The effect of mutant ubiquitin on proteasome function in relation to neurodegenerative disease
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CHAPTER I

General Introduction

The neuronal ubiquitin-proteasome system:
murine models and their neurological phenotype

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Abstract

The ubiquitin-proteasome system (UPS) is the main intracellular pathway for regulated protein turnover. This system is of vital importance for maintaining cellular homeostasis and is essential for neuronal functioning. It is therefore not surprising that impairment of this system is implicated in the pathogenesis of a variety of diseases, including neurological disorders, which are pathologically characterized by the presence of ubiquitin-positive protein aggregates. A direct correlation between intact neuronal functioning and the UPS is exemplified by a range of transgenic mouse models wherein mutations in components of the UPS lead to a neurodegenerative or neurological phenotype. These models have been proven useful in determining the role of the UPS in the nervous system in health and disease. Furthermore, recently developed in vivo models harbouring reporter systems to measure UPS activity could also substantially contribute to understanding the effect of neurodegeneration on UPS function. The role of the UPS in neurodegeneration in vivo is reviewed by discussing the currently available murine models showing a neurological phenotype induced by genetic manipulation of the UPS.

Introduction

Protein turnover plays an important role in the maintenance of cellular homeostasis and is involved in a diverse array of processes, ranging from endocytosis to signal transduction (Welchman et al., 2005; Mukhopadhyay and Riezman, 2007). Protein quality control, through degradation of aberrant or misfolded proteins, also contributes to a healthy intracellular environment (Hershko and Ciechanover, 1998). Degradation of proteins can be executed by various proteolytic systems, including lysosomal degradation, chaperone-mediated autophagy, and substrate-specific degradation by the ubiquitin-proteasome system (UPS). The focus of this review is on the role of the UPS in neurodegeneration in vivo.

The ubiquitin-proteasome pathway

The small 76-amino-acid ubiquitin protein was first discovered over three decades ago, by Goldstein and co-workers (Goldstein et al., 1975). Over the years, ubiquitin modification of substrate proteins emerged as a key regulator of many cellular processes, including protein degradation (Ciechanover et al., 1980; Hershko et al., 1980), and proved to be essential for cell viability in vitro (Finley et al., 1984) and in vivo (Ryu et al., 2007).
GENERAL INTRODUCTION

Substrate targeting by ubiquitin

The UPS is the main regulated intracellular proteolytic pathway and determines the stability of a broad array of proteins by a two-stage mechanism; first, substrates are tagged for degradation by covalent attachment of a chain of ubiquitin moieties, a process known as poly-ubiquitination. Subsequently, the ubiquitinated substrates are selectively targeted to the 26S proteasome, where protein degradation takes place and ubiquitin is recycled (for an extensive review of the UPS see (Glickman and Ciechanover, 2002)). Besides tagging proteins for degradation, additional roles for ubiquitin modification are currently emerging, mostly determined by the length and localization of the attached ubiquitin chain. These include regulation of chromatin structure, DNA repair and receptor endocytosis (Schwartz and Hochstrasser, 2003). Target substrates for proteasomal degradation are recognized by the enzymatic machinery of the UPS through intrinsic degradation signals (degrons, (Meinnel et al., 2006)) or by association with ancillary proteins, e.g. heat-shock proteins, leading to ubiquitination of the substrate (Cyr et al., 2002).

Proteasomal degradation shows a high level of substrate specificity, which is mainly achieved by the enzymatic cascade involved in substrate ubiquitination. This process employs a minimum of three different classes of enzymes performing subsequent tasks to covalently attach ubiquitin to a substrate protein. First, ubiquitin is activated by ATP-dependent cross-linking of its C-terminal glycine (G76) to the active site cysteine of ubiquitin activating enzyme E1, forming a high-energy thiolester intermediate. Subsequently, the activated ubiquitin is transferred from the active site of E1 to the active site of one of the E2 ubiquitin-conjugating enzymes present in the cell. Finally, the activated ubiquitin is transferred from the E2 to the target substrate which is bound to a ubiquitin ligase (E3 enzyme) (Figure 1). Until now, only a single conserved ubiquitin E1 enzyme was identified, in contrast to E2 enzymes, of which over fifty different variants are known (Semple, 2003). Only recently two additional E1 enzymes, Uba6 and UBE1L2, both present in vertebrates, were discovered (Jin et al., 2007; Pelzer et al., 2007). Substrate specificity is partially defined by the different E2s, combined with a broad array of hundreds of distinct E3 enzymes, which appear to be to some extent substrate-specific (reviewed in (Glickman and Ciechanover, 2002; Pickart, 2004)).

Of the E3 enzymes, three mechanistically distinct classes are described. The HECT (Homologous to E6-associated protein C-Terminus) domain E3 enzymes bind the E2-ubiquitin complex as well as the target substrate, serving as an intermediate docking station for transfer of the ubiquitin moiety from the E2 to a lysine residue in the substrate (Figure 1). The second class of E3s is the RING-finger (Really Interesting New Gene) motif containing E3s. RING-E3s transfer the ubiquitin moiety directly from the E2 enzyme to the target substrate (Figure 1). Some RING-E3s consist of a single subunit in which the substrate recognition site and the E2-binding site are united in one subunit. Other RING-E3 enzymes consist of multiple subunits, each holding distinct properties.
regarding substrate recognition, E2 binding and ubiquitin transfer (reviewed by Weissman, 2001). A third class of E3 enzymes is the U-box containing E3 enzymes (described in the next paragraph).

Figure 1  Ubiquitination cascade and proteasomal degradation. Ubiquitin (purple sphere) is activated by the E1 ubiquitin activating enzyme and transferred to a ubiquitin carrier, the E2 ubiquitin conjugating enzyme. Protein substrates to be targeted for degradation by the proteasome are recognized by one of the E3 ubiquitin ligase enzymes. In the case of RING-E3 ligases, the ubiquitin is directly transferred from the E3-bound E2 enzyme to a lysine residue in the substrate. For HECT-E3 enzymes, the ubiquitin is first transferred from the E2 to the E3 ligase and is subsequently attached to the substrate. Successive ubiquitin moieties are attached to the substrate-bound ubiquitin, forming a ubiquitin chain. With a K48-linked polyubiquitin chain of four or more ubiquitins the substrate is targeted to the 26S proteasome. Here, the ubiquitin chain is released and the substrate is degraded into small peptides by the 26S proteasome. Finally, the ubiquitin is recycled by release of free monomeric ubiquitin from the ubiquitin chain, an activity mediated by DUBs. See color section.
A ubiquitinated protein may be subjected to several subsequent rounds of ubiquitination on one or more lysine residues in the proximal ubiquitin moiety. These ubiquitin-ubiquitin linkages are made up of isopeptide bonds between an internal lysine residue in the bound ubiquitin and the C-terminal G76 of the consecutive ubiquitin moiety. Besides sequential attachment of single ubiquitin moieties to the substrate-bound ubiquitin, pre-formed ubiquitin chains can also be transferred as a whole to a substrate (Li et al., 2007). In some cases, a fourth ubiquitination enzyme, known as the ubiquitin chain elongation factor E4, is necessary, together with the E1, E2 and E3 enzymes to elongate a poly-ubiquitin chain to the desired length (Koegl et al., 1999). The defining motif of this E4 enzyme, designated the U-box motif, partially resembles the RING-finger domain and is also found in several other proteins which elongate ubiquitin chains. The capacity of these U-box-containing enzymes to generate ubiquitin chains in the absence of E3 enzymes indicates that they may also be addressed as a novel class of E3s (Hoppe, 2005).

Varying the ubiquitin linkage sites and chain lengths influences the fate of the ubiquitinated substrates. The ubiquitin protein contains seven conserved internal lysine residues, giving rise to numerous possible ubiquitin linkages. The minimal chain length for proteasomal targeting is a ubiquitin chain of four ubiquitins linked at the lysine at position 48 (K48) (Thrower et al., 2000). In vivo, ubiquitin linkages can also be formed at four additional internal lysine residues (K6, K11, K29 and K63), initiating processes other than degradation. For instance, K63 linkage of ubiquitin is implicated in ribosomal function and DNA repair (Spence et al., 2000).

**Proteasomal degradation**

Ubiquitinated substrates with a K48-linked ubiquitin chain of sufficient length are targeted to the 26S proteasome for degradation (Figure 1). The 26S proteasome is a ~2.5 MD multi-subunit protease complex, consisting of a 20S core particle flanked by at least one 19S regulatory particle. The proteolytic activity resides inside the 20S core, a barrel-shaped structure assembled of four stacked rings, with seven subunits residing in each ring. The two outer rings each contain seven α-subunits which guide substrates into the central proteolytic chamber composed of two inner rings of seven β-subunits. Three out of seven β-subunits show proteolytic active sites. The proteolytic activity can be specified into chymotrypsin-like, trypsin-like and peptidyl-glutamyl-peptide hydrolyzing activity in the β5, β2 and β1 subunits respectively. Also three alternative β subunits exist (β5i, β2i and β1i), and incorporation of these subunits into the 20S particle gives rise to the immuno-proteasome (in addition with 20S-PA28 association) involved in antigen presentation. The 19S complex consists of a base part attached to the 20S particle (containing six ATPases of the AAA family and two additional non-ATPase subunits) and a lid on top of the base made up of at least eight different subunits (reviewed in (Pickart and Cohen,
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The 19S proteasome particle has many specialized functions, which may be partly attributed to specific subunits, including substrate recognition and binding by the Rpt5 and Rpn10 subunits (Deveraux et al., 1994; Young et al., 1998; Lam et al., 2002) and ubiquitin chain removal by Rpn11, after which ubiquitin is recycled (Verma et al., 2002; Yao and Cohen, 2002). Presentation of substrates to the proteasome can be facilitated by proteasome-associated proteins, such as Rad23 and Dsk2, which tend to have a ubiquitin-like domain for proteasomal recognition and one or more ubiquitin-associated domains to bind to ubiquitin chains (reviewed in Elsasser and Finley, 2005).

**UPS-related processes**

An additional level of substrate selectivity (beyon E2 and E3 specificity) is achieved by spatial and temporal regulation of proteolytic degradation throughout the cell. The UPS exerts specialized functions, depending on the intracellular compartment in which it resides (e.g. at the origin of replication, mitotic spindle or synapse) or on time-dependent proteolytic regulation, the latter serving an important role in cell-cycle progression (reviewed in Pines and Lindon, 2005). An apparent example of a spatially regulated degradation mechanism is the endoplasmatic reticulum (ER)-associated degradation (ERAD) pathway. In the ER, proteins are glycosylated and folded before they are routed into the secretory pathway. ERAD is the main pathway for protein quality control of secretory proteins, to ensure that un- or misfolded proteins are degraded. In this system, substrates are first targeted from the ER to the ubiquitination machinery in the cytosol, where they are subsequently degraded by the 26S proteasome (reviewed by Meusser et al., 2005).

