The ubiquitin proteasome system in Huntington disease : impairment of the proteolytic machinery aggravates huntingtin aggregation and toxicity

Pril, R. de

Chapter 4

Modest proteasomal inhibition by aberrant ubiquitin exacerbates aggregate formation in Huntington disease

Remko de Pril, Barbara Hobo, Paula van Tijn, Raymund A.C. Roos, Fred W. van Leeuwen and David F. Fischer

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Abstract

UBB^{+1}, a mutant form of ubiquitin, is a substrate and an inhibitor of the proteasome which accumulates in the neuropathological hallmarks of Huntington disease (HD). *In vitro*, expression of UBB^{+1} and mutant huntingtin synergistically increase aggregate formation and polyglutamine induced cell death. We generated a UBB^{+1} transgenic mouse line expressing UBB^{+1} within the neurons of the striatum. In these mice lentiviral driven expression of expanded huntingtin constructs into the striatum results in a significant increase in neuronal inclusion formation. Although UBB^{+1} transgenic mice show neither a decreased lifespan nor apparent neuronal loss, they appear to be more vulnerable to toxic insults like expanded polyglutamine proteins due to a modest proteasome inhibition. These findings underscore the relevance of an efficient ubiquitin-proteasome system in HD.

Introduction

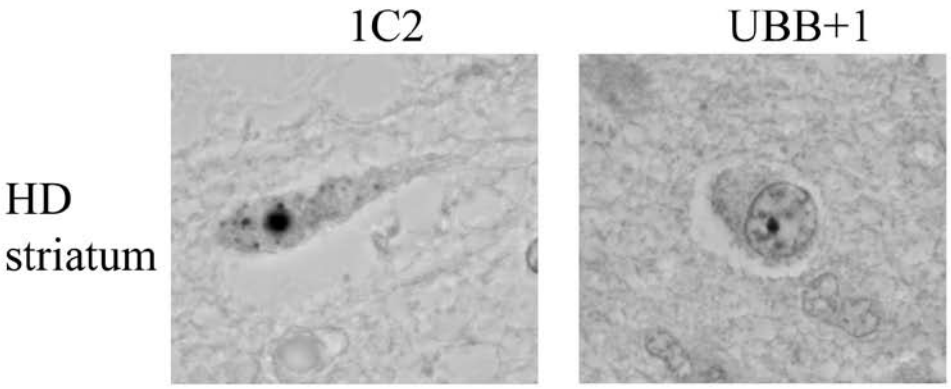
Huntington disease (HD) is an autosomal dominant neurodegenerative disorder that is caused by the expansion of a CAG trinucleotide (polyglutamine) repeat in the huntingtin gene. All polyglutamine expansion disorders are characterized by progressive neuronal dysfunction starting around mid-life and show an inverse correlation between polyglutamine repeat length and age at onset (Zoghbi and Orr, 2000). Expansion of this repeat in the huntingin protein to over 36 consecutive glutamines results in aberrant folding with a consequential toxic gain of function of the mutant protein. In HD, neuronal degeneration is particularly visible in the corpus striatum (cs) resulting in severe atrophy (Halliday et al., 1998). In addition to the repeat expansion there are other factors, including proteasomal activity and expression or recruitment of cellular chaperones, which could influence polyglutamine toxicity and disease development (Wexler et al., 2004).

Neuronal intranuclear inclusions (NIIs) of expanded polyglutamine protein are the most prominent pathological hallmark of polyglutamine diseases (DiFiglia et al., 1997). In HD, the huntingtin gene is cleaved to result in an N-terminal fragment containing the polyglutamine repeat, which is found in the inclusions (Sieradzan et al., 1999). Although recent observations point towards a protective role for formation of large inclusions, NIIs are still believed to correlate to toxic protein levels and severity of disease (Arrasate et al., 2004; Ross and Poirier, 2004).

