Neuronal dysfunction in a polyglutamine disease model occurs in the absence of ubiquitin–proteasome system impairment and inversely correlates with the degree of nuclear inclusion formation

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The accumulation of protein deposits in neurons, in vitro proteasome assays and over-expression studies suggest that impairment of the ubiquitin–proteasome system (UPS) may be a common mechanism of pathogenesis in polyglutamine diseases such as Huntington disease and spinocerebellar ataxias (SCAs). Using a knock-in mouse model that recapitulates the clinical features of human SCA7, including selective neuronal dysfunction, we assessed the UPS at cellular resolution using transgenic mice that express a green fluorescent protein (GFP)-based reporter substrate (Ub⁷⁶V-GFP) of the UPS. The levels of the reporter remained low during the initial phase of disease, suggesting that neuronal dysfunction occurs in the presence of a functional UPS. Late in disease, we observed a significant increase in reporter levels specific to the most vulnerable neurons. Surprisingly, the basis for the increase in Ub⁷⁶V-GFP protein can be explained by a corresponding increase in Ub⁷⁶V-GFP mRNA in the vulnerable neurons. An in vitro assay also showed normal proteasome proteolytic activity in the vulnerable neurons. Thus, no evidence for general UPS impairment or reduction of proteasome activity was seen. The differential increase of Ub⁷⁶V-GFP among individual neurons directly correlated with the down-regulation of a marker of selective pathology and neuronal dysfunction in SCA7. Furthermore, we observed a striking inverse correlation between the neuropathology revealed by this reporter and ataxin-7 nuclear inclusions in the vulnerable neurons. Altogether, these data show a protective role against neuronal dysfunction for polyglutamine nuclear inclusions and exclude significant impairment of the UPS as a necessary step for polyglutamine neuropathology.

INTRODUCTION

Expansion of the glutamine-encoding CAG triplet repeats in nine different genes is the cause of several degenerative neurological diseases (Huntington disease, SCA-1, -2, -3, -6, -7 and -17, DRPLA and SBMA) (1). A common feature of polyglutamine diseases is the neuronal accumulation of the mutant protein in nuclear or cytoplasmic inclusions (2,3). Similarly, expression of proteins containing long polyglutamine tracts in mice leads to protein aggregation, inclusions and neuronal dysfunction, thus providing an important model system for the study of disease pathology. These models have shown that the rate of neurological dysfunction is proportional to the length of the polyglutamine tract and expression level of the expanded protein. In fact, many investigators take advantage of this fact and use polyglutamine tracts much longer than normally observed in human disease, or over-express the mutant proteins, to observe neuropathology in the short lifespan of the mouse.

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Involvement of the ubiquitin–proteasome system (UPS) is strongly implicated in polyglutamine diseases (4). Polyglutamine inclusions and aggregates contain ubiquitin, molecular chaperones and components of the proteasome, suggesting that the mutant proteins are abnormally folded and may be targeted for degradation by the UPS (5–7). The continuous accumulation of these proteins suggests that they may be resistant to degradation by the UPS. A relationship between the UPS and polyglutamine diseases was bolstered by research showing that genes in the UPS and protein folding pathways are modifiers of neurodegeneration in disease models and by recent work that provides indirect evidence of UPS impairment in human patients (8–13). These observations have culminated in the hypothesis that impairment of the UPS may underlie the polyglutamine neurotoxicity and pathogenesis.

Support for the role of UPS impairment in polyglutamine neuropathology has been indirectly substantiated by two major findings. First, investigators using a GFP-based reporter of UPS activity observed near-complete impairment of the UPS by polyglutamine inclusions in an over-expression cell culture model (14). Impairment of UPS activity was confirmed in other over-expression cell culture models by examination of endogenous proteasome targets and in vitro proteasome activity assays (15,16). Second, recent work demonstrates that eukaryotic proteasomes fail to degrade polyglutamine sequences in vitro (17). These authors suggest that the failure of the proteasome to release undigested polyglutamine repeats may interfere with proteasome function. This idea is supported by recent work showing inefficient degradation of over-expressed polyglutamine proteins and sequestration of the proteasome by polyglutamine protein aggregates in cultured cells (18). However, contradicting data have been presented, showing that soluble polyglutamine-containing proteins with a strong degradation signal are efficiently degraded in cultured cells regardless of glutamine tract length and without proteasome impairment (19–21). Additionally, conditional polyglutamine disease models show rapid clearance of polyglutamine inclusions from neurons (22,23). Furthermore, measurement of extractable proteasome activity from transgenic mice expressing a mutant huntingtin fragment and from a knock-in Huntington disease model has revealed no change in proteasome activity by in vitro proteasome assays (24,25). Issues of soluble versus insoluble polyglutamine protein, expression level, protein and cellular context, and the nature of the degradation signal may justify these discrepancies. Most importantly, none of these studies examined UPS activity in the context of the vulnerable neuronal populations in vivo. Although measurement of extractable proteasome activity in the earlier mentioned mouse models comes close, the vulnerable neurons represented a minority of cells from the extracted tissues. Therefore, if proteasome impairment is restricted to the vulnerable neurons, then the reduction to extractable proteolytic activity would be small and likely undetectable. Moreover, besides reduction of proteasome activity, many other events can potentially impair the complex UPS under pathological conditions (26). To resolve these issues, it is necessary to examine UPS activity specifically in the vulnerable neuronal population during disease progression.