Apart from functioning as a degradation signal, ubiquitination regulates a myriad of other processes in the cell, also dependent on the characteristics of the ubiquitin chain. Mono-ubiquitination of proteins regulates distinct cellular functions, including histone regulation, retrovirus budding, transcriptional regulation and endocytosis. Via the latter pathway substrates at the plasma membrane are modified by a single ubiquitin moiety on a lysine residue (mono-ubiquitination) or by multiple mono-ubiquitination of several internal lysine residues (multi-ubiquitination) for internalization and intracellular routing via the late endosomes (also known as multivesicular bodies) to the lysosome (reviewed in Haglund et al., 2003; Mukhopadhyay and Riezman, 2007). Also ubiquitin-like proteins, of which the best known are NEDD8 (neuronal precursor cell expressed, developmentally down-regulated 8) and SUMO (small ubiquitin-like modifier), modify substrates by a similar cascade of E1 and E2 enzymatic activity and regulate protein function, ranging from E3-ligase activity regulation by NEDD8 to transcriptional regulation, and possibly even antagonize ubiquitination by SUMO modification of substrates (Welchman et al., 2005).
Deubiquitination
Substrate ubiquitination is a reversible process and both the ubiquitination and deubiquitination processes are tightly regulated by specific enzymes. The distinct enzymatic activity which reverses ubiquitination is performed by deubiquitinating enzymes (DUBs), which serve various functions by their ability to cleave ubiquitin moieties. The approximately one hundred known DUBs belong to the family of proteases and are subdivided into five subclasses, i.e. (1) the ubiquitin C-terminal hydrolases-UCHs, (2) the ubiquitin specific proteases-USPs, (3) the ovarian tumor proteases-OTUs, (4) the Machado-Joseph Disease protein domain proteases-MJDs and (5) the JAB1/MPN/Mov34 metalloenzyme-JAMM motif proteases (Nijman et al., 2005). DUBs are responsible for cleaving ubiquitin precursors, rescuing target substrates from degradation, cleaving the ubiquitin chain at the proteasome entrance, and for disassembly of unanchored ubiquitin chains generating single ubiquitin moieties. Some DUB activities are physically linked to the 26S proteasome such as ubiquitin chain release by the Rpn11 19S subunit (Verma et al., 2002; Yao and Cohen, 2002). The 19S-associated DUB UCHL5 (UCH37) removes ubiquitin only from the distal end of substrate bound poly-ubiquitin chains, editing the length of the ubiquitin chain (Lam et al., 1997). Other DUBs have more general functions, like recycling of ubiquitin chains to generate free ubiquitin moieties by the DUB isopeptidase-T. DUBs seem to possess (partial) substrate specificity as specific targets or involvement in specific pathways are found for an increasing number of DUBs (reviewed in (Amerik and Hochstrasser, 2004; Nijman et al., 2005)).

The UPS in the nervous system
As described in the previous section, the UPS plays an important role in maintaining cellular homeostasis, determines the turnover rate of many short-lived proteins, and recent studies increasingly recognize an essential role for the UPS in cell cycle progression (Reddy et al., 2007; Stegmeier et al., 2007). However, the UPS also fulfills specific tasks in post-mitotic neurons, including turnover of long-lived proteins, synaptic development and maintenance of established synaptic connections (Table 1, for a detailed review of the neuronal UPS see (Yi and Ehlers, 2007)).

The UPS in neuronal development and plasticity
During development, axons have to locate to their appropriate targets in other areas of the nervous system, a process known as axon guidance. A growth cone is located at the outer end of the developing axon and determines the axonal branching pattern. In these growth cones resides an active UPS machinery, which, together with various guidance molecules,
regulates growth cone behavior by inducing rapid changes in local protein levels (Campbell and Holt, 2001). Also guidance receptors can be regulated via ubiquitination (Myat et al., 2002) and the UPS also plays a role in formation of new growth cones and in axonal regeneration after axotomy (Verma et al., 2005). In addition, modification of established axonal connections can be regulated by the UPS. This process of local degeneration of the distal ends of an axon, axon pruning, requires both the ubiquitination machinery and proteasomal activity in Drosophila (Watts et al., 2003).

The formation of synapses also requires a tight balance between protein ubiquitination and deubiquitination. The UPS not only plays an important role in the development of synapses, it also regulates synaptic transmission and synaptic strength, for instance by regulating the levels of neurotransmitter receptors (reviewed in (Yi and Ehlers, 2007)). Turnover of many other proteins within the post-synaptic density is mediated by ubiquitin-dependent degradation and an increasing number of substrates for ubiquitination and proteasomal degradation are currently being elucidated (Ehlers, 2003). It is thus not surprising that the UPS is involved in the modulation of synaptic plasticity, first shown in Aplysia, where long-term facilitation was eliminated after inhibition of the proteasome (Hegde et al., 1997). More recently, it was shown that a tight balance between protein synthesis and degradation determines the expression of hippocampal late long-term potentiation (LTP) (Fonseca et al., 2006). Thus, as summarized in Table 1, ubiquitination and proteasomal degradation of proteins by the UPS as well as post-translational ubiquitin modification of proteins may have an influence on the development of synapses and synaptic strength on both the pre- and post-synaptic side of the synaptic cleft (reviewed in (DiAntonio and Hicke, 2004; Yi and Ehlers, 2007)).

**UPS in neurodegenerative diseases**

As the UPS fulfills an important role in many processes in neurons during development as well as in fully differentiated neurons, it is not surprising that a diminished function of the UPS is implicated in a broad array of neurological diseases. A direct linkage between UPS...
malfunction and disease pathogenesis is the accumulation of ubiquitin conjugates and other UPS-related components in the neuropathological hallmarks of many neurodegenerative diseases, including Alzheimer’s disease (AD), Parkinson’s disease (PD), polyglutamine diseases, such as Huntington’s disease (HD) and spinocerebellar ataxias (SCAs), amyotrophic lateral sclerosis and prion disease (reviewed by (Ciechanover and Brundin, 2003)). The discovery of familial variants of PD caused by genetic mutations in UPS-related genes (Kitada et al., 1998; Leroy et al., 1998) substantiated the importance of the UPS in neurodegenerative disease. However, for most diseases the precise mechanism by which (altered) UPS activity mediates disease progression is not fully understood.

There are currently two main views on the biological relevance of the ubiquitin-containing aggregates in disease: (1) they can be seen as the result of effective ubiquitination of target proteins followed by a reduced activity of the proteasome or (2) they could be a protective mechanism of the cell to confine aberrant or misfolded proteins to inert inclusions. Intracellular aggregated proteins are often sequestered into inclusion bodies (IBs), which regularly contain components of the UPS and, in some cases, molecular chaperones and intermediate filaments (reviewed in (Kopito, 2000)). In mammalian cells, aggregates can be actively transported via the microtubuli network to the aggresome, a specialized IB-like ubiquitin-rich structure at the microtubule organizing centre (Johnston et al., 1998). Through sequestration into aggresomes, aggregation-prone proteins can be removed from the cell via transfer to the lysosome, possibly by an autophagic process (Taylor et al., 2003). It is conceivable that not the IBs themselves but rather the early protein aggregate-intermediates (oligomers) are toxic. These oligomers are physically separated from other cellular compartments when trapped into IBs. Indeed, aggregated proteins can inhibit the UPS (Bence et al., 2001; Diaz-Hernandez et al., 2006) before IBs are present (Bennett et al., 2005). Also, in a cell line model of HD, the formation of IBs is predictive of increased cell survival and decreases the aberrant protein load in the cell (Arrasate et al., 2004). Obtaining conclusive evidence on the precise contribution of ubiquitin-containing aggregates and IB formation to disease pathogenesis will remain a challenge; the protein aggregation seems to result from UPS malfunction; however, these aggregated proteins also hold UPS inhibitory properties themselves.

General factors influencing UPS function could contribute to the pathogenesis of neurodegeneration. During aging, a main risk factor for many of these diseases, proteasome activity progressively declines in various tissue types, including nervous tissue (Keller et al., 2000). Oxidative stress can influence proteasome activity levels, either by oxidative modification of the 26S proteasome itself or by increased levels of oxidative proteins which interact with the proteasome during aging (reviewed in (Carrard et al., 2002; Keller et al., 2002)). In turn, decreased chymotryptic proteasome activity hyper-sensitizes cells to oxidative stress, resulting in accumulation of ubiquitinated proteins (Li et al., 2004). ER stress negatively influences proteasome activity and so potentially contributes to the
aberrant protein accumulation seen in neurodegenerative disorders (Menendez-Benito et al., 2005). Furthermore, decreased ribosome functioning and impairments in protein synthesis are associated with early AD (Ding et al., 2005), two processes which can be induced by inhibition of proteasome activity (Ding et al., 2006; Stavreva et al., 2006).

In the following sections, the relation between the UPS and disease pathogenesis will be briefly discussed for three types of neurodegenerative disorders: AD and other tauopathies, PD, and HD and other polyglutamine diseases.

Alzheimer’s disease and other tauopathies

AD is the most common form of dementia and characterized by extracellular plaques consisting of amyloid-β (Aβ), and intraneuronal tangles and neuropil threads consisting of hyperphosphorylated tau (Hardy and Selkoe, 2002). Already two decades ago it was observed that neurofibrillary tangles are ubiquitin positive, as well as the dystrophic neurites surrounding the plaques (Mori et al., 1987; Perry et al., 1987) (Figure 2). There are now many indications that the UPS is involved in the pathogenesis of AD (reviewed by (de Vrij et al., 2004; Scheper and Hol, 2005)). It has been shown that proteasome activity is diminished in affected brain areas of AD patients, such as the hippocampus and temporal cortex (Keller et al., 2000) and that the enzymatic ubiquitination machinery is defective in cortical brain areas in AD (Lopez Salon et al., 2000). The two predominant proteins accumulating in AD directly diminish UPS activity; i.e. paired helical filaments (tau) isolated from AD brain inhibit the proteasome via the 20S core (Keck et al., 2003) and Aβ holds proteasome inhibitory properties in a cell-free system (Gregori et al., 1995), in neuronal primary cultures (Lopez Salon et al., 2003), and also in several AD transgenic mouse models (Oh et al., 2005; Almeida et al., 2006; Tseng et al., 2007). The UPS inhibition observed in AD is not an intrinsic property of the 20S core, as purified 20S from AD brain does not show a decreased proteasome activity (Gillardon et al., 2007). Aβ toxicity could be mediated by the E2-conjugating enzyme E2-25K, via ubiquitination of E2-25K substrates which affect proteasome activity in concert with Aβ (Song et al., 2003). Immunisation of triple transgenic AD mice, showing Aβ and tau pathology, with Aβ antisera reverses amyloid pathology as well as early hyperphosphorylated tau pathology. This latter process was shown to be mediated through the proteasomal pathway (Oddo et al., 2004), stressing the importance of this pathway in the neuropathology of AD. UCHL1, a DUB responsible for the recycling of ubiquitin, is associated with AD tangle pathology. The level of soluble UCHL1 protein is inversely correlated to the number of tangles in the brains of AD patients (Choi et al., 2004). Indeed, soluble UCHL1 protein levels are reduced in the hippocampus of an AD transgenic mouse model with APP and PS mutations, accompanied by a decrease in hydrolyase activity. The defects in synaptic functioning and in learning and memory present in this AD model can be reversed by exogenous neuronal
expression of UCHL1 (Gong et al., 2006). For a more detailed description of the role of UCHL1 and UCHL3 in neuronal function, see section “UCH-Lx mutant mice”.

