Mutant ubiquitin and the proteasome in Alzheimer's disease

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Mutant ubiquitin and the proteasome in Alzheimer's disease

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Table of contents

General introduction

Experimental chapters

Discussion

CHAPTER I

Protein quality control in Alzheimer's disease by the ubiquitin proteasome system

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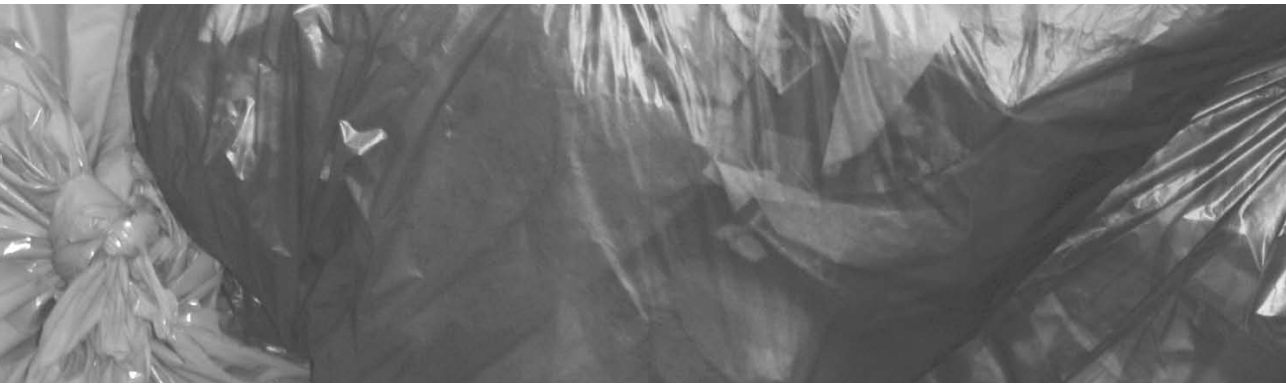

ABSTRACT

The ubiquitin proteasome system (UPS) is the major protein quality control system in eukaryotic cells. Many neurodegenerative diseases are characterized by aggregates and inclusions of aberrant proteins, implying a sub-optimal functioning or defective UPS. The last few years have seen increasing evidence for the involvement of the UPS in neurodegenerative disorders, including Alzheimer's disease (AD). Notably, decreases in proteasome activity were detected in several cortical areas in AD patients. In addition, proteins that accumulate in the classical hallmarks of AD were linked to UPS function. This review specifically discusses the involvement of the UPS in AD pathogenesis. First, a detailed overview of the UPS is presented, after which AD pathology and its relation to the UPS is discussed.

TABLE OF CONTENTS

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia, and affects more than twenty million people worldwide. About 25 to 50 percent of the population aged 85 years and over has AD (Hebert et al., 2003; Ott et al., 1995). Many genetic and non-genetic factors have been implicated in the pathogenesis of AD, but for the non-familial forms the initial cause remains elusive (Rocchi et al., 2003). In the last few years, evidence has accumulated that supports the premise that the ubiquitin proteasome system (UPS) plays a role in many neurodegenerative diseases, including AD. In non-pathological conditions the UPS is involved in a vast array of cellular processes, including protein trafficking, antigen presentation and protein degradation of short-lived proteins, such as transcription factors and synaptic proteins (Hershko and Ciechanover, 1998).

The UPS is also a major player in cellular protein quality control, and is involved in the degradation of misfolded and other aberrant proteins. Most neurodegenerative diseases are characterized by intracellular deposits of aggregated and mis-processed proteins, many of which are proteasomal components and substrates. Furthermore, several mutations in UPS components have been associated with neurodegenerative diseases (Ciechanover and Brundin, 2003), and it is therefore highly conceivable that the UPS is involved in the neuropathogenesis of these diseases.

In relation to AD pathology, the accumulation of ubiquitinated proteins in the neuropathological hallmarks of AD is the pre-eminent characteristic of the disease. Moreover, UPS activity diminishes in the cortex and hippocampus of AD brain and also decreases with aging (Keck et al., 2003; Keller et al., 2000a; Keller et al., 2000c; Lopez Salon et al., 2000). The exact mechanism that impairs the UPS in AD remains obscure, although there are several indications that the general mechanisms underlying AD might have a direct effect on the UPS. In recent years, many excellent general reviews on the UPS have appeared (Berke and Paulson, 2003; Glickman and Ciechanover, 2002; Keller et al., 2002; Pickart, 2004; Shringarpure and Davies, 2002) as well as on its role in neurodegenerative diseases (Ciechanover and Brundin, 2003; Ding and Keller, 2001b; Hernandez et al., 2004; Lang-Rollin et al., 2003; Moore et al., 2003). The present review will focus specifically on Alzheimer's disease pathology and its relation to protein quality control by the UPS.

ALZHEIMER'S DISEASE & PROTEIN QUALITY CONTROL 2

The aberrant and misprocessed proteins that accumulate in AD brain constitute the neuropathological hallmarks of AD. The two most pronounced hallmarks are neurofibrillary tangles (NFT), formed by intracellular accumulations of the hyperphosphorylated protein tau, and plaques, which are extracellular deposits of the 40-42 amino acid amyloid peptide (Aβ), processed from the Amyloid Precursor Protein (APP) (Braak et al., 1998). In 1987, Mori et al. and Perry et al. were the first to describe the presence of ubiquitin in paired helical filaments, the major components of the tangles in AD brains (Mori et al., 1987; Perry et al., 1987). Since then, many others have confirmed these data and ubiquitin immunostaining is now used in many neuropathological labs for diagnosing neurodegenerative diseases. The presence of ubiquitin and ubiquitinated proteins in AD brain were the initial clues suggesting that the UPS was involved in the pathogenesis of AD. Later, our research group discovered an aberrant form of ubiquitin (UBB^{+1}), which also accumulates in the neuropathological hallmarks of AD (Van Leeuwen et al., 1998b). This UBB^{+1} is translated from ubiquitin-B mRNA, which contains a dinucleotide deletion near a GAGAG-repeat (Van Leeuwen et al., 1998b). The two nucleotides are likely to be deleted during or post-transcription, since the mutation cannot be detected in the *UbB* gene of AD patients. UBB^{+1} accumulates in the earliest affected brain areas of patients with AD, such as neurons in the transentorhinal hippocampal cortex area (Van Leeuwen et al., 1998b). All three proteins mentioned above, i.e. tau, amyloid peptide and UBB^{+1}, have in common that they accumulate in AD brain, and all were reported to affect the proteasomal pathway (Fig.1). The connection between these proteins and the UPS in AD will be discussed in detail in paragraph 4.

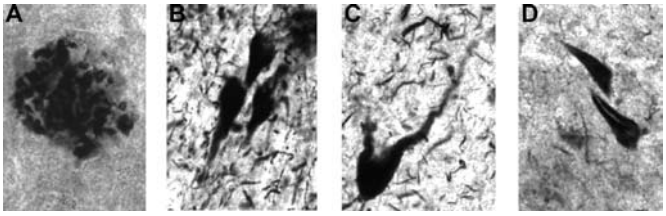

Fig. 1 Proteins that accumulate in Alzheimer's disease. Vibratome hippocampal sections of a 92-year-old female AD patient stained for **A** ßA4, **B** MC1 tau, **C** Ub (DAKO), **D** UBB^{+1} (ubi2).

Direct evidence for involvement of the UPS in AD is abundant (see table 1): (i) ubiquitinated proteins accumulate in AD brain, (ii) proteasome subunit immunoreactivity is detected in disease-related areas, (iii) proteasome activity is decreased in AD brain (Keller et al., 2000a; Lopez Salon et al., 2000) and (iv) different UPS-related mRNA expression profiles were observed in studies with AD brain tissues. The majority of the gene expression profiling studies rarely address UPS components, except for a non-significant difference in Ub expression (Colangelo et al., 2002; Ginsberg et al., 2000). One study with six AD patients mentions down-regulation of the 20S proteasome α5 subunit, the 19S regulator S1 subunit, and a relatively unknown E2 enzyme, similar to Drosophila's bendless gene product (Loring et al., 2001). Another study shows an age-regulated slight down-regulation of three E2 enzymes in frontal brain tissue (Lu et al., 2004). The ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) is also down-regulated in the superior temporal gyrus of five early AD samples, compared to samples of non-demented individuals (Pasinetti, 2001). In contrast, another study reported an upregulation of UCH-L1 in AD (Wang et al., 2003). However, the latter study used only three non-confirmed AD samples, and the brain regions studied were not disclosed.

The UPS controls the levels of most cytosolic and nuclear proteins, while the lysosomal system is responsible for the removal of secretory and internalized proteins. Alterations in lysosomal function are also implicated in AD pathology (Nixon et al., 2000). Another major protein quality control system is present in the endoplasmic reticulum (ER) of cells. All proteins that function in the ER or Golgi apparatus, in the endosomal-lysosomal system, or at the plasma membrane, as well as secretory proteins are first translocated to the ER while they are being synthesized by cytosolic ribosomes. In the ER, the nascent polypeptide chains are bound by ER-resident chaperones that assist protein folding and at first prevent the protein from folding until the entire protein is translated. These ER chaperones are mostly heat shock proteins, like Bip (binding protein), and their levels increase in response to stress. Once a protein is correctly folded, it is further transported to the Golgi apparatus. Proteins that fail to fold correctly, however, are retrotranslocated from the ER to the cytosol, and are then recognized by ER-specific E3 ligases, that mediate polyubiquitination of the misfolded protein on the cytoplasmic side of the ER, and subsequent

Protein	Function	Implications	Ref
E1	Ubiquitinating enzyme	Decreased levels and activity in AD	(Lopez Salon et al., 2000)
E2-25K	Ub conjugating enzyme	Mediates Aβ toxicity *in vitro*. Ubiquitinates UBB+1 and huntingtin	(Song et al., 2003)
E2 enzymes	Ub conjugating enzyme	Downregulated in AD or aging	(Loring et al., 2001; Lu et al., 2004)
CHIP	Ub ligase	Serves as E3 enzyme for phosphorylated tau	(Petrucelli et al., 2004; Shimura et al., 2004)
UCH-L1	Deubiquitinating enzyme and dimerised Ub ligase activity	Downregulated in AD brain Accumulates in subpopulation of tangles Oxidatively modified in AD brain	(Pasinetti, 2001) (Lowe et al., 1990) (Castegna et al., 2002)
UBB^{+1}	Unknown, translated from mutant mRNA	Accumulates in AD (also in other tauopathies and HD). Both substrate and inhibitor of the proteasome	(Lindsten et al., 2002; Van Leeuwen et al., 1998b)
20S β subunits	Protein degradation	All three proteolytic activities are decreased in AD affected brain areas	(Keller et al., 2000a)
20S α5 subunit	Confining proteolytic chamber	Downregulated in AD brain	(Loring et al., 2001)
S6b	19S ATPase	Immunoreactivity in neurofibrillary tangles	(Fergusson et al., 1996)
S1	19S non-ATPase	Downregulated in AD brain	(Loring et al., 2001)
Amyloid β	Unknown, product of APP processing	Aβ1-42 accumulates in plaques, but is also found intraneuronally. Inhibits proteasomal activity *in vitro*	(Gregori et al., 1995)
APP	Unknown, membrane-spanning glycoprotein	C-terminus of APP is degraded by proteasome	(Nunan et al., 2003)
Presenillin	Essential component of γ-secretase complex	Degraded by proteasome	(Fraser et al., 1998; Kim et al., 1997)
Pen-2	Essential component of γ-secretase complex	Degraded by proteasome through ERAD, only in absence of functional PS	(Bergman et al., 2004; Crystal et al., 2004)
Tau	Microtubule associated protein	Accumulates in neurofibrillary tangles as PHF tau, is mono-ubiquitinated. PHF tau inhibits the proteasome *in vitro*. Normal tau is a proteasome substrate	(David et al., 2002; Keck et al., 2003)
ApoE ε4	Lipid transport and cholesterol homeostasis	Gene dosage dependent risk factor for AD. Associated with decreased Aβ clearance and increased oxidative stress in AD. In macrophages and hepatocytes, apoE is degraded by the proteasome	(Wenner et al., 2001)
LRP receptor	Receptor for apoE and mediates Aβ clearance	Cytosolic fragment processed by proteasome	(May et al., 2002)

Table 1. Proteins related to UPS and AD.

degradation of the protein by the proteasome. This process is called ER-associated degradation (ERAD) (Hampton, 2002). Various types of cellular stresses can cause accumulation of unfolded proteins in the ER. The cell reacts to this ER stress with a so-called unfolded protein response (UPR), which involves up-regulated expression of UPR target genes encoding ER resident chaperones, and also genes involved in ERAD and proteasomal degradation. Notably, a γ-secretase component related to AD is degraded by ERAD. The γ-secretase complex is a key enzyme in the production of Aβ. The γ-secretase component Pen-2 is degraded by ERAD in the absence of another γ-secretase component, namely Presenillin (PSEN) (Bergman et al., 2004; Crystal et al., 2004). In addition, *PSEN1* mutants linked to familial AD increase the susceptibility of neuronal cell cultures to ER stress (Imaizumi et al., 2001; Katayama et al., 1999). This increased vulnerability, due to mutant *PSEN1*, was caused by decreased mRNA induction of ER chaperones by the UPR. A disturbed function of ER stress transducers, the stress sensors of the ER, was implicated in the mediation of the effect of mutant *PSEN1* (Katayama et al., 1999). In a later study, however, these effects could not be reproduced, and it was shown that the increased UPR activity was independent of *PSEN* expression (Sato et al., 2000). Moreover, the latter study also showed that Bip levels are not significantly different in brains of individuals with *PSEN1*-linked familial AD or sporadic AD compared to levels in control brains.

In conclusion, protein quality control through ERAD in the ER and by the UPS in the cytosol are tightly coupled processes. Both may be involved in pathogenesis of AD, although the subject requires more research.

3 THE UBIQUITIN PROTEASOME SYSTEM

3.1 Ubiquitin

Ubiquitin (Ub) was first described in 1975 (Goldstein et al., 1975) as a highly conserved ubiquitous protein, hence its name. It is a protein of 76 amino acids found in the cytosol and nucleus of cells, and expressed in all eukaryotic cells, where it is synthesised from multiple genes. It is transcribed in several forms: the first is a family

of Ub-fusion genes, in which a single Ub-encoding sequence is fused, in frame, with a carboxyterminal extension protein (CEP). The extensions encoded by *Ub-CEP* genes (or *UbA* genes) have been demonstrated to encode essential ribosomal proteins, such as S27a and L40 (Kirschner and Stratakis, 2000; Redman and Rechsteiner, 1989). The other Ub genes are polyubiquitin genes, i.e. *UbB* and *UbC*. These encode head-to-tail repeats of the Ub sequence (Muller and Schwartz, 1995). When Ub RNA is translated, the protein is cleaved by carboxyterminal Ub hydrolases, liberating free Ub monomers into the cytosol.

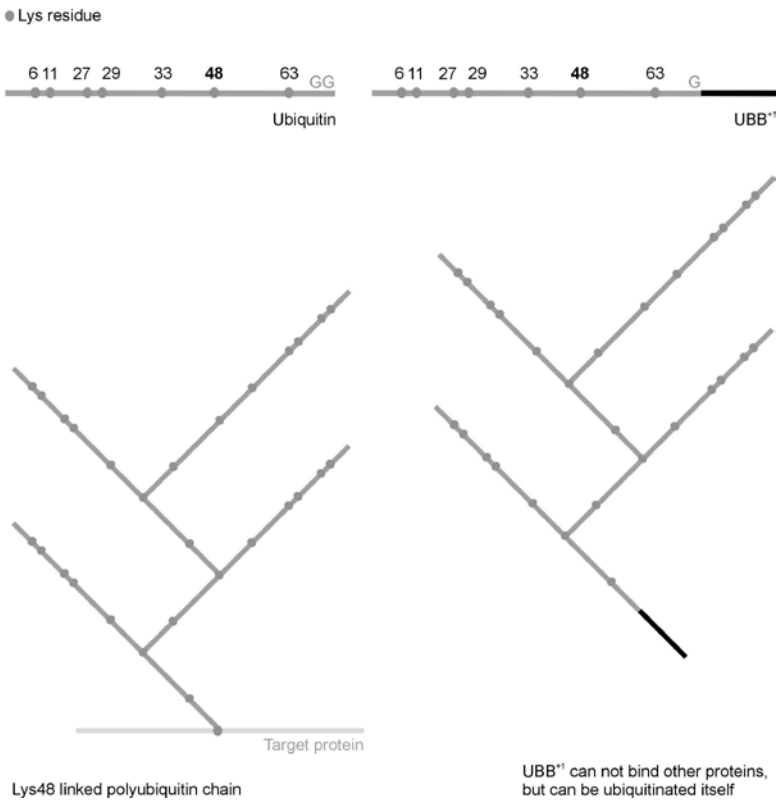

Fig. 2 Polyubiquitin chains are formed by the addition of multiple ubiquitin molecules through Gly-Lys interactions

As far as its role in protein degradation is concerned, Ub is usually linked to substrates through isopeptide bonds between the C-terminal Gly residue of Ub and the ε-amino groups of lysine (Lys) residues in proteins (Fig.2). However, there are also reports of proteins that are ubiquitinated without the requirement for Lys residues (Bloom et al., 2003). Through a complex enzymatic machinery, branched chains of multiple Ubs can be attached to a protein. These covalently attached poly-Ub chains are formed by the sequential addition of mono-Ub to a Lys residue of substrate-bound Ub (Fig.2). Ub has seven lysines, making several 'mixed' topologies of a poly-Ub chain possible. The first poly-Ub chain that was discovered had its Ub moieties conjugated through Lys48 (Chau et al., 1989), which turned out to be the most common linkage for targeting substrates for degradation by the 26S proteasome. Other poly-Ub chains involve Lys63, which appears to have a distinct role in DNA repair (Spence et al., 1995), and Lys29 (Varshavsky, 1997). A proteomics study revealed the Lys polyubiquitin chain patterns in yeast. All 7 Lys in Ub were targets for Ub modification, of which Lys48, followed by Lys63, were the most frequently targeted for Lys-linked polyubiquitin chain building. The remaining five Lys were significantly less involved in polyubiquitin chain formation (Peng et al., 2003). The key to specific recognition of Lys48 linked Ub chains probably lays in the three-dimensional conformation of the polyubiquitin structure. This is supported by the finding that repeated patches in the chain formed by three hydrophobic residues on the surface of Ub, Leu8, Ile44 and Val70, are essential for the recognition of Lys48 linked chains by the proteasome (Beal et al., 1996). The conformation of Lys48 linked (Ub)4, which is the minimal signal for efficient targeting to the proteasome (Thrower et al., 2000), gives better access to the hydrophobic patches of the Ub units than the (Ub)2 conformation (Varadan et al., 2002).

3.2 Ub-like proteins

There is a growing list of Ub-like proteins that have similar posttranslational modifying properties as Ub (Schwartz and Hochstrasser, 2003). SUMO (Small Ubiquitin-like MOdifier) and NEDD8 (Neural precursor cell-Expressed Developmentally Down-regulated - also known as Rub1 (Related to Ub 1)), are the most well known Ub-like proteins. They are covalently attached to substrates through their carboxyl termini, just like Ub (Hochstrasser, 2000).

However, Ub itself seems to be the only Ub-like protein that forms chains.

The reversible conjugation of SUMO participates in nuclear transport, transcriptional regulation, chromosome segregation and cell-cycle control and is essential for viability, although only in budding yeast (Muller et al., 2001). NEDD8 is more similar in sequence to Ub (Whitby et al., 1998). It is translated as an 81-amino acid protein, of which the C-terminal five amino acids following a Gly-Gly dipeptide are cut off. The only known substrates of NEDD8 are cullins, which represent subunits in E3 ligase complexes such as SCF (Skp1, Cullin, F-box proteins). NEDD8 attachment to the cullin subunit of SCF complexes is necessary for the activity of the ligase. NEDD8 itself might be degraded by the proteasome via interaction with the nuclear NUB1 protein (Kamitani et al., 2001), which is interferon-inducible and can bind the proteasome regulatory subunit S5a. Together, these findings suggest that NEDD8 and Ub are involved in several common pathways, although their functional association is negligible.

Interestingly, NEDD8 was recently found to accumulate in almost all Ub-containing inclusions found in several diseases (Dil Kuazi et al., 2003). NEDD8 immunoreactivity was found in (i) Mallory bodies, which are hepatocyte inclusions associated mostly with alcoholic liver diseases, and contain Ub, intermediate filament, and αB crystalline and (ii) Rosenthal fibres, which are found in astrocytes in Alexander's disease and in astrocytomas, and consist of GFAP aggregates (Gordon, 2003). In AD, however, NEDD8 was only found in some tangles in a subpopulation of AD patients, while Ub immunoreactivity is found in all AD patients and tangles are increasingly ubiquitinated with maturation (Bancher et al., 1989). The staining pattern of NEDD8 seems reminiscent of reported UCH-L1 staining in AD brain (Lowe et al., 1990), which was also commonly found in ubiquitinated inclusions in brain, but not routinely in AD tangles. Perhaps these staining patterns reflect early tau pathology in AD.

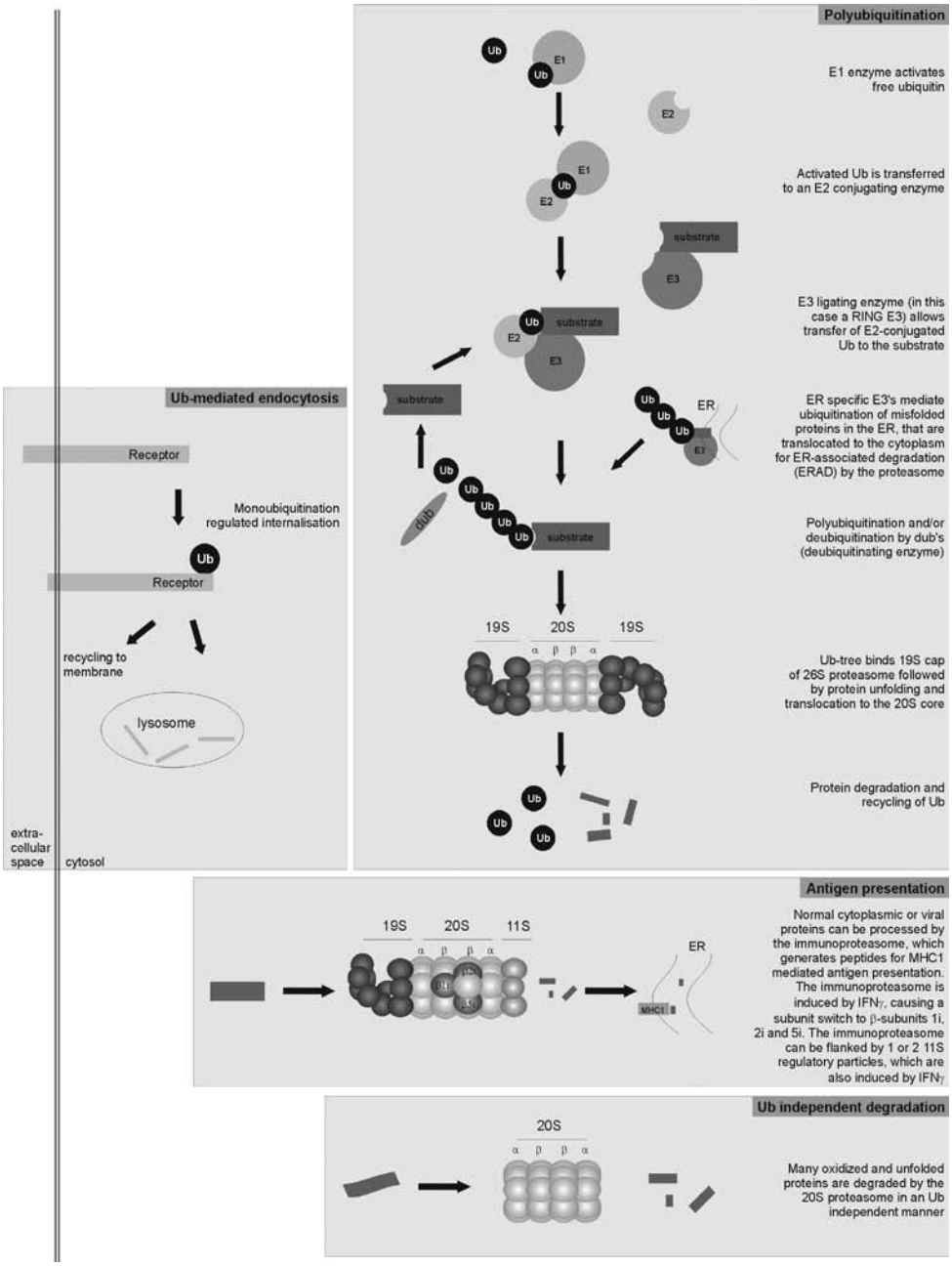

Fig. 3 Main functions of ubiquitin and the proteasome in the eukaryotic cell

Monoubiquitination 3.3

Besides targeting proteins for degradation by the proteasome, which requires at least four Ub-moieties attached to a substrate (Thrower et al., 2000), mono-ubiquitination serves as a modification for entirely different functions in, for instance, histone regulation, virus budding, membrane protein internalisation and trafficking (Haglund et al., 2003; Hicke, 2001). Histones H2A and H2B are monoubiquitinated in yeast and mammalian cells, which is needed for meiosis. Monoubiquitination of the Gag polyprotein, which is common to all retroviruses, is required for virus budding (Hicke, 2001). In mammalian cells, the epidermal growth factor receptor and interleukin-2 receptor α chain are examples of membrane proteins that are monoubiquitinated. Monoubiquitination of these proteins not only mediates their internalization but also targets them to the late endosomal/lysosomal compartment (Fig.3) (Haglund et al., 2003). The proteasome is largely uninvolved in these monoubiquitination processes. Hypothetically, disturbed Ub modification of, for instance, neurotransmitter receptors, could interfere with neuronal transmission. Studies with *C. elegans* demonstrated that Ub-mediated endocytosis of AMPA glutamate receptors might regulate the strength of synaptic transmission (Burbea et al., 2002; Turrigiano, 2002). It remains unclear if mammalian AMPA receptors also require monoubiquitination for endocytosis, which might implicate a role for this process in AD. However, it has also been demonstrated that proteasome activity rather than monoubiquitination is required for this process (Patrick et al., 2003), probably through polyubiquitin-mediated degradation of proteins that normally prevent internalization of glutamate receptors, such as PSD-95 (Colledge et al., 2003).

Ubiquitination machinery 3.4

The UPS is the major system for intracellular protein degradation in eukaryotes (Glickman and Ciechanover, 2002). Ub tags proteins for degradation by poly-ubiquitination. The multi-enzyme cascade responsible for polyubiquitination involves Ub-bound thioesters with distinct classes of enzymes (Fig.3): E1 (Ub-activating enzyme), E2 (Ub-conjugating enzymes) and E3 (Ub protein ligases) enzymes (Weissman, 1997). The E1 enzyme activates Ub in an ATP-dependent reaction that generates a high-energy E1-thiol-ester-Ub intermediate. The activated Ub is then transferred to an E2 enzyme. Ub activation

by the E1 enzyme forms an important and unique starting point of the UPS. In yeast, only one E1 enzyme was identified (*UBA1*), and its inactivation is lethal (McGrath et al., 1991). The human homolog of this E1 gene encodes two isoforms of the enzyme. The longer E1 displays cell-cycle dependent nuclear localization and phosphorylation (Grenfell et al., 1994; Handley-Gearhart et al., 1994). Recently, several mouse Ub-associated proteins with E1 domains were described (Semple, 2003). However, it is unknown if these proteins constitute different functional E1 enzymes. In a human genome search for E1 enzymes, three genes were identified, suggesting that there could be more than one functional E1 enzyme in humans (www.ensembl.org, search done 13-05-2004). The activity of E1 is decreased in AD brain (Lopez Salon et al., 2000).

E2s are conjugating enzymes that catalyse the covalent attachment of Ub to target proteins or transfer the activated Ub to an E3-Ub intermediate. Eleven E2s were identified in yeast, more than thirty in flies, and there are definitely many more in vertebrates (Glickman and Ciechanover, 2002). The E2 enzyme E2-25K/Hip2 was recently implicated in AD, as its levels were found to increase in cell lines upon Aβ treatment. In AD brain the enzyme seems to be upregulated as well (Song et al., 2003). Each E2 interacts with a number of ligases (E3s).

E3s are Ub-protein ligases, which are responsible for the specific recognition of a wide variety of substrates of the UPS. E3s display the greatest variety among the enzyme components of the pathway, explaining the high specificity of this hierarchical system. Most E3s can be classified into two major groups: HECT (Homologous to the E6-AP C Terminus)-domain- and RING (Really Interesting New Gene) finger-containing E3s, and several minor groups (Glickman and Ciechanover, 2002). The HECT-domain E3s accept Ub transferred from E2 enzymes by forming another high-energy thiol ester bond between an active site Cys residue and Ub. Ub is subsequently transferred to the ligase-bound substrate. The RING finger E3s serve more as a bridge that brings together the E2 and the substrate to the proximity that allows for efficient transfer of Ub from the E2 to the substrate. After initial ubiquitin ligation by E3s, chain elongation can proceed by action of a different ligase, termed E4 (Ufd2 in yeast) (Koegl et al., 1999). *UFD2a* is the mammalian homolog of *Ufd2* (Kaneko et al., 2003). The U box domain in the E4 enzyme mediates the interaction with Ub-conjugated targets. It was reported that

other mammalian proteins with U box domains were able to mediate ubiquitination of substrates in combination with E1 and E2 enzymes, without the need for other E3 components (Hatakeyama et al., 2001). This suggests that U box proteins form a distinct class of E3 enzymes, and that some may function as E4 enzymes by mediating further ubiquitination of E3 ubiquitinated substrates.

The E3 enzyme E6-AP (E6-associated protein)was the first UPS component found in which mutations were linked directly to the cause of a disease. Mutations in the *E6-AP* gene lead to the neurodevelopmental disease Angelman syndrome (Kishino et al., 1997). Moreover, loss of function of the enzyme in spinocerebellar ataxia 1 (SCA1) mice enhances neuropathology (Cummings et al., 1999). Parkin is another example of an E3 enzyme that is specifically involved in neurological disease, in this case the neurodegenerative Parkinson's disease (PD). Mutations in *parkin* are estimated to account for about 50% of all autosomal recessive early-onset Parkinsonism cases (Zhang et al., 2000). Parkin is self-ubiquitinated, promoting its own degradation, and also ubiquitinates the synaptic proteins CDCerl-1 (Zhang et al., 2000), synphillin-1 (Chung et al., 2001), and a novel form of O-glycosylated α-synuclein (Shimura et al., 2001). α-Synuclein is the main constituent of Lewy body inclusions found in PD brains and synphilin-1 is known to bind α-synuclein. PD patients with *parkin* mutations were thought not to develop Lewy bodies, suggesting that normal parkin would be involved in the formation of Lewy bodies (Hayashi et al., 2000; Ishikawa and Takahashi, 1998; Mori et al., 1998; Zhang et al., 2000). However, this assumption is based on only a few autopsies of autosomal recessive juvenile Parkinsonism cases. One study describes Lewy bodies in early-onset PD with confirmed *parkin* mutations (Farrer et al., 2001). Although the *parkin* mutations in this study are different from the ones described earlier, the presence of Lewy bodies challenges the hypothesis of parkin function in these inclusions. CHIP (carboxyl terminus of the Hsc70-interacting protein) was recently found to serve as a Ub ligase for phosphorylated tau, a protein that is relevant to AD (Petrucelli et al., 2004; Shimura et al., 2004) (see chapter 4.1 for details).

In summary, the following findings support a link between the process of ubiquitination and AD pathology:(i) E1 levels are lower in

AD brains (Lopez Salon et al., 2000). (ii) E2-25K/Hip2 is implicated in Aβ-mediated toxicity (Song et al., 2003) and (iii) some E3 ligases are linked to AD, such as the U box protein CHIP, which ubiquitinates AD-type phosphorylated tau (Shimura et al., 2004).

3.5 Deubiquitinating enzymes

Modification of proteins by Ub is a reversible process (Fig.3). Deubiquitinating enzymes (DUBs) therefore play a significant role in maintaining the steady state levels of free ubiquitin, and in controlling the stability of Ub-conjugated proteins. All known DUBs are cysteine proteases that specifically hydrolyze the isopeptide bond after the C-terminal Gly residue of Ub. There are two distinct categories of DUBs, namely UCHs (ubiquitin C-terminal hydrolases) and UBPs (ubiquitin specific proteases). UCHs are small proteins that remove short peptides from the C-terminus of Ub, while UBPs are responsible for cleaving the isopeptide bond linking Ub-Ub or Ub-protein. UBPs can also cleave biosynthetic linear fusions of Ub (Chung and Baek, 1999; Wilkinson, 2000). Mutations in *UCH-L1* demonstrate the importance of deubiquitinating enzymes in neuronal protein quality control, as they are associated with Parkinson's disease (PD) and cause gracile axonal dystrophy in mice (Saigoh et al., 1999). In PD, UCH-L1 also seems to have a role in the ubiquitination of α-synuclein, but only when one or more Ub molecules are already attached to the protein. Moreover, a dimerised form of the UCH-L1 enzyme has Ub-Ub ligase activity through Lys63 residues in Ub (Liu et al., 2002). The S18Y polymorphism of UCH-L1 is associated with a lower risk for PD, probably caused by a shift in the balance between the ligase and hydrolase activities of the enzyme (Liu et al., 2002). Not much is known about the involvement of DUBs in AD pathology. In one study UCH-L1 was found to accumulate in a minority of tangles in a subpopulation of AD patients (Lowe et al., 1990). In addition, UCH-L1 was found to be oxidatively modified in AD brain (Castegna et al., 2002). Impaired function of ubiquitin hydrolases can affect many proteins, the degradation of which is normally regulated by a balance between ubiquitination and deubiquitination. For AD pathology, a shift in this balance regarding UBB^{+1} processing, would possibly lead to more UBB^{+1} accumulation and thereby to more proteasome inhibition (Lindsten et al., 2002).

Proteasomes 3.6

Polyubiquitinated proteins are targeted for degradation by the proteasome, which is a large enzymatic complex found in all eukaryotic cells. There is a large diversity in proteasome composition, as this complex contains several different components and many interchangeable subunits (Fig.3).

The proteolytic core of the proteasome or "20S proteasome" is a 28 subunit multi-catalytic particle consisting of four heptagonal rings. The two outer rings consist of 7 α-subunits each and the two inner rings of 7 β-subunits each. Three of the seven types of β-subunits in each ring (six in total) confine the catalytic activity to the proteasome. The β-subunits 1, 2 and 5 exhibit "peptidylglutamyl-peptide hydrolyzing" (PGPH), trypsin-like and chymotrypsin-like activity respectively. The PGPH activity is also known as "post-acidic" or "caspase-like" activity (Kisselev et al., 1999; Kisselev et al., 2003). The three catalytic sites interact allosterically. For example, inhibitors of the caspase-like activity stimulate the trypsin-like activity but do not affect the chymotrypsin-like activity. In addition, substrates of the caspase-like activity allosterically inhibit the chymotrypsin-like activity (Kisselev et al., 2003).

The proteasome active sites are confined to a proteolytic chamber, which is thought to be controlled by a regulatory gating mechanism. This mechanism protects proteins from spontaneous degradation by the catalytic activities. In yeast, the N-termini of α-subunits in the 20S proteasome form a 'plug' at the entrance of the 20S barrel by interacting tightly with the N-terminus of the α-3 subunit. Access to the barrel can only be achieved by substantial structural rearrangement, which is established by the binding of regulators, such as 19S (Groll and Huber, 2003).

The 20S core can be capped at each end by several multimeric components, of which the 19S regulatory particle is the most important for degradation of ubiquitinated substrates. Together, the 20S and 19S particles give rise to the 26S proteasome, which as a whole is responsible for the degradation of polyubiquitinated proteins (Ciechanover, 1994). The regulatory 19S complex consists of at least 20 subunits and has multiple functions within the 26S proteasome (Ferrell et al., 2000). This complex is composed of two parts, the so-called base and lid. The base consists of two non-ATPase subunits (S1 and S2) and six ATPase subunits, some of

which have been found to attach directly to the α-ring of the 20S complex and whose probable function is to open the central channel. The ATPase subunits, too, are most likely to be involved in the unfolding of substrates and their translocation into the 20S central channel (Braun et al., 1999). The six ATPase subunits form a ring, in which they interact in pairs: S4 binds to S7, S6b to S8 and S6a to S10b (Richmond et al., 1997). Studies on the archaebacterial homologue of the 19S base regulatory complex, PAN, indicate that the substrates are first unfolded at the surface of the ATPase ring before being transported into the 20S proteasome (Navon and Goldberg, 2001). In AD as well as in other neurodegenerative diseases, immunoreactivity of the S6b ATPase subunit was found in neurofibrillary tangles, plaque neurites and neuropil threads (Fergusson et al., 1996).

The S5a subunit is a non-ATPase subunit that interacts with both the lid and the base of the 19S particle, stabilizing their interaction (Fu et al., 2001). The S5a binds polyubiquitin chains at two independent binding sites in its C-terminus (Young et al., 1998). Recognition of polyubiquitinated chains is not likely to be restricted to the S5a subunit, as this subunit is dispensable in yeast. The S6a subunit also binds polyubiquitinated chains under modulation of ATP hydrolysis (Lam et al., 2002). The lid of the 19S complex consists of eight non-ATPase subunits arranged in a disk-like shape. The function of the lid subunits remains unclear, but they are essential for proteolysis of ubiquitinated substrates. Isopeptidases are also present in the lid and catalyze the release of free Ub (Lam et al., 1997).

The proteasome degrades cytosolic proteins as well as proteins that fail to pass protein quality control in the ER. Misfolded proteins in the ER are recognized by ER-specific E3 ligases that mediate polyubiquitination of the misfolded protein on the cytosolic side of the ER and are subsequently degraded by the proteasome (Hampton, 2002).

The 20S proteasome can also be associated with one or two 11S (PA28) particles, which consist of α- and β-subunits that can be induced by interferon γ (IFNγ) and result in so-called immunoproteasomes. The association of 11S and 20S particles is thought to also lead to a rearrangement of α-subunit chains, resulting in the widening of the openings to the 20S barrel, thereby facilitating the access of substrates and the exit of peptide fragments at the other end of the proteolytic chamber. In response to IFNγ, hybrid proteasomes can

also assemble into particles consisting of 20S proteasomes with a 19S particle at one end and an 11S particle at the other (Cascio et al., 2002). As a result of this switch in regulatory particles, there is an increase in ATP-independent degradation of small peptides, but not proteins (Whitby et al., 2000). In mammals, IFNγ also induces changes in the 20S proteasome; the three catalytic β-subunits in 20S particles are replaced by IFNγ inducible subunits, namely LMP2 (βi1), LMP10 (βi2) and LMP7 (β5i) (Rivett et al., 2001). Proteasomes (not necessarily associated with 11S regulators) are referred to as immunoproteasomes when they contain the inducible subunits, as they are involved in cleaving peptides for MHC class I (major histocompatibillity complex 1) antigen presentation. MHC class I mediated antigen presentation is responsible for displaying self-proteins and intracellular viral proteins at the cell-surface. In this process, proteins are partially degraded to peptides by 26S, hybrid or immunoproteasomes in the cytosol (Goldberg et al., 2002). Each of the different types of proteasomes gives rise to a variety of peptides. These peptides are further trimmed by TPPII peptidase and aminopeptidases (Reits et al., 2004). The trimmed peptides are subsequently translocated to the ER via the TAP transporter and bind to MHC class I receptor proteins for transport to the cell surface. Oxidative modification might make proteins susceptible to degradation by the immunoproteasome, facilitating peptide generation and antigen presentation (Teoh and Davies, 2004). A significant increase in LMP2 and LMP7 subunits was found in Huntington's disease (HD), indicating an induction of the immunoproteasome (Diaz-Hernandez et al., 2003). This increase correlates with a rise in 20S proteasome activity assayed with fluorogenic substrates that are processed in a Ub independent manner. The immunoproteasome induction seemed to take place specifically in degenerating neurons in both *huntingtin* transgenic mice (HD94) and HD patient brain extracts (Diaz-Hernandez et al., 2003). Also cell lines expressing polyglutamine expansion constructs show an increase in LMP2 subunit expression (Ding et al., 2002). These results imply that the immunoproteasome can be involved in neurodegeneration. Moreover, LMP2 knock-out mice exhibit differences in brain function (Martin et al., 2004) and non-toxic levels of oxidative stress cause an up-regulation of immunoproteasome subunits in neurons (Ding et al., 2003). Immunoproteasomes could also play a role in AD, as inflammatory processes are thought to be involved in AD pathology (McGeer and McGeer, 2003). Hypothetically, an up-regulation of

immunoproteasome subunits might lead to decreased functioning of 26S proteasomes, and might thus contribute to the accumulation of ubiquitinated substrates.

In summary, the following findings support a link between proteasome function and AD pathology: (i) the three catalytic activities of the proteasome are decreased in AD brains (Keller et al., 2000a), (ii) AD affected brain areas display immunoreactivity for the 19S subunit S6b (Fergusson et al., 1996) and (iii) S1, a non-ATPase 19S subunit, and α5, a 20S proteasome subunit, were found to be down-regulated in a gene expression profile of six AD patients (Loring et al., 2001).