Spinocerebellar ataxia 7 (SCA7) is an autosomal dominant neurodegenerative disease characterized by ataxia, retinal degeneration and brainstem dysfunction. SCA7 is caused by the expansion of the glutamine-encoding CAG repeat in SCA7. In normal individuals, the repeat length ranges from 4 to 35 repeats, whereas affected individuals have between 37 and 460 repeats (27–29). As with other polyglutamine diseases, the age of onset is inversely proportional to repeat length. Repeat lengths less than 60 typically cause adult onset disease, repeat lengths greater than 60 cause juvenile onset and repeat lengths greater than 200 cause infantile onset. Clinically, infantile SCA7 is among the most severe forms of polyglutamine diseases and is associated with very large repeat expansions (27,29).

We have generated a knock-in model of SCA7 by inserting 266 glutamine-encoding CAG repeats (instead of the usual five CAG repeats) into the endogenous SCA7 mouse gene (30). This polyglutamine disease model displays high fidelity to the pathological features of the human disease including expression of a single mutant allele by endogenous control elements, a polyglutamine repeat size within the range observed in humans, considerable overlap in the specific vulnerable neurons and shared symptoms with patients who have similar repeat sizes (30). The mice (Sca7266Q/5Q) develop molecular changes in the retina at 4 weeks and functional impairments of the retina and cerebellum at 5–6 weeks. The visual and neurological functions of these mice progressively deteriorate; by 14 weeks (C57Bl/6j genetic background), the animals die due to brainstem dysfunction. There is a gradual accumulation of insoluble ataxin-7 aggregates concomitant with disease progression. Like other polyglutamine diseases, ataxin-7 nuclear inclusions form in both affected and unaffected cells of the brain and retina. The cone and rod photoreceptor neurons, whose nuclei are found in the outer nuclear layer of the retina, are the most sensitive to SCA7 pathology. In fact, although ataxin-7 nuclear inclusions eventually form in all three nuclear layers of the retina, significant dysfunction, apoptosis and cell loss are confined to the photoreceptors. A striking decrease of abundant photoreceptor specific transcripts, first in the cones and then in the rods, precedes cell loss and detectable functional impairment of the photoreceptors and is the earliest known pathological feature. Furthermore, disease progression is marked by the severe and rapid loss of the photoreceptor outer segments, a decrease in photoreceptor inner segment (IS) mass and ONL photoreceptor cell count, with little to no detectable loss of cells, both neurons and glia, located in the other layers of the retina. The retinal neuropathology of SCA7 presents a unique opportunity to study polyglutamine neurotoxicity in a tissue where the vulnerable neurons are a majority (>70%) of the cells (31).

A prominent mechanism by which UPS impairment has been proposed to occur in polyglutamine disease is through direct blockage of the 20S core proteasome by polyglutamine sequences (17,32). The mechanism suggests that the longer the polyglutamine tract, the more severe the proteasome impairment might be. Thus under this mechanism, infantile SCA7 with its very large polyglutamine tract would be the most sensitive disease for detecting such an impairment. The UbG76V-GFP transgenic mouse has been developed as an
in vivo reporter of the UPS (33). The Ub^{G76V}-GFP reporter protein is an N-terminal ubiquitin mutant (Ub^{G76V}) in frame with the enhanced green fluorescent protein (GFP), widely expressed at high levels by a chimeric CMV-IE enhancer, chicken β-actin promoter. The reporter is rapidly processed and degraded by the cellular UPS, so steady state levels are very low under wild-type conditions. Impairment to the UPS in vivo causes an accumulation of the Ub^{G76V}-GFP reporter protein, sensitive to both the extent and duration of inhibition (33). Alterations in core proteasome activity and general UPS activity are detectable by this reporter, whereas impairment to some target-specific UPS components (e.g. a specific ubiquitin ligase not active on the reporter) may not be detected (33,34).

Here, we use this reporter to assess impairment of the UPS at cellular resolution in a mouse model of the polyglutamine disease SCA7.

RESULTS

Using an in vivo reporter to assess the UPS in SCA7

Male Sca^7266Q/5Q (henceforth simplified to Sca^7266Q) mice were crossed with female Ub^{G76V}-GFP/1^+ transgenic mice to generate experimental animals for assessment of the UPS. As an initial assessment of the UPS, Ub^{G76V}-GFP/1^+ /Sca^7266Q/5Q (henceforth simplified to Ub^{G76V}-GFP^+), Ub^{G76V}-GFP/1^+ /Sca^7266Q and Ub^{G76V}-GFP/1^-/Sca^7266Q (henceforth called non-transgenic) littermates were analyzed by western blot analysis (Fig. 1A and B, first lanes). Analysis of the UPS reporter levels in retinas of 6-week-old animals (early stage of pathogenesis) revealed no significant difference between Ub^{G76V}-GFP^+ and Ub^{G76V}-GFP^- Sca^7266Q animals (Fig. 1A). However, by 13 weeks (terminal stage of pathogenesis), there was a significant increase in reporter levels in Ub^{G76V}-GFP^+ Sca^7266Q retinas (Fig. 1B). This was due to the general blockade of the UPS in the initial phase of the SCA7 pathology, it raised the possibility of an impairment to the UPS at the final stages of disease.