The ubiquitin proteasome system (UPS) has been widely implicated in neurodegenerative diseases (Schwartz and Ciechanover, 2009). In HD, impairment of the proteasome has been suggested to affect disease progression and this involvement is reflected by localization of ubiquitin and components of the UPS to NIIs (DiFiglia et al., 1997; Schmidt et al., 2002). Furthermore, expanded polyglutamine proteins have been shown to directly inhibit the proteasome and lead to the upregulation of poly-ubiquitinated proteins that are targeted for degradation (K48 linked) (Bence et al., 2001; Bennett et al., 2007).

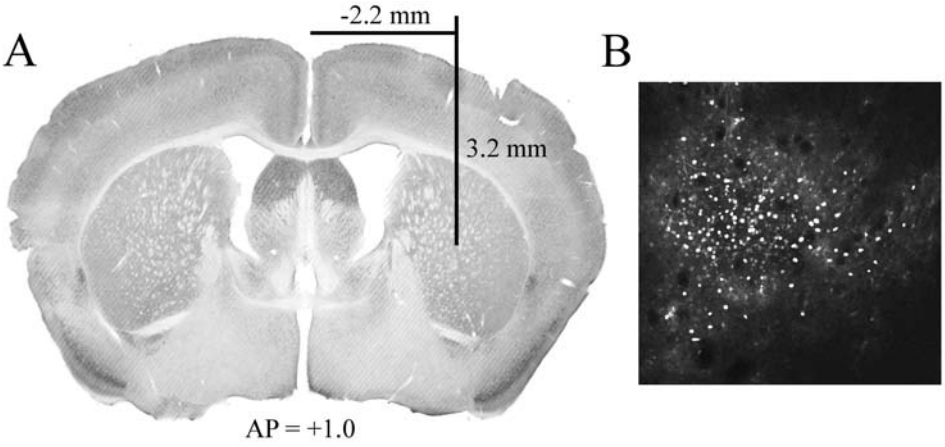
An aberrant form of ubiquitin (UBB^{+1}) was found to accumulate in the hallmarks of HD and spinocerebellar ataxia 3 (de Pril et al., 2004; Fischer et al., 2003). UBB^{+1} can no longer ubiquitinate substrate proteins, and was shown to be a reporter for proteasomal dysfunction (Fischer et al., 2003). Although UBB^{+1} can be ubiquitinated and degraded by the proteasome, at higher concentrations it also inhibits proteasomal degradation of cellular proteins and leads to cell death in neuroblastoma cells (van Tijn et al., 2007). To investigate the effect of UBB^{+1} expression *in vivo*, we generated transgenic mice neuronally expressing UBB^{+1} in different areas of the brain, including the striatum (Fischer et al., 2009). Even though no overt atrophy or degeneration was observed in these mice, proteasome activity in the cerebral cortex is decreased to about 80% of wild-type levels.

Figure 1: UBB^{+1} localizes to Huntingtin inclusions in human caudate nucleus of the corpus striatum.



Staining for 1C2, against expanded polyglutamine protein, and UBB^{+1} present NIIs in the cs of a HD patient (#4). For details see (de Pril et al., 2004). Scale bar is 20 µm.

Figure 2: Unilateral injection of UBB^{+1} transgenic line 3413 and wildtype littermates.



Coronal 50 µm vibratome section of a line 3413 UBB^{+1} transgenic displaying the injection site (A) and overview of the HD-Q43 injection in the striatum(B).

Previously, we found an increased aggregate formation and aggravation of polyglutamine induced cell death mediated by UBB^{+1} *in vitro* (de Pril et al., 2004). Furthermore, we detected UBB^{+1} protein expression in NIIs within the cs and cortex in post mortem brain material of HD. Therefore the aim of this study was to investigate the influence of UBB^{+1} on aggregate formation *in vivo* and test whether a modest proteasomal inhibition has a significant impact on the neuropathology of HD.