3.7 Ub-independent degradation

The 26S proteasome degrades polyubiquitinated proteins, but is also known to catalyze Ub-independent protein degradation. The first protein shown to be degraded by the 26S proteasome in a Ub-independent manner was ornithine decarboxylase (ODC) (Murakami et al., 1992). ODC requires ATP and antizyme for its degradation (Coffino, 2001). The targeting role of ubiquitin is replaced by antizyme, which binds to ODC and contains an N-terminal domain that stimulates its degradation by the 26S proteasome. ODC itself also contains a C-terminal region that promotes its recognition by the proteasome (Murakami et al., 2000). Other examples of proteins that are degraded by the proteasome in a Ub-independent manner are c-Jun (Jariel-Encontre et al., 1995), calmodulin (Tarcsa et al., 2000), troponin C (Benaroudj et al., 2001) and p53 (Asher et al., 2002). Hydrophobic stretches of amino acids in calmodulin and troponin C might substitute for Ub and be sufficient for recognition by the proteasome (Benaroudj et al., 2001). Artificially targeting a protein to the proteasome by bypassing the need for its ubiquitination, is sufficient for degradation (Janse et al., 2004). For example, p21Cip1 can be degraded by the 26S as well as the 20S proteasome. p21Cip1 was thought to be degraded in a Ub-independent manner, because Lys-mutations in the protein did not abolish its degradation (Sheaff et al., 2000). However, p21Cip1 is not degraded in a Ub-independent manner, but rather in a Lys-independent manner. Apparently, p21Cip1 can be ubiquitinated at its N-terminus on residues other than Lys (Bloom et al., 2003). This ubiquitination mechanism might

also apply to other proteins thought to be degraded independently of Ub.

20S proteasomes are more abundant than any other proteasome forms in the cell. Most unfolded proteins, short-lived regulatory proteins and oxidatively damaged, misfolded, mutated, or otherwise damaged proteins are susceptible to degradation by the 20S proteasome (Orlowski and Wilk, 2003). Oxidized proteins are particularly relevant to neurodegenerative disorders, such as AD and ALS, and seem to be degraded by the 20S proteasome in a Ub-independent manner (Grune et al., 2003). While mild oxidation was found to inactivate the Ub-dependent system and 26S proteasome activity, it did not affect the activity of the 20S proteasome. Furthermore, yeast strains defective in 26S proteasome assembly or lacking the genes encoding subunits of the 19S regulatory particle, are more effective in degrading oxidized proteins than wild type strains (Inai and Nishikimi, 2002). In addition, cells with a thermolabile E1 enzyme effectively degraded oxidized proteins in an ATP-independent manner, and their degradation was blocked by proteasome inhibitors (Shringarpure et al., 2003).

Involvement of Ub-independent protein degradation in neurodegenerative diseases is supported not only by its link to oxidative stress, but also by its relevance to the degradation of two *in vivo* substrates of the 20S proteasome, namely the Parkinson's disease-related unfolded protein α-synuclein (Tofaris et al., 2001) and the Alzheimer's disease-related microtubule binding protein tau (David et al., 2002).

4 AD PATHOGENESIS AND THE UPS

Years of debate have still not provided a conclusive answer to the question of the order of events leading to AD pathology (Braak and Del Tredici, 2004; Hardy, 2004; Mudher and Lovestone, 2002; Price and Morris, 2004; Schonheit et al., 2004; Zhu et al., 2004a; Zhu et al., 2004b). There is major controversy in the field between so-called Baptists and Tauists, that each proclaim an exclusive initiating role for either Aβ or tau pathology in AD (Lee, 2001; Mattson, 2004). The UPS is emerging as well as an important competitor in the pathogenesis of AD. Over the years, evidence has accumulated for a primary role of Aβ, preceding tangle formation. An argument in favor of this hypothesis is that Aβ injections in FTDP-17 (frontotemporal dementia with parkinsonism linked to chromosome 17) mice induce more severe tangle pathology (Gotz et al., 2001). Recent immunization clinical trials with anti-Aβ antibodies in AD patients have shown promising results, such as a decrease in plaque load and in the speed with which dementia progresses (Hock et al., 2003). However, the mechanism by which Aβ is cleared remains elusive and there are problems with inflammatory reactions due to immunization. The large cohort of 300 immunized patients was halted in 2002 because 17 of the patients developed aseptic meningoencephalitis (Schenk, 2002). This response could not be successfully treated and thus remains a major concern for such therapies. In a triple transgenic mouse model of AD, Aβ immunization reduced the amounts of Aβ, resulting in a subsequent clearance of tau pathology (Oddo et al., 2004). These findings argue in favor of the amyloid cascade hypothesis.

Despite the importance of Aβ, there are also strong arguments for at least an equally important involvement of tau pathology. Tangle formation seems to be a very important factor in the progress of dementia, as it is characteristic of several types of dementia, collectively named tauopathies. Mice that are transgenic for human AD-associated mutant *APP* only, show some phosphorylated tau immunohistochemistry, but fail to develop paired helical filaments (PHFs) (Sturchler-Pierrat et al., 1997). In addition, hippocampal neurons from homozygous tau knockout mice do not degenerate in the presence of fibrillar Aβ, while neurons of human tau transgenic mice or of wild-type mice do (Rapoport et al., 2002). Moreover, Aβ also

accumulates in senile plaques in normal human brains during aging (Funato et al., 1998), and insoluble Aβ was found in young, healthy control subjects. In several studies, tangle pathology was found to correlate better with the state of dementia in AD patients than with plaques (Arriagada et al., 1992; Bierer et al., 1995; Giannakopoulos et al., 2003). Overall, both amyloid and tau pathology seem to be crucial to the long process of developing AD.

As mentioned earlier, the substantial role the UPS plays in AD pathology is increasingly recognized. For example, proteasome activity was found to be lower in AD brains than in age-matched controls (Keller et al., 2000a; Lopez Salon et al., 2000). In addition, high levels of Ub were detected in brain homogenates and cerebrospinal fluid samples (both lumbar punctions and *post mortem*) of AD patients (Kudo et al., 1994). Moreover, protein inclusions in AD brains generally contain ubiquitinated proteins and are specifically immunoreactive to at least one of the 19S regulatory proteasome subunits, S6b (Fergusson et al., 1996). These characteristics are not specific for AD, and are detected in other neurodegenerative diseases as well. Intriguingly, tau and Aβ, the two major players in AD pathology, as well as the mutant form of ubiquitin, UBB^{+1}, were found to alter proteasome activity. These findings strongly support the relevance of altered proteasomal degradation in AD.

Tau 4.1

Tau is a microtubule-associated protein of ~55 kDa. Besides playing a role in the stabilization of axon microtubules, tau has also been shown to interact with the actin cytoskeleton and plasma membrane and to play a role in neurite outgrowth, enzyme anchoring and intracellular vesicle transport regulation (Friedhoff et al., 2000). The *tau* gene (17q21) contains fifteen exons, which can give rise to six different isoforms through alternative splicing. The C-terminus of tau contains three or four repeats, which constitute the microtubule binding domain (Buee et al., 2000; Spillantini and Goedert, 1998).

In AD, tau is thought to be dissociated from microtubules because of its hyperphosphorylation, which probably affects axonal transport. Hyperphosphorylated and abnormally folded tau accumulates in neurons as paired helical filaments (PHF), leading to neurofibrillary tangles (NFT) and eventually ghost-tangles. More than 25 potential

phosphorylation sites have been identified in PHF-tau isolated from AD brain, and multiple protein kinases appear to be involved in hyperphosphorylation of tau, including GSK3β, cdk5, MAPK and PKA (Geschwind, 2003; Johnson and Bailey, 2002; Morishima-Kawashima et al., 1995).

Although tangles are to some extent also found in non-demented individuals, tangle formation seems to be a rather late event in AD and is also associated with other neurodegenerative diseases, collectively known as tauopathies. A particular set of tauopathies, termed frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), is caused by mutations in the tau gene. In these cases, mutations in *tau* seem to be sufficient to cause dementia, and FTDP-17 brains lack Aβ pathology. More than 25 unique mutations in the *tau* gene were linked to neurodegenerative diseases (Johnson and Bailey, 2002). However, *tau* mutations are not linked to any known form of AD, supporting the notion that PHF formation is a rather distal event in the disease process.

The tau protein normally exists as an unfolded protein and was suggested to be degraded by the 20S proteasome *in vitro*, both from the N to C and from the C to N-terminus (David et al., 2002). This implies that tau, just like other unfolded proteins, can be degraded by the 20S proteasome in an Ub independent manner. In PHFs, however, tau was reported to be mono-ubiquitinated (Morishima-Kawashima et al., 1993), but to our knowledge these results were not confirmed by other studies, and the *in vivo* ubiquitination of normal tau was not conclusively demonstrated. The monoubiquitinated form of tau could hypothetically reflect a deubiquitinated state of polyubiquitinated tau. Phosphorylated tau extracted from AD brain was recently found to be ubiquitinated *in vitro* by the E2 enzyme UbcH5B and a CHIP/Hsc70 complex as the E3 ligase, the latter being immunodetected in tau aggregates (Petrucelli et al., 2004; Shimura et al., 2004). AD type tau phosphorylation seems to be the recognition signal for the E3 ligase, resulting in the subsequent targeting of tau to the proteasome. Although tau aggregates were detected in CHIP transduced cells, ubiquitination by CHIP rescued cells from phosphorylated tau induced cell death. These findings suggest that the soluble aberrant tau, rather than aggregated tau, is toxic to cells. On the other hand, PHF-tau has been suggested to bind, and thereby inhibit, the proteasome in AD brain (Keck et al., 2003). Indeed, tau protein co-immunoprecipitated with 20S

proteasome subunits. In addition, in that particular brain area, a positive correlation was found between the amount of proteasome-bound tau and the extent of proteasome inhibition. PHF tau isolated from AD brain also significantly inhibited proteasomal activity *in vitro* (Keck et al., 2003). This inhibition was caused by the aggregation rather than the phosphorylation state of tau. Other aggregated proteins, like polyglutamine protein aggregates, were also reported to inhibit the proteasome (Bence et al., 2001). It is not clear if this phenomenon is simply due to clogging of the proteasome or if other mechanisms are involved.

Amyloid β 4.2

Amyloid β (Aβ) is deposited as extracellular senile plaques in AD brain. Aβ1-40 and Aβ1-42 are formed by sequential processing steps of APP (Morishima-Kawashima and Ihara, 2002). APP can be cleaved by α-secretase in the Aβ domain, which results in the release of APPsα from the cell and the retention of a membrane bound C83 fragment. If C83 is subsequently cleaved by γ-secretase, this liberates a γ-stub into the cytosol and leads to secretion of p3. In the Aβ-forming proteolytic pathway, APP is first cleaved by β-secretase, leading to secretion of APPsβ and leaving C99 in the membrane. Subsequently, γ-secretase cleavage of C99 produces the 40 or 42 amino acid Aβ peptide. The two additional amino acids that distinguish Aβ42 from Aβ40 are hydrophobic, rendering Aβ42 more prone to aggregation than the 40 amino acid form.

β-secretase cleavage is performed by BACE1 (β-site APP cleaving enzyme), which is a type-I membrane bound aspartyl protease located preferentially in endosomes and also in the Golgi apparatus and ER (Huse et al., 2000). γ-Secretase consists of a complex of four enzymatic proteins, namely presenilin (PSEN), nicastrin, Aph-1 and Pen-2 (De Strooper, 2003). The familial mutations linked to AD in genes for *APP*, *PSEN1* and *PSEN2* are all associated with increased Aβ42 (and Aβ40) production. The mutations in *APP* all flank the Aβ region of the molecule, probably affecting β- and γ-secretase cleavage (Haass et al., 1994). More than seventy mutations in the *PSEN1* gene and six in the *PSEN2* gene are linked to familial AD, and they all lead to enhanced production of Aβ42 (Borchelt et al., 1996; Jankowsky et al., 2004). Transgenic mice that express human *APP* and *PSEN1* mutations develop amyloid depositions

and impaired memory function, which are correlated to selectively reduced expression of synaptic plasticity-related genes (Dickey et al., 2003).

Presenilins were also linked to the UPS. PSEN1 and PSEN2 are subjected to proteasomal degradation, most likely through ERAD (Fraser et al., 1998; Kim et al., 1997). Moreover, Pen-2, another member of the γ-secretase complex, is also a proteasome substrate under certain conditions (Bergman et al., 2004). PSEN is responsible for the subcellular localization of Pen-2. In the absence of PSEN, Pen-2 failed to be transported to post-ER compartments, where further assembly of the γ-secretase complex occurs, was retained in the ER, and was subsequently efficiently degraded by ERAD. Apparently, PSEN regulates the levels of its binding partner Pen-2 by posttranslationally preventing its degradation by the proteasome (Crystal et al., 2004). Hypothetically, proteasome inhibition would lead to enhanced PSEN and Pen-2 levels, resulting in increased γ-secretase activity and more Aβ production.

In vitro studies demonstrated that the C-terminal part of APP can also be processed by the 20S proteasome, which decreased γ-secretase processing (Nunan et al., 2003). Together, these findings support the premise that an AD-associated decline in proteasome activity would lead to increased γ-secretase APP processing, which would result in elevated Aβ levels.

The origin and mechanism of Aβ-mediated toxicity remain elusive. Both extracellular and intracellular Aβ have been widely discussed as mediators of neurotoxicity. Most Aβ, Aβ40 in particular, is secreted from cells. However, there are several reports on the toxicity of intracellular Aβ, formed either by processing APP in the ER, leading to lysosomal Aβ, or by endocytosis of extracellular Aβ (Hartmann, 1999; Ida et al., 1996; Nagele et al., 2002; Pasternak et al., 2004). In addition, injected Aβ1-42 or cDNA-expressing cytosolic Aβ1-42 was found to be specifically cytotoxic to human neurons (Zhang et al., 2002). Moreover, several studies hypothesize that plaques are formed by the remains of cells that died of Aβ accumulation, and that act as seeds for more aggregation of extracellular Aβ (D'Andrea et al., 2002; D'Andrea et al., 2001; Pasternak et al., 2004). In human brain, intracellular accumulation of Aβ42 was found in neurons in AD-vulnerable brain regions of patients with mild cognitive impairment (Gouras et al., 2000). These intraneuronal accumulations seemed to precede tangle formation, as they were more numerous than

hyperphosphorylated tau-containing neurons. Moreover, the amount of intracellular Aβ staining decreased with increasing cognitive impairment, possibly due to cell death. In transgenic mice carrying a familial AD-associated mutant *PSEN1*, neurodegeneration was linked to a significant increase in neurons accumulating Aβ42 intracellularly, while plaque formation was not detected (Chui et al., 1999). Intraneuronal accumulation of Aβ was also detected in a triple transgenic mouse model of AD expressing human tau, APP and presenilin (Oddo et al., 2003). These mice show a defect in synaptic plasticity that precedes extracellular accumulation of Aβ and tangle formation. The defective synaptic plasticity was claimed to be caused by intraneuronal accumulation of Aβ, which was detected as one of the first neuropathological manifestations, preceding tangle and plaque formation. Moreover, Aβ immunization in these mice decreased the levels of both intra- and extracellular Aβ and, most intriguingly, also cleared early tau pathology (Oddo et al., 2004). The clearance of tau pathology in these mice was mediated by the proteasome, which is a first indication of UPS involvement in transgenic AD mouse models. Immunization-induced reduction of Aβ preceded the clearance of tau pathology, suggesting that Aβ interferes with proteasomal activity, and its removal alleviates this impairment, resulting in degradation of tau (Oddo et al., 2004). The mechanism by which Aβ mediates proteasome inhibition remains unclear.

It was reported earlier that Aβ can bind to the 20S core of the proteasome and inhibit its activity in a 20-200 μM range *in vitro* (Gregori et al., 1995; Gregori et al., 1997). *In vivo*, however, a physical interaction between Aβ and the proteasome seems unlikely, as proteasomes are only located in the cytosol and nucleus (Wojcik and DeMartino, 2003), while Aβ is produced in the secretory pathway, which means that its intracellular localization is mainly confined to processing or re-uptake compartments such as ER, Golgi apparatus, endosomes and secretory vesicles. It is therefore unlikely that proteasome inhibition by Aβ is caused by physical association of Aβ with proteasomes. Moreover, a small pool of cytosolic Aβ produced by ERAD-mediated translocation of ER localized Aβ is degraded by the proteasome rather than inhibiting it (Schmitz et al., 2004). Cytosolic Aβ that might originate from Golgi apparatus deformation, atrophy and disruption in AD (Stieber et al., 1996) is therefore likely to be degraded by the proteasome as well.

A study that presents a possible mechanism for indirect proteasome inhibition by Aβ demonstrates that the toxicity of extracellular Aβ in neuronal cell lines is mediated by the E2 Ub-conjugating enzyme E2-25K/Hip2 (Song et al., 2003). E2-25K expression was increased upon Aβ treatment, and antisense E2-25K cDNA abolished Aβ-induced toxicity. These findings seem to be biologically relevant, as the E2 enzyme was also found to be up-regulated in AD brain and Swedish mutant *APP* transgenic mice (Tg2576) and co-localized with Aβ-immunoreactivity. E2-25K functions both as an E2 Ub-conjugating enzyme and as an unusual Ub ligase to produce Ub-Ub and unanchored poly-Ub chains, without further requirement of other E3 ligases (Chen and Pickart, 1990). Intriguingly, E2-25K is also capable of ubiquitinating UBB^{+1} (Lam et al., 2000), which accumulates in AD brains. Other mechanisms that could mediate proteasome inhibition indirectly by Aβ are induction of ROS (Reactive Oxigen species) (Kanski et al., 2002; Miranda et al., 2000), lipid peroxidation products (Butterfield et al., 2002; Shringarpure et al., 2000) and mitochondrial dysfunction (Canevari et al., 2004).

4.3 Mutant ubiquitin

UBB^{+1} is a mutant Ub resulting from molecular misreading of the *ubiquitin-B* gene (Van Leeuwen et al., 1998b). This mutant ubiquitin accumulates in the neuritic plaques and tangles in AD patients and in non-demented elderly controls with initial AD pathology. UBB^{+1} lacks the C-terminal Gly of wild type Ub and instead has a 19 amino acid extension. This mutant Ub can be ubiquitinated but cannot be covalently attached to other proteins (Fig.2) (De Vrij et al., 2001; Lam et al., 2000). UBB^{+1} behaves like an ubiquitin-fusion-degradation (UFD) substrate and is therefore a target for the proteasome. However, UBB^{+1} is also a potent and specific inhibitor of the proteasome (Lam et al., 2000; Lindsten et al., 2002). Proteasome inhibition by UBB^{+1} requires a certain threshold concentration to be reached, which implies that other pathogenic mechanisms that interfere with proteasomal degradation precede the accumulation of UBB^{+1}. As mentioned above, proteasome inhibition by Aβ may be mediated by its up-regulation of E2-25K (Song et al., 2003). An increase in E2-25K levels would lead to a rise in ubiquitinated UBB^{+1}, which in turn would inhibit the proteasome and lead to neurodegeneration. However, it is unlikely that the toxicity induced by Aβ and E2-25K is mediated only by UBB^{+1}, as, to our knowledge,

accumulation of endogenous UBB^{+1} protein has not been found in neuronal cell lines (De Vrij, unpublished data).

All in all, due to its dual substrate/inhibitor properties, UBB^{+1} seems to be an endogenous marker for proteasome inhibition, not only in AD but also in other (but not all) neurodegenerative diseases (De Pril et al., 2004; Fischer et al., 2003). UBB^{+1} may therefore be an important determinant of neurotoxicity, contributing to an environment that favors the accumulation of misfolded proteins.

Oxidative stress 4.4

Many oxidized proteins and lipid peroxidation products accumulate in AD brains, suggesting that oxidative stress is an important event in AD (Zhu et al., 2004a). For instance, as mentioned earlier, UCH-L1 was found to be oxidatively modified in a proteomics study of AD brain (Castegna et al., 2002).

The exact cause for oxidative stress in AD brain remains unclear. Aβ is likely to contribute to the onset of oxidative stress in AD brain, as Aβ generates free radicals through metal-catalyzed reactions that result in neuronal death (Miranda et al., 2000). Free radicals peroxidize membrane lipids and oxidize proteins. In general, such oxidized proteins are degraded by the proteasome. A relationship between oxidative stress and proteasome function is supported by the finding that the familial form of amyotrophic lateral sclerosis (ALS) is associated with a mutation in the superoxide dismutase (*SOD*) gene (Rosen, 1993). Histological characteristics of the disease are the presence of Bunina bodies, which are round eosinophilic inclusions, and of Ub-immunoreactive filamentous skein-like inclusions in motor neurons (Van Welsem et al., 2002). *SOD1* normally scavenges oxygen radicals, thereby preventing oxidative stress. Increased levels of oxidatively modified proteins and a parallel increase in proteasome activity were observed in cell lines that stably express a mutant form of human *SOD* (Aquilano et al., 2003). These cells were more sensitive to proteasome inhibition, which caused programmed cell death and accumulation of neuronal nitric oxide synthase (nNOS). The proteasome activity of these cells was, most likely, up-regulated to keep nNOS levels down and prevent oxidative stress-induced cell death. In a different study, proteasome inhibition in cells expressing a mutant SOD form caused the formation of aggregates containing ubiquitinated or nitrated α-

tubulin, SOD, α-synuclein and 68K neurofilaments. In these cells, the NOS inhibitor L-NAME prevented the viability loss and aggregation, suggesting that nitration of proteins plays an important role in the observed aggregation and cell death (Hyun et al., 2003).

In summary, there seems to be a cross-talk between proteasomal activity and oxidative stress-related mechanisms, also involving aggregate formation, at least in the familial ALS form caused by *SOD* mutation. The presence of oxidized proteins in AD is probably related to proteasome function as well. The increased levels of oxidized proteins in AD are more likely to be caused by a preceding decrease in proteasome activity, as a decline in proteasome function is observed in AD brains, rather than an increase, as is the case in ALS. In neuroblastoma cells however, oxidative stress can inhibit the proteasome and proteasome inhibitors increase the toxicity of oxidative stressors (Ding and Keller, 2001a).

4-Hydroxy-2-nonenal (4-HNE) is an aldehydic product of membrane lipid peroxidation, which is generated following exposure of neuronal membranes to Aβ. 4-HNE levels are significantly elevated in cerebrospinal fluid of AD patients compared to control subjects (Lovell et al., 1997). Interestingly, 4-HNE binds directly to tau and inhibits its dephosphorylation (Mattson et al., 1997), suggesting that 4-HNE is associated with tau hyperphosphorylation in degenerating neurons in AD brain. This premise is supported by the finding that isolated PHF from AD brain also contain HNE-protein conjugates (Perez et al., 2002). Intracellular tau aggregates can be induced in SH-SY5Y cells by adding okadaic acid (a phosphatase inhibitor) and 4-HNE (Perez et al., 2002), suggesting that both phosphorylation and oxidative modification are required for tau filament formation. Furthermore, the *in vitro* HNE modification of Aβ augments its ability to inhibit the 20S proteasome (Shringarpure et al., 2000).

4.5 ApoE

ApoE is the major lipoprotein within the CNS, where it is synthesized mostly by astrocytes. ApoE is critical for lipid transport and cholesterol homeostasis within the brain. There are three major isoforms of apoE - apoE2, 3 and 4 - which originate from three different APOE alleles (*APOE ε2, 3* and *4*), and their combinations lead to six possible genotypes. The prevalence of *APOE ε4* alleles is associated with an increased risk of developing AD in a gene

dosage-dependent manner (Corder et al., 1993; Poirier et al., 1993; Saunders et al., 1993). The prevalence and number of *APOE ε4* alleles also negatively affect the age of onset of AD and the pace of memory decline. In contrast, the *APOE ε2* allele is associated with a decreased risk for AD and a delayed age of onset (Roses, 1995). This difference may be explained by the reduced ability of apoE4 to clear Aβ compared to apoE2 (Yang et al., 1997). Amyloid plaque density, modulation of Aβ42 induced oxidation (Lauderback et al., 2002), decreased activity of neurons in the nucleus basalis in AD patients and controls (Dubelaar et al., 2004; Salehi et al., 1998), membrane phospholipid metabolite alterations (Klunk et al., 1998), and increased susceptibility to focal ischemia were all correlated in a dose-dependent manner with *APOE* genotypes. Moreover, promoter polymorphisms and transcription alterations of *APOE* were also suggested to influence AD risk (Laws et al., 2003).

ApoE was shown to be degraded by the proteasome in macrophages and hepatocytes (Wenner et al., 2001). Interestingly, neuron-specific intracellular processing of apoE, preferentially apoE4, occurs in AD brains and cultured neurons (Huang et al., 2001). The resulting cytosolic fragment induces NFT-like inclusions and is associated with increased tau phosphorylation (Brecht et al., 2004).

The low density lipoprotein receptor-related protein (LRP) is a receptor for apoE that was also shown to mediate Aβ clearance in an apoE isoform-specific manner (Beffert et al., 1999). Proteolytic processing of LRP by a γ-secretase-like activity is directly related to the proteasome, as the resulting cytosolic fragment is degraded by the proteasome (May et al., 2002). In the light of all these results, it would be interesting to investigate if the prevalence of *APOE ε4* alleles is related to different levels of proteasome activity.

UPS and aging 4.6

The strongest risk factor for developing AD is aging. During aging, many alterations occur in the brain that are not related to pathology, but apparently provide an environment in which AD is more likely to develop. The UPS is one of the systems that is also affected by normal aging. Proteasome activity and expression are decreased in aged rats in heart, kidney, lung and liver (Keller et al., 2000b), and are most pronounced in the central nervous system (hippocampus, cortex and spinal cord), with the exception of brain stem and cerebellum (Keller

et al., 2000b; Keller et al., 2000c). Increased 20S proteasome levels were detected in age-related muscle atrophy (Husom et al., 2004). This increase was not due to more constitutive proteasomes, but to an increase in immunoproteasomes, demonstrated by higher levels of cytokine-induced β-subunits LMP2 and LMP7 in aged muscle. The total proteasome activity did not differ between ages, suggesting a lower specific activity of individual proteasomes (Husom et al., 2004).

Oxidation of proteins is considered a hallmark of cellular aging. As the 20S proteasome is the major proteolytic enzyme responsible for removal of oxidized proteins, the age-related changes in proteasome subunit composition and activity probably relate to the oxidative state of the tissue. Oxidative injury and lipid oxidation products, such as 4HNE, inhibit the *in vitro* activity of the proteasome (Keller et al., 2000b).

Other measurements of age-related changes in proteasome content or activity have yielded varied results in many different tissues, ranging from no change to decreased or increased amounts of specific subunits. An interesting opportunity to gain further insight into the effect of age on proteasome activity will be to study aged transgenic Ub^{G76V}-GFP mice, that allow monitoring proteasome activity *in vivo* (Lindsten et al., 2003).

Although more research is required regarding AD pathology, all the results above show that aging might cause a cellular environment with decreased protein quality control capacity, which will favor a situation in which AD-related mechanisms can lead to neurodegeneration.

4.7 UPS, learning and memory

The major clinical manifestation in AD is memory loss. Massive synapse dysfunction, which occurs at an early clinical stage of AD is detected particularly in the molecular layer of the dentate gyrus, and correlates with cognitive impairment (Masliah et al., 1992; Masliah et al., 1991; Sze et al., 1997; Terry et al., 1991). In addition, neuroplasticity failure has been implicated in AD pathology (Mesulam, 1999) and Aβ was reported to have synaptotoxic effects in wild type human *APP* transgenic mice (Mucke et al., 2000). Intriguingly, synaptic plasticity was added to the large number of

cellular processes regulated by proteasomal activity (Cline, 2003; Hegde and DiAntonio, 2002).

In agreement with an important role for proteasome inhibition in neuronal functioning, primary neurons seem particularly vulnerable to proteasome inhibition (Laser et al., 2003; Pasquini et al., 2000; Qiu et al., 2000). This is in contrast to neuronal cell lines, in which proteasome inhibition induces neurite outgrowth rather than cell death (Giasson et al., 1999; Laser et al., 2003). The postmitotic state of neurons or the importance of UPS functioning for synaptic signaling might explain this selective vulnerability to proteasome inhibition.

UPS components are present in synapses and regulate synaptic plasticity and transmission within a timeframe of several minutes. Moreover, ubiquitin, E1 Ub activating enzyme, and proteasomes are present in *Xenopus* retina growth cones (Campbell and Holt, 2001). In this system axon guidance factors induce the levels of ubiquitinated proteins to double within as little as five minutes. Mice that carry a mutation in *E6-AP* Ub ligase show impaired LTP and context-dependent learning (Jiang et al., 1998). Interestingly, the human *E6-AP* gene (also known as *UBE3A*) is mutated in Angelman syndrome, which is characterized by mental retardation (Kishino et al., 1997). Additional evidence for the involvement of the UPS in memory formation was found in rats, where bilateral infusion of the proteasome inhibitor lactacystin into the CA1 region of the hippocampus caused complete retrograde amnesia for one-trial inhibitory advanced learning (Lopez-Salon et al., 2001).

During insect metamorphosis the UPS is essential for reconstruction of neuronal dendritic trees and axonal projections (Watts et al., 2003). In *Drosophila*, inhibition of the UPS leads to accumulation of Dunc13a, which regulates the strength of synaptic transmission through effects on synaptic vesicle priming. Excess Dunc13a results in increased synaptic transmission in the neuromuscular junction (Speese et al., 2003). *Drosophila* lines that over-express the deubiquitinating protease fat facets (faf) show synaptic overgrowth and defects in neurotransmitter release (DiAntonio et al., 2001). In *Aplysia*, the UPS was found to play a role in both pre- and postsynaptic transmission. Proteasome inhibitors increased the glutamate evoked response, neurite outgrowth, and the number of presynaptic boutons (Zhao et al., 2003a).

All these studies reflect the ability of the UPS to drastically influence synaptic transmission and plasticity in a neuronal network. Changes in substrate degradation can alter the number of synaptic boutons, while endocytosis of glutamate receptors - which is also dependent on proteasome activity (Patrick et al., 2003) - regulates the strength of synaptic transmission. These changes are of great impact on the physiological state of a neuron, because substrate degradation and endocytosis of receptors are not quickly reversible, like phosphorylation for instance, but require protein translation and transport to be reversed. It looks like the UPS is well on its way to join phosphorylation as a common mechanism for regulating neuronal activity.

5 CONCLUDING REMARKS

Although further insights into the neuropathology of AD are rapidly being gained, it is still not clear what is causing the disease, how it can be prevented or whether it can be cured.

Research in the past few years has focused on whether Aβ or tau has a more prominent role in AD pathology, although they are both indispensable to the process of developing AD. Besides these two major players in the field, the UPS is emerging as a clear co-factor in the development of AD, and in most other neurodegenerative diseases. Apparently, neurons are particularly vulnerable to factors that compromise UPS function. This might be due to the significant role that the UPS appears to play in synaptic transmission and plasticity. However, it remains unclear to what extent UPS aberrations found in neurodegenerative diseases are primary causes or secondary phenomena that result from other causes. The latter is not hard to imagine, as the UPS plays such an essential role in almost all processes that are essential for cell viability. One could argue that any severe alteration in one of these processes would have an effect on the UPS. Mutations in UPS components directly leading to neurodegeneration suggest a more prominent role in effectively causing some of the diseases. In PD, for instance, mutations in *parkin*, an E3 enzyme, seem to cause the majority of autosomal recessive early-onset PD cases.

ALS neuropathology shows an interesting link between proteasomal activity and oxidative stress-related mechanisms. This link might have implications for AD, where oxidative stress is also strongly associated with pathology. In contrast to ALS, however, where oxidized proteins probably activate the proteasome, proteasome inhibition probably precedes the accumulation of oxidized proteins in AD.

In AD pathology, UBB^{+1} is a clear-cut example of an aberrant UPS component, which compromises UPS function. However, because UBB^{+1} is also an efficient substrate for the proteasome, it seems more likely that other factors besides UBB^{+1} precede and cause the initial proteasomal inhibition in AD brain. After initial proteasome inhibition, UBB^{+1} can accumulate and promote further proteasome inhibition. The recent link between Aβ-mediated toxicity and proteasome activity, through the E2-25K enzyme and UBB^{+1}, points to an even more prominent role of the UPS in AD pathology than was previously thought. Moreover, in the last few years, many AD pathology-related proteins were shown to be degraded by the proteasome: tau, PSEN1 and PSEN2, the γ-secretase component Pen-2, apoE, oxidized proteins and the C-terminus of APP and of LRP. The decrease in proteasomal activity detected in AD brain is therefore related to all these different aspects of AD pathology. In addition, mostly *in vitro* studies demonstrated that the following molecules impair proteasome function: UBB^{+1}, Aβ, PHF tau and oxidized proteins. An overview of all proteins related to AD and the UPS is listed in table 1. All these complex interactions make it difficult to hypothesize what is actually causing proteasome inhibition in AD, and to what extent this proteasome inhibition is crucial to the development of AD pathogenesis. However, there is strong evidence supporting the premise that the UPS plays a central role in AD pathology. Unraveling the temporal course of AD neuropathology is important in this matter. Studies of mouse models related to AD failed to address the contribution of the UPS to the neuropathology of the mice. The recently described transgenic Ub^{G76V}-GFP mouse provides a model for monitoring proteasome activity *in vivo* (Lindsten et al., 2003). Crossing these mice with mice carrying familial AD mutations will provide important *in vivo* information about the involvement of the UPS in AD.

If proteasome inhibition is an early event in AD, we speculate that mutations or a shift in subunit composition might be the cause

of proteasome inhibition. So far, proteasome activity and subunit composition have not yet been correlated with the severity of AD pathology. There is supporting evidence for a possible change in subunit composition in several neurodegenerative diseases. For example, altered subunit composition occurs in Huntington's disease (Diaz-Hernandez et al., 2003). Moreover, 19S subunit S6b immunoreactivity was detected in several neuropathologies (Fergusson et al., 1996). On the other hand, many of the other AD pathological events, such as Aβ and PHF formation, were shown to inhibit the proteasome, which could therefore also be starting points of the proteasomal inhibition seen in AD. Accumulation of oxidized proteins and UBB^{+1} probably follow rather than cause initial proteasome inhibition. Once accumulated, however, these proteins are likely to promote proteasome inhibition and neurodegeneration.

In conclusion, although it is clear that alterations in UPS function are key factors in the final development of AD pathology, more research is needed to elucidate the exact mechanisms and order of events concerning the role of the UPS in AD.

Acknowledgements

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Scope and outline of this thesis

Ubiquitin B $^{+1}$ (UBB^{+1}) was discovered to accumulate in the pathological hallmarks of Alzheimer's disease (AD) along with other frameshifted proteins, i.e. APP^{+1} (amyloid precursor protein +1) and GFAP^{+1} (glial fibrillary acidic protein +1). The mutant form of ubiquitin seemed particularly intriguing, since its aberrant C-terminal structure clearly predicted a dysfunction, compared to normal ubiquitin. Moreover, the ubiquitin proteasome system is impaired in AD brains, as well as in most other neurodegenerative diseases. In parallel to AD-related studies, UBB^{+1} was found to accumulate in many other diseases as well.

The main focus of the studies described in this thesis was the question whether UBB^{+1} plays a role in proteasome inhibition and neurodegeneration in AD. The involvement of the ubiquitin proteasome system in AD is reviewed in **Chapter 1**.

The specific objectives of this thesis were:

1 To investigate the influence of UBB^{+1} on cellular functioning.

2 To determine if, and by what mechanisms, UBB^{+1} inhibits proteasomal degradation.

3 To characterize other AD-related mechanisms that may have synergistic effects on proteasome inhibition by UBB^{+1}.

The first objective was studied in **Chapter 2** in biochemical studies demonstrating that UBB^{+1} lacks the capacity to ubiquitinate other proteins, but can be ubiquitinated itself. The ubiquitinated form of UBB^{+1} was found to be relatively stable compared to other ubiquitinated proteins. Overexpressing UBB^{+1} in human neuroblastoma cell lines revealed apoptotic-like cell death specifically caused by UBB^{+1}.

In **Chapter 3** we provide more insight into the mechanism by which UBB^{+1} induces this apoptotic-like cell death, addressing objective number two. With the use of a green fluorescent protein (GFP)-based reporter system for proteasome inhibition we demonstrated that ubiquitinated UBB^{+1} is a potent and specific inhibitor of proteasomal activity. This study also revealed that UBB^{+1} has

seemingly paradoxical properties, as in low concentrations, UBB^{+1} appeared to be an efficient substrate for the proteasome.

In **Chapter 4** this paradox was partly explained by demonstrating a threshold effect of UBB^{+1} accumulation in an organotypic mouse cortex slice culture model. Quantification in human neuroblastoma cells confirmed that UBB^{+1} only inhibits the proteasome after it exceeds a critical level, causing an ongoing irreversible UBB^{+1} accumulation, thereby creating a negative feedback loop in proteasome inhibition. This conclusion led to our understanding that in AD affected cells apparently have reached the threshold of UBB^{+1} accumulation, as the protein is clearly present in the pathological hallmarks. However, since UBB^{+1} is a substrate at low concentrations, it is not likely that UBB^{+1} forms the initial trigger for proteasome inhibition in AD pathogenesis.

In **Chapter 5** Aβ peptide intermediates were studied as possible initiators of proteasome inhibition, addressing objective number three. Cytosolic Aβ was found not to inhibit the proteasome. However, indirect proteasome inhibition by Aβ through ER-associated degradation (ERAD) mechanisms redirecting ER-located Aβ to the cytoplasm in a modified form, or extracellular Aβ still remain candidates for this putative function.

Chapter 6 discusses the results presented in this thesis and addresses future research questions and directions. In addition, preliminary results are presented on human post mortem cortex slice cultures and on AD-related mechanisms other than Aβ-processing that could influence proteasome activity.

CHAPTER 2

Mutant ubiquitin expressed in Alzheimer's disease causes neuronal death

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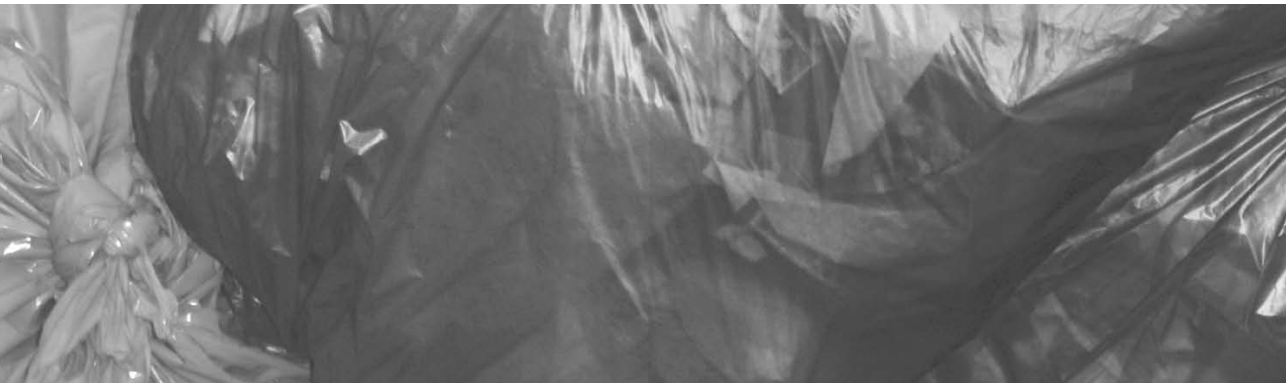

ABSTRACT

Ubiquitin-B^{+1} (UBB^{+1}) is a mutant ubiquitin that accumulates in the neurons of patients with Alzheimer's disease (AD). Here we report on the biochemical and functional differences between ubiquitin and UBB^{+1} and the effect of the mutant protein on neuronal cells. UBB^{+1} lacks the capacity to ubiquitinate, and although it is ubiquitinated itself, UBB^{+1} is not degraded by the ubiquitin-proteasomal system and is quite stable in neuronal cells. Overexpression of UBB^{+1} in neuroblastoma cells significantly induces nuclear fragmentation and cell death. Our results demonstrate that accumulation of UBB^{+1} in neurons is detrimental and may contribute to neuronal dysfunction in AD patients.

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia, affecting a large group of the elderly. Many genetic and nongenetic factors have been implicated in the pathogenesis of AD (Cummings et al., 1998b). We recently demonstrated that dinucleotide deletions in mRNA occur in AD brains, resulting in translation and accumulation of frameshifted so-called +1 proteins which may affect neuronal functioning. The process by which these dinucleotide deletions form is termed molecular misreading (Van Leeuwen et al., 1998b). Several examples of these aberrant +1 proteins have been found in the hallmarks of AD. Molecular misreading of the *ubiquitin B* gene results in UBB^{+1} protein, which accumulates in the earliest affected brain areas of patients with AD (Braak and Braak, 1991) (e.g. pyramidal cells in the CA1 region of the hippocampus, and neurons in the subiculum and cortex of AD patients) (Van Leeuwen et al., 1998a). Recently, Lam et al. (Lam et al., 2000) and we (Van Leeuwen et al., 1998a; Van Leeuwen et al., 2000) proposed that UBB^{+1} in the diseased aging brain could result in a dominant negative inhibition of the major proteolysis system, the ubiquitin-proteasomal system (Ciechanover, 1998). This blockade of protein degradation might contribute to the process of neuropathology in AD.

The ubiquitin-proteasomal system is involved in many cellular processes, such as cell cycle, apoptosis, endocytosis and ATP-dependent proteasomal breakdown of proteins (Ciechanover, 1998). Ubiquitin (Ub) tags proteins for degradation by conjugating to substrates through isopeptide bonds between the carboxy-terminal glycine residue of Ub and the ε-amino groups of lysine residues in proteins. Branched multi-Ub chains are formed by the sequential addition of mono-Ub to a lysine residue of substrate-bound Ub (Varshavsky, 1997). Such a multi-Ub chain serves as a targeting signal, resulting in degradation of the protein by the 26S proteasome (Ciechanover, 1998).

