exon1-C4 for 48 h showed IBs that were labeled with ReAsH (red, R). Following a chase of 8 or 20 h, newly-synthesized mHtt(Q97) exon1-C4 was labeled with FlAsH (green, F), which was localized in a new layer around the existing IB induced by previously synthesized mHtt fragments. (B) Cross section of a mHtt(Q97)exon1 initiated IB showing differences in fluorescence pixel intensities of FlAsH and ReAsH labeling after a chase of 20 h. (C) Fluorescence pulse-chase experiments show redistribution of proteasomes in mHtt(Q77)exon1 induced IBs. Cells were transfected with untagged mHtt(Q77)exon1 to induce IBs and the proteasome subunit PSMB4-C4 which was subsequently labeled with ReAsH (red, R). Following a chase of 8 or 20 h, newly-synthesized PSMB4-C4 was labeled with FlAsH (green, F), showing an exchange between old (R) and newly-synthesized (F) proteasomes in yellow. (D) Cross section of C4-tagged proteasomes in the mHtt initiated IBs showing large overlap in fluorescence pixel intensities of FlAsH and ReAsH labeling after a chase of 20 h. (E) Fluorescence pulse-chase experiment shows sequestration of Q99 peptides into IBs. Cells expressing Ub-Q99-C4 for 48 h were labeled with ReAsH (red, R). Following a chase of 8 or 20 h, newly-synthesized Q99 peptides were labeled with FlAsH (green, F), which was localized in a new layer around the existing Q99 peptide in IBs (red, R). (F) Fluorescence pulse-chase experiment shows redistribution of proteasomes in polyQ peptide IBs. Cells containing untagged Q104-peptide IBs co-expressed the proteasomal subunit PSMB4-C4 for 48 h which was labeled with ReAsH (red, R). Following a chase of 8 or 20 h, newly-synthesized PSMB4-C4 was labeled with FlAsH (green, F), showing an exchange between old (R) and newly-synthesized (F) proteasomes with colocalization in yellow. Scale bar = 5 µm.
Our observation that proteasomes which are recruited into IBs remain active and accessible seems in contrast to earlier reports that suggest that proteasomes can become impaired due to unsuccessful attempts to degrade polyQ proteins. When examining polyQ protein degradation by proteasomes in vitro and in living cells, it was suggested that proteasomes may actually be unable to degrade the polyQ repeat present in the proteins. The undigested, expanded polyQ peptide derived from a degraded mHtt fragment may be unable to diffuse out of the proteasome, resulting in proteasomal impairment. A stable interaction between fluorescently-tagged proteasomes and fluorescent mHtt fragments was indeed observed by FRET microscopy [5], although this could also be due to a reversible interaction when proteasomes act on mHtt in IBs, as shown by FLIM microscopy (Fig. 1C). Importantly, our fluorescent pulse-chase experiments show that these interactions are reversible since there is an exchange of proteasomes at the periphery if IBs, in contrast to mHtt and polyQ peptides that are irreversibly trapped in IBs.

PolQ fragments that enter the proteasome appear to be efficiently degraded, since less aggregation is observed when polyQ proteins like mHtt are targeted towards the proteasome [21]. This indicates that the proteasome can indeed deal with polyQ proteins, which is in line with our results that proteasomes recruited into IBs remain catalytically active and accessible for substrates, a competition between regular UPS substrates and aggregating mHtt as substrates for the proteolysis network might affect the turn-over of other intracellular proteins, as recently proposed [20]. As discussed earlier, this would explain the increase in half-lives of various short-lived UPS reporters [19].
MATERIALS AND METHODS

DNA Constructs. mHtt(Q97)exon1 was generated by replacing the C-terminal GFP sequence of polyQ-expanded mHttexon1-GFP (kindly provided by Prof. RR. Kopito, Stanford University, USA) for a tetracycline C4-tag (FLNCPCGCCMEP) and a stop codon [14]. Ub-Q103 was generated as described previously [7]. Ub-Q99-C4 was generated by introducing a C4-tag after GFP-Ub-Q99 using two annealed C4-primers, forward 5'-GATGCTTCTTAATGTTTGCCGTTGGTGATAGGAACCTTAAT-3' and reverse 5'-CTATGTTACCGGTCATACAAACAGGACCATTATAGAGAAAC-3'. PSMB4-mRFP was kindly provided by Prof. NP. Dantuma (Karolinska Institute, Stockholm, Sweden), and the PSMB4-GFP and PSMB4-C4 constructs were generated by replacing the mRFP for an eGFP or C4-tag.