Ubiquitinated deposits can also be found in the disease hallmarks of many other diseases with tau pathology (taupathies, (Lee et al., 2001)). Ubiquitination of soluble tau occurs on three internal lysine residues and the resulting polyubiquitin chain is primarily linked via K48, indicating that tau is indeed a target for degradation by the UPS (Cripps et al., 2006). Tau ubiquitination is mediated by the U-box E3/E4 CHIP (C-terminal Heat-shock protein 70 Interacting Protein), together with Hsp70. CHIP binds tau through its microtubule-binding domain, thereby allowing the ubiquitination of tau, which ultimately leads to an increase in the level of insoluble aggregated tau (Petrucelli et al., 2004; Shimura et al., 2004). Together, these data implicate an important role for the UPS and the chaperone-folding system in the pathogenesis of AD. The possible cross-talk between these systems might be mediated by CHIP (also see section “CHIP KO mice”).

Parkinson’s disease
The main neuropathological feature of PD is degeneration of the dopaminergic neurons in the substantia nigra (SN) pars compacta, accompanied by cytoplasmic protein inclusions. These inclusions, known as Lewy bodies, contain many ubiquitinated normal and aberrant proteins, including α-synuclein, neurofilaments and components of the UPS machinery (reviewed in (Ciechanover and Brundin, 2003) (Figure 2). A direct linkage between UPS malfunction and neurodegenerative disease is probably best exemplified by PD, as autosomal-dominant mutations in UPS enzymes appear to be causative for a percentage of PD cases. The most common form of familial PD, Autosomal Recessive-Juvenile Parkinsonism (AR-JP), results from mutations in the RING-E3 ligase parkin, which most likely leads to a decrease of function of this enzyme (Kitada et al., 1998). Parkin is a RING-E3 enzyme capable of forming various types of ubiquitin linkages on substrate proteins (K48-linked, K63-linked and (multiple) mono-ubiquitination), indicating a role for this enzyme beyond substrate poly-ubiquitination routing substrates to proteasomal degradation (reviewed by (Moore, 2006)). The Pael receptor is one of the many parkin substrates (together with the E4 activity of CHIP to establish poly-ubiquitination) and defective ubiquitination induces misfolding of this receptor leading to activation of the unfolded protein response in the ER (Imai et al., 2001; Imai et al., 2002). Also the α-synuclein interacting protein synphilin-1 is a parkin substrate for K48- and K63-linked ubiquitination (Chung et al., 2001; Lim et al., 2005). Various parkin knockout (KO) mouse models have been developed, showing varying severity of PD pathology, described in section “Parkin KO mice”.

A mutation residing in the DUB UCHL1 gene leads to a diminished function of this enzyme and could be causative of autosomal dominant familial PD in one German family
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(Leroy et al., 1998). The in vitro physiological function of UCHL1 is to cleave small ubiquitin C-terminal adducts (Larsen et al., 1996), and also to provide single ubiquitin molecules to ubiquitinate substrate proteins (Larsen et al., 1998), further described in section “UCH-Lx mutant mice”. A decrease of catalytic activity of UCHL1 through the PD mutation could putatively lead to a shortage of free ubiquitin. On the other hand, dimeric UCHL1 contains E3 activity, which enhances α-synuclein ubiquitination. A polymorphism for UCHL1, which decreases this ligase activity, has a possible protective effect on PD (Liu et al., 2002). In agreement with these observations, UCHL1 protein levels are decreased in sporadic PD and the protein is a target for oxidative damage, the latter possibly interfering with its deubiquitinating activity (Choi et al., 2004). As is the case in AD, in PD, the activity of the proteasome is modestly decreased in the affected brain areas (McNaught and Jenner, 2001). Also the composition of the proteasome is altered, as there is a selective substantial decrease of α-subunits and 19S regulatory particles in the dopaminergic neurons in the SN (McNaught et al., 2002; McNaught et al., 2003).

Huntington’s disease and other polyglutamine diseases

The family of polyglutamine (polyQ) diseases is characterized by an expansion of CAG tri-nucleotide repeats in genes, such as huntingtin and the ataxin genes, leading to an extension of the encoded polyQ stretch. The best-known polyQ disease is HD, an autosomal dominant neurological disorder caused by an expanded CAG repeat in the huntingtin

![Image](image_url)

**Figure 2**  *Ubiquitin-positive pathology in neurodegenerative disease.* One of the neuropathological hallmarks in AD is accumulation of abnormally phosphorylated tau in the intra-neuronal tangles (A). These tangles also contain the ubiquitin protein (B). In PD patients, α-synuclein (C) and ubiquitin (D) co-localize in intra-neuronal aggregates (Lewy bodies). Immuno-stainings were performed on 200 μm hippocampal vibratome sections of a 92-year-old female AD patient (A, B) or on 6 μm paraffin sections of the gyrus cinguli of an 80-year-old female patient with Lewy bodies (C, D) using antibodies directed against abnormally phosphorylated tau (MC-1; Davies, P., New York, USA), ubiquitin (Z0458, Dako) and α-synuclein (clone KM51, Novacastra). Scale bar = 0.025 mm.
gene. The pathological hallmarks of HD consist of nuclear and cytoplasmic huntingtin aggregates gathered into IBs. Besides mutant huntingtin, these IBs also contain many other proteins, including ubiquitin and UPS related proteins, indicating a role for the UPS in HD pathology (reviewed in (Valera et al., 2005)). The current views on how huntingtin aggregates and IBs interfere with UPS function are still quite controversial. In vitro, polyQ aggregates, although resistant to proteasomal degradation, do not induce UPS inhibition (Verhoef et al., 2002), although in other studies aggregated huntingtin does induce inhibition of the UPS (Bence et al., 2001; Bennett et al., 2005; Bennett et al., 2007) and relocalization of proteasome components to the huntingtin aggregates leads to a decrease in UPS activity (Jana et al., 2001). Also, proteasomal changes are observed in HD, resulting in a decrease of proteasome activity in the brain of HD patients (Seo et al., 2004) and the induction of 20S immuno-proteasome subunits (Diaz-Hernandez et al., 2003). However, total proteasome content and activity are unaltered compared to non-diseased subjects in this study. Contradicting these observations are results from transgenic mouse studies showing that UPS impairment is not necessarily connected to polyQ pathology in a SCA-7 model (Bowman et al., 2005) and that proteasome activity remains unaltered in HD transgenic mice (Bett et al., 2006).

CHIP is one of the proteins able to suppress polyQ toxicity, again showing a link between the UPS, the chaperone system and a neurodegenerative disease process (Miller et al., 2005). Also the ubiquitin conjugating E2 enzyme E2-25K (or huntingtin interacting protein 2) interacts directly with huntingtin and may mediate ubiquitination of this protein (Kalchman et al., 1996). Recently it was shown that E2-25K is involved in polyQ aggregate formation and mediates polyQ-induced cell death, depending on the C-terminal catalytic domain of the enzyme essential for ubiquitination of substrate proteins. In human postmortem brain, the E2-25K enzyme is present in a subset of the neuronal IBs found in HD and SCA3 patient material, again indicating the relevance of ubiquitination for the pathology of polyQ disease (de Pril et al., 2007).

In 2006, Diaz-Hernandez et al. showed that the observed UPS inhibition in HD could be dissected into differential effects of aggregated huntingtin on 20S and 26S proteasome activity. Ubiquitinated filamentous huntingtin aggregates from HD transgenic mice co-localized with the 19S proteasome cap particles, resulting in 26S, but not 20S, proteasome inhibition. In addition, ubiquitin and mutant huntingtin positive filamentous aggregates isolated from human HD patients inhibited 26S proteasome activity without altering 20S activity. In contrast, intact IBs isolated from the same HD mice did not induce UPS inhibition in the 26S or 20S proteasome fraction. These data suggest that the ubiquitinated form of filamentous aggregates could be detrimental to the neuron, whereas entrapment of these aggregates into larger IBs could be cytoprotective (Diaz-Hernandez et al., 2006). Ubiquitinated inclusions are also found in other polyQ diseases, indicating a general mechanism of UPS involvement in polyQ pathology. A number of SCAs are caused by
polyQ expansion in ataxin genes. A puzzling correlation between UPS dysfunction and polyQ disease is found in SCA-3 (Machado-Joseph disease), which results from a polyQ expansion in ataxin-3, a protein with deubiquitinating activity and able to bind ubiquitinated proteins through its ubiquitin interacting motif (UIM) (Burnett et al., 2003; Burnett and Pittman, 2005). An overview of murine models harboring ataxin-3 mutations is given in section “Ataxin-3 transgenic mice”.

Mutant ubiquitin (UBB+1)

Another indication of UPS malfunction in neurodegenerative disease is the occurrence of a mutant form of ubiquitin in many neurodegenerative disorders. This mutant ubiquitin (UBB+1) results from a di-nucleotide deletion in the ubiquitin B mRNA. The resulting UBB+1 protein has a 19 amino acid C-terminal extension, with the ubiquitin C-terminal G76, normally required for ubiquitinating substrates, mutated in Y76. UBB+1 accumulates in the neuropathological hallmarks of AD and Down syndrome patients (van Leeuwen et al., 1998; van Leeuwen et al., 2006). Moreover, UBB+1 co-localizes with huntingtin aggregates in HD and with ataxin-3 in SCA-3 (De Pril et al., 2004).

