The Ubiquitin Proteasome System in Huntington disease

Impairment of the proteolytic machinery aggravates huntingtin aggregation and toxicity

Remko de Pril

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Neurodegenerative diseases are characterized by progressive dysfunction of the nervous system. These devastating illnesses commonly correlate with atrophy of the affected areas in the brain or peripheral nervous system. Several of these disorders including Alzheimer disease (AD), amyotropic lateral sclerosis (ALS) and Parkinson disease (PD) are sporadic although some familial forms are caused by gene mutations.

The polyglutamine diseases consist of a family of neurodegenerative disorders that are caused by the excessive expansion of a CAG repeat in a transcribed gene. Consecutive translation of this CAG repeat results in the formation of proteins containing a pathological polyglutamine repeat. To date, nine disorders have been identified with polyglutamine expansions in different proteins that correspond with distinct clinical presentation (Table 1). In all these diseases an inverse correlation is found between polyglutamine expansion and age of disease onset.

Huntington Disease (HD) is the best known and most extensively studied of the polyglutamine diseases with an incidence of one in every 10-20,000 inhabitants of the western world. This accounts for an estimated 1300 HD patients in the Netherlands and even more people that are currently at risk. The huntingtin protein (Htt) – encoded by the *HTT* gene – functions in vesicle transport and normally contains between 6 and 27 glutamine repeats. Persons with over 36 consecutive glutamines in Htt, usually develop HD around midlife. An intermediate repeat length between 36 and 39 results in disease development in some people whereas others are spared (McNeil et al., 1997; Rubinsztein et al., 1996; Zoghbi and Orr, 2000). Although there is a clear correlation between longer polyglutamine repeat lengths and earlier disease onset, patients with equal pathological expansion display enormous variation in clinical manifestation and a clear disparity between repeat length and subcortical atrophy (Halliday et al., 1998). Apparently, other factors, including genetic modifiers such as the wild-type *HTT* allele can influence development of the polyglutamine diseases by modifying the mutant protein toxicity (Aziz et al., 2009; Wexler et al., 2004). These differences indicate that cellular mechanisms modulate the neurotoxic properties of the mutant Htt protein.

The striatum and frontal cortex are the areas most affected in HD demonstrating obvious pathology. Especially the medium spiny neurons of the striatum are affected and most of these neurons are lost over the course of the disease. Within the remaining striatal neurons, neuronal intranuclear inclusions (NII) are identified that contain the mutant Htt protein. In addition to the NII several dystrophic neurites have been identified that resemble axonal processes (DiFiglia et al., 1997). Large polyglutamine expansions to more than 55 sometimes arise *de novo* that cause a juvenile form of the disease, which in general starts before the age of 20 years, causes more widespread neuropathology and faster disease progression. Apparently, further expansion of the

Table 1: Polyglutamine expansion disorders.

Disease	Protein	Function	Localization	Pathological expansion (intermediate alleles)	Affected areas
HD	Huntingtin	functions in transport of synaptic vesicles along microtubules	cytoplasmic	40 - 121 (36 - 39)	striatum and frontal cortex
SCA1	Ataxin-1	possible role in synaptic plasticity and learning	nuclear	29 - 82	cerebellum
SCA2	Ataxin-2	functions in long term potentiation and fear behavior	cytoplasmic	36 - 63 (32 - 34)	cerebellum and brain stem
SCA3	Ataxin-3	de-ubiquitinating enzyme	cytoplasmic	62 - 84	basal ganglia, brain stem and spinal cord
SCA6	α1A calcium channel subunit	subunit of a voltage dependent calcium channel	cell-membrane	21 - 33	cerebellum and brain stem
SCA7	Ataxin-7	possible histone acetyltransferase activity	nuclear	37 - 130 (28 - 35)	cerebellum, inferior olive and cranial nerve nuclei
SCA17	TATA binding protein	general transcription initiation factor	nuclear	47 - 55	striatum, thalamus, frontal and temporal cortex
DRPLA	Atrophin-1	nuclear receptor corepressor	cytoplasmic	49 - 84	cerebral cortex, globus pallidus, striatum, cerebellar cortex and in the subthalamic, red and dentate nuclei
SBMA	Androgen receptor	steroid hormone receptor that activates transcription upon androgen binding	nuclear and cytoplasmic	38 - 62	anterior horn cell, bulbar neuron and dorsal root ganglion cell

polyglutamine repeat lowers the threshold for toxicity of the expanded protein. As a result, different neuronal populations with lower expression levels become affected by the aberrant protein.

The spinocerebellar ataxias (SCAs) comprise a heterogeneous group of disorders that share cerebellar atrophy and variable degeneration of brain stem and spinal cord. The gene products of SCA1, 2, 3 and 7 all encode proteins with diverse cellular functions (Table 1). SCA17 which was most recently discovered, contains an expansion in the TATA-binding protein (TBP) a general transcription initiation factor (Nakamura et al., 2001). Affected regions of the SCAs include the cerebellum and spinal cord with additional affected areas depending on the expanded gene as listed in Table 1. In the channelopathy SCA6, neurodegeneration is caused by a small CAG expansion (to 19-30 repeats) in a calcium channel subunit that presumably causes a change of function (Zhuchenko et al., 1997). In contrast, the other eight polyglutamine diseases are characterized by a gain of function mechanism whereby the expanded polyglutamine repeat acquires a toxic conformation (Scherzinger et al., 1997). Nonetheless, loss of the functional protein in addition to expression levels possibly contributes to the tissue specific distribution and phenotype of the disease.

Spinobulbar muscular atrophy (SBMA) is the only polyglutamine disease that shows an X-linked pattern of inheritance in contrast to the autosomal dominant inheritance of the other diseases (Kennedy et al., 1968). The CAG repeat is located in the androgen receptor, a steroid hormone receptor that activates transcription upon binding of androgen. Dentatorubropallidoluysian atrophy (DRPLA) in turn, is caused by a polyglutamine expansion in the atrophin-1 protein that is widely expressed and thus causes a more extensive pathology (Koide et al., 1994).

In addition to the polyglutamine expansion disorders several triplet repeat diseases exist that are caused by repeat expansion in non-transcribed regions. These diseases also display autosomal dominant inheritance and include SCA8 and 12 as well as Friedreich ataxia (reviewed in (Everett and Wood, 2004)). Repeat expansion in these disorders results in disruption of gene expression causing the disease symptoms. In contrast to the polyglutamine expansion disorders these are not represented by a toxic gain of function mechanism that results in neuronal toxicity.

Since the discovery of the mutation responsible for HD, extensive research has been performed using genetic models in cell-lines as well as transgenic animals. These model systems have resulted in many findings that have lead to a better understanding of human disease in HD and other polyglutamine diseases. This review will summarize several of the important aspects leading to neurodegeneration in HD with a specific focus on the ubiquitin proteasome system (UPS).

HD animal models

Animal models are being used extensively to gain a better insight in the characteristics of genes and disorders and study early disease development. Over the past years several HD mouse models were made that show some resemblance to the characteristics of human disease (Heng et al., 2008). These transgenic animals have enabled the study of cells in their physiological context as well as early events in the development of HD. The R6/2 transgenic line contains the first exon of *HTT* with a repeat expansion of 144 CAG and is the most extensively studied model (Mangiarini et al., 1996). These mice display a very rapid disease progression with severe atrophy which is however not selective for regions affected in HD. R6/1 mice display similar characteristics although slower disease progression due to lower expression levels of mutant *HTT* and a repeat of 116 CAG. N171-82Q transgenic mice contain not only the first but also the second exon of Htt with 82 glutamines and display a less severe phenotype which includes striatal atrophy (Schilling et al., 1999).

In contrast, the YAC128 mice contain the full length *HTT* construct with a repeat of 120 CAGs under the endogenous promoter. Consequently, this model does show a better representation of HD with comparable regional brain atrophy (Van Raamsdonk et al., 2005). The same accounts for the HdhQ94, HdhQ111, HdhQ140 and $Hdh^{(CAG)150}$ knock in mice that contain an expanded CAG repeat within the endogenous mouse *HTT* gene ranging from 94 to 150 repeats (Menalled et al., 2002). Similarly, the BACHD transgenic mice show a significantly delayed onset of neurodegenerative signs and may be a better model for HD compared to R6/2 (Gray et al., 2008).

Further transgenic models have been developed in other organisms that might more closely mimic human disease development in HD. A transgenic rat model has been generated that contains a large rat *HTT* fragment with a moderate repeat of 51 CAG (von Horsten et al., 2003). These rats resemble human HD with late-onset and progressive phenotype which includes cognitive impairment and motor dysfunction. Neuropathology includes the formation of NII and striatal shrinkage. A transgenic HD model in a rhesus macaque is currently being developed containing exon 1 of *HTT* with 84 CAG repeats (Yang et al., 2008). These monkeys demonstrate clinical HD features including dystonia and chorea. Neuropathology includes NII and neuropil aggregates and early death in monkeys carrying higher copy numbers.

The mouse homolog of *HTT* is essential during early development and null-mice that lack functional Htt die at embryonic day 7.5 (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). Conditional knock-down of *HTT* results in progressive neurodegeneration demonstrating an important function of Htt also in the adult brain (Dragatsis et al., 2000). In contrast, knock-down of *HTT* in Drosophila does not affect viability although long term survival and mobility are affected (Zhang et al., 2009). However, loss of Drosophila *HTT* does result in decreased complexity of axonal termini and increased susceptibility of animals to mutant Htt expression.

Altogether, these data indicate an important function of Htt in development and neuroprotection and indicates that loss of normal Htt function possibly contributes to specific neuropathology.

Protease cleavage

In HD, nuclear inclusions consist primarily of N-terminal fragments of mutant Htt. Nuclear translocation potentially requires processing of the full length 348 kDa protein into smaller fragments in order to enter the nucleus. Caspase cleavage of wild type and mutant Htt at position 552 occurs in vivo before the onset of neurodegeneration (Wellington et al., 2002). Indeed, in transgenic mice, caspase-6-mediated cleavage of Htt is required for the onset of neuronal dysfunction (Graham et al., 2006). Within the NII even smaller fragments are detected suggesting further processing of the mutant protein before translocation. Small fragments arise from cleavage at position 167 resulting in an N-terminal fragment that exerts increased aggregation and toxicity (Ratovitski et al., 2009).

Nuclear translocation

Despite the diverse functions of the polyglutamine proteins in the cytoplasm and the nucleus, all polyglutamine expansion disorders are represented by nuclear inclusions. Toxicity of expanded polyglutamine proteins is increased upon translocation to the nucleus supporting an important contribution of the intracellular location to pathogenesis (Peters et al., 1999; Saudou et al., 1998). Potentially, deregulation of transcription factors is responsible for the increased toxicity of nuclear expanded polyglutamine protein. The X-linked recessive inheritance of SBMA supports this notion as the androgen receptor is translocated to the nucleus upon binding of testosterone. Also in SBMA transgenic mice, neuronal dysfunction is specific for males but can be induced in females by administration of testosterone whereas castration rescues motor neuron deficits in males (Chevalier-Larsen et al., 2004).

Changing the intracellular localization of other polyglutamine proteins similarly affects the toxicity of mutant proteins demonstrating a clear influence of compartmental interactions (Nucifora et al., 2003; Peters et al., 1999). *In vivo*, inactivation of the nuclear localization signal in ataxin-1 resulted in decreased Purkinje cell pathology and these mice did not demonstrate significant motor abnormalities (Klement et al., 1998). Additional nuclear targeting of mutant Htt resulted in comparable degeneration demonstrating that the disturbance of nuclear mechanisms accounts for an important part of the neuropathology (Schilling et al., 2004). These results demonstrate that translocation to the nucleus contributes to neuropathology in SBMA, SCA1 as well as HD.

Transcriptional deregulation

One aspect of the toxic gain of function of mutant Htt is thought to be linked to deregulation of gene expression in neurons. A wide array of transcription changes has been detected in HD mouse models that show some overlap but are in part specific for each model (Chan et al., 2002; Luthi-Carter et al., 2002; Luthi-Carter et al., 2000; Luthi-Carter et al., 2002; Sipione et al., 2002). Nevertheless, these studies clearly demonstrate the effect of expanded Htt on gene expression in HD models. In human HD brain, the transcriptional changes parallel the pathology of HD with more severe changes in the caudate nucleus followed by the cortex (Hodges et al., 2006). mRNA changes are observed in very diverse pathways that range from neuronal signalling and neurotransmitter receptors to homeostasis and ion channels.

Expanded Htt has been shown to have a stronger affinity for binding directly to DNA and will thereby prevent the binding of transcription factors to the promoters (Benn et al., 2008). Additionally, this direct binding results in a more open chromatin structure which also affects transcription. Wild-type Htt has been shown to interact with repressor element-1 transcription factor / neuron restrictive silencer factor (REST/NRSF) (Zuccato et al., 2003). This interaction is disturbed through expansion of the polyglutamine repeat resulting in decreased expression of neuronal genes like brain derived neurotrophic factor (BDNF) which contain a neuron restrictive silencer element (NRSE).

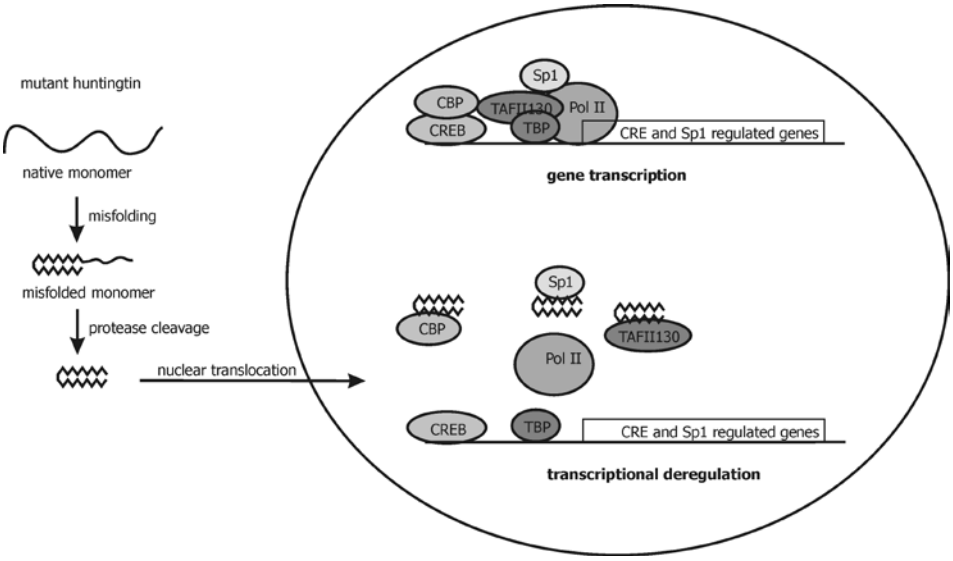
In contrast, expansion of the polyglutamine repeat in Htt results in an increased interaction with the transcription factor Specificity protein-1 (Sp1) (Li et al., 2002). This binding thereby disrupts the normal promoter binding of Sp1 resulting in decreased expression of several genes including the D2 dopamine receptor and nerve growth factor receptor (NGFR) (Dunah et al., 2002; Li et al., 2002). A schematic representation of transcriptional deregulation is given in Figure 1. Overexpression of Sp1 partially rescues the toxicity of mutant Htt and the decrease in neurite extension demonstrating that these effects are indeed caused by insufficiency of Sp1. In SCA1, polyglutamine binding protein-1 (PQBP-1) has been shown to bind mutant ataxin-1 resulting in decreased phosphorylation of polymerase-II and reduction in basal transcription (Okazawa et al., 2002).

cAMP responsive element binding protein (CREB) and family members (CREM) are essential for neuronal development and affect neuronal survival (Mantamadiotis et al., 2002). In HD, CREB binding protein (CBP, a histone acetyltransferase) has been shown to be partially recruited to NIIs potentially affecting transcription regulation. Expansion of the polyglutamine repeat *in vitro,* results in a reduction of CRE mediated transcription and specific toxicity which can partially be rescued by cAMP overexpression (Wyttenbach et al., 2001). Besides the histone acetyltransferase CBP, other transcriptional co-activators and histone modification enzymes have been implicated in transcriptional dysregulation by mutant Htt. These include HDAC4

and HDAC6 (Dompierre et al., 2007; Steffan et al., 2001; Thomas et al., 2008), SIRT2 (Luthi-Carter et al., 2010) and PGC1α (Cui et al., 2006; Strand et al., 2007; Weydt et al., 2006).

Furthermore, the TBP transcriptional co-activator $TAF_{II}130$ has been shown to interact directly with expanded polyglutamine repeats (Shimohata et al., 2000). TBP is required for CREB- and Sp1-dependent transcriptional activation and $TAF_{II}130$ binding to expanded polyglutamines can thereby affect both CRE and Sp1 dependent transcriptional activation. In addition to direct interaction $TAF_{II}130$, CREB, TBP and Sp1 have been shown to be recruited to NII thereby affecting the transcription regulation. However, in HD mouse models it was shown that these transcription factors do not show considerable localization to the NII and in addition normal expression levels of the soluble forms were found (Yu et al., 2002). These differences in co-localization could be caused by prolonged Htt expression in patients in contrast to the short term exposure to extensive repeats in transgenic animals. Also in juvenile HD more pronounced aggregate formation is detected that results in a different pattern of disease progression. Potentially, interaction of transcription factors with soluble Htt has a more profound effect on gene expression in patients.

Figure 1: Transcriptional deregulation in HD.



Htt containing an expanded polyglutamine repeat is misfolded and cleaved into smaller fragments that can enter the nucleus. Within the nucleus Htt was shown to interact with several transcription factors including CBP, $TAF_{II}130$ and Sp1. The increased association of mutant huntingtin results in deregulation of normal gene transcription.

Htt function and interactions

Although disease development is clearly triggered by the expanded polyglutamine repeat in Htt, the precise function of Htt is still unknown. Several interaction partners have been identified that suggest diverse biological functions of Htt in transcription, vesicle transport and neuroprotection. A number of large scale interaction studies have also been performed that have yielded many interactors of Htt with little biological validation (Goehler et al., 2004; Kaltenbach et al., 2007).

Huntingtin interacting protein 1 (Hip1) interacts with Htt and clathrin coated vesicles implicating Htt in vesicle transport. Clathrin binding to Hip1 and Hip1r (Hip1 related) reduces the affinity for actin binding, suggesting a role for Hip1 and Hip1r in vesicle budding (Wilbur et al., 2008). Expansion of the polyglutamine repeat has been shown to reduce the binding of Htt to Hip1 (Kalchman et al., 1997). This reduced interaction results in increased levels of available Hip1 which consequently interacts with another interaction partner Hip1 protein interactor (Hippi) to recruit caspase-8 and activate apoptosis (Gervais et al., 2002). In addition, this interaction results in increased translocation of the Hip1/Hippi complex to the nucleus to activate transcription of caspase-1 (Banerjee et al., 2010). Consequently, the polyglutamine expansion in Htt will indirectly result in increased caspase-1 and -8 dependent apoptosis.

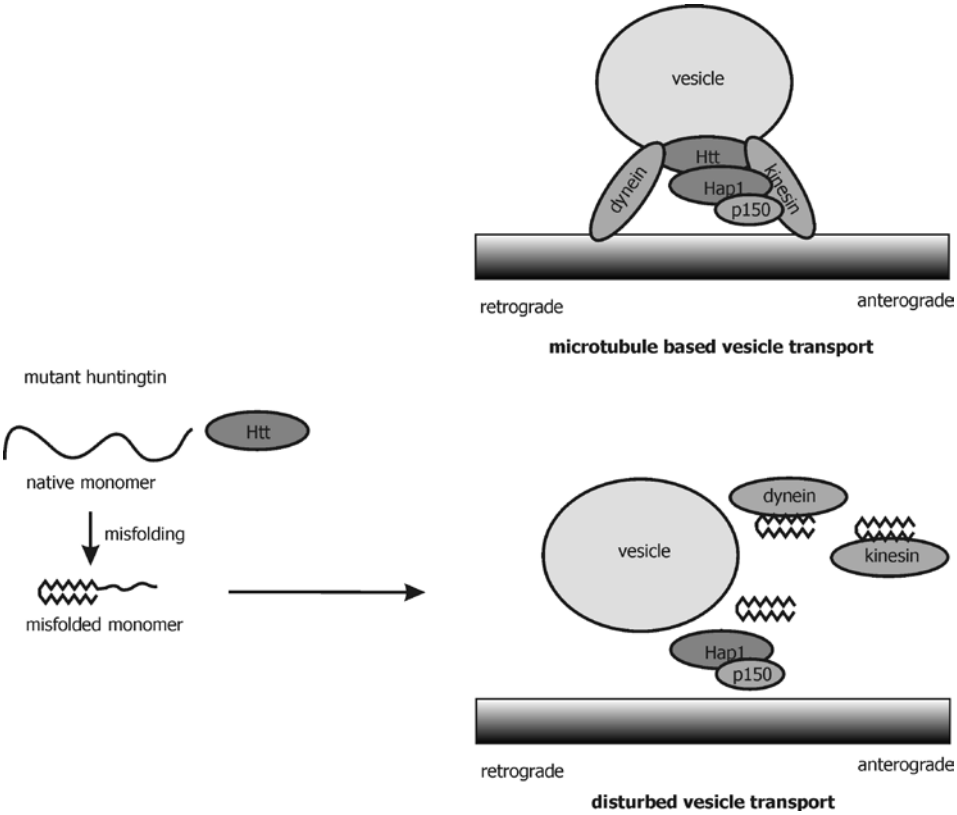
Wild-type Htt functions in vesicle transport by binding to dynactin and motor proteins dynein and kinesin. Phosphorylation by Akt at serine 421 (S421) results in recruitment of kinesin-1 to the dynactin complex on microtubules and vesicles (Colin et al., 2008). This kinesin recruitment promotes anterograde transport of vesicles including BDNF cargoes and dephosphorylation results in detachment of kinesin and retrograde transport. Additionally, wild-type Htt has been implicated in vesicle trafficking of proteins from the Golgi to the extracellular space (Strehlow et al., 2007). Accordingly, Htt knock-down results in a downregulation of extracellular proteins that are involved in matrix, cell adhesion, receptor binding or hormone activity.

Mutant Htt was shown to inhibit both anterograde and retrograde fast axonal transport without visible aggregates (Szebenyi et al., 2003). Interestingly, both a reduction and polyglutamine expansion of Htt in Drosophila resulted in axonal transport defects (Gunawardena et al., 2003). Although large axonal aggregates have been detected that could directly block vesicle transport these defects are most likely caused by an interaction with the motor proteins. This binding of dynein and kinesin to aggregation prone Htt could result in depletion of motor proteins resulting in increased stalling and axonal accumulation of transport vesicles (Sinadinos et al., 2009). A schematic representation of the disturbed vesicle transport is given in Figure 2. More specifically, Htt expansion and proteolysis both result in disruption of BDNF vesicular transport potentially affecting neurotrophic support through cortico-striatal projections (Gauthier et al., 2004). Phosphorylation of S421 of mutant Htt restores both the anterograde and retrograde transport of vesicles by normalizing the interaction with

p150^{Glued} and microtubules (Zala et al., 2008). Akt or IGF-1 can thereby compensate for the transport defect by phosphorylating Htt.

Huntingtin associated protein 1 (Hap1) has been shown to be responsible specifically for transport of BDNF cargoes through interaction with kinesins and p150^{Glued}. In addition, amino acid substitution of threonine for methione at position 441 in Hap1 results in a delay in the age at onset of HD in human (Metzger et al., 2008). The methionine substitution thereby results in tighter binding of Htt to Hap1 and reduces soluble Htt which prevents toxicity of the expanded polyglutamine protein. The polyglutamine expansion in Htt interferes with the function of Hap1, dynein and kinesin and thereby disturbs axonal transport which is essential for normal functioning of neurons.

Figure 2: Disturbed microtubule based vesicle transport.



Both a reduction of Htt levels as well as polyglutamine expansion result in disturbed retrograde and anterograde vesicle transport. Htt most likely interacts directly with the motor proteins dynein and kinesin resulting in depletion of these proteins and increased vesicle stalling. Also Hap1 binding is disturbed which results in decreased transport of BDNF vesicles. Tighter binding of mutant Hap1^{T441M} results in a delay in the age at onset of HD suggesting that polyglutamine expansion disturbs normal binding of Htt to Hap1.

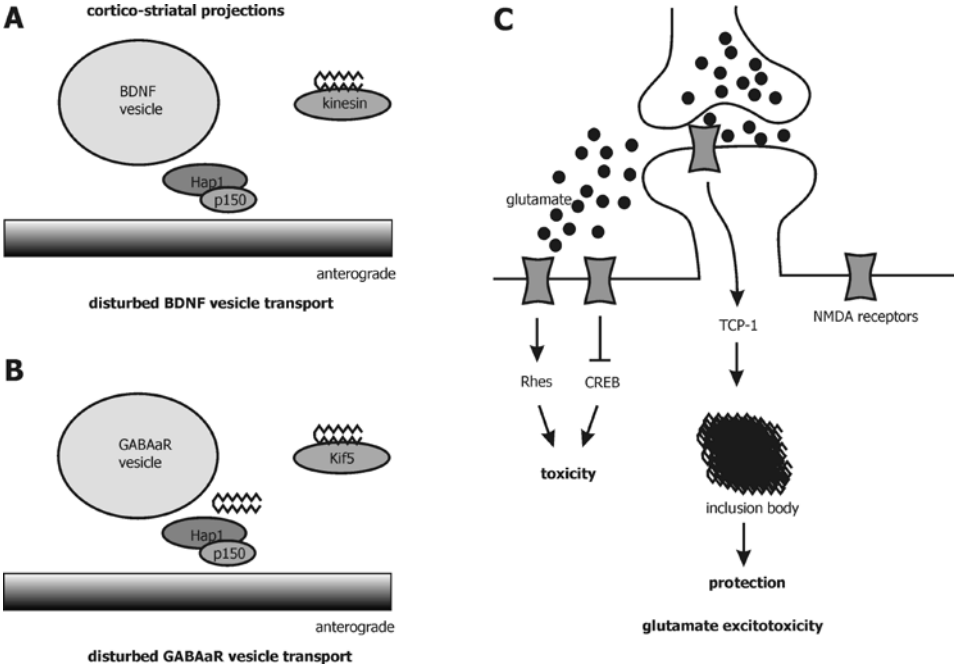
The neuroprotective effect of Htt can be greatly governed by the indispensable function of wild-type Htt in neuronal vesicle transport. In addition, wild-type Htt has been shown to inhibit caspase-3 activation *in vivo* which is disturbed by polyglutamine expansion or *HTT* knock-down (Zhang et al., 2006). P21-activated kinase-2 (Pak2) was shown to bind Htt which prevents cleavage of Pak2 by caspase-3 and -8 (Luo and Rubinsztein, 2009). The constitutive active C-terminal fragment Pak2-p34 induces cell death and is detected in response to different death stimuli. In contrast, Pak1 interaction with Htt increases oligomerization of both wild-type as well as expanded Htt (Luo et al., 2008). This interaction thereby causes an increase in aggregate formation of expanded Htt as well as polyglutamine-induced cell death. Altogether, these Htt interactions indicate important functions for wild-type Htt and provide insight in an additional loss of function due to polyglutamine expansion.

Neurotransmitter trafficking and activity

Synaptic activity through NMDAR promotes inclusion formation of mutant Htt through upregulation of T complex-1 (TCP-1) – part of the chaperonin TCP-1 ring complex – and thereby increases survival (Okamoto et al., 2009). Extrasynaptic stimulation of NMDAR, including glutamate excitotoxicity increases cell death through upregulation of Rhes – a small guanine nucleotide binding protein – and downregulation of CREB resulting in lower PGC-1α which is neuroprotective (Cui et al., 2006; Subramaniam et al., 2009). A schematic representation of disturbed neurotransmitter activity and trafficking is given in Figure 3. Interestingly, this demonstrates that synaptic activity ameliorates the toxicity of mutant Htt in addition to increased vulnerability of these neurons to excitotoxic insults. Furthermore, a direct interaction has been shown for Htt with postsynaptic density protein-95 which interacts with the NR2B subunit of NMDAR and this interaction is increased upon repeat expansion (Fan et al., 2009). Inhibition of binding to NR2B reduced NMDAR surface expression on medium spiny neurons and consequentially the susceptibility to excitotoxicity.