UBB^{+1} is a putative target for ubiquitination by wild-type Ub as it still contains the lysine residue at position 48. On the other hand, the UBB^{+1} protein is probably not able to participate actively in forming multi-ubiquitin trees itself, as it lacks the carboxyl-terminal glycin moiety. Recently, ubiquitinated UBB^{+1} has been found to inhibit the proteasome in a cell-free system (Lam et al., 2000). Inhibition of the ubiquitination process or a dominant negative effect on proteasomal

breakdown is likely to cause problems in protein degradation and therefore in neuronal functioning.

Here, we report on the biochemical properties of UBB^{+1} in an *in vitro* system and on the differential effects of wild-type Ub and UBB^{+1} on cellular functioning achieved by overexpressing these proteins in the human neuroblastoma cell line SK-N-SH. Our data show that UBB^{+1} was ubiquitinated but, in contrast to other ubiquitinated proteins, was not degraded. Moreover, we show that UBB^{+1} changed cell morphology, followed by neuronal cell death.

MATERIALS AND METHODS

In vitro experiments with UBB^{+1}

Rabbit reticulocytes were used as the source of enzymes participating in the Ub degradative pathway. ATP- and ubiquitin-depleted fraction II was prepared from rabbit reticulocytes as described by Hershko et al (Hershko et al., 1982).

Ubiquitin conjugation experiments (Fig. 1) were performed according to Gregori et al (Gregori et al., 1995). Recombinant his-tagged UBB^{+1}, purified on a Ni+ column and ubiquitin wild-type (wtUb) were iodinated using the iodogen method (Pierce, Rockford, IL). The specific activities were 3 x 10^4 cpm/μg and 6.4 x 10^4 cpm/μg for UBB^{+1} and wtUb, respectively. Approximately 1 μM of wtUb or UBB^{+1} was used in the reaction mixture in the presence or absence of ATP. At the indicated times, an aliquot of the reaction mixture was removed, the reaction was stopped by the addition of gel electrophoresis sample buffer containing 1% SDS and 0.5% β-mercaptoethanol, boiled for 3 minutes, and subjected to gel electrophoresis. The gel was dried and ubiquitin conjugates were visualized by autoradiography.

The conditions for ubiquitin and ATP-dependent degradation of iodinated substrates have been described elsewhere (Gregori et al., 1995). Lysozyme was used as the control for the degradation reaction. Lysozyme-specific radioactivity was 10^5 cpm/μg. Unlabeled

ubiquitin (10 μM) was added to the reaction mixture in addition to iodinated UBB^{+1} and wtUb, both at the final concentration of 1 μM (Fig. 2). Iodinated lysozyme concentration was 15 μM. During degradation, digested proteins were reduced to amino acids and small peptides that are acid-soluble. Radioactivity in the acid-soluble fraction was measured with a gamma counter and reported as the percentage of total radioactivity. The results shown in Fig. 2 represent the average values and the SE of the mean of a triplicate experiment.

Cell lines

SK-N-SH neuroblastoma cells were cultured in high-glucose Dulbecco's modified Eagle medium (Life Technologies, Grand Island, NY) containing 10% foetal calf serum (FCS) (Life Technologies) and supplemented with 100 U/ml penicillin (Life Technologies) and 100 μg/ml streptomycin (Life Technologies) (DMEM-10% FCS). Cells were cultured on poly-L-ornithine-hydrobromide (Sigma, St. Louis, MO)-coated glass coverslips in 24-wells plates (Nunc, Roskilde, Denmark) 1 day prior to infection. In one experiment 0.2% gelatin coating was used on the glass coverslips, which gave similar results.

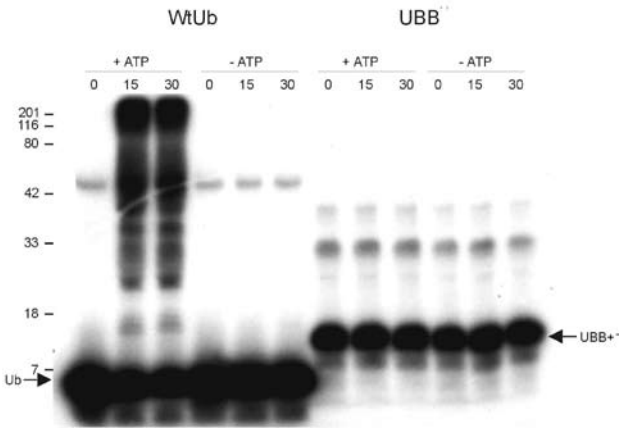

Fig.1 UBB^{+1} is not able to conjugate proteins. Autoradiograph of iodinated wtUb (left panel) and iodinated UBB^{+1} (right panel) incubated with fraction II of rabbit reticulocyte lysate in the presence (+) or absence (-) of ATP at different times (indicated in minutes). It is clear that in the presence of ATP, only wtUb is able to conjugate proteins. Arrows indicate monomeric wtUb and UBB^{+1}.

Viral constructs

First generation recombinant adenoviral vectors were constructed, purified and titered as described elsewhere (Hermens et al., 1997). All three viral vectors (AdLacZ, AdUBB^{+1} and AdwtUb) are based on the Ad5 mutant *dl309* (Jones and Shenk, 1979) and use the cytomegalovirus immediate early (CMV) promoter to drive transgene expression.

Titration of double CsCl gradient-purified Ad-CMV-LacZ, Ad-CMV-UBB^{+1} and Ad-CMV-wtUb on the permissive cell line 911 (Fallaux et al., 1996) revealed titres of 1 x 10^9, 4 x 10^9 and 1 x 10^{10} plaque forming units/ml, respectively. The presence of replication of competent adenovirus (RCA) in the adenoviral UBB^{+1} stock was determined by standard procedure titration on A549 cells (Hermens et al., 1997). No cytopathic effect in relation to RCA was observed.

Infections of neuroblastoma cells

One day prior to infection, neuroblastoma cells were cultured in 24-wells plates (2 x 10^5 cells/well). The next day the cells were differentiated by adding medium with 4 μM all-trans retinoic acid (Sigma) (Slack et al., 1992). Cells were infected 4 h later with a multiplicity of infection (MOI) of 10 with Ad-CMV-wtUb, Ad-CMV-UBB^{+1}, or Ad-CMV-LacZ as a control. Medium containing the adenoviral vectors was left on for 2 h, after which medium was replenished with normal medium containing retinoic acid. Cells were fixed at different times after infection (1, 3, and 6 days).

Western blots

Pellets of neuroblastoma cells were resuspended in suspension buffer (0.1 M NaCl, 0.01 M Tris-HCl pH 7.6, 1 mM EDTA pH 8.0) containing protease inhibitors PMSF and leupeptin, in concentrations of 100 μM and 10 μg/ml respectively, or in SDS loading buffer containing 1mM NEM (Sigma).

All samples were loaded onto 15% SDS-PAGE gels and then transferred semi-dry onto nitro-cellulose. Blots were then probed with rabbit antisera to wtUb (anti-wtUb, 1:500, DAKO, Carpinteria, CA) or UBB^{+1} (Ubi3^{+1} serum, 05/08/97, 1:1000, epitope: YADLREDPDRQ).

Subsequently, blots were incubated with anti-rabbit HRP (DAKO, 1:1000) and Lumilight ECL (Boehringer, Mannheim, Germany) chemiluminescence.

Pulse chase assay

SK-N-SH cells were cultured in 3.5cm dishes (Nunc) and differentiated with retinoic acid when confluency was reached. Cells were infected with the different Ad vectors as described previously. One day after infection, cells were incubated in medium lacking methionine and cysteine for 1 h, after which the cells were labeled by incubating them with medium containing 100 μCi Tran^{35}S-label for 4 h at 37 and 5% CO_2. After labeling, the medium was replaced by DMEM-10% FCS medium. Cells were harvested at different times in buffer (10 mM Tris, 0.15 M NaCl, 0.1% Nonidet P40, 0.1% Triton-x-100, 20 mM EDTA, pH 8.0) containing 0.1% SDS and protease inhibitors. For immunoprecipitation, anti-UBB^{+1} antibody Ubi3^{+1} (1:1000) and protein-A Sepharose beads were added to the Ad-UBB^{+1}-infected cell lysates. This suspension was shaken overnight at 4 . The next day, beads were spun down and the supernatant was replaced with buffer containing 0.1% SDS. The pellet was rinsed three times in buffer and twice in 10 mM Tris-HCl, pH 8.0. Subsequently, loading buffer was added to the Sepharose beads, after which the samples were boiled and loaded on a 15% SDS-PAGE gel. Gels were visualized using a PhosphorImager.

Immunofluorescence

After infection, cells were fixed in 4% formalin in phosphate buffered saline (PBS), pH 7.4, for 1 h or longer. In between staining steps, cells were rinsed with PBS, pH 7.4. Rabbit polyclonal anti-UBB^{+1} (Ubi3^{+1} serum, 05/08/97), rabbit polyclonal anti-wtUb (Sigma) and monoclonal anti-β-gal (gal-13, Sigma) antibodies were diluted 1:500, 1:100 and 1:350, respectively. The secondary anti-rabbit-Cy3 and anti-mouse-Cy3 were diluted 1:200 in Supermix containing 0.05M Tris, 0.9% NaCl, 0.25% gelatin and 0.5% Triton-X-100, pH 7.4. Nuclei of cells were stained with TO-PRO-3 (Molecular Probes, 1:500). Coverslips were mounted in Mowiol+ (0.1M Tris-HCl pH8.5, 25% glycerol, 10% w/v mowiol 4-88 and 0.1% anti-fading w/v 1,4-diazabicyclo-(2,2,2)-octane). Images were acquired by confocal

laser scanning microscopy (Zeiss 410) with three different lasers emitting at 488, 543 and 633 nm to excite FITC, Cy3 and TO-PRO-3, respectively. For quantification experiments the cells were stained as described above except for the nuclear staining, which was performed with Hoechst (Biorad 10 μg/ml).

Quantification of cells

SK-N-SH cells were quantified by hand with ImagePro software (Media Cybernetics, Silver Springs, MD). Images were acquired with a Sony XC-77CC black/white camera through a Zeiss axioskop with a Plan-Neofluar objective (40x oil lens). For each coverslip, a Cy3 image and the corresponding Hoechst image were acquired in five fields. The experiment was performed in triplicate. The total number of cells was quantified by counting the nuclei, which were visualised by Hoechst staining. The number of transduced cells was quantified by counting the immunopositive cells in the Cy3 images. In an overlay of the Hoechst and Cy3 images, fragmented nuclei in immunopositive cells were counted. After summation of the number of cells of five fields per coverslip, averages were calculated for three coverslips per condition. A two-factor ANOVA, based on α=0.05 was performed for each graph. In case of significant effects in virus and time or in virus-time interaction, multiple comparison was performed to find significance between the different groups. The experiment was repeated in duplicate, which gave similar results.

RESULTS

UBB^{+1} has lost the ability to participate in tagging proteins for degradation by the proteasome

Recombinant His-tagged UBB^{+1} was produced in *Escherichia Coli*. The purified protein was used to study whether UBB^{+1} is able to conjugate proteins in a rabbit reticulocyte cell-free system (Hershko

et al., 1982). Iodinated UBB^{+1} or wtUb were mixed with fraction II of rabbit reticulocyte lysate in the presence or absence of ATP. The reactions were stopped at different times (Fig. 1). WtUb was covalently conjugated to proteins only in the presence of ATP, as indicated by the appearance of Ub-containing bands with a molecular mass higher than monomeric Ub (8 kDa). When wtUb was substituted with UBB^{+1} (11 kDa), no conjugates were observed, indicating that this mutant Ub was not able to conjugate to proteins. These results are consistent with the fact that UBB^{+1} lacks a functional carboxyl-terminal residue (glycine 76) (Bamezai and Breslow, 1991).

UBB^{+1} is not degraded by the ATP-dependent proteasome pathway

UBB^{+1} is an abnormal protein; as such, it could be a substrate of the ubiquitin-proteasome dependent degradation pathway. We tested this possibility in the fraction II *in vitro* system. Radioiodinated UBB^{+1} was incubated with fraction II and Ub in the presence and absence of ATP. As positive and negative controls for the degradation reaction, we used radioiodinated lysozyme substrates and wtUb, respectively (Fig. 2). Figure 2A shows that lysozyme was efficiently degraded in an ATP-dependent manner. WtUb (Fig. 2B) was not significantly degraded either with or without ATP. With radioiodinated UBB^{+1} (Fig. 2C) we observed no ATP-dependent proteasomal degradation of the mutant protein. However, UBB^{+1} was degraded in an ATP- and proteasome-independent reaction, possibly by a proteolytic activity present in fraction II. In these experiments, lysozyme is clearly the only protein that is degraded by an ATP-dependent proteasomal activity (compare the final amount of protein degraded in Fig. 2D). The results in Fig. 2 indicated that UBB^{+1} was not a substrate of the ATP-dependent ubiquitin-proteasome pathway in the *in vitro* system.

UBB^{+1} is ubiquitinated in neuronal cells

To determine whether UBB^{+1} is processed in cells, we transduced the human neuroblastoma cell line SK-N-SH with adenoviral vectors (Hermens et al., 1997) encoding UBB^{+1} (AdUBB^{+1}) or wtUb (AdwtUb) as a control. Expression of both constructs was driven by the CMV promoter to ensure high expression of the proteins of interest. SK-N-SH cells were infected with AdUBB^{+1} or AdwtUb with an MOI of 10 for 2 h, resulting in high expression of either protein with an

efficiency of more than 60% of the cells. Cultures were harvested 1 day after infection. Western blots of transfected cells probed with either anti-UBB^{+1}or anti-wtUb antibodies confirmed production of the proteins. In the Ad-wtUb infected cells, a monomeric Ub band with an approximate molecular weight of 8 kDa (Fig. 3) and a high molecular mass smear, representing multiple ubiquitinated proteins, were detected. AdUBB^{+1}-infected cell lysates probed with UBB^{+1} antibody showed an 11 kDa UBB^{+1} band and higher molecular mass bands (+/- 19 kDa and 36-50 kDa) most likely representing ubiquitinated forms of UBB^{+1} (Fig. 3, asterisks). Ubiquitination of UBB^{+1} is likely to occur, since the protein contains a lysine at position 48, which is known to be a target for ubiquitination (Pickart, 1998). Lam et al. have recently shown that in 293T cells transfected with UBB^{+1}, the mutant ubiquitin is also ubiquitinated (Lam et al., 2000). We found a more pronounced ubiquitination of UBB^{+1} than that reported by Lam et al. (Lam et al., 2000), which is most likely due to the efficient transduction by the Ad vector.

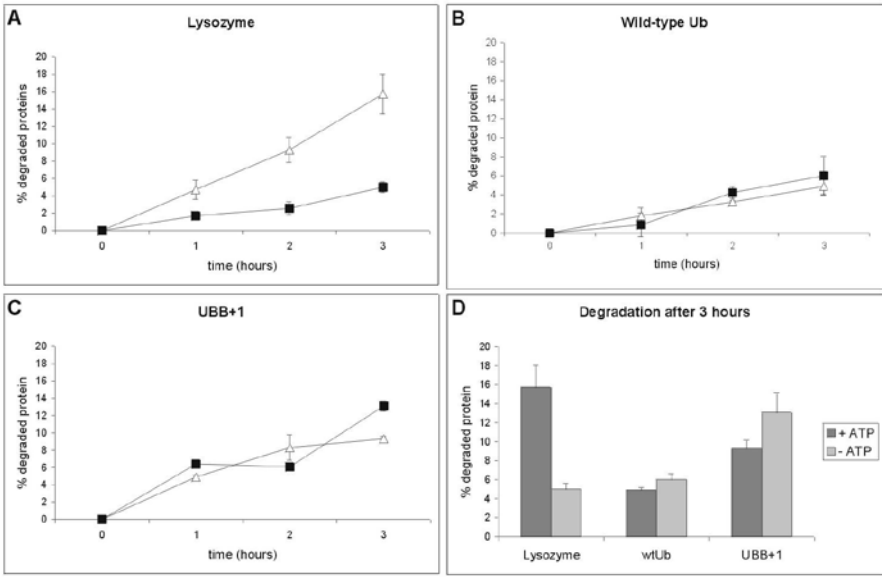

Fig.2 UBB^{+1} is not degraded by the proteasomal machinery. Degradation was determined as the acid soluble material released during the course of the reaction and is depicted as percentage of protein after incubation with reconstituted reticulocyte lysate in the presence (△) and absence (■) of ATP (+/- SE). **A** Time course of the degradation of iodinated lysozyme. **B** Time course of the degradation of iodinated wtUb. **C** Time course of the degradation of iodinated UBB^{+1}. **D** Three-hour incubation results from a direct comparison of the degradation rates. UBB^{+1} was not a substrate of the ubiquitin-proteasome pathway.

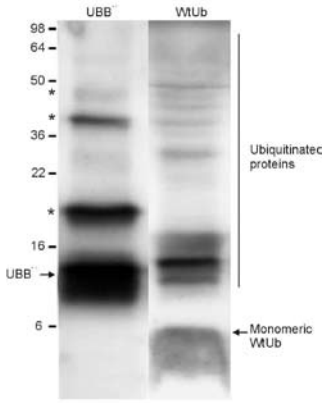

Fig.3 UBB^{+1} is ubiquitinated. Western blot on transduced SK-N-SH cell lysates. Left lane: AdUBB^{+1}-infected SK-N-SH cells after 1 day of infection probed with anti-UBB^{+1} showing that UBB^{+1} (11 kDa, arrow) is ubiquitinated (* = ubiquitinated forms of UBB^{+1}). As a control, a blot on LacZ transduced cell lysate was probed with anti-UBB^{+1}, which did not show bands (data not shown). Right lane: Control of AdwtUb infected cell lysate probed with anti-wtUb, showing multiple ubiquitinated proteins.

Ubiquitinated UBB^{+1} is stable

Pulse-chase assays were performed to investigate the stability of UBB^{+1} and its ubiquitinated forms, since the protein might be degraded after ubiquitination. SK-N-SH cells were transduced with AdUBB^{+1}, then labeled with [^{35}S]-methionine/cysteine for 4 h. Cells were harvested at different times after labeling.

Immunoprecipitation of AdUBB^{+1}-infected cell lysates with anti-UBB^{+1} antibody resulted in a pattern of bands (Fig. 4a, left lane) similar to that observed on the Western blots of infected cells. Immunoprecipitation of AdUBB^{+1} transduced cell lysates with anti-wtUb antibody also strongly suggest that UBB^{+1} is ubiquitinated. Several discrete high molecular weight bands of multimeric UBB^{+1} were observed, but the momomeric form of UBB^{+1} was not detected with the anti-wtUb antibody (Fig. 4A, right panel). One possible explanation is a conformational difference between monomeric forms of UBB^{+1} and wtUb, which would prevent UBB^{+1} from being recognized by the anti-wtUb antibody used in this experiment.

All UBB^{+1} conjugate bands were stable for more than 3 h, indicating that UBB^{+1} and ubiquitinated UBB^{+1} were barely degraded (Fig. 4B).

The specificity of the assay was confirmed by immunoprecipitation with anti-UBB^{+1} of AdLacZ- and AdwtUb-infected cell lysates. No bands as observed in Fig. 4B were detected in those experiments (data not shown).

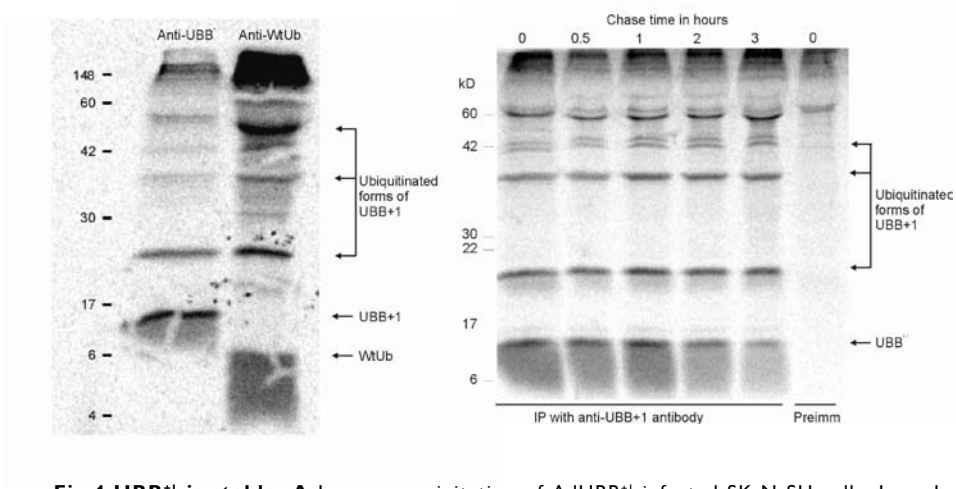

Fig.4 UBB^{+1} is stable. A Immunoprecipitation of AdUBB^{+1}-infected SK-N-SH cells. Lane 1: immunoprecipitation was done with anti-UBB^{+1}; lane 2: with anti-wtUb. The anti-wtUb antibody does not recognise the UBB^{+1} band but does stain the higher bands, confirming the ubiquitination of UBB^{+1}. **B** Pulse chase assay on AdUBB^{+1}-infected SK-N-SH cells. Cells were chased at different times after infection (t in hours). The image was acquired with a PhosphorImager after immunoprecipitation (IP) with anti-UBB^{+1} antibody or preimmune serum was performed. UBB^{+1} and its ubiquitinated forms were barely degraded after 3 hours.

UBB^{+1} overexpression induces cell death

It has been shown that UBB^{+1} inhibits the 26S proteasome in a cell-free system (Lam et al., 2000). Inhibition of the proteasome has been found to cause apoptosis in several cell lines (Pasquini et al., 2000; Qiu et al., 2000). To study the possible toxicity induced by UBB^{+1} in neuronal cells, SK-N-SH cells were transduced with either of the adenoviral vectors AdUBB^{+1}, AdwtUb, or AdLacZ as a control. Cells were fixed at different times after transduction, stained for proteins with fluorescent antibodies, and analyzed by confocal laser scanning microscopy.

SK-N-SH cells transduced with UBB^{+1} and immunopositive for anti-UBB^{+1} staining were morphologically different from cells transduced with wtUb. UBB^{+1} positive cells exhibited vesicle-like extensions (Fig. 5A), whereas wtUb and LacZ transduced cells had a normal appearance (Fig. 5B). Cells were also stained with the nuclear dye TO-PRO, revealing occasional nuclear fragmentation in UBB^{+1} transduced cells (Fig. 6), but no fragmentation of nuclei was seen in LacZ or wtUb transduced cells (data not shown). Both vesicle-like extensions and nuclear fragmentation are reminiscent of apoptosis. From 1 to 6 days after transduction, fewer UBB^{+1} immunopositive cells were observed whereas the number of wtUb transduced cells remained stable over time, indicating that UBB^{+1} transduced cells had died (Fig. 7). Quantification of the cells after AdUBB^{+1} infection revealed a clear and significant decrease ($p= 0.012$) in the total number of cells on day 6 vs. day 1, whereas no significant decrease was observed in AdLacZ- or AdwtUb-infected cells (Fig. 8A). The number of immunopositive UBB^{+1} transduced cells significantly decreased ($p=0.014$) on day 6 after infection (Fig. 8B), indicating

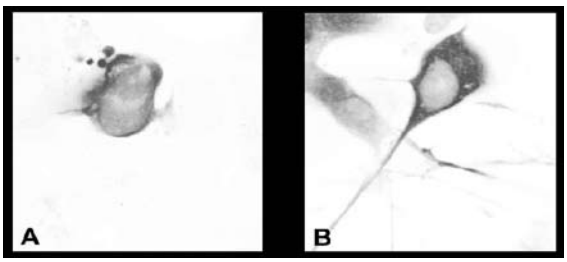

Fig.5 Overexpression of UBB^{+1} changes cell morphology. A AdUBB^{+1} transduced SK-N-SH cell, stained with anti-UBB^{+1}. **B** AdLacZ transduced SK-N-SH cell, stained with anti-Gal13. There is a clear visible difference between cells in panels A and B. UBB^{+1} transduced cells are rounded and show vesicle-like extensions whereas LacZ transduced cells show normal dendrites.

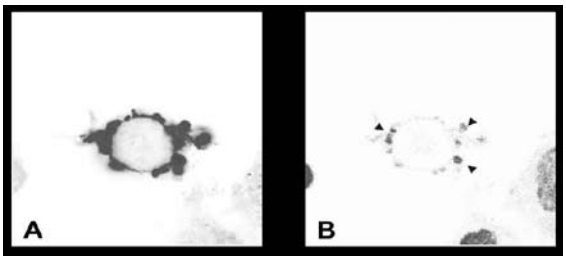

Fig.6 UBB^{+1} transduced cells show nuclear fragmentation. AdUBB^{+1} transduced SK-N-SH cell. **A** Anti-UBB^{+1}staining. **B** Nuclear TO-PRO staining of the same cell. The nucleus of this infected cell is fragmented (arrowheads).

that the decrease in the total number of cells was due to the death of UBB^{+1} transduced cells. The virus-time interaction of the analysis for the number of immunopositive cells was also significant ($F=2.63$, $p=0.04$). Moreover, the immunopositive UBB^{+1} transduced cells still present in the culture on day 6 after infection showed a significant percentage of fragmented nuclei compared with day 1 ($p=0.001$). This was a clear and significant increase compared with the wtUb and LacZ transduced cells on day 6 ($p=0.001$) (Fig. 8C). Western blots of $AdUBB^{+1}$-infected cell lysates showed a decrease in UBB^{+1} and its ubiquitinated forms at later times after infection, due to cell death, whereas wtUb was expressed equally well (data not shown). These data indicate that overexpression of UBB^{+1} triggers apoptosis in neuronal cells.

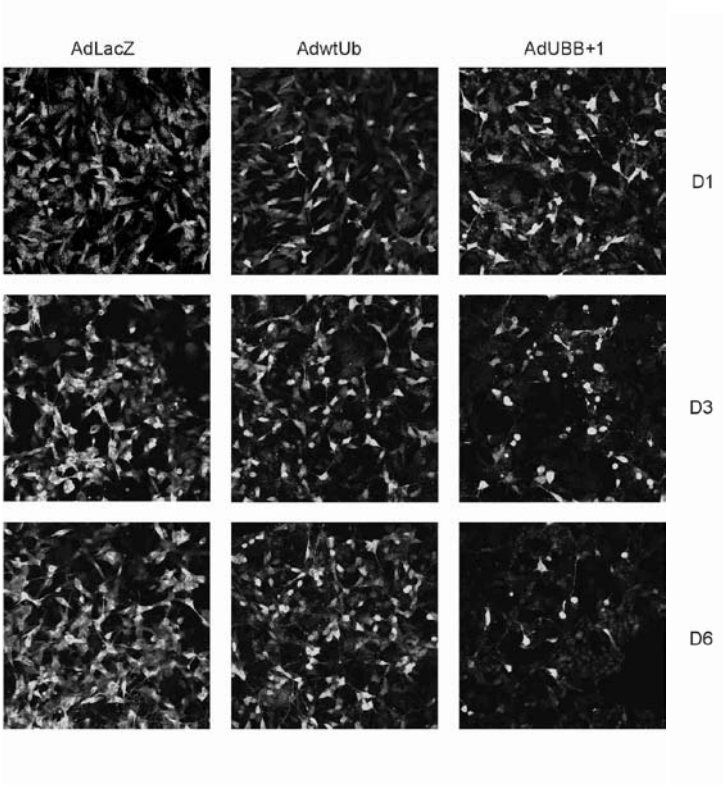

Fig.7 UBB^{+1} transduced cells die at later times after infection. SK-N-SH cells infected with Ad-LacZ, AdwtUb or $AdUBB^{+1}$ at different times after infection (days 1, 3, and 6). Ad-LacZ-infected cells were stained with anti-Gal13, AdwtUb-infected cells with anti-wtUb, and $AdUBB^{+1}$-infected cells with anti-UBB^{+1} antibody. The number of UBB^{+1} transduced cells clearly decreases at later times after infection whereas the control infections show remarkably less cell death.

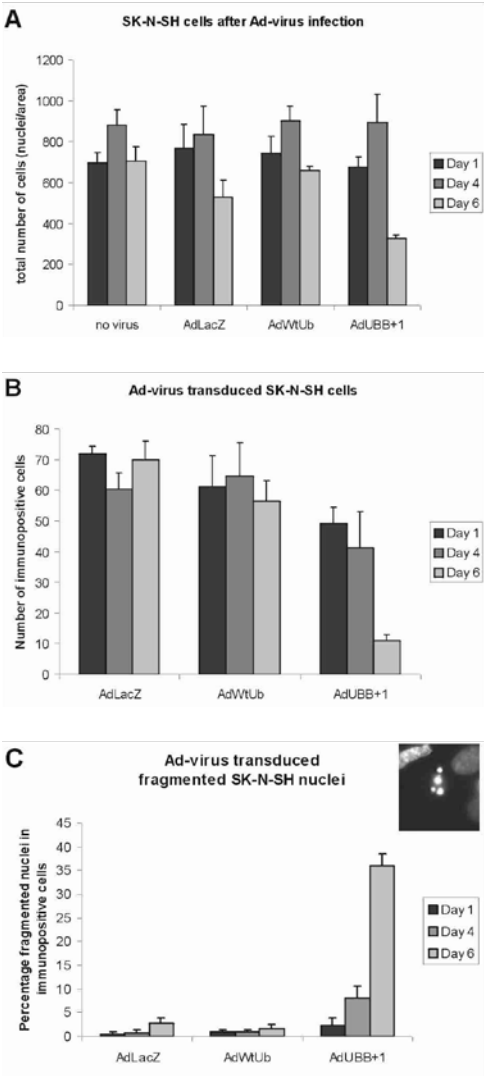

Fig.8 Quantification of UBB^{+1} induced cell death A Quantification of total number of cells by counting nuclei stained with Hoechst. The total amount of cells is significantly decreased 6 days after AdUBB^{+1} infection compared with day 1 ($p= 0.012$), whereas no significant decrease was observed in AdwtUb- or AdLacZ-infected cells. **B** Quantification of Ad-virus transduced cells by counting immunopositive cells (stained for either LacZ, wtUb or UBB^{+1}). Two-factor ANOVA showed a significant virus-time interaction ($F=2.62$, $p=0.04$, $p(time)=0.06$, $p(virus)<0.01$). The amount of UBB^{+1} transduced cells significantly decreased 6 days after infection vs. day 1 ($p=0.014$), indicating that UBB^{+1} transduced cells die. **C** Quantification of fragmented nuclei of immunopositive transduced cells depicted as percentage of nuclei in immunopositive cells. UBB^{+1} transduced cells showed a significant percentage of fragmented nuclei ($p=0.001$) on day 6 after infection, indicating that UBB^{+1} transduced cells had died in an apoptotic fashion.

DISCUSSION

Our results reveal that UBB^{+1} is defective in ubiquitinating proteins (Fig. 1). This mutant ubiquitin was not conjugated to proteins in reticulocyte fraction II, which contains all required elements of the ubiquitin-proteasome machinery. That UBB^{+1} is unable to conjugate to proteins is not surprising, since the carboxyl-terminal glycine at position 76 (Gly76) of Ub, which is lacking in UBB^{+1}, is essential in conjugating to other proteins (Varshavsky, 1997). The lack of conjugating properties of UBB^{+1} indicates that the mutant protein is not able to participate in targeting proteins for degradation. This finding agrees with data on the inability of the Des-75-76 Ub molecule (lacking the Gly75 and 76 residues) to conjugate to substrates (Bamezai and Breslow, 1991) and the inability of UBB^{+1} to bind to E1-activating enzyme (Lam et al., 2000).

Furthermore, we show that UBB^{+1} is not degraded by the ATP-dependent proteasome in reticulocyte lysate (Fig. 2), nor in neuronal cells (Fig. 4B). Surprisingly, we found that although UBB^{+1} is ubiquitinated, it is refractory to degradation in neuroblastoma cells, as UBB^{+1} and its ubiquitinated forms were stable for at least 3 h (Fig. 4B). Normally, most proteins targeted for degradation by the proteasome by ubiquitination are degraded in minutes (Johnson et al., 1992; Lenk and Sommer, 2000).

If UBB^{+1} is not degraded by the proteasome, why is it ubiquitinated? One possibility is that UBB^{+1} is targeted for degradation by ubiquitination but somehow inhibits the proteasome (discussed later). On the other hand, ubiquitination of UBB^{+1} may not represent a targeting signal but be part of the preassembly of multi-Ub chains. *In vivo*, unanchored multi-Ub chains are formed by conjugating (E2) and ligating (E3) enzymes that are specific for Ub. This preassembly of multi-Ub chains enables fast multi-ubiquitination and subsequent degradation of target proteins (Mastrandrea et al., 1999). UBB^{+1} could theoretically be incorporated into a preassembled multi-Ub chain, as this is formed by conjugation to either Lys29 or Lys48 (Mastrandrea et al., 1999), which are both present in UBB^{+1}. However, UBB^{+1} primed Ub chains will not be degraded by isopeptidase T (Wilkinson et al., 1995) as are normal multi-Ub chains because this enzyme requires a carboxyl-terminal Gly residue at the proximal end of the chain (Lam et al., 2000; Wilkinson et al., 1995).

Similarly, isopeptidase T is not effective in the disassembly of Des-75-76 Ub terminated chains (Amerik et al., 1997).

Our experiments show that high expression of UBB^{+1} induces massive cell death in human neuroblastoma cells, as seen by the sharp and significant decrease in the number of UBB^{+1} transduced cells in time (Fig. 8B). The decrease in UBB^{+1} immunopositive cells was accompanied by a decrease in total cell number (Fig. 8A), indicating that the UBB^{+1} transduced cells die. In addition, the morphology of UBB^{+1} immunopositive cells, i.e. vesicular extensions and nuclear fragmentation (Fig. 8C), show that UBB^{+1} transduced cells are dying in an apoptotic fashion, since apoptosis is normally characterised by blebbing, cell shrinkage, and nuclear condensation and fragmentation (McGahon et al., 1995).

The induction of cell death by UBB^{+1} is in line with the hypothesis that this mutant Ub inhibits the proteasome. Inhibition of the proteasome through other mechanisms has been shown to cause apoptosis in many studies, including apoptosis in neuronal cells. In cultured cerebellar granule cells, for example, inhibition of the proteasome by lactacystin results in apoptosis and activation of caspase-3 (Pasquini et al., 2000). In primary cultured cortical neurons, proteasome inhibitors carbobenzoxy-Leu-Leu-Leu-aldehyde and lactacystin both induced caspase-3 mediated apoptotic neuronal death (Qiu et al., 2000). Based on these results, the apoptotic death induced by UBB^{+1} can be explained by a direct inhibitory effect of UBB^{+1} on the proteasome.

In the pathology of AD, increasing evidence emerges for a role of apoptosis. However, the evidence is limited because cell loss is a dynamic process that almost certainly occurs over many years; therefore, neuropathologic studies may not have adequate sensitivity to detect relatively rapid processes such as apoptosis. In several studies, however, DNA laddering and terminal dUTP-mediated nick-end labeling were found in brains of AD patients vs. brains of nondemented control patients (Anderson et al., 2000; Su et al., 1994). Moreover, evidence for apoptosis decision cascades (Cotman, 1998) and a correlation between caspase activation and neurofibrillary tangle formation (Rohn et al., 2001) were recently found to be involved in AD.

It was reported recently that the proteasomal activity in brains of AD patients is diminished (Keller et al., 2000a). The activity of

the proteasome was significantly decreased in the parahippocampal gyrus, the superior and middle temporal gyri, and the inferior parietal lobe of AD brains, which are regions showing severe degenerative alterations in AD and UBB^{+1} staining. Moreover, ubiquitination of cerebral proteins was found to be defective in AD (Lopez Salon et al., 2000), suggesting that the Ub-proteasome degradation machinery is involved in the pathogenesis of AD. These data are, however, purely descriptive and do not indicate a molecular cause for the down-regulation of the proteasomal activity. In contrast, our earlier data on the neuronal accumulation of UBB^{+1} in AD patients (Van Leeuwen et al., 1998b), in combination with the data of Lam et al. (Lam et al., 2000) on the inhibition of the proteasome by UBB^{+1} and our present data on the stability of UBB^{+1} and induction of apoptotic cell death, strongly suggest that UBB^{+1} is a key protein in causing the proteasomal inhibition in AD brains.

In summary, we have shown that UBB^{+1} does not exhibit the degrading and conjugating properties of wtUb and that accumulating levels of this mutant protein in neuronal cells are detrimental and cause neuronal apoptosis. We also show that UBB^{+1} is ubiquitinated and barely degraded in neuronal cells. The finding that UBB^{+1} 1) is expressed in AD brains, 2) lacks the ability to ubiquitinate, 3) inhibits proteasomal activity (Lam et al., 2000) and 4) induces apoptotic cell death in neurons shows that this mutant Ub can interfere with normal neuronal functioning and probably contributes to neurodegeneration. Therefore, we propose that molecular misreading of the *ubiquitin B* gene is an early event in the pathogenesis of AD. The gradual and slow accumulation of UBB^{+1} protein will eventually inhibit the proteasomal activity in neurons, interfering with normal neuronal functions and resulting in neuronal loss.

Acknowledgements

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CHAPTER 3

Mutant ubiquitin found in neurodegenerative disorders is a ubiquitin fusion degradation substrate that blocks proteasomal degradation

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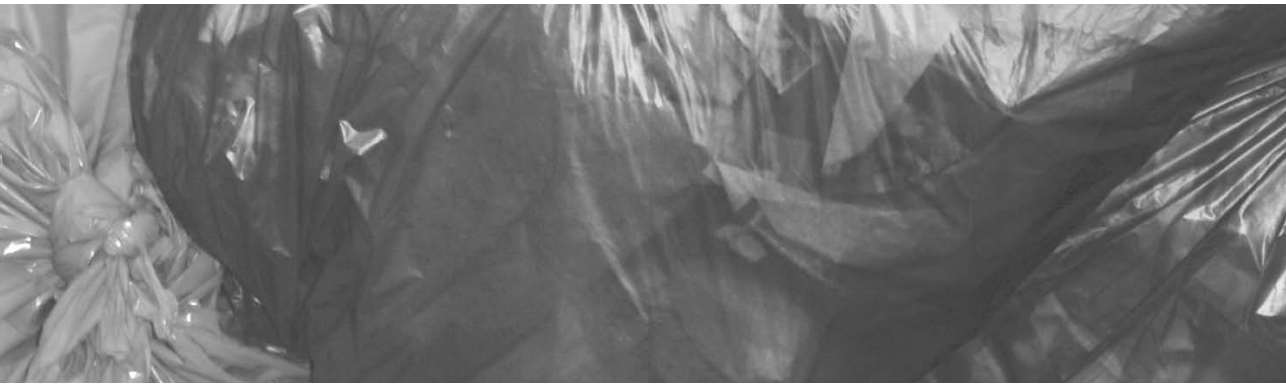

ABSTRACT

Loss of neurons in neurodegenerative diseases is usually preceded by the accumulation of protein deposits that contain components of the ubiquitin/proteasome system. Affected neurons in Alzheimer's disease often accumulate UBB^{+1}, a mutant ubiquitin carrying a 19–amino acid C-terminal extension generated by a transcriptional dinucleotide deletion. Here we show that UBB^{+1} is a potent inhibitor of ubiquitin-dependent proteolysis in neuronal cells, and that this inhibitory activity correlates with induction of cell cycle arrest. Surprisingly, UBB^{+1} is recognized as a ubiquitin fusion degradation (UFD) proteasome substrate and ubiquitinated at Lys 29 and Lys 48. Full blockade of proteolysis requires both ubiquitination sites. Moreover, the inhibitory effect was enhanced by the introduction of multiple UFD signals. Our findings suggest that the inhibitory activity of UBB^{+1} may be an important determinant of neurotoxicity and contribute to an environment that favors the accumulation of misfolded proteins.

INTRODUCTION

A broad array of human neurodegenerative diseases share strikingly similar histopathological features that may hold the key to their molecular pathogenesis (Sherman and Goldberg, 2001). A common finding is the presence of insoluble proteinaceous deposits, such as the neurofibrillary tangles and neuritic plaques of Alzheimer's disease, the Lewy bodies of Parkinson's disease, and the intranuclear inclusions of Huntington's disease, that differ in their protein content but invariably contain components of the ubiquitin/proteasome system (UPS) (Schwartz and Ciechanover, 1999). As this cellular proteolytic machinery is involved in the clearance of misfolded proteins, this has led to the suggestion that a chronic imbalance between their generation and processing may be the primary cause for the formation of protein deposits (Cummings et al., 1998a; Sherman and Goldberg, 2001). This model is further supported by the identification of inactivating mutations in a ubiquitin ligase (Kitada et al., 1998) and a deubiquitinating enzyme (Leroy et al., 1998) as the cause for rare familial forms of Parkinson's disease as well as genetic mouse models of neurodegeneration (Saigoh et al., 1999). Moreover, the cellular toxicity correlated with nuclear inclusions can be suppressed by components of the UPS (Fernandez-Funez et al., 2000), confirming the role of this proteolytic pathway in the clearance of their precursors.