UBB+1 has lost the ability to ubiquitinate substrates as it lacks the C-terminal glycine. In turn, UBB+1 is a ubiquitin-fusion degradation (UFD) substrate for proteolytic degradation: UBB+1 is poly-ubiquitinated and directed to the 26S proteasome for degradation (De Vrij et al., 2001; Lindsten et al., 2002). Furthermore, ubiquitinated UBB+1 is refractory to deubiquitination by the DUB isopeptidase-T (Lam et al., 2000). When the protein is expressed at high levels, it is a potent inhibitor of the UPS. These dual UPS substrate/inhibitory properties of UBB+1 are dose-dependent; UBB+1 shifts from a proteasome substrate at low expression levels to a UPS inhibitor at high expression levels (van Tijn et al., 2007). At these high expression levels, UBB+1 induces cell cycle arrest, neuritic beading and mitochondrial stress, apoptotic-like cell death and a heatshock response (De Vrij et al., 2001; Lindsten et al., 2002; Hope et al., 2003; Tan et al., 2007). The frequency of UBB+1 mRNA is very low (1:10^5 to normal ubiquitin mRNA) and UBB+1 mRNA levels of diseased and control patients do not differ significantly (Fischer et al., 2003; Gerez et al., 2005). As the UBB+1 protein is only found in diseased subjects and aged subjects showing neuropathology, accumulation of the protein could reflect a downstream feature of disease pathogenesis. These data suggest that UBB+1 normally is degraded and that UPS inhibition, induced by e.g. disease pathology, initiates accumulation of the protein. In human, the accumulation of UBB+1 can be seen as an endogenous marker for proteasomal dysfunction (Fischer et al., 2003).

It is interesting to note that UBB+1 accumulation is disease-specific as the protein is present in all tauopathies studied, but it is not present in synucleinopathies such as PD (Fischer et al., 2003). UBB+1 was shown to accumulate occasionally in α-synuclein con-
taining neuronal aggregates in the entorhinal cortex in subjects with combined multiple system atrophy and AD (Terni et al., 2007). It must be noted that in this brain area, α-synuclein pathology coexisted with tau inclusions, thus it is conceivable that UBB^+1 only accumulated in neurons containing both tau and α-synuclein. This discrepancy between tauopathies and synucleinopathies concerning UBB^+1 accumulation could reflect an intrinsic difference in the disease mechanisms; UPS inhibition might be a general disease characteristic in tauopathies leading to accumulation of many substrates, including UBB^+1. In the case of synucleinopathies, dysfunction of a specific UPS component, e.g. an E3 ligase, will result in a substrate-specific degradation impairment or a transient (partial) decrease of UPS activity (Fischer et al., 2003; Hol et al., 2005). We have generated several UBB^+1 transgenic mouse models to further elucidate the effect of UBB^+1 expression on neuronal functioning. Similar to our previous observations in cell lines, we observed that high levels of neuronal UBB^+1 expression led to a modest decrease in proteasome activity in vivo, accompanied by cognitive deficits, further indicating that intact proteasome activity is a prerequisite for proper neuronal functioning (Fischer et al., 2004; van Tijn et al., 2005).

**Mouse models of the UPS**

Recognition of the pivotal role of the UPS in many cellular pathways has led to the introduction of an array of in vivo models with an altered UPS. These were generated through two different approaches; (1) altered UPS function resulting from expression of (aberrant) proteins which modify UPS activity or (2) mutation of an intrinsic component of the UPS machinery, e.g. an E3 or DUB, resulting in a phenotype with disturbed UPS function. Targeting of transgene expression or protein knockdown to specific tissues make these in vivo models essential in understanding the mechanism of UPS action. They also provide an opportunity to test potential therapeutic agents which target the UPS in vivo. In this review, we will focus on mouse models with an alteration of components of the UPS machinery leading to a neurological phenotype (section “Mouse models of neurodegenerative disease induced by an altered UPS”, Table 2). In addition, we will briefly discuss the current developments in monitoring UPS activity in vitro and in vivo using UPS reporter proteins (section “Mouse models to measure UPS activity in vivo”, Table 3).

**Mouse models of neurodegenerative disease induced by an altered UPS**

**Parkinson’s disease mouse models**

The majority of the neurodegenerative genetic mouse models for PD harbor mutations in genes which encode proteins associated with PD (e.g. α-synuclein and parkin). Most mouse models expressing PD mutations show only a partial PD phenotype; mice with
parkin mutations show pathology found in the early stages of PD, including defects in the nigrostriatal pathway without massive loss of DA neurons (reviewed in (Fleming et al., 2005)). In 2004, McNaught et al. published a controversial PD model in rat directly linking the UPS to the development of PD pathology. Systemic injection of UPS inhibitors in adult rats induced a Parkinsonian phenotype, exemplified by gradual progressive motor deficits. In addition, these rats were reported to show PD associated DA cell loss in the SN pars compacta, as well as neuronal degeneration in other brain regions affected by PD. Lewy body-like neuronal IBs containing α-synuclein and ubiquitin were shown to be present in the affected brain areas (McNaught et al., 2004). However, this promising model for PD has become increasingly controversial, as attempts by various laboratories to replicate the abovementioned results have only been partially successful (Beal and Lang, 2006). Several other studies did show that direct infusion of UPS inhibitors in the rat striatum or SN induced degeneration of nigrostriatal DA cells accompanied by neuronal inclusions resembling Lewy bodies (McNaught et al., 2002; Fornai et al., 2003; Miwa et al., 2005).

**Parkin KO mice**

A subset of PD mouse models have a partial deletion of the Park2 gene (similar to human familial autosomal recessive juvenile Parkinson (AR-JP) caused by PARK2 mutations), leading to loss of function of the parkin RING-finger E3 protein. The pathology observed in human AR-JP closely resembles the pathology of idiopathic PD (motor impairments accompanied by DA neuron loss in the SN), but Lewy body pathology is absent.

The first two parkin KO models were engineered by deleting exon 3 of the Park2 gene, giving rise to the absence of the parkin protein (Goldberg et al., 2003; Itier et al., 2003). In both models, brain morphology and cellular structure in the SN were normal without nigrostriatal DA neuron loss. Modest modifications in the DA system were present; parkin KO mice showed increased levels of extracellular DA in the striatum, as well as a decreased synaptic excitability of the striatal neurons, probably arising from a postsynaptic deficit. Parkin KO mice performed poorly in the beam transversal task, a behavioral task sensitive to nigrostriatal deficits. The levels of parkin E3 ligase substrates CDCrel-1, synphilin-1 and also levels of α-synuclein were unaltered (Goldberg et al., 2003), whereas proteomic analysis of ventral midbrain tissue of these mice revealed changes in proteins involved in regulation of mitochondrial function and oxidative stress (Palacino et al., 2004). In the parkin exon 3 deletion model described by Itier et al., striatal levels of DA transporter protein were decreased. DA levels were increased in the limbic system, as well as DA metabolism by monoamine oxidase. Also pre-synaptic electrophysiological changes (inhibition of glutamate release) were found in the hippocampus. Compared to wild-type mice, these mice showed a decline in exploratory behavior and decreased alternation in a T-maze (Itier et al., 2003). Proteomic analysis of brain tissue
from these mice revealed 12 classes of differentially regulated proteins, the main functional category being energy metabolism proteins. Also changes in protein processing pathways were present, including UPS-mediated protein degradation. Parkin E3 ligase substrates such as CDCrel-1 and synaptotagmin I were slightly up-regulated (Periquet et al., 2005).

In contrast, disruption of the nigrostriatal DA system was absent in a PD model with a mutation in parkin exon 7 encoding the first RING domain (Von Coelln et al., 2004). In this parkin KO model a significant reduction of catecholaminergic neurons in the locus coeruleus was observed already at 2 months of age, accompanied by a loss of noradrenalin in selective target regions of locus coeruleus axonal projections (olfactory bulb and spinal cord). This resulted in a decreased acoustic startle response, a behavioral process mediated by noradrenergic neurotransmission (Von Coelln et al., 2004). The mechanism by which loss of the E3 function of parkin modulates neuron loss in the locus coeruleus is not yet understood. Analysis of known parkin substrates in these mice showed an increase in only one parkin substrate, the aminoacyl-tRNA synthetase cofactor p38/JTV-1 (Ko et al., 2005). The loss of neurons in the locus coeruleus may well mimic early PD pathology, as in human sporadic PD neuron loss is more pronounced in the locus coeruleus than in the SN and pathology arises earlier in this brain region during PD progression (Braak et al., 2003; Zarow et al., 2003).

Several years ago, the spontaneous mouse model quaking viable (qkv) was found to harbor a ~1 Mb deletion on mouse chromosome 17 resulting in the altered splicing of the quaking gene. In addition, a proximal region was deleted containing the first five coding exons of Park2, the Park2 co-regulated gene (Pacrg) and the joint Park2/Pacrg promoter region. This deletion resulted in the absence of Park2 and Pacrg mRNA as well as the absence of the resulting proteins in homozygous qkv mice (Lockhart et al., 2004). The main phenotype of qkv mice consists of demyelinization of the central nervous system in combination with locomotor deficiencies and tremor of the hind limbs (Sidman et al., 1964). Together with the previously described parkin KO models, these mice also showed an altered DA regulation resulting in an increased DA metabolism, but without changes in the DA levels in the nigrostriatal and mesolimbic systems (Nikulina et al., 1995). Contrary to the neuron loss in the locus coeruleus in the exon 7 parkin KO mice, qkv mice showed an increased noradrenergic neuron count in the locus coeruleus (Le Saux et al., 2002).

In contrast to the earlier observations in parkin-deficient mice, an exon 2 parkin KO model with a deleted ubiquitin-like domain did not exhibit a Parkinsonian phenotype even though functional parkin protein could not be detected (Perez and Palmiter, 2005). This discrepancy may originate in the different genetic backgrounds, which influences e.g. behavioral performance. Varying types of parkin mutations could also induce different splicing patterns of the parkin gene. A possible explanation for the absence of a PD phe-
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notype in the parkin exon 2 KO mice could be a redundancy between E3 ligases present in the mouse brain, which compensates for the loss of parkin E3 ligase activity (Perez and Palmiter, 2005). Already in the parkin KO models which do show a Parkinsonian phenotype, contrasting observations indicate a variable effect of the parkin E3 deletion, such as the decrease in neurons in the locus coeruleus in the exon 7 mutant (Von Coelln et al., 2004), as opposed to the increase in locus coeruleus neurons in the qk mice (Le Saux et al., 2002). It remains to be investigated whether loss of parkin activity has an effect on proteasome function in vivo, as does the precise mechanism by which loss of an E3 ligase induces a partial PD phenotype.

UCH-Lx mutant mice
At approximately 6 months of age, the spontaneous autosomal recessive mutant gracile axonal dystrophy (gad) mice develop sensory ataxia, followed by tremor, moving difficulties and hind-limb muscle atrophy, leading to premature death (Yamazaki et al., 1988). On a neuropathological level, the primary defects in the gad mice include axonal dystrophy of the gracile tract and degeneration of the gracile nucleus in the medulla. The axons show a “dying back” degenerative phenotype and spheroid body formation occurs in the nerve terminals of the axons in the gracile nucleus surrounded by projections of reactive astrocytes (reviewed in (Kwon and Wada, 2006)). A decade later, it was shown that this phenotype resulted from an in-frame deletion of exons 7 and 8 of the Uchl1 gene, coding for the ubiquitin C-terminal hydrolase Uchl1 (Saigoh et al., 1999).