Inhibitory synaptic transmission is regulated by $GABA_A$ receptor density at synapses. Trafficking of $GABA_AR$ is performed by kinesin family motor protein 5 (Kif5) whereby Hap1 functions as adaptor protein for linking Kif5 to the receptor vesicles (Twelvetrees et al., 2010). Since mutant Htt demonstrates a stronger affinity for Hap1 binding compared to wild-type Htt the polyglutamine expansion is likely to interfere with normal transport of the receptor vesicles to the synapse. Strikingly, mutation of Ubiquitin specific protease-14 causes ataxia and results in increased expression of $GABA_AR$ at the surface of Purkinje cells increasing inhibitory signalling which disrupts normal motor coordination (Lappe-Siefke et al., 2009; Wilson et al., 2002). These findings underline the importance of regulation of the $GABA_AR$ signalling for normal coordination in HD patients.

Figure 3: Disturbed neurotransmitter trafficking and activity in HD.



Htt interaction partner Hap1 is responsible for transport of BDNF cargoes. (A) Binding of mutant Htt to kinesin and disturbed binding of Hap1 results in increased vesicle stalling and decreased neurotrophic support from the cortex towards the striatum. (B) Similarly, transport of $GABA_AR$ vesicle transport is disturbed resulting in decreased receptor density at the synapses. (C) Mutant Htt results in increased extrasynaptic NMDAR which results in glutamate excitotoxicity. Synaptic NMDA activation results in TCP-1 upregulation which promotes Htt inclusion formation. Extrasynaptic activity causes cell-death through Rhes upregulation as well as CREB downregulation.

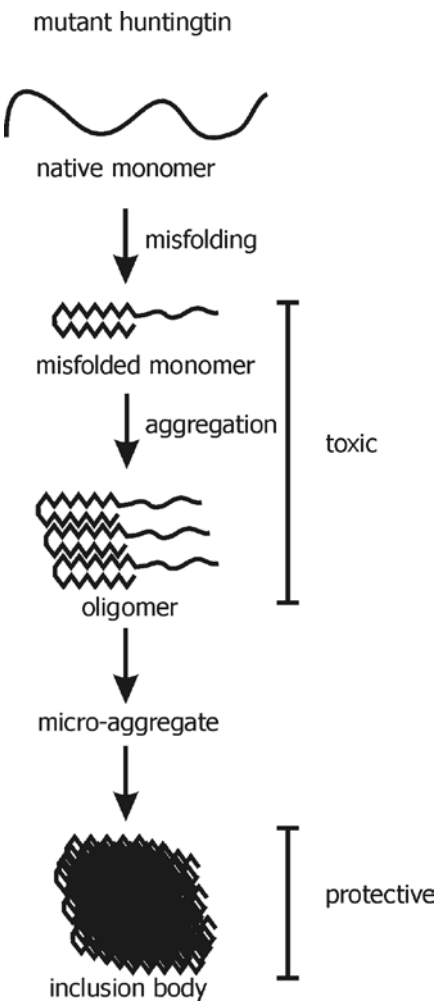
Inclusion bodies

Polyglutamine expansion disorders are characterized by the formation of intranuclear as well as cytoplasmic inclusions or aggregates suggesting a direct correlation between neurodegeneration and aggregate formation. However, several studies have shown that the formation of inclusions is actually beneficial, supposedly through efficient storage of aggregation prone proteins (Arrasate et al., 2004; Saudou et al., 1998). A schematic representation of aggregation is given in Figure 4. Most likely, the detrimental effects of polyglutamine proteins are caused by their ability to aggregate and not by the full blown inclusions that function as protective storage mechanisms. Aggregation prone proteins are much more likely to interact with and disrupt cellular function of transcription factors, UPS components and chaperones in their soluble state.

Interestingly, in HD transgenic mice the behavioural phenotype as well as cellular dysfunction preceded the formation of NII or even microaggregates (Menalled et al., 2002). This demonstrates *in vivo* that neuronal function is affected by the presence of expanded polyglutamine but visible aggregate formation is not required for disruption of cellular homeostasis. Additionally, lack of the self association region of ataxin-1 resulted in similar neurodegeneration in SCA1 transgenics but these mice did not demonstrate any visible aggregates (Klement et al., 1998). Moreover, inducible HD mice demonstrated that acute expression of expanded polyglutamine proteins resulted in inhibition of the UPS (Ortega et al., 2010). This UPS impairment was rescued by the formation of inclusion bodies and aggregation inhibitors resulted in sustained impairment of the UPS.

Figure 4: Misfolding of expanded Htt and formation of inclusion bodies.

Polyglutamine expansion in the Htt protein results in misfolding of the polyglutamine protein. Misfolded monomers tend to aggregate with other polyglutamine containing proteins resulting in oligomeric forms of the protein. The monomeric as well as oligomeric forms are most likely to interfere with normal cellular functions eventually resulting in toxicity. Further protein aggregation results in the formation of inclusion bodies which are actually protective as they decrease the levels of soluble misfolded protein.



Autophagy

The main protein degradation systems of the cell are the UPS and autophagy. Autophagosomes are responsible for the uptake of misfolded aggregated proteins and subsequent fusion with lysosomes for degradation of these proteins. Autophagy is involved in the degradation of mutant Htt in cellular models (Bjorkoy et al., 2005; Qin et al., 2003; Rideout et al., 2004).

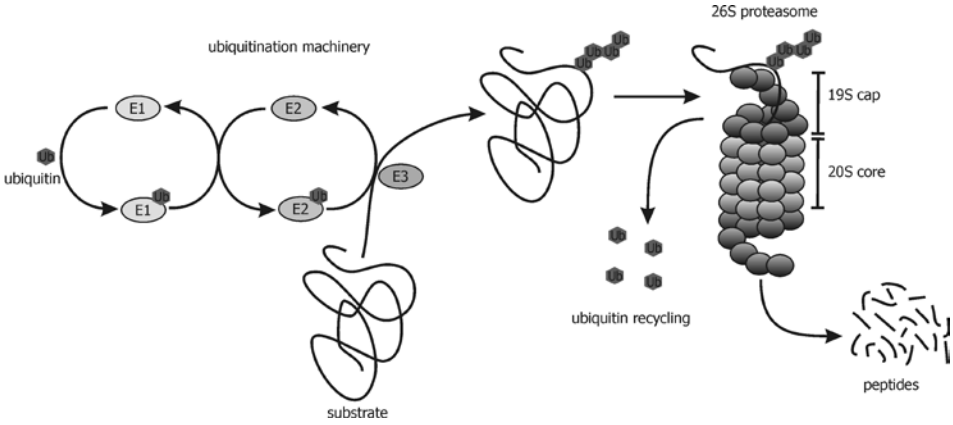
Induction of autophagy was shown to be beneficial in animal models of several polyglutamine diseases and reduces neuropathology as well as phenotypic performance (Menzies et al., 2010; Pandey et al., 2007; Ravikumar et al., 2004). Clearance of aggregated proteins involves a direct interaction of the inclusions with p62/sequestosome-1 and requires microtubules and histone deacetylase 6 (HDAC6) (Bjorkoy et al., 2005; Iwata et al., 2005). Interestingly, p62 has been shown to bind directly to LC-3 as well as to ubiquitinated inclusions requiring its ubiquitin binding motif in order to facilitate autophagic degradation (Donaldson et al., 2003; Pankiv et al., 2007). Autophagic components as well as lysosomes require an intact microtubule cytoskeleton and HDAC6 could act directly through deacetylation of tubulin. Additionally, HDAC6 is required for deacetylation of Hsp90 and could therefore indirectly affect autophagy through activation of Hsp90 target proteins (Kovacs et al., 2005). Furthermore, specific acetylation of mutant but not wild type Htt results in increased association with autophagosomes through interaction with p62 (Jeong et al., 2009). This acetylation is likely to be regulated by the histone acetyltransferase domain of CBP and overexpression of CBP increases acetylation and consecutive degradation of mutant Htt.

Upon autophagy impairment, p62 accumulates and binds to poly-ubiquitinated targets resulting in inhibition of proteasomal degradation of these ubiquitinated targets (Korolchuk et al., 2009). The decreased proteasomal degradation can consecutively be rescued by down regulation of p62 suggesting that the impairment is indeed caused by direct binding of the substrates. Normally, p62 will shuttle these substrates to the autophagosome but prevents transport to the proteasome. Through nucleocytoplasmic shuttling p62 is also involved in transport of poly-ubiquitinated targets to promyelocytic leukemia bodies within the nucleus (Pankiv et al., 2010). Within the nucleus, both E3 ubiquitin ligases San1p and UHRF-2 – ubiquitin like with PHD and Ring finger domain-2 – can ubiquitinate Htt and thereby enhance intranuclear degradation of expanded polyglutamine proteins (Iwata et al., 2009). Altogether, this indicates that aberrant proteins can be targeted for degradation within the nucleus and protein quality control is not restricted to the cytoplasm alone. Lower levels of protein quality control proteins as well as the inability of autophagy to clear nuclear proteins can however contribute to preferential aggregation of polyglutamine proteins within the nuclear compartment.

Ubiquitin proteasome system

Several links have been revealed between neurodegenerative diseases in general and the UPS (Ciechanover and Brundin, 2003). Genetic mutations in UPS components cause several forms of familial neurodegeneration and ubiquitin is found in the hallmarks of most neurodegenerative diseases including AD, PD and polyglutamine diseases. Furthermore, the UPS is involved in cellular protein quality control and responsible for the degradation of the aberrant proteins that accumulate in neurodegeneration. The UPS is a complex, tightly controlled system for the degradation of excessive or aberrant intracellular proteins. It contributes to cellular homeostasis by regulating the expression of essential proteins in a temporal and spatial pattern. The substrate to be degraded is tagged by multiple ubiquitin molecules followed by translocation to the proteasome where the protein is proteolytically processed. See also Figure 5 for a schematic representation of ubiquitination and the UPS.

Figure 5: The ubiquitin proteasome system.



Ubiquitin is activated by an E1 enzyme followed by transfer to an E2 ubiquitin conjugating enzyme and transfer to the E3 bound substrate. Currently two human E1s have been identified, over fifty E2s and hundreds of E3 enzymes indicative of increased substrate specificity of ubiquitin transfer. Consecutive ubiquitin moieties are attached to the ubiquitin on the substrate to form a poly-ubiquitin chain, which functions in different cellular signaling pathways including proteasomal targeting. Ubiquitinated substrates are transported to the proteasome where they are unfolded and de-ubiquitinated by the 19S cap for insertion into the 20S proteolytic core for degradation into small peptides. These peptides are subsequently degraded by cytoplasmic proteases into amino acids and recycled.

Ubiquitination

The ubiquitin conjugation is accomplished by a cascade of proteins that activate (E1), conjugate (E2) and ligate (E3) ubiquitin to the target proteins (reviewed in (Pickart,

2001)). An E1 ubiquitin-activating enzyme binds ubiquitin at an internal cysteine residue via a high-energy thiol-ester bond in an ATP dependent reaction. Ubiquitin then transfers to one of several E2 ubiquitin-conjugating enzymes via another high-energy thiol-ester bond. Finally, ubiquitin transfers to a lysine residue of the substrate that is specifically bound by an E3 ubiquitin-ligating enzyme. For most ubiquitin signalling, including proteasomal degradation, multiple ubiquitin proteins are linked to form a poly-ubiquitin chain on the target protein (Thrower et al., 2000).

There are many E3 enzymes that recognize specific target sequences and are subdivided into four distinct families on the basis of their binding domains (Kim and Huibregtse, 2009; van Wijk et al., 2009). Firstly, the HECT-domain E3s – for homologous to E6-AP C-terminus – generate an additional high-energy thiol-ester bond on an internal cysteine residue, before transfer of ubiquitin to the substrate. Secondly, the RING finger containing E3s – for really interesting new gene – catalyze the direct transfer of ubiquitin from E2 to the substrate by an active site of cysteines and histidine residues around two zink ions. Further families containing U-box and PHD – for plant homeo domain – probably catalyze the ubiquitin transfer in a similar way. Linking of additional ubiquitin proteins to form the polyubiquitin chain is regulated by the same cascade although in some cases a different ligase (E4) catalyzes chain elongation.

This cascade is involved in all ubiquitination reactions, irrespective of whether the bound ubiquitin will signal proteasomal targeting, protein expression or endocytosis. Importantly, the formation of different ubiquitin trees by linkage to another ubiquitin lysine residue on positions 6, 29, 48 or 63 confers part of the signalling specificity (Pickart and Fushman, 2004; Xu et al., 2009). Ubiquitin chains that are attached by K48 linkage are known to specifically target proteasomal degradation whereas K63 linkage is involved in trafficking and translation. The lysine residue within the substrate that is ubiquitinated possibly contributes to this specificity. Importantly, ubiquitination is involved in several cellular processes which indicates an important role for the ubiquitination machinery in the cells.

Proteasomal degradation

Recruitment to the proteasome by multi-ubiquitin chain binding proteins (MCBP) confers additional substrate specificity. Proteins such as Rad23, Dsk2 and Ddi1 shuttle specific substrates to the proteasome by binding to the ubiquitin chains with a ubiquitin-associated (UBA) domain and to the proteasomes with a ubiquitin-like (UbL) domain (Elsasser et al., 2004; Kim et al., 2004; Verma et al., 2004). The proteasome subunit Rpn10 similarly contains the ability to bind multi-ubiquitin chains, although in yeast this subunit is dispensable suggesting involvement of other factors in binding ubiquitinated targets to the proteasome (van Nocker et al., 1996). In addition, the Rpt5 subunit has been shown to bind ubiquitin chains in intact proteasomes requiring ATP-hydrolysis (Lam et al., 2002).

The proteasome itself consists of a multi-subunit complex that is normally subdivided in two regulatory 19S cap structures on both sides of the 20S proteolytic core. Upon binding of the ubiquitinated substrate to the 19S cap, the ubiquitin molecules are recycled and the substrate is unfolded, chaperoned and consecutively inserted into the 20S core. The core contains trypsin-like, chymotrypsin-like and peptidyl-glutamyl peptide hydrolizing (PGPH) activities which are responsible for specific cleavage of the substrate into small peptide fragments.

Alternatively, association of the 20S core with one or two 11S structures can be induced by interferon-γ and the resulting immunoproteasome is involved in MHC class I antigen presentation. In addition, interferon-γ induces the LMP2, LMP7 and MECL-1 subunits that replace the normal catalytic β-subunits in the 20S core. These modifications result in the exit of larger peptides from the proteasome that are eventually used for antigen presentation (Fruh et al., 1994). In HD, neuronal induction of the immunoproteasome was detected showing increased levels of LMP2 and LMP7 (Diaz-Hernandez et al., 2003). Strikingly, this potentially accounts for the observed increase in both trypsin- and chymotrypsin-like activities without affecting the PGPH activity. This could either be induced by inflammatory cytokines like interferon-γ released by reactive glia or a direct response of the neuron to cope with the expanded polyglutamine repeats which are difficult to degrade. However, deletion of the proteasome activator REGγ, which suppresses the PGPH activity, failed to improve proteasomal activity or neuropathological symptoms in R6/2 mice (Bett et al., 2006). Thus, activation of the proteasomal PGPH activity is not sufficient to rescue cells from high levels of mutant huntingtin.

Coaggregation of proteins and chaperones

In polyglutamine diseases, the inclusions contain ubiquitin or ubiquitinated proteins which indicates that the aggregating proteins are targeted for degradation by the proteasome (DiFiglia et al., 1997; Paulson et al., 1997). In addition, inclusions in polyglutamine diseases have been shown to recruit at least parts of the 26S proteasome and expanded polyglutamines inhibit proteasomal activity *in vitro* (Bence et al., 2001; Schmidt et al., 2002).

Several proteins coaggregate with NIIs in polyglutamine diseases but most notable are the chaperone proteins including heat-shock proteins and components of the UPS and autophagy pathway. These clearance mechanisms include proteins involved in refolding, degradation and autophagy. Alternatively, these proteins could function in the efficient assembly of aggresomes which function to clear the cell of detrimental aggregation prone proteins. Both p62 as well as non-expanded ataxin-3 are sequestered into aggregates requiring their ubiquitin binding motifs (Donaldson et al., 2003). This specific co-aggregation suggests an active interaction of these proteins with ubiquitinated proteins.

The stress response or heat-shock proteins (Hsp) are involved in refolding of misfolded proteins or alternatively degradation by the proteasome. Polyglutamine aggregates of several disorders, including SBMA and SCA1, have been shown to contain Hsps (Cummings et al., 1998; Stenoien et al., 1999). Additionally, overexpression of Hsp40 decreased aggregation of expanded polyglutamine proteins. *In vitro* both Hsp40 and Hsp70 reduce aggregation of expanded HD exon-1 through direct binding to the polyglutamine repeat and separating the monomeric form (Muchowski et al., 2000; Wacker et al., 2004). *In vivo*, decrease of Hsp70 in R6/2 mice resulted in increased neuropathology and decreased survival although an increase was only observed of inclusion bodies and not of fibrillar aggregates (Wacker et al., 2009). Overexpression of Hsp70 indeed ameliorated the neuropathology and phenotype of transgenic mice of both SBMA and SCA1 (Adachi et al., 2003; Cummings et al., 2001). Most likely, Hsps respond to the misfolded protein by promoting proteasomal degradation of the expanded polyglutamine repeat protein (Bailey et al., 2002). Conversely, Hsp70 overexpression in R6/2 mice failed to improve the neurological phenotype despite the slight delay in aggregate formation (Hay et al., 2004). Interestingly, pharmacological induction of the heat shock response did improve solubility of polyglutamine proteins through sustained chaperone induction.

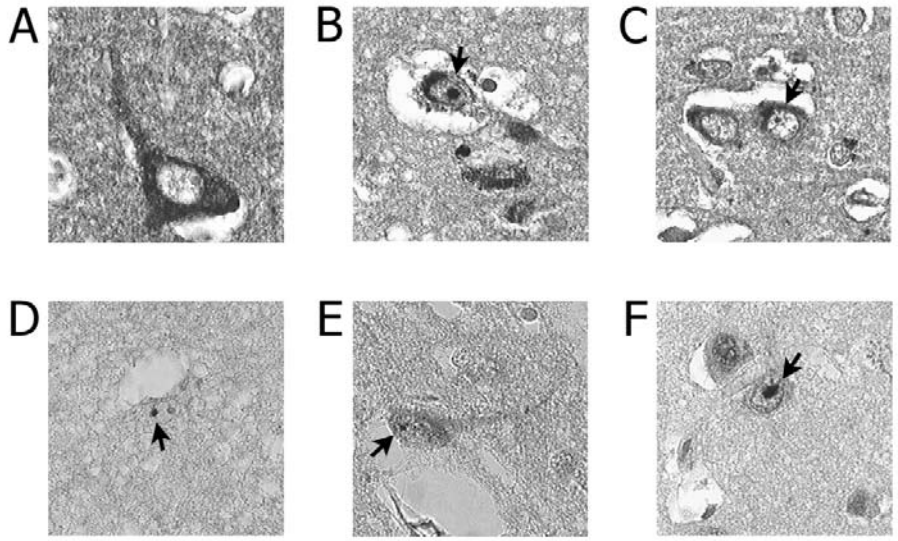
Furthermore, C-terminus of Hsp70 interacting protein (CHIP; a U-box E3-ligase) was shown to interact with expanded polyglutamine protein (Jana et al., 2005). Importantly, overexpression of CHIP increased ubiquitination of expanded Htt and ataxin-3 providing a direct link between the heat shock response and proteasomal degradation of expanded polyglutamine proteins. As a result, both aggregation as well as toxicity of expanded polyglutamine proteins was decreased *in vitro* and *in vivo* (Miller et al., 2005). Interestingly, overexpression of CREB and Hsp70 in Drosophila additively suppresses polyglutamine toxicity (Iijima-Ando et al., 2005). These results clearly demonstrate differential defects leading to toxicity in polyglutamine diseases as well as a lack of compensatory mechanisms.

Proteasome inhibition

In polyglutamine diseases there are several indications of UPS impairment and a contribution of the UPS to neuropathology. *In vitro*, expanded polyglutamine proteins cause a relocation of the 20S proteasome core from the cytoplasm to the insoluble inclusions (Jana et al., 2001). This change results in a decrease in proteasome activity and consecutively diminishes degradation of normal cellular proteins like p53 resulting in increased cell death. Furthermore, expanded polyglutamines can directly inhibit the proteasome through direct binding (Bence et al., 2001; Verhoef et al., 2002). In addition, the ability of aggregate forming cells to respond to secondary stress-insults like heat shock is decreased (Ding et al., 2002).

In SCA3 and HD patients, besides ubiquitin also subunits of the 26S proteasome have been shown to be recruited to NIIs (Figure 6) (Chai et al., 1999; Schmidt et al., 2002). In SCA1 transgenic mice, the Purkinje cell pathology was aggravated by mutation of the E6-AP ubiquitin ligase (Cummings et al., 1999). Altogether, these findings point towards an involvement of the UPS in the pathogenesis of polyglutamine diseases and to an enhancement of neurodegeneration by further impairment of the UPS (Ciechanover and Brundin, 2003).

Figure 6: Colocalization of proteasome subunits with NIIs.



Immunohistochemical staining shows that several proteasome subunits are translocated into the NIIs in HD. (A-B) Apparent upregulation of 20S subunits β1 (A) and β1i (B) in the frontal cortex of HD but colocalization is only observed for the β1i subunit. (C) Similar upregulation is found for 11S subunit REGβ as well as colocalization with NII. Several 19S subunits colocalize with NII including Rpt2 / S4 (D), Rpt3 / S6B (E) and Rpn2 / S1 (F).

Proteasome inhibition in R6/2 mice demonstrates controversial results. Both the ubiquitin proteasome reporters GFPu as well as UbGFP failed to show an accumulation of these reporters in R6/2 mice despite accumulation of ubiquitin conjugates, suggested to be caused by compensatory mechanisms (Bett et al., 2009; Maynard et al., 2009; Ortega et al., 2010). Interestingly, in these and other studies a buildup of large ubiquitin conjugates was detected pointing towards a disruption in the degradation machinery in these mice (Bennett et al., 2007). Possibly, large polyglutamine fragments are incapable of entering the proteasome *in vivo* and consequently do not result in clear inhibition of the 26S activity. Inducible mouse models have shown that UPS impairment is seen upon acute expression of polyglutamine whereas this was not detected upon chronic expression of expanded polyglutamine (Ortega et al., 2010). In Htt knock-in mice with endogenous expression levels of expanded Htt no

inhibition of the UPS or autophagy activation was detected (Li et al., 2010). However, pharmacological inhibition of the UPS did result in a larger buildup of N-terminal mutant Htt compared to inhibition of autophagy, demonstrating a more important function for the UPS in the clearance of Htt.

In vitro it has been shown that expanded polyglutamine proteins result in direct inhibition of the proteasome and the repeats themselves are difficult to degrade (Bence et al., 2001; Holmberg et al., 2004). It would be interesting to test a mouse model with later onset HD in order to discern whether the prolonged expression of mutant Htt does result in decreased proteasome activity. Furthermore, the early expression of exon 1 of *HTT* with an extreme repeat expansion could account for compensatory mechanisms to deal with the mutant protein. Although expanded polyglutamine proteins have been shown to inhibit the proteasome, further expansion will result in more aggregation prone proteins with potentially decreased ability to enter and thereby block the proteasome. Most likely, certain aggregation intermediates do have a detrimental effect other than the proteasome causing neuropathology in these mice.

Aberrant ubiquitin - UBB^{+1}

Ubiquitin is a highly expressed protein that is essential for cellular function. Mutations in other components of the UPS result in several forms of familial neurodegeneration. In AD, an aberrant form of ubiquitin (UBB^{+1}) accumulates in the neuropathological hallmarks of the disease (van Leeuwen et al., 1998). This UBB^{+1} protein is formed by a dinucleotide deletion (ΔGU), leading to a +1 reading frame in the mRNA, and subsequent translation to a protein with an aberrant C-terminus. Thus far UBB^{+1} protein has been found in the hallmarks of several neurodegenerative diseases, including AD and other tauopathies, whereas it was not detected in synucleinopathies and young control patients without pathology (Fischer et al., 2003; van Leeuwen et al., 1998). The aberrant transcript however, in contrast to the protein, appeared to be present even in young controls. Under normal circumstances, neurons can apparently cope with UBB^{+1}, and accumulation of this protein reflects proteasomal dysfunction in different neuropathological disorders (Fischer et al., 2003).

In vitro studies have shown that, 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 (Lindsten et al., 2002; van Tijn et al., 2007). UBB^{+1} can no longer ubiquitinate substrate proteins, and was shown to be a reporter for proteasomal dysfunction (Fischer et al., 2003). In addition, UBB^{+1} has been implicated to mediate neurodegeneration via downstream interaction with the E2-25K ubiquitin conjugating enzyme, which induces amyloid-β neurotoxicity *in vitro* (Ko et al., 2010; Song et al., 2003). Furthermore, E2-25K is highly expressed in the brain and was found in a yeast two-hybrid screen to interact with Htt in a repeat-independent matter (Kalchman et al., 1996). In this perspective, UBB^{+1}

might accelerate disease progression and increase the severity of neurodegeneration. Notably, the proteasome activity is indeed decreased in AD (Keck et al., 2003; Keller et al., 2000), strengthening the idea that UBB^{+1} accumulation is intimately related to impairment of the proteasome (Ciechanover and Brundin, 2003; Tank and True, 2009). Interestingly, neurotoxicity of mutant Htt has also been shown to increase upon ageing suggesting a decreasing cellular capacity to handle aberrant proteins (Diguet et al., 2009). Altogether, different proteins start to accumulate at later stages in life indicating similar mechanisms that lead to the build up and consecutive neurotoxic properties.

Concluding remarks and aim of this thesis

The UPS is essential for normal cellular function by degrading proteins that are no longer required, become damaged or are aberrant. Degrading surplus proteins is especially important for non-dividing cells like neurons that lack the capability to replace ageing cells (Keller et al., 2002). Strikingly, decreased activity of the UPS has been shown upon ageing and aberrant proteins like UBB^{+1} are only detected in aged individuals (van Leeuwen et al., 1998). Despite expression from development, neurodegenerative disorders including the polyglutamine diseases manifest themselves around mid-life suggesting a buildup of aberrant proteins or cellular injury (Ciechanover and Brundin, 2003). It appears likely that factors like the UPS that become affected upon ageing influence development of these disorders.

The aim of this thesis was to elucidate the contribution of the UPS to neurodegeneration in HD. Several aspects of neurodegeneration in HD with a specific focus on the UPS are reviewed in **Chapter 1**.

Chapter 2 reviews the discovery of molecular misreading which occurs on GAGAG motifs in different genes. The frameshift mutant of ubiquitin B (UBB^{+1}) is described including the contribution to disease development.

UBB^{+1} was shown to accumulate in AD and is an *in vivo* marker for proteasomal inhibition in neurodegenerative disorders. As described in **Chapter 3**, we wanted to elucidate whether the proteasome is also impaired in HD and SCA3 *in vivo* which would lead to the accumulation of UBB^{+1}. In addition, we investigated whether UBB^{+1} could affect neurodegeneration in an *in vitro* model for HD.

UBB^{+1} transgenic mice show a mild inhibition of the proteasome. The aim of **Chapter 4** was to examine whether this UPS inhibition by UBB^{+1} influences Htt aggregation *in vivo*. In general, we wanted to clarify whether a modest inhibition of the proteasome could have a significant impact on the neuropathology of HD.

The ubiquitin conjugating enzyme E2-25K has been shown to interact directly with Htt independent of polyglutamine repeat length. In **Chapter 5**, we questioned whether the sub-cellular localization of E2-25K was altered in disease as a result of the polyglutamine expansion. Additionally, we examined whether the interaction of Htt with E2-25K is involved in neurodegeneration in HD.

Chapter 6 discusses the different findings of this thesis as well as further research and perspectives.