The demonstration that components of the UPS often are involved in neurodegeneration prompted us to examine whether a general impairment of the proteolytic machinery may contribute to the pathology. Recently, an aberrant form of ubiquitin was found in affected neurons of patients with different tauopathies such as sporadic and familial Alzheimer's disease, Down syndrome (Van Leeuwen et al., 1998b), progressive supranuclear palsy (Fergusson et al., 2000), Pick's disease, frontotemporal dementia, argyrophilic grain disease, and the polyglutamine disorder Huntington's disease (unpublished data), but not in synucleinopathies, such as Lewy body disease and multisystem atrophy (Van Leeuwen et al., 1998b). Ubiquitin is generated from precursor proteins consisting of tandem ubiquitin moieties that are cleaved into monomeric ubiquitin by ubiquitin C-terminal hydrolases (Wilkinson, 2000). Due to a mechanism known as molecular misreading (Van Leeuwen et al., 2000), a dinucleotide deletion can occur within the mRNA encoding the ubiquitin B precursor resulting in a +1 frame shift close to

the C-terminus of the first ubiquitin moiety (Van Leeuwen et al., 1998b). Translation of the shifted open reading frame results in the product UBB^{+1} that comprises the first ubiquitin moiety with a 19–amino acid extension. Because the cleavage site of the ubiquitin C-terminal hydrolase is absent in UBB^{+1}, the extension is not removed. The aberrant C terminus prevents the activation and conjugation of UBB^{+1}, but due to the unaffected lysine residues, the mutant ubiquitin may serve as a scaffold for ligation of wild-type ubiquitin molecules (Van Leeuwen et al., 2000). Synthetically ubiquitinated UBB^{+1} was shown to inhibit proteasomal degradation *in vitro*, and therefore it was hypothesized that its expression in neurons may disturb ubiquitin-dependent proteolysis (Lam et al., 2000). Using two different green fluorescent protein (GFP)-based reporters that allow monitoring of ubiquitin-/proteasome-dependent proteolysis in living cells (Dantuma et al., 2000b), we show that UBB^{+1} acts as a strong inhibitor of the proteasome *in vivo* and induces a general accumulation of ubiquitinated substrates and cell cycle arrest. Surprisingly, UBB^{+1} is recognized as a ubiquitin fusion degradation (UFD) substrate and accordingly ubiquitinated at both Lys29 and Lys48 residues of its ubiquitin moiety. The inhibitory capacity relies on its recognition as a UFD substrate, as substitutions of either lysine residue releases the blockade while the inhibitory activity is further activated by enhancement of the UFD signal.

RESULTS

UBB^{+1} inhibits the ubiquitin/proteasome system in living cells

Two previously characterized GFP-based proteasome substrates carrying an N-end rule (Ub-R-GFP) or a UFD (Ub^{G76V}-GFP) degradation signal (Dantuma et al., 2000b) were used to monitor ubiquitin-/proteasome-dependent proteolysis in UBB^{+1}-expressing cells. The N-end rule degradation signal triggers ubiquitination close to the N terminus of the GFP reporter once the ubiquitin moiety of the fusion is cleaved by endogenous ubiquitin C-terminal hydrolases (Varshavsky, 1996), whereas the UFD signal includes the N-

terminal uncleavable ubiquitin moiety Ub^{G76V} that serves as target for polyubiquitination (Johnson et al., 1995). Because UBB^{+1} mainly has been found in neurons, the reporters were stably transfected in the SH-SY5Y neuroblastoma cell line. In addition, we used a previously characterized HeLa transfectant that constitutively expresses the Ub^{G76V}-GFP reporter (Dantuma et al., 2000b). Reporter expressing SH-SY5Y and HeLa cells were transiently transfected with FLAG-tagged ubiquitin (^{FLAG}Ub) or UBB^{+1}, and were analyzed in parallel for expression of these proteins and activity of the UPS as assessed by accumulation of the GFP fluorescence. Microscopic and flow cytometric analysis revealed accumulation of the Ub^{G76V}-GFP and Ub-R-GFP reporters in cells expressing detectable amounts of UBB^{+1}, whereas overexpression of ^{FLAG}Ub had no effect (Fig. 1A). Flow cytometric analysis of HeLa cells that accumulated the Ub^{G76V}-GFP reporter revealed a 60-fold increased fluorescence intensity (unpublished data), compared with a 100-fold increase in the same assay after treatment with potent inhibitors of the proteasome (Dantuma et al., 2000b; Myung et al., 2001). It is noteworthy that even though the vast majority of UBB^{+1}-positive cells accumulated the Ub^{G76V}-GFP reporter, the percentage of fluorescent cells was ~1–2% of the total population, which is surprisingly low, as transfection efficiencies between 20 and 40% were routinely obtained in these HeLa cells (see below).

Kinetics of Ub^{G76V}-GFP accumulation in UBB^{+1}-positive cells showed that after 10 h, ~1/2 of the UBB^{+1}-expressing cells had elevated levels of the Ub^{G76V}-GFP proteasome substrate, which further increased to 80% at 20 h posttransfection (Fig. 1B). Thus, the expression of UBB^{+1} preceded the accumulation of GFP. Only background fluorescence was detected in cells expressing ^{FLAG}Ub (Fig. 1B). In order to study whether the elevated Ub^{G76V}-GFP steady state levels are due to delayed turnover of this proteasome substrate in response to UBB^{+1}, we evaluated the clearance of the accumulated proteasome substrate after blocking protein synthesis with cycloheximide. We would like to emphasize that the cycloheximide treatment will block not only the expression of Ub^{G76V}-GFP, but also of UBB^{+1}. To validate the experimental set up, we first tested the clearance of Ub^{G76V}-GFP from cells in which the GFP substrate had been accumulated during a short incubation with the reversible proteasome inhibitor MG132. Incubation with the proteasome inhibitor resulted in an ~10-fold induction of GFP fluorescence. After removing MG132 and blocking protein synthesis with cycloheximide the cells degraded

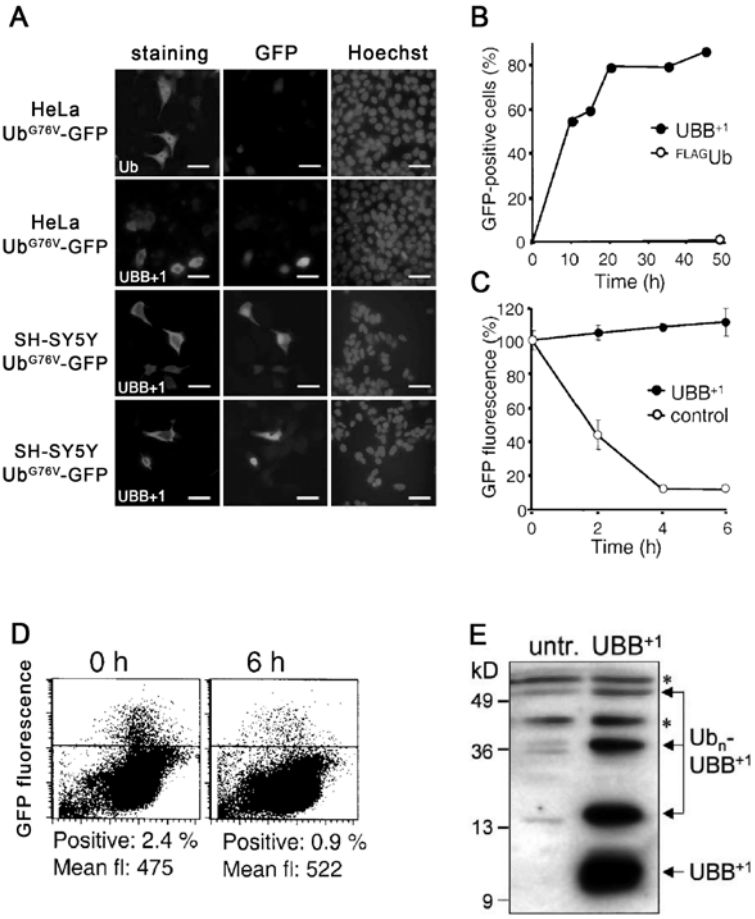

Fig. 1 UBB^{+1} inhibits the ubiquitin/proteasome pathway *in vivo*. **A** HeLa and SH-SY5Y cell lines stably expressing Ub^{G76V}-GFP or Ub-R-GFP were transfected with ^{FLAG}Ub or UBB^{+1}. Cells were stained with an anti-FLAG or anti-UBB^{+1} antibody and nuclei were counterstained with Hoechst 33258. Representative micrographs show expression of ^{FLAG}Ub and UBB^{+1} (left, red), fluorescence of Ub^{G76V}-GFP or Ub-R-GFP (middle, green), and counterstaining with Hoechst 33258 (right , blue). Bar, 20 µm. **B** Ub^{G76V}-GFP HeLa cells were transfected with UBB^{+1} or ^{FLAG}Ub harvested at indicated time points and analyzed by fluorescence microscopy. The results are expressed as percentage of UBB^{+1}- or ^{FLAG}Ub-positive cells accumulating Ub^{G76V}-GFP. **C** Protein synthesis was blocked in Ub^{G76V}-GFP HeLa cells transfected with UBB^{+1} by administration of 50 µg/ml cycloheximide. As a control, cycloheximide was added to Ub^{G76V}-GFP HeLa cells in which the reporter had been accumulated by a 2.5-h treatment with the reversible proteasome inhibitor MG132. The mean fluorescence intensity of the GFP fluorescent population was determined at the indicated time points by flow cytometry. Mean fluorescence when cycloheximide was administrated was standardized as 100%. Triplicate values of representative experiment. **D** Flow cytometric analysis of Ub^{G76V}-GFP fluorescence in UBB^{+1} -transfected cells upon administration of cycloheximide at time points 0 and 6 h (as in C). Percentage GFP-positive cells and their mean fluorescence intensity are indicated. **E** Western blot analysis with an anti-UBB^{+1} antibody of cell lysates of untransfected and UBB^{+1} transfected HeLa cells. Molecular mass marker and bands corresponding to UBB^{+1} and ubiquitinated UBB^{+1} are indicated. (*) Nonspecific immunoreactive bands.

the accumulated Ub^{G76V}-GFP within 4 h (Fig. 1C). In sharp contrast, the mean fluorescence intensity of cells transfected with UBB^{+1} did not decline over a 6-h period, but rather showed a modest increase. Although the percentage GFP fluorescent cells declined in both the control and the UBB^{+1}-transfected cells, after 6 h, we observed that only in the UBB^{+1}-transfected cells was there still a substantial amount of cells with accumulated Ub^{G76V}-GFP (Fig. 1D; unpublished data). These data show that the accumulated Ub^{G76V}-GFP has a prolonged half-life in cells transfected with UBB^{+1}. The decrease of GFP fluorescent cells upon blockage of protein synthesis in UBB^{+1}-transfected cells also suggests that newly synthesized proteins are required to maintain a full blockage on the UPS.

Western blot analysis of UBB^{+1}-transfected HeLa and SH-SY5Y cells revealed the presence of unmodified UBB^{+1}, as well as three slower migrating bands (Fig. 1E; unpublished data). This pattern corresponds to that found in earlier studies in which the bands were identified as conjugates of UBB^{+1} with one, two, or three ubiquitin moieties (De Vrij et al., 2001; Lam et al., 2000).

Expression of UBB^{+1} induces accumulation of polyubiquitinated proteins and cell cycle arrest

In subsequent experiments we analyzed the ubiquitination status of accumulating proteasome substrates in UBB^{+1}-expressing cells. Ub^{G76V}-GFP HeLa cells were transiently transfected with UBB^{+1} and then sorted by flow cytometry based on GFP fluorescence intensity. Western blots of lysates from GFP-positive and -negative cells probed with an anti-ubiquitin antibody demonstrated that elevated GFP levels correlated with a general accumulation of polyubiquitinated proteins (Fig. 2A), corresponding to an approximately twofold increase in the intensity of the smear of polyubiquitin adducts (Fig. 2B). Thus, UBB^{+1} is likely to affect an event downstream of polyubiquitination.

Impairment of the UPS accompanied by the accumulation of polyubiquitinated proteins, as observed in cells treated with inhibitors of the proteasome, normally results in induction of apoptosis often preceded by arrest in the G2/M phase of the cell cycle (Dantuma et al., 2000b; Lee and Goldberg, 1998). Therefore, 48 h posttransfection we analyzed the cell cycle distribution of UBB^{+1}-transfected Ub^{G76V}-GFP HeLa cells emitting background

(Ub^{G76V}-GFP^{low}), moderately elevated (Ub^{G76V}-GFP^{medium}) or high levels of GFP fluorescence (Ub^{G76V}-GFP^{high}). Ub^{G76V}-GFP^{low} cells displayed a cell cycle distribution comparable to that of untransfected cells, whereas a larger proportion of the Ub^{G76V}-GFP^{medium} and Ub^{G76V}-GFP^{high} cells were found in the G2/M phase, which is indicative for cell cycle arrest (Fig. 2C). A similar G2/M arrest was observed in parental HeLa cells expressing UBB^{+1}, excluding the possibility that

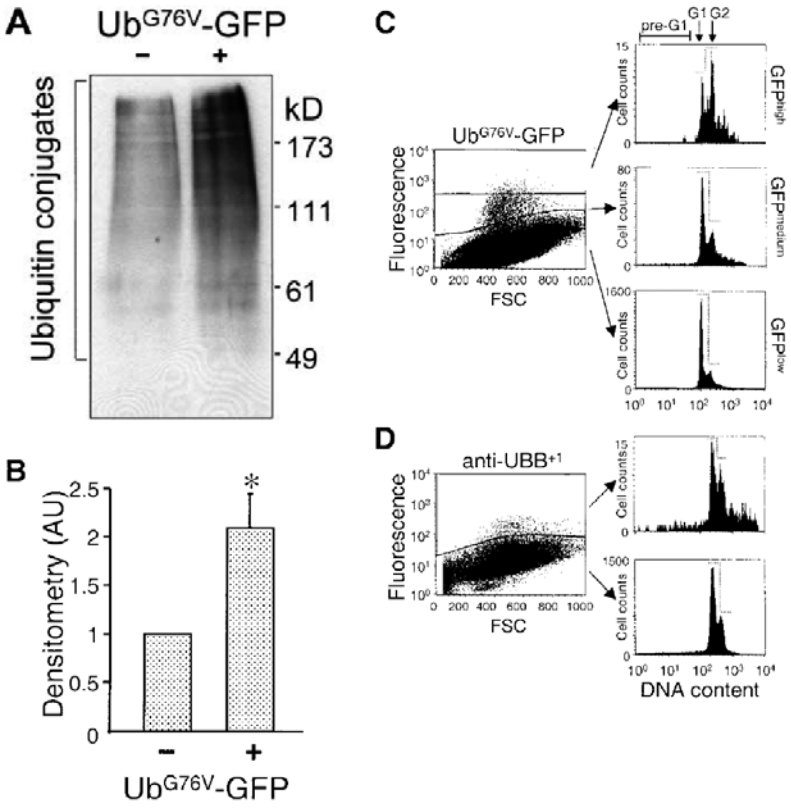

Fig.2 UBB^{+1} induces accumulation of polyubiquitinated proteins and G2/Mcell cycle arrest. A Ub^{G76V}-GFP HeLa cells were transfected with UBB^{+1} and 40,000 high fluorescent and 40,000 low fluorescent cells were sorted by flow cytometry 48 h posttransfection. Cell lysates of these populations were analyzed by Western blot probed with an anti-ubiquitin antibody. Molecular mass marker is indicated. **B** Quantitative analysis of antiubiquitin immunoreactivity by densitometry from three independent experiments as described in A. **C** Flow cytometry analysis of Ub^{G76V}-GFP HeLa cells transiently transfected with UBB^{+1} on the left. The cell cycle distribution, analyzed by propidium iodide staining, of the Ub^{G76V}-GFP^{high}, Ub^{G76V}-GFP^{medium}, and Ub^{G76V}-GFP^{low} fluorescence are illustrated to the right. One representative experiment out of three. **D** Flow cytometric analysis of UBB^{+1} -transfected parental HeLa cells stained with an anti-UBB^{+1} antibody (left). The cell cycle distribution of the UBB^{+1} - positive and negative population is shown (right).

accumulation of the GFP reporter may be responsible for the effect (Fig. 2D). Within the time frame of our transient transfection, we did not observe a significant increase of apoptotic cells in the GFP positive populations.

UBB^{+1} is a UFD substrate

To test whether physiological ubiquitination is required for the inhibitory activity of UBB^{+1} *in vivo*, we generated the mutant UBB$^{+1/K48R}$ in which the common ubiquitin conjugation site Lys48 was substituted with Arg. Surprisingly, UBB$^{+1/K48R}$ was still subject to ubiquitination in SH-SY5Y and HeLa cells (Fig. 3A; unpublished data), suggesting that an alternate ubiquitination site may be used. Targeting of substrates for proteasomal degradation may also occur via the less common ubiquitination site Lys29. To date, this site has only been described for UFD substrates in yeast in which both Lys29 and Lys48 of the N-terminal ubiquitin moiety are targets for polyubiquitination (Johnson et al., 1995; Koegl et al., 1999). Therefore, we compared UBB^{+1} mutants carrying Lys29 Arg and Lys48 Arg substitutions. Indeed, both UBB$^{+1/K29R}$ and UBB$^{+1/K48R}$ were equally efficiently ubiquitinated, whereas ubiquitin conjugation was virtually abrogated in the double mutant UBB$^{+1/K29,48R}$ (Fig. 3A). Furthermore, substitution of either lysine residue was sufficient to induce a significant increase in the steady state levels of the mutant protein. The effect was most dramatic with the UBB$^{+1/K29R}$ mutant (Fig. 3A), suggesting that this ubiquitination site may preferentially target UBB^{+1} for proteasomal degradation. Surprisingly, we observed consistently higher levels of UBB$^{+1/K29R}$ as compared with UBB$^{+1/K29,48R}$ in both HeLa and neuroblastoma cells. Although we did not fully understand this observation, subsequent analysis confirmed that this is not due to proteasomal degradation of the double mutant (Fig. 3C; unpublished data).

Paradoxically, we observed that the UBB^{+1} is a potent inhibitor of the UPS, whereas proteins carrying a UFD signal are normally rapidly degraded by the proteasome (Johnson et al., 1992; Johnson et al., 1995). As noted above, we observed in transient transfections an unanticipated low percentage of cells with detectable levels of the UBB^{+1} protein. This prompted us to investigate the possibility that the UBB^{+1} may be degraded in a fraction of the cells. To this end, we constructed a plasmid in which UBB^{+1} expression and GFP expression

are driven by the CMV and SV40 promotors, respectively, which allowed us to identify all transfected cells by the GFP fluorescence. Microscopic examination showed that only ~5% of the transfected cells expressed detectable amounts of UBB^{+1} (Fig. 3B, top). Inclusion of the specific proteasome inhibitor lactacystin (Fig. 3B, bottom) or epoxomicin (unpublished data) resulted in accumulation of UBB^{+1} in a great part of transfected cells. Western blot analysis confirmed the increase of UBB^{+1} in response to lactacystin and epoxomicin and showed that proteasomal degradation of UBB^{+1} was abrogated when Lys29 and Lys48 were substituted with Arg residues (Fig.

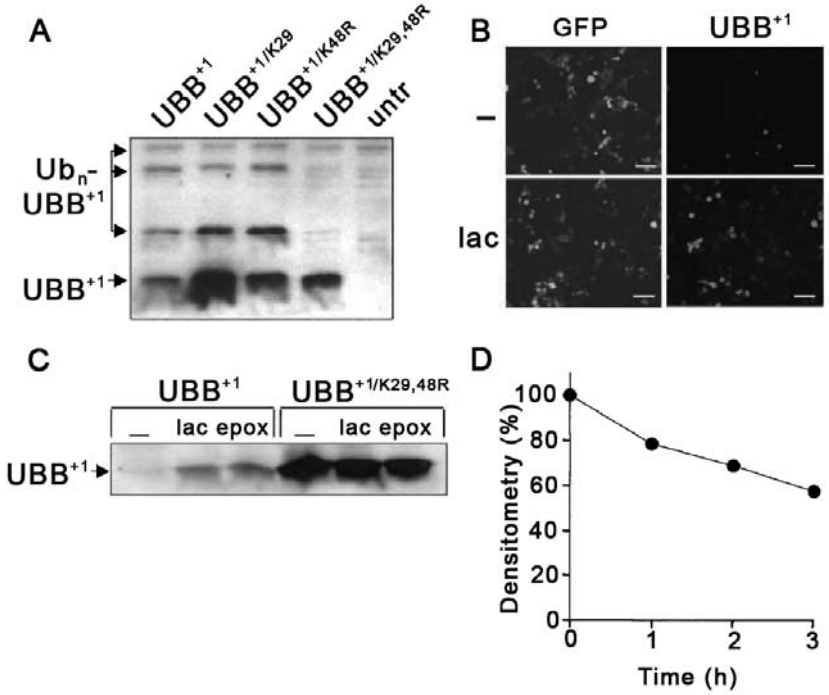

Fig.3 UBB^{+1} is a UFD substrate. A Western blot analysis with an anti-UBB^{+1} antibody of cell lysates from HeLa cells transfected with UBB^{+1}, $UBB^{+1/K29R}$, $UBB^{+1/K48R}$, $UBB^{+1/K29,48R}$. Products corresponding to unmodified and ubiquitinated UBB^{+1} are indicated. **B** Micrographs of HeLa cells transfected with pCMS-UBB^{+1} /GFP that were left untreated (top) or incubated for 16 h with 30 μM lactacystin (bottom). Transfected cells were identified by GFP expression (left) and transfected cells expressing detectable levels of UBB^{+1} were visualized by immunostaining (right). Bars, 100 μm. **C** Western blot analysis with an anti-UBB^{+1} antibody of the steady-state levels of UBB^{+1} and $UBB^{+1/K29,48R}$ in transiently transfected HeLa cells that were left untreated or incubated the proteasome inhibitors lactacystin (30 μM) or epoxomicin (500 nM). **D** The turnover of UBB^{+1} was determined by pulse-chase analysis in SK-N-SH neuroblastoma cells transduced with lenti-UBB^{+1}. Intensity of the UBB^{+1} band was quantified with a phosphoimager and the intensity at time point 0 was standardized as 100%. (A–D) One representative experiment out of three. **See Color figures.**

3C). Pulse-chase analysis of neuroblastoma cells transduced with a lentiviral vector encoding UBB^{+1} revealed that the UBB^{+1} levels declined over the 3-h period monitored, which is in line with the notion that UBB^{+1} is degraded in many cells (Fig. 3D). These data, together with the experiment shown in Fig. 1, C and D, indicate that whereas the fast majority of UBB^{+1}-expressing cells turnover the mutant ubiquitin, it remains stable in a fraction of the cells due to a general blockage of the UPS. Therefore, we conclude that UBB^{+1} is an authentic UFD substrate and degraded accordingly by the UPS in many cells.

Ubiquitination as a UFD substrate is required for a full inhibitory activity

Next, we tested whether ubiquitination at specific sites is required for the inhibitory activity of UBB^{+1}. UBB^{+1} mutants lacking the Lys29, Lys48, or both ubiquitination sites were transiently transfected in SH-SY5Y cells expressing the GFP reporters and the activity of the UPS was monitored by measuring GFP-accumulation. Mutation of both Lys29 and Lys48 abrogated the accumulation of both GFP reporters in the neuroblastoma cells confirming that ubiquitination is critical for the inhibitory effect (Fig. 4, A and B). Surprisingly, substitutions of single lysine residues had different effects on the degradation of UFD and N-end rule substrates. The single lysine mutants UBB$^{+1/K29R}$ and UBB$^{+1/K48R}$ were still able to inhibit the degradation of Ub^{G76V}-GFP, although the inhibitory effect was strongly compromised. In contrast, substitution of either lysine residue was sufficient to fully abrogate the effect of UBB^{+1} on accumulation of the Ub-R-GFP reporter, demonstrating that both ubiquitination sites are required to block the degradation of N-end rule substrates. Thus, efficient inhibition of the UPS can only be accomplished by UBB^{+1} containing both ubiquitination sites.

Lys29 or Lys48 residues can independently target an authentic UFD substrate for degradation

The intriguing finding that UBB^{+1} needs both lysine residues for optimal inhibitory activity brought up the question whether these two ubiquitination sites act in concert or independently in targeting substrates to the proteasome. This question is difficult to address

with UBB^{+1}, as the different UBB^{+1} mutants with lysine substitutions were shown to differ in their capacity to inhibit the proteasome; therefore, changes in the turnover of these mutants can be due to targeting as well as inhibitory events. For this reason we turned to the Ub^{G76V}-GFP reporter, which is a designed UFD substrate that allows easy evaluation of proteasomal degradation (Dantuma et al., 2000b). We used a previously described flow cytometric assay in which HeLa cells were transiently transfected with the different

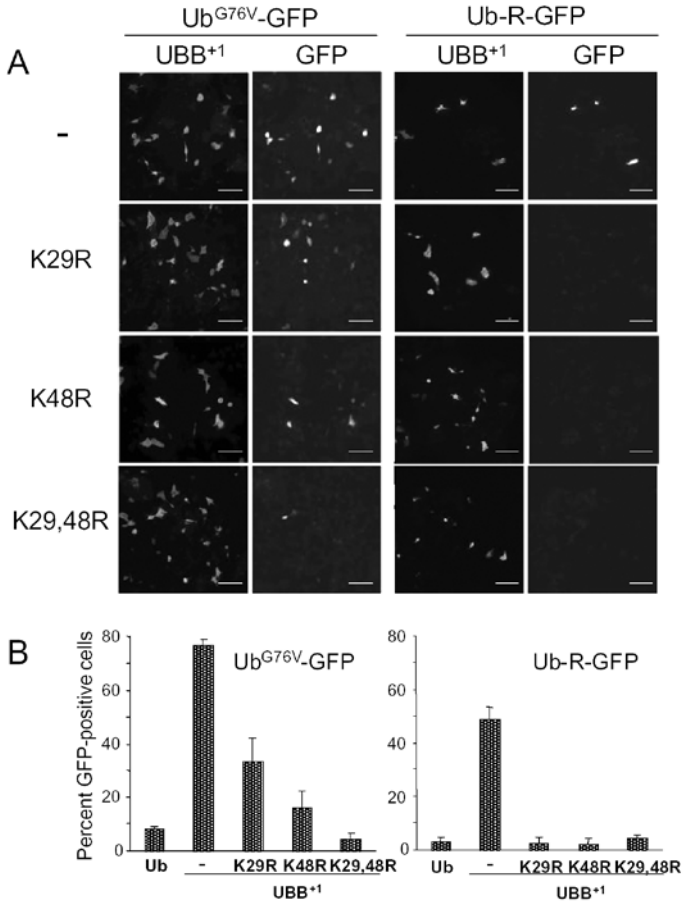

Fig.4 Inhibitory activity of UBB^{+1} requires ubiquitination at Lys29 and Lys48. (A) Micrographs of Ub^{G76V}-GFP (left) and Ub-R-GFP SH-SY5Y cells (right) transfected with UBB^{+1}, UBB$^{+1/K29R}$, UBB$^{+1/K48R}$, or UBB$^{+1/K29,48R}$. The cells were stained for UBB^{+1} (left) and analyzed for GFP fluorescence (right). Bars, 100 μm. (B) Quantification of three independent experiments as shown in A. The results are expressed as the percent of the UBB^{+1} expressing cells with accumulated Ub^{G76V}-GFP or Ub-R-GFP levels. **See Color figures.**

Ub^{G76V}-GFP mutants and the percentage of GFP fluorescent cells in the absence or presence of the proteasome inhibitor carboxybenzyl-leucyl-leucyl-leucine-vinyl sulfone (Z-L3-VS; (Bogyo et al., 1997)) was determined (Dantuma et al., 2000a). Substitution of both Lys29 and Lys48 residues in Ub^{G76V}-GFP completely abrogated proteasomal degradation of the GFP reporter (Fig. 5, A and B), confirming that these two lysines are the sole ubiquitination sites targeting for degradation. We observed that substitution of Lys29 resulted in a partial stabilization, whereas removal of Lys48 did not stabilize the protein. These data show that each of these two ubiquitin trees can function as an autonomous signal that target a model UFD substrate to the proteasome. Yet, similar to the situation in yeast (Johnson et al., 1995; Koegl et al., 1999), the Lys29 tree appears to be more effective than Lys48 in targeting a UFD for degradation.

Enhancement of the UFD signal strengthens the inhibitory activity of UBB^{+1}

Because UBB^{+1} is a target as well as an inhibitor of the UPS, we asked whether the inhibitory activity could be reversed by modifications that may enhance its degradation. UFD signals can be turned into a more potent degradation signal by introducing multiple tandem organized uncleavable ubiquitin moieties (Stack et al., 2000). Therefore, we inserted one or two additional uncleavable ubiquitin (Ub*) moieties at the N terminus of UBB^{+1} and generated the Ub*-UBB^{+1} and Ub*$_2$-UBB^{+1} constructs (Fig. 6A). However, unexpectedly, enhancement of the UFD signal did not result in accelerated turnover of UBB^{+1}, as reported with other UFD substrates (Stack et al., 2000), but instead a dramatic accumulation of UBB^{+1} was observed (Fig. 6 B). The effect was most apparent with Ub*$_2$-UBB^{+1}, in which in addition high-molecular mass species were observed in the stacking gel, implying that polyubiquitin trees are conjugated to UBB^{+1}.

Next, we compared the effect of UBB^{+1}, Ub*-UBB^{+1}, and the Ub*$_2$-UBB^{+1} on proteasomal degradation in HeLa and SH-SY5Y cells. In line with the positive correlation between the number of N-terminal ubiquitin moieties and the amounts of UBB^{+1}, Ub-UBB^{+1}, or Ub*$_2$-UBB^{+1} accumulating in transfected cells, we found a dose-dependent correlation between the number of ubiquitin moieties and the accumulation of Ub^{G76V}-GFP in HeLa cells (Fig. 6C) and Ub^{G76V}-GFP and Ub-R-GFP in SH-SY5Y cells (unpublished data).

Thus, targeting for ubiquitin-/proteasome-dependent degradation is crucial for the inhibitory activity of UBB^{+1}, and enhancement of its degradation signal paradoxically increases its stability and strengthens its inhibitory activity resulting in a more severe inhibition of proteasomal degradation.

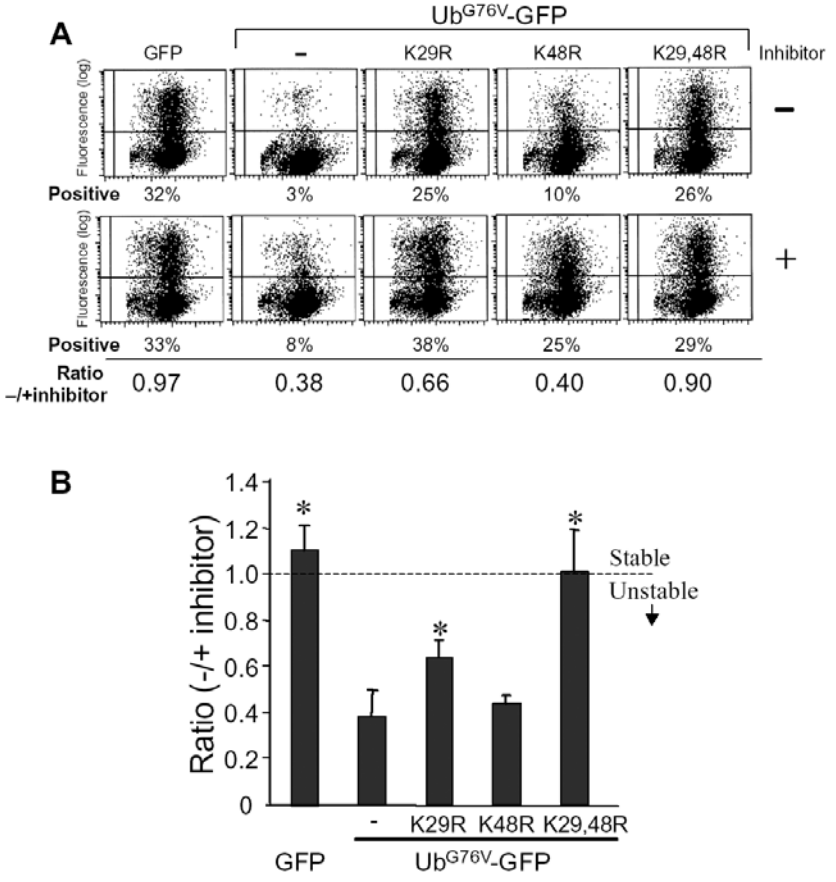

Fig.5 Lys29 and Lys48 can independently target a UFD substrate for degradation. A Dot plots of flow cytometric analysis of HeLa cells transiently transfected with GFP, Ub^{G76V}-GFP, $Ub^{K29R/G76V}$-GFP, $Ub^{K48R/G76V}$-GFP, and $Ub^{K29,48R/G76V}$-GFP. Half of the cells were left untreated and the other half was incubated for 16 h with 10 μM of the proteasome inhibitor Z-L3-VS. The percentage GFP-positive cells and the ratio between the percentage of fluorescent cells in samples untreated/inhibitor-treated are indicated. **B** Quantification of three independent experiments as shown in A. Values significantly different from the Ub^{G76V}-GFP sample are marked with asterisks (t test, P = 0.05). Mean +/- SD of three independent experiments. Ratios <1 indicate proteasomal degradation of the protein.

No impaired proteasomal degradation in response to overexpression of other substrates

A possible explanation for the inhibitory activity of UBB^{+1} is that overexpression of proteasome substrates will saturate the system and competitively affect degradation of the Ub-R-GFP and Ub^{G76V}-GFP substrates. To address this issue, we designed substrates whose expression was driven by the CMV promotor similar to the UBB^{+1} constructs. These substrates were ^{FLAG}Ub-R-nfGFP and ^{FLAG}Ub^{G76V}-nfGFP, which are based on a nonfluorescent variant of GFP (nfGFP), and ^{FLAG}p53. Ub^{G76V}-GFP HeLa cells expressing the substrate were identified by the FLAG tag present on each of the substrates. Microscopic and flow cytometric analysis demonstrated

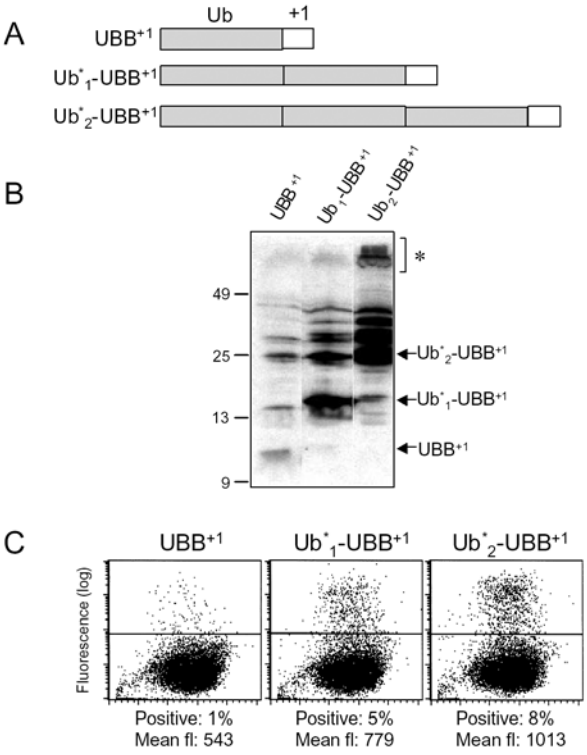

Fig.6 Targeting UBB^{+1} for proteasomal degradation enhances its inhibitory effect. A Schematic illustration of the UBB^{+1}, Ub*–UBB^{+1}, and Ub*$_2$-UBB^{+1} constructs. **B** Western blot analysis with anti-UBB^{+1} antibody of cell lysates of HeLa cells transfected with UBB^{+1}, Ub*–UBB^{+1}, Ub*$_2$-UBB^{+1}. Molecular mass marker and bands corresponded to unmodified and ubiquitinated UBB^{+1} proteins as well as high molecular mass UBB^{+1} are indicated. **C** Flow cytometric analysis of GFP fluorescence of Ub^{G76V}GFP HeLa cells transfected with UBB^{+1}, Ub*–UBB^{+1}, and Ub*$_2$-UBB^{+1}. The percentage of cells with accumulated GFP and the mean fluorescence intensity of this population are indicated at the bottom.

that only UBB^{+1} was able to block degradation of the GFP substrate, whereas none of the other three substrates had an effect on Ub^{G76V}-GFP levels (Fig. 7). It is noteworthy that even the nonfluorescent variant of the Ub^{G76V}-GFP substrate itself did not induce accumulation. Hence, the inhibitory effect of UBB^{+1} is not simply due to saturating the UPS by overexpression of a substrate.

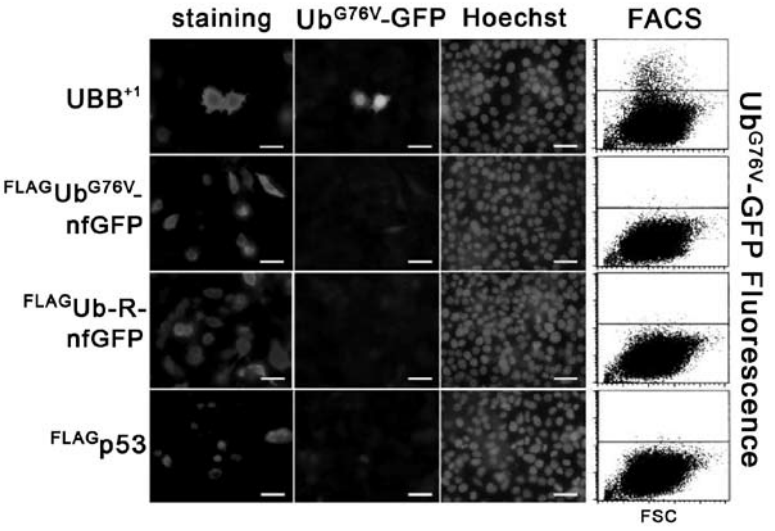

Fig.7 Overexpression of other proteasome substrates does not inhibit turnover of Ub^{G76V}-GFP. Ub^{G76V}-GFP HeLa cells were transiently transfected with UBB^{+1}, $^{FLAG}Ub^{G76V}$-nfGFP, ^{FLAG}Ub-R-nfGFP and ^{FLAG}p53. UBB^{+1} transfected cells were stained with the anti-UBB^{+1} antibody while the nonfluorescent $^{FLAG}Ub^{G76V}$-nfGFP and ^{FLAG}Ub-R-nfGFP constructs and ^{FLAG}p53 were stained with a FLAG-specific antibody. Representative micrographs of the immunostaining (left, red), the Ub^{G76V}-GFP fluorescence (middle, green), and the Hoechst 33258 counterstaining (right, blue) are shown. Note that as expected the $^{FLAG}Ub^{G76V}$nfGFP and ^{FLAG}Ub-R-nfGFP give a homogenous staining in the cytosol and nucleus, whereas ^{FLAG}p53 is localized in the nucleus. To the left are shown flow cytometric analysis of the GFP fluorescence upon transfection with the different constructs. **See Color figures.**

DISCUSSION

In the present study we show that an abnormal component of the UPS, which has been detected in a broad variety of neurodegenerative diseases, can inhibit proteasomal degradation in neuronal cells. Interestingly, all the pathologic conditions for which expression of UBB^{+1} has been described, including several tauopathies and a polyglutamine disorder, are characterized by the accumulation of

insoluble deposits formed by aggregated proteins (Sherman and Goldberg, 2001). Under normal conditions, misfolded proteins are efficient substrates of ubiquitin-/proteasome-dependent proteolysis, and a key question has been the nature of the primary events that favors their accumulation rather than rapid clearance in affected neurons. Our data show that UBB^{+1} is a powerful inhibitor of this proteolytic pathway *in vivo*. The effect was sufficient to induce cell cycle arrest at the G2/M boundary, at least under the conditions of overexpression achieved in our transient transfection assays. A particularly important aspect of our findings is the demonstration that UBB^{+1} is not only an inhibitor, but also a target of the UPS. Interestingly, it has been shown that whereas UBB^{+1} transcripts are present in both normal and affected brains, the protein product has only been detected in affected neurons of individuals suffering from neurodegenerative disorders (unpublished data). Notably, we observed that only a small population of the transfected cells expressed detectable levels of the UBB^{+1} protein followed by accumulation of the GFP substrates, whereas the majority of the cells destroy the UBB^{+1} by proteasomal degradation. Using an adenovirus based transduction method in neurons, which accomplishes massive expression of UBB^{+1}, and an *in vitro* degradation assay, it was recently shown that UBB^{+1} is a rather stable and toxic protein (De Vrij et al., 2001). Conceivably, the UPS can cope with low levels of UBB^{+1} but accelerated proteasomal targeting, by elevated steady-state levels or by enhancement of the UFD signal, obstructs ubiquitin-/proteasome-dependent proteolysis of this aberrant ubiquitin. Alternatively, the cells that accumulate UBB^{+1} and the GFP substrates have a suboptimal UPS, making them more sensitive to the inhibitory effect of UBB^{+1}. We envision that *in vivo* slight changes in the efficiency of proteolysis, as may be achieved in selected neurons by the production of β-amyloid peptide in Alzheimer's disease (Gregori et al., 1995; Keller et al., 2000a), or the formation of insoluble aggregates in polyglutamine disorders (Bence et al., 2001; Jana et al., 2001), may be sufficient to initiate a process resulting in accumulation of UBB^{+1} that will eventually lead to cellular intoxication by a general inhibition of the UPS and ultimately to cell death.

Detailed analysis of the requirements for the inhibitory effect of UBB^{+1} revealed some unexpected characteristics. It was acknowledged earlier that UBB^{+1}, even though it cannot be conjugated to substrates (Van Leeuwen et al., 1998b), can serve

as a recipient for polyubiquitination. Therefore, it was postulated that polyubiquitinated UBB^{+1}, similar to free polyubiquitin trees (Piotrowski et al., 1997), can block proteolysis of proteasome substrates (Lam et al., 2000). Indeed, we confirm that ubiquitination of UBB^{+1} is required for its inhibitory activity *in vivo*. However, several lines of evidence argue that ubiquitinated UBB^{+1} does not simply act as a free polyubiquitin tree but is instead an aberrant UFD substrate. First, we show that UBB^{+1} is ubiquitinated both at Lys29 and Lys48, a pattern that is unique for UFD substrates (Johnson et al., 1995; Koegl et al., 1999). Second, UBB^{+1} is structurally similar to a UFD substrate, as it has an N-terminal uncleavable ubiquitin moiety linked to a C-terminal extension. Third, our data clearly demonstrate that UBB^{+1} is degraded by the proteasome in a large number of the transfected cells.