To assess changes in Ub^{G76V}-GFP levels in specific layers of the retina, we used laser confocal microscopy on 16 μm frozen sections of retina to observe the native GFP fluorescence. We can detect Ub^{G76V}-GFP protein by its native fluorescence in Ub^{G76V}-GFP^- mouse retina as visualized by comparison with the non-transgenic control (Fig. 2A). We observed a significant increase in GFP fluorescence, specifically in the ONL of 13-week-old Ub^{G76V}-GFP^+ Sca^7266Q mice (Fig. 2A). To confirm the increase of Ub^{G76V}-GFP reporter protein, we used laser confocal microscopy on replicate 16 μm frozen retina sections stained for GFP by immunofluorescence visualized with Cy3 to avoid overlap with the emission spectrum of native GFP fluorescence. Again, 13-week-old Ub^{G76V}-GFP^+ Sca^7266Q mice showed a specific increase in reporter levels in the ONL of the retina when compared with Ub^{G76V}-GFP^- littermates (Fig. 2B). Comparison of the Ub^{G76V}-GFP images with Toto-3 nuclear stained images revealed that Ub^{G76V}-GFP is both cytoplasmic and nuclear, but excluded from the more densely nuclear stained heterochromic core of the ONL photoreceptors.

To determine how much Ub^{G76V}-GFP protein levels change during SCA7 pathogenesis and to quantify the difference, we have measured native GFP fluorescence levels in retina of Ub^{G76V}-GFP^- and Ub^{G76V}-GFP^+ Sca^7266Q and non-transgenic littermate sets at 5, 7, 9, 11, 13 and 14 weeks of age (Sca^7266Q animals die at 13–14 weeks). To optimize quantitative measurements, samples in each set were prepared and analyzed side-by-side in identical fashion. Although GFP immunofluorescence gave a higher signal-to-noise ratio, native fluorescence was determined to be optimal for quantitative analysis due to significantly lower signal variability. The fluorescent signal from the Ub^{G76V}-GFP reporter in Ub^{G76V}-GFP^- and Ub^{G76V}-GFP^+ Sca^7266Q animals was detected significantly higher than the background signal of the non-transgenic control for all data sets (t-test, P < 0.005) (Fig. 3A and B). Analysis across time points revealed an increase in Ub^{G76V}-GFP protein levels in the ONL over the course of pathogenesis (Fig. 3A). In early-stage pathogenesis (5 and 7 weeks), reporter protein levels were not significantly higher in the Ub^{G76V}-GFP^- Sca^7266Q mice when compared with Ub^{G76V}-GFP^- mice. By mid-stage pathogenesis (9 and 11 weeks), Ub^{G76V}-GFP^+ Sca^7266Q ONL reporter levels were consistently and significantly elevated, on average 1.6-fold above Ub^{G76V}-GFP^- ONL reporter levels (t-test, P < 0.001). By late-stage pathogenesis (13 and 14 weeks), Ub^{G76V}-GFP^- Sca^7266Q ONL reporter levels were further elevated from Ub^{G76V}-GFP^- Sca^7266Q mid-stage levels and were on average 3.2-fold above Ub^{G76V}-GFP^- ONL levels. In contrast, inner nuclear layer (INL) levels were not substantially different between Ub^{G76V}-GFP^- and Ub^{G76V}-GFP^- Sca^7266Q animals over the time course of pathogenesis (Fig. 3B). Elevation of Ub^{G76V}-GFP levels over the course of disease was confirmed in an independent Sca^7266Q line of mixed C57Bl/6J and 129SV/EV background (data not shown). We also observed an increase in Ub^{G76V}-GFP protein in the IS of Ub^{G76V}-GFP^+ Sca^7266Q animals when compared with Ub^{G76V}-GFP^- animals (Fig. 2A and B). However, the loss of IS mass in the Sca^7266Q animals made the follow-up measurements of this difference problematic.
Figure 2. Ub^{G76V}-GFP reporter levels are increased specifically in photoreceptors. The sum images of z-section confocal image scans of retina cryosections of littermate animal sets. The IS, ONL and inner nuclear layer (INL) are indicated on the left; genotype and age are indicated on the right. Photoreceptor nuclei reside in the ONL. (A) Left panels in blue show Toto-3 nuclear stain image; right panels in green show native GFP fluorescence. There is a specific increase in Ub^{G76V}-GFP levels in the ONL. (B) Left panels in blue show Toto-3 nuclear stain image; right panels in green show anti-GFP immunofluorescence using a Cy3-conjugated secondary antibody for visualization. There is a specific increase in Ub^{G76V}-GFP levels in the ONL.
An increase in UbG76V-GFP mRNA levels accounts for the increase in protein levels

Steady state levels of the UbG76V-GFP protein are determined by a balance between protein production and protein degradation. For changes in UbG76V-GFP protein levels to be an accurate measure of UPS activity, changes in protein production must be minimal relative to changes in protein degradation in the samples being compared. Because many abundant photoreceptor specific transcripts are significantly down-regulated in the Sca7266Q mice, we wanted to determine whether changes in the levels of the reporter mRNA were influencing our measurement of UPS activity. Our predominant concern was that UbG76V-GFP mRNA levels would be decreased in the Sca7266Q mice leading to an underestimate of UPS impairment. The UbG76V-GFP mRNA levels were similar between UbG76V-GFP+ and UbG76V-GFP+ Sca7266Q animals at an early stage of pathogenesis (5 weeks). However, contrary to expectations, we observed a significant increase in UbG76V-GFP mRNA levels in late-stage pathogenesis (12.5 weeks) in UbG76V-GFP+ Sca7266Q animals by northern blot (Fig. 4A). Furthermore, the difference in UbG76V-GFP mRNA levels in total retina at late stages of pathogenesis matched, or were perhaps slightly greater than, the difference in UbG76V-GFP protein levels for total retina at late stages determined by both confocal microscopy and western blot (Fig. 4B). Thus, the levels of reporter mRNA reflected the levels of reporter protein at both early and late stages of pathogenesis. To determine whether the increase in UbG76V-GFP mRNA levels was specific to the ONL, like the increase in UbG76V-GFP protein levels was, we performed in situ hybridization on 16 μm frozen retinal sections. We observed a striking increase in UbG76V-GFP mRNA levels in the ONL and IS (where the protein production machinery of the photoreceptor cells resides) in UbG76V-GFP+ Sca7266Q animals by hybridization (data not shown). The changes in UbG76V-GFP mRNA levels were remarkably similar to the changes in UbG76V-GFP protein levels.