Chapter 2

Conformational diseases: an umbrella for various neurological disorders with an impaired ubiquitin-proteasome system

Remko de Pril, David F. Fischer and Fred W. van Leeuwen

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Abstract

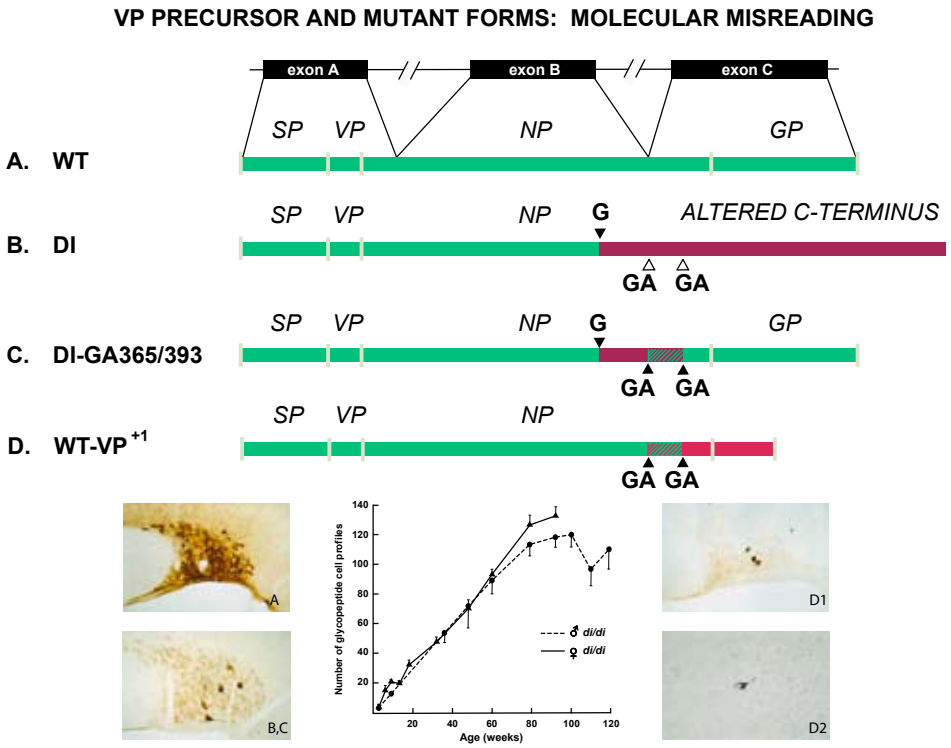
It is increasingly appreciated that failures in the ubiquitin-proteasome system play a pivotal role in the neuropathogenesis of many neurological disorders. This system, involved in protein quality control, should degrade misfolded proteins, but apparently during neuropathogenesis, it is unable to cope with a number of proteins that, by themselves, can consequently accumulate. Ubiquitin is essential for ATP- dependent protein degradation by the proteasome. Ubiquitin^{+1} (UBB^{+1}) is generated by a dinucleotide deletion (ΔGU) in UBB mRNA. The aberrant protein has a 19 amino acid extension and has lost the ability to ubiquitinate. Instead of targeting proteins for degradation, it has acquired a dual substrate-inhibitor function; ubiquitinated UBB^{+1} is a substrate for proteasomal degradation, but can at higher concentrations inhibit, proteasomal degradation. Furthermore, UBB^{+1} protein accumulates in neurons and glial cells in a disease-specific way, and this event is an indication for proteasomal dysfunction. Many neurological and non-neurological conformational diseases have the accumulation of misfolded proteins and of UBB^{+1} in common, and this combined accumulation results in the promotion of insoluble protein deposits and neuronal cell death as shown in a cellular model of Huntington disease.

Introduction

Only at the 9th Alzheimer meeting in Philadelphia, 2004, "conformational diseases" were acknowledged as a group of disorders that share a common feature: the accumulation of insoluble protein deposits in the affected cells (Carrell and Lomas, 1997). To this group belong many age-related neurodegenerative disorders, such as tauopathies (e.g. Alzheimer and Pick disease), synucleinopathies (e.g. Lewy body disease) and polyglutamine diseases (e.g. Huntington disease, and several spinocerebellar ataxias) (Ciechanover and Brundin, 2003; Landles and Bates, 2004). In addition, in non-neuronal disorders inclusions are present as well (e.g. alcoholic liver disease, inclusion body myositis and α1-antitrypsin deficiency (Askanas and Engel, 2003; French et al., 2001; Lomas and Carrell, 2002; McPhaul et al., 2002). Although the deposits vary in protein composition, shape and localization, each of these structures (e.g. aggregates, aggresomes or inclusions) is mainly composed of insoluble misfolded proteins (e.g. Huntingtin, α-synuclein or hyperphosphorylated tau), different molecular chaperones (e.g. heat shock proteins) and various components of the ubiquitin-proteasome (UPS) (e.g. E3 ligases such as CHIP, 19S and 20S proteasomal subunits of the 26S proteasome) (McDonough and Patterson, 2003; Sherman and Goldberg, 2001). The presence of these factors suggests that these insoluble proteins are misfolded and have been targeted for degradation, but instead of being properly refolded or proteolytically degraded, they accumulate in insoluble protein deposits (Alves-Rodrigues et al., 1998). The coexistence of heat shock proteins and UPS compounds is expected, since one of the fundamental tasks of the UPS is to degrade damaged or abnormal proteins and to protect cells during stress responses. If the capacity of the UPS is exceeded (e.g. during aging (Keller et al., 2004)), autophagy, essential for the elimination of aggregates, is induced. Autophagy is considered a highly regulated but non-selective pathway by which cytoplasmic constituents are degraded in lysosomes (see (Cuervo, 2004; Nixon, 2005; Ross and Pickart, 2004)). In the past few years excellent reviews on cross-talk between the UPS (Keller et al., 2004; Pickart and Cohen, 2004) and autophagy (Shintani and Klionsky, 2004) in relation to neurodegeneration have appeared (Ciechanover and Brundin, 2003; de Vrij et al., 2004; McNaught, 2004; Ross and Poirier, 2004).

In the present minireview the discovery of an unexpected type of mutation ("molecular misreading") and the contribution of one of the resulting aberrant proteins (UBB^{+1}) to proteasomal dysfunctioning will be discussed in relation to various conformational diseases that have an impaired UPS (Song and Jung, 2004).

Figure 1: Molecular misreading.



Schematic representation of the vasopressin (VP) gene, its precursor protein and its mutant forms. The VP gene was cloned in the early eighties and consists of 429 nucleotides divided over three exons. After splicing, a precursor protein is translated at the endoplasmatic reticulum which is enzymatically processed within granules and subsequently transported to the nerve terminals. In the normal situation (A) a wild-type (wt) VP precursor is synthetized in the supraoptic nucleus (SON) that can be posttranslationally processed and packaged in neurosecretory granules that are subsequently axonally transported towards the neural lobe. The homozygous Brattleboro rat (B) lacks a single G base in exon B by which an out of frame mutant protein is formed with a polylysine tail that cannot be processed properly. As a result a severe diabetes insipidus (DI) develops. The volume of urine formed daily can reach 70% of the body weight. This trait is autosomally recessive and inherited in a simple Mendelian manner. In the SON and the paraventricular nucleus surprisingly an age-dependent increase of solitary neurons with a revertant VP phenotype (C, diagram) was found (van Leeuwen et al., 1989) that appeared to be due to a second mutation (ΔGA) in downstream located GAGAG motifs. As a result the VP (mutant) precursor (i.e. the glycoprotein-containing part) can be processed and the neurosecretory granules can be axonally transported again towards the neural lobe (Evans et al., 1996). As GAGAG motifs are also present in the wt-VP gene of rat and human (D), a similar process can take place and change the normal VP precursor in an aberrant one. This was shown in neurons of the SON of the rat (D1) and the human (D2).

Discovery of molecular misreading

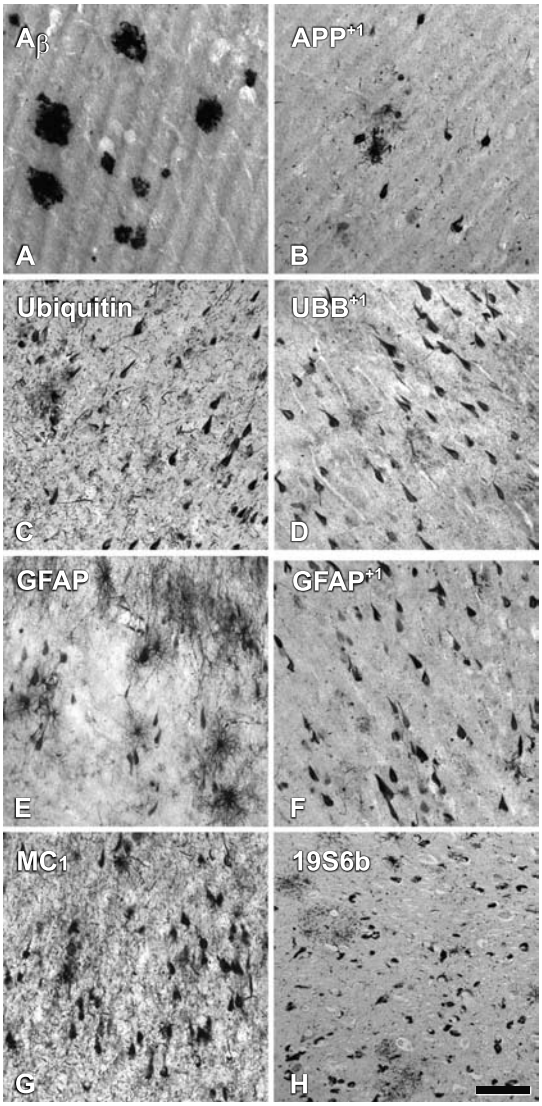
Molecular misreading of genes (i.e. the inaccurate conversion of genomic information into aberrant proteins) was demonstrated in the vasopressin (VP) gene, which is highly expressed in magnocellular neurons of the hypothalamo-neurohypophyseal system. The first occurring knockout ever presented was that of VP, discovered in the city of Brattleboro (Vermont, USA (Valtin, 1982); Figure 1). Gene cloning revealed the mutation; a single G residue deletion in exon B results in a VP precursor with a very sticky polylysine C-terminus which remains in the endoplasmatic reticulum. Antibodies raised against all parts of the VP precursor (e.g. the very C-terminal part of the VP precursor; glycopeptide) showed an intense staining of VP cells (Figure 1A). As a specificity control for these antibodies, the homozygous Brattleboro rat was used. Indeed, many neurons in the magnocellular hypothalamic nuclei showed no reaction (Lu et al., 1982). However, surprisingly, solitary neurons were intensely stained with glycopeptide antibodies (Figure 1B,C). These neurons, which had a revertant VP phenotype, were shown to increase in number with advancing age by the diseased state (diabetes insipidus) of these animals (Evans et al., 1994). Follow-up analysis of the mRNA of these cells revealed dinucleotide deletions (ΔGA) located downstream of the G deletion, in GAGAG motifs. Consequently, at these points the normal reading frame was restored.

Subsequently, the question was raised whether this mutation is a peculiarity of the Brattleboro rat or a more general phenomenon. The wild-type VP genes of rat and human have the same GAGAG motifs. Indeed, antibodies directed against the predicted VP sequences in the -2 or +1 reading frame resulted in an intense staining of magnocellular neurons (Figure 1, D1 and D2; (Evans et al., 1996)). Thus, a similar dinucleotide deletion (ΔGA) is likely to occur in wild-type sequences and is not restricted to homozygous Brattleboro rats. It is important to realize that in these cases an abnormal precursor protein is created out of a normal one. These abnormal +1 proteins are potentially functionally different (Figure 1D).

Which transcripts have GAGAG motifs?

The next step was to see if other genes do have GAGAG motifs or other simple repeats. The chance to encounter a GAGAG motif is 1:1024, and there are many genomic sequences which have such a potential, error-prone site. We focused on Alzheimer's disease associated genes such as β amyloid precursor protein and ubiquitin B, of which indeed the respective +1 proteins were found to coexist in the hallmarks of all cases of Alzheimer's disease (including sporadic ones) and Down syndrome (van Leeuwen et al., 1998). The proposed dinucleotide deletions were found as well. The existence of these dinucleotide deletions was confirmed independently (van den Hurk et al., 2001). Several +1 proteins appeared to coexist (e.g. Amyloid Precursor Protein, Ubiquitin and GFAP; Figure 2) due to molecular misreading and exon skipping (Hol

Figure 2: Various +1 stainings in comparison to their wild-type form or conventional markers.



Fifty-µm-thick vibratome section of the hippocampus of an Alzheimer patient (AD, #96115, 90 years old) showing Aβ, APP^{+1}, ubiquitin, UBB^{+1}, GFAP and GFAP^{+1}. Abnormal Tau is present in the hallmarks of AD (G, neurofibrillary tangles, neuropil threads and dystrophic neurites) similar to APP^{+1}, Ubiquitin, UBB^{+1} and remarkably with GFAP and GFAP^{+1} (Hol et al., 2003). Note in panel E that GFAP also reacts with astrocytes. In panel H the 19S regulator subunit 6b is present in the same hallmarks indicating its upregulation (patient #88028, paraffin section, (Zouambia et al., 2008)) and proteasomal dysfunction that can be explained by the presence of UBB^{+1} by which the proteasome is clogged up.

et al., 2003). We subsequently focused upon ubiquitin B because of the potentially devastating effects of both a gain or loss of function of the resulting +1 protein in different cellular processes (Ciechanover and Brundin, 2003).

Ubiquitin B and molecular misreading

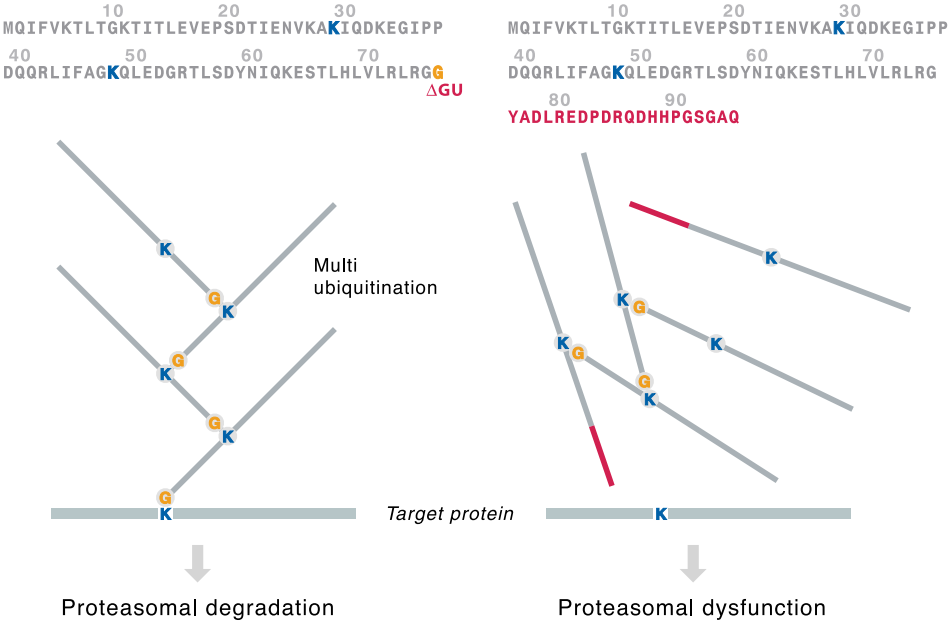
Human ubiquitin B is a protein localized on chromosome 17p11.2 and it is one of the best conserved eukaryotic proteins. From yeast to human, only at three positions does the amino acid sequence show variation in the 76 residues long protein, indicating its functional relevance. Ubiquitin B is synthetized in a three repeat from which 76 amino acids are cleaved off. The ubiquitin molecule harbors a number of lysine moieties, of which #29 and #48 are involved in ATP-dependent multi-ubiquitination, a process that triggers transport to the proteasome, followed by proteolysis. At the very C-terminus of ubiquitin a glycine moiety (#76) is present, which is essential for all biological function of ubiquitin B. A few nucleotides upstream of this C-terminal glycine moiety a GAGAG motif is present. The result of a dinucleotide deletion in this motif is a loss of the C-terminal glycine moiety and a 19 amino acids longer ubiquitin molecule called ubiquitin^{+1} (UBB^{+1}) (Figure 3). It was suggested that UBB^{+1} is unable to ubiquitinate and in fact, might be a substrate for ubiquitination (van Leeuwen et al., 1998), and disturb proteasomal degradation as mentioned in Vogel (Vogel, 1998).

Towards an effect of ubiquitin^{+1}

The functional relevance of UBB^{+1} has been clarified during the past five years. UBB^{+1} can be degraded at low concentrations (Lindsten et al., 2002), apparently after deubiquitination, but is also refractory to deubiquitination (Lam et al., 2000). Ubiquitinated UBB^{+1} furthermore appears to inhibit the proteasome potently (Lindsten et al., 2002). In case of high concentrations, neuronal cell death by apoptosis was found (De Vrij et al., 2001). Since deubiquitination of UBB^{+1} is a prerequisite for its entrance, subsequent unfolding, substrate recognition, chaperoning and channelling by 19S regulatory subunits to the 20S proteolytic core are possibly disturbed. Consequently, degradation of UBB^{+1} by the β 1, 2 and 5 subunits in the proteolytic core is impaired (Pickart and Cohen, 2004).

The next step to find out whether UBB^{+1} contributes to the neuropathogenesis of Alzheimer's disease, was to generate transgenic mice expressing UBB^{+1} regionally (hippocampus and cerebral cortex). This work has been executed successfully is in progress; we are currently using these transgenic UBB^{+1} mice to explore the downstream effects of UBB^{+1} (e.g. proteomic and behavioral analyses, long-term potentiation (LTP) and transcript and protein analysis of gene products involved in LTP, e.g. CREB; (Hegde, 2004)). Indeed, UBB^{+1} induces expression of heat shock proteins, as shown in human neuroblastoma cells (Hope et al., 2003), and a behavioral

Figure 3: Multiubiquitination of proteasome substrates.



Simplified and schematic representation of how ubiquitin acts via multiubiquitination through a number of enzymatic steps (E_1, E_2 and E_3) (left panel, for details see (Ciechanover and Brundin, 2003)). The lysine moieties at positions 29 and 48 are involved in the multiubiquitination and degradation. At the C-terminus of ubiquitin a GAGAG motif is present. It was shown that adjacent to this motif a dinucleotide deletion (ΔGU) occurs resulting in an extension of 19 amino acids (right panel, for further details see (Gerez et al., 2005)). Due to the dinucleotide deletion, the G_{76} moiety at the C-terminus, essential for binding to a target protein, is not synthetized. Consequently this molecule cannot ubiquitinate. In fact, it has become a substrate for degradation and contains a ubiquitin fusion degradation signal as well. It has been shown that ubiquitinated UBB^{+1} protein inhibits the proteasome (Lindsten et al., 2002) and acts synergistically with polyglutamine repeats with regard to aggregation and cell death in a cellular model of HD (de Pril et al., 2004).

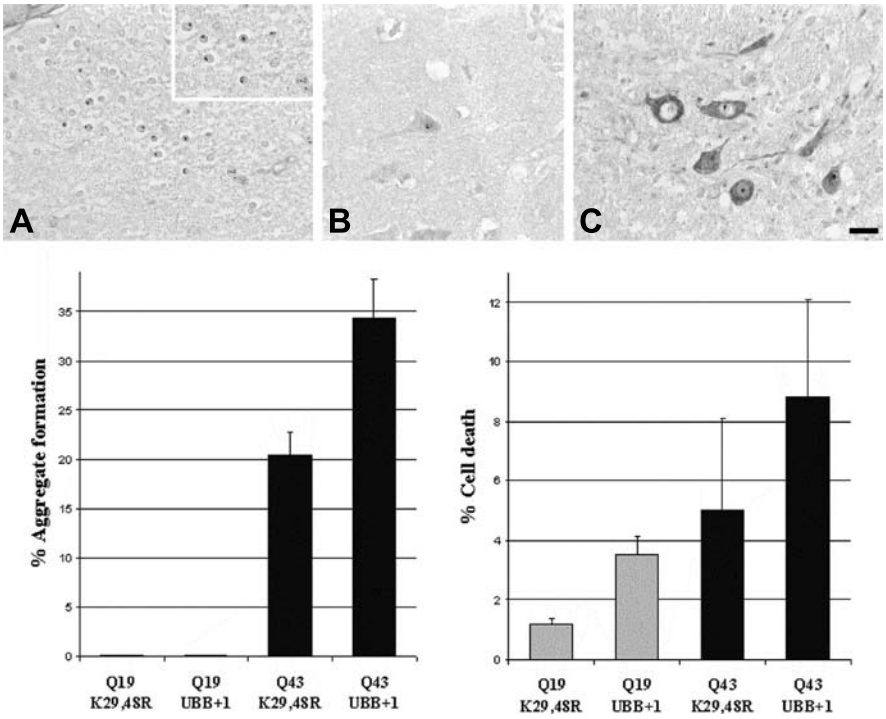
phenotype was found accompanied by other proteomic changes in these mice (Fischer et al., 2009). In this manner UBB^{+1} mouse lines have become available with a life-long genetically encoded inhibition of the proteasome. These unique lines can be used for crossing with other Alzheimer transgenic lines and other mouse models that are currently being used for other types of tauopathies (e.g. frontotemporal dementia) and Huntington disease. UBB^{+1} is present in the hallmarks of other tauopathies (Fischer et al., 2003) and polyglutamine diseases (Figure 4A-C) (de Pril et al., 2004). It was shown in a cellular model for Huntington disease (HD) that UBB^{+1} has a strong synergistic effect on aggregate formation and cell death (Figure 4D) (de Pril et al., 2004). These results are in agreement with the conclusion of Wexler et al. (Wexler et al., 2004), who reported that approximately 40% of the variance remaining in onset age is attributable to genes other than the HD gene and 60% is environmental.

Fischer et al. (Fischer et al., 2003) showed in the rat brain that UBB^{+1} requires the lysine moieties of position 29 and 48 for its proteasomal degradation. It appeared that under normal circumstances in both the rat brain and in young non-demented control patients UBB^{+1} can be degraded rapidly and only accumulates upon proteasomal impairment. In other words, the accumulation of UBB^{+1} in various tauopathies and polyglutamine diseases, but not in synucleinopathies, points to UBB^{+1} as a marker for proteasomal dysfunction (de Pril et al., 2004; Fischer et al., 2003). The latter results indicate a difference between tauopathies and polyglutamine diseases at one side and synucleinopathies at the other one. In synucleinopathies the ubiquitin machinery may be disturbed, as shown in rare forms of Parkinson's disease; the E3 ligase Parkin, the deubiquitinating enzyme ubiquitin carboxy terminal hydrolase UCH-L1, α-synuclein and DJ1 are mutated in familial forms of Parkinson's disease (Ciechanover and Brundin, 2003; Giasson and Lee, 2003). In tauopathies and several polyglutamine diseases, accumulation of UBB^{+1} (possibly following a failure to deubiquitinate; (Lam et al., 2000)) and other substrates of the proteasome (e.g. Aβ, huntingtin) inhibit the proteasome (Lindsten et al., 2002). However, whether or not the ubiquitin machinery or the proteasome itself is impaired, both in tauo- and synucleinopathies, and in polyglutamine diseases, proteasomal dysfunction is the outcome in all these neurological disorders.

Molecular misreading in non-neuronal cells

Again using the VP system, we were able to address the question whether molecular misreading occurs outside of neuronal cells. In order to do so we used transgenic mice in which the rat VP gene is expressed ectopically in epithelial structures of secretory organs under the control of the mouse mammary tumor virus long terminal repeat promotor. Indeed, VP was found in the gonadal system (e.g. testis and epididymis). Using antisera specific for frameshifted VP (VP^{+1}), we were able to show that in the principal cells of the caput of the epididymis VP^{+1}, VP and other parts of the VP precursor coexist. This result was supported by specific in situ hybridization

Figure 4: UBB^{+1} accumulates in polyglutamine diseases and enhances aggregate formation and cell death.



UBB^{+1} was found to accumulate in all thirty cases of Huntington disease tested (A, striatum, #4 of (de Pril et al., 2004), having 59 glutamine repeats, insert shows various intranuclear inclusions (blow-up of A, 1.3x) and B, frontal cortex of #5 in (de Pril et al., 2004), having 41 glutamine repeats). The same reaction was found in six cases of spinocerebellar ataxia-3 (C, pons of #36 in (de Pril et al., 2004), having a polyglutamine repeat length 24-70). Note the intense staining in the intranuclear inclusions as well as the cytoplasmic accumulation of UBB^{+1}. Digital recordings of 6-µm-thick paraffin sections were made by the extended-depth program by which different pictures were combined to give one in-focus composite image (Imagepro 5.0, Media Cybernetics, Silver Springs, USA). Bar = 20 µm. D, In a cellular model of HD we found that UBB^{+1} not only increases the aggregate formation of expanded polyglutamine repeats but in addition has a synergistic effect on polyglutamine-induced cell death. Differentiated SH-SY5Y neuroblastoma cells were lentivirally infected with constructs with truncated huntingtin fragments containing 19 or 43 glutamine repeats (HA-Q-GFP) in combination with either UBB$^{+1;K29,48R}$ or UBB^{+1}. Cells were quantified for aggregate formation 4 days after infection (left panel). Note that huntingtin with 19 glutamines does not result in the formation of aggregates. Constructs with 43 glutamines show a marked increase in the number of aggregate forming cells in the presence of UBB^{+1}. Cell-death was assessed by a cell survival assay 4 days after infection (right panel). Polyglutamine-GFP positive cells were analyzed by flow cytometry for the percentage of cell death under different conditions (de Pril et al., 2004). Q19 with UBB$^{+1;K29,48R}$ shows the background percentage of cell death due to infection or culturing. A minor increase in cell death was observed with either expansion of the polyglutamine repeat or co-infection of UBB^{+1} with Q19. A marked increase in cell death was observed after coinfection of UBB^{+1} with the construct with 43 glutamines (n=3; P<0.005; for details see (de Pril et al., 2004)).

(Van Leeuwen et al., 2000). Thus, VP^{+1} is formed in non-neuronal cells (molecular misreading), due to molecular misreading of the transgene. Subsequently, frameshifted ubiquitin (UBB^{+1}) was found in inclusions of various human diseases and suggests a similar mechanism as found in neuronal cells: the Mallory bodies of hepatocytes during cirrhosis in alcoholic liver disease (McPhaul et al., 2002), in hepatocytes of patients with an α1-antitrypsin deficiency (Wu et al., 2002) and in aggregates of inclusion body myositis (Fratta et al., 2004).

The unfolded protein response and the UPS

Many newly synthetized proteins are translocated into the lumen of the endoplasmic reticulum (ER), which is the place for their folding and assembly. Alterations in homeostasis by various cellular stressors that prevent protein folding cause an accumulation of misfolded proteins in the ER, which are referred to as types of ER stress. Eukaryotic cells can adapt, for survival, to deal with an accumulation of unfolded proteins in the ER by various signals from the ER lumen to the cytoplasm and the nucleus. This induction system is called unfolded protein response (UPR) and includes the transcriptional induction of UPR target genes (e.g. ER-resident chaperones, such as GRP78/BiP to facilitate protein folding), translational attenuation of global protein synthesis and ER-associated degradation (ERAD). In conformational diseases such as Alzheimer's disease, recent reports indicate that the UPR is involved (Katayama et al., 2004), and a link between ERAD and the UPS has been suggested (Kopito and Sitia, 2000; Kostova and Wolf, 2003). In addition, a proteasome independent ERAD pathway may exist (Donoso et al., 2005). So far no link between UBB^{+1} effects and the UPR activity has been reported.

Challenges for the next decade

- It is evident that the UPS contributes substantially to conformational diseases, such as Alzheimer's disease, during which "it stops delivery" (Miller and Wilson, 2003). This might reflect a primary or secondary event. Of course one should realize that Alzheimer's disease and other related inclusion diseases are multifactorial. The latter aspect needs to be kept in mind when generating transgenic mice. The step towards multiple transgenic mice has been taken (e.g. (Oddo et al., 2004)) and the numerous contributing factors to Alzheimer's disease can be organized in a temporal pattern (e.g. (Konishi et al., 2003; Muchowski and Wacker, 2005; Song and Jung, 2004)). The recently generated UBB^{+1} mouse lines are an obvious candidate for further crossings. Long term potential (LTP) measurements in the hippocampus of these mice and changes in gene and protein levels (e.g. CREB, synaptophysin and AMPA receptors, (Hegde, 2004)) as well as further proteomic analysis (Fischer et al., 2009) are currently under investigation.