Cell culture and transfection. HeLa, Mel JuSo (melanoma) and HEK293 cells were cultured in DMEM supplemented with 10 % fetal calf serum at 37°C in a 5 % CO2 atmosphere. HEK293 cells were transfected with jetPEI (Polyplus transfection), HeLa and Mel JuSo cells were transient transfected with HD Fugene as described by the manufacturer (Roche Applied Sciences, Mannheim, Germany). For confocal microscopy imaging cells were grown on 2 cm coverslips (Menzel Glaser, Braunschweig, Germany) in 6-well plates.

Biarsenical labeling and confocal imaging. At 48 h after transfection, HeLa and Mel JuSo cells were stained as described by Martin et al. [14]. Briefly, 1 mM ReAsH which was pre-incubated in 10 mM 1,2-ethanediethiol (EDT, Sigma-Aldrich, Sigma-Aldrich, Steinheim, Germany) in dimethyl sulfoxide (DMSO) for 10 min. Subsequently, cells were washed using PBS (GIBCO/Invitrogen, Breda, The Netherlands) and incubated for 45 min at 37°C with 1 μM ReAsH in Optimem (GIBCO/Invitrogen, Breda, The Netherlands), followed by 4 washes at RT in wash medium (complete DMEM medium with 1 mM EDT). Mel JuSo cells were fixed with 2 % paraformaldehyde (EMS, Hatfield, PA, USA) in 1x PBS (GIBCO/Invitrogen, Breda, The Netherlands) prior to labeling and HeLa cells were fixed for labeling. Samples were examined using a Leica TCS SP2 confocal microscope equipped with Ar/Kr laser and 40x or 63x objective (Leica Microsystems, Mannheim, Germany).

Native gel analysis. HEK293 cells were harvested in TSDG buffer (10 mM Tris pH 7.5, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl2, 0.1 mM EDTA and 8 % glycerol) and lysed by 3 freeze/thaw cycles in liquid nitrogen. After centrifugation (15 min, 20,817 x g) the concentration of the supernatant was determined by a Bradford protein assay (Serva, Heidelberg, Germany). Proteasomes were labeled in the lysate using 0.5 μM ABP2 (bodipy-epoxomicin) [22] for 1 h at 37°C. 4x Native sample buffer (20 mM Tris pH 8.0, 50 % glycerol, 0.1 % bromophenol blue) was added to 30 μg lysate. The samples were loaded on a 4-12 % Criterion XT Precast Bis-Tris gel (Biorad, Hercules, CA, USA) and ran for 4 h at 180V. Fluorescence imaging was performed on a Trio Thyspooon (GE Healthcare) using the 580 BP 30 filter to detect the bodipy-epoxomicin probe and mRFP, the 610 BP 30 filter to detect ReAsH and the 520 BP 40 filter for GFP detection. For western blotting the gels were incubated 0.5 h in transfer buffer with SDS added (25 mM Tris pH 7.5, 192 mM Glycine, 20 % MeOH and 0.1 % SDS) and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). α2 proteins were detected by the MCP236 antibody (1:1000) (kindly provided by Rasmus Hartmann-Petersen, Biologisk Institut, University of Copenhagen), and detection was done by the Odyssey detection system (LICOR Biosciences, Lincoln, NE, USA).

