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

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Chapter 1

Introduction

The ubiquitin proteasome system in Huntington disease

Cause and consequence of neuronal dysfunction

Polyglutamine diseases

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).

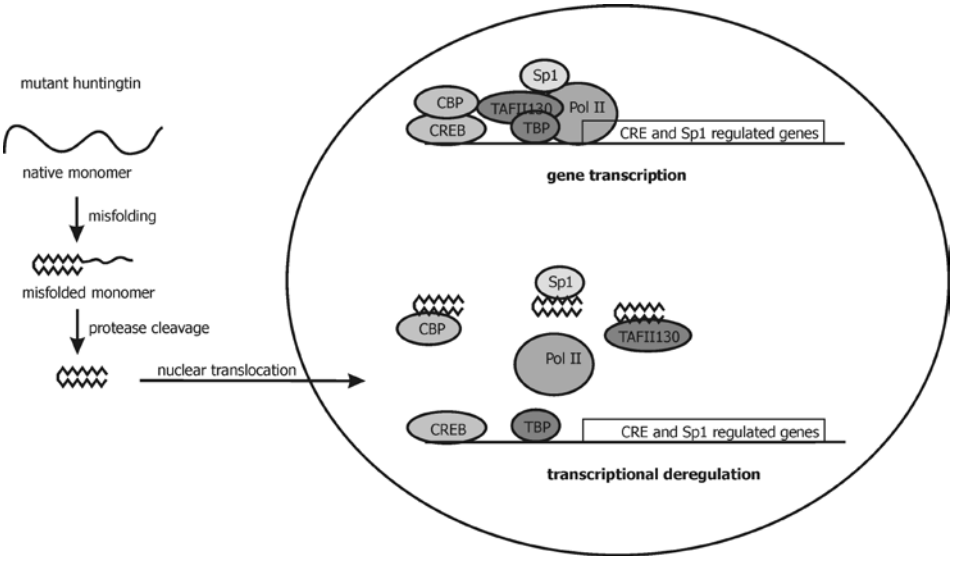
In contrast, expansion of the polyglutamine repeat in Htt results in an increased interaction with the transcription factor Specificity protein-1 (Sp1) (Li et al., 2002). This binding thereby disrupts the normal promoter binding of Sp1 resulting in decreased expression of several genes including the 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.

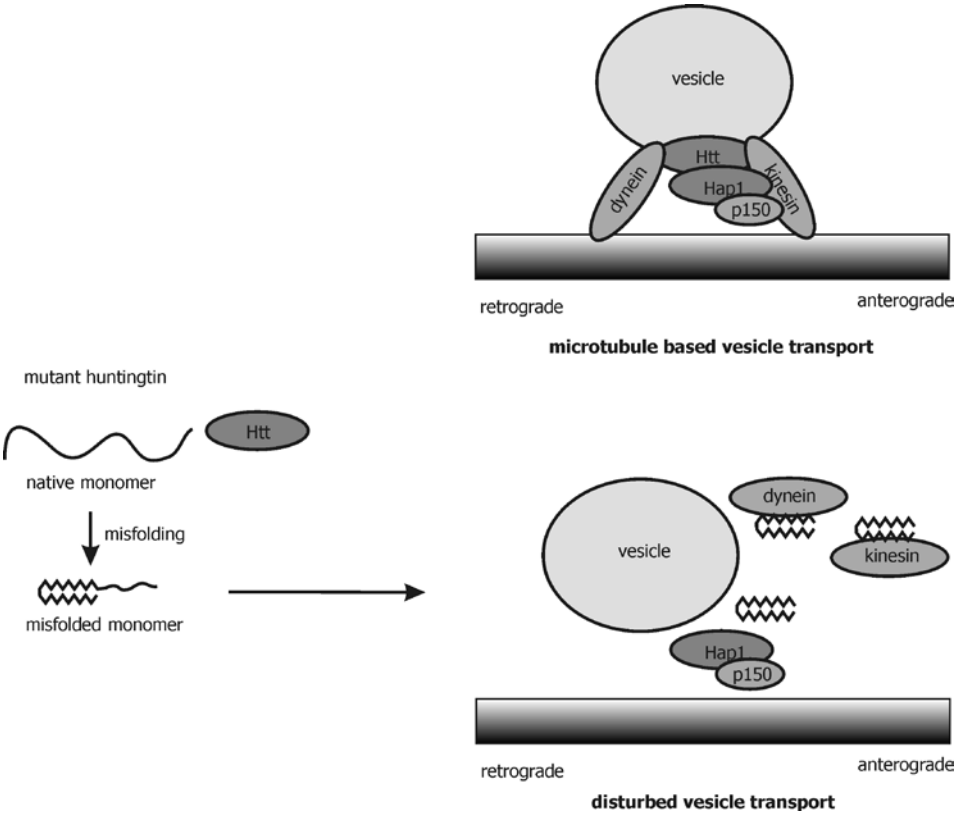
Wild-type Htt functions in vesicle transport by binding to dynactin and motor proteins dynein and kinesin. Phosphorylation by Akt at serine 421 (S421) results in recruitment of kinesin-1 to the dynactin complex on microtubules and vesicles (Colin et al., 2008). This kinesin recruitment promotes anterograde 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.