- Synaptic plasticity is also known to be affected in Alzheimer's disease (Terry and Katzman, 2001). The UPS and its inhibition by UBB^{+1} are attractive candidates for further research (Ehlers, 2003). The recently developed transgenic UBB^{+1} mice (Fischer et al., 2009) may contribute in this respect.
- Another challenge is trying to substantiate the effects of different cellular stressors (e.g. Aβ, E2-25K/Hip2, UBB^{+1}) by introducing them into cell lines followed by rescue experiments, i.e. silencing the effect of these proteins by means of RNA interference (Novina and Sharp, 2004), as shown by Song et al. (Song and Jung, 2004).
- The presence of UBB^{+1} outside the nervous system, in dividing cells, enabled experiments *in vitro* to assess the contribution of UBB^{+1} to aggresome formation (e.g. (Bardag-Gorce et al., 2003; French et al., 2001)). Indeed, when the capacity of the proteasome is exceeded, aggregate formation followed by lysosomal degradation has become an increasingly attractive subject of research (e.g. (Shintani and Klionsky, 2004)). Other possible mechanisms to cope with insoluble proteins are posttranslational modifications like sumoylation and neddylation (e.g. (Schwartz and Hochstrasser, 2003; Steffan et al., 2004)), the contribution of which to inclusion formation in the various conformational diseases needs to be assessed.
- Relevant essential subunits of the ubiquitin and deubiquination machinery as well as of essential proteasome subunits are now ready for analysis to reveal their contribution to neuropathogenesis (e.g. (Cookson, 2004; Guterman and Glickman, 2004; McDonough and Patterson, 2003; Petrucelli et al., 2004)).
- It is clear that proteasomal activators are badly needed (without side-effects). Proteins as activators of the 20S proteolytic core are known as PA28 and PA200, and are obvious targets for the development of even more potent molecules mobilizing the proteolytic machinery (Rechsteiner and Hill, 2005). Alternatively elucidation of deubiquitinating enzymes (DUBS) by screening RNAi libraries might result in DUBS promoting deubiquitination of ubiquitinated UBB^{+1} (Brummelkamp et al., 2003).

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Chapter 3

Accumulation of aberrant ubiquitin induces aggregate formation and cell death in polyglutamine diseases

Remko de Pril, David F. Fischer, Marion L.C. Maat-Schieman,
Barbara Hobo, Rob A.I. de Vos, Ewout R. Brunt, Elly M. Hol,
Raymund A.C. Roos and Fred W. van Leeuwen

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Abstract

Polyglutamine diseases are characterized by neuronal intranuclear inclusions of expanded polyglutamine proteins, indicating failing protein degradation. UBB^{+1}, an aberrant form of ubiquitin, is a substrate and inhibitor of the proteasome, and was previously reported to accumulate in Alzheimer disease and other tauopathies. Here we show accumulation of UBB^{+1} in the neuronal intranuclear inclusions and the cytoplasm of neurons in Huntington disease and spinocerebellar ataxia type 3, indicating inhibition of the proteasome by polyglutamine proteins in human brain. We found that UBB^{+1} not only increased aggregate formation of expanded polyglutamines in neuronally differentiated cell lines, but also had a synergistic effect on apoptotic cell death due to expanded polyglutamine proteins. These findings implicate UBB^{+1} as an aggravating factor in polyglutamine-induced neurodegeneration, and clearly identify an important role for the ubiquitin-proteasome system in polyglutamine diseases.

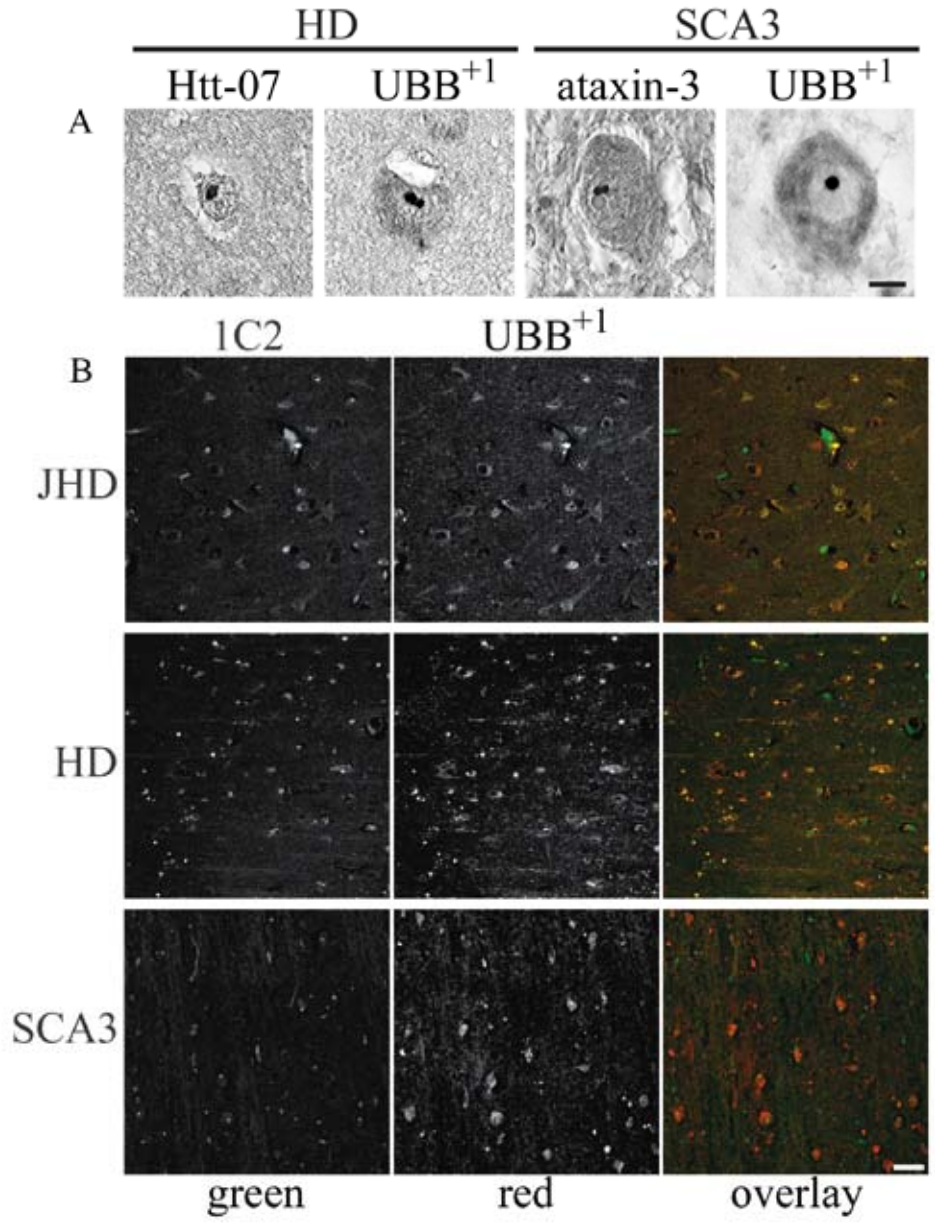
Introduction

At least nine different neurodegenerative diseases are known that are caused by the expansion of a CAG repeat in the coding region of a transcribed gene, including the spinocerebellar ataxias (SCAs) and Huntington disease (HD) (Nakamura et al., 2001; Zoghbi and Orr, 2000). All these CAG expansion diseases are characterized by progressive neuronal dysfunction starting around adult-life and resulting in severe neurodegeneration. In the channelopathy SCA6, neurodegeneration is caused by a small CAG expansion (to 19-30 repeats) in a calcium channel subunit that presumably causes a change of function (Zhuchenko et al., 1997). The other eight diseases are probably caused by a gain of function of the proteins carrying the expanded polyglutamine repeat. The pathological repeat length for these genuine polyglutamine expansion disorders starts around 40 glutamine repeats in the affected gene, with increasing severity and earlier manifestation upon greater expansion (Zoghbi and Orr, 2000).

One of the hallmarks of the pathology of polyglutamine diseases is the formation of neuronal intranuclear inclusions (NIIs) in the affected areas of the brain (DiFiglia et al., 1997). Although many of the proteins carrying the polyglutamine repeat have a cytoplasmic function, upon polyglutamine expansion they all form intranuclear inclusions that contain at least the expanded polyglutamine fragment. In HD, for instance, the NIIs contain only the N-terminal part of huntingtin with the polyglutamine stretch (DiFiglia et al., 1997; Maat-Schieman et al., 1999; Sieradzan et al., 1999; Zhou et al., 2003). The major pathological difference between the polyglutamine diseases is the regional distribution of neurodegeneration. In HD the striatum is the most severely affected area and the cortex is affected to a lesser extent (Halliday et al., 1998). In SCA3, in contrast, neuronal degeneration occurs primarily in the nuclei of the brainstem and the spinal cord (Takiyama et al., 1994). This regional specificity is probably caused by differences in expression levels of the respective disease genes among the various brain regions or different vulnerability of various types of neurons. In addition to the repeat expansion, other factors, such as proteasomal activity and expression or recruitment of cellular chaperones, probably influence polyglutamine toxicity and disease development (Chan et al., 2002; Wexler et al., 2004; Willingham et al., 2003).

NIIs contain ubiquitin or ubiquitinated proteins, which indicates that the aggregating proteins are targeted to, but not degraded by, the proteasome (DiFiglia et al., 1997; Paulson et al., 1997). In vitro studies have furthermore shown that expanded polyglutamines can directly inhibit the proteasome (Bence et al., 2001; Verhoef et al., 2002). In addition, in SCA3 patients, subunits of the 26S proteasome have been shown to be recruited to NIIs (Chai et al., 1999; Schmidt et al., 2002). Finally, in SCA1 transgenic mice, the Purkinje cell pathology was aggravated by mutation of the E6-AP ubiquitin ligase (Cummings et al., 1999). All these findings point towards an involvement of the ubiquitin-proteasome system (UPS) in the pathogenesis of

Figure 1: UBB^{+1} colocalizes with polyglutamine proteins in HD and SCA3.



Staining of paraffin sections of the frontal cortex of HD patients and the pons of SCA3 patients for UBB^{+1} and respectively Htt-07 and α-ataxin-3 (A). Pictures show staining of NIIs with UBB^{+1}, distinct from the nucleoli, in both polyglutamine diseases. Magnification bar is 10μm. Double stainings show UBB^{+1} staining in all inclusions positive for 1C2 in juvenile as well as late onset HD and SCA3 (B). Magnification bar is 50μm. Note also the cytoplasmic staining for UBB^{+1} in both disorders (A and B).

polyglutamine diseases and to an enhancement of neurodegeneration by further impairment of the UPS (Ciechanover and Brundin, 2003).

We previously reported that, in Alzheimer disease (AD), an aberrant form of ubiquitin (UBB^{+1}) accumulates in the neuropathological hallmarks of the disease (van Leeuwen et al., 1998). This UBB^{+1} protein is formed by a dinucleotide deletion (ΔGU), leading to a +1 reading frame in the mRNA, and subsequent translation to a protein with an aberrant C-terminus. Thus far UBB^{+1} protein has been found in the hallmarks of several neurodegenerative diseases, including AD and other tauopathies, whereas it was not detected in synucleinopathies and young control patients without pathology (Fischer et al., 2003; van Leeuwen et al., 1998). The aberrant transcript however, in contrast to the protein, appeared to be present even in young controls. Under normal circumstances, neurons can apparently cope with UBB^{+1}, and accumulation of this protein reflects proteasomal dysfunction in different neuropathologal disorders (Fischer et al., 2003).

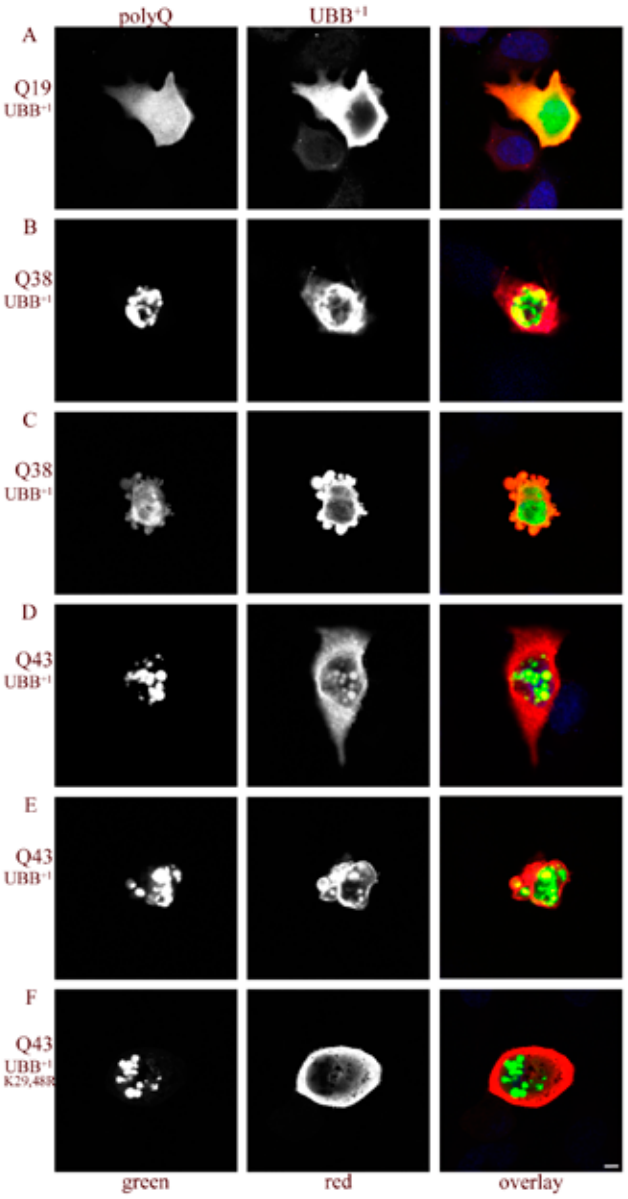
In vitro studies have shown that, although UBB^{+1} can be degraded by the proteasome (Fischer et al., 2003; Lindsten et al., 2002), at higher concentrations it inhibits proteasomal degradation of cellular proteins and leads to cell death in neuroblastoma cells (De Vrij et al., 2001; Lam et al., 2000). In addition, UBB^{+1} has recently been implicated to mediate neurodegeneration via downstream interaction with the E2-25K ubiquitin conjugating enzyme, which induces amyloid-β neurotoxicity *in vitro* (Song et al., 2003). In this perspective, UBB^{+1} might accelerate disease progression and increase the severity of the disease. Notably, recent reports show that the proteasome activity is indeed decreased in AD (Keck et al., 2003; Keller et al., 2000), strengthening the idea that UBB^{+1} accumulation is intimately related to impairment of the proteasome (Ciechanover and Brundin, 2003).

Different findings point towards defective protein degradation in polyglutamine diseases. We examined post-mortem brain material of HD and SCA3 for the accumulation of UBB^{+1}, as a marker for proteasomal impairment. Moreover, we used a cellular model for polyglutamine disease to study the contribution of UBB^{+1} to disease progression, i.e. polyglutamine aggregation and cell death.

Results

To identify NIIs in different brain areas of HD and SCA3, we used antibodies against the N-terminal fragment of the huntingtin protein or full length ataxin-3 respectively (Figure 1A). In addition, we used an ubiquitin antibody and the 1C2 antibody to identify the inclusions. SCA6 showed staining of cytoplasmic aggregates with the α1A-subunit antibody (data not shown).

Figure 2: UBB^{+1} colocalizes with expanded polyglutamines in vitro.



HeLa cells co-transfected with plasmids with a truncated huntingtin fragment containing 19, 38 or 43 glutamine repeats (HA-Q-GFP) together with constructs for UBB^{+1} or UBB$^{+1;K29,48R}$, were fixed 4 days post transfection. All cells that were transfected with the construct with 19 glutamines showed a diffuse cellular polyglutamine staining (A; green; left panel). Constructs with 38 and 43 glutamine repeats result in the formation of multiple polyglutamine aggregates (respectively B-C, and D-F; green; left column). Co-transfection with UBB^{+1} gave cytoplasmic staining for UBB^{+1} (A-E) (in red; middle column) with all constructs. In addition, UBB^{+1} co-aggregates with the polyglutamine constructs Q38 (B and C) and Q43 (D and E) whereas UBB$^{+1;K29,48R}$ only shows cytoplasmic staining despite aggregate formation of Q43 (F). The right column shows the overlay of UBB^{+1} and polyglutamine, with faint To-Pro nuclear staining in blue. Magnification bar is 10μm.

With antibodies against the UBB^{+1} protein, we detected immunopositive NIIs in the cortex (Figure 1A) and striatum of all HD patients (*N*=30). We found accumulation of UBB^{+1} not only in adult-onset patients but already in juvenile HD. Furthermore, we detected UBB^{+1} immunopositive NIIs in all SCA3 patients (*N*=6), in all the areas investigated (Figure 1A). Besides localization to the NIIs, we also found cytoplasmic staining for UBB^{+1} in the affected neurons in HD and SCA3 (Figure 1A). In contrast, in the SCA6 patients (*N*=2) we did not find any UBB^{+1} staining in the affected regions (data not shown). To ascertain whether UBB^{+1} localizes to all or only a subset of NIIs stained with 1C2 we performed double immunofluorescent stainings on tissue sections of both HD and SCA3. We found colocalization of polyglutamine proteins and UBB^{+1} in all the inclusions that were found in both HD and SCA3 (Figure 1B). Accumulation of UBB^{+1} is thus not specific for HD but a general phenomenon found in polyglutamine diseases.

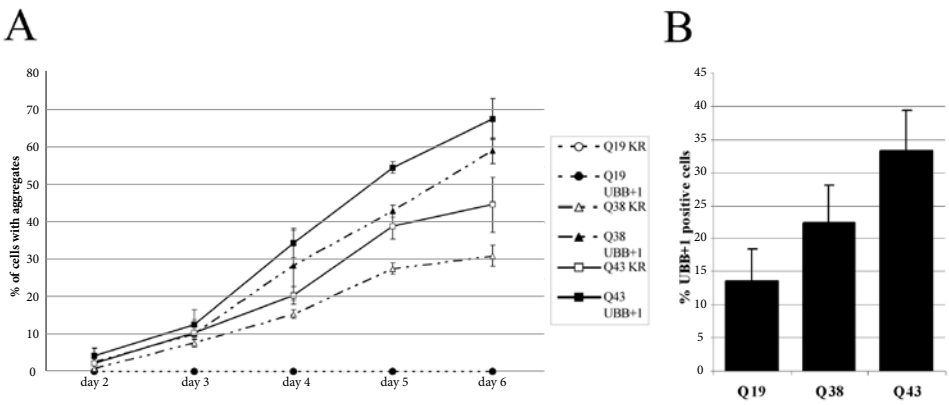
Besides being a marker for proteasome impairment, *in vitro*, UBB^{+1} inhibits the proteasome (Lam et al., 2000; Lindsten et al., 2002). Constructs with expanded polyglutamine repeats can be used to mimic many of the features of polyglutamine diseases in cell lines, including aggregate formation and interaction with other proteins (Cummings et al., 1999; de Cristofaro et al., 1999; Hackam et al., 1999; Lunkes and Mandel, 1998).

To investigate the impact of UBB^{+1} protein on aggregate formation we used plasmids with a truncated huntingtin fragment containing 19, 38 or 43 glutamine repeats (HA-Q19-GFP, HA-Q38-GFP and HA-Q43-GFP) in combination with plasmids containing either UBB^{+1} or a lysine mutant of UBB^{+1} (UBB$^{+1;K29,48R}$), that does not inhibit the proteasome (Lindsten et al., 2002). Co-transfection of UBB^{+1} with Q19 resulted in distribution of Q19 throughout the cells and cytoplasmic staining for UBB^{+1} (Figure 2A). However, besides accumulation of UBB^{+1} in cells with expanded polyglutamine proteins we found colocalization with the inclusions of Q38 (Figure 2B and C) and Q43 (Figure 2D and E). The nuclear localization of the aggregates in these cells was similar to what was observed in post-mortem brain material of the different polyglutamine diseases. In addition, a number of cells display a clear apoptotic morphology, with fragmented nuclei (Figure 2C and E), in agreement with previous findings for UBB^{+1} or expanded polyglutamines alone (De Vrij et al., 2001; Lunkes and Mandel, 1998). In contrast to UBB^{+1}, after transfection with UBB$^{+1;K29,48R}$ we found no colocalization with the aggregating polyglutamines (Figure 2F; Q43). It is remarkable that this latter ubiquitin mutant is not incorporated in the inclusions, although cytoplasmic levels are higher than for UBB^{+1} transfected cells (Lindsten et al., 2002).

For functional characterizations we used neuronally-differentiated human neuroblastoma cells (SH-SY5Y) (Encinas et al., 2000). Cell-proliferation markedly influences the aggregate formation and cell-death (Yoshizawa et al., 2001), which makes differentiated cells a more useful model for functional assays. In our experiments, HeLa cells showed comparable aggregate formation, but due to proliferation and susceptibility, much lower levels of cell-death (data not shown).

We quantified the aggregate formation after lentiviral transduction of SH-SY5Y neuroblastoma cells with polyglutamine vectors in combination with UBB^{+1} or the double lysine mutant of UBB^{+1} (Figure 3A). The truncated huntingtin fragments with 38 or 43 glutamines show a marked increase in aggregate formation under the influence of UBB^{+1} in comparison to $UBB^{+1;K29,48R}$. Accumulation of UBB^{+1} causes an increase in polyglutamine protein and thus enhances the aggregate formation. For the huntingtin fragment with 38 glutamines we even find a doubling of the number of aggregate forming cells due to UBB^{+1} from 4 days on (Figure 3A). Statistical analysis by three-way ANOVA confirmed a highly significant correlation between the aggregate formation, the influence of UBB^{+1} and the time course (P<0.001). Immunofluorescent stainings furthermore show a preferential accumulation of UBB^{+1} in the cells with the expanded huntingtin fragments (Figure 3B). Quantification of the cells 4 days after infection showed a clear increase in the amount of UBB^{+1} expressing cells upon expansion of the polyglutamine repeat (Figure 3B). Statistical analysis by one-way ANOVA confirmed a highly significant correlation between the repeat expansion and UBB^{+1} accumulation (P=0.001).

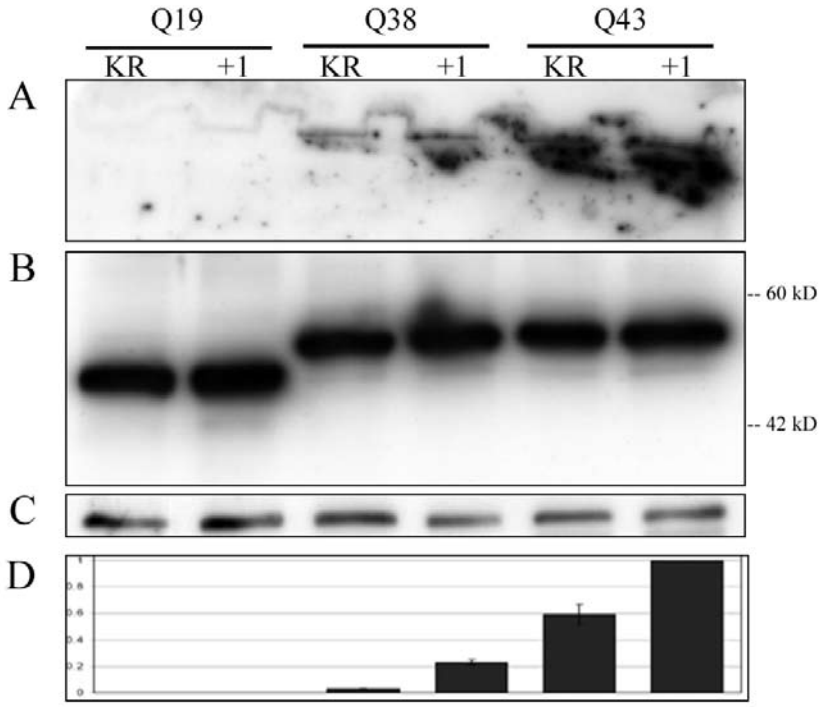
Figure 3: UBB^{+1} induces aggregate formation and preferentially accumulates with expanded polyglutamines.



Differentiated SH-SY5Y neuroblastoma cells were lentivirally infected with constructs with truncated huntingtin fragments containing 19, 38 or 43 glutamine repeats (HA-Q-GFP) in combination with either $UBB^{+1;K29,48R}$ (KR) or UBB^{+1} (+1). Cells were quantified for aggregate formation every 24 hours after infection (A). Note that huntingtin with 19 glutamines does not result in the formation of aggregates, resulting in two overlapping lines in this graph. Constructs with 38 and 43 glutamines both show a marked increase in the number of aggregate forming cells in the presence of UBB^{+1}. Statistical analysis by three-way ANOVA demonstrated a strong correlation between the aggregate formation of expanded polyglutamines, the influence of UBB^{+1} and the time course (P<0.001). Expression of UBB^{+1} was quantified 4 days after infection (B). The expression of expanded huntingtin fragments causes a preferential accumulation of UBB^{+1} in these cells. Statistical analysis by one-way ANOVA showed a highly significant correlation between repeat expansion of the huntingtin fragments and the UBB^{+1} accumulation (P=0.001). All experiments were performed in triplicate.

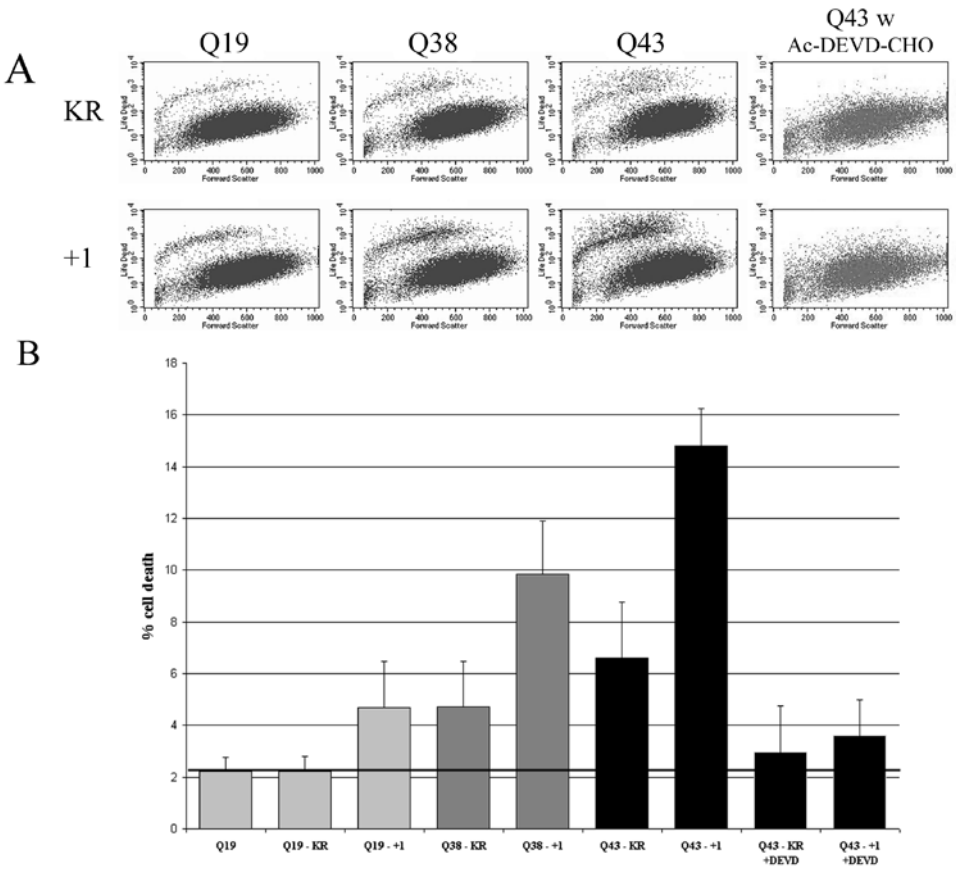
We made cell lysates 6 days after infection and analyzed these by Western blotting (Figure 4). Western blots probed with an anti-HA antibody showed, due to UBB^{+1}, a threefold increase in SDS-insoluble aggregate fraction at the top of the gel with both 38 and 43 glutamines (Figure 4A). The amounts of soluble polyglutamine protein however, show only a slight increase for all repeat lengths (Figure 4B). The bars (Figure 4D) represent the relative amount of aggregates (Figure 4A) after correction for β-actin (Figure 4C). Statistical analysis by two-way ANOVA confirmed that UBB^{+1} has a pronounced, highly significant synergistic effect on the aggregate formation of constructs with 38 and 43 glutamines (P<0.001).