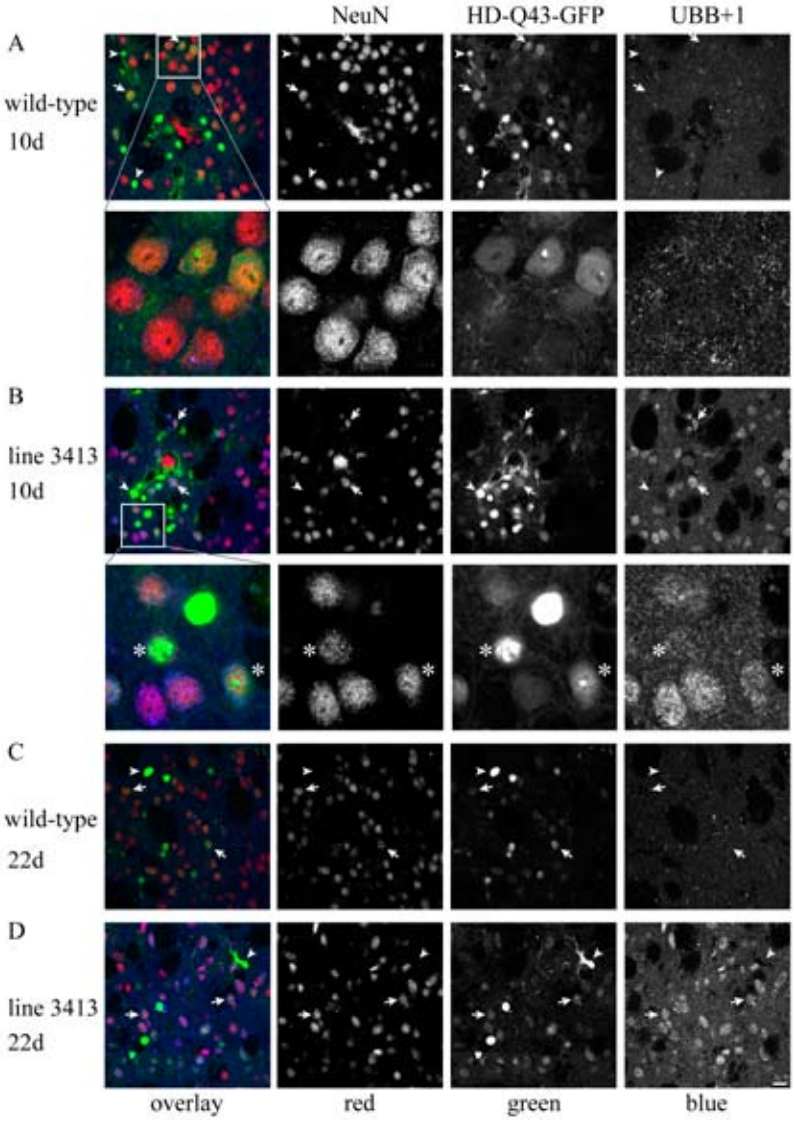
Results

The main pathological changes in HD manifest primarily within the neurons of the cs and frontal cortex. Within the striatum, severe atrophy is observed resulting in only a small set of neurons that remain which display NIIs. In parallel with the frontal cortex (de Pril et al., 2004), we find that NIIs in the cs accumulate aberrant ubiquitin along with the expanded polyglutamine protein (Figure 1). Both the 1C2 antibody, raised against expanded polyglutamine (Trottier et al., 1995), and UBB^{+1} antibodies identify inclusions in HD sections. Since the cs is the most severely affected area in HD we selected this area for our *in vivo* study.

UBB^{+1} transgenic mice line 3413 express UBB^{+1} under the CamKIIα promoter, and show transgene expression in neurons in the cortex, hippocampus, amygdala and striatum (Fischer et al., 2009). We performed unilateral injections in the striatum of male, 6-month old UBB^{+1} transgenic mice and wild-type littermate controls with lentiviral vectors encoding a GFP-tagged huntingtin fragment containing a pathological repeat of 43 glutamines (HD-Q43; Figure 2). As control, we used a similar lentiviral vector encoding a non-pathological repeat length of 19 glutamines (HD-Q19). To identify the neuronal cell population expressing UBB^{+1}, tissue sections were stained with the neuron specific marker NeuN and anti-UBB^{+1}.