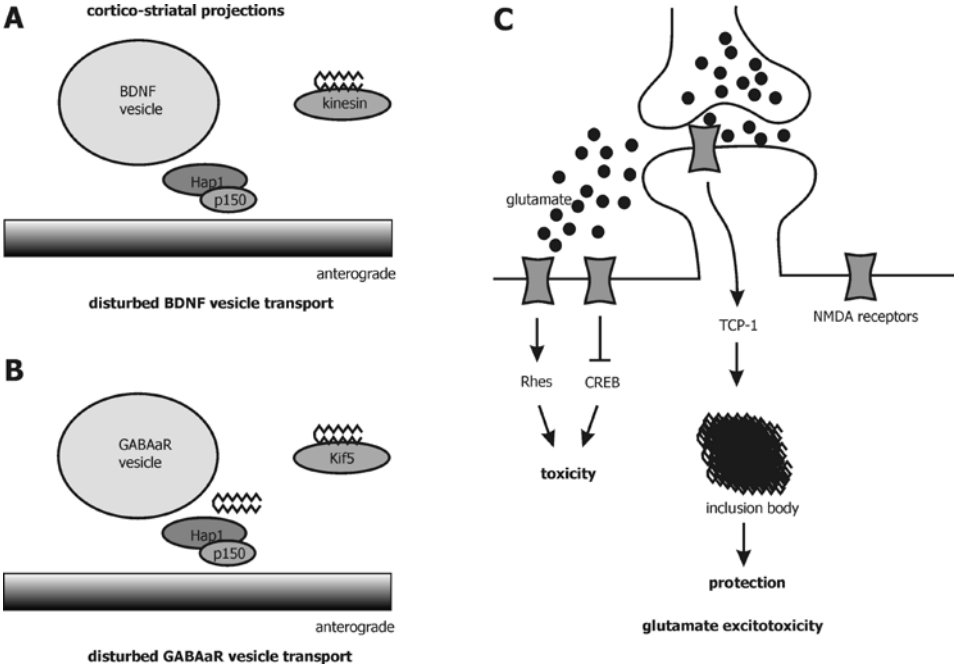
The neuroprotective effect of Htt can be greatly governed by the indispensable function of wild-type Htt in neuronal vesicle transport. In addition, wild-type Htt has been shown to inhibit caspase-3 activation *in vivo* which is disturbed by polyglutamine expansion or *HTT* knock-down (Zhang et al., 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.

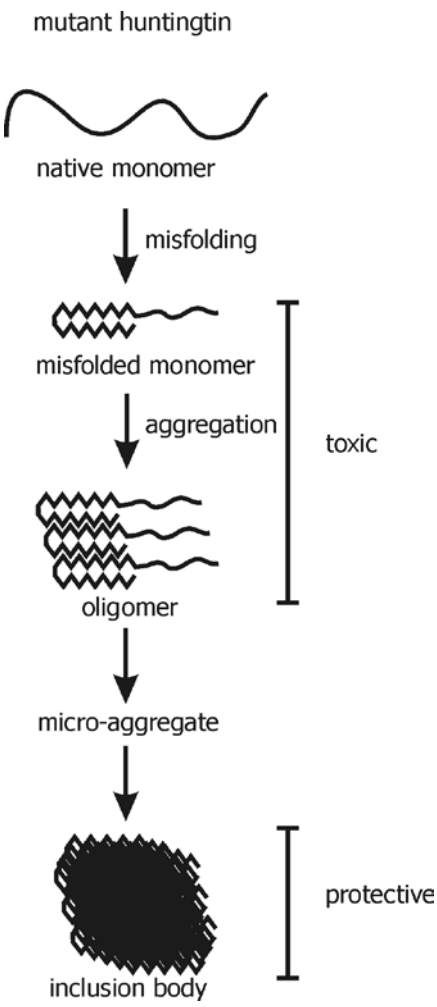
Inclusion bodies

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).