Figure 4: UBB^{+1} increases aggregate formation of expanded polyglutamines.



Differentiated SH-SY5Y neuroblastoma cells were lentivirally infected with constructs with truncated huntingtin fragments containing 19, 38 or 43 glutamine repeats (HA-Q-GFP) in combination with either UBB$^{+1;K29,48R}$ (KR) or UBB^{+1} (+1), and harvested 6 days after infection. Especially with 38 glutamines a marked increase is seen in the SDS-insoluble fraction that is retained at the top of the gel when cells are co-infected with UBB^{+1} (A). The levels of soluble polyglutamine protein show a minor increase (B, around 60kD). Panel (C) shows a β-actin staining as a loading control. Bars show the relative integrated optical densities of the aggregate fraction after correction for loading (D). Statistical analysis by two-way ANOVA showed a strong synergistic effect of UBB^{+1} on aggregate formation by expanded polyglutamines (P<0.001). All experiments were performed in triplicate.

Figure 5: UBB^{+1} synergistically aggravates polyglutamine-induced cell death.



Cell death of differentiated SH-SY5Y neuroblastoma cells, as assessed by a cell survival assay 6 days after infection, with constructs with 19, 38 or 43 glutamines (HA-Q-GFP) in combination with UBB$^{+1;K29,48R}$ (KR) as a control or UBB^{+1} (+1). Polyglutamine-GFP positive cells were analyzed by flow cytometry for conversion of red-fluorescent dye (A; representative FACS analysis). Scatter plots clearly show the differential distribution of living cells (lower population) and dead cells (higher population; bright red-fluorescent). The bars (B) show the percentage of cell death that was observed under different conditions. Q19 shows the background percentage of cell death due to infection or culturing and no increase was observed in combination with UBB$^{+1;K29,48R}$ (B). The horizontal line signifies the percentage of background cell death. A minor increase in cell death was observed with expansion of the polyglutamine repeat. Co-infection with UBB^{+1} caused a small increase in cell death of Q19, but a marked increase was observed with constructs of both 38 and 43 glutamines. Statistical analysis by two-way ANOVA showed a strong synergistic effect of UBB^{+1} on cell death by expanded polyglutamines (P<0.001). Caspase inhibition with Ac-DEVD-CHO resulted in a marked decrease of cell-death due to UBB^{+1} as well as due to expanded polyglutamines. Statistical analysis revealed no significant increase in cell-death in comparison to the background level. All experiments were performed in triplicate.

To investigate the effect of the expression of the different proteins on the viability of neuroblastoma cells we performed a cell-survival assay (Figure 5). The scatter plots show a representative FACS analysis of the polyglutamine expressing cells (Figure 5A). Dead cells show a marked increase in red-fluorescence due to conversion of the reactive dye (higher population). Cell death due to Q19 represents the background level of cell death due to infection and culturing of the cells, whereas Q19 itself is not toxic (de Cristofaro et al., 1999). Combination of Q19 with $UBB^{+1;K29,48R}$, which was used as a control, does not affect cell death. Expansion of the polyglutamine repeat resulted in a moderate increase in cell death when combined with $UBB^{+1;K29,48R}$. We measured a small but significant increase for the plasmid with 38 glutamines, whereas the expansion to 43 caused a significant three-fold increase in cell death. Upon addition of UBB^{+1} a moderate and significant increase in cell death was found for the Q19 construct. In contrast to previous experiments (De Vrij et al., 2001), lentiviral transduction resulted in only moderate cell death for UBB^{+1} (Figure 4B) due to degradable levels of UBB^{+1} protein (Fischer et al., 2003). However, UBB^{+1} in combination with longer glutamine repeats caused a much more pronounced increase in cell death for Q38 as well as for Q43 constructs. Statistical analysis by two-way ANOVA confirmed that the combination of UBB^{+1} with constructs of 38 and 43 glutamines has a pronounced, highly significant synergistic effect on the cell death mechanism ($P<0.001$). This synergistic effect manifests itself as an increase of the absolute effect of UBB^{+1} on cell death with increasing length of the Hungtingtin fragment. Caspase inhibition by Ac-DEVD-CHO resulted in a marked decrease in cell-death in our polyglutamine model, indicating that cell death due to UBB^{+1} as well as due to expanded polyglutamines occurs via an apoptotic pathway (Figure 4B). The percentage of cell death with caspase inhibitor did not significantly increase above the background level.

Discussion

Previously we reported that the accumulation of UBB^{+1} in tauopathies is a marker for proteasomal dysfunction (Fischer et al., 2003). Here we show that this accumulation is not only indicative of proteasome impairment, but that UBB^{+1} apparently also contributes to the pathogenesis of polyglutamine diseases. The accumulation of UBB^{+1} protein enhances aggregate formation in a cellular model of polyglutamine disease. In addition, the toxicity of UBB^{+1} in combination with expanded polyglutamines is not only additive but clearly works synergistically. It thus suggests that both toxic proteins somehow intervene with the same or analogous pathways to compromise cellular function, and eventually cause cell death.

Molecular misreading of the *UBB* gene is a general process that occurs both in neurological diseases and in control individuals (Fischer et al., 2003). We performed a ligase chain reaction (LCR), as described earlier, to confirm the presence of $UBB^{\Delta GU}$ transcripts in the mRNA from frontal cortex tissue of three different HD patients

(data not shown) (Fischer et al., 2003). The GU deletion was present in all HD patients tested, indicating that at the level of the mRNA there is no difference between HD patients, controls or any of the previously tested neurological diseases (Fischer et al., 2003). Differences in UBB^{+1} staining are probably the result of decreased proteasomal activity due to disease-related proteins or ageing (Carrard et al., 2002; Fischer et al., 2003; Keck et al., 2003; Keller et al., 2000; Zhou et al., 2003). Neurological diseases such as Parkinson disease, in which impairment of the proteasome is not demonstrated, do not lead to UBB^{+1} accumulation (Fischer et al., 2003; Furukawa et al., 2002).

Both HD and SCA3 form NIIs, and we demonstrated here that, independent of their protein context, huntingtin and ataxin-3 respectively, these aggregates contain UBB^{+1} (Figure 1). Moreover, UBB^{+1} not only localizes to the inclusions; the cytoplasmic immunoreactivity for this aberrant protein is increased as well (Figure 1A), indicating high protein levels that potentially impair the proteasome. Accumulation of UBB^{+1} reflects in vivo proteasomal impairment in the polyglutamine disorders, which is in agreement with previous in vitro results (Bence et al., 2001; Chai et al., 1999; Verhoef et al., 2002). What is striking is the accumulation of UBB^{+1} in all tested HD and SCA3 patients, irrespective of polyglutamine length and age, showing that UBB^{+1} can already accumulate at a young age (Figure 1B). The high stability of ubiquitinated UBB^{+1} contributes to the accumulation in disease, and it has been suggested that even low levels of misreading can result in toxic protein levels (Lam et al., 2000). We did not find UBB^{+1} in SCA6, suggesting proper functioning of the proteasome in this channelopathy. Indeed the proteasome has not been implicated in disease progression in SCA6, and the inclusions, which are only present in the cytoplasm, are not ubiquitinated (Ishikawa et al., 1999).

Although the mechanism by which different cellular proteins are recruited into NIIs is unknown, the presence of UBB^{+1} in NIIs in human tissue and in a cellular model implicates a similar mechanism. Accumulation of UBB^{+1} will probably start in the cytoplasm, followed by translocation into the NIIs. Interestingly, we showed that ubiquitination on lysine 29 or 48 is not only required for degradation of UBB^{+1}, in agreement with previous results (Lindsten et al., 2002), but is also needed for its translocation to the inclusions. Polyubiquitin chains of targeted proteins are bound to the S6a regulatory subunit of the 19S cap, and it is likely that both the UBB^{+1} resistance to degradation and its proteasomal impairment resemble binding to this subunit but lack of processing through the 20S core (Lam et al., 2002; Lam et al., 2000). Components of the proteasome, and especially the 19S cap structure, have been shown to be present in NIIs and could thus explain the localization of UBB^{+1} into inclusions (Chai et al., 1999; Schmidt et al., 2002).

In addition to human brain material, we also found accumulation and co-aggregation of the proteasome substrate UBB^{+1} with expanded polyglutamines in a cellular model (Figure 2). Moreover, the proteasome inhibitor UBB^{+1} causes an increase in aggregate formation of expanded polyglutamines (Figure 3 and 4). We suggest that inhibition of

the degradation machinery is central to development of polyglutamine diseases and causes accumulation of different aberrant proteins.

We demonstrated that, UBB^{+1} not only increases aggregate formation, but also has a synergistic effect on apoptotic cell-death by expanded polyglutamines (Figure 5). Ubiquitinated UBB^{+1} and expanded polyglutamine proteins have been shown to inhibit the proteasome in vitro (Bence et al., 2001; Lindsten et al., 2002; Verhoef et al., 2002), and we demonstrated here that both factors enhance the effect of the other aberrant protein. The mutual decrease of proteasome activity probably leads to further accumulation of aberrant proteins, and eventually cell death. The fact that ubiquitination of UBB^{+1} is required for its proteasomal inhibition (Lindsten et al., 2002), its toxicity (De Vrij et al., 2001) and its contribution to aggregate formation and polyglutamine-induced cell death suggests that polyubiquitination is a prerequisite for both aggregate formation and cell-death.

Although the genetic basis of polyglutamine diseases is known, a number of questions remain unanswered as to the precise cause of toxicity and whether aggregates are either beneficial or toxic (Michalik and Van Broeckhoven, 2003). Even between individuals with similar repeat length, there are differences as to the age at onset, the progression, and the extent of atrophy (Halliday et al., 1998; McNeil et al., 1997; Rubinsztein et al., 1996; Zoghbi and Orr, 2000). Recent reports indicate that genetic and familial factors account for a great deal of variation of the age at onset in HD (Li et al., 2003; Wexler et al., 2004). Possible candidates include proteasome subunits and cellular chaperones (Chan et al., 2002; Willingham et al., 2003) and, as described in the present paper, also factors such as UBB^{+1} can act as modifiers of both the onset and severity of the disease.

From the present study it is evident that UBB^{+1} not only accumulates in polyglutamine diseases, indicating impairment of the proteasome, but probably plays a role in neurodegeneration itself. In addition to co-aggregation with polyglutamine proteins, ubiquitinated UBB^{+1} synergistically aggravates polyglutamine-induced aggregate formation, and especially cell-death. Targeting of UBB^{+1} to the proteasome by ubiquitination is thereby required for degradation, toxicity and colocalization with the NIIs. Consequently, the accumulation of UBB^{+1} in HD and SCA3 post-mortem tissue, in both the inclusions and the cytoplasm of neurons, implicate UBB^{+1} as an aggravating factor in polyglutamine-induced neurodegeneration, and clearly identifies an important role for the ubiquitin-proteasome system in polyglutamine diseases. These results indicate that proteasomal activation or removal of its blockades is a promising avenue for the treatment of polyglutamine diseases.

Table 1. Clinicopathological information of Huntington, SCA 3 and SCA 6 patients.

Patient number	Age	Sex	Postmortem delay (h)	Brain weight (g)	Fixation duration (days)	CAG repeat expansion	Vonsattel grade	Areas investigated
HD*								
1	11	f	21	1240	21	22 - 84	3	frontal cortex
2	20	f	36	860	21	17 - 86	4	frontal cortex
3	33	f	144	1220	122	20 - 52	3	frontal cortex
4	39	m	10	na	363	15 - 59	4	frontal cortex, striatum
5	40	m	16	1400	124	10 - 41	3	frontal cortex
6	41	f	24	1150	42	16 - 46	3	frontal cortex, striatum
7	49	m	120	1120	186	21 - 51	4	frontal cortex
8	49	f	na	na	186	17 - 47	3	frontal cortex
9	50	m	4	1230	31	20 - 47	3	frontal cortex
10	51	m	4	1260	2	27 - 45	3	frontal cortex
11	51	f	5	900	14	19 - 46	4	frontal cortex
12	51	f	74	1100	na	15 - 43	3	frontal cortex
13	52	f	20	840	31	16 - 53	4	frontal cortex
14	55	m	24	1250	124	22 - 47	2-3	frontal cortex
15	56	m	32	1250	2	18 - 49	3	frontal cortex
16	57	f	72	1200	162	16 - 43	3	frontal cortex
17	58	f	34	1170	7	24 - 43	3	frontal cortex
18	60	m	12	1200	248	20 - 43	3	frontal cortex
19	60	m	na	na	35	26 - 47	4	frontal cortex
20	61	f	17	1050	na	17 - 45	3	frontal cortex
21	61	f	24	840	21	21 - 47	4	frontal cortex
22	62	m	12	1200	62	20 - 43	3	frontal cortex
23	63	f	10	1100	58	17 - 45	3	frontal cortex, striatum
24	64	f	60	1080	7	19 - 42	3	frontal cortex
25	66	m	28	1125	78	21 - 40	3-4	frontal cortex
26	66	f	na	950	107	20 - 44	3-4	frontal cortex, striatum
27	68	f	12	1130	62	20 - 42	3	frontal cortex
28	69	f	24	na	155	21 - 40	3	frontal cortex
29	69	m	48	1200	62	16 - 42	3	frontal cortex
30	76	m	14	970	81	15 - 46	3	frontal cortex
SCA3*								
31	34	m	na	1598	28	20 - 75		medulla oblongata
32	51	m	24	1244	21	27 - 72		hippocampus, mesencephalon
33	59	f	12	1290	21	20 - 72		hippocampus, pons
34	62	m	21	1236	21	23 - 70		hippocampus, pons
35 ***	65	m	<12	1258	28	27 - 72		pons
36 ***	66	m	14	1220	21	24 - 70		pons
SCA6*								
37	69	m	46	1395	28	**		hippocampus, cerebellum
38	76	m	24	1418	120	22		cerebellum

* all cases positive with htt-07, ataxin-3 and A6RPT-C respectively
** not available, but most probably 21 or 22
*** brothers
na - not available

Materials and Methods

Patients

Autopsy material was obtained from the Huntington bank (Leiden University Medical Center, The Netherlands) for HD cases, and the Laboratory of Pathology East Netherlands (Enschede, The Netherlands) and University Hospital Groningen (Groningen, The Netherlands) for SCA cases (for details see Table 1). We analyzed striatal tissue from 4 different HD patients and frontal cortex tissue of in total 30 HD patients with polyglutamine expansion of various lengths. Furthermore, we examined the hippocampus, pons, mesencephalon and medulla oblongata of 6 different SCA3 patients and the hippocampus and cerebellum of two SCA6 patients. All brain areas were fixed in formaldehyde and subsequently embedded in paraffin.

Immunohistochemistry

Paraffin sections (6 μm) were immunohistochemically labeled with different antibodies using the peroxidase-anti-peroxidase method with nickel intensification as described previously (van Leeuwen et al., 1998). The 1C2 monoclonal antibody against expanded polyglutamine repeats (Trottier et al., 1995) (1:10,000; Chemicon, Temecula, USA), polyclonal Htt-07 antiserum against the N-terminus of huntingtin (1:100)(Maat-Schieman et al., 1999), polyclonal anti-ataxin-3 antibody (1:2000) (Paulson et al., 1997) and polyclonal A6RPT-C against the C-terminus of the α1A-subunit of the voltage dependent Ca channel (1:100) (Ishikawa et al., 1999) were used for the detection of the respective disease proteins. UBB^{+1} was detected using antisera against the C-terminal part of the protein (Ubi2A, 1:1000, bleeding 020698 and Ubi2+1, 1:1000, bleeding 010994; see references for details on epitope and specificity (Fischer et al., 2003; van Leeuwen et al., 1998)).

For double stainings, we subjected paraffin sections to irradiation with a broad spectrum lamp for at least 24 hours to reduce autofluorescence (Hol et al., 2003; Neumann and Gabel, 2002). We then deparaffinized sections and incubated them with the first antibodies overnight in a humid chamber at 4°C. We enhanced the UBB^{+1} signal using the avidin-biotin-complex and tyramine procedure (Kerstens et al., 1995) (Sigma-Aldrich, St.Louis, USA) and stained with streptavidin-conjugated-Cy5 (Jackson Laboratories, West Grove, USA). 1C2 was directly visualized using donkey-anti-mouse-Cy2. Signal specificity was demonstrated by swapping fluorescent dyes and omission of primary antibodies to exclude aspecific staining or signal enhancement. Images were obtained using a confocal laser scanning microscope (Zeiss 510).

Cell lines:

HeLa cells were cultured in low-glucose Dulbecco's modified Eagle medium containing 10% fetal calf serum, supplemented with 10000 IU/ml penicillin and 10

mg/ml streptomycin (all Invitrogen, Grand Island, NY). Cells were cultured on 0.2% gelatin-coated glass coverslips in 24-well plates (Nunc, Roskilde, Denmark) 1 day before transfection.

SH-SY5Y neuroblastoma cells (ATCC:CRL-2260) were cultured in high-glucose Dulbecco's modified Eagle medium containing 15% fetal calf serum, supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (all Invitrogen). Cells were cultured in 6-well plates (Nunc) 1 day before infection. Caspase inhibition was performed by addition of 100 µM Ac-DEVD-CHO (Sigma) to the culture medium every two days.

Transfections

We performed transfections with the calcium-chloride method, using 0.5µg plasmid DNA of each of the respective plasmids for 24 well plates (Naldini et al., 1996). Polyglutamine plasmids containing a truncated *huntingtin* fragment with different polyglutamine repeats (19, 38 and 43) flanked by a HA tag and green fluorescent protein (GFP) reporter sequence were provided by Dr. de Christofaro (de Cristofaro et al., 1999). Vectors for ubiquitin, UBB^{+1} and a lysine mutant of UBB^{+1} in which both lysine 29 and 48 are mutated into an arginine residue ($UBB^{+1;K29,48R}$) were described earlier (Lindsten et al., 2002).

Cells were fixed 4 days after transfection and stained for UBB^{+1} (Ubi3, 1:500, bleeding 050897 (De Vrij et al., 2001)). Subsequently cells were incubated with donkey-anti-rabbit-Cy3 (1:200; Jackson laboratories) and ToPro-3 nuclear staining (1:200, Molecular Probes, Leiden, The Netherlands). After staining for UBB^{+1}, pictures were obtained using a confocal laser scanning microscope (Zeiss 510).

Infections

cDNAs for UBB^{+1}, $UBB^{+1;K29,48R}$, HA-Q19-GFP, HA-Q38-GFP and HA-Q43-GFP were cloned in the lentiviral transfer plasmid pRRLsin-PPThCMV-GFP-pre (Naldini et al., 1996). VSV-G pseudotyped lentivirus was produced by cotransfection of the transfer plasmid and helper plasmids (pCMVdeltaR8.74 and pMD.G.2) in 293T cells. Medium was harvested 24 and 48 h after transfection and concentrated by ultracentrifugation. Virus pellets were resuspended in PBS containing 0.5% bovine serum albumin (Sigma). Stocks were titered with a HIV-1 p24 coat protein ELISA (NEN Research, Boston, USA). Lentiviral vectors were used to infect SH-SY5Y neuroblastoma cells at a multiplicity of infection of 50.

Quantification of cells

For aggregate formation, cells were counted manually at different time intervals after infection. The number of aggregate forming cells was quantified in three randomly

selected fields per experiment. Experiments were performed in triplicate and statistics was performed using three-way ANOVA, testing the interaction between repeat expansion, UBB^{+1} protein expression and time course. UBB^{+1} immunopositive cells, were counted after fixation and immunofluorescent staining for UBB^{+1}. The number of UBB^{+1} immunopositive cells were quantified in three randomly selected fields per experiment. Experiments were performed in triplicate and statistics was done using one-way ANOVA, testing the significance of repeat expansion on UBB^{+1} accumulation.

Western blots

We harvested neuroblastoma cells (SH-SY5Y) 6 days after infection in cold PBS. Cell-pellets were resuspended in lysis-buffer (1% NP40, 0.1 M NaCl, 0.01 M Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) containing protease inhibitors; 100 mM PMSF and 10 mg/ml leupeptin. We performed cell-lysis at 4°C for 30 minutes and passed samples through a 25G needle to ensure complete lysis. All samples were loaded onto SDS-PAGE gels and transferred semi-dry onto nitrocellulose (Schleicher & Schuell, Dassel, Germany). Polyglutamine proteins were detected using a monoclonal antibody directed against the HA tag (12CA5, 1:100, culture supernatant) (Field et al., 1988). Blots were additionally probed with a monoclonal β-actin antibody (JLA2.0, 1:500; Developmental Studies Hybridoma Bank, Iowa, USA) as a control for the amount of cell-lysate loaded. Subsequently, blots were incubated with anti-mouse HRP (1:1000; DAKO, Glostrup, Denmark) followed by Lumilight ECL (Perkin Elmer, Norwalk, USA) chemiluminescence. The integrated optical density of the bands was determined by image analysis with Image Pro Plus.

Cell survival assay

We assessed SH-SY5Y cell-survival using a Life-Dead® kit (red fluorescence; Molecular Probes, Leiden, The Netherlands) and analyzed on a flow cytometer (Becton Dickinson, Palo Alto, USA) according to the manufacturers protocol. In short, we harvested lentivirally infected cells (see before) 6 days after infection, washed in PBS and incubated them for 30 minutes with the fluorescent reactive dye. Subsequently, we fixed cells with 4% formaldehyde for 15 minutes and resuspended in PBS-BSA (1x PBS pH-7.6, 0.5% BSA, 0.1% NaN_3). We analyzed polyglutamine-GFP expressing cells by flow cytometry for conversion of the Life-Dead® kit reactive dye. At least 10^4 polyglutamine-GFP positive cells were examined per sample. Dead cells showed a marked increase of red-fluorescence over living cells due to conversion of the dye (Figure 4A). Experiments were performed in triplicate and statistics were done using two-way ANOVA, testing the interaction between UBB^{+1} protein expression and repeat expansion.

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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

Mol Cell Neurosci **43:** 281-286

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

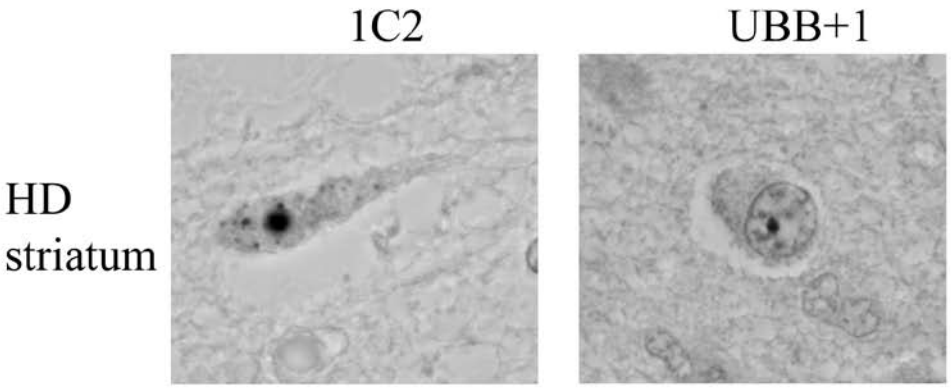
Huntington disease (HD) is an autosomal dominant neurodegenerative disorder that is caused by the expansion of a CAG trinucleotide (polyglutamine) repeat in the huntingtin gene. All polyglutamine expansion disorders are characterized by progressive neuronal dysfunction starting around mid-life and show 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).

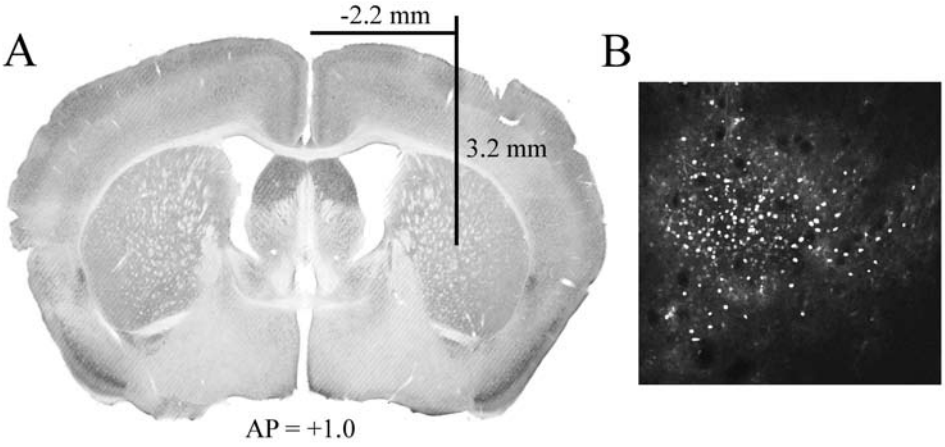
An aberrant form of ubiquitin (UBB^{+1}) was found to accumulate in the hallmarks of HD and spinocerebellar ataxia 3 (de Pril et al., 2004; Fischer et al., 2003). UBB^{+1} can no longer ubiquitinate substrate proteins, and was shown to be a reporter for proteasomal dysfunction (Fischer et al., 2003). 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.

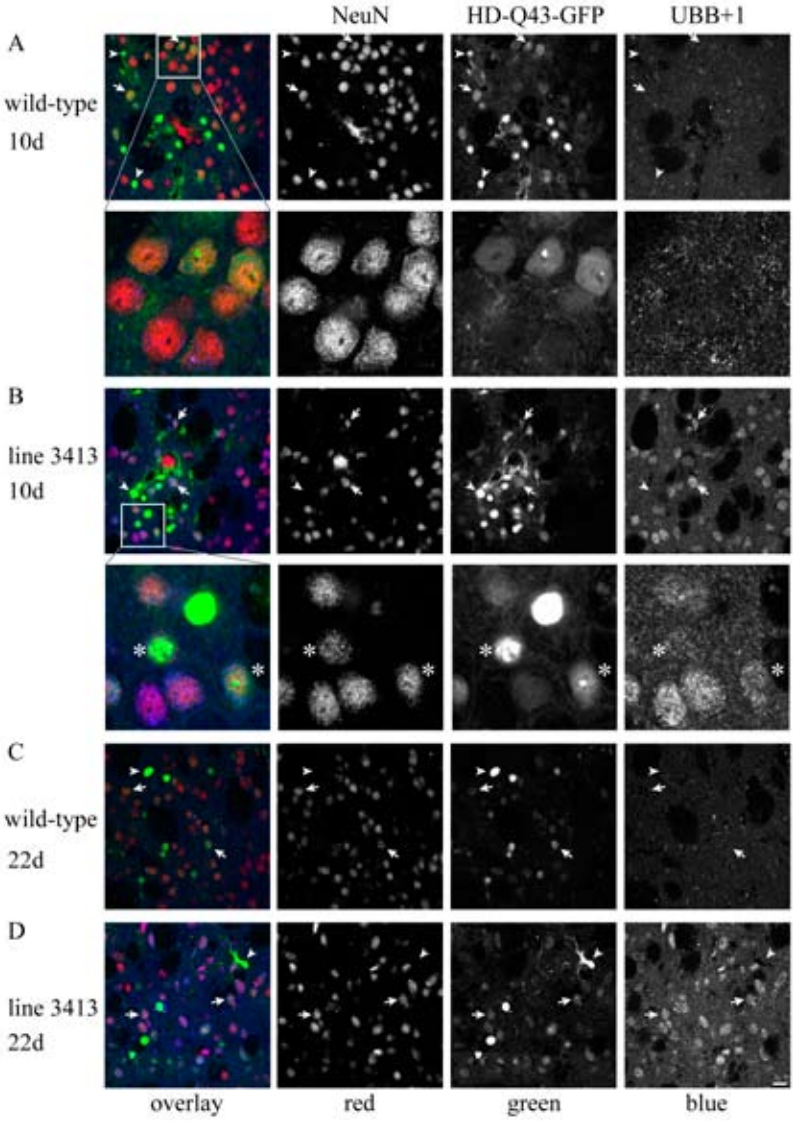
Results

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.