This mouse model was the first to link a defective ubiquitination machinery to a neurodegenerative phenotype in vivo. The deletion in the Uchl1 gene resulted in the formation of Uchl1 protein lacking the catalytic site residue. However, the phenotype of gad mice does not resemble a Parkinsonian phenotype, as might be expected since a UCHL1 mutation in human has been identified in one PD family. Interestingly, abnormal accumulation of proteins did occur in this model, such as a diffuse intracellular accumulation of amyloid precursor protein, and probably also Aβ. To some extent this resembles AD pathogenesis, where Aβ deposition in fibrillary and dense-core plaques takes place accompanied by axonal degeneration and glial cell activation (Ichihara et al., 1995). In addition, ubiquitinated “dot-like” structures and increased proteasome immunoreactivity were observed in the affected brain regions in the gad mouse, further connecting malfunctioning of the UPS to this model (Wu et al., 1996; Saigoh et al., 1999). The small DUB Uchl1 is normally involved in generating monomeric ubiquitin (Larsen et al., 1998) and associates with mono-ubiquitin in vivo, preventing its degradation by extending the half-life of the protein (Osaka et al., 2003). In line with these observations, loss of Uchl1 function in gad mice induced a 20-30% decrease in neuronal mono-ubiquitin levels. In mice overexpressing Uchl1, the opposite effect was found, i.e. an increase in free mono-ubiquitin levels. It is possible that these decreased levels of free ubiquitin affect the ubiquitination of substrates...
and initiate accumulation of proteins which would normally be degraded by the UPS (Osaka et al., 2003).

When a mutant form of human UCHL1, found in familial PD patients (UCHL1 I93M), is expressed in a transgenic model, a different phenotype emerges. High expression levels of UCHL1 I93M led to a 30% reduction in TH-positive DA neurons in the SN at 20 weeks of age and also to a decrease in DA levels in the striatum, accompanied by neuropathological features, such as cytoplasmic inclusions positive for UCHL1 and ubiquitin and dense-core vesicles in neurons of the SN. Unlike Lewy bodies, the ubiquitin-positive inclusions were not eosinophilic and did not stain for α-synuclein. These mice showed a mild behavioral defect in spontaneous voluntary movement. This model is the first mouse model for familial PD which shows DA cell loss in the SN. However, the 30% decrease in DA cell number appears to be insufficient to induce a complete PD phenotype in these mice (Setsuie et al., 2007).

The ubiquitin C-terminal hydrolases UCHL1 and UCHL3 display 52% sequence identity and are probably functionally redundant to some extent. However, the expression patterns differ; the Uchl3 transcript is widely present, whereas Uchl1 expression is confined to brain and testis. As Uchl1 deficient mice (gad mice) show a neurological phenotype, it could be expected that loss of Uchl3 also induces a phenotype. However, mice lacking functional Uchl3, due to a deletion of exons 3-7 in the Uchl3 gene, did not show an overt developmental or adult phenotype and no histological defects were found in any of the tissues studied, including brain tissue (Kurihara et al., 2000). Further analysis did show a small defect in the dorsal root ganglion cell bodies and dystrophic axons were found in the nucleus tractus solitarius and area postrema (Kurihara et al., 2001). When these Uchl3Δ3-7 mice were crossed with gad mice, the neurodegenerative phenotype of the gad mice was enhanced and accompanied by an increased weight loss, probably due to dysphagia. A loss of function of either Uch1 or Uchl3 thus led to a distinct phenotype, showing that these DUBs have specific functions. In a double knockout, these phenotypes were enhanced, hinting that Uch1 and Uchl3 also have overlapping functions in maintaining neuronal homeostasis in specific areas of the brain (Kurihara et al., 2001).

Ap-uch, the Aplysia orthologue of Uchl3, is required for long-term facilitation in Aplysia, showing a direct connection between the ubiquitin pathway and memory formation (Hegde et al., 1997). Using several learning and memory paradigms, Wood et al. investigated if murine Uchl3 also plays a role in this process. Indeed, Uchl3Δ3-7 mice showed a significant learning deficit and impaired spatial reference memory in a watermaze, as well as impaired working memory coupled to a slightly impaired reference memory in a radial maze without deficits in long-term potentiation. The direct relation between loss of Uchl3 and defective learning and memory is not clear. A decreased availability of ubiquitin due to defective deubiquitination could affect processes regulated by mono-ubiquitination, and alter proteasomal turnover of as yet undefined substrates of Uchl3 (Wood et al., 2005).
Although a direct correlation between Uchl1 and learning and memory has not been established in the gad mice, it is known that inhibition of Uchl1 activity in mice inhibits hippocampal LTP and that transduction of Uchl1 protein can reverse synaptic defects and contextual memory deficits in AD transgenic mice (Gong et al., 2006). These observations further establish a role for the UCHs in cognitive function.

**Usp14 KO mice (ataxia mice)**

The spontaneous recessive mouse mutant ataxia has provided new insights in the relation of the UPS to synapse function. In these ataxia mice (ax^J), an insertion was found in intron 5 of the Usp14 gene, resulting in a decrease of Usp14 gene expression in the brain of mice to 5% of the Usp14 expression levels in wild-type mice (Wilson et al., 2002). This is reflected in greatly reduced levels (90%-100%) of Usp14 protein expression in brain extracts from homozygous mutants (Wilson et al., 2002; Anderson et al., 2005). ax^J/ax^J mice showed neurological deficits starting with tremors, followed by paralysis of the hind limbs and by death at ~10 weeks of age. Only mild developmental defects were found in the CNS, including under-development of the corpus callosum, hippocampus, dentate gyrus and some regions in the brainstem (D’Amato and Hicks, 1965; Burt, 1980). ax^J/ax^J mice showed no overt neuropathology, including absence of neuronal cell loss and ubiquitin-positive pathology. Wilson et al. demonstrated that ax^J/ax^J mice showed pre-synaptic defects in synaptic transmission at the NMJ, indicative of defective neurotransmitter release. In addition, these mice showed alterations in short-term plasticity in the CA1-CA3 circuit of the hippocampus, also pointing to a pre-synaptic defect (Wilson et al., 2002).

The deficits found in ax^J mice could give more information about the role of the UPS in synaptic transmission in the normal as well as the diseased brain, as synaptic deficits are an early pre-clinical event in e.g. AD (Coleman et al., 2004). USP14 normally functions as a proteasome-associated DUB (Borodovsky et al., 2001). Ubp6 (the yeast homologue of Usp14) appears to have a dual role in proteasome function; it delays substrate degradation by partial inhibition of the proteasome, whilst simultaneously deubiquitinating the same substrate (Hanna et al., 2006). It is not yet understood to what extent the deubiquitinating activity or the proteasome inhibitory activity of Usp14 contributes to the neurological phenotype of these mice. It is conceivable that Usp14 regulates local turnover of substrates involved in synaptic transmission and so induces the synaptic deficits; possible target proteins could be related to synaptic vesicle trafficking and sorting, vesicle docking or endocytosis (Wilson et al., 2002).

In the brain of ax^J/ax^J mice, the levels of monomeric ubiquitin were decreased by 30-40%, indicating that Usp14 is required for maintaining a pool of free ubiquitin (Anderson et al., 2005). Usp14 normally associates with proteasomes extracted from brain tissue in wild-type mice. This association was lost in ax^J mice, most likely attributable to the very
low levels of Usp14 protein. However, this did not affect the proteolytic activity of the proteasome measured with fluorogenic 20S proteasome substrates. In contrast to the observations in yeast, where Usp14 homologue Ubp6 represents the predominant ubiquitin-hydrolyzing activity of the proteasome (Leggett et al., 2002), only a small decrease was found in the ubiquitin hydrolyzing activity in ax1 mice (Anderson et al., 2005). It is thus conceivable that other DUBs, such as the proteasome-associated DUB Uchl5 (synonym UCH37), compensate for the decreased Usp14 levels to maintain ubiquitin hydrolysis at the proteasome. The ataxia phenotype of ax1 mice, including the decreased levels of monomeric ubiquitin and motor deficits, could be rescued by transgenic neuronal expression of recombinant Usp14 (Crimmins et al., 2006).

**Ataxin-3 transgenic mice**

One of the polyQ diseases with a direct link to the UPS is Machado-Joseph disease, also known as SCA-3. This disease is characterized by progressive motor problems due to motor neuron defects. This most common autosomal dominant ataxia is caused by an expansion of the polyQ repeat in the ataxin-3 \((ATXN3)\) gene. Neuropathologically, neuron loss is present in the spinal cord and in several brain regions, such as the brainstem and basal ganglia. IBs are found in the surviving neurons, containing aggregated mutant polyQ protein and UPS associated proteins including ubiquitin (reviewed in (Zoghbi and Orr, 2000)). The normal ataxin-3 protein (with a polyQ repeat length of 12-41) was identified as a protein which binds to ubiquitin through UIM domains (Donaldson et al., 2003) and shows DUB activity, possibly in the N-terminal Josephin domain (Burnett et al., 2003).

Mutant ataxin-3 has a polyQ repeat of 62-84 repeats. Due to alternative splicing, different isoforms of mutant ataxin-3 are formed (Ichikawa et al., 2001), including mjd1a (Kawaguchi et al., 1994) and ataxin-3c (Schmidt et al., 1998).

In the first SCA-3 mouse models, expression of an mjd1a cleavage fragment containing an expanded polyQ stretch (Q79) induced motor deficiencies, including an ataxic phenotype and gait disturbance starting at 4 weeks of age, whereas expression of a control Q35 fragment as well as full-length mjd-Q79 did not induce any phenotype (Ikeda et al., 1996). In the affected mice, the cerebellum was severely atrophic and neuronal Purkinje cell loss was observed, while the cortex morphology appeared normal without neuropathology. It should be noted that in this model transgene expression was driven by the L7 promoter, giving rise to high expression levels in cerebellar Purkinje cells (Ikeda et al., 1996). However, in human SCA-3 pathology the affected cerebellar cells are mainly located in the dentate nuclei (Koeppen, 2005) and no neuronal IBs are found in Purkinje cells (Koyano et al., 2002).

This mjd1a cleavage fragment was not found in a transgenic model expressing a YAC construct encoding the full-length \(ATXN3\) gene with an expanded polyQ stretch and flank-
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In diseased mice, the normally predominantly cytoplasmic ataxin-3 protein was relocated to neuronal ubiquitinated intranuclear IBs in the pontine and dentate nucleus, two areas affected by SCA-3. Areas not implicated in SCA-3 pathology (e.g. hippocampus and striatum) were devoid of IBs. In the mice with an expanded polyQ stretch, motor deficiencies were accompanied by degeneration of the dentate and pontine nuclei and slight atrophy of the Purkinje cell layer (Cemal et al., 2002). Generally, the phenotype in this model was milder and showed slower progression than in the mjd1a-Q79 cleavage model.