An independent assay confirms normal levels of proteasome activity

The change in UbG76V-GFP protein levels observed in UbG76V-GFP+ Sca7266Q animals was matched by a change in UbG76V-GFP mRNA levels, indicating no significant impairment of UPS activity. We considered the possibility that proteasome activity may have been reduced in the Sca7266Q retina, but left unnoticed in the reporter mice because it did not reach the critical threshold for functional impairment of the UPS. To test this possibility, we measured the extractable chymotrypsin-like proteasome activity in wild-type and Sca7266Q littermates at the final stages of pathogenesis. Two independent measurements of extractable proteasome activity failed to detect a decrease in the proteasome activity in the retina (Fig. 6). In fact, a slight elevation in proteasome activity was observed in both sample pairs. This assay also failed to detect a decrease in proteasome activity in another vulnerable tissue of Sca7266Q animals, the cerebellum (data not shown).

UbG76V-GFP levels as a marker of polyglutamine neuropathology

A marker of SCA7 pathogenesis and photoreceptor dysfunction is the progressive down-regulation of photoreceptor specific transcripts, such as Rhodopsin (30). As the increase in UbG76V-GFP mRNA and protein levels observed is specific to the vulnerable photoreceptor neurons, while less vulnerable neurons of the retina show no substantial change, UbG76V-GFP reporter levels seem to be a marker of selective neuropathology.
Examination of the UbG76V-GFP protein pattern revealed a non-uniform accumulation in the ONL. To determine whether the variation in UbG76V-GFP levels reflects differences in the degree of neuronal dysfunction and pathology between individual neurons, we compared UbG76V-GFP levels with the levels of rhodopsin in photoreceptors by immunofluorescence. By late (14 weeks)-stage pathogenesis, rhodopsin mRNA levels have been reduced for several weeks, allowing sufficient time for protein levels to decline. Although the majority of rhodopsin is located in the outer segment, staining in the ONL is detectable in both wild-type and mutant retina (data not shown). The photoreceptors with the highest levels of UbG76V-GFP reporter consistently displayed relatively low levels of rhodopsin (Fig. 7A), whereas photoreceptors with the highest levels of rhodopsin consistently displayed relatively low levels of UbG76V-GFP (Fig. 7B). To quantify this relationship, the mean fluorescent signal of rhodopsin and UbG76V-GFP for individual photoreceptors in the ONL was measured for ONL cells with the highest intensity of rhodopsin signal and ONL cells with highest intensity of UbG76V-GFP signal (see Materials and Methods). The 95% confidence interval for both measures of the two classes of cells was plotted on an XY graph to illustrate the inverse relationship (Fig. 7C). The mean UbG76V-GFP and mean rhodopsin signals were inversely correlated and significantly different (t-test, P < 0.0001) between the class of photoreceptors with the brightest rhodopsin staining and the class of photoreceptors with the brightest UbG76V-GFP signal. Therefore, the increase in UbG76V-GFP reporter levels in individual photoreceptors directly correlates with the down-regulation of rhodopsin, a marker of selective neuropathology and neuronal dysfunction.

**Strong inverse relationship between UbG76V-GFP detected neuropathology and ataxin-7 nuclear inclusions**

Nuclear inclusions are a hallmark feature of polyglutamine disease. To determine how the abundance of the UbG76V-GFP reporter in individual photoreceptor neurons of the ONL correlated with the accumulation of inclusions, we stained the retinal sections for ataxin-7 nuclear inclusions by immunofluorescence. We observed a strong inverse correlation between UbG76V-GFP protein levels and the size and intensity of ataxin-7 nuclear inclusions from mid (9 weeks) to late (14 weeks) stages of pathogenesis. For example, at mid-stage pathogenesis, the photoreceptor nuclei with the highest intensity nuclear inclusions had the weakest UbG76V-GFP signal (Fig. 8A), whereas the cells with the strongest UbG76V-GFP signal had weaker staining or lacked ataxin-7 nuclear inclusions (Fig. 8A). The confounding effect of multiple cells on the same z-axis makes visualization of this observation in two-dimensional reconstructions of the confocal images difficult, as a photoreceptor cell with a bright nuclear inclusion may lie behind a cell with a bright UbG76V-GFP signal. Volume rendering three-dimensional reconstructions of the ataxin-7 nuclear inclusions and UbG76V-GFP signal revealed the robustness of this observation (data not shown). Examination of three-dimensional reconstructions revealed that photoreceptors with the brightest ataxin-7 nuclear inclusions invariably had relatively weak UbG76V-GFP reporter levels, whereas photoreceptors with the strongest UbG76V-GFP reporter levels invariably had the weakest stained or lacked detectable ataxin-7 nuclear inclusions altogether. This inverse correlation is in stark contrast with the ataxin-7 staining pattern in the less vulnerable cells of the INL, where the UbG76V-GFP reporter levels were unchanged from wild-type and independent of the nuclear inclusions (data not shown). To quantify the inverse correlation in the photoreceptors, the mean fluorescent signal of ataxin-7 and UbG76V-GFP for individual photoreceptors in the ONL was...
measured for ONL cells with high intensity ataxin-7 nuclear inclusions and ONL cells with high intensity Ub\textsuperscript{G76V}-GFP signal at mid (9 weeks) and late (14 weeks) stages of pathogenesis (see Materials and Methods). The 95% confidence interval for both measures of the two classes was plotted on an \(XY\) graph to illustrate the inverse relation (Fig. 8B). The mean Ub\textsuperscript{G76V}-GFP and mean ataxin-7 signals were inversely correlated and significantly different