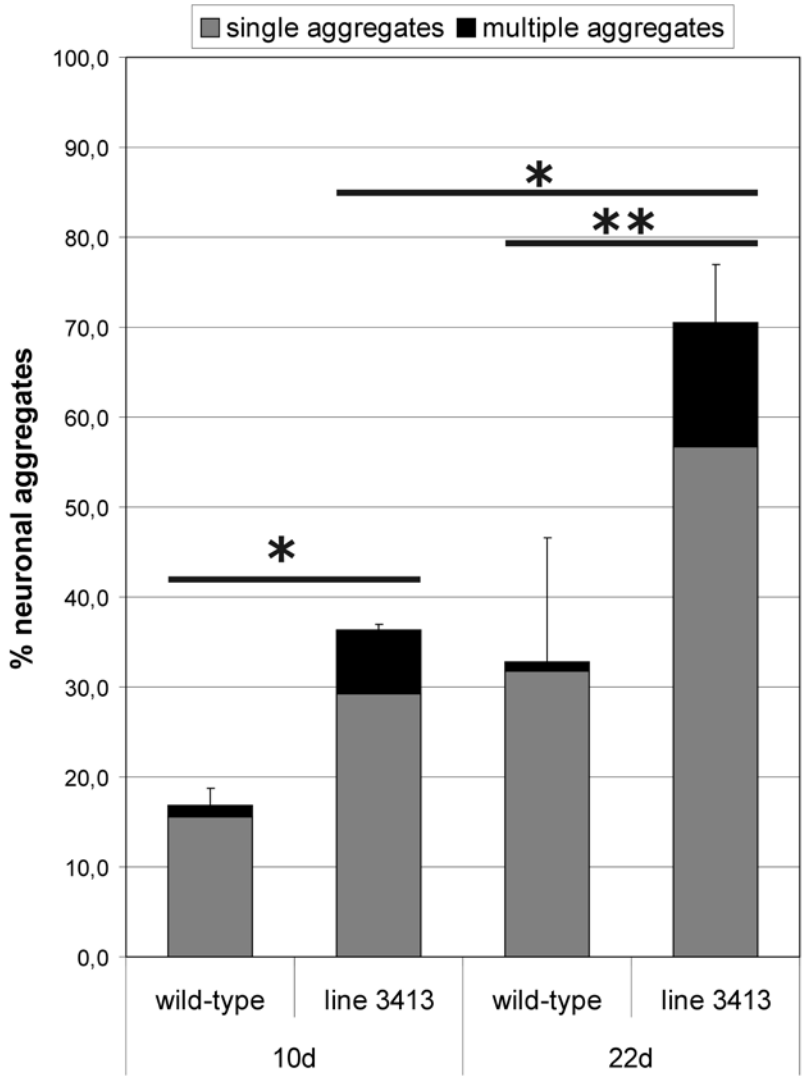
induce neuropathology by itself. We clearly show here that in the presence of a second insult such as polyglutamine expression, UBB^{+1}-mediated proteasome inhibition leads to an exacerbated neuropathology.

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.

Acknowledgements

We thank Drs. M.L. Maat-Schieman (Department of Neurology, Leiden University Medical Center, Leiden, The Netherlands) for tissue sections and G. de Fluiter, M. Cozijnsen, G. Adema, L. Vis and C. Levelt (Netherlands Institute for Neuroscience) for animal care, T. de Christofaro (Universita di Napoli, Italy) for sending of polyglutamine plasmids and L. Naldini (Institute for Cancer Research, Italy) for lentiviral plasmids. Financial support was given by the Prinses Beatrix Foundation (MAR 99-0113), the ISAO/IARF grants 01504, Matty Brand Stichting and Hersenstichting Nederland (13F05.11 15F07(2).48 and 2008(1).17).

Chapter 5

Ubiquitin-conjugating enzyme E2-25K / Hip-2 increases aggregate formation and cell death in polyglutamine diseases

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

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Abstract

Polyglutamine diseases are characterized by neuronal intranuclear inclusions of expanded polyglutamine proteins, which are also ubiquitinated, indicating impairment of the ubiquitin proteasome system. E2-25K (Hip2), an ubiquitin-conjugating enzyme, interacts directly with huntingtin and may mediate ubiquitination of the neuronal intranuclear inclusions in Huntington Disease. E2-25K could thus modulate aggregation and toxicity of expanded huntingtin. Here we show that E2-25K is involved in aggregate formation of expanded polyglutamine proteins and polyglutamine-induced cell death. Both a truncated mutant, lacking the catalytic tail domain, as well as a full antisense sequence, reduce aggregate formation. Strikingly, both E2-25K mutants also reduced polyglutamine-induced cell death. In post-mortem brain material of both Huntington Disease and SCA3, E2-25K staining of polyglutamine aggregates was observed in a sub-set of neurons bearing intranuclear neuronal inclusions. These results demonstrate that targeting by ubiquitination plays an important role in the pathology of polyglutamine diseases.

Introduction

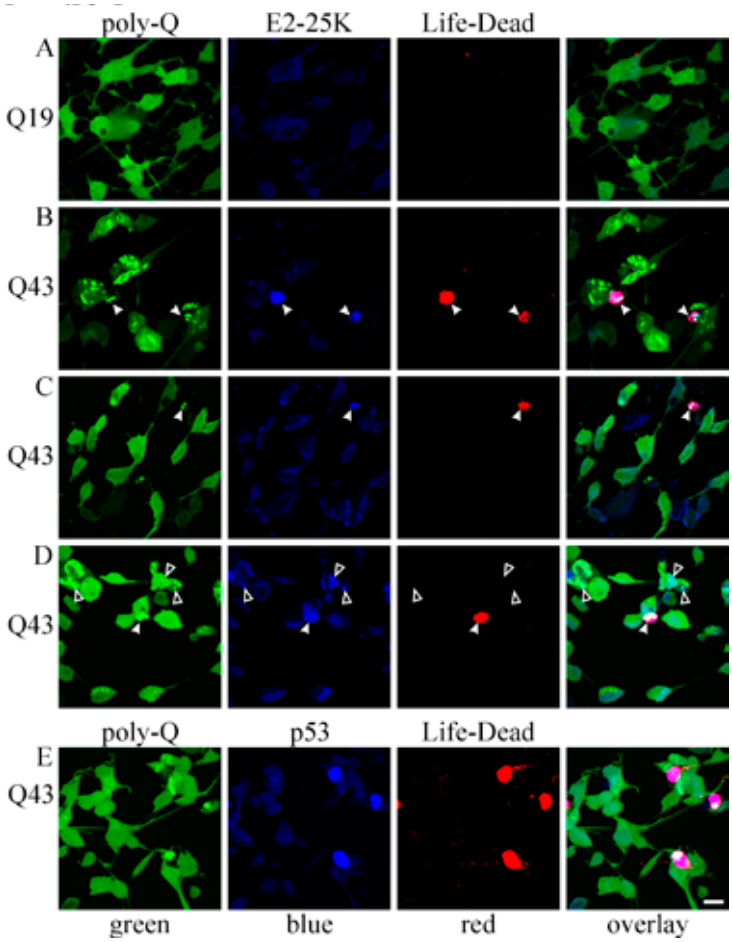
Several neurodegenerative diseases have been shown to be caused by the pathogenic expansion of a polyglutamine repeat, including the Spinocerebellar ataxias (SCAs) and Huntington disease (HD) (Nakamura et al., 2001; Zoghbi and Orr, 2000). Expansion of the repeat above a critical length results in severe neurodegeneration accompanied by the pathological formation of neuronal intranuclear inclusions (NIIs) in the affected areas of the brain (DiFiglia et al., 1997). Although there is still an ongoing debate as to whether these inclusions are detrimental, accumulating evidence points to the favourable formation of large aggregates as a means of sequestering these aberrant proteins (Arrasate et al., 2004; Saudou et al., 1998). However, since the aggregation prone properties of these proteins are the underlying cause of the disease (Perutz et al., 1994; Scherzinger et al., 1997), presumably an early stage of aggregate formation initiates toxicity.

Several studies have implicated the ubiquitin-proteasome system (UPS) in the pathogenesis of polyglutamine diseases and demonstrate an enhancement of neurodegeneration by further impairment of the UPS (reviewed by (Ciechanover and Brundin, 2003)). The fact that NIIs incorporate ubiquitin or ubiquitinated proteins indicates that the aggregating proteins are targeted to, but not efficiently degraded by, the proteasome (DiFiglia et al., 1997; Paulson et al., 1997). *In vitro* studies have furthermore shown that expanded polyglutamines can directly inhibit the proteasome (Bence et al., 2001; Verhoef et al., 2002), resulting in apoptotic cell death (de Pril et al., 2004; Li et al., 2000). In addition, in SCA3 patients, subunits of the 26S proteasome have been shown to be recruited to NIIs (Chai et al., 1999; Schmidt et al., 2002). Finally, in SCA1 transgenic mice, the Purkinje cell pathology was aggravated by mutation of the E6-AP ubiquitin ligase although the number of NIIs was reduced (Cummings et al., 1999).

Recently, we reported that an aberrant form of ubiquitin (UBB^{+1}) accumulates in the NIIs and the cytoplasm of neurons within the affected areas of HD and SCA3 (de Pril et al., 2004). The accumulation of UBB^{+1} in post-mortem brain material of different neuropathological disorders acts as a marker for proteasomal impairment (de Pril et al., 2004; Fischer et al., 2003). Moreover, we demonstrated that UBB^{+1}, being a substrate and an inhibitor of the proteasome, enhances aggregation of expanded polyglutamine proteins and synergistically aggravates polyglutamine-induced cell death. These findings demonstrate the importance of an efficient UPS in neurodegenerative diseases such as the polyglutamine disorders.

Ubiquitin-conjugating enzyme E2-25K (or Hip2; Huntingtin interacting protein 2) is highly expressed in the brain and was found in a yeast two-hybrid screen to interact with huntingtin in a repeat-independent matter (Kalchman et al., 1996). Recently, E2-25K has been implicated in the mediation of amyloid-β neurotoxicity and proteasome inhibition *in vitro* (Song et al., 2003). Despite the interaction with huntingtin and the

Figure 1: E2-25K colocalizes with polyglutamine aggregates in dead cells.



Differentiated SH-SY5Y neuroblastoma cells were lentivirally transduced with truncated huntingtin constructs containing 19 or 43 glutamine repeats (Qxx-HTT-GFP). A cell death assay was performed 4 days post transduction. Cells were subsequently fixed and stained for endogenous E2-25K. Cells that are transduced with the Q19-HTT-GFP construct show a diffuse cytoplasmic staining for E2-25K (A; blue; panel 2) and cytoplasmic localization of Q19-HTT-GFP (green; left panel). Upon transduction with Q43-HTT-GFP we once more find cytoplasmic staining for E2-25K (B-D; blue panel 2) and the formation of aggregates of expanded polyglutamines (green; left panel). Cells that are subject to expanded polyglutamine induced cell death (red; panel 3; cell death reporter) show upregulation or nuclear translocation of E2-25K and coaggregation of the enzyme with the expanded polyglutamine proteins (closed arrowheads). Normally, even cells with a high aggregate load (C) do not show coaggregation of E2-25K. Occasionally we do find aggregates that are positive for E2-25K and are not stained by the cell death fluorescent reagent (D; open arrowheads) suggesting that upregulation or nuclear translocation and consecutive coaggregation is one of the events preceding apoptosis. Staining for p53 shows diffuse staining in most cells and a clear colocalization with the cell death reporter (E).The right column shows the overlay of E2-25K (or p53), cell death and poly-Q. Magnification bar is 50 µm.

importance of E2-25K as part of the UPS machinery, there have been no reports on the influence of E2-25K on HD or even its sub-cellular localization in this disease.

In the present paper we studied the role of E2-25K in aggregate formation and cell death in polyglutamine disease.

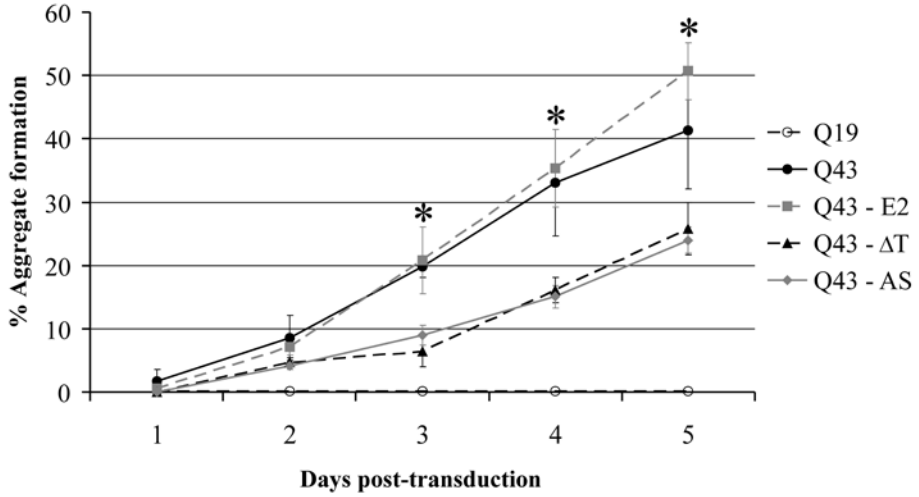
Results

We used neuronally differentiated SH-SY5Y cell lines, which were transduced with a lentiviral vector expressing a truncated huntingtin fragment containing 19 or 43 glutamine repeats. Staining of these cells for endogenous E2-25K showed a diffuse cytoplasmic staining in all cells (Figure 1A-D; panel 2). However, we noticed that, although E2-25K did colocalize in some of the aggregates, this was not the case for all aggregate-forming cells. The morphology of these cells suggested that this might reflect the viability of the cells and lead us to perform a cell death assay. Surprisingly, it appeared that the ubiquitin-conjugating enzyme E2-25K was found in the aggregates primarily in the cells that were in the process of dying (Figure 1B-D; closed arrowheads). Occasional colocalization of E2-25K with the polyglutamine aggregates in living cells, suggests that the upregulation or nuclear translocation of E2-25K and its subsequent colocalization with polyglutamine aggregates, precedes cell death (Figure 1D; open arrowheads). However, using Western blot analysis we were unable to demonstrate an obvious upregulation of the enzyme over the entire population of cells (data not shown). Staining for the tumour suppressor protein p53 demonstrates a clear upregulation and colocalization with the cell death reporter (Figure 1E). Indeed, p53 has been shown to play an important role in determining whether a cell will go into apoptosis and has been shown to coaggregate with polyglutamine inclusions (Levine, 1997; Steffan et al., 2000; Suhr et al., 2001).

To test the influence of E2-25K on aggregate formation and cell-viability we constructed lentiviral vectors containing either the full length E2-25K enzyme, the enzyme lacking its C-terminal catalytic domain or the complete anti-sense sequence. These constructs were cotransduced with the truncated huntingtin fragments. The E2-25K protein contains a tail region that is necessary for the generation of K48 linked polyubiquitin chains (Haldeman et al., 1997). The E2-25K protein lacking its tail region still interacts with ubiquitin activator E1. Expression of the truncated enzyme will thus compete for activated ubiquitin without tagging target proteins for degradation. Furthermore, the full anti-sense sequence of the E2-25K mRNA has been shown to be sufficient to knock down the expression of E2-25K in cell lines (Song et al., 2003).

We quantified the aggregate formation after lentiviral transduction of SH-SY5Y neuroblastoma cells with polyglutamine vectors in combination with one of the E2-25K vectors. The truncated huntingtin fragment with 43 glutamines fused to green fluorescent protein (Q43-HTT-GFP) shows a marked time-dependent increase

Figure 2: E2-25K mediates aggregate formation of expanded polyglutamines.



*Differentiated SH-SY5Y neuroblastoma cells were lentivirally transduced with constructs with truncated huntingtin fragments containing 19 or 43 glutamine repeats (Qxx-HTT-GFP) in combination with either E2-25K, a truncated Δtail mutant, or the full antisense sequence. Cells were quantified for aggregate formation every 24 hours after transduction. Upon addition of E2-25K there is no significant increase in aggregate formation of Q43-HTT-GFP, probably due to sufficient levels of endogenous E2-25K in the cells. Addition of either the Δtail mutant or the antisense sequence of E2-25K results in a highly significant decrease in aggregate formation. E2-25K clearly mediates the ubiquitination and subsequent aggregate formation of the expanded polyglutamines. Statistical analysis by three-way ANOVA confirmed that the combination of E2-25K with constructs of 43 glutamines has a pronounced, highly significant effect on the aggregate formation in time *P<0.001.*

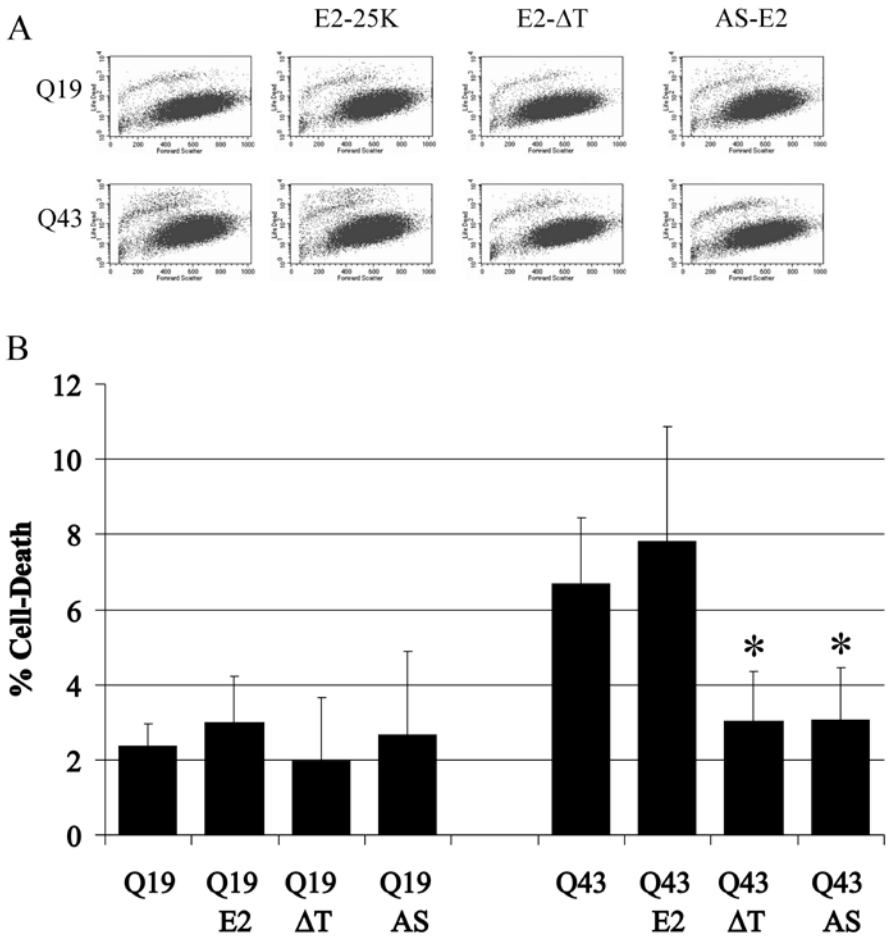
in aggregate formation compared to the Q19-HTT-GFP construct (Figure 2). Co-transduction with the E2-25KΔtail mutant or with the antisense sequence reduces the aggregate formation of Q43-HTT-GFP, compared to a mock-treated condition or with addition of E2-25K ($P<0.001$). Co-transduction of Q43-HTT-GFP with E2-25K did not enhance aggregate formation; apparently the levels of endogenous E2-25K are not rate-limiting for aggregate formation.

Since it is still under debate whether aggregate formation is either beneficial or detrimental we measured the viability of the cells. As we showed previously there is a low level of cell death due to viral transduction and culturing that is represented by the levels of Q19-HTT-GFP (de Pril et al., 2004). Here we found that co-expression of E2-25K and Q19-HTT-GFP does not result in an effect on cell death (Figure 3B). Co-expression of either the Δtail mutant or the antisense sequence with Q19-HTT-GFP neither had an effect on these levels.

As we have shown previously, expansion of the polyglutamine repeat results in a marked increase in cell death (de Pril et al., 2004). Overexpression of E2-25K in combination with Q43-HTT-GFP does not increase cell death, as was also seen for aggregate formation. Co-expression of either the Δtail mutant or the antisense sequence with Q43-HTT-GFP however resulted in a significant decrease in the levels of cell death (Figure 3B; $P<0.05$). Inhibition of E2-25K thus results in a reduction of expanded polyglutamine toxicity to background levels.

We found that E2-25K has a role in both aggregate formation and cell death by expanded polyglutamine proteins and colocalizes in aggregates in a cell-culture model. To reveal whether these findings are relevant for Huntington Disease, and for other polyglutamine diseases, we stained post mortem brain material of both HD and Spinocerebellar Ataxia type 3 (SCA3) for E2-25K reactivity. All patient material was characterized previously and found to be immunopositive for ubiquitin, the UBB^{+1} protein, and for the respective expanded polyglutamine proteins (see Table 1 of (de Pril et al., 2004)). We found that a fraction of NIIs in all cases of both HD (Figure 4A-C; closed arrowheads) and SCA3 (Figure 4D) were immunopositive for E2-25K. To our knowledge this is the first evidence that E2-25K is indeed localized in the inclusions in polyglutamine diseases. Double staining of the polyglutamine antibody 1C2 with E2-25K shows that, in contrast to many cellular proteins that reportedly coaggregate in all NIIs (Mitsui et al., 2002), E2-25K is not found in all the inclusions (Figure 4C; open arrowhead). The presence of E2-25K in part of the NIIs indicates a differential mechanism that might reflect the disease state of the respective aggregate bearing neurons. This finding corresponds to our *in vitro* observations, that co-aggregation of E2-25K with polyglutamine aggregates marks cell death and coincides with p53 upregulation. Indeed, staining for the tumour suppressor protein p53 showed an upregulation and coaggregation in the E2-25K-positive neurons suggesting that these neurons are likely to proceed into apoptosis (Levine, 1997). The finding of E2-25K localization to polyglutamine aggregates in disease, underlines the importance of E2-

Figure 3: E2-25K mediates polyglutamine-induced cell death.



*Cell death of differentiated SH-SY5Y neuroblastoma cells, as assessed by a cell survival assay 6 days after transduction. Constructs with 19 or 43 glutamine repeats (Qxx-HTT-GFP) were cotransduced with either E2-25K, a truncated Δtail mutant or the full antisense sequence of E2-25K. Polyglutamine-GFP positive cells were analyzed by flow cytometry for conversion of red-fluorescent cell death reporter (A; representative FACS analysis). Scatter plots clearly show the differential distribution of living cells (lower population) and dead cells (higher population; bright red-fluorescent). The bars (B) show the percentage of cell death that was observed under different conditions. Q19-HTT-GFP shows the background percentage of cell death due to transduction or culturing. Expansion of the polyglutamine repeat to Q43-HTT-GFP gives a marked increase in cell death that is not changed by addition of E2-25K. Both Δtail as well as the antisense sequence give a significant reduction in the levels of cell death. Statistical analysis by two-way ANOVA confirmed that cotransduction of both the Δtail and the antisense E2-25K constructs with Q43-HTT-GFP caused a pronounced, significant decrease of the cell death compared with Q43-HTT-GFP with wt E2-25K or mock treated *P<0.05.*

25K for HD and also SCA3 and demonstrates that it is not restricted to our *in vitro* model.

Discussion

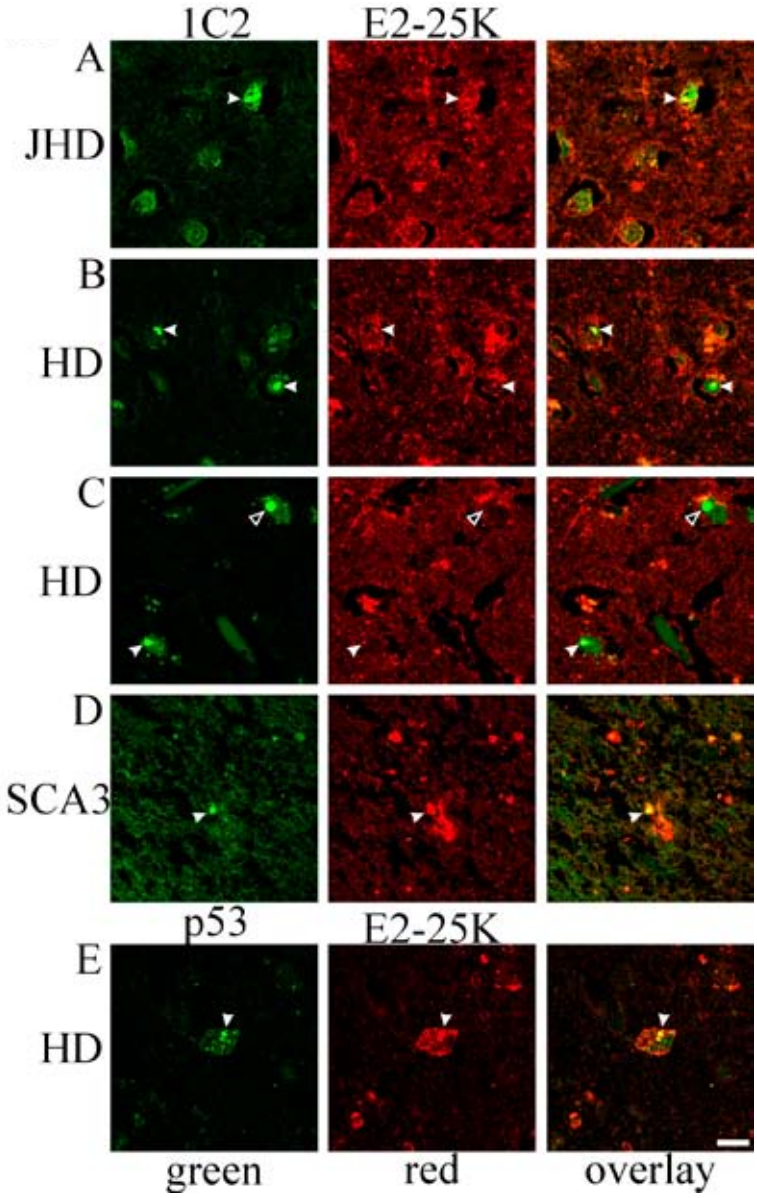
We previously showed that NIIs in HD and SCA3 were immunopositive for the respective expanded polyglutamine proteins (huntingtin and ataxin 3) as well as for ubiquitin and UBB^{+1} (de Pril et al., 2004). The aberrant UBB^{+1} protein appeared to be present in all inclusions and in addition showed a diffuse cytoplasmic staining indicating impaired proteasomal function in the affected neurons (Fischer et al., 2003). The ubiquitination machinery is an obvious candidate to promote the degradation of aberrant proteins such as expanded polyglutamines or UBB^{+1}. Ubiquitination is performed by a cascade of enzymes that activate (E1), conjugate (E2) and ligate (E3) ubiquitin to target proteins (Glickman and Ciechanover, 2002; Pickart, 2001). E2 proteins play an indispensable role in the ubiquitination of proteins that are to be degraded and interact specifically with a subset of E3 and target proteins.

The ubiquitin-conjugating enzyme E2-25K was reported to be expressed in all areas of the brain with higher levels in the areas that are affected in HD, i.e. the striatum and frontal cortex (Kalchman et al., 1996). Interaction of E2-25K with huntingtin, as found in a yeast two hybrid screen, was however not affected by repeat length. So far there have not been any reports on the influence of this interaction on disease and the localization in HD. Furthermore, E2-25K was shown to be involved in Aβ-mediated neurodegeneration and could play a role in Alzheimer pathogenesis (Song et al., 2003).

Staining of neuronally differentiated cell-lines for E2-25K shows a diffuse cytoplasmic localization in all cells. Interestingly, the E2-25K protein coaggregates only in part of the polyglutamine inclusions and this appeared to coincide with cell death. Aggregate forming cells that go into apoptosis might upregulate E2-25K in an attempt to rid the cells of the overload of aberrant proteins resulting in coaggregation in these cells. This indicates that E2-25K is an important factor in polyglutamine-induced neurodegeneration. It is remarkable that Kalchman et al found no obvious upregulation of E2-25K on Western blot of the frontal cortex of HD patients compared to controls, despite the relatively high expression in the frontal cortex and striatum of controls (Kalchman et al., 1996). However, an upregulation in the remaining neurons might be masked by neuronal loss in these areas.

Here we demonstrate that ubiquitination by E2-25K enhances the aggregate formation of expanded polyglutamine proteins. Although we do not find an increase in aggregate formation upon addition of E2-25K, both the Δtail mutant as well as antisense E2-25K give a large decrease in the aggregate load. The lack of increase upon addition of E2-25K can be explained by sufficient endogenous protein that is present in these

Figure 4: E2-25K colocalizes with the NIIs in HD and SCA3.