Even though the related UFD reporter Ub^{G76V}-GFP seems to be susceptible to inhibition by UBB^{+1} with a single ubiquitination site to some extent, blockage of degradation of the Ub-R-GFP reporter required the Lys29 as well as Lys48 residues.

One possible explanation is that the pool of inhibitory UBB^{+1} consists of molecules bearing two ubiquitin trees. Binding of both trees to acceptor sites in the proteasome may be required to achieve interactions sufficiently tight to prevent access to other polyubiquitinated substrates. It is noteworthy that in the crystal structure of ubiquitin the Lys29 and Lys48 residues are localized on opposite faces of the molecule and would structurally allow double ubiquitin trees (Cook et al., 1994). It is also possible that the two sites act cooperatively in optimizing ubiquitination, as suggested by the recent finding that in yeast the polyubiquitination factor E4/UFD2 requires Lys48 in a UFD signal in order to accommodate efficient polyubiquitination at Lys29 (Koegl et al., 1999). Interestingly, a recombination event in the gene encoding the murine homologue of *E4/UFD2* may underlie the delayed Wallerian nerve degeneration observed in a mouse strain (Conforti et al., 2000). The experiments with the Ub^{G76V}-GFP substrate strongly support the model based on a tight interaction between UBB^{+1} bearing two ubiquitin trees and the proteasome, as both lysine residues can independently target this model UFD substrate to the proteasome, suggesting that Lys29 and Lys48 can each bear a functional ubiquitin tree. Several studies suggest that the rate of polyubiquitination determines the duration of the interaction between a substrate and the proteasome (Lam et al.,

1997; Thrower et al., 2000), and it is likely that regardless of whether these two lysine residues are required for the formation of double ubiquitin trees or more efficient polyubiquitination at Lys29, the outcome is a polyubiquitinated UBB^{+1} that cannot be rapidly released from the proteasome. The combination of a tightly bound but poorly degradable proteasome substrate may clog the system by obstructing access to other substrates, especially when the UBB^{+1} feed in large amounts to the proteasome. It is tempting to speculate that in its short C-terminal extension may lie the reason for the inhibitory activity of UBB^{+1}, either because it is too short to allow efficient tethering of the recruited UBB^{+1} into the cavity of the proteasome as has been proposed for another UFD substrate with a short extension (Johnson et al., 1992), or due to the presence of specific residues that stabilize the structure and hamper unfolding (Lee et al., 2001). Notably, during the revision of this manuscript, it was reported that introduction of stable structures within a proteasome substrate can turn an otherwise normal substrate into a potent inhibitor (Navon and Goldberg, 2001). An alternative possibility is that UBB^{+1} interferes more dramatically with degradation of the Ub^{G76V}-GFP substrate because these proteins are both UFD substrates and may well be targets for the same ubiquitin ligase. Accordingly, the stabilized UBB^{+1} may competitively inhibit the ubiquitination of Ub^{G76V}-GFP.

Our model deviates from an earlier presented model that proposed poor deubiquitination of UBB^{+1} as a possible cause for inhibition of the UPS. Although we show that UBB^{+1} can indeed inhibit the proteasome *in vivo*, and that this inhibitory activity relies on ubiquitination of UBB^{+1}, in accordance with the *in vitro* data (Lam et al., 2000), our results warrant a reevaluation of some of the observations in this earlier study. In the light of our results it is not surprising that ubiquitinated UBB^{+1} is less efficiently disassembled than free polyubiquitin trees by isoT, considering that this deubiquitination enzyme is highly specific for free polyubiquitin trees rather then ubiquitinated substrates (Wilkinson et al., 1995). It will be interesting to compare in a similar deubiquitination assay if UBB^{+1} is also more refractory to deubiquitination when compared with an authentic UFD substrate. The length dependence of the ubiquitin tree is another puzzling aspect. We confirmed that the bulk of UBB^{+1} in cell lysates contains one, two, or at most three conjugated ubiquitin moieties, whereas in the *in vitro* assay, UBB^{+1} with synthetically linked Lys48 tetraubiquitin was used, which fulfill much better the minimal length requirement for inhibitory polyubiquitin (Thrower et al.,

2000). However, the interaction between substrates simultaneously ubiquitinated at Lys29 and Lys48 and the proteasome is not well understood, and it is possible that with these unique trees UBB^{+1} can interact with the proteasome while bearing only a limited number of ubiquitins.

The critical significance of the UFD nature of UBB^{+1} is further emphasized by the finding that introduction of multiple UFD signals had a dramatic enhancing effect on its inhibitory activity. Contrary to what we had expected on the basis of previously reported data (Stack et al., 2000), addition of one or two uncleavable ubiquitin moieties resulted in further accumulation of UBB^{+1} and a stronger inhibition of the UPS. Thus, in line with the hypothesis that cells can cope only with a certain level of ubiquitinated UBB^{+1}, when this level is increased by accelerating targeting UBB^{+1} starts to accumulate and further inhibits its own degradation. The inhibitory activity of UBB^{+1} may then establish a destructive feedback loop, which may ultimately result in overall inhibition of the UPS.

In conclusion, we have provided evidence that UBB^{+1} acts as a potent inhibitor of the UPS in neuronal cells, and we have uncovered some important features of its mechanism of action. It remains to be seen whether and under what conditions this impaired proteolysis contributes to the generation of the protein aggregates that characterize many UBB^{+1}-associated pathologies. Finally, of paramount importance will be the identification of factors that can override the inhibitory effect of UBB^{+1}.

MATERIALS AND METHODS

Plasmid construction

All UBB^{+1} and ubiquitin open reading frames were expressed from a CMV promoter in the mammalian expression vectors pcDNA3 (Invitrogen), pBKCMV (Stratagene), EGFP-N1, or pCMS-EGFP (CLONTECH Laboratories, Inc.). The FLAG-tagged ubiquitin construct, ^{FLAG}Ub, was generated by PCR amplification of ubiquitin

from UBB^{+1} and subsequent in-frame ligation into a FLAG-containing vector. Construction of the modified UBB^{+1} constructs Ub*-UBB^{+1} and Ub*$_2$-UBB^{+1} was based on a UBB^{+1} plasmid in which an *NheI* site was introduced in between the ubiquitin moiety and the +1 extension of UBB^{+1} (this also introduced a D79S amino acid substitution, although that did not affect its inhibitory capacity). The UBB^{+1} (*NheI*) was digested with *NheI*, and PCR-amplified Ub^{G76V} was ligated between the ubiquitin moiety and +1 extension. This procedure was repeated once to generate the Ub*$_2$-UBB^{+1} construct. Lys to Arg substitutions in the different constructs were introduced by PCR amplification. nfGFP was constructed by introducing the amino acids substitution Y67R in the chromophore of GFP using PCR amplification. ^{FLAG}Ub^{G76V}-nfGFP, ^{FLAG}Ub-R-nfGFP, and ^{FLAG}p53 were generated by insertion of a double stranded oligonucleotide encoding the FLAG epitope as described previously (Heessen et al., 2002).

Transfections and tissue culture

The human cervical epithelial carcinoma line HeLa and neuroblastoma cell line SH-SY5Y were cultured in Iscove's modified Eagle's medium and high-glucose Dulbecco's modified Eagle medium, respectively, supplemented with 10% fetal calf serum (Life Technologies), 10 U/ml penicillin, and 10 μg/ml streptomycin. HeLa and SH-SY5Y cells were transiently transfected with Lipofectamine (Life Technologies) and calcium phosphate method, respectively. Cells were analyzed 48 h posttransfection unless stated otherwise. Stably transfected cell lines were selected in the presence of 0.5 mg/ml geneticin (Sigma-Aldrich) and screened for GFP fluorescence upon administration of proteasome inhibitors. Where indicated transfected cells were treated the reversible proteasome inhibitor MG132 (Affinity) or the irreversible proteasome inhibitors lactacystin, epoxomicin (Affinity) or Z-L3-VS, a gift from Dr. Hidde Ploegh (Harvard Medical School, Boston, MA) (Bogyo et al., 1997).

Western blot analysis

Cell lysates were fractionated on SDS-PAGE and transferred to Protan BA 85 nitrocellulose filters (Schleicher & Schuell). The filters were blocked in PBS supplemented with 5% skim milk and 0.1% Tween-20, were and incubated with rabbit polyclonal antibody

specific to UBB^{+1} (Ubi-3, 050897; (Van Leeuwen et al., 1998b)), ubiquitin (Dako), or GFP (Molecular Probes). After subsequent washings and incubation with peroxidase-conjugated goat anti–rabbit serum, the blots were developed by enhanced chemiluminiscence (ECL; Amersham Pharmacia Biotech). Quantification of Western blot bands was performed by densitometry (Molecular Dynamics).

Pulse-chase analysis

Neuroblastoma cells, SK-N-SH, were cultured and differentiated with retinoic acid. Differentiated SK-N-SH cells were transfected with a lentiviral based vector (Naldini et al., 1996b), containing the UBB^{+1} open reading frame (lenti-UBB^{+1}). 24–48 h after transduction, cells were incubated in medium lacking methionine and cysteine for 1 h, and were subsequently metabolically labeled by incubating them with medium containing 100 μCi Tran^{35}S-label for 4 h. After the labeling period, medium was replaced by Dulbecco's modified Eagle medium with 10% FCS medium. Cells were washed, chased with culture medium, and harvested at the indicated time points in 10 mM Tris, 0.15 M NaCl, 0.1% NP40, 0.1% Triton X-100, 20 mM EDTA, pH 8.0 buffer containing 0.1% SDS and protease inhibitors. UBB^{+1} was immunoprecipitated overnight at 4 with anti-UBB^{+1} antibody Ubi-3 (1:1,000), and protein-A Sepharose beads were added to the UBB^{+1} infected cell lysates. Analysis and quantification of the pulse-chase experiments were performed with the usage of a phosphoimager and the software package Imagequant software.

Fluorescence microscopy and flow cytometry

For fluorescence microscopy, the cells were grown and transfected on coverslips. After rinsing in PBS and fixation in 4% paraformaldehyde, immunostaining was performed using an anti-UBB^{+1} rabbit polyclonal antibody or anti-FLAG mouse monoclonal antibody (M5; Sigma-Aldrich). After subsequent washing steps with PBS, cells were incubated with the secondary antibodies labeled Alexa Fluor 594 (Molecular Probes) or Texas red (Dako). All antibodies were diluted in 50 mM Tris, pH 7.4, 0.9% NaCl, 0.25% gelatin, and 0.5% Triton X-100. Cells were counterstained with Hoechst 33258 (Molecular Probes). Fluorescence was analyzed using a LEITZ-

BMRB fluorescence microscope (Leica) and images were captured with a Hamamatsu cooled CCD camera. For quantitative analysis, 100–200 UBB^{+1} or ^{FLAG}Ub-positive cells per sample were scored for GFP fluorescence. Flow cytometry was performed with a FACSort flow cytometer (Becton Dickinson) and data were analyzed with CellQuest software. For analysis of cell cycle distribution, cells were harvested 2 d post transfection and fixed in 1% paraformaldehyde. After two washings in PBS, the cells were permeabilized with 70% ethanol and then incubated with propidium iodide. Flow cytometric analysis of the stability of Ub^{G76V}-GFP mutants was performed as described before (Dantuma et al., 2000a).

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CHAPTER 4

Critical levels of Alzheimer associated mutant ubiquitin cause a shift from substrate to inhibitor of the proteasome

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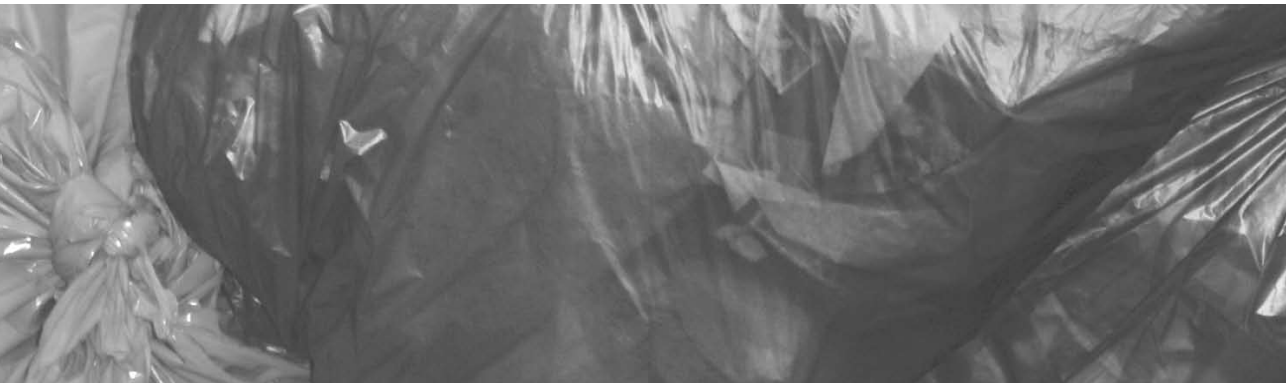
Submitted



ABSTRACT

UBB^{+1} is a mutant ubiquitin that accumulates in the pathological hallmarks of Alzheimer's disease. Besides being an efficient proteasome substrate, UBB^{+1} can also act as a potent inhibitor. To explain this paradox, we hypothesized that UBB^{+1} contributes to proteasome inhibition only after exceeding a threshold of accumulation. This hypothesis was studied in organotypic cortex cultures of mice. A GFP-based proteasome substrate, Ub^{G76V}-GFP, was used to monitor proteasome activity. Like Ub^{G76V}-GFP, UBB^{+1} was efficiently degraded in cortex cultures after lentiviral transduction, and only accumulated after additional proteasome inhibition. After washing out the reversible inhibitor MG132, proteasome activity was restored, as demonstrated by regained capacity to degrade Ub^{G76V}-GFP. UBB^{+1} however, remained accumulated in many cells, corroborating our hypothesis. In cortex cultures of Ub^{G76V}-GFP transgenic mice it was demonstrated that accumulated UBB^{+1} inhibits the proteasome in this system. These results indicate that accumulated UBB^{+1} in Alzheimer brain may have reached a critical threshold from which UBB^{+1} can contribute to proteasome inhibition and neurodegeneration.

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia, affecting an increasingly large group of elderly (Cummings et al., 1999; Selkoe, 2001). In the last few years, evidence has accumulated that supports the premise that the ubiquitin proteasome system (UPS) plays a role in many neurodegenerative diseases, including AD (Ciechanover and Brundin, 2003).

The UPS normally is responsible for the majority of protein degradation in the cell. Ubiquitin (Ub) tags proteins for degradation through a complex enzymatic machinery, consisting of Ub activating (E1), Ub conjugating (E2) and Ub ligating (E3) enzymes. Through this pathway, isopeptide bonds are formed between the C-terminal Gly residue of Ub and the ε-amino groups of Lys residues in proteins. By the sequential addition of mono-Ub to a Lys residue of substrate-bound Ub, a polyubiquitin tree is formed, which targets the protein for degradation by the 26S proteasome - for a review see (Glickman and Ciechanover, 2002). The proteasome is a large enzymatic complex, consisting of a 20S core, which can be flanked by several regulatory particles, such as 19S (Ferrell et al., 2000). The 20S and 19S particles together form the 26S proteasome, which is mainly responsible for the degradation of polyubiquitinated proteins.

UBB^{+1} is a mutant form of Ub which is the result of a dinucleotide deletion in the mRNA of the *ubiquitin-B* gene (Van Leeuwen et al., 1998b). UBB^{+1} accumulates in the neuropathological hallmarks of AD and other neurodegenerative diseases, with the exception of synucleinopathies (De Pril et al., 2004; Fischer et al., 2003). UBB^{+1} has paradoxical properties; on the one hand it acts as a ubiquitin-fusion-degradation (UFD) substrate for the proteasome, on the other hand however, it is a specific inhibitor of the proteasome when expressed at high levels (Lindsten et al., 2002) and eventually causes apoptotic-like cell death (De Vrij et al., 2001). We hypothesized that high levels of UBB^{+1} accumulation will cause a shift in UBB^{+1} properties from proteasome substrate to proteasome inhibitor after exceeding a threshold.

The balance between substrate and inhibitor properties of UBB^{+1} was found to be highly variable between different systems (De Vrij et al., 2001; Fischer et al., 2003; Lindsten et al., 2002). In rat brain it was shown that injected lentivirus encoding UBB^{+1} intriguingly did not lead to accumulation of the protein (Fischer et al., 2003), in contrast

to cell line studies. Therefore, we chose to study our threshold hypothesis in a system relevant to the situation in the human brain, where UBB^{+1} is most likely degraded in young control individuals and only accumulates in certain specific neurodegenerative diseases (De Pril et al., 2004; Fischer et al., 2003). We made use of organotypic cortex slice cultures of mice, which represent an elegant system to study brain cells in their original context.

Transgenic mice expressing a previously characterized GFP-based proteasome reporter, Ub^{G76V}-GFP (Dantuma et al., 2000b; Lindsten et al., 2003; Neefjes and Dantuma, 2004), were used to validate the ability to manipulate proteasome activity in our organotypic cortex culture system. The proteasome reporter carries a UFD-signal consisting of an uncleavable Ub moiety that is target for polyubiquitination and subsequent degradation by the proteasome, comparable to UBB^{+1} (Lindsten et al., 2002). Therefore, cells that express this proteasome reporter will only accumulate GFP when proteasomal degradation is inhibited. For a direct comparison of this reporter substrate with lentivirally induced UBB^{+1}, organotypical cortex slice cultures of wild type C57BL/6 mice were transduced with lentiviral vectors encoding either Ub^{G76V}-GFP or UBB^{+1}. The results of using a reversible proteasome inhibitor that allows recovery of proteasome activity after strong inhibition supported our threshold hypothesis for UBB^{+1}. Quantification of this effect was performed in human neuroblastoma cells.

RESULTS

Viral transduction in organotypical mouse cortex cultures

In AD, UBB^{+1} accumulates mostly in neurons, while in other neurodegenerative diseases, such as progressive supranuclear palsy, UBB^{+1} also clearly accumulates in glial cells of white matter (Fischer et al., 2003). Organotypical cortex slice cultures form an elegant system to study different types of brain cells in their original context. In this study, C57BL/6 mouse cortex slice cultures were efficiently transduced with viral vectors encoding

several proteins of interest. Transduction of wildtype cortex slice cultures with lentivirus (LV) encoding Ub-M-GFP - a control fusion protein resulting in a stable form of GFP (Dantuma et al., 2000b) - resulted in many immunopositive cells (Fig.1) two days after transduction. The population of cells that was transduced consisted mainly of astrocytes, although also neuronal cells were observed, as demonstrated by NeuN/GFAP double staining in LV-Ub-M-GFP transduced cultures (Fig.2).

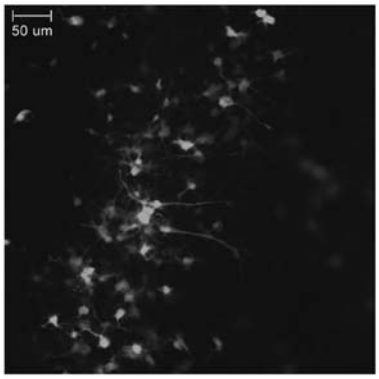


Fig.1 Successful lentiviral transduction of organotypic mouse cortex slice cultures. Wild type organotypic mouse cortex slice cultures were transduced with LV encoding the stable control GFP fusion protein Ub-M-GFP, GFP stained with αGFP/Cy2. **See Color figures.**

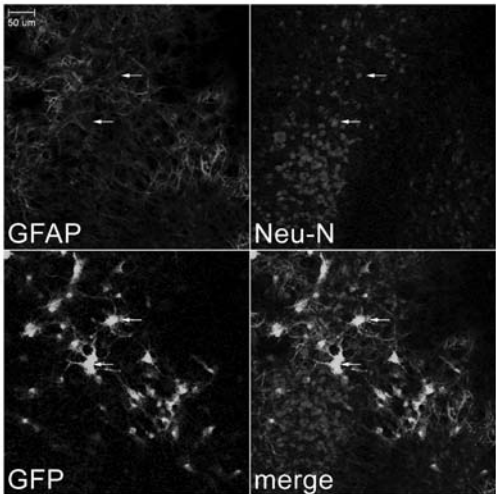


Fig.2 Lentivirus targets a heterogeneous cell population in cortex slice cultures. NeuN (blue) and GFAP (red) double staining on LV-Ub-M-GFP transduced cultures revealed mostly GFAP labeled GFP positive cells, but also NeuN positive neurons. Arrows indicate transduced NeuN positive neurons. **See Color figures.**

The proteasome reporter system in mouse cortex slice cultures

To verify if the proteasome reporter system works in our culture system, organotypic cortex slices of Ub^{G76V}-GFP/2 transgenic mice (Lindsten et al., 2003) were cultured and subsequently treated with the proteasome inhibitor epoxomicin. GFP staining confirmed that the reporter only accumulated in the cortex slice cultures after inhibitor treatment (Fig.3).

In order to compare lentivirally induced UBB^{+1} to Ub^{G76V}-GFP, also lentiviral vectors of Ub^{G76V}-GFP were produced and applied to cortex cultures of wild type mice. Lentiviral transduction of the proteasome reporter only resulted in accumulation of Ub^{G76V}-GFP after treatment of the cultures with epoxomicin (Fig.4). Lentiviral transduction of wildtype cortex slice cultures with UBB^{+1} intriguingly also did not lead to accumulation of the protein (Fig.4), in contrast to lentiviral transduction of cell lines (Lindsten et al., 2002). Apparently, UBB^{+1} was very efficiently degraded in cortex slice cultures, as demonstrated by the accumulation of UBB^{+1} after proteasome inhibition by epoxomicin (Fig.4). The lysine-mutant of UBB^{+1}, $UBB^{+1K29,48R}$, is not a target for ubiquitination (Lindsten et al., 2002) and therefore accumulated in transduced cortex cultures regardless of proteasome inhibition, as expected (Fig.4).

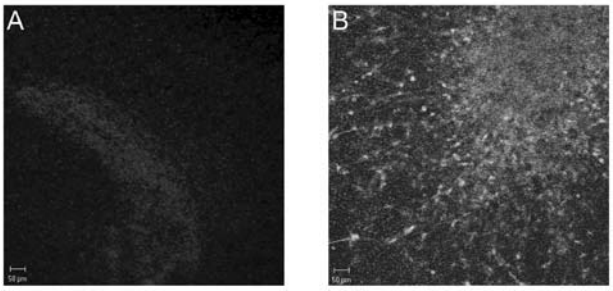


Fig.3 The proteasome reporter system in cortex cultures of Ub^{G76V}-GFP transgenic mice. A Ub^{G76V}-GFP transgenic cortex cultures without treatment. **B** Ub^{G76V}-GFP transgenic cortex cultures treated with 1 μM epoxomicin. The reporter substrate only accumulates after proteasome inhibition. **See Color figures.**

UBB^{+1} threshold in mouse cortex cultures

To test our threshold hypothesis for UBB^{+1} we made use of the reversible proteasome inhibitor MG132 that allows reactivation of the proteasome after strong inhibition. Applying MG132 to

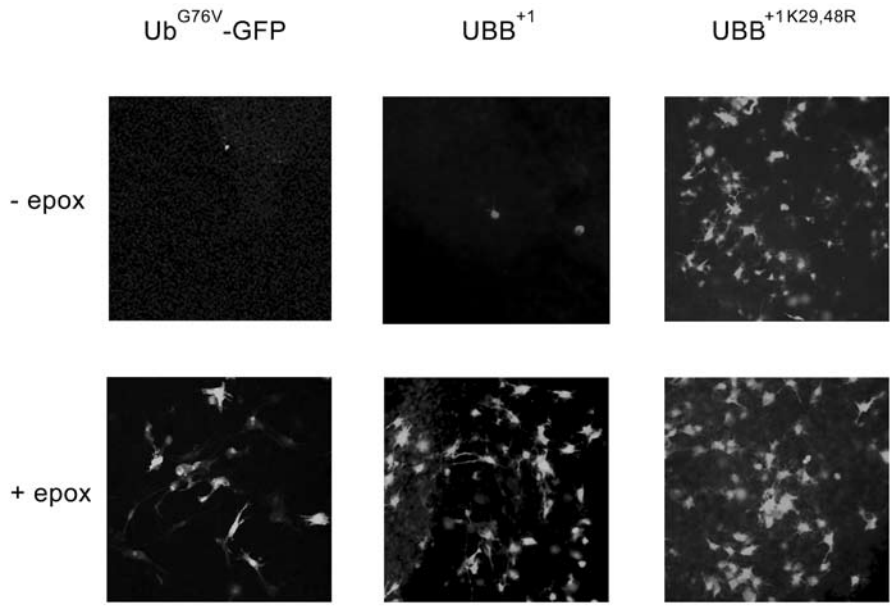


Fig.4 UBB^{+1} is degraded by the proteasome in cortex cultures. Both the reporter protein Ub^{G76V}-GFP (green) and UBB^{+1}(red) accumulate after treatment with 1 μM epoxomicin overnight. The lysine mutant of UBB^{+1} also accumulates without inhibitor treatment. **See Color figures.**

lentivirally transduced cultures for 16 hours resulted in strong accumulation of both Ub^{G76V}-GFP and UBB^{+1} (Fig.5). When MG132 treated cultures were rinsed and allowed to recover, proteasome activity was restored, as demonstrated by the regained capacity to completely degrade the GFP reporter protein (Fig.5). Nevertheless, this restored proteasome activity was not capable of degrading the accumulated UBB^{+1} in all cells (Fig. 5). The number of cells containing accumulated UBB^{+1} after washout of the inhibitor was increased compared to the number of UBB^{+1} positive cells after initial transduction. These experiments indicate that although UBB^{+1} is processed more efficiently in mouse cortex cultures than it is in human neuroblastoma cells, UBB^{+1} is less efficiently degraded than Ub^{G76V}-GFP, which can be due to the fact that it inhibits the proteasome system. Intrinsically, the ability of UBB^{+1} to inhibit its own degradation in this system, does not prove that it also inhibits the proteasome in general, like we found in cell lines (Lindsten et al., 2002). Therefore, we also transduced cortex cultures of Ub^{G76V}-

GFP transgenic mice to study if accumulated UBB^{+1} also results in accumulation of the reporter in this system. However, similar to wild type cortex slice cultures, lentiviral UBB^{+1} transduction did not lead to UBB^{+1} accumulation unless additional proteasome inhibitors were applied. This treatment would certainly lead to GFP accumulation in the transgenic cultures regardless of UBB^{+1} expression, making it impossible to distinguish proteasome inhibition by UBB^{+1}. Therefore, we found a way to induce higher expression levels of UBB^{+1}, which, according to our threshold hypothesis, might trigger UBB^{+1} accumulation by itself. Such high expression levels of UBB^{+1} were achieved by making use of adenoviral in stead of lentiviral transduction of UBB^{+1}. We confirmed the increased expression of adenoviral compared to lentiviral UBB^{+1} by transducing 293 cells with equal multiplicities of infection of either LV-UBB^{+1} or Ad-UBB^{+1} and comparing protein levels on western blot - performed as described in (De Vrij et al., 2001). Adenoviral transduction resulted in 4- to 5-fold expression compared to lentiviral transduction (Fig.6A). Transduction of Ub^{G76V}-GFP transgenic cortex cultures with Ad-UBB^{+1} indeed resulted in accumulation of UBB^{+1} in many cells

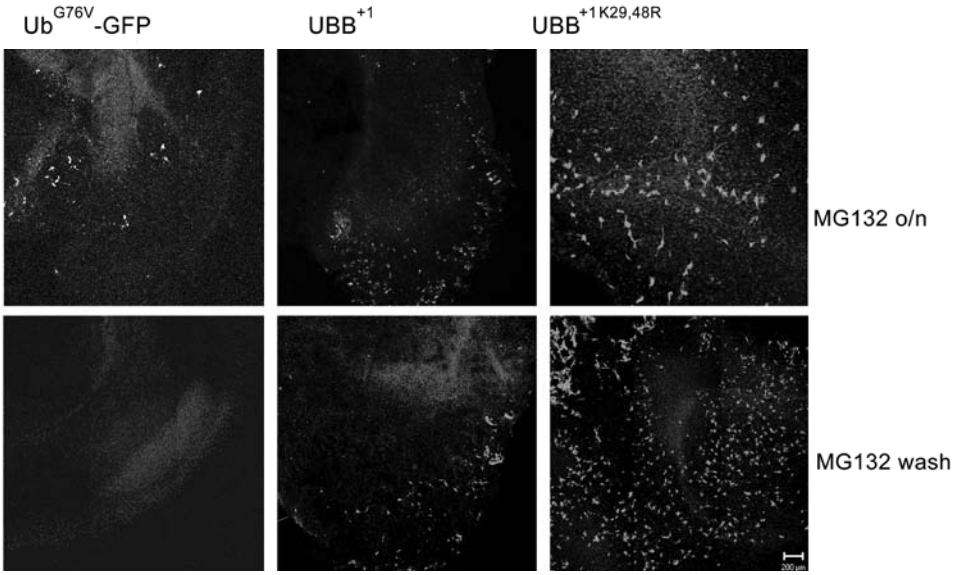


Fig.5 The threshold effect of UBB^{+1} accumulation in cortex cultures. 16 hour incubation of transduced cultures with the reversible inhibitor MG132 results in accumulation of both UBB^{+1} and Ub^{G76V}-GFP. Washing out the reversible inhibitor reactivates the proteasome, as shown by the degradation of Ub^{G76V}-GFP. UBB^{+1} however, remains accumulated in a considerable amount of cells. **See Color figures.**

(Fig.6C), in contrast to lentiviral transduction (Fig.6B). Most cells that were positive for UBB^{+1} clearly accumulated the GFP reporter (Fig.6D), indicating that UBB^{+1} is capable of inducing proteasome inhibition in this system.

The threshold effect that was observed with lentiviral transduction followed by reversible proteasome inhibition was difficult to quantify in this culture system. Therefore, experiments in human neuroblastoma cells were designed to quantify the threshold hypothesis for UBB^{+1} accumulation.

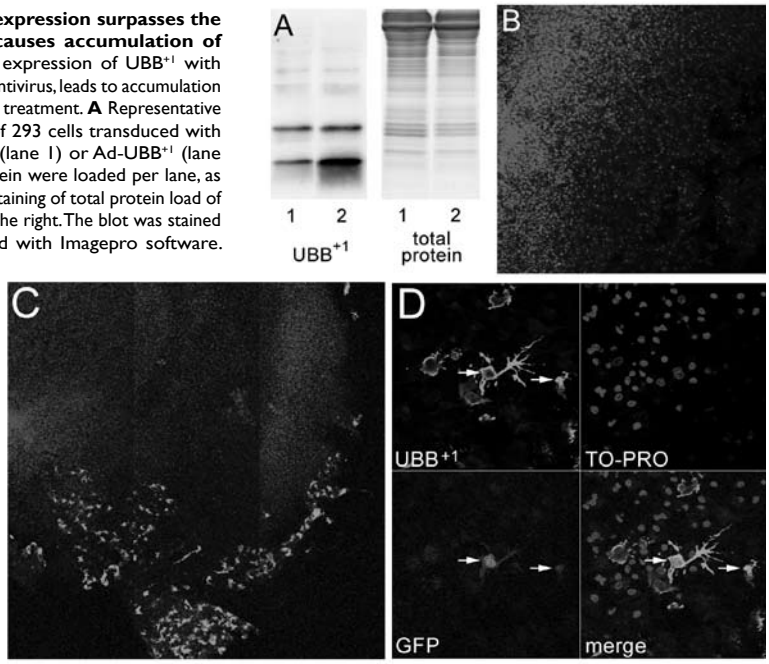


Fig.6 High Ad-UBB^{+1} expression surpasses the threshold level and causes accumulation of Ub^{G76V}-GFP. Increased expression of UBB^{+1} with adenovirus compared to lentivirus, leads to accumulation of UBB^{+1} without inhibitor treatment. **A** Representative western blot of lysates of 293 cells transduced with equal MOI of LV-UBB^{+1} (lane 1) or Ad-UBB^{+1} (lane 2). Equal amounts of protein were loaded per lane, as confirmed by Coomassie staining of total protein load of the same lanes shown on the right. The blot was stained with Ubi3 and quantified with Imagepro software. Organotypic cortex slice cultures of Ub^{G76V}-GFP transgenic mice were transduced with **B** LV-UBB^{+1}, or **C** Ad-UBB^{+1}. LV-UBB^{+1} transduction did not induce accumulation of UBB^{+1}, while Ad-UBB^{+1} did result in many UBB^{+1} immunopositive cells. **D** UBB^{+1} accumulation after adenoviral transduction leads to accumulation of Ub^{G76V}-GFP (arrows). **See Color figures.**

UBB^{+1} threshold in human neuroblastoma cells

Human neuroblastoma cells were transduced with lentiviral vectors encoding UBB^{+1}, UBB$^{+1K29,48R}$ or Ub^{G76V}-GFP. Three days after transduction cells were treated with the irreversible proteasome inhibitor epoxomicin or the reversible inhibitor MG132. Inhibitors were left on for 16 hours or washed out after five hours of treatment and grown for 16 hours in normal medium. All conditions were fixed at the same time (Fig.7).

The lysine mutant of UBB^{+1}, $UBB^{+1K29,48R}$ is not degraded by the proteasome and therefore accumulated in all transduced cells (36.7% of total cell number) (Fig.7). Without inhibitor treatment UBB^{+1} was degraded in the majority of transduced cells, but accumulated in a considerable amount of them (8.6% of total, ~25% of transduced cells), as expected (De Vrij et al., 2001; Lindsten et al., 2002). The GFP reporter Ub^{G76V}-GFP was completely degraded by the proteasome in virtually all cells (0.9% of total). After treatment with either proteasome inhibitor overnight all three proteins accumulated to about the same levels (~40% of total), demonstrating that transduction levels between the different lentiviral constructs were comparable. Washing out the reversible inhibitor MG132 led to almost complete remission of the accumulation of Ub^{G76V}-GFP (1.7% of total). UBB^{+1} however, remained significantly more accumulated (17.2% of total, p=0.007) in this condition than in the control situation (8.6% of total) (Fig.7). These results show that proteasome inhibition by UBB^{+1} indeed seems subject to a threshold level of UBB^{+1} accumulation. Accumulated UBB^{+1} was capable of sustaining proteasome inhibition by itself in cells that were able to degrade UBB^{+1} before treatment with the reversible inhibitor.

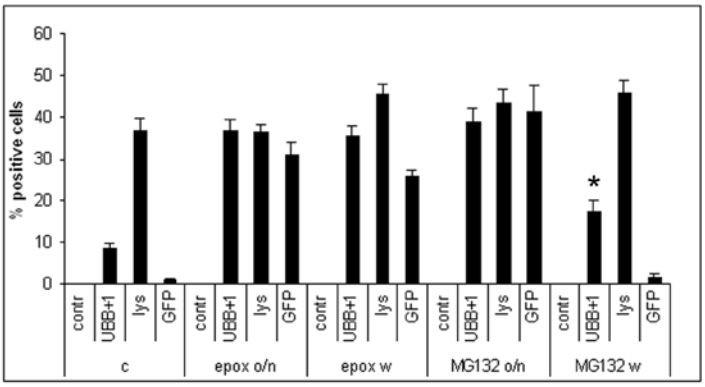


Fig.7 Quantification of the threshold effect of UBB^{+1} in human neuroblastoma cells. Reversible proteasome inhibition in lentivirally transducted SH-SY5Y cells. No virus (contr), LV-UBB^{+1} (UBB^{+1}), LV-$UBB^{+1K29,48R}$ (Lys) and LV-Ub^{G76V}-GFP conditions were treated with: no inhibitor (c), MG132 overnight (o/n) or MG132 for 6 hours, which was allowed to wash out overnight (MG132 w) and the same treatments with the irreversible inhibitor epoxomicin. The reporter protein Ub^{G76V}-GFP was almost completely degraded in cells after washout of the inhibitor (1.7% accumulation), while UBB^{+1} remained significantly more accumulated (17.2%, p=0.007, asterisk) than in the control situation. Results of two representative experiments, each performed in duplo.

DISCUSSION

UBB^{+1} has paradoxical properties as both a substrate and an inhibitor of the proteasome. The differences in UBB^{+1} processing between cell lines, cortex slice cultures and in vivo experiments, further complicate the understanding of the mechanisms of action of UBB^{+1}. These differences can be partly explained by the threshold hypothesis as shown in this study. In mouse cortex slice cultures, UBB^{+1} was found to accumulate in more cells after reversed proteasome inhibition than in the control situation and compared to the UFD reporter protein. This effect was quantified in neuroblastoma cells and found to be significant. An interesting issue that remains to be resolved is why UBB^{+1} accumulates relatively easy in human neuroblastoma, HeLa or 293 cells after lentiviral transduction, but is degraded in virtually all cells in mouse cortex slice cultures transduced with the same virus. This effect was also observed earlier in lentiviral UBB^{+1} injection in rat hippocampus, which also showed degradation of UBB^{+1} (Fischer et al., 2003). Human to murine species differences between the cortex cultures and neuroblastoma cell lines were ruled out as a cause for this discrepancy as human SH-SY5Y and rat N2a neuroblastoma cells did not show different numbers of UBB^{+1} positive cells after lentiviral transduction (results not shown). A more probable explanation could be a difference in transduction efficiency of lentivirus in cell lines versus cortex cultures. In earlier reports, it has also been described that higher expression levels are obtained in transfection of tumorigenic cells versus normal cells (Wadia et al., 2004). In contrast to lentiviral UBB^{+1}, adenoviral induced UBB^{+1} expression leads to accumulation of the protein not only in cell lines, but also in cortex slices. Adenoviral expression is stronger than lentiviral expression, as we demonstrate in this study in cell lines. In cortex slice cultures, this increased expression of UBB^{+1} apparently surpasses the threshold that lentiviral expression does not reach in this system. Alternatively, the difference in accumulation of UBB^{+1} in cortex slice cultures transduced with lentivirus compared to adenovirus could also reflect an influence of certain viral proteins expressed by the adenoviral vector or a difference in tropism of the viruses. In the latter case, adenoviral transduction would target a subpopulation of cells that is more vulnerable to proteasome inhibition than lentivirally transduced cells are. It is known from earlier reports that lentivirus is more capable of transducing neurons than adenovirus is (Ehrengruber et

al., 2001). Besides this difference, in which lentivirus only seems to have a broader tropism than adenovirus, it is not known if adenovirus could transduce an entirely different cell population than lentivirus. In our cultures, no obvious difference was observed between cells transduced with lentivirus or with adenovirus. Therefore, we believe that the higher expression levels of adenovirally transduced UBB^{+1} compared to lentiviral transduction are responsible for the difference in UBB^{+1} accumulation.

In AD brain, proteasomal inhibition is found in affected brain areas (Keck et al., 2003; Keller et al., 2000a). The threshold for UBB^{+1} accumulation is clearly reached in AD brain, as UBB^{+1} immunoreactivity is highly associated with the pathological hallmarks of AD. However, since UBB^{+1} normally is an efficient substrate for the proteasome, it is not likely that UBB^{+1} accumulation forms the initial trigger for proteasome inhibition in AD. Other AD-related mechanisms probably precede UBB^{+1} accumulation and are more prone to elicit inhibition of the proteasome. UBB^{+1} will then serve as an endogenous reporter of proteasome inhibition, as suggested earlier (Fischer et al., 2003). However, besides this reporter function, we now show that UBB^{+1} will most likely contribute to proteasome inhibition in the cells in which it accumulates. PHF-tau, Aβ and oxidative stress have all been implicated to play a role in proteasome inhibition (Gregori et al., 1995; Keck et al., 2003; Keller et al., 2000b; Zhu et al., 2004a) and therefore might form the initial trigger for proteasome inhibition and UBB^{+1} accumulation in AD. Recently, an interesting link was demonstrated between Aβ mediated toxicity and proteasome activity, through the E2-25K enzyme and UBB^{+1} (Song et al., 2003).

In summary, the current study indicates that UBB^{+1} properties shift from proteasome substrate to inhibitor after a critical level of accumulation is reached. It is notable that the experiments are performed in a physiologically relevant setting of organotypic cortex slice cultures, in which sub threshold levels UBB^{+1} are efficiently degraded. In our view, this situation is comparable to AD neuropathology, in which UBB^{+1} probably is efficiently degraded until the threshold is finally reached after a gradual increase in UBB^{+1} levels due to proteasome inhibition by other AD-related mechanisms. The present study indicates that once this threshold is reached, UBB^{+1} will act as a potent inhibitor of the proteasome. Therefore, we hypothesize that UBB^{+1} is involved in neurodegeneration in AD

by contributing to proteasome inhibition once a threshold of UBB^{+1} accumulation is reached.