Figure 5. Ub\textsuperscript{G76V}-GFP mRNA levels are specifically increased in photoreceptors. Representative \textit{in situ} hybridization for Ub\textsuperscript{G76V}-GFP mRNA (purple) with an eosin counter stain (pink). IS, ONL and INL are indicated on the left of each panel; genotype and age are indicated on the right. Photoreceptor nuclei reside in the ONL, and photoreceptor protein translation occurs in the IS. Both layers show a specific increase in Ub\textsuperscript{G76V}-GFP mRNA levels.
DISCUSSION

We have examined the UPS in a mouse model of the polyglutamine disease SCA7 (specifically the infantile form); in this model, the real-time progression of disease and selective neuronal vulnerability is similar to what is seen in infant patients with similar polyglutamine expansions (30). We have examined the neurons most vulnerable to this disease with an in vivo reporter that assays UPS activity at the resolution of individual cells. Because the increase in UbG76V-GFP protein levels were more than matched by an increase in UbG76V-GFP mRNA levels, and because in vitro assays of extractable chymotrypsin-like proteasome activity were at normal levels, in fact possibly higher in the mutants, our experiments rule out significant impairment of the UPS in the Sca7266Q model. Furthermore, no changes in UbG76V-GFP reporter levels are observed at 5 and 7 weeks of age, despite documented photoreceptor dysfunction as measured by electrophysiological and molecular studies (30). It is difficult to determine the minimal level of UPS impairment necessary to detect an increase in UbG76V-GFP reporter levels, as relating independent measures of proteasome activity (e.g. extractable chymotrypsin-like proteasome activity) to changes in UPS activity on in vivo substrates is problematic (26). However, previous work has shown that the UbG76V-GFP transgenic reporter line is sensitive to both the time and degree of proteasome impairment (33). Twenty hours after treatment with proteasome inhibitors at the concentration necessary to

$t$-test, $P < 0.0001$ at both 9 and 14 weeks) between the class of photoreceptors with the brightest ataxin-7 nuclear inclusions and the class of photoreceptors with the brightest UbG76V-GFP signal.

Figure 6. Proteasome activity is not decreased in an in vitro proteasome assay. Chymotrypsin-like proteasome activity in total retinal protein extracts of wild-type (5Q) and Sca7266Q (266Q) animals in two littermate pairs. Arbitrary in vitro activity relative to total protein concentration of each extract is graphed, with error bars indicating the sum of the linear regression errors. No impairment in proteasome activity was detected.

![Figure 6](image_url)

Figure 7. UbG76V-GFP levels in the ONL are a marker of neuronal dysfunction. The down-regulation of Rhodopsin in photoreceptors is a marker of pathology and neuronal dysfunction in SCA7. The photoreceptor cells of the ONL were analyzed by confocal imaging of 14-week-old UbG76V-GFP+ Sca7266Q retina cryosections stained by immunofluorescence for rhodopsin and UbG76V-GFP. (A) Representative single optical section of a photoreceptor cell classified to the group of cells having the brightest UbG76V-GFP staining. Single channel and merged images are shown. (B) Representative single optical section of a photoreceptor cell classified to the group of cells having the brightest rhodopsin staining. Single channel and merged images are shown. (C) A two-point XY plot showing the quantitative confocal measurement of 14-week-old UbG76V-GFP+ Sca7266Q retinas, plotting the arbitrary mean rhodopsin signal (Y-axis) and the arbitrary mean anti-GFP immunofluorescent signal (X-axis) from two groups of photoreceptors, those with the brightest rhodopsin staining (red square) versus those with the brightest UbG76V-GFP signal (green circle). Error bars indicate the 95% confidence interval for the mean value of the rhodopsin signal (error bars along the Y-axis) and the UbG76V-GFP signal (error bars along the X-axis) for individual photoreceptors of each group ($n = 22$). Photoreceptor cells with the highest levels of UbG76V-GFP exhibit the weakest levels of rhodopsin, whereas photoreceptor cells with the least degree of rhodopsin down-regulation show the lowest levels of UbG76V-GFP. A strong inverse correlation in both rhodopsin and UbG76V-GFP signals is observed between the two groups.
detect statistically significant changes in extractable proteasome activity, many mouse tissues showed substantial increases in UbG76V-GFP reporter levels; moreover, proteasome impairment in primary cultures showed a 10-fold increase after 16 h. Because photoreceptor dysfunction and pathology persist for at least 9 weeks (from 5 weeks of age till death at 14 weeks of age) in the Sca7 266Q mutant line, this allows substantial time for potential reporter accumulation. Furthermore, our analysis indicated that we could detect very small changes, such as an increase of just 1.5-fold in ONL UbG76V-GFP protein levels, at a high statistical confidence. The high sensitivity of our assay, coupled with the ability to examine UPS activity at the cellular level, should have greatly facilitated our ability to detect impairments to the UPS (34). It is thereby remarkable that we fail to detect significant UPS impairment in the most vulnerable neuronal population even at the terminal stages of pathogenesis.