Double stainings were performed on paraffin sections of HD patients and SCA3 patients for E2-25K (red; middle panel) and 1C2 (green; left panel). Besides cytoplasmic staining, E2-25K staining was found in part of the inclusions positive for 1C2 in frontal cortex tissue of juvenile HD patients (JHD; A), and HD (B-C; closed arrowheads) as well as the pons of SCA3 (D). However, E2-25K does not localize to all the NIIs (C; open arrowhead; HD) and appears to be more prone to colocalize with the cytoplasmic aggregates that are found (A-D). p53 staining of HD frontal cortex shows colocalization with E2-25K (E) suggesting that there is in fact a link between cell death and E2-25K coaggregation in disease. Magnification bar is 50μm.

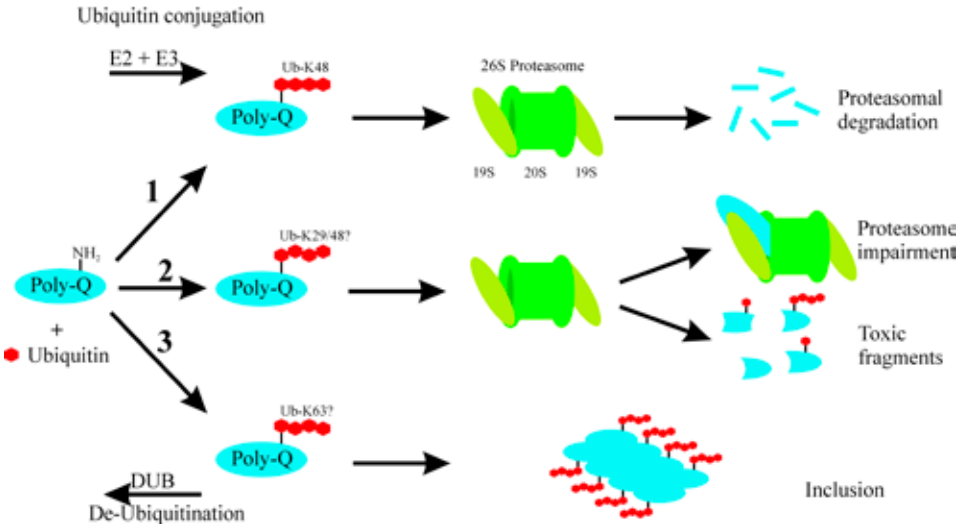
cells, as we did not use a knock-out cell line. It appears that ubiquitination by E2-25K is a stimulatory mechanism for aggregate formation in polyglutamine disorders. This suggests that ubiquitination is either needed for the translocation to the inclusions or indirectly, for the formation of more aggregation prone fragments.

Analysis of cell survival showed that E2-25K mediates polyglutamine-induced cell death. For a normal repeat length (Q19) no change in the levels of cell death was found upon addition of either E2-25K, the Δtail mutant or the antisense sequence. Expansion of the glutamine repeat to Q43 results in an increase in cell death, and addition of E2-25K has no effect, which demonstrates that endogenous E2-25K is not a limiting factor in either the toxicity or the degradation of polyglutamine fragments in the cell. However, addition of either the Δtail mutant or the antisense sequence results in a drop of toxicity to background levels. The Δtail mutant that lacks its functional domain for polyubiquitination will still interact with E1 and accept the activated ubiquitin but will no longer transfer the ubiquitin molecule to target proteins like huntingtin (Haldeman et al., 1997). Apparently this mutant has a similar dominant negative effect on ubiquitination, as does the knock-down of E2-25K by use of the full antisense sequence. These results show that ubiquitination of E2-25K targets does not only influence the aggregate formation of expanded polyglutamine proteins but more importantly triggers polyglutamine-induced cell death. Ubiquitination supposedly results in the targeting of the expanded polyglutamines to the proteasome, resulting in either proteasome impairment or the formation of toxic fragments.

Staining of post mortem brain material of both HD and SCA3 for E2-25K showed that here as well, a differential staining is found between the NIIs. Although we do find staining of aggregates in both disorders and all patients examined, not all aggregates are immunopositive for E2-25K. These results are in agreement with our finding in neuronally differentiated cell lines where E2-25K preferentially coaggregates with expanded polyglutamine proteins in apoptotic cells (Figure 1). E2-25K thus appears to indicate the disease state of the respective neurons. Colocalization of E2-25K seems to coincide with polyglutamine-induced neuronal death, which occurs both in HD as well as in SCA3 (Munoz et al., 2002; Vonsattel et al., 1985).

The relevance of ubiquitination is furthermore demonstrated by the general finding of ubiquitin immunoreactivity in the neuropathological hallmarks of numerous neurodegenerative diseases including HD and SCA3 (DiFiglia et al., 1997; Paulson et al., 1997). In addition, we have shown for aberrant ubiquitin (UBB^{+1}) that it is necessary to ubiquitinate this protein to be translocated to the inclusions, since a mutant that lacks the lysine moieties at positions 29 and 48 of the ubiquitin molecule does not coaggregate and is no longer toxic (de Pril et al., 2004). Mutating the lysine residues at positions 6, 9 and 15 in a truncated fragment of huntingtin similarly reduces neuropathology (Steffan et al., 2004).

Figure 5: Model for targeting of expanded polyglutamines by differential ubiquitination.



Ubiquitination of proteins can play different roles in polyglutamine diseases directly or indirectly affecting polyglutamine toxicity. The ubiquitin conjugation by a certain set of E2 and E3 is proposed to regulate the position of ubiquitin attachment to the target protein and the ubiquitin lysine linkage. Their role in one of these pathways can explain the differences that are found between all ubiquitination proteins that have been described for involvement in polyglutamine pathology (Table 1). Role 1: Ubiquitination results in targeting of the aberrant proteins to the proteasome where they are properly degraded. Role 2: Ubiquitination results in proteasomal targeting but inefficient degradation of aberrant proteins, causing either impairment of the UPS or the formation of more toxic fragments. Role 3: Ubiquitination is followed by transport of the expanded polyglutamine proteins to inclusions as a non-toxic storage for aberrant proteins.

We propose here that ubiquitination of proteins plays different roles in polyglutamine diseases directly or indirectly affecting polyglutamine toxicity (Figure 5). The best known function for ubiquitination is targeting of the aberrant proteins to the proteasome where they are properly degraded. The second possibility is proteasomal targeting but inefficient degradation of aberrant proteins, causing impairment of the UPS or the formation of more toxic fragments. The third option is a role in transporting the expanded polyglutamine proteins to inclusions as a non-toxic storage for aberrant proteins. Indeed, ubiquitination has different effects on polyglutamine-induced pathology and toxicity depending on the ubiquitination enzyme that is targeted (Table 1). For example, E4B, a mammalian chain assembly factor (E3), is required to degrade the expanded form of ataxin 3 and is able to prevent aggregate formation of polyglutamines and even neurodegeneration in a Drosophila model of SCA3 (Matsumoto et al., 2004). In contrast, mutation of E6-AP (Ube3a), an ubiquitin ligase (E3), was shown to aggravate the Purkinje cell pathology in SCA1 transgenic mice but to reduce the number of NIIs (Cummings et al., 1999).

This synopsis recapitulates many different mechanisms that are involved in the cellular digestion of polyglutamine proteins (Table 1). The formation of different ubiquitin trees by linkage to another ubiquitin lysine (K6, 29, 48 or 63) will result in a different cascade, i.e. transport to the proteasome or to inclusions (Pickart and Fushman, 2004). The specificity of ubiquitin binding proteins for a certain ubiquitin chain linkage will eventually define the translocation of the target proteins (Verma et al., 2004). The location of ubiquitin attachment on expanded polyglutamines or other target proteins itself might influence the recognition and binding to the proteasome and thereby the subsequent degradation. The specific transport of aberrant proteins to non-toxic inclusions making use of the cytoskeleton also implies specific targeting to these inclusions and is likely to be regulated by ubiquitination (Taylor et al., 2003). Cells are accordingly protected from higher levels of free polyglutamine proteins that would have a higher probability to interfere with cellular processes eventually resulting in neuronal death (Arrasate et al., 2004). We propose that E2-25K is involved in the second pathway, whereby the unfavourable formation of ubiquitin trees results in an increase in either proteasome impairment or the formation of toxic fragments.

Our results demonstrate that E2-25K is a contributing factor in mediating aggregate formation and cell death. These findings illustrate the importance of ubiquitination for the cellular clearance or storage of toxic proteins that extends beyond the proteasome itself. The reduced toxicity of expanded polyglutamines in the absence of functional E2-25K makes it an attractive target in polyglutamine diseases. A small ubiquitin-like modifier (SUMO; a post-translational modifier) has recently been shown to modify E2-25K (Pichler et al., 2005), impairing ubiquitin chain formation by interfering with E1 interaction, making SUMO an interesting molecule to use for gene therapy. Interference with SUMOylation has already been shown to enhance neurodegeneration in a Drosophila polyglutamine model (Chan et al., 2002), which could be linked to E2-25K. Controversially however, reduction of SUMOylation has

Table 1: Ubiquitination and polyQ toxicity.

Gene	LOF / overexpression	Enzyme	Aggregation	Toxicity	Role	References
UBC	LOF	Ub	+	N.D.	1,2,3	(Nollen et al., 2004)
Uba1	LOF	E1	+	N.D.	1,2,3	(Nollen et al., 2004)
E2-25K	LOF	E2	-	-	2	**this paper**
UbcE2D2	LOF	E2	N.D.	+	1/3	(Fernandez-Funez et al., 2000)
Ubc2EH	LOF	E2	N.D.	+	1/3	(Fernandez-Funez et al., 2000)
Cdc34	LOF	E2	-	+	3	(Saudou et al., 1998)
Ube3a	LOF	E3	-	+	3	(Cummings et al., 1999)
E4B	LOF	E3	+	N.D.	1/2	(Matsumoto et al., 2004)
E4B	overexpression	E3	-	-	1	(Matsumoto et al., 2004)
CHIP	overexpression	E3	-	-	1	(Jana et al., 2005)
Parkin	overexpression	E3	-	-	1	(Tsai et al., 2003)
UCH-L3	LOF	DUB	N.D.	+	2	(Fernandez-Funez et al., 2000)
Ataxin-3	overexpression	DUB	-	-	2	(Warrick et al., 2005)
UCH-L8	LOF	DUB	+	N.D.	3	(Nollen et al., 2004)

Overview of the involvement of ubiquitination enzymes in polyglutamine aggregation and toxicity. Numerous studies have been conducted on the influence of a loss-of-function (LOF) or overexpression of ubiquitination enzymes in polyglutamine models. Indicated is the influence of the respective change in expression on the polyglutamine aggregation and toxicity. Related enzymes appear to result distinctly in an increase (+) or decrease (-) of aggregate formation and toxicity. Their influence on these processes implicates the ubiquitination enzymes in either of three different pathways: (1) Proteasomal targeting followed by proper degradation of polyglutamine fragments. (2) Proteasomal targeting resulting in either UPS impairment or the formation of more toxic fragments. (3) Formation of innocuous inclusions. See also Figure 5 for a schematic representation.

N.D.: not determined
DUB: de-ubiquitination enzyme

been shown to reduce pathology in another Drosophila polyglutamine model, and in cell-lines expression of SUMO accelerates polyglutamine-induced cell death (Steffan et al., 2004; Terashima et al., 2002).

The UPS remains an attractive candidate for therapy in polyglutamine diseases and other neurodegenerative disorders. Shifting the balance from UPS impairment and formation of toxic fragments to harmless inclusions and efficient degradation would be a promising avenue in solving these severe diseases.

Materials and Methods

Transduction

Polyglutamine plasmids containing a truncated *huntingtin* fragment with different polyglutamine repeats (19 and 43) flanked by a HA tag and green fluorescent protein (GFP) reporter sequence were provided by Dr. de Cristofaro (de Cristofaro et al., 1999). E2-25K, E2-25K-Δtail and E2-25K-antisense constructs were identical to those described by Song et al. (Song et al., 2003).

cDNAs for HA-Q19-HTT-GFP, HA-Q43-HTT-GFP, E2-25K, E2-25K-Δtail and E2-25K-antisense, were cloned in the lentiviral transfer plasmid pRRLsin-PPThCMV-GFP-pre (Naldini et al., 1996). VSV-G pseudotyped lentivirus was produced by cotransfection of the transfer plasmid and helper plasmids (pCMVdeltaR8.74 and pMD.G.2) in 293T cells. Medium was harvested 24 h after transfection and concentrated by ultracentrifugation. Virus pellets were resuspended in PBS containing 0.5% bovine serum albumin (Sigma). Stocks were titered with a HIV-1 p24 coat protein ELISA (NEN Research, Boston, USA). Lentiviral vectors were used to infect SH-SY5Y neuroblastoma cells at a multiplicity of infection of 50.

Cell lines

Human SH-SY5Y neuroblastoma cells (ATCC:CRL-2260) were cultured in high-glucose Dulbecco's modified Eagle medium containing 15% fetal calf serum, supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (all Invitrogen). Cells were cultured in 6-well plates (Nunc) 1 day before transduction with addition of 4 μM all-trans retinoic acid (Sigma) for differentiation.

Immunocytochemistry

Cells were cultured on collagen-coated glass coverslips and subjected to a Live-Dead® assay (red fluorescence; Molecular Probes, Leiden, The Netherlands) 4 days after transfection. Cells were subsequently fixed and stained for E2-25K (1:400) and visualized with donkey-anti-rabbit-Cy5 (1:200; Jackson laboratories). After staining

for E2-25K, pictures were obtained using a confocal laser scanning microscope (Zeiss 510).

Quantification of cells

For aggregate formation, cells were counted manually at different time intervals after transduction. The number of aggregate forming cells was quantified in three randomly selected fields per experiment. Experiments were performed in triplicate and statistics was performed using three-way ANOVA, testing the interaction between repeat expansion, E2-25K protein expression and time course.

Cell survival assay

We assessed SH-SY5Y cell death using a Live-Dead® kit (red fluorescence; Molecular Probes, Leiden, The Netherlands) and analyzed on a flow cytometer (Becton Dickinson, Palo Alto, USA) according to the manufacturer's protocol. In short, we harvested lentivirally transduced cells (see before) 6 days after transduction, washed them in PBS and incubated them for 30 minutes with the fluorescent reactive dye. Subsequently, we fixed cells with 4% formaldehyde for 15 minutes and resuspended in PBS-BSA (1x PBS pH-7.6, 0.5% BSA, 0.1% NaN_3). We analyzed polyglutamine-GFP expressing cells by flow cytometry for conversion of the Live-Dead® kit reactive dye. At least 10^4 polyglutamine-GFP positive cells were examined per sample. Dead cells showed a marked increase of red-fluorescence over living cells due to conversion of the dye (Figure 3A). Experiments were performed in triplicate and statistics were done using two-way ANOVA, testing the interaction between E2-25K protein expression and repeat expansion.

Human post-mortem tissue

Autopsy material was obtained from the Huntington bank (Leiden University Medical Center, The Netherlands) for HD cases, and the Laboratory of Pathology East Netherlands (Enschede, The Netherlands) and University Hospital Groningen (Groningen, The Netherlands) for SCA cases (for details see Table 1 of (de Pril et al., 2004)). We analyzed striatal tissue from 4 different HD patients and frontal cortex tissue of in total 30 HD patients with polyglutamine expansion of various lengths. Furthermore, we examined the hippocampus, pons, mesencephalon and medulla oblongata of 7 different SCA3 patients. All brain areas were fixed in formaldehyde and subsequently embedded in paraffin.

Immunohistochemistry

For double stainings, we subjected paraffin sections (6 µm) to irradiation with a broad spectrum lamp for at least 24 hours to reduce autofluorescence (Hol et al., 2003; Neumann and Gabel, 2002). We then deparaffinized sections and incubated them with

the first antibodies overnight in a humid chamber at 4°C. We used 1C2 monoclonal antibody against expanded polyglutamine repeats (Trottier et al., 1995) (1:10,000; Chemicon, Temecula, USA) monoclonal p53 (1:400; Chemicon) and E2-25K (1:400; Affinity) antibody. We enhanced the E2-25K signal using the avidin-biotin-complex and tyramine procedure (Kerstens et al., 1995) (Sigma-Aldrich, St.Louis, USA) and stained with streptavidin-conjugated-Cy5 (Jackson Laboratories, West Grove, USA). 1C2 and p53 were directly visualized using donkey-anti-mouse-Cy2. Signal specificity was demonstrated by swapping fluorescent dyes and omission of primary antibodies to exclude aspecific staining or signal enhancement. Images were obtained using a confocal laser scanning microscope (Zeiss 510).

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Chapter 6

Discussion

UBB^{+1} frameshift mutation

Frameshift mutations in short repetitive motifs such as GAGAG (ΔGU, ΔGA) during mRNA transcription were originally discovered in the rat vasopressin gene (Evans et al., 1994). The consecutive search for potential genes that are affected by this frameshift resulted in the identification of amyloid precursor protein and ubiquitin-B as targets of what was dubbed "molecular misreading" (van den Hurk et al., 2001; van Leeuwen et al., 1998). Not only aberrant transcripts for both genes were detected but also the resulting proteins were detected in the brains of Alzheimer Disease (AD) and Down Syndrome (DS) patients. The localization of these aberrant proteins specifically in the neuritic plaques, neuropil threads and tangles, charisteristic of AD pathology pointed towards an involvement in disease progression. Subsequent studies demonstrated that UBB^{+1} inhibits the proteasome and can potentially worsen disease development (Chapter 3 and (van Tijn et al., 2007)). Although UBB^{+1} can be degraded at low concentrations it was shown to be a marker for proteasomal inhibition most likely following the accumulation of disease-related proteins or ageing (Carrard et al., 2002; Fischer et al., 2003; Keck et al., 2003; Keller et al., 2000; Zhou et al., 2003). In synucleopathies and control subjects, in which impairment of the proteasome has not been consistently demonstrated, the transcript was detected but the aberrant protein was apparently degraded (Fischer et al., 2003; Furukawa et al., 2002). Lentiviral injection of UBB^{+1} constructs in rat brains indeed confirmed that healthy neurons are capable of degrading the protein without obvious detrimental effects (Fischer et al., 2003). Interestingly, UBB^{+1} transgenic mice – with a low, constitutive level of proteasomal inhibition – do not develop an overt neurological phenotype but only show mild defects in contextual memory (Fischer et al., 2009). These findings corroborate that expression of UBB^{+1} alone is not sufficient to cause disease at the low levels of misreading that are detected in humans but merely contributes to development and course thereof after initial onset (Gerez et al., 2005).

In two independent UBB^{+1} transgenic mouse lines several proteins were found to be differentially expressed that are also changed in AD brain or transgenic models (Fischer et al., 2009). These findings support the idea that the proteasome is inhibited in AD, potentially resulting in a dysregulation of energy metabolism (Salehi and Swaab, 1999). Altogether, this underlines the importance of efficient proteasomal degradation for neurodegenerative diseases such as AD for maintaining normal protein levels within the cell. It is conceivable that the accumulation of UBB^{+1} is caused by a failing protein quality control system under neurodegenerative conditions. Although UBB^{+1} is normally tagged by ubiquitin, the subsequent degradation by the proteasome is apparently failing within these neurons. The minor extension of the C-terminus of the UBB^{+1} protein potentially results in a difficult substrate for entry into the proteasome (Verhoef et al., 2009). Although ubiquitin chains have been shown to effectively activate opening of the proteasome the short C-terminal extension could still cause an intrinsic hurdle for degradation (Peth et al., 2009). However, the specific accumulation of UBB^{+1} in many neurodegenerative disorders with impaired

proteasome function marks this very protein as an indicator as well as a potential contributor for neurodegeneration.

The UPS in neurodegeneration

The UPS is responsible for the foremost degradation of cellular proteins that are aberrant, become redundant or are under regulatory turnover. Ubiquitination is performed by a cascade of enzymes that activate (E1), conjugate (E2) and ligate (E3) ubiquitin to target proteins (Glickman and Ciechanover, 2002; Pickart, 2001). Ubiquitin functions in tagging proteins for different pathways including proteolytic processing and endocytosis. Multi-ubiquitination by K48 or K29 ubiquitin chains tag the protein for degradation by the proteasome. The ubiquitin chain binds to the 19S part of the proteasome which contains de-ubuiquination activity (DUB) resulting in recycling of ubiquitin, and chaperone activity, which results in unfolding of the proteasome substrate. The unfolded protein is released into the 20S core for proteolytic processing by the tryptic, chymotryptic and PGPH-like activities. Small peptides exit the proteasome for further processing by proteases and recycling of the resulting amino acids. Efficient degradation of proteins by the UPS is essential for normal cellular homeostasis and ridding the cell of aberrant proteins.

The UPS has been implicated in several neurodegenerative disorders as either the primary cause or showing consequential inhibition (Ciechanover and Brundin, 2003). Many of these disorders including Parkinson Disease (PD), AD and the polyglutamine diseases manifest themselves at later stages in life. The activity of the UPS has been shown to decrease upon ageing and could thus contribute to the late onset of neurodegeneration (Carrard et al., 2002). Aberrant - disease associated - proteins can be degraded by an efficient functioning UPS but start to accumulate once this system starts to deteriorate (Zhou et al., 2003). Especially polyglutamine diseases are characterized by life-long expression of an aberrant protein from a mutant allele. The mostly adult age-at-onset of these disorders suggests a gradual buildup of toxic proteins or alternatively a gradual deterioration of the degradation machinery of these proteins over time as the explanation for the late onset.

The UPS in Huntington Disease

Huntington Disease (HD) and other polyglutamine diseases are characterized by the pathological gain of function of an expanded repeat beyond a threshold of around 36 glutamines. All polyglutamine disorders are characterized by degeneration of specific neuronal subtypes based on expression levels and potentially the function of the respective proteins. Repeat expansion in structurally distinct proteins results in similar aggregation-prone proteins that affect normal cellular function by interfering with transcription and transport processes. Neuronal Intranuclear Inclusions (NII)

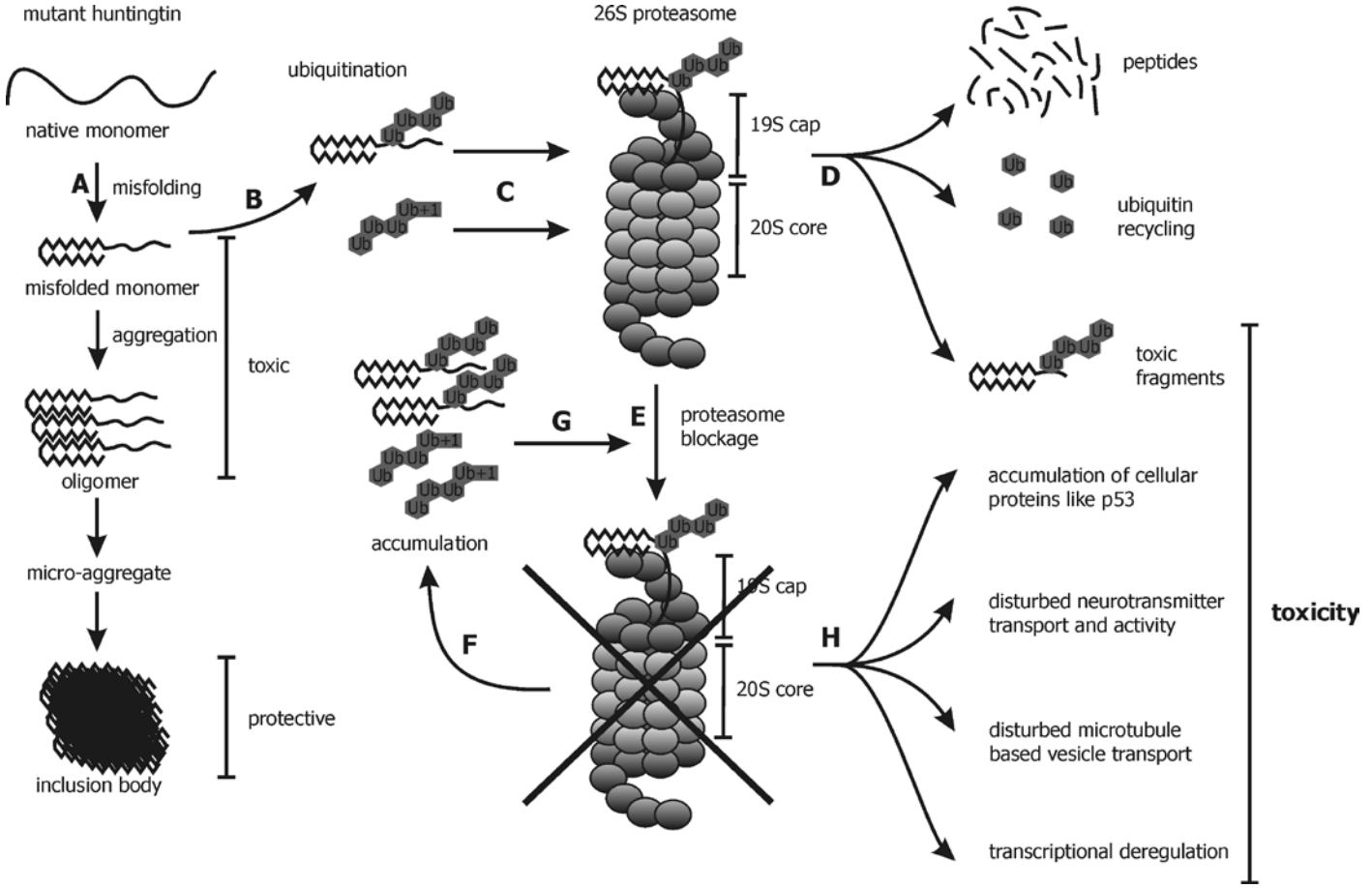
containing at least part of the expanded polyglutamine protein are a common finding among these diseases and contain several chaperone proteins including heat-shock proteins and components of the UPS.

Polyglutamine proteins are targeted to the proteasome by ubiquitination and can be efficiently degraded (Michalik and Van Broeckhoven, 2004). However, NIIs have been shown to contain ubiquitin or ubiquitinated proteins suggesting a failure of the proteasome to degrade all expanded polyglutamine protein rather than a failure of the ubiquitination machinery (DiFiglia et al., 1997). In addition, components of the proteasome have been detected in NIIs in HD and SCA3 (Chapter 1; Figure 6 (Chai et al., 1999; Schmidt et al., 2002)). Although expanded polyglutamine repeats can be degraded by the proteasome they have been shown to directly inhibit the proteasome (Bence et al., 2001; Chai et al., 1999; Verhoef et al., 2002). The repetitive sequence is potentially difficult to unfold for insertion into the 20S core and subsequent proteolytic processing might be inefficient within this sequence (Holmberg et al., 2004; Venkatraman et al., 2004). As a result the targeting to the proteasome is causing inhibition of the proteasome which instigates a further upregulation of aberrant and other cellular proteins that need to be degraded. Consequently, the proteasomal inhibition results in a deregulation of cellular protein levels causing disruption of normal processes within the respective neurons. Mutating the lysine residues at positions 6, 9 and 15 in a truncated fragment of huntingtin reduces proteasomal targeting and corresponding neuropathology (Steffan et al., 2004). Similarly, ubiquitination of UBB^{+1} at lysine 29 or 48 is required for its proteasomal inhibition and toxicity (Chapter 3 and (van Tijn et al., 2007)). A schematic representation of the mechanisms involved in proteasomal inhibition resulting in neuronal dysfunction is given in Figure 1. These findings demonstrate the essential function of the proteasome in maintaining protein levels below a toxic threshold. An increasing burden of aberrant proteins or decline in efficiency of the UPS can result in accumulation of these and other proteins. Decreasing the proteasomal targeting of these proteins relieves their inhibitory effect on the 26S proteasome.

In the R6/2 mice, an accumulation of different proteasome reporters was not detected despite the high expression levels of Htt exon-1 with 144 glutamine repeat (Bett et al., 2009; Maynard et al., 2009). However, an accumulation of large ubiquitin conjugates was detected in these mice suggesting inefficient degradation (Bennett et al., 2007). Interestingly, UPS impairment is detected upon acute expression of expanded Htt suggesting activation of compensatory mechanisms in the R6/2 model due to high mutant Htt expression throughout life (Ortega et al., 2010). In HD, similar compensatory mechanisms might be able to cope with the aberrant protein at early age but gradual decline of the UPS or other mechanisms could eventually result in Htt accumulation and corresponding neurodegeneration.