MATERIALS AND METHODS

Organotypic cortex slice cultures

Organotypic cortex slice cultures were made from five day old C57BL/6 wild type or C57BL/6 transgenic Ub^{G76V}-GFP/2 mice (Lindsten et al., 2003). The mice were decapitated; the brain was removed as a whole from the skull and transferred to ice cold Gey's Balanced Salt Solution (GBSS; Sigma) containing 5.4 mg/ml glucose, 100 U/ml penicillin and 100 μg /ml streptomycin (Gibco). The meninges were removed and the brain was cut into two hemispheres. The fronto-parietal part of the hemisphere was sliced into 300 μm coronal sections using a tissue chopper (McIlwain). The first four slices per hemisphere were excluded from the experiments. The slices were cultured on an air-fluid interface on culture plate inserts (Millipore; 0.4 μm pore size; 30 mm diameter; 3 cultures per insert) on 1 ml culture medium containing 50% Minimum Essential Medium alpha (MEMα; Gibco), 25% HBSS (Gibco), 25% horse serum (Gibco), 6.5 mg/ml glucose, 2 mM glutamine (Gibco) and penicillin/ streptomycin (100 U/ml, 100 μg/ml; Gibco). The slices were cultured in an incubator at 37 with 5% CO_2. Viral transduction of cultures was achieved by applying 1 x 10^6 transducing units of virus in a 10 μl droplet of culture medium on top of the slices. Treatment with proteasome inhibitors epoxomicin and MG132 (Affinity, UK) was performed in the same manner in concentrations of 1 μM and 10 μM, respectively. Proteasome inhibitors were applied for six hours and subsequently either left on or washed out overnight. Slices were stained free floating by incubation in Supermix, containing 0.05M Tris, 0.9% NaCl, 0.25% gelatine and 0.5% Triton-X-100, pH 7.4, with rabbit polyclonal anti-UBB^{+1} antibody (Ubi3 serum, 05/08/97,1:1000 (De Vrij et al., 2001)), followed by secondary anti-rabbit Cy3 antibody (1:800). Rabbit polyclonal αGFAP (DAKO), monoclonal αGFP (Chemicon) and monoclonal NeuN (Chemicon) antibodies

were used in dilutions of 1:4000, 1:500 and 1:400 respectively, followed by Cy2 and Cy3 staining (1:800). Nuclei were visualised with TO-PRO (1:1000, Molecular Probes). Subsequently, slices were mounted in mowiol (0.1M Tris-HCl pH8.5, 25% glycerol, 10% w/v mowiol 4-88) and analyzed with confocal laser scanning microscopy (Zeiss 510).

Viral constructs

First generation recombinant adenoviral vectors AdUBB^{+1} and AdwtUb were generated, purified and titered as described elsewhere (De Vrij et al., 2001; Hermens et al., 1997). Adenoviral vectors were based on the Ad5 mutant *dl309* (Jones and Shenk, 1979) and employed the cytomegalovirus immediate early (CMV) promoter to drive transgene expression. Titration of double CsCl gradient-purified Ad-CMV-UBB^{+1} and Ad-CMV-wtUb on the permissive cell line 911 (Fallaux et al., 1996) revealed titres of 1x10^{9} plaque forming units/ml.

DNA encoding GFP, Ub-M-GFP, Ub^{G76V}-GFP, UBB^{+1} or the control lysine mutant of UBB^{+1}, UBB$^{+1K29,48R}$, was cloned into the lentiviral transfer plasmid pRRLsin-ppThCMV. Lentivirus was produced according to Naldini et al (Naldini et al., 1996a; Naldini et al., 1996b) by co-transfecting the transfer lentiviral plasmid, the VSV-G envelope plasmid (pMD.G.2) and the packaging plasmid (pCMVΔR8.74) into 293 T human kidney cells using a calcium phosphate method. After two days the lentivirus (LV) was harvested, filtered and further concentrated by ultra-centrifugation at 20000 rpm at 16 for 2.5 h. The virus was then resuspended in phosphate buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 0.24 g/l KH2PO4, 1.44 g/l Na2HPO4; pH 7.4) + 0.5% BSA (Sigma). Virus titres were determined with an HIV-1 p24 core profile ELISA kit (Perkin Elmer Life Sciences, USA) and correlated to titres determined by counting GFP fluorescent cells of an LV-Ub-M-GFP stock. In this way titres of adenoviral and lentiviral stocks could be correlated.

Cell lines

SH-SY5Y human neuroblastoma cells were cultured in high-glucose Dulbecco's modified Eagle medium (Gibco) containing 10% foetal

calf serum (FCS) (Gibco) and supplemented with 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco) (DMEM-10% FCS). Cells were cultured on 0.2% gelatin coated glass coverslips in 24-wells plates (Nunc, Denmark) one day prior to lentiviral transduction. The next day the cells were transduced with lentiviral vectors with a multiplicity of infection (MOI) of 10. Medium containing the lentiviral vectors was left on overnight, after which medium was replenished with culture medium containing 4 μM all-trans retinoic acid (Sigma).

After transduction cells were fixed in 4% formalin in phosphate-buffered saline (PBS), pH 7.4 for one hour or longer. In between staining steps cells were rinsed with PBS, pH 7.4. Rabbit polyclonal ubi3 (anti-UBB^{+1}) was used in a dilution of 1:1000 overnight in Supermix. The secondary anti-rabbit Cy3 antibody was diluted 1:200 and nuclei of cells were stained with TO-PRO-3 (Molecular Probes, 1:1000). Coverslips were mounted in mowiol. Images were acquired by confocal laser scanning microscopy (Zeiss 510) with three different lasers emitting at 488, 543 and 633 nm to excite GFP, Cy3 and TO-PRO-3, respectively. Cells were quantified by hand with ImagePro software (Media Cybernetics, Silver Springs, MD). For each coverslip, a Cy3 image and the corresponding TO-PRO image were acquired in five fields. The experiments were performed in duplicate. The total number of cells was quantified by counting the nuclei. The number of transduced cells was quantified by counting the immunopositive cells in the Cy3 or GFP images. Statistics was performed by applying single-factor ANOVA between groups, based on α=0.05.

Acknowledgements

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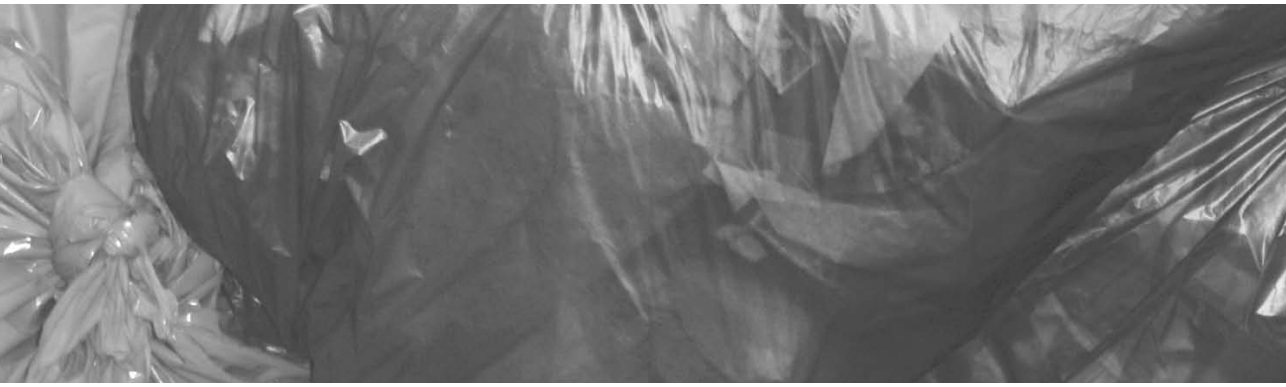
CHAPTER 5

Aβ40 and 42 peptides expressed in the cytosol are efficiently degraded and do not inhibit the proteasome

Femke M.S. de Vrij, David F. Fischer, Jacqueline A. Sluijs,
Fred W. van Leeuwen and Elly M. Hol



ABSTRACT

The modes of action and location of amyloid β-protein (Aβ)-processing and -toxicity in Alzheimer's disease (AD) still remain obscure. In this study, cytosolic Aβ peptide was studied as a putative initiator of proteasome inhibition in AD, since it was shown that Aβ can bind and inhibit the proteasome in a cell-free system. Proteasome inhibition by Aβ could explain the accumulation of ubiquitinated proteins in AD, such as mutant ubiquitin, which also acts as a proteasome inhibitor once critical levels of accumulation are reached. To study the effect of intracellular Aβ on the proteasome, constructs encoding fusion proteins of ubiquitin and Aβ were generated that produce strictly cytosolic Aβ1-40, Aβ1-42 or scrambled Aβ (Aβscr) after proteolytic cleavage of the fusion protein by ubiquitin hydrolases. These constructs were compared to an endoplasmic reticulum (ER)-targeted form of Aβ. Cytosolic Aβ was efficiently degraded in transduced cells, partly depending on the proteolytic activities of the proteasome and insulin-degrading enzyme. Although more research is needed to elucidate the mechanisms of Aβ degradation, we can conclude that cytosolic Aβ did not inhibit the proteasome nor cause toxicity in cell lines containing a green fluorescent protein (GFP)-based proteasome reporter system.

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia, affecting an increasingly large group of elderly (Cummings et al., 1998b). The pathological hallmarks of AD are neurofibrillary tangles formed by the intraneuronal accumulation of paired helical filament (PHF) tau and extracellular plaques, consisting of aggregated amyloid-β peptide (Aβ) (Morishima-Kawashima and Ihara, 2002). Another characteristic of AD is proteasomal inhibition in affected brain areas (Keller et al., 2000a). As in most other neurodegenerative diseases, the ubiquitin proteasome system (UPS) is thought to play an important role in the pathogenesis of AD.

A mutant form of ubiquitin, UBB^{+1}, has been identified as an endogenous reporter of proteasome inhibition in AD brain (Fischer et al., 2003). At low levels, UBB^{+1} is an efficient substrate for the proteasome but in AD brain UBB^{+1} is accumulated in the neuropathological hallmarks of the disease. At high expression levels UBB^{+1} was found to be a specific and potent inhibitor of proteasomal activity and to eventually induce cell death (De Vrij et al., 2001; Lindsten et al., 2002). Hence, proteasome inhibition by UBB^{+1} is subject to a threshold of UBB^{+1} accumulation (De Vrij et al., unpublished results). Once accumulated, UBB^{+1} exerts its inhibitory effect on the proteasome and probably contributes to proteasome inhibition as it is found in AD. However, being a substrate at low concentrations, it is not likely that UBB^{+1} is the initial cause of this inhibition. Therefore, we decided to look for other AD-related mechanisms that initiate proteasome inhibition and UBB^{+1} accumulation. In this study, Aβ pathology was studied as a possible candidate for this function. There are several indications that Aβ could be responsible for initial proteasome inhibition in AD. Interestingly, Aβ was found to inhibit and bind the proteasome *in vitro* (Gregori et al., 1995; Gregori et al., 1997). Moreover, a link was recently described between Aβ toxicity and UBB^{+1} accumulation (Song et al., 2003).

Much debate has come to pass in AD literature about the mechanism of Aβ induced toxicity, plaque formation, and about which aggregational state of Aβ is the primary toxic form. There are theories emerging that state that plaques are formed by the remains of dead cells that died of intracellular Aβ accumulation, which act as seeds for more aggregation of extracellular Aβ (D'Andrea et al., 2002; D'Andrea

et al., 2001; Pasternak et al., 2004). In line with these theories, injected Aβ1-42 or cDNA expressing cytosolic Aβ1-42 was found to be specifically cytotoxic to human neurons (Zhang et al., 2002). *In vivo* however, it remains unclear if Aβ is actually present in the cytosol and nuclei of cells, the only cellular compartments were proteasomes are located (Wojcik and DeMartino, 2003). Several reports describe intracellular Aβ accumulation in AD brain material or transgenic mice (Chui et al., 1999; Gouras et al., 2000; Oddo et al., 2003). However, these reports do not limit its location down to the cytosol. Aβ is a product of the proteolysis of the transmembrane protein APP by β- and γ-secretases, which occurs in the secretory and endocytic pathways. Aβ is not always completely secreted into the extracellular space, but is also found in the ER (Cook et al., 1997; Hartmann, 1999; Hartmann et al., 1997). A way for Aβ to get into the cytosol is described in several studies in which the translocation of induced Aβ with a signal peptide from the ER to the cytosol through ER associated degradation (ERAD) was reported (Buckig et al., 2002; Schmitz et al., 2004). However, after this translocation to the cytosol, in these experiments the peptide was degraded by the proteasome rather than inhibiting it.

In order to elucidate these conflicting results, we designed constructs that produce cytosolic Aβ without a requirement for extra methionine residues or signal peptides. These constructs were compared to ER-targeted Aβ peptide (SP-Aβ) and tested for their putative ability to inhibit the proteasome.

MATERIALS AND METHODS

HA-Ub-Aß constructs

Different vectors containing sequences encoding three different Ub-Aβ fusion proteins were produced: HA-Ub-Aβ1-40, HA-Ub-Aβ1-42 and HA-Ub-Aβscrambled (HA-Ub-Aβscr). For the scrambled peptide, an earlier described scrambled sequence was used (Malin et al., 2001), which has been shown not to have any specific effect.

The Ub sequence was PCR-amplified from pBluescript-Ub using two primers ranging from an inserted *BamHI*-HA-tag sequence at the N-terminus of Ub up to the *SalI* restriction site in the Ub sequence. For generation of the Aβ part, four oligonucleotide sequences were ligated together with T4 ligase. One sense primer was designed to match the Ub sequence from the *SalI* site up to the Ub C-terminus, directly followed by 60 nucleotides of the Aβ sequence. Another sense primer encoded the remaining part of the Aβ sequence followed by an *ApaI* restriction site on the N-terminus. The two antisense primers were designed to overlap 20 nucleotides with the sense primers.

The *BamHI*-HA-Ub-SalI was cloned into the *BamHI/SalI* digested pBluescript vector. The ligated Aβ fragments were cloned into *SalI/ApaI* digested pBluescript. Subsequently, the *BamHI/SalI* Ub fragment was cloned into the three different *BamHI/SalI* digested pBluescript-Aβ vectors. The resulting constructs pBlue-HA-Ub-Aβ1-40, pBlue-HA-Ub-Aβ1-42 and pBlue-HA-Ub-Aβscr were verified by sequencing. CMV-promoter controlled pcDNA3 constructs of the three variants were made for transfection purposes by subcloning the *BamHI/ApaI* HA-Ub-Aβ fragments into *BamHI/ApaI* digested pcDNA3. Lentiviral constructs were obtained by cloning *BamHI/BssHII* HA-Ub-Aβ fragments from pBlue into a *BamHI/MluI* digested pHR2 transfer lentivector. The three lentiviral vectors LV-HA-Ub-Aβ1-40, LV-HA-Ub-Aβ1-42 and LV-HA-Ub-Aβscr were also checked by sequencing.

The signal-peptide-containing Aβ construct, pcDNA1-SP-Aβ42 was kindly provided by A. Schmitz.

Lentivirus production

Lentivirus was produced according to Naldini et al (Naldini et al., 1996a; Naldini et al., 1996b) by co-transfecting the transfer lentivectors (original transfer plasmid pRRLsin-ppThCMV) LV-HA-Ub-Aβ1-40, LV-HA-Ub-Aβ1-42 and LV-HA-Ub-Aβscr with the VSV G envelope plasmid (pMD.G.2) and the packaging plasmid (pCMVΔR8.74) into 293 T human kidney cells using a calcium phosphate method. After two days, the lentivirus was harvested, filtered and further concentrated by ultra-centrifugation at 20,000 rpm at 16 for 2.5 h. The virus was then resuspended in phosphate buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 0.24 g/l KH2PO4,

1.44 g/l Na2HPO4; pH 7.4) + 0.5% BSA (Sigma). Virus titres were determined with an HIV-1 p24 core profile ELISA kit (Perkin Elmer Life Sciences).

In vitro translation of Ub-Aß constructs

A TNT-T7 coupled reticulocyte lysate system (Promega) was used to generate ^{35}S-methionine labeled products of the pcDNA3-HA-Ub-Aβ constructs. 25 μl of TNT rabbit reticulocyte lysate was incubated with TNT reaction buffer, RNA polymerase, amino acid mixture minus methionine, Redivue ^{35}S-methionine (Amersham Biosciences), ribonuclease inhibitor and 1μg of DNA template in 50 μl of nuclease-free water for 90 minutes at 30 . Of the labeling reaction, 0.5 μl was loaded onto a 15% SDS-PAGE gel, which was subsequently visualized with a phosphoimager.

Bacterial production of fusion proteins

Electrocompetent *Escherichia coli* (*E.coli*) *Bl21DE3* bacteria were transformed by electroporation with 10 ng DNA of the three pcDNA3-HA-Ub-Aβ constructs and pcDNA3-UBB^{+1} as a positive control. Transformations were incubated for 1 h in 1ml SOC medium at 37 . Subsequently, 100 μl of the reaction was plated on LB-agar plates containing 1% glucose and 0.1 mg/ml ampicillin, which were incubated at 37 overnight. The next day colonies were picked and grown in 5 ml LB medium each with 1% glucose. At an optic density of 1, cells were pelleted and washed in normal LB. Subsequently, half of the cultures were grown in LB containing 1% glucose and half in LB medium containing 1 mM IPTG. After 1-3 h at 37 , cells were pelleted and resuspended in 200 μl SDS loading buffer for western blotting.

Cell lines

SH-SY5Y human neuroblastoma cells and 293 T human embryonic kidney cells were cultured in high-glucose Dulbecco's modified Eagle medium (Gibco) containing 10% foetal calf serum (FCS) (Gibco) supplemented with 10 U/ml penicillin (Gibco) and 10 μg/ml streptomycin (Gibco) (DMEM-10% FCS). Cells were cultured

on 0.2% gelatin-coated glass coverslips in 24-wells plates (Nunc, Denmark) one day prior to calcium-phosphate transfection or lentiviral transduction. The next day the cells were transfected by calcium-phosphate method or transduced with lentiviral vectors with a multiplicity of infection (MOI) of 10. Cells were replenished after 16 h with normal medium (293 T cells) or medium containing 4 μM all-trans retinoic acid (Sigma) (SH-SY5Y cells).

Immunocytochemistry

After transduction, cells on coverslips were fixed in 4% formalin in PBS, pH 7.4 for 1 h or longer. In between staining steps cells were rinsed with PBS, pH 7.4. Subsequently, cells were incubated for 16 h in Supermix, containing 0.05M Tris, 0.9% NaCl, 0.25% gelatine and 0.5% Triton-X-100, pH 7.4, with primary antibodies 6E10 (Sigma), αHA-tag - clone 12CA5 (Field et al., 1988), ubi3 (serum 05/08/97 (De Vrij et al., 2001)) in dilutions of 1:500, 1:100 and 1:1000, respectively. The secondary Cy3 antibodies were diluted 1:200 and nuclei of cells were stained with TO-PRO-3 (Molecular Probes, 1:1000) for 1 h in Supermix. Coverslips were mounted in Mowiol (0.1M Tris-HCl pH8.5, 25% glycerol, 10% w/v mowiol 4-88) and analyzed with confocal laser scanning microscopy (Zeiss 510).

Western blotting

Cells were harvested in suspension buffer (0.1 M NaCl, 0.01 M Tris-HCl pH 7.6, 1 mM EDTA pH 8.0) containing protease inhibitors PMSF and leupeptin in concentrations of 100 μM and 10 μg/ml, respectively, and 0.5% Triton-X-100. After centrifugation, the supernatant (soluble fraction) was separated from the pellet. The pellet was subsequently sonicated and resuspended in loading buffer (insoluble fraction). After boiling in loading buffer for 5 minutes, samples were loaded on 15% SDS-PAGE gels and transferred semi-dry onto nitrocellulose. Blots were probed with primary antibodies for 16 h at 4 . Subsequently, blots were incubated with HRP-conjugated secondary antibodies and Lumilight ECL (Boehringer) fluorescence.

RESULTS

Validation ubiquitin-amyloid fusion constructs

Three ubiquitin (Ub) fusion constructs were made for Aβ1-40, Aβ1-42 and Aβscr. Ub fusion constructs consist of a Ub sequence, with another sequence fused directly to its C-terminus, similar to real Ub fusion genes such as polyubiquitin genes or *Ub-CEP* genes that encode ribosomal subunits (Hershko and Ciechanover, 1998; Kirschner and Stratakis, 2000; Redman and Rechsteiner, 1989). The design of the different constructs is shown in figure 1 (Fig. 1A). The fused protein products of such sequences are generally cleaved right after the Ub C-terminus by Ub hydrolases, providing that the N-terminus of the fused protein permits cleavage (Fig. 1B). The advantage of using such fusion proteins is that the cleaved protein will have the exact amino acid sequence as *in vivo*, without need for an additional methionine residue for initiation of translation. An HA-tag was added to the N-terminus of the Ub moiety. The pBlue and lentiviral constructs of Aβ1-40, Aβ1-42 and Aβscr were checked by sequencing.

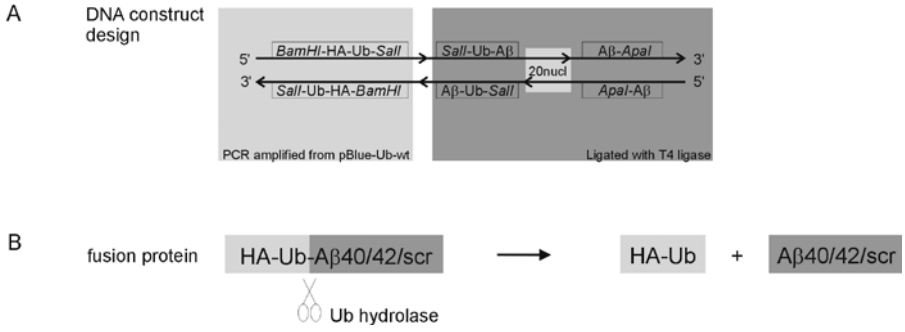


Fig.1 HA-Ub-Aß fusion construct design. A Design of the DNA constructs of HA-Ub-Aß1-40, HA-Ub-Aß1-42 and HA-Ub-Aßscr. **B** Schematic representation of HA-Ub-Aß fusion proteins. Cleavage by Ub hydrolases results in cytosolic HA-tagged Ub and Aß1-40, Aß1-42 or Aßscr.

E. coli bacteria do not contain Ub hydrolases or any other UPS enzymes (Layfield et al., 1999). Therefore, *E. coli Bl21DE3* bacteria expressing the HA-Ub-Aβ constructs should produce the fused protein of about 17 kDa. Indeed on western blots of bacterial lysates all three proteins were found in their fused form (Fig.2). All three proteins stained with an anti-HA-tag antibody. The Aβ directed 6E10 antibody only recognized the Aβ1-40 and 1-42 constructs and not the scrambled construct, as expected. The type of modification yielding three protein bands of approximately the same weight for all constructs was not further examined in this study. However, the results clearly indicate that the fusion proteins are produced in the transformed bacteria, containing both the HA-Ub and the Aβ moieties.

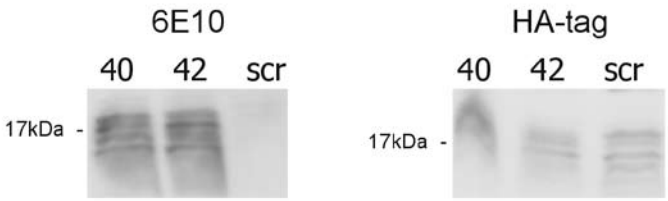


Fig.2 HA-Ub-Aß fusion proteins produced in bacteria. *E. coli Bl21DE3* bacteria lysates transformed with the Aß fusion constructs. Bacteria do not express Ub hydrolases, therefore the fusion proteins are not cleaved after translation, demonstrating the correct translation of the fusion products. The Aß directed antibody 6E10 recognizes both Aß1-40 and Aß1-42 fusion proteins, while the Aßscr is not recognized (left panel). The HA-tag directed antibody detects all three fusion proteins (right panel).

Next, the constructs were subjected to *in vitro* translation experiments in rabbit reticulocyte lysate. Methionine labeling of the produced proteins revealed a band of approximately 8 kDa, corresponding to free Ub (Fig. 3). The protein migrated faster than the 11 kDa control protein UBB^{+1} in the same gel, excluding the possibility of misinterpretation of the molecular weight marker. Therefore, we can conclude that the fusion protein was translated and subsequently cleaved by Ub hydrolases present in reticulocyte lysate. The fusion protein was not detected in the gel, indicating a large efficiency of processing of the protein, which will be discussed later. The cleaved Aβ product however, could not be detected on the gel, even though it also contains one methionine residue, just like Ub. This indicates that the Aβ peptides 1-40, 1-42 and scr might be degraded in reticulocyte lysate.

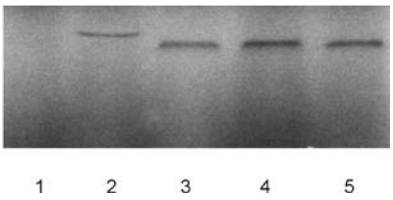


Fig.3 HA-Ub-Aß fusion contructs are cleaved and Aß is degraded in rabbit reticulocyte lysate. pcDNA3 constructs encoding the HA-Ub-Aß fusion proteins were subjected to *in vitro* translation in rabbit reticulocyte lysate. Lane 1, empty pcDNA3; Lane 2, pcDNA3-UBB^{+1}. Lane 3-5, pcDNA3-HA-Ub-Aß1-40/42 and scr, respectively. The methionine labeling of the produced proteins reveales a band of approximately 8 kDa, corresponding to Ub. The fusion proteins are cleaved efficiently in rabbit reticulocyte lysate. The Aß parts of the fusion proteins were not detected, indicating that they might have been degraded.

Expression in cell lines

Cell line transduction studies were performed to study the effects of the fusion proteins (Fig. 4). Staining of transduced neuroblastoma cells or 293 cells with anti-HA-tag antibody, 2 or 3 days after transduction, revealed many positive cells, indicating high transduction efficiency (Fig. 4). Staining with 6E10 or other Aβ antibodies (MCB, C42C, G42C, B42, 4G8, 6F3D - kindly provided by H. Yamaguchi) (not shown) however, revealed only very few positive cells (Fig.4). This indicates that Aβ might be degraded in these cells, just as in reticulocyte lysate. No difference in cell viability between transduced cells or non-transduced cells was observed.

On western blots of cells harvested 2 days after transfection, a faint 6E10 immunopositive band migrating at approximately 16 kDa was detected, which could correspond to the fusion protein (Fig. 5). However, this band was not observed in the same samples and on the same gel with an anti-HA antibody (Fig. 5). Moreover, Ub fusion proteins are generally cleaved very efficiently. The control protein Ub-M-GFP used in proteasome reporter substrate studies (Dantuma et al., 2000b; Neefjes and Dantuma, 2004), for example, is cleaved so efficiently, that cell lysate of Ub-M-GFP transduced neuroblastoma cells only show the cleaved M-GFP protein and not the fusion protein, as shown in figure 6 (Fig. 6). The M-GFP band in these samples corresponds to the GFP band of LV-GFP transduced cells while the uncleavable Ub^{G76V}-GFP proteasome substrate migrates at the size of the fusion protein. Therefore, we can conclude that Ub

fusion proteins, such as Ub-M-GFP and the HA-Ub-Aβ constructs are cleaved in such an efficient way in neuroblastoma cells, that the fusion protein is not detected on western blot. These results indicate that the 16 kDa band found with 6E10 antibody in LV-HA-Ub-Aβ1-40 and 1-42 transduced cells might consist of an aggregated form of Aβ peptide, which is resistant to degradation. Similar sizes of Aβ aggregates have also been described elsewhere in literature (Chu et al., 1998; Dyrks et al., 1992).

The Aβ derived from the fusion constructs is apparently aggregated in a small amount of transduced cells, which would explain the few immunopositive cells and the faint western blot band. Judging from the amount of HA-tagged Ub that is found in these cells however, one would expect to find much higher levels of Aβ.

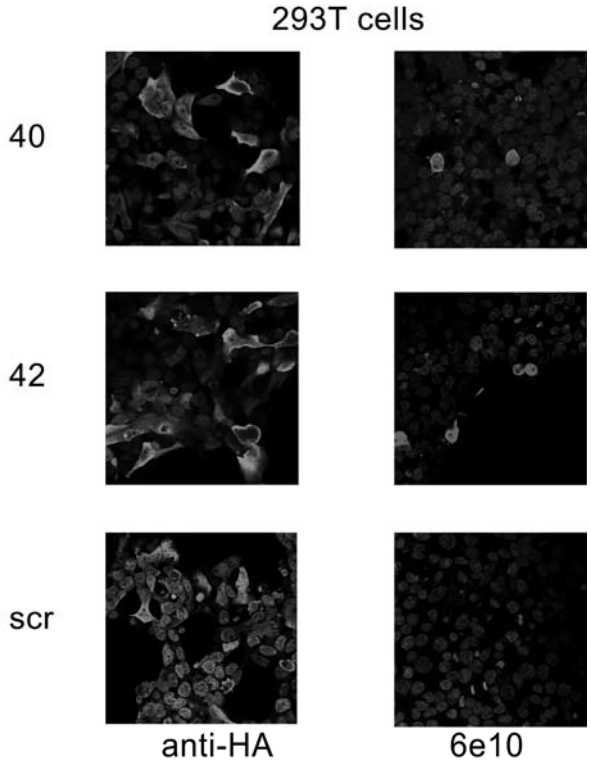


Fig.4 Expression of HA-Ub-Aß in cell lines. 293T were transduced with lentiviral vectors encoding HA-Ub-Aß1-40/ 42 or scr and stained for HA-tag (anti-HA) or Aß (6E10) two days later. The amount of HA-tag positive cells indicates high transduction efficiency. In contrast, only very few cells are positive for Aß1-40 of Aß1-42, suggesting that Aß is degraded after cleavage of the fusion proteins. Aßscr is not recognized by 6E10, corresponding to the lack of 6E10 immunopositive cells in the HA-Ub-Aßscr transduced cells. **See Color figures**

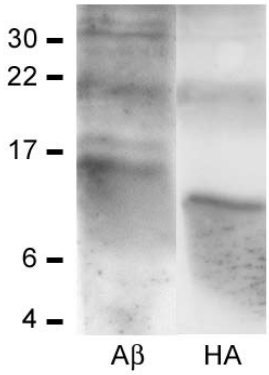


Fig.5 Western blot analysis of HA-Ub-Aß in 293T cells. 293T cells transfected with pcDNA3-HA-Ub-Aß1-42 were subjected to western blot analysis. In cell lysate, 6E10 revealed a 16 kDa band, which was not detected by the anti-HA antibody, indicating that the 16 kDa band is not the fusion protein, but might be an aggregated form of Aß1-42.

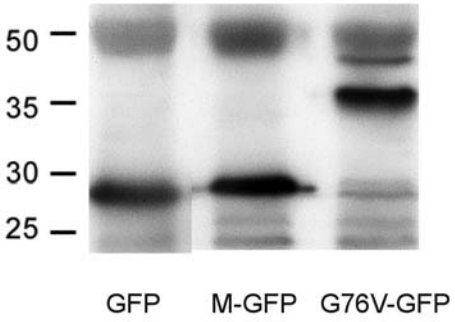


Fig.6 Fusion proteins are generally cleaved very efficiently by Ub hydrolase. Expression of the fusion protein Ub-M-GFP leads to such efficient cleavage by Ub hydrolases, that the fusion protein is not detected on western blot (middle lane). Only the M-GFP part is found, corresponding in size to normal GFP (left lane), while expression of the uncleavable fusion protein Ub^{G76V}-GFP produces a band corresponding to the complete fusion protein (right lane).

Cytosolic Aß does not inhibit the proteasome

Also human neuroblastoma SH-SY5Y cells stably expressing the Ub^{G76V}-GFP protein as a proteasome activity reporter were transduced with the Aβ fusion constructs and the SP-Aβ construct. SP-Aβ was reported to reach the cytosol through ER-associated degradation (Buckig et al., 2002; Schmitz et al., 2004). The proteasome reporter system was used to reveal a possible inhibitory effect of cytosolic Aβ on the proteasome, as was reported earlier to exist *in vitro* (Gregori et al., 1995; Gregori et al., 1997). However, no significant accumulation of the GFP reporter was found in the transduced or transfected cells after 1 to 4 days (Fig. 7). These results indicate that cytosolic Aβ1-40, Aβ1-42, Aβscr and SP-Aβ (results not shown) do not inhibit the proteasome in our reporter system.

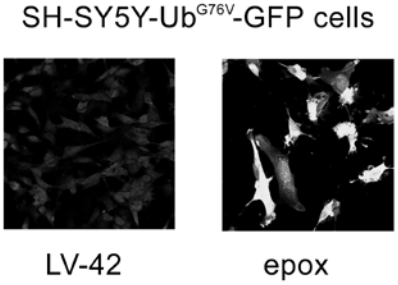


Fig.7 Intracellular Aß does not inhibit the proteasome. Lentiviral transduction with LV-HA-Ub-Aß1-42 of proteasome reporter cells expressing Ub^{G76V}-GFP, did not lead to accumulation of the GFP reporter after 1-4 days after transduction. As a control, 5 h epoxomicin treatment of these cells resulted in strong GFP reporter accumulation. LV-HA-Ub-Aß1-40, LV-HA-Ub-Aßscr and pcDNA3-SP-Aß42 expression also did not result in accumulation of the reporter (not shown). **See Color figures.**

Is cytosolic Aß degraded?

A logical explanation for the lack of Aβ in most transduced cells would be efficient degradation of monomeric Aβ. Several possible mechansims by which cytosolic Aβ could theoretically be degraded were studied. The proteasome and insulin degrading enzyme (IDE) have both been suggested to degrade cytosolic Aβ (Schmitz et al., 2004). Epoxomicin treatment of SP-Aβ transfected cells resulted in a clear increase in monomeric Aβ in the insoluble lysate fraction (Fig. 8). The soluble fraction of SP-Aβ expressing cells demonstrated a smear of Aβ-positve bands after epoxomicin treatment, ranging from monomeric to the 16kDa oligomeric state of Aβ. The accumulation of monomeric SP-Aβ after proteasome inhibition suggests that SP-Aβ is normally either very efficiently secreted and somehow stalled in the ER after proteasomal inhibition, or that the construct is normally very efficiently targeted to the proteasome through ERAD. Although the latter has been suggested earlier (Schmitz et al., 2004), our results showing such a large difference in the presence of monomeric Aβ after proteasome inhibition, emphasize the need for more experiments studying the processing of SP-Aβ.

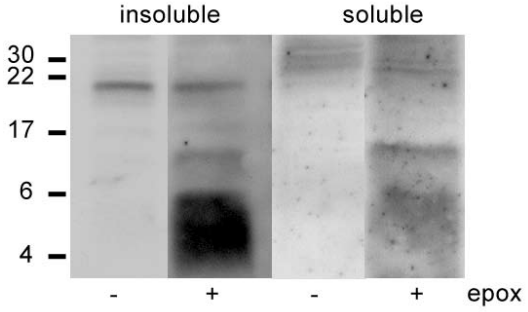


Fig.8 SP-Aß is degraded by the proteasome. Western blots of 293T cells transfected with pcDNA3-SP-Aß42 show hardly any specific 6E10 positive protein bands. Treatment of transfected cells with 100 nM of the proteasome inhibitor epoxomicin, resulted in a strong increase in monomeric Aß in the insoluble lysate fraction. In the soluble lysate fraction, a smear of Aß conjugates, ranging from monomeric to a 16 kDa form was observed.

Epoxomicin treatment of cells transfected with pcDNA3-HA-Ub-Aβ1-42 did not result in increased amounts of monomeric Aβ. However, there was an increase in the 16 kDa oligomeric form of Aβ, mainly in the insoluble fraction (Fig. 9A). Also treatment with bacitracin, an inhibitor of IDE produced slightly more pronounced bands of this oligomeric form in HA-Ub-Aβ1-42 transfected cell lysate. The soluble fraction only showed minor increases in the 16kDa Aβ form after epoxomicin or bacitracin treatment (Fig. 9B). The minor increases found with the HA-Ub-Aβ constructs suggest that monomeric Aβ is still degraded quite efficiently in epoxomicin or bacitracin treated cells.

As a control, a concentration range of Aβ peptide in water was stained on western blot, in order to determine the minimal amount of detectable Aβ in this system. An amount of 50 fmol of Aβ was readily visible on blots (Fig. 10A). The amount of Aβ that is present in samples of transduced cells was estimated by determining the amount of ubiquitin that was found in similar blots. We quantified the amount of unconjugated Ub in samples, which is an underestimation of the total amount, as Ub forms a conventional Ub smear on blot by conjugating to many other proteins. Cell lysates of Aβ fusion protein transduced cells and non-transduced cells were compared to known Ub concentrations of samples of recombinant Ub in water (Fig. 10B). An amount of approximately 1 pmol free Ub was calculated to be present in the transduced samples in addition to endogenous Ub. As the HA-Ub is derived from a fusion protein with Aβ, exactly the same amounts of Aβ should be present in the sample. Since 50 fmol of Aβ was readily visible on blots, the calculated amounts should not be so difficult to detect. Therefore, our results indicate that monomeric cytosolic Aβ is efficiently degraded. Although a slight increase in oligomeric forms of Aβ is found in HA-Ub-Aβ1-42 transfected cells after inhibition of the proteasome or IDE, the monomeric form of Aβ was never found in these lysates. In contrast, the monomeric Aβ peptide is readily detectable after proteasome inhibition in SP-Aβ42 transfected cells.

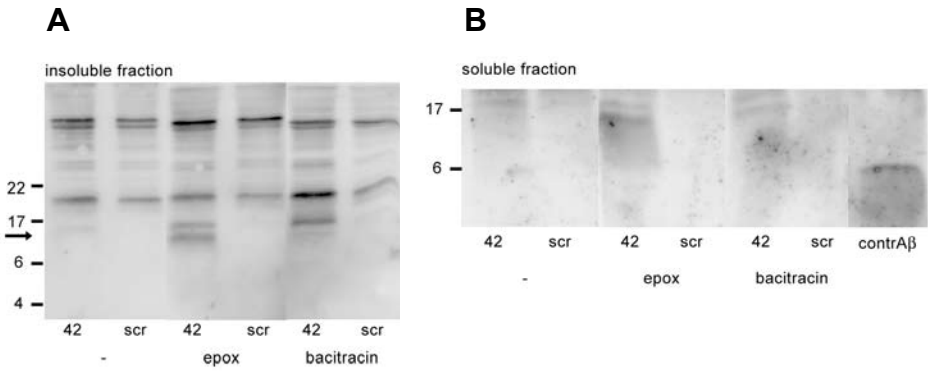


Fig.9 Cytosolic Aß1-42 derived from fusion proteins is partly degraded by the proteasome and by IDE. Western blot analysis of pcDNA3-HA-Ub-Aβ1-42 transfected 293T cells. **A** 16 h treatment of transfected cells with 100 nM epoxomicin or 200 μM bacitracin resulted in an increase in an oligomeric 16 kDa form of Aβ1-42. **B** These treatments only produced a slight increase of the 16 kDa form in the soluble fraction of the transfected cell lysates. In contrast to SP-Aß42 experiments, the monomeric for of Aß1-42 was not observed in any of the cell lysates.

DISCUSSION

In this study we have developed constructs that elegantly produce a cytosolic form of Aβ1-40, Aβ1-42 or a scrambled form of Aβ. Based on *in vitro* experiments in literature, cytosolic Aβ could inhibit the proteasome. However, most of these studies either inject possibly aggregated and rather high concentrations of Aβ, or are performed in cell-free systems. In studies that show DNA mediated expression of Aβ in cells, a clear toxic effect is observed, but the presence of Aβ in the cytosol was never confirmed. Moreover, these constructs usually contain an extra methionine residue, which is not present in endogenous Aβ, to provide a start codon for translation. Addition of such a stabilizing residue in front of a peptide that is only 42 residues in size might have strong effects on the biochemistry of the peptide. Because we found contradicting results with our experiments, we thoroughly checked our constructs. First, all DNA constructs were checked by sequencing. Next, bacterial production of the fused protein confirmed that the entire fusion protein was translated. The three fusion proteins for Aβ1-40, Aβ1-42 and Aβscr migrated with the corresponding size on western blot and all stained with the anti-HA antibody. More importantly, the Aβ1-40 and Aβ1-42

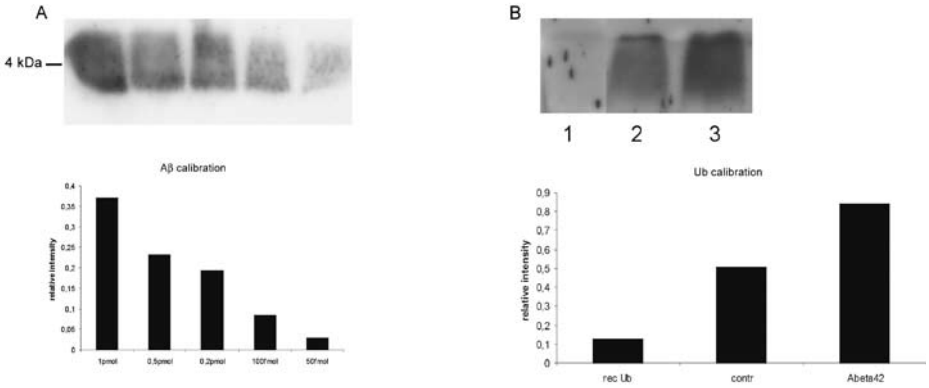


Fig.10 Calibration of detectable amount of Aß and Ub. A Concentration range of Aß peptide in water, stained on western blot with 6E10. 50 fmol is readily detectable on western blot. **B** Calculation of the amount of extra HA-Ub that is induced by the fusion constructs by comparison to known concentrations of recombinant Ub in water. The extra amount of Ub in addition to endogenous Ub (middle lane) in the transfected cell lysates was calculated to be approximately 1 pmol. Aß is produced by the fusion proteins in equimolar amounts as HA-Ub, indicating that 1pmol of Aß should be present in these cell lysates, which is well within the detectable range.

fusion proteins both clearly stained with 6E10 antibody, while the Aβscr fusion protein did not.