Confocal FLIM. We used EGFP and the red biarsenical fluorophore ReAsH as a FRET pair. Data was acquired using an Olympus IX81 inverted microscope with a FV1000- and confocal detection head, controlled by FV2.3 software. Donor fluorophores were excited using a 440 nm 20 MHz pulsing laser diode (Picoquant, Berlin, Germany), as controlled by a Sepiall laser driver unit (Picoquant, Berlin, Germany) and attenuated 10 times by a neutral density filter. The light was guided via a D405/480/560/635 primary dichroic mirror (Chroma, Brattleboro, VT, USA) through a water immersed 60x UPlanS-apo objective (NA 1.2) into the sample. Cellular samples were grown on 24 mm round coverslips (Menzel Glaser, Braunschweig, Germany) fixed in 2 % paraformaldehyde (EMS, Hatfield, PA, USA) and stored in PBS (GIBCO/Invitrogen, Breda, The Netherlands). The emission light was guided via a size-adjustable pinhole, set at 120 μm, through the Olympus detection box to the fibre output channel. The optical fibre was coupled to a custom-made detection box (Picoquant, Berlin, Germany) containing MPD avalanche photodiodes. The light was guided into one of the MPDs and filtered by a 525/45 emission filter (Chroma, Brattleboro, VT, USA). The photon arrival times were recorded by a Picoharp 300 time-correlated single-photon counting system (Picoquant, Berlin, Germany) and analysed in SymPhoTime 5.13 software (Picoquant, Berlin, Germany). For each ROI the fluorescence decay curve was fitted using a mono-exponential decay fit, including the estimated instrument response function (IRF) and a background term. For data analysis only fluorescence decays having more than 10.000 registered photons in the peak were considered and the quality of the fit was judged by visual inspection of the fitresiduals and the normalized Chi2 value.

Soluble and insoluble fraction analysis. HEK293 cells were lysed in 1.5 % SDS buffer (70 mM Tris pH 6.8, 1.5 % SDS, 20 % glycerol) and sonicated by the Soniprep150 (Sanyo, Leicester, UK), 50 mM EDT was added and samples were boiled for 10 min prior to 1 h centrifugation at 20,817 x g at RT. The pellet fraction, representing the insoluble fraction, was incubated with 10 μl 100 % formic acid, incubated at 37°C for 30 min and lyophilized for o/n in a speedvac (Eppendorf, Hamburg, Germany). 1.5 % SDS buffer was added to the pellet, following the soluble and insoluble fractions, which were supplemented with 0.05 % bromophenol blue, were loaded on a 12.5 % SDS-page gel. Polyglutamine stretches were detected by the 1C2 antibody (MAB 1574, 1:1000, Millipore, Bedford, MA, USA), α2 detection was done by the MCP236 antibody (1:1000, kindly provided by R. Hartmann-Petersen, University of Copenhagen, Denmark) and β-actin detection was done by N-21 (130656, 1:1000, Santa Cruz, Santa Cruz, CA, USA). For activity labeling cells were incubated in complete medium with 0.5 μM ABP2 at room temperature after harvesting. Subsequently, cells were washed in PBS (GIBCO/Invitrogen, Breda, The Netherlands), collected and treated like the above mentioned procedure. After running the samples on a 12.5 % SDS gel, the wet gel slab was scanned for fluorescence and further subjected for western blotting.

Pulse-Chase experiments. HeLa cells were labeled with ReAsH as describes above to stain the pre-existing pool of C4-tagged proteins. After washing the cells were incubated at 37°C for 8 or 20 h in 20 % fetal calf serum supplemented DMEM. After the chase, newly synthesized proteins were labeled with
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FLASH by the same procedure. Subsequently, cells were fixed in 2 % paraformaldehyde (EMS, Hatfield, PA, USA), embedded in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA) and analyzed by confocal microscopy, using Huygens essentials software (BioVision technologies, Exton, PA, USA) to determine fluorescence intensities of the cross sections.

Proteasome activity labeling in living cells. Mel Ju50 cells were incubated for 1 h at 37°C in serum free medium supplemented with 0.25 μM ABP1 (BodypFL-Ahx3LVS) [16]. Cells were subsequently washed 3 times in PBS (GIBCO/Invitrogen, Breda, The Netherlands) and fixed in 2 % paraformaldehyde (EMS, Hatfield, PA, USA) prior to ReAsH staining.