In HD patients, 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.,

Figure 1: Mechanism for proteasomal impairment leading to neuronal dysfunction in Huntington disease.



Htt containing an expanded polyglutamine repeat is misfolded (A) followed by aggregation into oligomers and eventually inclusion bodies. (B) Aberrant proteins including mutant Htt and UBB^{+1} are ubiquitinated and targeted to the 26S proteasome (C) for degradation. These proteins can be degraded by the proteasome (D), resulting in the release of small peptides and recycling of ubiquitin. However, inefficient degradation of these polyglutamine proteins can result in the formation of toxic fragments with an increased tendency to interfere with normal cellular function. In addition, misfolded polyglutamine proteins are difficult to degrade possibly due to their aggregated state which makes them problematic to unfold. Also UBB^{+1} is a difficult substrate for proteasomal degradation due the minor extension of the C-terminus of the protein. Being difficult substrates, both UBB^{+1} and mutant Htt can thus inhibit the proteasome (E). Proteasome impairment will result in a further accumulation of aberrant and misfolded proteins (F) resulting in a negative feedback loop for proteasomal impairment (G). Proteasome inhibition will result in the accumulation of cellular proteins as well as increased levels of aberrant proteins that can interfere with cellular functions (H). Toxicity can result from several pathways including transcriptional deregulation, impaired vesicle transport, disturbed neurotransmitter activity and accumulation of proteins like p53 directly causing cell death.

2004). Expanded Htt leads to the accumulation of substrate proteins that are targeted for proteasomal degradation showing evidence of UPS inhibition in HD (Bennett et al., 2007). It is likely that Htt is ubiquitinated and binds to the proteasome but the nature of this kind of substrate makes is difficult to unfold and degrade. Consequently, this will result in inhibition of the proteasome and bound substrates could account for the inability to reactivate these proteasomes.

Toxicity and neurodegeneration

As discussed in Chapter 1, the precise nature of specific neuronal dysfunction is still unclear but at least in part caused by the gain of function of the polyglutamine repeat. Inefficient degradation of these proteins results in increased levels of aggregation prone proteins that potentially interfere with other cellular functions. To date it is unclear which aggregation state of the protein is causing toxicity but either single misfolded protein or small complexes are more likely to interfere with other cellular processes. Inclusion bodies (IB) have been shown to function as protective storage sites for expanded proteins resulting in decreased levels of aggregation intermediates (Arrasate et al., 2004; Ortega et al., 2010). As a result of IB formation the activity of the proteasome is restored suggesting efficient recruitment of polyglutamine proteins (Mitra et al., 2009). Clearly, this points towards aggregation intermediates as the cause of polyglutamine-induced toxicity. Either single misfolded molecules or soluble aggregating multimers interfere with cellular processes including proteasomal degradation, transcription and transport.

UBB^{+1} accumulation

In line with other neurodegenerative disorders where proteasome inhibition was reported we detected accumulation of UBB^{+1} in HD and SCA3 (Chapter 3). UBB^{+1} accumulates in the cytoplasm and locates to the NII potentially as a result of association with proteasome components that are present in NIIs (Chapter 1: Figure 6) (Chai et al., 1999; Schmidt et al., 2002). Polyubiquitin, binding to the S6a subunit of the 19S cap, but lacking processing through the 20S core, potentially causes the UBB^{+1} resistance to degradation and its proteasomal impairment (Lam et al., 2002). Ubiquitination on lysine 29 or 48 is required for degradation of UBB^{+1} as well as its translocation to the inclusions (Chapter 3 and (Lindsten and Dantuma, 2003)). In SCA6 we did not detect UBB^{+1} accumulation, pointing towards either efficient degradation or lack of accumulation of this kind of aberrant proteins (Chapter 3). SCA6 contains a shorter repeat expansion supposedly disturbing the normal function of the α1A calcium channel subunit, i.e. loss of function, in contrast to the gain of function of the other disorders. Proteasome inhibition has not been implicated in disease development in this channelopathy and the cytoplasmic inclusions are not ubiquitinated (Ishikawa et al., 1999). This demonstrates that colocalization of UBB^{+1} with polyglutamine proteins is dependent on the proteasomal impairment and not on the inclusion formation per se.

Synergistic effect of UBB^{+1}

Within a cellular model for HD we demonstrated that addition of UBB^{+1} results in an increase in aggregate formation and polyglutamine induced toxicity (Chapter 3). UBB^{+1} itself is toxic when expressed at high levels and we clearly detected a synergistic increase in toxicity. Potentially, the additional proteasomal inhibition by UBB^{+1} results in increased levels of expanded Htt which is not efficiently degraded. These increased levels of aggregation prone or fibrillar Htt are causing increased aggregate formation in parallel to toxicity. Within the brain of HD and SCA3 patients it is likely that an additional inhibition of the UPS aggravates disease development as the machinery is no longer able to degrade the aberrant proteins. 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; Zhou et al., 2003). Proteasomal targeting of UBB^{+1} through ubiquitination is thereby required for its proteasomal inhibition, toxicity and contribution to aggregate formation and polyglutamine-induced cell death (Chapter 3 and 4 and (De Vrij et al., 2001; Lindsten et al., 2002)).

In the cerebral cortex of UBB^{+1} transgenic mice only a mild inhibition of the proteasome was detected as a result of the transgene expression (Fischer et al., 2009). Accordingly only mild phenotypic defects were found even in aged mice. Expression of expanded Htt in these UBB^{+1} transgenic mice did however result in a significant increase in aggregate formation compared to wild-type littermates (Chapter 4). The mild UPS inhibition in the transgenic mice results in increased accumulation of Htt and consequential aggregate formation. *In vitro* we detected an accompanying increase of polyglutamine induced toxicity which can potentially be extrapolated to the *in vivo* situation in mice and humans. Several studies have pointed at the protective role of inclusion bodies in polyglutamine diseases presumably by efficient storage of the aggregation prone proteins (Arrasate et al., 2004; Ortega et al., 2010). Strikingly, we detected a marked increase in the formation of multiple aggregates within the transgenic mice which points at a deregulation in the formation of the inclusions. Protective formation of inclusion bodies is likely to be contained at a single site which minimizes additional interactions. Supposedly, the UPS is the primary system for degradation of aberrant proteins and we indeed find that proteasomal inhibition by UBB^{+1} clearly contributes to accumulation of expanded Htt.

Function of E2-25K in HD

E2-25K or Hip2 is an ubiquitin conjugating enzyme that has been shown to interact with Htt and in addition mediates Aβ-induced toxicity (Kalchman et al., 1996; Song et al., 2003). Either anti-sense or dominant negative E2 constructs lacking the catalytic tail domain increase polyglutamine-induced toxicity and aggregation. We did not show an increased ubiquitination by E2-25K of Htt and can thus not exclude

an indirect effect of E2-25K inactivation on toxicity (Chapter 5). However, the accompanying increase in aggregate formation points towards decreased degradation of Htt that results in the deterioration. Furthermore, the direct interaction between Htt and E2-25K as well as higher expression levels in the striatum and frontal cortex suggests a direct effect of E2-25K on Htt (Kalchman et al., 1996). We did not detect a further increase in Htt aggregation or toxicity upon addition of UBB^{+1} which points towards a differential effect of both E2-25K and UBB^{+1} (data not shown). Specifically, UBB^{+1} is thought to directly inhibit the proteasome itself whereas E2-25K affects ubiquitination of substrate proteins. An indirect inhibition of the proteasome and not the UPS as a whole by E2-25K would potentially result in an additive effect of UBB^{+1} on Htt induced toxicity.

It appears that ubiquitination of E2-25K-targets influences the aggregate formation of expanded polyglutamine proteins but more importantly triggers polyglutamine-induced cell death. Ubiquitination supposedly results in the targeting of the expanded polyglutamines to the proteasome, resulting in either proteasome impairment or the formation of toxic aggregation prone fragments. Interestingly, E2-25K showed a differential staining between NIIs in post mortem brain material of HD and SCA3 and in neuronally differentiated cell lines (Chapter 5). It appears that E2-25K preferentially coaggregates with expanded polyglutamine proteins in apoptotic cells. Colocalization of E2-25K and polyglutamine protein potentially coincides with polyglutamine-induced neuronal death, which occurs both in HD as well as in SCA3 (Munoz et al., 2002; Vonsattel et al., 1985). Consequently, E2-25K might be upregulated or alternatively activated upon cellular stress that precedes apoptosis in these neuronal cells. In AD, colocalization was found of UBB^{+1} with E2-25K in the cerebral cortex (Song et al., 2003). Furthermore, functional E2-25K was required for UBB^{+1} induced toxicity in a cellular model. Altogether, these results could alternatively indicate that ubiquitination by E2-25K has a pro-apoptotic function that is activated upon severe proteasomal impairment.

Other UPS components

The UPS is widely implicated in neurodegenerative disorders either through mutations in components of this system or consequential inhibition (Ciechanover and Brundin, 2003). Mutations in parkin – an E3 ubiquitin ligase – are responsible for autosomal dominant PD (Kitada et al., 1998). Interestingly, overexpression of parkin decreased toxicity of polyglutamine expanded Atxn3 by direct interaction with the expanded polyglutamine protein and the proteasome (Tsai et al., 2003). This interaction potentially facilitates degradation of expanded polyglutamine proteins and was shown to result in decreased inhibition of the proteasome. Atxn3 itself is a de-ubiquitinating enzyme which decreases aggregation and toxicity of polyglutamine proteins (Warrick et al., 2005). The mammalian chain assembly factor E4B, is required to degrade the expanded form of Atxn3 and is able to prevent aggregate formation of polyglutamines

and even neurodegeneration in a Drosophila model of SCA3 (Matsumoto et al., 2004). In contrast, mutation of the ubiquitin ligase E6-AP (Ube3a), aggravates the Purkinje cell pathology in SCA1 transgenic mice but reduces the number of NIIs (Cummings et al., 1999).

Although the proteins responsible for degradation of mutant Htt have still to be determined, several proteins were shown to influence Htt aggregation and toxicity. Knock-down or dominant negative E2-25K resulted in reduced aggregation and toxicity (Chapter 5). A dominant negative Cdc34, another ubiquitin conjugating enzyme, reduced aggregate formation resulting in increased toxicity (Saudou et al., 1998). Overexpression of the ubiquitin ligase Hrd1 increases ubiquitination and consecutive degradation of mutant Htt (Yang et al., 2007). CHIP, another chain elongation enzyme, increases ubiquitination and decreases aggregation, resulting in improved survival (Jana et al., 2005). Altogether, these differences indicate diverse functions of ubiquitination on the same Htt protein resulting in respective signaling pathways. Potentially, the ubiquitination site on Htt could determine whether the substrate can be efficiently unfolded and degraded by the proteasome. Other ubiquitin chain linkages like K63 chains can direct the mutant protein into inclusion bodies for protective storage. Further studies will be required to investigate the chain specificity and whether these proteins influence other substrates or have a direct effect on Htt.

Potential therapy

In HD, proteasomal inhibition is caused by a genetic mutation that causes impairment of the degradation machinery. Consequently, activation of proteolytic processing within these cells would be a promising avenue for treating this type of proteinopathies. Increased clearance of aggregation prone Htt will prevent the harmful interaction between these proteins and other cellular components. 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). Activation of autophagy with known mediators like rapamycin could be highly beneficial for polyglutamine disorders (Ravikumar et al., 2004). UPS activation could potentially be even more advantageous as this system appears to account for a greater extent of physiological degradation of Htt (Li et al., 2010). Whereas to date several proteasome inhibitors have even made it to the market, only few activators of this pathway are known (Orlowski and Kuhn, 2008). A small-molecule inhibitor of Ubiquitin specific protease-14 results in activation of proteasome activity by inhibiting de-ubiquitination by Usp14 on the proteasome (Lee et al., 2010). However, mutation of Usp14 results in ataxia as a result of abnormal $GABA_A$ receptor turnover (Lappe-Siefke et al., 2009; Wilson et al., 2002). Also pharmacological activation of specific E3 enzymes would be a promising therapy as several enzymes including CHIP and Hrd1, were shown to reduce aggregation and toxicity (Jana et al., 2005; Yang et al., 2007). The reduced toxicity of expanded Htt in the absence of functional E2-25K also makes

this an attractive target for screening (Chapter 5). Shifting the balance from UPS impairment and formation of toxic fragments to harmless inclusions and efficient degradation would be a promising avenue in solving these severe diseases.

Several screens have been performed in order to identify modulators of Htt aggregation or polyglutamine-induced toxicity. Gene knock-down approaches in cell models as well as model organisms have yielded a great deal of insight in the pathways that are involved in disease development (Doumanis et al., 2009; Fischer et al., 2008; Nollen et al., 2004; Zhang et al., 2010). Target selection and consecutive screening are likely to identify new molecules to treat these disorders. Other approaches have used compound screens to directly identify disease modifying small molecules (Desai et al., 2006; Fecke et al., 2009). Alternatively, specifically preventing expression from the mutant allele would avoid the formation of the disease protein. RNAi based methods aimed at either the polyglutamine repeat or specific SNPs have identified several oligomers that decrease expanded Htt expression (Hu et al., 2009; Miller et al., 2003; Pfister et al., 2009; Zhang et al., 2009). Hopefully, over the coming years either of these methods will deliver a therapy to treat or alleviate these diseases with high unmet need.

Concluding remarks

HD is an autosomal dominant genetic disorder that manifests itself around mid-life. Despite expression of the expanded polyglutamine protein throughout life the symptoms take decades to develop. The UPS is crucial for the degradation of this type of aberrant proteins and is impaired in HD brain (Seo et al., 2004). UBB^{+1} accumulates in the cytoplasm and NII in HD and SCA3 but not in SCA6 (Chapter 3). Consequently, UBB^{+1} functions as a reporter for proteasome impairment in these diseases as a result of the pathological gain of function of the polyglutamine repeat. UBB^{+1} is targeted to the proteasome and thereby also inhibits proteasomal degradation. Both in a cellular model but also in transgenic mice, expression of UBB^{+1} resulted in an apparent increase in polyglutamine aggregation (Chapter 3 and 4). The decreased degradation of expanded Htt resulted in a synergistic increase in polyglutamine induced cell-death. Although UBB^{+1} is not causing disease it is clearly a contributing factor after initial proteasome inhibition by other factors.

E2-25K is a potential candidate for ubiquitination of mutant Htt and was shown to colocalize with UBB^{+1} in AD (Song et al., 2003). Functional knock-down of E2-25K indeed relieved polyglutamine induced aggregation and toxicity (Chapter 5). These findings illustrate the importance of ubiquitination for the cellular clearance or storage of toxic proteins that extends beyond the proteasome itself. Further research is needed to elucidate the sequence of events leading to proteasome inhibition and subsequent neuronal dysfunction. Importantly, the present thesis demonstrates the importance of an efficient UPS for the degradation of different aberrant proteins that lead to neurodegeneration.

Abbreviations

AD	- Alzheimer disease
ALS	- amyotropic lateral sclerosis
AMPA	- α-amino-3-hydroxy-5 methyl-4-isoxazolepropionicacid
BDNF	- brain derived neurotrophic factor
CBP	- CREB binding protein
CHIP	- C-terminus of Hsc70 interacting protein
CREB	- cyclic AMP response element binding protein
DRPLA	- dentatorubropallidoluysian atrophy
DS	- Down syndrome
DUB	- deubiquitinating enzyme
ERAD	- endoplasmatic reticulum associated degradation
GFAP	- glial fibrillaric acid protein
Hap1	- huntingtin associated protein 1
HD	- Huntington disease
HECT	- homologous to E6-AP, E3-domain type
Hip1	- huntingtin interacting protein 1
Hip1R	- Hip1-related
Hippi	- Hip1 protein interactor
Hsp	- heat shock protein
Htt	- huntingtin protein
LTP	- long-term potentiation
MCBP	- multiubiquitin chain binding proteins
NEDD8	- neural precursor cell-expressed developmentally down-regulated gene
NGFR	- nerve growth factor receptor
NII	- neuronal intranuclear inclusions
PA 28/200	- proteasome activator 28 / 200
Pak-1/2	- p21-activated kinase-1 / 2
PD	- Parkinson disease
PGPH	- peptidyl-glutamyl peptide hydrolizing
PHD	- plant homeo domain, E3-domain type
PQBP-1	- polyglutamine binding protein-1
RING	- really interesting new gene, E3-domain type
SBMA	- spinobulbar muscular atrophy
SCA	- spinocerebellar ataxia
Sp1	- specificity protein-1
SUMO	- small ubiquitin-like modifier
TBP	- TATA-binding protein
UBA	- ubiquitin-associated domain
UBB^{+1}	- ubiquitin-B^{+1}
UbL	- ubiquitin like domain
UCH-L1	- ubiquitin C-terminal hydrolase L1
UHRF-2	- ubiquitin like with PHD and Ring finger domain-2
UPR	- unfolded protein response
UPS	- ubiquitin proteasome system

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Summary

Huntington disease (HD) is the best know of the polyglutamine disorders which are caused by the excessive expansion of a CAG repeat in a transcribed gene. It is estimated that there are 1300 HD patients in the Netherlands and even more people that are currently at risk. Translation of the CAG repeat results in proteins with an expanded polyglutamine repeat. Expansion of this repeat above a threshold of 36 results in an aggregation prone protein leading to disease onset around mid-life. In all polyglutamine diseases an inverse correlation is found between repeat expansion and age of disease onset. Neuronal intranuclear inclusions (NII) which contain at least part of the expanded protein are identified as hallmarks of the polyglutamine diseases. Inclusions have been suggested to function as protective storage sites implying that soluble misfolded proteins are the primary cause of toxicity. The aggregation prone properties of these proteins are causing the pathological gain of function due to interference with normal cellular function.

The ubiquitin proteasome system (UPS) is responsible for the main protein degradation within the cell. The UPS contributes to cellular homeostasis by regulating the expression of essential proteins and degradation of excessive proteins. In addition, the UPS is involved in cellular protein quality control and responsible for the degradation of aberrant proteins that accumulate in neurodegeneration. Also expanded polyglutamine proteins can be degraded by the UPS in order to protect the cell from this aggregation prone protein. However, the expanded repeat is also difficult to degrade causing impairment of the proteasome and consequential accumulation.

The general introduction in **Chapter 1** gives an overview of several of the important aspects leading to neurodegeneration in HD and specifically the contribution of the UPS.

Chapter 2 describes the discovery of molecular misreading which occurs on GAGAG motifs in different genes. The frameshift mutant of ubiquitin B (UBB^{+1}) is formed by a dinucleotide deletion (ΔGU) in the mRNA. The mutant ubiquitin protein that is formed can no longer ubiquitinate substrate proteins but is a target for ubiquitination and subsequent proteasomal degradation. UBB^{+1} accumulates in several neurodegenerative diseases and is an *in vivo* marker for proteasomal inhibition.

In **Chapter 3**, we investigated the accumulation of UBB^{+1} in HD and Spinocerebellar ataxia-3 (SCA3) and potential contribution to neurodegeneration. UBB^{+1} was found to accumulate in the cytoplasm and NII in the affected brain regions of HD and SCA3 demonstrating *in vivo* proteasome inhibition in these disorders. In a cellular model of HD we observed that UBB^{+1} results in inhibition of the proteasome which causes increased aggregate formation. In addition, a synergistic increase in polyglutamine

induced cell death was found upon expression of UBB^{+1}. These findings implicate UBB^{+1} as an aggravating factor in polyglutamine induced neurodegeneration and stresses the importance of the UPS for degradation of aberrant polyglutamine and UBB^{+1} proteins.

UBB^{+1} transgenic mice show a mild inhibition of the proteasome. In **Chapter 4** we tested the influence of this UPS inhibition by UBB^{+1} on Htt aggregation *in vivo*. Expression of expanded polyglutamine protein in the striatum of the UBB^{+1} transgenic mice showed a strong increase in NII formation compared to wildtype littermates. These results demonstrate *in vivo* that minor differences in UPS capacity can have major detrimental effects on the neuropathology of HD.

The ubiquitin conjugating enzyme E2-25K has been shown to interact directly with Htt independent of polyglutamine repeat length. In **Chapter 5**, we investigated the localization of E2-25K in HD as well as the contribution to neurodegeneration. E2-25K colocalizes with a subset of NII in HD brain as well as with aggregates in apoptotic cells *in vitro*. Dominant negative E2-25K – lacking the catalytic tail domain – as well as an antisense construct decreased aggregate formation of expanded Htt. Additionally, mutant and antisense E2-25K reduced polyglutamine-induced cell death. These findings show that ubiquitination of E2-25K-targets contributes to aggregate formation as well as neuronal cell death in HD.

Finally, in **Chapter 6** the different findings of this thesis are discussed as well as further research and perspectives.

In conclusion, the findings of this thesis illustrate the importance of the UPS for the cellular clearance of toxic proteins involved in neurodegeneration. The precise mechanism of specific neuronal dysfunction in HD is still unclear but is triggered by the gain of function of the polyglutamine repeat. Impairment of the UPS results in the further accumulation of aberrant proteins and subsequent neuronal dysfunction. Since HD is caused by protein expression from a mutant allele, more efficient degradation could protect the neurons from harmful polyglutamine proteins.

Samenvatting

De ziekte van Huntington (HD) is de meest bekende van de polyglutamine ziektes die worden veroorzaakt door de expansie van een CAG herhaling in een getranscribeerd gen. In Nederland zijn er naar schatting 1300 patienten met HD en een nog groter aantal die het risico lopen de ziekte te ontwikkelen. Translatie van de CAG herhaling resulteert in huntingtine eiwit met een verlengde reeks glutamines in de N-terminus. Expansie van deze glutamine reeks boven een drempel van 36 resulteert in een eiwit dat aggregeert en leidt tot start van de ziekte rond middelbare leeftijd. In alle polyglutamine ziektes wordt een omgekeerde correlatie gevonden tussen glutamine verlenging en leeftijd bij aanvang van de ziekte. Neuronale intranucleaire inclusies (NII) die tenminste een gedeelte van het verlengde eiwit bevatten worden gevonden als kenmerk van de polyglutamine ziektes. Van deze inclusies is gesuggereerd dat ze functioneren als beschermende opslag terwijl de oplosbare verkeerd gevouwen eiwitten de voornaamste reden zouden zijn van de toxiciteit. De afwijkende eiwitvouwing van deze eiwitten veroorzaakt de uiteindelijke pathologie door verstoring van het normale functioneren van de cel.

Het ubiquitine proteasoom systeem (UPS) is verantwoordelijk voor de voornaamste eiwit afbraak binnen de cel. Het UPS draagt bij aan het cellulaire evenwicht door de regeling van de expressie van essentiele eiwitten en afbraak van overbodige eiwitten. Daarnaast is het UPS betrokken bij de cellulaire kwaliteits controle van eiwitten en verantwoordelijk voor de afbraak van foutieve eiwitten die ophopen bij neurodegeneratie. Ook verlengde polyglutamine eiwitten kunnen worden afgebroken door het UPS om de cel te beschermen tegen dit misgevouwen eiwit. De verlengde reeks glutamines is echter moeilijk af te breken wat resulteert in remming van het proteasoom en eiwit ophoping als gevolg daarvan.

De algemene introductie in **Hoofdstuk 1** geeft een overzicht van verschillende van de belangrijke aspecten die leiden tot neurodegeneratie in HD en in het bijzonder de bijdrage van het UPS.

Hoofdstuk 2 beschrijft de ontdekking van moleculaire leesfouten die optreden in GAGAG motieven in verschillende genen. De frameshift mutant van ubiquitine B (UBB^{+1}) wordt gevormd door een dinucleotide (ΔGU) deletie in het mRNA. Het mutant ubiquitine eiwit dat wordt gevormd kan niet langer substraat eiwitten ubiquitineren maar is zelf wel een substraat voor ubiquitinatie en daaropvolgende proteasomale afbraak. UBB^{+1} hoopt op bij verschillende neurodegeneratieve ziekten en is een *in vivo* marker voor proteasoom remming.

In **Hoofdstuk 3**, hebben we de ophoping onderzocht van UBB^{+1} bij HD en spinocerebellaire ataxie-3 (SCA3) en tevens de mogelijke bijdrage aan neurodegeneratie.

UBB^{+1} blijkt op te hopen in het cytoplasma en NII in de aangetaste hersengebieden van HD en SCA3 en toont daarmee *in vivo* aan dat het proteasoom geremd is bij deze ziekten. In een cellulair model van HD vonden we dat UBB^{+1} resulteert in remming van het proteasoom wat leidt tot een toename in de vorming van inclusies. Bovendien werd een synergistische verhoging van polyglutamine geïnduceerde celdood gevonden als gevolg van UBB^{+1} expressie. Deze bevindingen impliceren dat UBB^{+1} de polyglutamine geïnduceerde neurodegeneratie versterkt en onderstreept het belang van het UPS voor de afbraak van misgevouwen polyglutamine en UBB^{+1} eiwitten.

UBB^{+1} transgene muizen laten een milde remming van het proteasoom zien. In **Hoofdstuk 4** hebben we getest wat de invloed is van deze remming van het UPS door UBB^{+1} op de vorming van Htt inclusies *in vivo*. Expressie van verlengde polyglutamine eiwitten in het striatum van UBB^{+1} transgene muizen toonde een sterke toename in de vorming van NII in vergelijking met verwante wildtype muizen. Deze resultaten laten *in vivo* zien dat minimale verschillen in de UPS capaciteit sterke nadelige effecten kunnen hebben op de neuropathologie kenmerkend voor HD.

Van het ubiquitine conjugerende enzym E2-25K is aangetoond dat het een directe interactie aangaat met Htt onafhankelijk van de polyglutamine lengte. In **Hoofdstuk 5**, hebben we de lokalisatie van E2-25K onderzocht en tevens de bijdrage aan neurodegeneratie. E2-25K co-lokaliseert met een gedeelte van de NII in HD hersenen en tevens met inclusies in apoptotische cellen *in vitro*. Dominant negatieve E2-25K – zonder het katalytische staart domein – alsook een antisense construct verminderden de aggregaat vorming van verlengd Htt. Daarnaast reduceerden de mutant en antisense E2-25K constructen de polyglutamine geïnduceerde celdood. Deze bevindingen laten zien dat ubiquitinatie van E2-25K substraten bijdraagt aan zowel de inclusie vorming als neuronale celdood bij HD.

Tot slot worden in **Hoofdstuk 6** de verschillende bevindingen van dit proefschrift besproken evenals vervolg onderzoek en toekomst perspectieven.

De bevindingen van dit proefschrift illustreren het belang van het UPS voor de cellulaire afbraak van toxische eiwitten die betrokken zijn bij neurodegeneratie. Het precieze mechanisme van specifieke neuronale verstoring in HD is nog altijd onbekend maar wordt veroorzaakt door de verkregen functie van de polyglutamine repeat. Remming van het UPS resulteert in verdere ophoping van foutieve eiwitten en uiteindelijk neuronaal disfunctioneren. Doordat HD wordt veroorzaakt door eiwit expressie van een mutant allel zou efficiëntere afbraak van of mRNA of eiwit de neuronen kunnen beschermen tegen schadelijke polyglutamine eiwitten.

Curriculum vitae

Remko de Pril werd geboren op 22 september 1975 te Woerden. Na het atheneum op het Ichthus College te Enschede begon hij in 1993 met de studie scheikunde aan de Rijksuniversiteit Leiden. Bij de vakgroep Moleculaire Genetica aan de Rijksuniversiteit Leiden werd een stage gevolgd over nucleotide excisieherstel van cyclobutaan-pyrimidine dimeren onder begeleiding van Prof. Dr. J. Brouwer en Dr. M. Tijsterman. Vervolgens werd een stage gevolgd binnen het instituut voor celbiologie van de Eidgenössische Technische Hochschule te Zürich (Zwitserland), over het telomeer positie effect op de chromatine organisatie van het *URA3* gen onder begeleiding van Prof. Dr. F. Thoma. In 1999 werd het doctoraal diploma scheikunde behaald met als afstudeerrichting biochemie. In 2000 kwam Remko in dienst van het Nederlands Instituut voor Hersenonderzoek als onderzoeker in opleiding, onder begeleiding van Prof. Dr. R.A.C. Roos, Dr. F.W. van Leeuwen en Dr. D.F. Fischer. De resultaten van het onderzoek naar de invloed van het ubiquitine proteasoom systeem op de ziekte van Huntington staan beschreven in dit proefschrift. Sinds 2005 is Remko werkzaam als wetenschappelijk onderzoeker bij Galapagos.

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