At 10 days after injection, expression of non-pathogenic HD-Q19 in the striatum leads to cytoplasmic localization of the protein in both the 3413 line as well as their wildtype littermates (not shown). On the other hand, lentiviral expression of the expanded HD-Q43 resulted in the formation of NIIs at 10 days after injection in both wildtype (Figure 3A) and transgenic mice (Figure 3B). We observed clear expression of huntingtin-GFP fusion protein in neurons within a range of 400 μm surrounding the injection site (Figure 2B) and did not observe differences between line 3413 and wild-type littermates in the number of transduced cells (Table 1). As we did not use a neuron-specific promoter or viral backbone, GFP expression was also detected in astrocytes and oligodendrocytes. These non-neuronal cells do not express the UBB^{+1} transgene under the CamKIIa promoter and were excluded from the analysis. However, it can be clearly observed from the images, as shown in Figure 4, that these cells express higher levels of HD-Q43 compared to the neuronal cell population and therefore appear more prone to aggregate formation (Figure 2A; arrowheads). *In vitro* studies have indeed shown that aggregate formation is cell type

Figure 3: Expanded huntingtin expression in the striatum of mouse line 3413.



UBB^{+1} transgenic line 3413 and their wild-type littermates were injected with lentiviral HD-Q43-GFP (green) constructs into the striatum. Mice were sacrificed at 10 (A, B) and 22 days (C, D) post injection and stained for neuronal marker NeuN (red) and the UBB^{+1} transgene (blue). Representative images are shown of all groups displaying marked aggregate formation from 10 days onwards. The arrows show examples of NIIs within the neurons. In line 3413, we find that the neurons express UBB^{+1} and part of the transduced cells show aggregate formation of HD-Q43 (B; arrows). Magnifications of inserts clearly show NII formation in the striatum of injected mice. Arrowheads indicate high expression levels and aggregate formation in non-neuronal cells that do not express UBB^{+1}. Scale bar is 20 µm.

specific and influenced by expression levels of polyglutamine proteins resulting in extensive aggregate formation in astrocytes and oligodendrocytes (Yoshizawa et al., 2001).

We quantified the formation of NIIs in image stacks through the tissue sections of the striatum, as shown in Table 1. In addition, we assessed the number of neurons with cytoplasmic expression of HD-Q43. We found a highly significant (2.2-fold) increase in the percentage of UBB^{+1} positive neurons showing inclusion formation already at 10 days post injection (Table 1 and Figure 4; $P<0.001$). These results demonstrate that UBB^{+1} transgenic mice show a marked decrease in their ability to degrade aberrant polyglutamine protein. Also the percentage of neurons displaying multiple aggregates was markedly enhanced in transgenic animals compared to the wildtype littermates (5.2-fold; $P<0.001$), showing that the neurons in the transgenic mice are much more vulnerable to protein accumulation.

22 days after HD-Q43 injection, we observed an increase in the number of NIIs compared to 10 days in wild type as well as transgenic mice (Table 1; Figure 4). The percentage of neurons displaying inclusions increased to over 70% for the transgenic animals, in contrast to approximately 33% for the wildtype littermates, which is still 4% lower than the transgenic animals at 10 days. Altogether, line 3413 demonstrates a significant increase in the formation of aggregates compared to wild type littermates at 22 days ($P=0.01$) as well as over time ($P<0.001$). The number of neurons with multiple aggregates also showed a significant increase to 14%, compared to wild type animals at 22 days ($P<0.01$). It appears that wild type animals are able to cope differently with the toxic polyglutamine protein compared to UBB^{+1} transgenics, and that the inclusions are formed in a more organized manner, i.e. not distributed over the cell, but rather localized in single inclusion bodies (Figure 3).