Although there could be UPS impairment in the Sca7266Q model below detection of the UbG76V-GFP reporter, it is noteworthy that another proteasome targeted GFP reporter easily detected UPS impairment upon over-expression of a polyglutamine containing protein in cultured cells (14). Additionally, significant impairment of extractable chymotrypsin-like in vitro proteasome activity was detected after 3 days of expression of polyglutamate proteins in cultured cells (15,16,25). We employed similar methods of UPS activity determination and failed to detect impairment. In vitro assays from other cell culture and mouse models have also failed to detect impairment of proteasome activity (24,25,35). Specifically, investigation of a knock-in model of Huntington disease did not reveal impairment to proteasome activity (25). However, cell-type specific proteasome impairment could not be ruled out as a mitigating factor, as the vulnerable neurons represent a minority of cells in the brain.

Figure 8. Ataxin-7 nuclear inclusions are inversely correlated with UbG76V-GFP levels. (A) Representative section of a sum image of five slices from z-section confocal image scan of the ONL from a 9-week-old UbG76V-GFP° Sca7266Q retina cryosection stained by immunofluorescence for ataxin-7 nuclear inclusions and native GFP fluorescence of UbG76V-GFP. Photoreceptors with the brightest ataxin-7 nuclear inclusions have low UbG76V-GFP levels, whereas those with the brightest UbG76V-GFP levels have either weakly stained or undetectable ataxin-7 nuclear inclusions. Single channel and merged images are shown. (B) A two-point XY plot showing the quantitative confocal measurement of 9- and 14-week-old UbG76V-GFP° Sca7266Q retinas, plotting the arbitrary mean ataxin-7 signal (Y-axis) and the arbitrary mean native GFP fluorescent signal (X-axis) from two groups of photoreceptors, those with the brightest nuclear inclusions (red square) versus those with the brightest UbG76V-GFP signal (green circle). Error bars indicate the 95% confidence interval for the mean value of the ataxin-7 signal (error bars along the Y-axis) and the UbG76V-GFP signal (error bars along the X-axis) for individual photoreceptors of each group (9 weeks, n = 25; 14 weeks, n = 14). A strong inverse correlation in both ataxin-7 and UbG76V-GFP signals is observed between the two groups.
regions examined (24,25). Therefore, by examining UPS activity specifically in the vulnerable neuronal population, using established methods comparable to those previously used to show impairment in over-expression models, we conclude that significant UPS impairment is not required or detected in an authentic model of SCA7 polyglutamine neuropathology. Although we obviously cannot exclude the possibility of UPS impairment in other polyglutamine diseases or other polyglutamine genetic models (e.g. transgenic over-expression models), current theory as to the mechanism of proteasome impairment does not provide a reason to justify UPS impairment being disease specific. The mechanisms used to explain proteasome impairment focus on the polyglutamine tract itself (3,4,17,18,32). Thus, showing that any polyglutamine disease can occur in the absence of detectable UPS impairment is broadly applicable.

The approximately equivalent changes in UbG76V-GFP protein and mRNA indicate that the UbG76V-GFP protein levels are an indirect measure of UbG76V-GFP transcription (or possibly mRNA stability). Interestingly, another GFP reporter targeted for proteasomal degradation has been developed as an in vivo reporter of transcriptional activity (36). Li et al. showed that the short half-life of their GFP reporter was instrumental in detecting changes in promoter activity, as the high steady state levels found with wild-type GFP masked increases due to transcriptional induction. It is tempting to speculate that the short half-life of the UbG76V-GFP reporter in our in vivo model aided in the detection of small transcriptional aberrations. It is intriguing that the earliest sign of pathology in the Sca7266Q mice is a down-regulation of abundant photoreceptor specific transcripts (30,37). The SCA7 gene has recently been demonstrated to encode a subunit of the GCN5 histone acetyltransferase complex, STAGA, implicating its normal function to be in transcriptional regulation (38). The fact that transcriptional changes are the earliest event in SCA7 pathogenesis suggests that the mechanism for UbG76V-GFP reporter mRNA changes in SCA7 is likely to be transcriptional. In fact, a transcriptional pathology has been implicated for numerous polyglutamine diseases (39).

We propose two possible scenarios to explain the increase in UbG76V-GFP mRNA levels. One possibility is that neuronal stress, secondary to SCA7 neuropathology, is causing an increase in expression of the transgenic reporter. Another possibility is that the down-regulation of cell-specific transcription in the vulnerable neurons might free up the general transcriptional machinery, increasing its availability for expression of the transgenic reporter. This idea is consistent with our observation that photoreceptors with the highest level of reporter exhibit significantly lower levels of rhodopsin than neighboring cells with lower levels of the reporter.