The experiments in reticulocyte lysate confirmed that the fusion protein can be cleaved by isopeptidases, as a clear band corresponding in size to Ub was detected on the radioactive gel. However, this experiment should theoretically also show the cleaved Aβ part of the protein. The lack of this band was the first indication that all three Aβ forms can be quickly degraded by a system present in the reticulocyte lysate system. In a study of several years ago, *in vitro* translation of an Aβ construct was also described (Dyrks et al., 1992). In wheat germ extract the translated 4 kDa Aβ product could readily be detected on SDS PAGE gels. In reticulocyte lysate however, the authors reported that the translation products were hardly detectable. The cause of this lack of detection was attributed to inefficient translation. The current results however, point towards a possible Aβ degrading mechanism in reticulocyte lysate, because the fusion correlate of the protein was detected in this system, ruling out unsuccessful translation.

One of the main differences between wheat germ extract and reticulocyte lysate is the presence of the UPS in the latter. Indeed, our experiments with the proteasome inhibitor epoxomicin show

that the UPS is partly responsible for degrading the Aβ product of the fusion proteins. Regarding the possibility of proteasomal degradation of Aβ peptides, our constructs might be specifically susceptible to this type of degradation. If Aβ would be degraded through ubiquitination, the functional Ub that is produced also from the fusion protein, might aid in ubiquitinating Aβ. The functionality of the cleaved HA-Ub is shown in figure 11, were co-transfection of the Aβ fusion constructs and UBB^{+1} leads to a shift in the bands of ubiquitinated UBB^{+1}, corresponding to ubiquitination by the slightly larger HA-tagged Ub (Fig.11).

Taken together, these results strongly indicate that the fusion proteins are produced as we designed them and are cleaved accordingly. Drawing this conclusion, consequently we must acknowledge that cytosolic Aβ does not inhibit the proteasome in the proteasome activity reporter system that we use. HA-tag positive cells of the Ub^{G76V}-GFP cell line should also contain intracellular Aβ, but did not accumulate the GFP reporter. Indirect inhibition of the proteasome by extracellular Aβ (Song et al., 2003), or through disease mechanisms causing modified, aggregated intracellular Aβ to inhibit the proteasome, still provide interesting clues to study putative proteasome inhibition by Aβ.

Future experiments should address higher and longer expression of the Aβ fusion constructs in Ub^{G76V}-GFP reporter cell lines, as 6E10 positive cells were not observed in transduction experiments in these cells, whereas 293 cells did show few positive cells. Also further immunocytochemical and biochemical examination of the differences between expression of cytosolic Aβ and of the SP-Aβ is needed, which will result in new insights in intracellular Aβ processing.

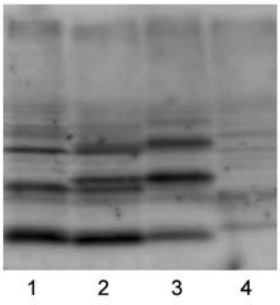


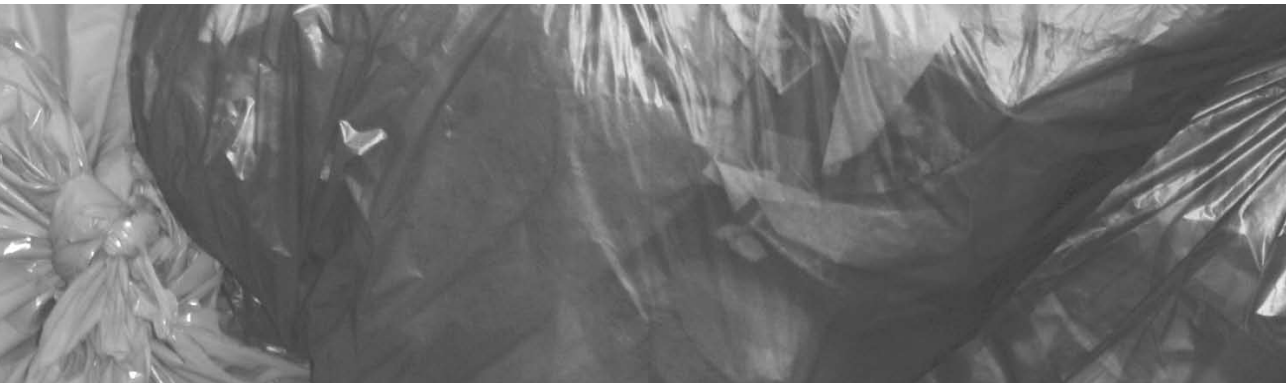
Fig.11 HA-Ub produced from the fusion proteins is functional. SH-SY5Y cells were transduced with lentiviral vectors encoding UBB^{+1}, HA-Ub-Aß1-40 or HA-Ub-Aß1-42. Cell lysates were stained on western blot with anti-UBB^{+1} antibody Ubi3. Lane 1, LV-UBB^{+1}; Lane 2, LV-UBB^{+1} + LV-HA-Ub-Aß1-40; Lane 3, LV-UBB^{+1} + LV-HA-Ub-Aß1-42; Lane 4, LV-HA-Ub-Aß1-42. HA-Ub functionally ubiquitinates UBB^{+1} in the combined transductions, leading to slightly slower migrating forms of ubiquitinated UBB^{+1}.

Acknowledgements

We would like to thank L. Naldini (University of Torino, Torino, Italy) for the use of the lentiviral system, A Schmitz (Rheinische Friedrich-Wilhelms-Universitat, Bonn, Germany) for the SP-Aβ construct and H. Yamaguchi (Gunma University, School of Health Sciences, Maebashi, Japan) for the amyloid antibodies.

CHAPTER 6

Discussion



The origin and mechanism of action of UBB^{+1}

After finding GAGAG motifs to be hotspots for mRNA dinucleotide deletions in rat vasopressin transcripts (Evans et al., 1994), ubiquitin-B +1 (UBB^{+1}) and amyloid precursor protein +1 (APP^{+1}) were discovered in a search for aberrant mRNA's containing such GAGAG motifs in Alzheimer's disease (AD) (Van Leeuwen et al., 1998b). The mechanism that generates dinucleotide deletions in the mRNA remains unknown. RNA polymerase slippage or posttranscriptional RNA editing-type mechanisms are possible candidates for causing these mutations, but their frequency seems to be so low that detection of the mechanism has proven to be extremely difficult (Gerez, 2005). A genetic origin of the mutations was ruled out as extensive searching failed to reveal the dinucleotide deletions in DNA (Evans et al., 1994; Van Leeuwen et al., 1998b). Despite the low frequency of so-called molecular misreading, the proteins that result from the mutant RNA clearly accumulate in AD. However, the aberrant mRNAs can also be found in brains of control individuals, and an increase in molecular misreading - related to aging or AD could not be detected (Gerez, 2005). The accumulation of +1 proteins in AD brain is therefore likely to be caused by defects in protein quality control systems rather than RNA quality control systems.

For UBB^{+1} protein, this implies that the protein is probably always produced, but normally is efficiently degraded. UBB^{+1} indeed is a substrate for proteasomal degradation. More specifically, it behaves as a ubiquitin fusion degradation (UFD) substrate (Chapter 3). However, UBB^{+1} is not a simple substrate, because at high concentrations it specifically and potently inhibits the proteasome (Chapter 3, (Li et al., 2004)). Eventually, high expression of UBB^{+1} even induces apoptotic cell death (Chapter 2 and (De Pril et al., 2004)), which is interesting in light of the putative apoptotic events in AD brain (Anderson et al., 2000; Cotman, 1998; Rohn et al., 2001; Su et al., 1994). In summary, UBB^{+1} is capable of forming a negative feedback loop in the protein quality control system (UPS) that is responsible for its own degradation as well as for that of other aberrant proteins, that accumulate as a result (for a review, see Chapter 1).

Ongoing efforts are made to elucidate the exact mechanism by which UBB^{+1} inhibits the proteasome. It is tempting to speculate that the aberrant C-terminal part of UBB^{+1} is responsible for its inhibitory

effect. To elucidate this, we performed experiments with the 20 amino acid +1 peptide in proteasome activity assays *in vitro*. The +1 peptide did not seem to inhibit proteasome activity in these assays, although these results were not conclusive (data not shown). The insertion of the UBB^{+1} 20 amino acid aberrant C-terminus at the end of another proteasome substrate would indicate if the +1 moiety can cause stabilization of this protein and if the protein gains inhibitory capacity towards the proteasome. For instance, the nonfluorescent GFP form used in Chapter 3 could be complemented with the +1 moiety at its C-terminus and studied in the GFP reporter-expressing neuroblastoma cells.

Alternatively, the inhibitory capacity of UBB^{+1} could be concealed in the length of the aberrant C-terminus, meaning that it would simply be too short to allow proper insertion into the proteasome channel. Lengthening of the substrate between the Ub moiety and the +1 moiety would theoretically overcome this problem. However, enhancement of the UFD signal, by adding more uncleavable Ub moieties at the front of UBB^{+1}, surprisingly increased its inhibitory capacity (Chapter 3), while other UFD proteins are more rapidly degraded after this procedure (Stack et al., 2000). Before completely ruling out the length of UBB^{+1} as the cause for its inhibitory capacity, more substrates should be studied. Hypothetically, application of a repeat in between the N-terminus and the +1 moiety of UBB^{+1} or inserting an N-end rule degron in front of the N-terminus might lead to conclusive answers without relying only on UFD-properties.

At the time of the *in vitro* experiments in Chapter 2, it was believed that the lack of ATP-dependent degradation of UBB^{+1} indicated that UBB^{+1} was not a substrate for the proteasome. We now know that UBB^{+1} in fact is a substrate for the proteasome in low concentrations. In retrospect, it can be concluded that the levels of recombinant UBB^{+1} added to the *in vitro* reaction most likely inhibited the proteasome, thereby preventing its own degradation.

UBB^{+1} and the catalytic activities of the proteasome

The proteasome displays three catalytic activities, the β-subunits 1, 2 and 5 exhibit "peptidylglutamyl-peptide hydrolyzing" (PGPH), trypsin-like and chymotrypsin-like activity respectively (see Chapter

1 for an extensive description of the proteasome). In AD, the PGPH and the chymotrypsin-like activities were found to be down-regulated (Keller et al., 2000a).

UBB^{+1} mainly seems to inhibit the chymotrypsin-like activity, as its degradation can largely be prevented by the proteasome inhibitor epoxomicin, the prime inhibitor of this activity. However, in our studies we noticed that UBB^{+1} transduction in cells that stably express Ub^{G76V}-GFP (a reporter for proteasome activity, see Chapter 3) induced higher accumulation of the reporter than epoxomicin treatment alone (data not shown). This suggested that UBB^{+1} might also affect other activities of the proteasome. The different proteolytic activities of the proteasome are subject to complicated allosteric interactions (Kisselev et al., 1999; Kisselev et al., 2003). Elucidating the effects of UBB^{+1} on specific proteolytic activities of the proteasome may provide more insight into the mechanism of inhibition and possibly into the variability in UBB^{+1}-effects between different systems.

In preliminary experiments performed in our lab, we studied a variety of proteasome inhibitors. The proteasome inhibitor YU102 specifically inhibits the PGPH activity of the proteasome (Kisselev et al., 2003; Myung et al., 2001). This inhibitor was shown to inhibit the degradation of PGPH substrates, but to have no effect on general protein degradation (Myung et al., 2001). Also Z-Val-Ala-Asp fluoromethyl ketone (Z-VAD-FMK) (Sigma), a caspase inhibitor that was reported to inhibit the chymotrypsin-like activity of the proteasome (Beyette et al., 1998), was studied in UBB^{+1} or Ub^{G76V}-GFP transduced neuroblastoma cells. Two days after transduction, treatment of UBB^{+1} transduced cells with 50 μM Z-VAD-FMK resulted in a significant increase ($p=0.003$) in UBB^{+1} immunopositive cells (Fig. 1). Cell death has not reached significance in the UBB^{+1} transduced control group at this time (not shown), ruling out a rescue of cell death as the cause for the increase in immunopositive cells. This indicates that Z-VAD-FMK indeed inhibits the proteasome, causing UBB^{+1} to accumulate in more cells. The same treatment in Ub^{G76V}-GFP transduced cells did not result in a significant increase in GFP-positive cells (Fig.1).

Z-VAD-FMK did not affect the viability of UBB^{+1} transduced cells, even at later times (data not shown), in contrast to results with other caspase inhibitors that do rescue cells from UBB^{+1} induced apoptosis (De Pril et al., 2004). The caspase inhibitor used in the latter study

was N-Acetyl-Asp-Glu-Val-Asp-al (Ac-DEVD-CHO), which inhibits caspase-3, while Z-VAD-FMK is a more general non-selective caspase-inhibitor, inhibiting capase-1- and -3-related proteases. The lack of rescue from UBB^{+1} induced apoptosis by Z-VAD-FMK could be caused by the dual effects of Z-VAD-FMK in UBB^{+1} transduced cells. Since Z-VAD-FMK treatment leads to increased accumulation of UBB^{+1}, this will also induce more apoptosis, thereby masking a possible apoptosis-rescuing mechanism of Z-VAD-FMK.

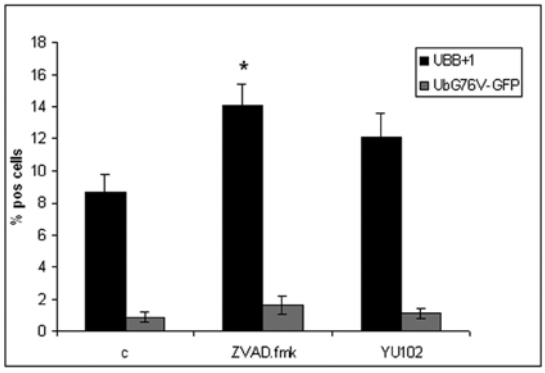


Fig. 1 Z-VAD-FMK inhibits the proteasome, causing accumulation of UBB^{+1} but not of Ub^{G76V}-GFP. SH-SY5Y cells were transduced with LV-UBB^{+1} or LV-Ub^{G76V}-GFP. Transduced cells were treated with vehicle (c), 50 μM Z-VAD-FMK or 10 μM YU102. Z-VAD-FMK treatment resulted in a significant increase in the amount of UBB^{+1} accumulating cells ($p=0.003$).

After treatment of LV-UBB^{+1}-transduced cells with 10 μM YU102, a trend towards an increase in the amount of immunopositive cells was seen, while the GFP reporter did not accumulate after treatment with this inhibitor (Fig. 1), as was also reported earlier (Myung et al., 2001). Although the effect on UBB^{+1} accumulation was not significant with any concentration used, together with the Z-VAD-FMK experiments these results indicate that UBB^{+1} is processed differently than the UFD GFP reporter protein and therefore does not behave as a 'normal' UFD substrate. Future studies on this subject will require improved, highly specific inhibitors and control substrates for each of the three proteasome activities, which are currently not available.

UBB^{+1} in human post mortem cortex cultures

UBB^{+1} has profoundly different effects in cell lines (Chapters 2, 3 and (De Pril et al., 2004)) compared to rodent organotypical cortex

cultures (Chapter 4) or *in vivo* expression in mice (Fischer et al., 2003; Van Leeuwen et al., 1998b). Causes for this discrepancy might be differences in the assemblage of proteasomes between systems, differences in the capacity distribution pattern of the three catalytic activities of the proteasome, or differences caused by the proliferative versus postmitotic state of the transduced cells. Alternatively, differences in the contribution of lysosomal degradation in maintaining protein homeostasis may also underlie the diverse susceptibilities to proteasome-inhibiting stimuli in different systems. For substrates other than UBB^{+1}, it has also been described that the lysosomal system can compensate for degradation when the capacity of the proteasome is no longer sufficient.

The variable effects of UBB^{+1} in different animal systems tempted us to study UBB^{+1} accumulation and proteasome activity in a setting relevant for AD. We performed pilot experiments on fresh human postmortem brain slice cultures of AD patients and control individuals. Brain material was obtained at autopsy by the Netherlands Brain Bank (coordinator Dr. R. Ravid), and cultured according to a procedure developed by Verwer et al. (Verwer et al., 2002) (see legend of Fig. 2 for details). Unfortunately, this complicated procedure yielded poor results with low reproducibility. However, although discussion about the outcome is purely speculative, the results of one experiment with a 64-year-old male control individual were promising and will be discussed further.

After two days in culture, human brain slice cultures were transduced with lentiviral vectors encoding the proteasome reporter Ub^{G76V}-GFP, GFP or UBB^{+1}. Slices in the one successful pilot experiment

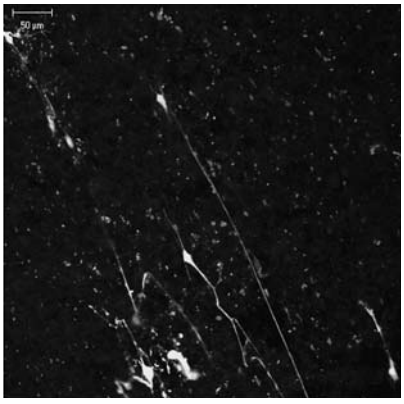


Fig.2 Successful transduction of human post mortem cortex culture with LV-GFP. Within a maximum of 8 h postmortem delay, 250 µm slices of frontotemporal cortex were made with a tissue chopper and put into culture free-floating in medium, which consisted of Neurobasal A medium (Gibco), complemented with 0,1 mg/ml vitamin C, B27 supplement (Gibco), 10 U/ml penicillin and 10 µg /ml streptomycin (Gibco). Lentiviral vectors were applied by dropping a 20 µl drop of medium containing 10^6 transducing viral particles on top of a dry slice, which was replenished with medium after five minutes. At several different times after transduction, slices were fixed for 1 h in 4% formalin, after which they were stained free-floating as described previously for cortex slices of mice (Chapter 4). **See Color figures.**

showed nice transduction of cells with GFP, some of which had clear neuronal morphology (Fig. 2). Transduction of slices with UBB^{+1} in this experiment surprisingly resulted in many immunopositive cells, while this was never observed in cortex slices of mice. UBB^{+1} immunopositive cells had a large and unusual appearance (Fig. 3). Some resembled neurons, while others looked more like large atypical astrocytes. The emergence of UBB^{+1} immunostaining in the slices indicates that these cells must have suffered from impaired proteasomal activity or are very sensitive to proteasome inhibition by UBB^{+1}. However, if many cells already suffered from impaired proteasomal activity in the slices, one would expect more positive cells after transduction of slices with Ub^{G76V}-GFP. Instead, only very few positive cells were found; three cells in two slices to be precise, suggesting that proteasomal activity is intact in most transduced cells. However, these cells may have had only slight proteasome inhibition, which would trigger UBB^{+1} accumulation but is still sufficient to degrade Ub^{G76V}-GFP.

A subset of transduced slices was treated with the proteasome inhibitor epoxomicin. Theoretically, this should lead to accumulation

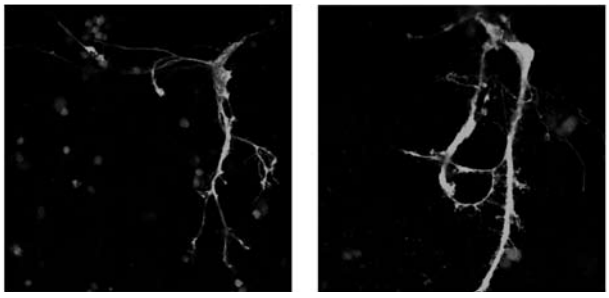


Fig.3. Transduction of human post mortem cortex cultures with LV-UBB^{+1} results in accumulation of UBB^{+1} in large cells. Ubi3 staining for UBB^{+1} (red), TO-PRO staining for nuclei (blue). **See Color figures.**

of endogenous UBB^{+1} as well as accumulation of the induced UBB^{+1} or Ub^{G76V}-GFP. Accumulation of endogenous UBB^{+1} was not observed, but cells that accumulated UBB^{+1} after epoxomicin treatment seemed to be dying (Fig. 4), indicating that UBB^{+1}-accumulating cells in human cortex slices might indeed be very vulnerable to acute UBB^{+1}-induced toxicity. Ub^{G76V}-GFP did accumulate after proteasome inhibition by epoxomicin: 17 cells in two slices were observed

(Fig. 4), which indicates that proteasomes were still active in these postmortem cultures. Unfortunately, these intriguing results could not be replicated due to technical restraints, meaning that no final conclusions can be drawn from these preliminary experiments. A total of five patients were studied: two controls, two Alzheimer's and one Pick's disease patient (table 1). The slices of only one control individual showed successful lentiviral transduction. In slices of the other four patients, no immunopositive cells of any of the constructs were observed. Different culture treatments (e.g. types of media during transport and culture, refreshment etc.) and times of transduction and fixation did not improve the results, and these pilot experiments were too few to be correlated to postmortem delay, age, pH or sex. As these experiments are very time-consuming and the variables that determine success could not (yet) be determined, these experiments were not continued. However, it would be most interesting to study these cultures further, as they form an ideal system to study proteasomal activity in human disease-specific material. With the combined use of viral transduction and ir- or reversible proteasome inhibitors, proteasomal activity can be monitored and manipulated, in addition to conventional proteasome activity assays - such as the fluorescent peptide assay (Keller et al., 2000a). Working with human post mortem material is complicated since many variables cannot be controlled. The starting condition of the cultures is variable, due to disease, *post mortem* delay etc., and the viability of the slices is highly vulnerable in culture. For efficient transduction, a high viability of the slices is essential. As was also shown in Chapter 4, different viral vectors (i.e. lentivirus vs. adenovirus) can have profoundly different effects, for instance in mouse cortex slices. Moreover, efficient transduction also depends on cell surface receptor expression complimentary to the virus with which transduction is achieved. According to literature, Semliki Forest Virus (SFV) is highly capable of transducing neurons in organotypic cultures in a short timescale, whereas lentiviral vectors take much longer to express the protein of interest in neurons (Ehrengruber et al., 2001). SMV is highly cytotoxic, but novel SMV vectors have been developed that are less cytotoxic (Lundstrom et al., 2003), and especially in acute experiments these might prove to be an excellent tool to transduce neurons in human *post mortem* cultures.

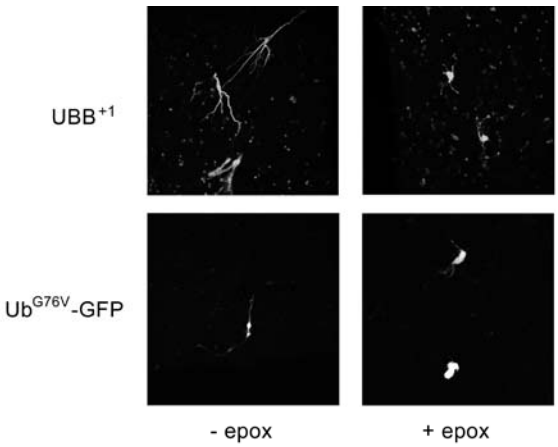


Fig.4 Proteasome activity is functional in one pilot experiment of human post mortem cortex cultures. UBB^{+1} accumulation seems to be highly toxic in these cells. Human post mortem cortex cultures were transduced with LV-UBB^{+1} or LV-Ub^{G76V}-GFP and subsequently treated with 1 µM epoxomicin. Ub^{G76V}-GFP (green) accumulated in 17 cells total in 2 slices, as opposed to 3 cells in the control situation. The amount of UBB^{+1} accumulating cells (red) decreased after epoxomicin treatment an they had a less healthy appearance than in the control situation. **See Color figures.**

NHB nr	Pathology	Sex	Age (years)	Post mortem delay (h)	pH
02-101	C	M	64	8,5	6,2
03-004	AD	F	80	6,2	7
03-017	AD	M	68	6	6,7
03-026	Pick's	F	54	5,4	6,2
03-074	C	F	61	5	nd

Table 1

Why does UBB^{+1} accumulate in different cell types in different diseases?

UBB^{+1} accumulates in several different neurodegenerative diseases (De Pril et al., 2004; Fergusson et al., 2000; Fischer et al., 2003), as well in as non-neurological diseases, such as chronic liver diseases (Riley et al., 2002). Interestingly, tauopathies are characterized by UBB^{+1} accumulation, while synucleinopathies are devoid of UBB^{+1} accumulation (Fischer et al., 2003). Also cell types that accumulate UBB^{+1} in these diseases differ. In AD, UBB^{+1} is mostly present in neurons. However, in progressive supranuclear palsy, glia accumulate UBB^{+1} as well (Fischer et al., 2003). These differences

in UBB^{+1} accumulation probably reflect a difference in vulnerability to proteasome inhibition of the different cell types in these diseases. More specifically, differences in the composition and different proteolytic activities of the proteasome in each disease and cell type might also account for the differences. In PD for instance, loss of proteasome α-subunits was found (McNaught et al., 2002), and also decreased proteasome activity in the substantia nigra (McNaught et al., 2003), UBB^{+1} however, does not accumulate in PD. Future research will need to elucidate these different vulnerabilities to proteasome inhibition.

Since neurons are post-mitotic cells, they might be more vulnerable to proteasome inhibition than other cells. Primary neuronal cultures, for instance, are known to be highly sensitive to proteasome inhibition (Laser et al., 2003; Pasquini et al., 2000; Qiu et al., 2000). In our lab we performed preliminary experiments to elucidate possible differences in sensitivity to proteasome inhibition between cell types in organotypic cortex slice cultures of Ub^{G76V}-GFP mice (Lindsten et al., 2003). The advantage of this system is that all cell types can be compared in the same environment. Different concentrations of the proteasome inhibitor epoxomicin were applied to slices, ranging from 100 nM to 1 μM (Fig. 5). Although a concentration-dependent increase in the amount of GFP accumulating cells in general was observed, unfortunately, no differences in GFP positive cell types could be observed. In fact, neurons did not seem to accumulate the reporter at all (Fig. 5). This could not be due to a lack of transgene expression in neurons, since, the transgene of the Ub^{G76V}-GFP mouse was designed under control of a CMV-β-actin promoter. Theoretically, this should mean that all cell types in the brain are able to express the GFP reporter. Moreover, primary neuron cultures of these mice have previously been shown to express the reporter (Lindsten et al., 2003). Alternatively, the lack of GFP accumulation in neurons in organotypic cortex slice cultures could be caused by a differential permeability of cell types to the proteasome inhibitor. More research is needed to answer these questions. Future studies in aged Ub^{G76V}-GFP transgenic mice might elucidate whether the GFP reporter will accumulate in the brain due to aging-induced proteasome inhibition and if neurons display increased vulnerability to this inhibition.

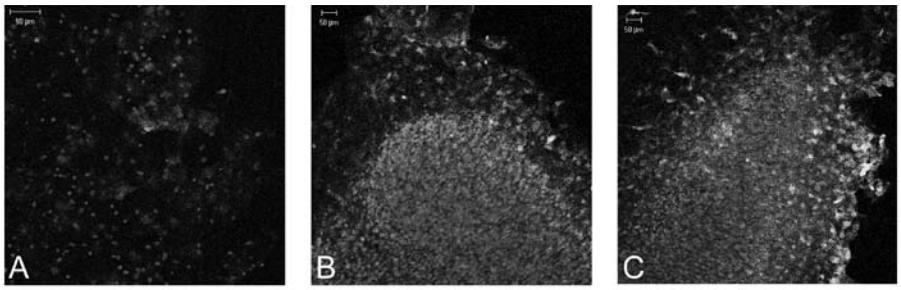


Fig.5 No concentration-dependent difference between cell types in vulnerability to proteasome inhibition. Transgenic Ub^{G76V}-GFP mouse cortex cultures were treated with epoxomicin in concentrations of **A** 100 nM, **B** 500 nM, **C** 1 μM. Cultures were stained for the neuronal marker NeuN (red), TO-PRO (blue) and GFP (green). **See Color figures.**

What are the mechanisms that cause initial proteasome inhibition in AD?

Since UBB^{+1} is a substrate for proteasomal degradation at sub threshold levels (Chapter 3, 4), it is not likely that UBB^{+1} forms the primary cause of proteasome inhibition in AD. Therefore, we decided to look for other AD-related mechanisms that could form the initial trigger for inhibition and would thus, with UBB^{+1}, have a synergistic effect on proteasome activity. In our view, the most likely candidates to play such a role were i) the intermediates of Aβ40 and Aβ42 peptide aggregates, ii) tau, phosporylated forms of tau or paired helical filament tau and iii) oxidative stress. These three candidates all have been implicated to inhibit the proteasome *in vitro*.

Amyloid ß

The effects of cytosolic Aβ peptide were extensively studied in Chapter 5. Our finding that cytosolic Aβ does not inhibit the proteasome is contradictory to *in vitro* (cell-free) results that were described earlier (Gregori et al., 1995; Gregori et al., 1997). To be able to inhibit the proteasome physically, Aβ must be in close contact with the proteasome, which is present in the cytosol and nucleus (Wojcik and DeMartino, 2003). However, *in vivo* it is unknown if Aβ as such is present in the cytosol, since Aβ is normally cleaved

from APP in the secretory pathway. Mechanisms that could explain the difference between a possible stable form of cytosolic Aβ and the Aβ produced by our fusion constructs need further study. For instance, if Aβ would reach the cytosol through ERAD (Buckig et al., 2002; Schmitz et al., 2004), then perhaps the secretase cleavage it undergoes before release can modify the peptide in such a way that it becomes stable or can form aggregates. Other explanations for a stable form of cytosolic Aβ could be stabilizing modifications occurring in the ER or chaperone-bound transport from the ER, which might protect the Aβ from degradation. In light of the latter theory, it is interesting that intracellular Aβ peptide containing a signal peptide was recently found to interact with several chaperones, localized in the ER or in the cytosol (Fonte et al., 2002).

Alternatively, our fusion construct (for details see Chapter 5) might induce a cytosolic form of Aβ that is more susceptible to degradation, due to intrinsic properties of the construct. Hydrolase cleavage of

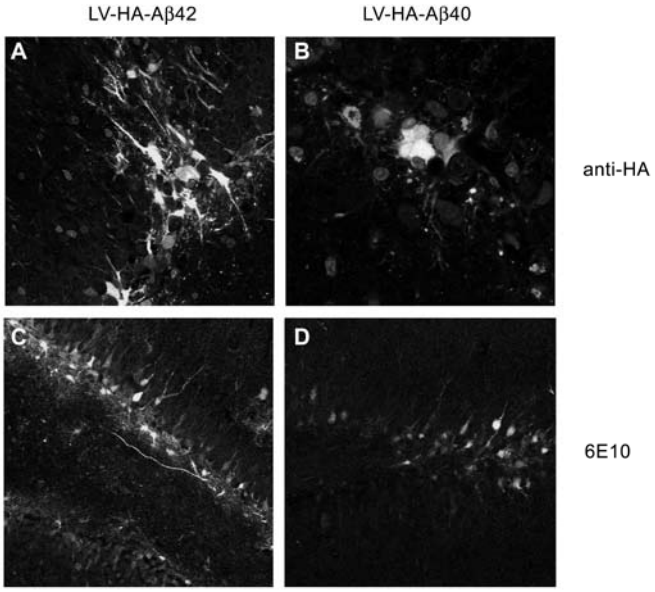


Fig.6 Injection of Aß fusion protein lentiviral vectors in rat hippocampus results in very few positive cells that have altered morphology. Adult Wistar rats were injected with $8x10^5$ TU LV-HA-Ub-Aß1-40 or LV-HA-Ub-Aß1-42 in 0,9 µl saline solution (0,2 µl/min) and coinjected with 0,1 µl with $8x10^5$ TU LV-GFP. After perfusion, 50 µm thick coronal vibratome sections were stained for anti-HA (red), GFP (green) and TO-PRO (blue). **See Color figures.**

the fusion protein might, for instance, allow direct degradation of the Aβ-part of the protein. In our experiments where hydrolases successfully cleaved the fusion proteins, neither the fusion protein itself nor the Aβ-part was found back, as if the Ub hydrolase itself immediately degrades the Aβ-part of the protein in the process of cleaving the fusion protein. Control experiments with inhibitors of Ub hydrolase, such as Ub aldehyde (Hershko and Rose, 1987) will have to elucidate this matter.

Injection of LV-HA-Ub-Aβ1-40 and LV-HA-Ub-1-Aβ42 in hippocampi of wild-type rats, revealed very little HA-tag positive cells, although the co-injected LV-GFP construct was evidently present (Fig. 6). The few HA-tag positive cells that were observed, displayed severe membrane blebbing and seemed to be unhealthy.

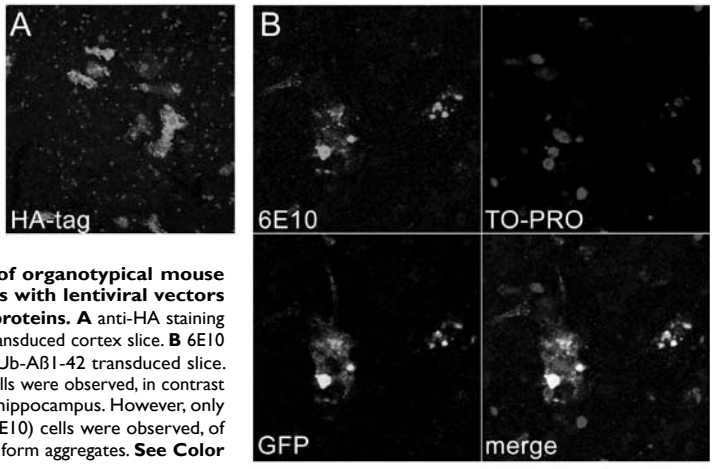


Fig.7 Transduction of organotypical mouse cortex slice cultures with lentiviral vectors encoding Aß fusion proteins. A anti-HA staining of LV-HA-Ub-Aß1-42 transduced cortex slice. **B** 6E10 positive cell in LV-HA-Ub-Aß1-42 transduced slice. Many HA-tag positive cells were observed, in contrast to LV-injections in rat hippocampus. However, only very few Aß positive (6E10) cells were observed, of which some seemed to form aggregates. **See Color figures.**

All this led us to believe that in the time-course of the experiment, the transduced cells had died. No differences were observed between Aβ1-40 or Aβ1-42 injections and the Aβ-scrambled condition was lost in this experiment, which means we cannot conclude that the postulated cell death is caused specifically by Aβ1-42 or Aβ1-40. Moreover, in organotypic cortex slice cultures of mice this toxic effect was not observed, demonstrating many HA-tag positive cells after LV-HA-Ub-Aβ1-42 transduction (Fig. 7A). In organotypic

cortex slice cultures of Ub^{G76V}-GFP transgenic mice, no accumulation of GFP was observed in LV-HA-Ub-Aβ1-42 transduced cells (not shown). After epoxomicin treatment, an occasional cell with 6E10- (a mononclonal antibody recognizing Aβ) and GFP-positive inclusions was observed (Fig. 7B). However, these results were not conclusive and need further study.

All together, the results with this cytosolic form of Aβ indicate that prudence is called for when interpreting results with artificially induced forms of cytosolic Aβ. Interesting as they may be, any *in vitro* cell-free results that show that Aβ binds and inhibits the proteasome (Gregori et al., 1995; Gregori et al., 1997), any implications of oxidatively modified Aβ having an even more pronounced effect on proteasome activity (Shringarpure et al., 2000) or any demonstrations of extracellular applied Aβ as a target for proteasomal degradation (Lopez Salon et al., 2003) are all physiologically irrelevant if Aβ *in vivo* is never located in the cytosol or nucleus and thus never in proximity of proteasomes. Re-uptake of extracellular Aβ could also be a way for Aβ to get into the cell (Ida et al., 1996; Nagele et al., 2002). However, internalized Aβ is most likely directed to endosomes for degradation, and will therefore not reach the cytosol either.

Moreover, much is still unknown about modifications and aggregational states that modulate toxicity of the Aβ peptide (Kayed et al., 2003; Lashuel et al., 2002). Our results indicate that Aβ peptide in the cytosol, should it ever get there, will be rapidly degraded, and therefore suggest that cytosolic Aβ does not inhibit the proteasome.

Extracellular Aβ was also implied to inhibit the proteasome indirectly (Song et al., 2003). Interestingly, this mechanism was mediated by the E2 enzyme E2-25K/Hip-2. E2-25K/Hip-2 ubiquitinates UBB^{+1}, which could explain the proteasome inhibition induced by extracellular Aβ. However, Aβ is also found in plaques in individuals without AD and in diseases where no accumulation of UBB^{+1} is found, which implies that Aβ does not necessarily inhibit the proteasome through UBB^{+1}.

Tau

Abnormal tau is the main constituent of neurofibrillary tangles in AD. This microtubule associated protein accumulates in tangles in a hyperphosphorylated form that aggregates as paired helical filaments (PHF). These PHFs were recently found to bind and inhibit the proteasome (Keck et al., 2003). Both PHFs, isolated from AD brain and PHFs assembled *in vitro*, had this effect on isolated proteasomes. On the other hand, normal unfolded tau has been implicated to be a substrate for the 20S proteasome (David et al., 2002) in a bidirectional and Ub-independent manner. To elucidate if tau might be responsible for initial proteasome inhibition in AD, we made use of two vectors encoding normal human four repeat (4R) tau (τwt) and a 4R mutant form of tau (τP301L) (kindly provided by P. Heutink, VU medical centre, Amsterdam). The mutant form of tau is associated with frontotemporal dementia and Parkinsonism linked to

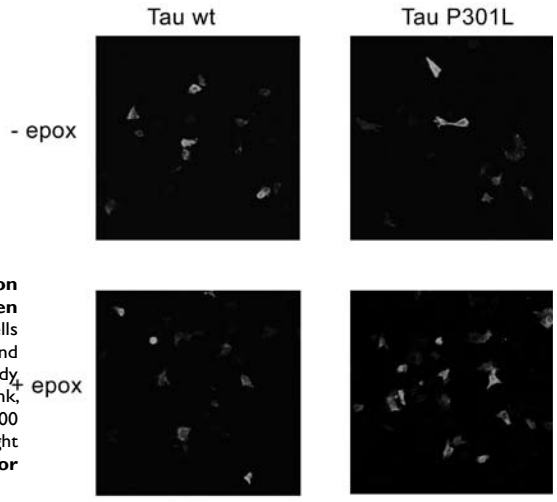


Fig.8 No differences in transduction efficiency or morphology between LVτ and LVτP301L cells. SH-SY5Y cells were transduced with LVτ or LVτP301L and subsequently stained with tau5a6 antibody (Developmental studies Hybridoma Bank, red). Treatment of transduced cells with 100 nM epoxomicin overnight, resulted in a slight increase in immunopositive cells. **See Color figures.**

chormosome 17 (FTDP-17) and is known to induce tangle formation in mice (Gotz et al., 2001; Lewis et al., 2000). Human neuroblastoma cells stably transfected with the Ub^{G76V}-GFP proteasome reporter (Dantuma et al., 2000b; Lindsten et al., 2002) were transduced with lentiviral vectors encoding the two types of tau. These preliminary experiments resulted in many positive cells, indicating high transduction efficiency (Fig. 8). No differences in transduction

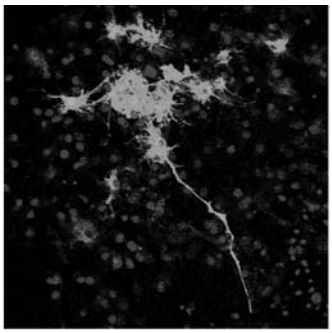


Fig.9 LVτP301L does not lead to proteasome inhibition in Ub^{G76V}-GFP transgenic mouse organotypic cortex slices. Cortex cultures were transduced with LVτP301L and subsequently stained with tau5a6 (red), GFP (green) antibodies and TO-PRO (blue). τP301L expression did not induce accumulation of the GFP reporter and therefore does not seem to inhibit the proteasome. **See Color figures.**

efficiency, cell survival or cell morphology were observed between the two constructs. In our experiments, when transduced cells were treated with the proteasome inhibitor epoxomycin, a slight increase in the amount of positive cells was observed (Fig. 8), indicating that in our system tau might be degraded by the proteasome to some extent. Again, no differences were observed between the two constructs. Transduction with neither of the two constructs resulted in accumulation of the GFP reporter, indicating that in this system neither soluble wild type nor mutant FTDP-17 tau inhibited the proteasome.

Transduction of organotypic cortex cultures of Ub^{G76V}-GFP transgenic mice with the tau lentiviral constructs resulted in many tau immunopositive cells, but also in this system, no accumulation of the GFP reporter was observed (Fig. 9).

Our results are in contrast with studies that show altered morphology and induction of apoptosis in τP301L transduced SH-SY5Y cells (Zhao et al., 2003b). Nevertheless, other studies have also observed that transduction of neuroblastoma cells with mutant tau did not result in aggregation of tau or filament formation (Ko et al., 2002; Lu and Kosik, 2001; Sahara et al., 2000). According to literature, only PHF tau is capable of inhibiting the proteasome (Keck et al., 2003). For now, we can conclude that soluble wild type tau and soluble P301L mutant tau in the cytosol do not inhibit the proteasome, at least not to an extent that causes accumulation of the GFP reporter. Accordingly, since PHF formation is probably a rather distal event in AD pathogenesis, combined with our results with soluble tau, it is not likely that tau pathology is responsible for the initial proteasome inhibition and UBB^{+1} accumulation in AD.