More recently a third transgenic model for SCA-3 was presented that expressed high levels of normal (Q20) or expanded (Q71) full length human mjd1a in the brain and spinal cord (Goti et al., 2004). The mjd1a-Q71 mice showed SCA-3-like pathology, including motor deficits, neuronal intranuclear inclusions and cell loss in the SN. These mice also showed weight loss accompanied by a decreased life-span. An ataxin-3 mjd1a cleavage fragment still containing the polyQ stretch was detected in the brains of the mjd1a-Q71 mice. Notably, there was an abundance of the cleavage fragment in affected mjd1a-Q71 transgenic mice compared to mjd1a-Q71 mice with a normal phenotype (Goti et al., 2004), which showed the toxicity of the fragment. It could be that this cleavage fragment recruits the full-length protein into the aggregates in vivo the same way it does in vitro (Haacke et al., 2006). The normal ataxin-3 protein is ubiquitinated and degraded by the UPS (Matsumoto et al., 2004; Berke et al., 2005) and has two or three functional UIMs mediating binding of the protein to ubiquitin chains. These UIMs are required and sufficient to localize ataxin-3 into aggregates in vitro (Donaldson et al., 2003). In Drosophila, the normal ataxin-3 function paradoxically protects against polyQ-induced neurodegeneration by diminishing the aggregation of the mutant ataxin-3 protein. This process requires an intact protease domain and UIMs of ataxin-3 and depends on normal proteasome function, indicating that the UPS mediates this protective effect (Warrick et al., 2005).

Ube3a transgenic mice

The UBE3A gene encodes for one of the first E3 ubiquitin ligases discovered, the ubiquitin-protein ligase E3A (E6-AP), which promotes degradation of several proteins including the p53 tumor suppressor protein in complex with the E6 viral protein (reviewed in Glickman and Ciechanover, 2002)). In Angelman Syndrome (AS), a neurological disorder associated with severe mental retardation, motor problems and seizures, the human UBE3A locus is mutated (by a maternal deletion) (Kishino et al., 1997). The chromosomal region where the UBE3A gene resides is subject to genomic imprinting, i.e. specific expression of the gene according to parental origin. In mice, this mechanism leads to expression of primarily the maternal allele in several brain regions, including the hippocampal neurons and Purkinje cells (Albrecht et al., 1997).
GENERAL INTRODUCTION

The two best-characterized mouse models with an AS phenotype are maternally deficient (m-/p+) for Ube3a. In the AS model developed by Jiang et al., the m-/p+ mice did not show gross phenotypic abnormalities, but displayed motor dysfunction with varying severity (Jiang et al., 1998). Also seizures were more readily induced in the m-/p+ mice or in mice with a total deficiency of Ube3a, as well as continuous abnormalities in EEG recordings in awake active mice. Ube3a protein expression was absent from the hippocampal neurons and Purkinje cells in m-/p+ mice, where this was not the case in wild-type or m+/p- mice, indicating that, indeed, the maternal allele is essential for expression in these brain regions. The hippocampus is important in learning and memory formation; therefore, context-dependent memory and LTP were measured in these mice (Jiang et al., 1998). Indeed, the m-/p+ mice were deficient in a fear-conditioning paradigm for context-dependent memory and showed a defective LTP response, possibly through increased phosphorylation of the calcium/calmodulin-dependent protein kinase II, a protein involved in calcium-dependent signal transduction pathways needed for LTP induction (Weeber et al., 2003). This is also exemplified by the rescue of the AS phenotype in Ube3a m-/p+ mice by introducing a mutation preventing the increased inhibitory phosphorylation of CamKIIα (van Woerden et al., 2007). In addition, the ubiquitination substrate for E6-AP, p53, was increased in the cytoplasm of hippocampal and cerebellar neurons (Jiang et al., 1998).

Similar results were found in a Ube3a maternal deficient AS model by Miura et al.; these mice also showed learning deficits, EEG abnormalities and motor dysfunction (Miura et al., 2002), as well as sleep disturbances in baseline conditions or after sleep deprivation (Colas et al., 2005) and altered Purkinje cell firing (Cheron et al., 2005). The only discrepancy between these two mouse models is the absence of p53 accumulation in the model by Miura et al. The origin of this difference is not yet fully understood, although it indicates that p53 degradation is not essential to the AS phenotype (Miura et al., 2002). The AS mouse models show that the UPS plays an important role in learning and memory, and in LTP formation in mice. Due to Ube3a loss of function, ubiquitination and subsequent degradation of as yet unidentified Ube3a substrates might be defective, as was shown for p53, which could influence learning and memory.

Other UPS defective models for neurodegeneration

Prion disease and mahoganoid mutant mice
Mutations in the prion protein gene are causative for several forms of spongiform neurodegeneration, including Creutzfeld-Jacob disease. The mutations cause conformational changes in the prion protein (PrP), leading to the misfolded PrP-scrapie variant (PrPsc), accumulating in extracellular prion-amyloid aggregates, accompanied by neuronal death, spongiform vacuolation and astrogliosis. There are several indications that the pathogene-
sis of prion disease is modulated by the UPS: (1) PrP can be ubiquitinated and is possibly an ERAD substrate (Yedidia et al., 2001), (2) UPS inhibition promotes aggregation of mutant PrP and also (3) promotes a cytosolic conversion of wild-type PrP to mutant PrP \textit{in vitro} (Ma and Lindquist, 2002; Ma et al., 2002). In addition, (4) prion infection reduces proteosome activity \textit{in vitro} and \textit{in vivo} (Kristiansen et al., 2007).

A null-mutation in the mouse \textit{Mgrn1} gene, responsible for the coat color mutation \textit{mahoganoid}, leads to a phenotype resembling prion disease neuropathology, including progressive spongiform neurodegeneration starting from 2 months of age. Vacuolation of the grey matter and astrogliosis begin around 11-12 months. No accumulation of PrP\textsubscript{sc} was observed in these mice. The Mgrn1 (mahogunin) mRNA has 4 isoforms which express a RING domain, possibly identifying mahogunin as a RING-E3 ubiquitin ligase. Indeed, mahogunin exhibits E3 ligase activity \textit{in vitro}, suggesting that defective substrate ubiquitination might underlie the prion disease phenotype \textit{in vivo} (He et al., 2003). When these \textit{Mgrn1} null mutant mice were analyzed for changes in protein expression, many mitochondrial proteins showed reduced expression levels. Indeed, mitochondrial activity was decreased, leading to mitochondrial dysfunction in animals by 1 month of age. Several other proteins were upregulated in the brain of these mutant mice. Possibly these proteins are normally targets for \textit{Mgrn1}-mediated ubiquitination and subsequent proteasomal degradation (Sun et al., 2007).

\textbf{CHIP KO mice}

The CHIP protein directly connects the chaperone system and the UPS with neurodegeneration. CHIP facilitates proteasomal degradation of Hsp70 bound phospho-tau through its E3 ligase activity (Petrucelli et al., 2004). The majority of CHIP KO mice (Dai et al., 2003) showed a motor deficit, were smaller than wild-type littermates and died prematurely on day 30-35 (Dickey et al., 2006). In these symptomatic CHIP KO mice the Hsp70 levels were decreased. The levels of soluble phospho-tau and total tau were significantly increased, confirming a role for CHIP in turnover of tau protein. Poly-ubiquitinated tau was absent in the CHIP KO model, even though the phospho-tau levels were high. Abnormal tau accumulation consisted of phospho-tau species, but not the conformationally altered tau which is seen in, for instance, AD.

In this model, CHIP proved to be essential for ubiquitination and degradation of phospho-tau. This model could be useful to dissect the role of the chaperone system in tau-related neurodegeneration in human, where the balance between phosphorylation of tau and ubiquitin-dependent degradation of phospho-tau is possibly regulated by CHIP. In disease, a diminished UPS function or increased levels of abnormally phosphorylated tau could disturb this balance. The levels of ubiquitinated phospho-tau then exceed the proteasome capacity for degradation, and ubiquitination of phospho-tau by CHIP will now mediate the aggregation of these tau species into ubiquitin-positive stable aggregates. The
latter mechanism might protect neuronal cells against the excess of possibly toxic soluble (hyper-) phospho-tau species (Dickey et al., 2006).

**Lmp2 KO mice**

In a mouse model lacking functional expression of the low mass protein 2 (Lmp2, Psmb9) β1i subunit of the immuno-proteasome, no substantial abnormalities were found. Proteasome activity levels in the brain remained unchanged, although changes in activity were observed in peripheral tissues (Van Kaer et al., 1994). Recently, proteasomal activity of Lmp2 KO brain tissue was studied in more detail, showing a lower chymotrypsin and peptidyl-glutamyl-peptide hydrolyzing activity at 4 months of age, as well as a more robust age-related decline in 20S and 26S proteasome activity at 12 months of age (Ding et al., 2006). At 3-4 months, these mice showed enhanced motor function accompanied by an increase in body weight. A direct correlation between loss of the Lmp2 proteasome subunit and the increase in motor function remains unclear (Martin et al., 2004).

**Atg KO mice**

Recently it was shown that disturbance of the autophagy system can also induce a neurodegenerative phenotype. Autophagy is a process involved in bulk protein turnover and is especially important for nutrient supply during starvation. Constitutive basal autophagy also plays a role in degradation of cytosolic proteins, as does the UPS, and declined macroautophagy as well as chaperone mediated autophagy are implicated in neurodegenerative disease (reviewed by (Nixon, 2006)). In mice deficient for neuronal autophagy-related 7 protein (Atg7, (Komatsu et al., 2006)) or autophagy-related 5 (Atg5, (Hara et al., 2006)) autophagy was impaired in cells of neural lineage. These mice showed a neurodegenerative phenotype, including motor deficits and massive neuron loss in several brain areas. Strikingly, in both the Atg7 and the Atg5 KO mice IBs containing ubiquitinated proteins appeared in a time-dependent manner, preceded by accumulation of ubiquitinated diffuse abnormal proteins in the Atg5 KO mice and without altering proteasome function in the Atg7 KO mice.