There has been considerable debate regarding the role of polyglutamine inclusion bodies in neuropathology (2,3). Previous work has shown that nuclear inclusions are not required for cell death or neuropathology, and genetic conditions that lead to decreased inclusion body formation in cultured neurons or transgenic mice, correlate with increased cell death and neuropathology (8,40 – 42). However, these studies could not control for susceptibility differences between neuronal subtypes or the altered genetic conditions of the neuronal populations examined. A recent study has elegantly demonstrated that polyglutamine nuclear inclusion formation promotes cell survival in neurons transfected with a polyglutamine GFP fusion protein (43). Given the protection against cell death in cultured neurons, this poses the question of whether nuclear inclusions also provide protection in vivo or from the selective neuronal dysfunction that gradually develops in disease and precedes neuronal death. In the Sca7266Q model, significant photoreceptor dysfunction occurs prior to cell loss (30). To determine whether nuclear inclusions play a protective role against neuronal dysfunction in vivo, and at the level of individual neurons, we examined the relationship between the ataxin-7 nuclear inclusions and UbG76V-GFP reporter levels. UbG76V-GFP reporter accumulation is a sensitive in vivo marker of disease pathology, given its specific and progressive accumulation in the vulnerable neurons, and the correlation between this accumulation and the loss of rhodopsin, a marker of photoreceptor dysfunction and neuropathology (Figs 3 and 7). We found a striking inverse correlation between nuclear inclusion load and the degree of neuropathology between individual photoreceptors (Fig. 8 and data not shown). Importantly, because we observe this effect among neighboring neurons of the same subtype, this protective effect cannot be simply explained by differences in the susceptibility or genetic state of the neuronal population. Lastly, ataxin-7 nuclear inclusion formation antagonizes neuronal dysfunction throughout pathogenesis, such that at any stage of disease, there is an inverse correlation between inclusions and neuropathology. Thus, within the vulnerable neuronal population, the cells with more extensive inclusion formation exhibit reduced pathology relative to those cells with a lesser degree of inclusion formation. Unfortunately, nuclear inclusion formation is insufficient to halt disease, as the neuropathology gradually overwhelms the photoreceptors despite continued inclusion formation.

Polyglutamine aggregation reactions are kinetically driven by a nucleation process, and the aggregation process can deplete soluble pools of the polyglutamine protein (2,3,43). The inverse relationship between the nuclear inclusions and UbG76V-GFP reporter levels is consistent with the neurotoxic species being either monomeric polyglutamine proteins or oligomers. This finding supports recent in vitro and cell culture observations pointing to a mechanism of transcription factor impairment, mediated through soluble polyglutamine proteins (44). Inhibition of polyglutamine aggregation is being pursued as a therapeutic approach to polyglutamine disease. Our findings do not necessarily obviate a therapeutic benefit for this approach as long as decreasing the aggregate load is accompanied by a global decrease in total cellular polyglutamine protein. It is obviously critical to ensure that the process of eliminating aggregates is not enhancing the soluble toxic pool of the mutant protein. In all likelihood, the relationship between the inclusions and concentration of the toxic polyglutamine species is complex and may explain the beneficial effect of some aggregation inhibitors in polyglutamine models (45,46). However, the robust in vivo inverse correlation we observed strongly excludes a causative role for polyglutamine nuclear inclusions in neuronal dysfunction and selective neuronal vulnerability. In summary, the data presented clarify the relationship between nuclear inclusions and polyglutamine-induced pathology in vulnerable neurons and
reveal, in an authentic disease model, that despite an established disease state, no impairment of the UPS is detected.

MATERIALS AND METHODS

Mouse genetics

Both the Sca7<sup>266Q</sup>/<sup>5Q</sup>C1 line (30) and Ub<sup>G76V</sup>-GFP/1 line (33) used in this study have been backcrossed with C57Bl/6J. Heterozygous male Sca7<sup>266Q</sup> mice (7–10-week-old) were crossed with two or three heterozygous female Ub<sup>G76V</sup>-GFP<sup>+</sup> mice (2–6-month-old) to generate experimental animals. Genotyping of the progeny was performed by PCR using the primer sets as previously described (30,33). Genotyping for all animals was confirmed after dissections. Data sets always included three animals, littermate paired Ub<sup>G76V</sup>-GFP<sup>+</sup> and Ub<sup>G76V</sup>-GFP<sup>+</sup> Sca7<sup>266Q</sup> animals, plus a non-transgenic age matched animal that was always almost a littermate.

Western blot

Total retinal protein was extracted from dissected retina by Dounce homogenization in TSTE buffer [150 mM NaCl, 50 mM Tris pH7.5, 0.5% Triton X-100, 2 mM EDTA and 1× protease inhibitor cocktail (Sigma P-8340)], incubated on ice for 4–6 h, centrifuged at 14 000 rpm for 30 min and supernatant pulled to a fresh tube, stored at −80°C till use. Equal protein was loaded and analyzed by standard western blot protocols. An affinity-purified polyclonal antibody against GFP (Novus Biologicals Cat No. 600–308) was used at a 1:1000 dilution. A mouse monoclonal antibody against Gapdh (Advanced Immuno Chemical Cat No. RGM2) was used at a 1:10 000 dilution. Western blots were developed using Pierce SuperSignal West Dura Extended Duration Substrate, according to manufacturer’s directions. Relative levels of full-length Ub<sup>G76V</sup>-GFP protein (47) to Gapdh protein were determined by densitometry.

Preparation of retinal cryosections for confocal analysis

All steps of tissue preparation, sectioning, slide preparation and staining were done side-by-side in identical fashion, with common reagents for each data set. The complete dissection of retina in cold PBS was performed in <5 min and retina placed in 4% paraformaldehyde on ice in the dark (diluted from 16% solution, Electron Microscopy Sciences), until all samples were ready for the next morning for confocal analysis. Immunofluorescence staining for Ub<sup>G76V</sup>-GFP or ataxin-7 nuclear inclusions was performed as previously described, except slides were kept in dark whenever possible (30). The rabbit anti-GFP antibody (Novus) was used at 1:1000, the mouse anti-rhodopsin antibody (Chemicon) was used at 1:200, the 1261 anti-ataxin-7 antibody (Cy-3 or Alex488) conjugated secondary antibodies (Jackson Immunochemical) were used at 1:400.