Discussion

Our results demonstrate the importance of the role the UPS plays in the pathogenesis of neurodegenerative disorders such as HD. Our previous findings that UBB^{+1}, which accumulates in NIIs in HD and SCA3 patients, aggravates both polyglutamine induced cell-death as well as aggregate formation in a cell culture model (de Pril et al., 2004) emphasized the relevance of protein homeostasis in polyglutamine diseases. Here, we demonstrate that efficient UPS function is equally important in an *in vivo* model, showing increased polyglutamine aggregate formation in the UBB^{+1} transgenic line. Transgenic mice line 3413 postnatally express UBB^{+1} in neurons, leading to a reduction in cortical proteasome activity (down to 80% of wild-type levels) accompanied by an altered proteomic profile that resembles the changes observed in Alzheimer's disease (AD) (Fischer et al., 2009). Strikingly, UBB^{+1} transgenic mice show a normal life-span, and absence of classical neuropathology. These data suggest that UBB^{+1}, which accumulates in many neurodegenerative disorders including AD and HD, does not

Table 1: Aggregate formation in the striatum of UBB^{+1} transgenic line 3413 and wildtype and mice.

		10 days post injection					22 days post injection				
		wildtype		3413 tg			wildtype		3413 tg		
Total HD-Q43 expression		788	(100%)	810	(100%)		389	(100%)	491	(100%)	
Cytoplasmic expression		654	(83%)	514	(63%)		238	(61%)	150	(31%)	
Aggregates	single	124	(16%)	234	(29%)	$P<0.001$	145	(37%)	276	(56%)	$P=0.01$
	≥ 2	10	(1.3%)	62	(7.7%)	$P=0.007$	6	(1.5%)	65	(13%)	$P<0.01$

Tissue sections of wild type mice and UBB^{+1} transgenic line 3413 were quantified for expression of expanded polyglutamine protein for either cytoplasmic expression, or the formation of single or multiple aggregates after injection with lentiviral HD-Q43-GFP.

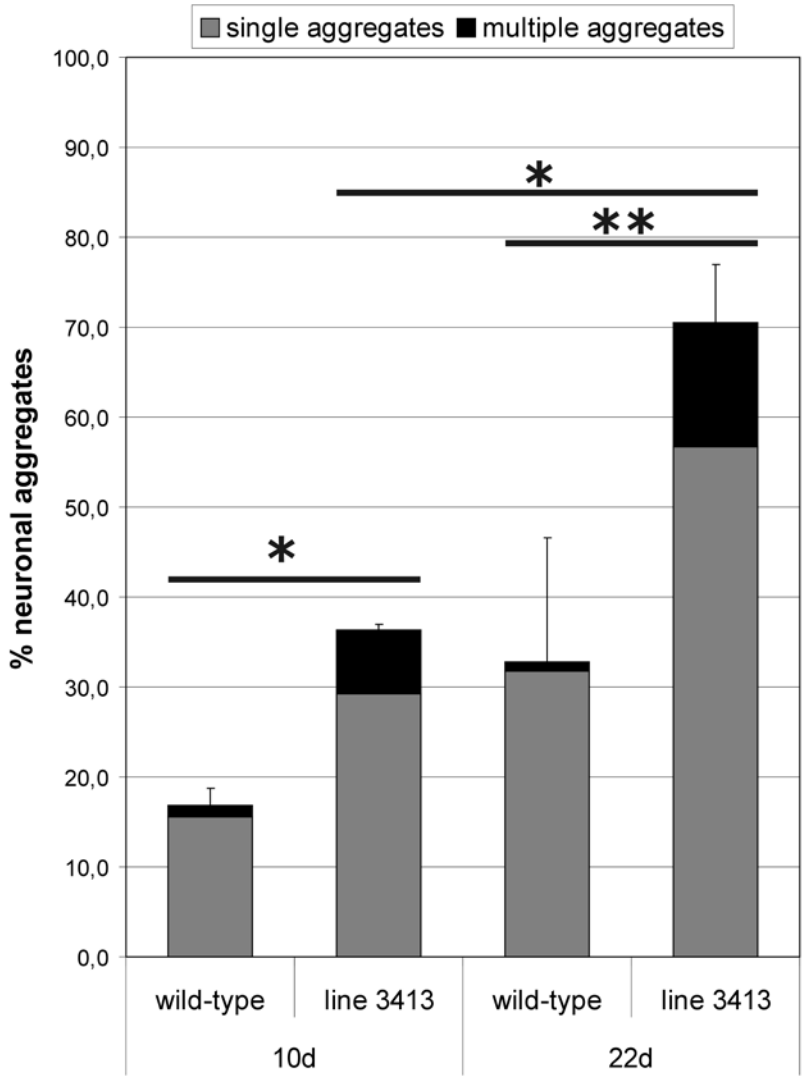
induce neuropathology by itself. We clearly show here that in the presence of a second insult such as polyglutamine expression, UBB^{+1}-mediated proteasome inhibition leads to an exacerbated neuropathology.