These two mouse models show that the autophagic pathway is important for maintaining intracellular homeostasis and that age-dependent accumulation of ubiquitinated proteins occurs even when the UPS is fully functional. Currently it is still undetermined if the UPS and autophagy operate separately or cooperatively in removing proteins from the intracellular environment in these mice (Hara et al., 2006; Komatsu et al., 2006). Conversely, UPS inhibition can induce autophagy in cell culture and also in Drosophila, possibly to alleviate ER stress induced by proteasome impairment (Ding et al., 2007; Pandey et al., 2007). In addition, autophagy was shown to be the compensatory degradation system for UPS impairment in a Drosophila model for the neurodegenerative disease spinal-bulbar muscular atrophy (Pandey et al., 2007).
### Table 2  
Mouse models for neurodegeneration with a defective UPS

<table>
<thead>
<tr>
<th>Model</th>
<th>Mutation</th>
<th>Function</th>
<th>Neuropathology</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>parkin</td>
<td>parkin</td>
<td>RING-E3</td>
<td>striatal DA level ↑, striatal synaptic excitability ↓, motorfunction ↓, levels of parkin substrates unaltered</td>
<td>(Goldberg et al., 2003)</td>
</tr>
<tr>
<td>KO</td>
<td>Δexon 3</td>
<td></td>
<td>decreased mitochondrial function</td>
<td>(Palacino et al., 2004)</td>
</tr>
<tr>
<td>parkin</td>
<td>Δexon 3</td>
<td>RING-E3</td>
<td>limbic DA level ↑, limbic DA metabolism ↑, striatal DA transporter levels ↓, hippoc. glutamate release inhibited, motorfunction ↓</td>
<td>(Itier et al., 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>protein levels of parkin substrates ↑, altered levels of UPS proteins</td>
<td>(Periquet et al., 2005)</td>
</tr>
<tr>
<td>parkin</td>
<td>Δexon 7</td>
<td>RING-E3</td>
<td>noradrenalin in spinal cord &amp; olf bulb ↓, acoustic startle response ↓, catecholaminergic neuron loss in locus coeruleus levels of parkin substrate p38/JTV-1 ↑, other substrates unaltered</td>
<td>(Von Coelln et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>protein levels of parkin substrates ↑, altered levels of UPS proteins</td>
<td>(Periquet et al., 2005)</td>
</tr>
<tr>
<td>parkin</td>
<td>Δexon 2</td>
<td>RING-E3</td>
<td>no DA or behavioral phenotype</td>
<td>(Perez and Palmiter, 2005)</td>
</tr>
<tr>
<td>quaking</td>
<td>I93M</td>
<td>DUB</td>
<td>sensory ataxia, locomotor deficits, premature death (6 months), axonal dystrophy of the gracile tract, gracile nucleus degeneration</td>
<td>(Yamazaki et al., 1988)</td>
</tr>
<tr>
<td>Uchl1</td>
<td>gac⁺</td>
<td>DUB</td>
<td>diffuse APP and Aβ accumulation, ubiquitin dot-like inclusions, proteasome immunoreactivity ↑, mono-ubiquitin levels ↓</td>
<td>(Ichihara et al., 1995)</td>
</tr>
<tr>
<td>KO</td>
<td>Uchl1</td>
<td></td>
<td>DA neuron loss in SN, striatal DA levels ↓, ubiquitin/UCHL1 positive inclusions, motorfunction ↓</td>
<td>(Wu et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Δexon 7-8</td>
<td></td>
<td></td>
<td>(Saigoh et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Osaka et al., 2003)</td>
</tr>
<tr>
<td>UCHL1</td>
<td>I93M</td>
<td>DUB</td>
<td>no gross phenotypic abnormalities</td>
<td>(Setsuie et al., 2007)</td>
</tr>
<tr>
<td>mutant</td>
<td></td>
<td></td>
<td>abnormal dorsal root ganglion cells, dystrophic axons</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>spatial learning and memory deficits</td>
<td>(Kurihara et al., 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Kurihara et al., 2001)</td>
</tr>
<tr>
<td>Uchl3</td>
<td>Uchl3</td>
<td>DUB</td>
<td>no gross phenotypic abnormalities</td>
<td>(Wood et al., 2005)</td>
</tr>
<tr>
<td>KO</td>
<td>Δexon 3-7</td>
<td></td>
<td>abnormal dorsal root ganglion cells, dystrophic axons</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>spatial learning and memory deficits</td>
<td></td>
</tr>
<tr>
<td>Usp14</td>
<td>ataxia¹</td>
<td>DUB</td>
<td>neurological deficits; tremor, paralysis, premature death (6-10 weeks), slight abnormalities CNS morphology synapti</td>
<td>(D’Amato and Hicks, 1965)</td>
</tr>
<tr>
<td>KO</td>
<td>IAP</td>
<td></td>
<td>tic transmission NMJ and hippoc. ↓, mono-ubiquitin levels ↓</td>
<td>(Wilson et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>exon 5</td>
<td></td>
<td></td>
<td>(Anderson et al., 2005)</td>
</tr>
</tbody>
</table>

¹ Overexpression of floxed alleles in Usp14 KO leads to disease.
### Table 2: Mouse models for neurodegeneration with a defective UPS (continued)

<table>
<thead>
<tr>
<th>Model</th>
<th>Mutation</th>
<th>Function</th>
<th>Neuropathology</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ataxin3</td>
<td>mjd1a-Q79 fragment</td>
<td>DUB</td>
<td>motorfunction ↓, cerebellar atrophy, Purkinje cell loss</td>
<td>(Ikeda et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>ataxin-3 polyQ YAC</td>
<td>DUB</td>
<td>motorfunction slightly ↓, pontine &amp; dentate nucleus atrophy, ubiquitin positive inclusions</td>
<td>(Cemal et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>mjd1a-Q71</td>
<td>DUB</td>
<td>motorfunction ↓, ubiquitin positive inclusions, neuron loss in SN, premature death, mjd1a cleavage fragment detected</td>
<td>(Goti et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ube3a</td>
<td>Ube3a Δexon 2 (maternal)</td>
<td>HECT-E3</td>
<td>motorfunction ↓, inducible seizures, EEG abnormalities, protein levels of Ube3a substrate p53↑, learning and memory deficits, LTP↓ misregulation of hippocampal CamKIIα</td>
<td>(Jiang et al., 1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ube3a Δexon 15-16 (maternal)</td>
<td>HECT-E3</td>
<td>motorfunction ↓, EEG abnormalities, unaltered protein levels of Ube3a substrate p53, learning and memory deficits, sleep disturbances, altered Purkinje cell firing</td>
<td>(Miura et al., 2002)</td>
</tr>
<tr>
<td>Mgrn1</td>
<td>mahoganoid non-agouti curly¹, Mgrn1 mutation intron 9</td>
<td>RING-E3</td>
<td>progressive spongiform neurodegeneration, vacuolation of gray matter, astrogliosis from 11-12 months, mitochondrial dysfunction</td>
<td>(He et al., 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Sun et al., 2007)</td>
</tr>
<tr>
<td>CHIP</td>
<td>CHIP Δexon 1-3</td>
<td>U-box E3</td>
<td>premature death (5 weeks), motorfunction ↓, soluble phospho-tau and total tau ↑, absence of poly-ubiquitinated tau</td>
<td>(Dickey et al., 2006)</td>
</tr>
<tr>
<td>Lmp2</td>
<td>Lmp2 Δexon 2 / intron 2 (partial)</td>
<td>20S β1-immuno subunit</td>
<td>proteasome activity ↓, protein oxidation ↑, motor function ↑</td>
<td>(Ding et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Martin et al., 2004)</td>
</tr>
<tr>
<td>Atg5</td>
<td>Atg5 Δexon 3</td>
<td>autoph. enzyme</td>
<td>motorfunction ↓, massive neuron loss, ubiquitin positive inclusions, diffuse accumulation of ubiquitinated proteins</td>
<td>(Hara et al., 2006)</td>
</tr>
<tr>
<td>Atg7</td>
<td>Atg7 mutation exon 14</td>
<td>autoph. enzyme</td>
<td>motorfunction ↓, massive neuron loss, ubiquitin positive inclusions</td>
<td>(Komatsu et al., 2006)</td>
</tr>
</tbody>
</table>

¹ spontaneous mutant
CHAPTER I

Mouse models to measure UPS activity in vivo

Correlation of proteasome activity with genetic or pharmacological manipulation in mouse models for neurological diseases can also specify the role of the UPS. The activity of the UPS may be monitored using fluorescent substrates, e.g. by monitoring proteolytic cleavage of small fluorogenic substrates. Various probes have been developed for the three different catalytic activities of the proteasome. Most of these substrates are directly processed by the 20S core, without the necessity of the 19S particle and do not require ubiquitination for degradation. The total proteasome content of a sample may also influence the level of measured activity. A second method of measuring UPS activity is monitoring the turnover of well-defined endogenous substrates of the UPS. In this case, the proteasome has to be fully assembled to degrade these substrates. It should be taken into account that proteasome activity is time and cell type-specific and also depends on the ubiquitination capacity (reviewed in (Lindsten and Dantuma, 2003)). Another caveat is that these substrates should be properly ubiquitinated and that they are translation dependent. There are indications that inhibition of the proteasome also affects protein synthesis (Ding et al., 2006), possibly affecting turnover of these substrates.

Vast progress was made in measuring UPS activity by the development of fluorescently tagged UPS reporter substrates (reviewed by (Neefjes and Dantuma, 2004)). These short-lived substrate proteins contain an artificial degron and are degraded by the proteasome. When the UPS is inhibited, these fluorescent substrates will accumulate and may be directly visualized and quantitatively analyzed (Table 3). Dantuma et al. developed various fluorescent reporter substrates by generating ubiquitin-green fluorescent protein (GFP) fusion proteins with a short half-life, which are based on the N-end rule degron. A UFD substrate was developed as well, by attaching an uncleavable ubiquitin moiety to GFP. In this case, the ubiquitin acts as an acceptor for additional ubiquitin moieties forming a K48 linked chain, after which the whole construct, including the GFP, is efficiently targeted for proteasomal degradation. These substrates (e.g. Ub-R-GFP and UbG76V-GFP) give very low background fluorescence in living cells and accumulate readily after treatment with UPS inhibitors (Dantuma et al., 2000). Analogous to these GFP-based substrates, various types of substrates with a yellow fluorescent protein (YFP) tag have also been developed, including a YFP-tagged ERAD substrate based on the T-cell receptor subunit CD3δ, an N-end rule substrate, a UFD substrate and a substrate containing a CL-1 degron (Menendez-Benito et al., 2005), based on the previously described GFP-CL1 (Bence et al., 2001). By developing a transgenic mouse line ubiquitously expressing the UbG76V-GFP construct, it also became feasible to monitor UPS activity in vivo. Transgene expression was confirmed in many tissues ranging from heart to brain tissue, the latter shown by accumulation of the reporter in cultured primary neurons after treatment with proteasome inhibitor and after infection with UBB1 (Lindsten et al., 2003); this also occurred in cortical organotypic cultures of these transgenic mice (van Tijn et al., 2007).
Using this transgenic model, the role of the UPS can be analyzed in vivo and further elucidated by cross breeding this line with neurodegenerative mouse models. One of the first examples hereof is a study by Bowman et al., in which the UbG76V-GFP mice were crossed with a transgenic model for SCA-7, showing that in UbG76V-GFPxSCA7 mice, IBs were not directly linked to UPS inhibition (Bowman et al., 2005).