Quantitative confocal image collection and data analysis

Image collection was done in a single sitting for each data set on a Zeiss LSM 510 confocal microscope. Identical confocal settings were used for all samples within a data set, the settings were adjusted for the data set to the Ub<sup>G76V</sup>-GFP<sup>+</sup> Sca7<sup>266Q</sup> animal sample to avoid pixel saturation and achieve optimal detection. Laser power was set to give minimal bleaching during z-section collection. Image collection of an area was done only once, and selection of the area to collect was done with the GFP excitation laser off to avoid collection bias. Z-section image collection was done at constant step interval (0.6 μm), through the full-depth of the 16 μm cryosection for the each channel, with slice count noted and kept within 15% variability for all image collections in the set. Quantitative analysis of the fluorescence signal was done with ImageJ software, using the sum image of the entire z-stack. Selection of the ‘region-of-interest’ (ROI) to measure was decided by marking the ROI from the Toto-3 nuclear stain channel image, then measuring the GFP channel in the matched image. Regions of the image were excluded where the nuclear stain revealed damage to the tissue from slide preparation, otherwise the entire retinal layer of interest (ONL, INL or total retina) was selected as the ROI. For analysis of total retinal levels, all layers were included in the ROI except the outer segment because of auto-fluorescence. The mean value of the ROI in the GFP channel was determined for each image collected. Then the average of means with standard error for each genotype of the data set was determined; the sample values for each genotype were then set relative to the Ub<sup>G76V</sup>-GFP<sup>+</sup> sample of each data set, defined as the unitary value. Analysis of independently analyzed replicate slides gave <20% variability in the determined relative values (data not shown). To quantify the inverse correlation between Ub<sup>G76V</sup>-GFP (Alexa488) and rhodopsin (Cy3) immunofluorescent signals, photoreceptors from single optical sections of multiple z-section images from a single animal were used; for the inverse correlation of Ub<sup>G76V</sup>-GFP native fluorescence and Ataxin-7 nuclear inclusions (Cy3), photoreceptors from constant optical depth sum images of multiple z-section images from a single animal were used. The inverse correlation between Ub<sup>G76V</sup>-GFP and rhodopsin or ataxin-7 nuclear inclusions was independently repeated with at least two animals. In choosing ONL cells with the highest intensity Cy3 staining or ONL cells with the highest intensity Ub<sup>G76V</sup>-GFP signal, we excluded cells when another photoreceptor in the same Z-axis had a high intensity signal in either channel that overlapped on the optical section image. To ensure that
all detectable ataxin-7 aggregates in a selected cell would be measured, only cells entirely contained within the optical section image were measured. The mean fluorescent signals for the individual photoreceptors of each class were measured for both the Ub<sup>G76V</sup>-GFP and Cy3 channel images, using a circular ROI selection in ImageJ software. All statistical analysis of data to determine the mean value, standard error, 95% confidence interval of the mean and t-test (two sample assuming unequal variances) were performed using Microsoft Excel.

**Northern blot**

Total retina RNA was prepared using both retinas from individual animals, using a Trizol (Invitrogen) extraction with Dounce homogenization, according to the manufacturer’s instructions. Northern blot was performed as previously described (30). The Gapdh probe was a 486 bp PCR fragment of the mouse cDNA; the GFP probe was from the plasmid pEGFP-N1 (Invitrogen). The relative levels of Ub<sup>G76V</sup> mRNA and Gapdh mRNA were determined by phosphoimager analysis.

**In situ hybridization**

The protocol for in situ hybridization on retina with a DIG-labeled anti-sense RNA probe was adapted for 16 μm cryosections, but otherwise performed as described (30). The EGFP probe, subcloned from the pEGFP-N1 vector into pBluescript II SK, was transcribed with T3 RNA polymerase from linearized vector to avoid vector sequence contamination. Tissue was prepared as described earlier for confocal analysis.

**In vitro proteasome activity**

The chymotrypsin-like activity of total retina extracted proteasomes from Sca7<sup>266Q</sup> and wild-type littersmates were measured using the fluorogenic substrate succinyl-Leu-Leu-Val-Tyr-AMC (Affiniti) in conditions very similar to those previously described (14). Both retinas of each animal were extracted in 400 μl of proteasome activity buffer [10 mM Tris pH 7.5, 1 mM EDTA, 20% glycerol, 2 mM ATP, 4 mM DTT, 0.5% Triton X-100 and 1× protease inhibitor cocktail (Sigma P-8340)] on ice by dounce homogenization, spun at 14 000 g supernatant collected on ice and assayed within 4 h. The substrate was used at 130 μM, and digestion was monitored over a period of 2 h with five time points on a fluorometer (Hoefer Pharmacia Biotech TKO 100). Proteasome independent digestion was very low as monitored by digestion of lactacystin. Chymotrypsin-like activity of total retina extracted proteasomes from Sca7<sup>266Q</sup> and wild-type littersmates were measured using a Trizol (Invitrogen) extraction with Dounce homogenization, according to the manufacture’s instructions. Northern blot was performed as previously described (30). The Gapdh probe was a 486 bp PCR fragment of the mouse cDNA; the GFP probe was from the plasmid pEGFP-N1 (Invitrogen). The relative levels of Ub<sup>G76V</sup>-GFP mRNA and Gapdh mRNA were determined by phosphoimager analysis.

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**References**