Differences in UPS efficiency or the level at which aberrant proteins like UBB^{+1} accumulate could account for the inter-patient variation in disease onset or extent of atrophy of HD (McNeil et al., 1997; Wexler et al., 2004). Expanded polyglutamine proteins lead to the accumulation of substrate proteins that are targeted for proteasomal degradation showing *in vivo* evidence of UPS inhibition in HD (Bennett et al., 2007). However, these accumulated proteins do show proper ubiquitination, hence the ubiquitination machinery does not appear to be affected. *In vitro* experiments have shown that aberrant proteins such as expanded polyglutamine and UBB^{+1} are difficult to degrade and thereby inhibit the proteasome (Bence et al., 2001; van Tijn et al., 2007). In HD, a reduction in proteasome activity was observed in all affected brain regions as well as an inability to activate the proteasomes that are present (Seo et al., 2004). This proteasomal inhibition by expanded polyglutamine proteins results in accumulation of other aberrant proteins which could account for the synergistic effect that we found in UBB^{+1} transgenic mice. In HD transgenic mouse line R6/2 no alteration in UPS activity could be detected although an upregulation of 20S activity was found in response to expanded huntingtin expression (Bett et al., 2009). Interestingly, the increase in 20S activity might correlate with activation of UPS activity in response to cellular stress in contrast to long time exposure to expanded polyglutamine proteins in symptomatic HD patients. The addition of other cellular stressors such as UBB^{+1} is therefore likely to better represent the disease phenotype that is found in patients and which might be aggravated by normal human ageing resulting in mid-life onset of HD in patients.

A mere 20% reduction in UPS activity in line 3413 resulted in a more than 2-fold induction in the number of NIIs. As NII appear to be protective in *in vitro* studies (Arrasate et al., 2004), we can not exclude this as a contributing factor in the observed increase in NII formation in UBB^{+1} transgenic mice. The clear effect of UPS inhibition by UBB^{+1} on polyglutamine aggregation makes it unlikely to be accounted for by more efficient protein storage at equal expression levels of huntingtin. Also the increase in multiple aggregates per cell does argue against protective aggresomes. Increased levels of expanded huntingtin, as a result of UPS inhibition by aberrant ubiquitin, are likely to result in increased aggregate formation in parallel with increased pathology as also observed in juvenile HD (Maat-Schieman et al., 1999).

Although we can not resolve the consequential accumulation of aberrant proteins, subtle differences, also in their expression, can play a role in the start of this deleterious process and affect the onset of disease. These findings underline the importance of the UPS for neurodegenerative disorders. Decreased proteasome activity disturbs the cellular machinery of protein homeostasis whereby target proteins are no longer efficiently degraded. The consecutive further increase of aberrant proteins

Figure 4: UBB^{+1} transgene expression increases aggregate formation of expanded huntingtin.