Activity labeling in gel. HEK293 cells were harvested in TSGD buffer (10 mM Tris pH 7.5, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl2, 0.1 mM EDTA and 8 % glycerol), lysed by three freeze/thaw cycles in liquid nitrogen and protein levels were determined by a Bradford assay (Serva, Heidelberg, Germany). 40 μg of cell lysates were incubated with 0.5 μM ABP1, 0.5 μM Epoxomicin or similar amounts of DMSO for 1 h at 37°C. Samples were loaded on a 4-12 % Criterion XT Precast Bis-Tris gel (Biorad, Hercules, CA, USA) and western blotting was performed as described above.

Activity labeling in vivo. Freshly frozen brains of 15-week-old R6/2 mice (mHtt(Q200)) and littermate controls were used (kindly provided by prof. G. Bates, London). 10 μm thick brain slices were mounted on 1 mm thick PEN membrane slides (Zeiss, Oberkochen, Germany), dried for 10 minutes at room temperature and stored at -20°C until further use. Sections were incubated for 10 minutes with 1 μM ABP1 in 10 mM PBS. After 3x washing with PBS, sections were fixed with 4 % paraformaldehyde (EMS, Hatfield, PA, USA). Next, sections were pre-incubated in permeabilization buffer (1 % BSA, 2 % FCS and 0.4 % Triton-X100) for 1 h and incubated with antibodies against the N-terminal region of mHtt-exon1 (sc-8767, 1:500, Santa Cruz, Santa Cruz, CA, USA) and the neuronal nuclear protein NeuN (Mab377, 1:500, Chemicon, Temecula, CA, USA) for 2 h. Sections were washed and incubated with Cy3- or Dylight649-labeled secondary antibodies (1:400, Invitrogen). After washing the samples were embedded in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA) on 24x60 mm cover slips. Images of the cortal and striatal region were analyzed by using a Leica TCS SP8 X Confocal Microscope (Leica Microsystems, Mannheim, Germany). The whole procedure was conducted at room temperature.

Acknowledgements

We like to thank Alicia Sanz Sanz, Andrea Lehmann and Dr. Frédéric Ebstein for their contributions in the experiments, Dr. R. Hartmann-Petersen for providing the MCP236 antibody, Prof. PM. Klötzlel for fruitful discussions, and Prof. CIF. van Noorden for critically reading the manuscript.

Supplementary fig. 1.
PSMB4 is efficiently incorporated in 20S proteasomes. HEK293 cells were transiently transfected with PSMB4-RFP (lane 2), PSMB4-GFP (lane 3) and PSMB4-C4 (labeled with ReAndH, lane 4), subjected to electrophorartion on a native gel and scanned for fluorescence. Activity based probe 2 (ABP2) (Bodyp-epoxomicin) labeled proteasomes were used as a marker for proteasome complexes (lane 1). The fluorescently-labeled subunits can be identified at similar levels of active proteasome labeling, confirming the incorporation into proteasomal complexes (lane 2-4). Right panel, presence of the 20S complex was confirmed by Western blotting against α2.

Supplementary fig. 2.
Proteasomes are not sequesterd in the insoluble fraction of 20S proteasomes. HEK293 cells were transiently transfected with mHtt(Q97) and lysed after 72 h in 1.5 % SDS containing buffer. The insoluble fraction, including aggregated proteins, was separated from the soluble fraction and solubilized using formic acid. Whereas polyQ Htt is present in both the soluble and insoluble fraction, proteasomes were only present in the soluble fraction (as detected by the α2 subunit of the proteasome).

Supplementary fig. 3.
All three catalytic sites remain equally active in cells with IBs as compared to wild type cells. HEK293 cells expressing Htt-exon1(Q200) or Ub-Q99-C4 were incubated with the ABP2 and similarly treated as described in Supplementary Figure S2. While mHtt(Q97) (arrow) and poly peptides (arrow heads) were detected in the insoluble SDS-fraction, proteasomes were only present in the soluble SDS-fraction. Activity labeling of proteasomes in cells containing polyQ initiated IBs or control cells showed similar efficiencies in labeling of the catalytic subunits.

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REFERENCES