Another reporter based on the UFD degradation signal is a β-lactamase fused to two UbG76V moieties. This reporter has an in vitro half-life of less than 10 minutes, which decreases even further when more uncleavable ubiquitins are added to the construct (Stack et al., 2000). This mechanism of creating a degradation signal by fusing UbG76V moieties to a substrate was also true for GFP and pro-caspase-3, making this poly-ubiquitin signal a

<table>
<thead>
<tr>
<th>Table 3</th>
<th>UPS reporter constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPS Reporter Construct</td>
<td>Degradation Signal</td>
</tr>
<tr>
<td>Ub-R-GFP; Ub-L-GFP</td>
<td>N-end rule</td>
</tr>
<tr>
<td>Ub-P-GFP; UbG76V-GFP</td>
<td>UFD</td>
</tr>
<tr>
<td>Ub-R-YFP</td>
<td>N-end rule</td>
</tr>
<tr>
<td>UbG76V-YFP</td>
<td>UFD</td>
</tr>
<tr>
<td>CD3δ-YFP</td>
<td>ERAD substrate</td>
</tr>
<tr>
<td>YFP-CL1</td>
<td>C-terminal CL1-degron</td>
</tr>
<tr>
<td>polyUb-β-lactamase</td>
<td>UFD</td>
</tr>
<tr>
<td>Ub-FL</td>
<td>UFD</td>
</tr>
<tr>
<td>GFP*</td>
<td>C-terminal CL1-degron</td>
</tr>
<tr>
<td>GFPdgn</td>
<td>C-terminal CL1-degron</td>
</tr>
<tr>
<td>EGFP-HC (GFP-HLA-A2)</td>
<td>ERAD</td>
</tr>
<tr>
<td>6xHisUb/GFP</td>
<td>-</td>
</tr>
<tr>
<td>6xHisUbK48R/GFP</td>
<td>-</td>
</tr>
<tr>
<td>6xHisUbK63R/GFP</td>
<td>-</td>
</tr>
</tbody>
</table>
general applicable mechanism to tag proteins for degradation (Stack et al., 2000). A similar model involving this principle employs a firefly luciferase-based reporter (Ub-FL) of which the N-terminus is fused to four Ub G76V moieties (Luker et al., 2003). Luker et al. showed that this reporter is degraded in cell lines, unless the UPS was inhibited. In Ub-FL xenografts re-implanted in mice, the background fluorescence was nearly undetectable. After treatment with clinically relevant doses of the UPS inhibitor bortezomib (used to treat multiple myeloma) UPS inhibition was visualized in vivo by measuring the bioluminescence of the Ub-FL xenografts. This approach also allowed multiple measurements of UPS activity over time (Luker et al., 2003), since the measurement can be performed with a lumino-scanner in living animals.

Besides these N-end rule and UFD based UPS reporters, also other UPS reporter constructs have been developed, such as the GFP-tagged ERAD substrate MHC class 1 heavy chain, when co-expressed with the human cytomegalovirus proteins US2 or US11 (Kessler et al., 2001; Fiebiger et al., 2002) and the UPS reporters based on the 16 amino acid long CL-1 degron fused to the C-terminus of GFP (Bence et al., 2001; Kumarapeli et al., 2005). Using this latter CL-1 reporter (GFP-unstable; GFPu), which has a protein half-life of 20-30 minutes, it was shown that protein aggregation induced by expression of a huntingtin fragment with an expanded polyQ repeat or a folding mutant involved in cystic fibrosis lead to accumulation of the GFPu reporter, indicating impairment of the proteasome (Bence et al., 2001). On the other hand, it was shown in a different study, using the previously mentioned Ub G76V-GFP and Ub-R-GFP reporters, that aggregate formation of polyQ proteins did not induce accumulation of these reporters (Verhoef et al., 2002). This discrepancy between the two reporter systems indicates that both might respond to a blockade of different components of the UPS pathway. Using GFPu it was also shown that, in cell lines, overexpression of PD-related mutant α-synuclein decreased UPS activity and increased the sensitivity to proteasome inhibition, an effect rescued by the E3 parkin (Petrucelli et al., 2002). In a transgenic line expressing a similar GFP reporter (GFPdgn), accumulation of GFP was present in many tissues after intravenous and intraperitoneal injection with the UPS inhibitor MG262. However, in contrast to the Ub G76V-GFP transgenic mice, a significant level of background baseline fluorescence was detected. This makes it possible to study not only UPS impairment by measuring accumulation of GFP, but also activation of the UPS, by observing decreases in GFP levels (Kumarapeli et al., 2005).

Furthermore, models have been engineered for monitoring the ubiquitination process. Tsirigotis et al. developed a mouse model expressing a 6xHis-Ub/GFP fusion protein under the UbC promoter to monitor protein ubiquitination. By analyzing epitope-tagged ubiquitin patterns and identifying the ubiquitinated proteins, more can be learned about general ubiquitination in vivo (Tsirigotis et al., 2001). In a similar fashion, transgenic lines were designed with 6xHisUb K48R/GFP or 6xHisUb K63R/GFP fusion constructs to study
defective K48 or K63 ubiquitination. Overexpressing Ub\textsuperscript{K48R} mediated a protective effect against viral insults (Zhang et al., 2003) and other cellular stressors (Gray et al., 2004), and delayed the onset of neurological disease symptoms in a familial amyotrophic lateral sclerosis mouse model (Gilchrist et al., 2005) and in a transgenic mouse model for SCA-1 (Tsirigotis et al., 2006).

**Concluding remarks**

Evidently, the UPS fulfills an important role in maintaining neuronal homeostasis by regulating a variety of processes and by serving as a protein quality control mechanism to rid the cell of aberrant or misfolded proteins. The latter is of especial importance for the pathogenesis of neurodegenerative disease, where abnormal accumulation of proteins can be found in the disease hallmarks. In many of these proteinaceous aggregates, components of the UPS machinery and chaperone proteins are present, indicating a direct link between the UPS and disease. Furthermore, the decreases in proteasome activity, found in, for instance, AD and PD, and the increasing evidence for ubiquitin modification of disease-related proteins, point to an important role for this system in neuropathogenesis. In sporadic forms of neurodegenerative disease it is not yet fully understood if malfunctioning of the UPS is a result of the disease progression or if it is an initial factor in disease onset. This issue is further complicated by the fact that many disease-related proteins are also ubiquitin-modified in a non-diseased state to exert their normal function or to regulate their half-life. The diversity of possible ubiquitin linkages on substrates, including (multiple) mono-ubiquitination and K48-linked and K63-linked poly-ubiquitination, also adds to the complexity of the role of the UPS in neurodegenerative disease.

The current advancements towards resolving the fundamental mechanisms of proteasomal degradation *in vivo*, using the increasing diversity of UPS model systems, will contribute to unraveling the role of the UPS in neuropathogenesis and in the pathogenesis of many other diseases. Further research could also elucidate if there are means by which altering the UPS with chemical compounds, such as UPS inhibitors, or by silencing specific components of the UPS machinery, might alter disease progression in a favorable way (Hol et al., 2006).

**Acknowledgements**

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CHAPTER I

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CHAPTER I

Scope and Outline
SCOPE

Scope and outline of this thesis

The ubiquitin-proteasome system (UPS) is of vital importance for maintaining intracellular homeostasis, as it is the main regulated pathway for degradation of aberrant, misfolded and short-lived proteins. Increasing evidence suggests that impairment of this pathway is involved in the pathogenesis of a broad array of neurodegenerative disorders, which are characterized by ubiquitin-positive protein aggregates. This is further exemplified by a range of murine models, wherein mutations in components of the UPS can induce a neurological phenotype, reviewed in Chapter 1.

Another compelling indication for malfunctioning of the UPS in neurodegenerative disease is the accumulation of a mutant ubiquitin, UBB+1, in the neuropathological hallmarks of tauopathies, including Alzheimer’s disease (AD), and in the hallmarks of Huntington’s disease. The aim of this thesis is to dissect the effects of UBB+1 on UPS function and relate this to neurodegenerative disease in vitro and in vivo.

In Chapter 2, we characterize the in vitro properties of UBB+1 regarding proteasome activity in cell lines and in organotypic cortex cultures. We demonstrate in a human cell line, using a green fluorescent based reporter for proteasome activity, that UBB+1 properties shift from proteasome substrate to a (reversible) proteasome inhibitor in a dose-dependent manner. Also in mouse organotypic cortex slices, UBB+1 accumulation and subsequent UPS inhibition is present only at high expression levels.

These findings are further corroborated in vivo in Chapter 3 and Chapter 4, where we describe the generation and characterization of transgenic mouse models neuronally expressing varying levels of UBB+1. We generated the high expression UBB+1 transgenic lines 3413 and 8630, showing accumulation of the UBB+1 protein mainly in the cortex, hippocampus and striatum, as described in Chapter 3. Although no overt neuropathology is present in these mice, line 3413 transgenic mice do show decreased cortical proteasome activity, accompanied by accumulation of ubiquitinated proteins and alterations in proteins involved in energy metabolism or organization of the cytoskeleton. UBB+1 induced proteasome inhibition also gives rise to a moderate spatial memory deficit in the Morris watermaze and in Pavlovian fear conditioning at the age of 9 months.

In Chapter 4, we describe the transgenic mouse line 6663, expressing low levels of UBB+1. Similar to our previous observations in vitro in Chapter 2, the UBB+1 protein is a substrate for proteasomal degradation at low expression levels in vivo and therefore, the UBB+1 protein is scarcely detectable in the brains of line 6663 transgenic mice. UBB+1 accumulates only after intracranial administration of proteasome inhibitors. Using this transgenic line 6663, we also provide in vivo confirmation that UBB+1 accumulation serves as an endogenous marker for proteasome inhibition.

The relation between UPS inhibition and cognitive behavior is further studied in Chapter 5. We show that the defect in spatial memory in the watermaze at 9 months of
age, described in Chapter 3, persists up to the age of 15 months. This defect is not accompanied by other gross neurological defects. Also motor coordination, assessed using the rotarod paradigm, is not affected in the 3413 UBB$^{+1}$ transgenic mice up to the age of 15 months.

In Chapter 6, we study the effect of UBB$^{+1}$ induced UPS inhibition on AD pathogenesis. Therefore, we crossed the 3413 transgenic mice with a transgenic mouse model for AD, expressing mutant amyloid precursor protein and mutant presenilin 1, showing cerebral amyloid deposition. By measuring the plaque burden in UBB$^{+1}$/AD triple transgenic mice during aging, we demonstrate that UBB$^{+1}$ induced proteasome inhibition significantly decreases the plaque load in the cortex and dentate gyrus at 6 months of age compared to AD control mice, without affecting the levels of UBB$^{+1}$ accumulation.

In Chapter 7, we critically discuss the results presented in this thesis and provide possible mechanisms for UBB$^{+1}$ induced proteasome inhibition. In addition, pathways underlying the cognitive deficits of the UBB$^{+1}$ transgenic mice are further addressed. Finally, the role of UBB$^{+1}$ accumulation and concomitant proteasome inhibition is discussed in relation to human neurodegenerative disease and suggestions for future research are provided.