Neuronal aggregate formation of HD-Q43-GFP was quantified at 10 and 22 days post injection. NeuN-positive cells were scored for cytoplasmic localization respectively aggregation of expanded huntingtin A significant increase was found in the number of NIIs that are formed in the UBB^{+1} transgenic mice at 10 and 22 days after injection compared to wildtype animals as well as in time (P<0.001; ** P=0.01). Also the number of neurons with multiple aggregates showed a marked increase (P<0.01).*

may eventually lead to neuronal death. Potentially, by disturbing the capacity of the UPS, other pathways involved in aberrant protein metabolism may be activated. Interestingly, impairment of the UPS in a Drosophila model of spinobulbar muscular atrophy resulted in a compensatory increase in autophagy to rescue aberrant protein-induced neurodegeneration (Pandey et al., 2007). It will be interesting to further evaluate the consequence of UBB^{+1} expression on the neuropathological changes in HD by crossing line 3413 with HD transgenic mouse lines. Double transgenics will more closely relate to the actual HD phenotype and enable a further investigation of the influence of an impaired UPS on HD development.

Materials and Methods

Mice

Male 6 month old mice of line 3413 (Fischer et al., 2009) were used in this study. For Q19 injections 2 mice were injected of every group. For expanded huntingtin constructs (Q43) 3 mice were used for all groups. Injected groups of line 3413 and littermate controls were sacrificed 10 or respectively 22 days after transduction. Mice were kept in group housing on a 12/12 h light-dark cycle with food and water ad libitum in specific pathogen free conditions. All animal experiments were performed conforming to national animal welfare law and under guidance of the animal welfare committee of the Royal Netherlands Academy of Arts and Sciences.

Striatal transductions

Mice were anesthetized with 10 ml/kg FFM (0.0787 mg/ml fentanyl citrate, 2.5 mg/ml fluanisone, 0.625 mg/ml midazolam in water). The skull was exposed and coordinates for infusion (1.0 mm anterior-posterior, -2.2 mm lateral; figure 2A) were read against bregma (Paxinos and Franklin, 2001). A hole was drilled through the skull, and the dura was punctured. An 80 µm glass needle was inserted 3.2 mm into the brain and $1*10^9$ transducing units of virus was injected in a total volume of 1 µl. Production of htt-GFP lentivrial vectors has been described in (de Pril et al., 2004). The skin was sutured, mice were administered 0.05 µg/g buprenorphine intramuscular as an analgesic and 10 µl/g 0.9% NaCl subcutaneously to prevent de-hydration. Mice were kept at 37°C until they had recovered, subsequently they were housed individually to prevent opening of the sutures.

Immunohistochemistry

Animals were given deep pentobarbital anesthesia (i.p.) and were perfused intra-cardially with phosphate-buffered saline (PBS) followed by PBS containing 4% paraformaldehyde. Brains were cut on a sectioning vibratome in 50 micron thick sections. Slices were stained free floating with rabbit polyclonal anti-UBB^{+1} (Ubi3

serum; 1:1000) and monoclonal NeuN (Chemicon; 1:400) diluted in Supermix (50 mM Tris, 150 mM NaCl, 0.25% gelatin and 0.5% Triton X-100, pH 7.4), followed by Cy5 and Cy3 staining (Jackson ImmunoResearch; 1:800) Nuclei were visualised with TO-PRO-3 (Molecular Probes; 1:1000). Subsequently, slices were mounted in mowiol (0.1 M Tris-HCl pH 8.5, 25% glycerol, 10% w/v Mowiol 4-88) images were acquired using confocal laser scanning microscopy (Zeiss 510) and accompanying software (Zeiss LSM Image Browser).

Quantification of transductions

Image stacks (5-7 per animal) were obtained by sectioning through the brain slices at 2 μm apart to obtain an optimal resolution. Transduced neurons were quantified on the basis of NeuN positive staining and GFP signal. Inclusions were qualified as clearly distinct protein accumulations of GFP tagged polyglutamine protein. Quantifications were performed blinded with respect to genotype of the mice and statistics was performed using two-way Anova. Neuronal aggregates were counted manually through these sections, blinded with respect to the genotype.

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