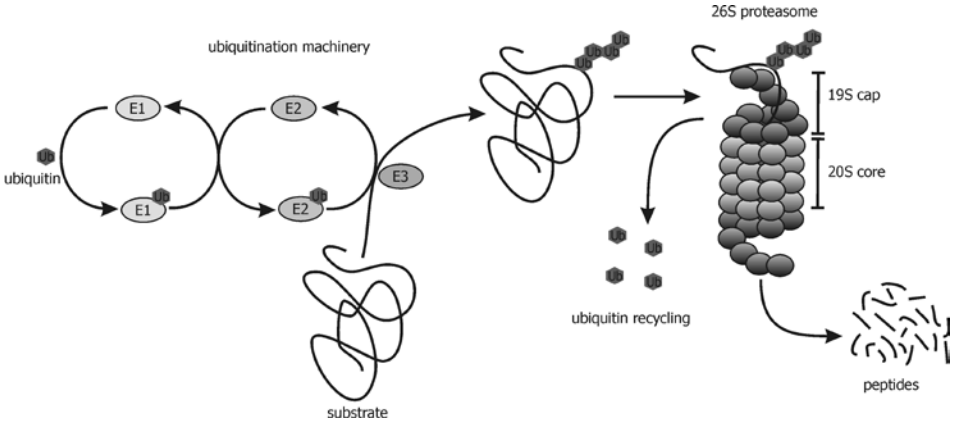
Induction of autophagy was shown to be beneficial in animal models of several polyglutamine diseases and reduces neuropathology as well as phenotypic performance (Menzies et al., 2010; Pandey et al., 2007; Ravikumar et al., 2004). Clearance of aggregated proteins involves a direct interaction of the 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.

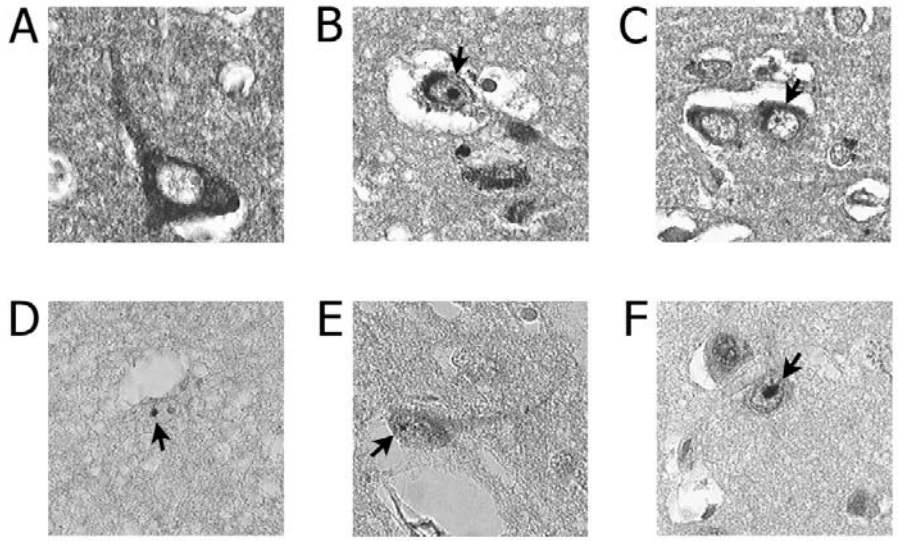
Furthermore, C-terminus of Hsp70 interacting protein (CHIP; a U-box E3-ligase) was shown to interact with expanded polyglutamine protein (Jana et al., 2005). Importantly, overexpression of CHIP increased ubiquitination of expanded Htt and ataxin-3 providing a direct link between the heat shock response 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.