Oxidative stress

Another mechanism that applies for a role in initial proteasome inhibition in AD is oxidative stress. Multiple lines of evidence demonstrate a clear involvement of oxidative stress in AD pathogenesis (Castegna et al., 2002; Lovell et al., 1997; Miranda et al., 2000; Zhu et al., 2004a). Interestingly, in our studies H_2O_2-induced oxidative stress was found to increase the inhibitory effect of UBB^{+1} in neuroblastoma cells synergistically, at concentrations that did not induce inhibition of the proteasome by itself (Fig. 10). 100 μM H_2O_2 was applied to SH-SY5Y cells that stably express the proteasome reporter construct Ub^{G76V}-GFP. Although this concentration did not induce accumulation of the GFP reporter by itself, 100 μM H_2O_2 together with UBB^{+1} resulted in a significant increase in GFP accumulating cells compared to untreated UBB^{+1} transduced cells. However, with hindsight, in the series of experiments in which these results were consequently found, cells were treated with miscalculated antibiotic concentrations. The antibiotic used for selection of stably transfected reporter cells, geneticin, was used in almost twice the concentration needed for selection. Therefore, these cells were in a worse physiological state than normally. The differences in the amount of UBB^{+1}-positive cells between experiments also indicates the high variability and sensitivity of UBB^{+1} accumulation. The experiments were then repeated in healthy reporter cells, with several oxidative stress inducing compounds: tert-butyl-hydroperoxide (tBHP), 4-hydroxynonenal (4HNE) and H_2O_2. Unfortunately, neither of them showed similar results as in the experiments with the mistreated cells. Apparently, in previous experiments the worsened physiological state of the cells made them more vulnerable to UBB^{+1} toxicity combined with oxidative stress. Oxidative stress induction alone however, never had any effect on the cells, including the mistreated cells. There are many indications in literature that oxidative stress might indeed inhibit proteasomal degradation (Ding and Keller, 2001a; Reinheckel et al., 2000). Our results, however, suggest that the proteasome reporter system we use is not affected by oxidative stress. Monitoring degradation of the GFP reporter is a very indirect way of measuring proteasomal activity. It is not known exactly by which exact proteasome conformation or by which of the three proteolytic activities the reporter is degraded. Their proteasomal degradation is likely to involve 19S activity as UFD substrates are ubiquitinated preferentially at Lys29 or Lys48

(Johnson et al., 1995). The 26S proteasome is thought to be more susceptible to oxidative stress than the 20S proteasome (Ding et al., 2003; Reinheckel et al., 2000), which suggests that oxidative stress might interfere with degradation of the reporter. However, the reporter apparently is such an efficient substrate for the proteasome that other toxic events induced by oxidative stress are limiting, causing cell death before accumulation of the reporter. Interestingly, UBB^{+1} expressing cells were recently found to be more resistant to oxidative stress, rather than more vulnerable (Hope et al., 2003). In a conditional expression model of UBB^{+1} in SH-SY5Y cells, UBB^{+1} induced expression of heat shock proteins, which protected the cells from tBHP-mediated oxidative stress. However, an inducible cell line of UBB^{+1} might represent a pre-selected cell that is able to cope with UBB^{+1}, and therefore is more able to quickly regulate heat shock proteins. Our results indicate that *in vivo*, an oxidative stress insult on vulnerable cells in an aged and diseased environment such as the AD brain, might have a synergistic effect with UBB^{+1} accumulation on proteasome inhibition.

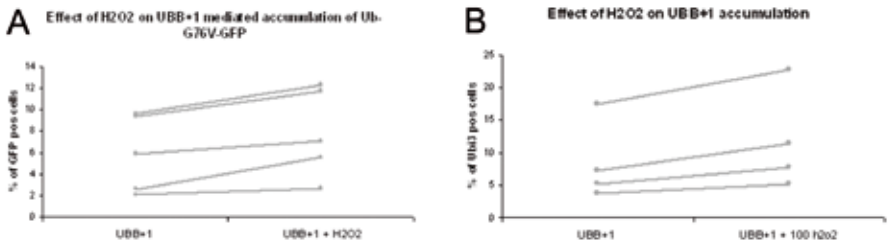


Fig.10 H_2O_2 induced oxidative stress has a synergistic effect on proteasome inhibition by UBB^{+1} in Ub^{G76V}-GFP expressing neuroblastoma cells in a worsened physiological state. Mistreated Ub^{G76V}-GFP SH-SY5Y cells were transduced with LV-UBB^{+1} and subsequently treated either with or without 100 μM H_2O_2. **A** GFP accumulating cells were quantified by FACS analysis. Lines represent results in duplo of 5 different experiments. **B** UBB^{+1} accumulating cells were quantified by counting ubi3 immunopositive cells. Lines represent results in duplo of 4 different experiments. Both GFP and UBB^{+1} positive cells were significantly increased after H_2O_2 treatment (paired Student's T-test).

Concluding remarks

The spatial and temporal course of events in the pathogenesis of AD has been the subject of endless debate (Braak and Del Tredici, 2004; Hardy, 2004; Mudher and Lovestone, 2002; Price and Morris, 2004; Schonheit et al., 2004; Zhu et al., 2004a; Zhu et al., 2004b). Proteasomal inhibition in AD so far has not been fully characterized in this respect. Future studies should elucidate possible correlations between proteasome activity and the state of dementia, neuropathological staging and *ApoE* genotype.

AD is a multifactorial disease which takes decades to develop. Therefore, impairment of the proteasome in AD brain is probably caused by a chronic low level inhibition, since acute proteasome inhibition is highly toxic. UBB^{+1} is already present in the pathological hallmarks in the brain quite early in AD, which means that UBB^{+1} must have surpassed the threshold level in these cells. UBB^{+1} thereby acts as an endogenous reporter for proteasome activity, indicating that proteasome inhibition is an early event in AD. The UBB^{+1} accumulating cells in early AD brain seem to be capable of handling threshold levels of UBB^{+1} for quite some time, or else they would all have died and disappeared from the late AD brain. Nevertheless, our studies have shown that UBB^{+1} can be an important contributor to proteasome inhibition and eventually to neurodegeneration, after it has passed an accumulation threshold (Chapter 4). Cytosolic Aβ was studied as a possible candidate for initiating proteasome inhibition in AD, but, surprisingly, we found that Aβ is degraded efficiently in the cytosol, even at high concentrations (Chapter 5). However, extracellular Aβ might contribute to proteasome inhibition through indirect mechansims, possibly involving UBB^{+1} and its ubiquitinating enzyme (Song et al., 2003). We have also found indications that oxidative stress might play an important role in mediating initial proteasome inhibition and UBB^{+1} accumulation in AD. Moreover, aging, the main risk factor for AD, is also known to decrease proteasome activity. More research is needed to further define the different contributions of AD-related mechanisms to proteasome inhibition, and to elucidate the possibly synergistic effects with UBB^{+1}.

In summary, although more research is needed to elucidate the order of events and initial causes for proteasome inhibition, it is clear that the UPS plays a key role in the pathogenesis of

AD, and we hypothesize that accumulated UBB^{+1} contributes to neurodegeneration through a negative feedback loop on proteasome function (Fig. 11).

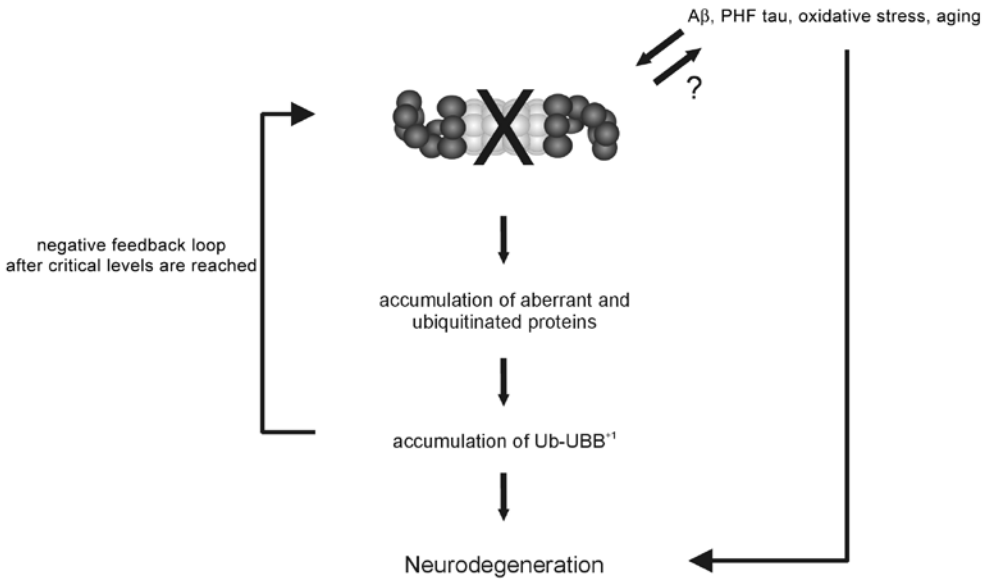


Fig.11 General hypothesis

Summary

Neurodegenerative diseases in general are characterized by intracellular accumulation of aberrant proteins in specific brain areas. The ubiquitin proteasome system (UPS) is normally responsible for protein quality control and performs the majority of protein breakdown in the cell. A defective UPS is more and more implicated as a common factor in neurodegenerative diseases and its activity is diminished in Alzheimer's disease brain. Affected neurons in Alzheimer's disease generally accumulate UBB^{+1}, a mutant ubiquitin carrying a nineteen amino acid C-terminal extension generated by a transcriptional dinucleotide deletion. Ubiquitin (Ub) is normally responsible for tagging proteins for degradation by the proteasome. Ub molecules form a branched tree of proteins by linking to substrates through isopeptide bonds between its C-terminal glycine residue and lysine residues in the substrate protein and in subsequent Ub proteins. This Ub tree represents a signal for protein breakdown by the proteasome. Chapter 1 is the general introduction of this thesis and presents a detailed view on the involvement of the UPS in Alzheimer's disease.

In chapter 2, the properties of UBB^{+1} are studied in a cell-free system and in human neuroblastoma cells. UBB^{+1} does not participate in tagging proteins for proteasomal breakdown, since it lacks the C-terminal glycine residue that is necessary for this process. UBB^{+1} is subject to ubiquitination itself, as the essential lysine residues are in the unchanged part of the protein. High expression of UBB^{+1} resulted in apoptotic-like cell death.

Chapter 3 describes the specific inhibitory effect of UBB^{+1} on the UPS, which therefore may contribute to proteasome inhibition as it is found in Alzheimer brain. In this study, a green fluorescent protein (GFP)-fusion-protein-based reporter system was used. The proteasome reporter system consists of fusion proteins of Ub and GFP with different linkers in between. The control fusion protein has a stable methionine residue in between, which results in a stable form of GFP after cleavage of the fusion protein by isopeptidases. The reporter construct does not contain a stable amino acid linker,

but has a glycine to valine mutation at the last amino acid of the Ub part of the fusion protein. Because of this mutation the reporter fusion protein is uncleavable to isopeptidases and is efficiently degraded as a whole by the proteasome. Therefore, cells expressing the latter construct will only accumulate GFP if proteasome activity is inhibited. In this system, UBB^{+1} specifically and potently caused accumulation of the GFP reporter, indicating that UBB^{+1} inhibits proteasome activity. Both proteasome inhibition and toxicity were found to be dependent on ubiquitination of UBB^{+1} on two of its lysine residues. Paradoxically, this property also leads to UBB^{+1} acting as a substrate for proteasomal degradation.

Chapter 4 discusses a probable explanation for this paradox, namely a threshold of UBB^{+1} accumulation that needs to be surpassed to actively inhibit proteasome activity. This threshold hypothesis was studied in organotypical mouse cortex slice cultures and quantified in neuronal cell lines. Below the threshold, proteasomes can deal with UBB^{+1}, but if (through other mechanisms) proteasome activity is decreased, UBB^{+1} accumulates and can contribute to further inhibition of proteasome activity. In Alzheimer's disease it seems that the threshold for UBB^{+1} accumulation is exceeded, as the protein clearly accumulates in affected brain areas. However, the mutant UBB^{+1} mRNA can also be found in control individuals and the protein is an efficient substrate for the proteasome at low concentrations. Therefore, it is not likely that UBB^{+1} forms the initial trigger for proteasome inhibition in Alzheimer's disease, but more probable that other Alzheimer-related mechanisms cause this inhibition.

Chapter 5 deals with intracellular amyloid-β peptide (Aβ) formation as one of those mechanisms, which has been implicated in proteasome inhibition in Alzheimer's disease before. By expressing Ub-Aβ fusion constructs in cell lines, a purely cytosolic form of Aβ was induced. However, cytosolic Aβ was not able to inhibit proteasome activity in the GFP proteasome reporter system.

Finally, chapter 6 forms a general discussion of the results presented in this thesis and describes suggestions for future research. Additionally, preliminary data on tau aggregation and oxidative stress as proteasome-inhibiting factors in Alzheimer's disease are presented. From these data, no effect of soluble tau on proteasome activity was observed so far. Interestingly, oxidative stress was found to synergistically inhibit the proteasome with UBB^{+1}, but only under certain conditions that need further study.

In Alzheimer brain, initial proteasome inhibition can lead to accumulation of UBB^{+1} up to critical levels, which can subsequently form an important contribution to further inhibition of the UPS in and eventually to neurodegeneration. Additionally, UBB^{+1} can be regarded as an endogenous reporter of proteasome activity. Intriguingly, UBB^{+1} only seems to accumulate in tauopathies and not in synucleinopathies. More research on the mechanism of accumulation and proteasome inhibition by UBB^{+1}, possibly synergistically with other processes, will provide more insight in discriminating between the different molecular backgrounds and pathogeneses of these diseases.

Samenvatting

Neurodegeneratieve ziekten worden in het algemeen gekenmerkt door ophopingen van afwijkende eiwitten in specifieke hersengebieden. Het systeem dat de kwaliteit van eiwitten bewaakt is het ubiquitine proteasoom systeem (UPS). Een goed werkend UPS is essentieel voor het opruimen van dergelijke afwijkende eiwitten. De laatste jaren is uit onderzoek gebleken dat dit systeem inderdaad een belangrijke rol speelt in de pathogenese van vele neurodegeneratieve ziekten, zoals de ziekte van Alzheimer, Parkinson en Huntington. In Alzheimer is een mutante vorm van ubiquitine (Ub) aangetoond, UBB^{+1}. Dit UBB^{+1} accumuleert in de pathologische kenmerken van de ziekte. Normaal is Ub verantwoordelijk voor het markeren van eiwitten die afgebroken dienen te worden, door een isopeptide binding aan te gaan met het substraat. Vervolgens ontstaat er een boom van Ub eiwitten, door een opeenvolging van isopeptide bindingen tussen Ub moleculen. Deze Ub boom vormt een uniek herkenningssignaal voor het proteasoom.

Hoofdstuk 1 is de algemene inleiding van dit proefschrift en geeft een gedetailleerd beeld van de betrokkenheid van het UPS in de ziekte van Alzheimer.

In hoofdstuk 2 worden de eigenschappen van UBB^{+1} onderzocht in een celvrij systeem en in humane neuroblastoma cellijnen. UBB^{+1} ontstaat door een dinucleotide deletie in het UBB mRNA, waardoor er een leesraamverschuiving optreedt. Deze leesraamverschuiving leidt tot een afwijkende C-terminus vanaf het laatste aminozuur van Ub tot en met een extra 20 aminozuren. In het UBB^{+1} eiwit ontbreekt dientengevolge het eindstandige glycine residue waarmee Ub aan substraten bindt. Hierdoor is UBB^{+1} niet in staat substraten te ubiquitineren. Het is echter wel zelf nog onderhevig aan modificatie door Ub moleculen, aangezien de lysine residuen die daarvoor nodig zijn, zich in het ongewijzigde deel van het eiwit bevinden. Hoge expressie van UBB^{+1} leidde uiteindelijk tot apoptotische celdood.

In hoofdstuk 3 wordt beschreven dat UBB^{+1} een specifiek remmend effect heeft op het UPS en dus mogelijk bij kan dragen aan de

proteasoom remming die gevonden wordt in het brein van Alzheimer patiënten. Bij dit onderzoek werd gebruik gemaakt van een uniek op GFP ('green fluorescent protein') gebaseerd proteasoom reporter systeem. Dit systeem bestaat uit fusie-eiwitten van Ub en GFP, met verschillende linkers tussen de twee eiwitten. Het controle fusie-eiwit heeft een stabiel methionine residue tussenin, waardoor een stabiele vorm van GFP onstaat na klieving van het fusie-eiwit door isopeptidases. Het proteasoom reporter fusie-eiwit heeft geen stabiel aminozuur tussenin, maar een gemuteerd aminozuur aan het einde van het Ub gedeelte van het eiwit. Hierdoor kan het fusie-eiwit niet gekliefd worden door isopeptidases en wordt het in zijn geheel afgebroken door het proteasoom. Cellen die dit construct tot expressie brengen, zullen dus alleen GFP ophopen als het proteasoom op een of andere manier geremd is. In dit systeem bleek UBB^{+1} specifiek tot ophoping van de GFP reporter te leiden, en dus remming van het proteasoom te veroorzaken. Proteasoom remming en celdood bleken beide afhankelijk van de ubiquitinering op twee lysine residuen van UBB^{+1}, aangezien deze effecten uibleven bij expressie van een dubbele lysine mutant van UBB^{+1}. In schijnbare tegenstelling, is het ook juist deze eigenschap die UBB^{+1} in lage concentraties tot een efficiënt substraat maakt voor het proteasoom. Aan de ene kant is UBB^{+1} dus een substraat voor het proteasoom, maar aan de andere kant kan het ditzelfde systeem ook specifiek en doeltreffend remmen.

In hoofdstuk 4 wordt een mogelijke verklaring voor dit paradoxale effect bediscussieerd, namelijk een drempelwaarde die UBB^{+1} moet bereiken om actief het proteasoom te kunnen remmen. Deze drempelwaarde werd in organotypische cortex kweken van muizen onderzocht en in neuronale cellijnen gekwantificeerd. Onder de drempel kan het proteasoom het UBB^{+1} verwerken, maar als er eenmaal (door andere oorzaken) een verminderde activiteit van het proteasoom ontstaat, accumuleert UBB^{+1} en kan het zelf bijdragen aan verdere remming van het proteasoom. Voor de ziekte van Alzheimer kan dus gesteld worden dat deze drempelwaarde overschreden is, aangezien het eiwit duidelijk ophoopt in het brein. Aangezien het mutante mRNA ook in controle individuen aanwezig is en UBB^{+1} in lage hoeveelheden afgebroken kan worden, is het echter niet waarschijnlijk dat UBB^{+1} de initiele oorzaak van de proteasoom inhibitie in Alzheimer is. Het is waarschijnlijker dat het begin van proteasoom remming te wijten is aan andere Alzheimer-gerelateerde mechanismen.

Hoofdstuk 5 behandelt 1 van deze mechanismen die al eerder in verband was gebracht met proteasoom remming, namelijk intracellulaire amyloid-β peptide (Aβ) vorming. Door expressie van Ub-Aβ fusie-eiwitten in cellijnen werd een puur cytosolaire vorm van Aβ geïnduceerd. Cytosolair Aβ bleek echter niet in staat het proteasoom te remmen in het GFP proteasoom reporter systeem.

Tenslotte wordt in hoofdstuk 6 een algemene discussie van de resultaten in dit proefschrift gepresenteerd en worden mogelijkheden voor vervolgonderzoek besproken. Daarnaast worden in dit hoofdstuk preliminaire data bediscussieerd van experimenten die tau en oxidatieve stress als proteasoom-remmende factor in de ziekte van Alzheimer onderzoeken. Deze data geven tot dusver geen aanleiding om oplosbaar tau deze rol toe te bedelen. Oxidatieve stress blijkt wel in bepaalde nader te bestuderen omstandigheden samen met UBB^{+1} een synergistisch remmend effect op het proteasoom te hebben.

In het Alzheimer brein kan initiële remming van het proteasoom dus leiden tot ophoping van kritische hoeveelheden UBB^{+1} die vervolgens in belangrijke mate bij kunnen dragen aan verdere remming van het proteasoom en uiteindelijk aan neurodegeneratie. UBB^{+1} kan bovendien gezien worden als een belangrijke endogene graadmeter voor proteasoom activiteit. Cellen die UBB^{+1} ophopen, moeten immers een minder goed functionerend UPS hebben. In dit aspect is het bijzonder interessant dat UBB^{+1} alleen in tauopathieën lijkt op te hopen en niet in synucleinopathieën. Verder onderzoek naar het ophopingsmechanisme en de remming van het proteasoom door UBB^{+1}, al dan niet synergistisch met andere processen, zou meer inzicht geven in het onderscheiden van de verschillende moleculaire achtergronden en neuropathogenese van deze ziekten.

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Color figures

Chapter 3

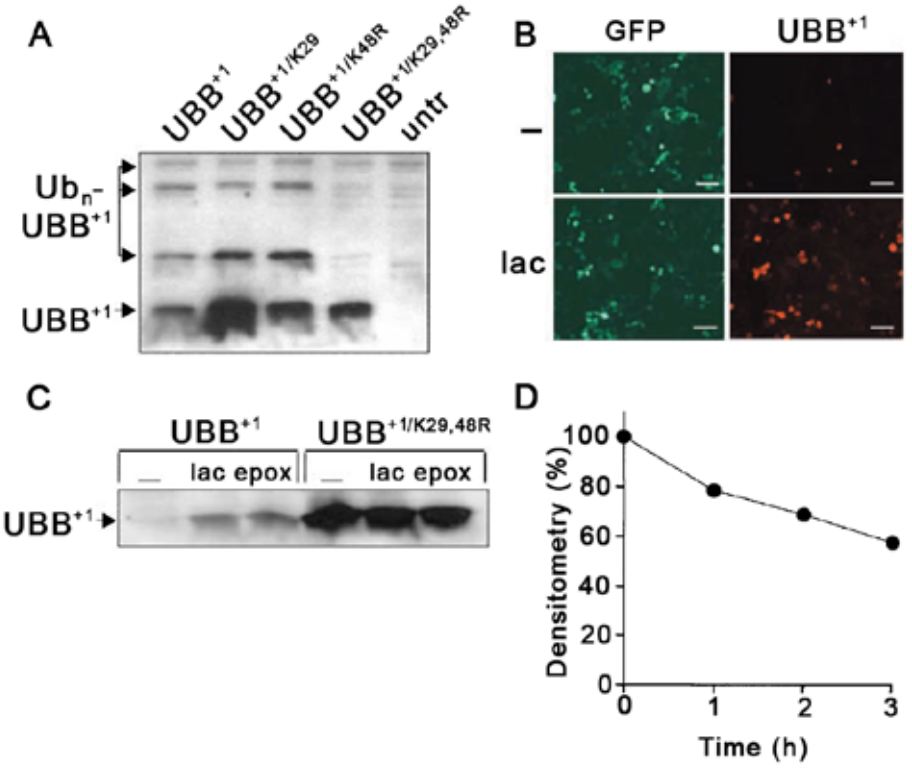

Fig. 3 UBB^{+1} is a UFD substrate. A Western blot analysis with an anti-UBB^{+1} antibody of cell lysates from HeLa cells transfected with UBB^{+1}, UBB$^{+1/K29R}$, UBB$^{+1/K48R}$, UBB$^{+1/K29,48R}$. Products corresponding to unmodified and ubiquitinated UBB^{+1} are indicated. **B** Micrographs of HeLa cells transfected with pCMS-UBB^{+1} /GFP that were left untreated (top) or incubated for 16 h with 30 μM lactacystin (bottom). Transfected cells were identified by GFP expression (left) and transfected cells expressing detectable levels of UBB^{+1} were visualized by immunostaining (right). Bars, 100 μm. **C** Western blot analysis with an anti-UBB^{+1} antibody of the steady-state levels of UBB^{+1} and UBB$^{+1/K29,48R}$ in transiently transfected HeLa cells that were left untreated or incubated the proteasome inhibitors lactacystin (30 μM) or epoxomicin (500 nM). **D** The turnover of UBB^{+1} was determined by pulse-chase analysis in SK-N-SH neuroblastoma cells transduced with lenti-UBB^{+1}. Intensity of the UBB^{+1} band was quantified with a phosphoimager and the intensity at time point 0 was standardized as 100%. (A–D) One representative experiment out of three.

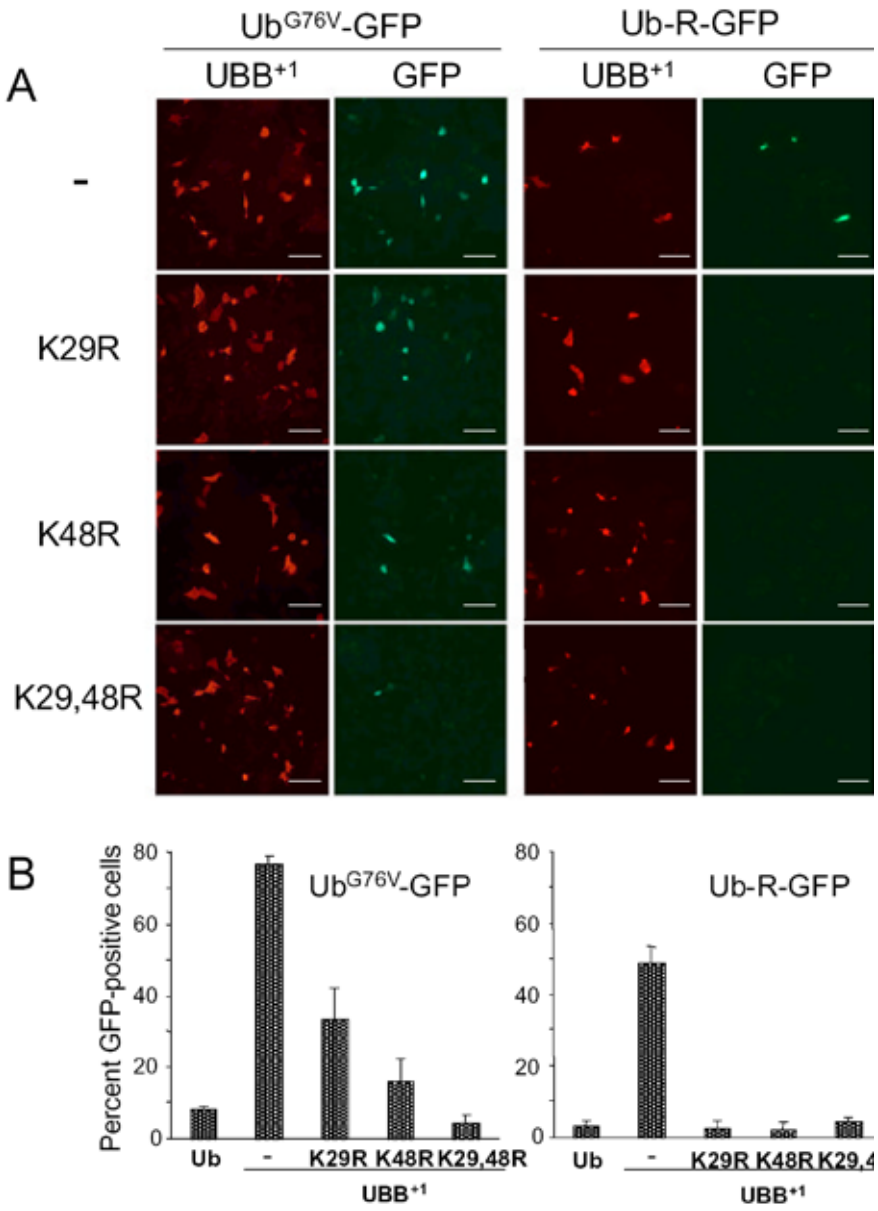

Fig. 4 Inhibitory activity of UBB^{+1} requires ubiquitination at Lys29 and Lys48. (A) Micrographs of Ub^{G76V}-GFP (left) and Ub-R-GFP SH-SY5Y cells (right) transfected with UBB^{+1}, UBB$^{+1/K29R}$, UBB$^{+1/K48R}$, or UBB$^{+1/K29,48R}$. The cells were stained for UBB^{+1} (left) and analyzed for GFP fluorescence (right). Bars, 100 µm. (B) Quantification of three independent experiments as shown in A. The results are expressed as the percent of the UBB^{+1} expressing cells with accumulated Ub^{G76V}-GFP or Ub-R-GFP levels.

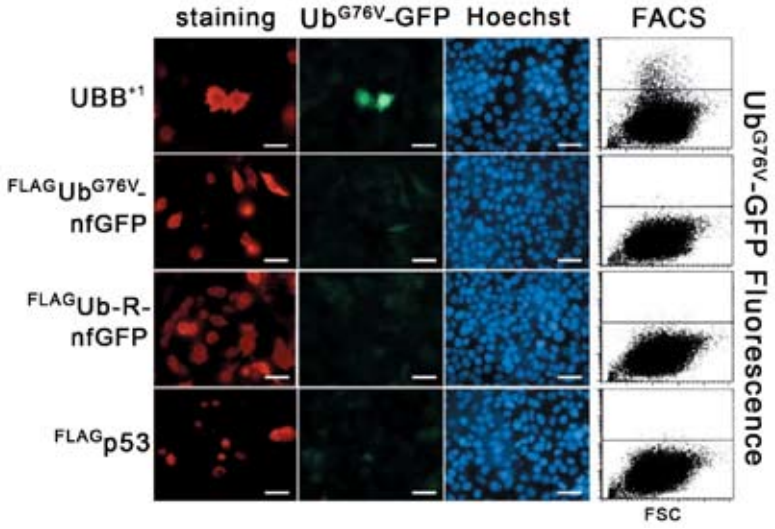

Fig. 7 Overexpression of other proteasome substrates does not inhibit turnover of Ub^{G76V}-GFP. Ub^{G76V}-GFP HeLa cells were transiently transfected with UBB^{+1}, $^{FLAG}Ub^{G76V}$-nfGFP, ^{FLAG}Ub-R-nfGFP and ^{FLAG}p53. UBB^{+1} transfected cells were stained with the anti-UBB^{+1} antibody while the nonfluorescent $^{FLAG}Ub^{G76V}$-nfGFP and ^{FLAG}Ub-R-nfGFP constructs and ^{FLAG}p53 were stained with a FLAG-specific antibody. Representative micrographs of the immunostaining (left, red), the Ub^{G76V}-GFP fluorescence (middle, green), and the Hoechst 33258 counterstaining (right, blue) are shown. Note that as expected the $^{FLAG}Ub^{G76V}$nfGFP and ^{FLAG}Ub-R-nfGFP give a homogenous staining in the cytosol and nucleus, whereas ^{FLAG}p53 is localized in the nucleus. To the left are shown flow cytometric analysis of the GFP fluorescence upon transfection with the different constructs.

Chapter 4

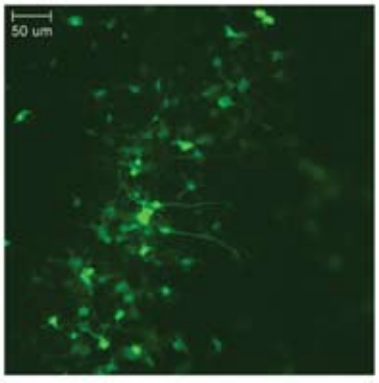

Fig.1 Successful lentiviral transduction of organotypic mouse cortex slice cultures. Wild type organotypic mouse cortex slice cultures were transduced with LV encoding the stable control GFP fusion protein Ub-M-GFP, GFP stained with αGFP/Cy2.

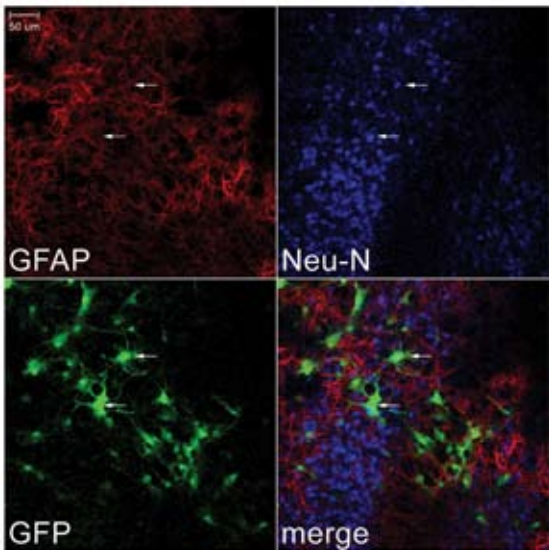

Fig.2 Lentivirus targets a heterogeneous cell population in cortex slice cultures. NeuN (blue) and GFAP (red) double staining on LV-Ub-M-GFP transduced cultures revealed mostly GFAP labeled GFP positive cells, but also NeuN positive neurons. Arrows indicate transduced NeuN positive neurons.

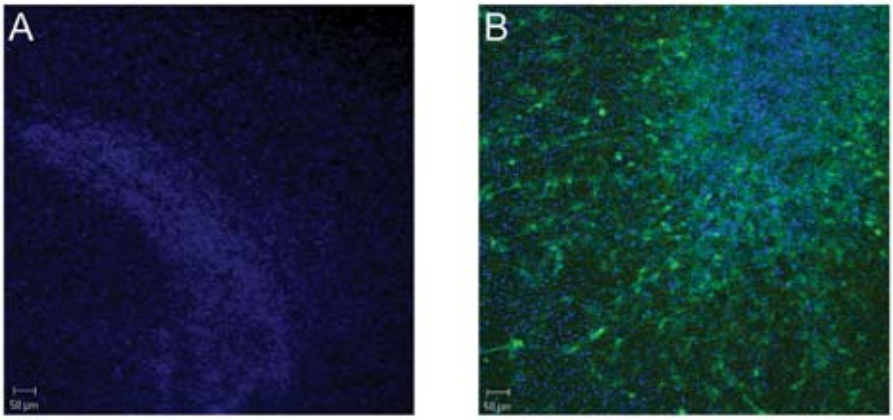

Fig.3 The proteasome reporter system in cortex cultures of Ub^{G76V}-GFP transgenic mice. A Ub^{G76V}-GFP transgenic cortex cultures without treatment. **B** Ub^{G76V}-GFP transgenic cortex cultures treated with 1 µM epoxomicin. The reporter substrate only accumulates after proteasome inhibition.

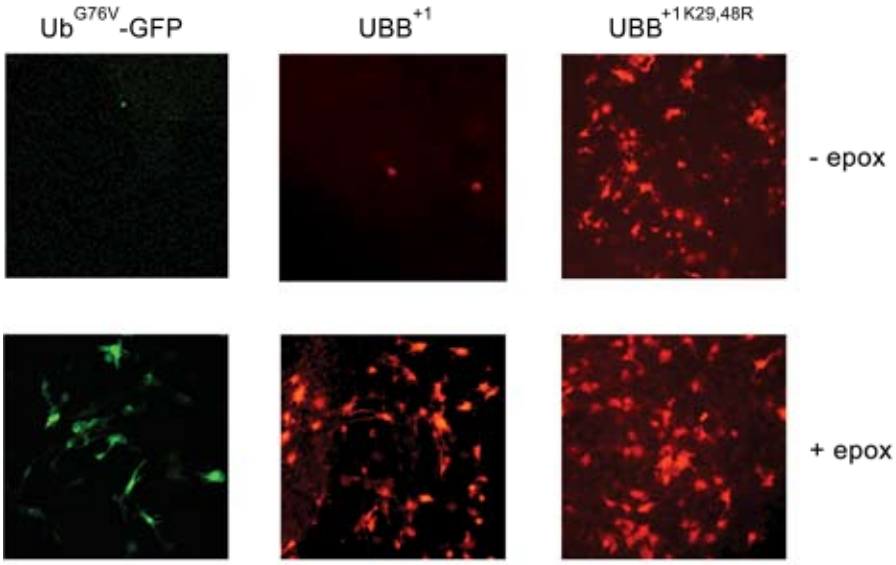

Fig.4 UBB^{+1} is degraded by the proteasome in cortex cultures. Both the reporter protein Ub^{G76V}-GFP (green) and UBB^{+1}(red) accumulate after treatment with 1 μM epoxomicin overnight. The lysine mutant of UBB^{+1} also accumulates without inhibitor treatment.

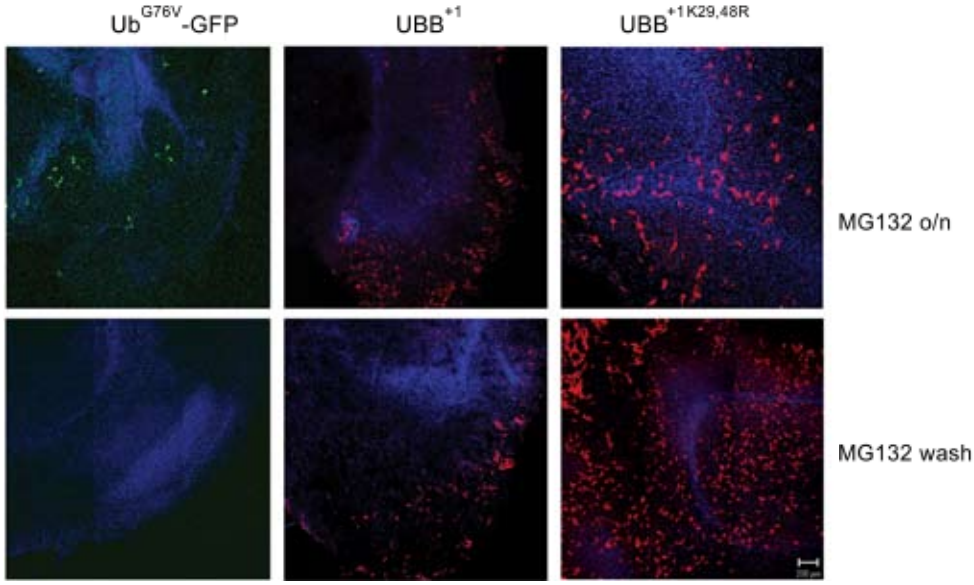

Fig.5 The threshold effect of UBB^{+1} accumulation in cortex cultures. 16 hour incubation of transduced cultures with the reversible inhibitor MG132 results in accumulation of both UBB^{+1} and Ub^{G76V}-GFP. Washing out the reversible inhibitor reactivates the proteasome, as shown by the degradation of Ub^{G76V}-GFP. UBB^{+1} however, remains accumulated in a considerable amount of cells.

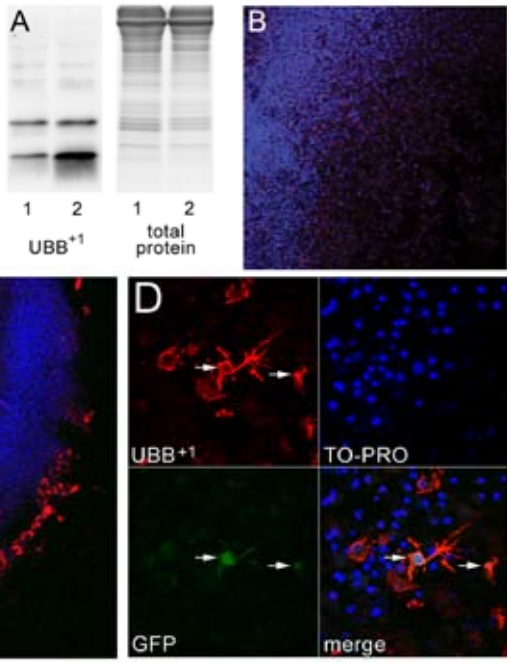

Fig.6 High Ad-UBB^{+1} expression surpasses the threshold level and causes accumulation of Ub^{G76V}-GFP. Increased expression of UBB^{+1} with adenovirus compared to lentivirus, leads to accumulation of UBB^{+1} without inhibitor treatment. **A** Representative western blot of lysates of 293 cells transduced with equal MOI of LV-UBB^{+1} (lane 1) or Ad-UBB^{+1} (lane 2). Equal amounts of protein were loaded per lane, as confirmed by Coomassie staining of total protein load of the same lanes shown on the right. The blot was stained with Ubi3 and quantified with Imagepro software. Organotypic cortex slice cultures of Ub^{G76V}-GFP transgenic mice were transduced with **B** LV-UBB^{+1}, or **C** Ad-UBB^{+1}. LV-UBB^{+1} transduction did not induce accumulation of UBB^{+1}, while Ad-UBB^{+1} did result in many UBB^{+1} immunopositive cells. **D** UBB^{+1} accumulation after adenoviral transduction leads to accumulation of Ub^{G76V}-GFP (arrows).

Chapter 5

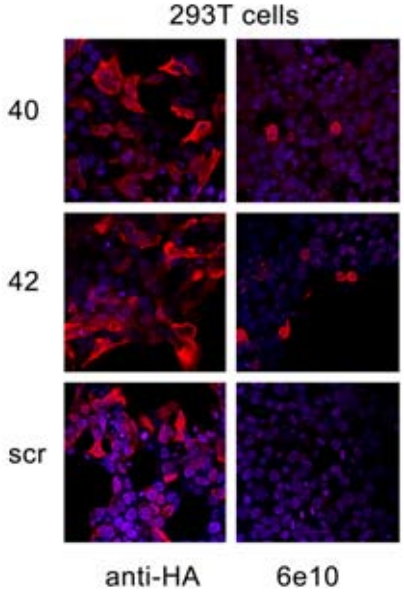

Fig.4 Expression of HA-Ub-Aß in cell lines. 293T were transduced with lentiviral vectors encoding HA-Ub-Aß1-40/42 or scr and stained for HA-tag (anti-HA) or Aß (6E10) two days later. The amount of HA-tag positive cells indicates high transduction efficiency. In contrast, only very few cells are positive for Aß1-40 of Aß1-42, suggesting that Aß is degraded after cleavage of the fusion proteins. Aßscr is not recognized by 6E10, corresponding to the lack of 6E10 immunopositive cells in the HA-Ub-Aßscr transduced cells.

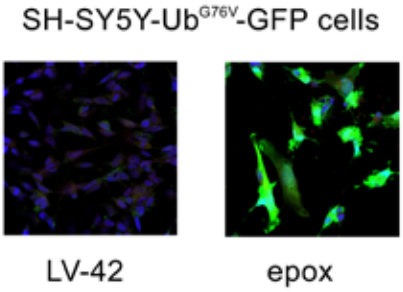

Fig.7 Intracellular Aß does not inhibit the proteasome. Lentiviral transduction with LV-HA-Ub-Aß1-42 of proteasome reporter cells expressing Ub^{G76V}-GFP, did not lead to accumulation of the GFP reporter after 1-4 days after transduction. As a control, 5 h epoxomicin treatment of these cells resulted in strong GFP reporter accumulation. LV-HA-Ub-Aß1-40, LV-HA-Ub-Aßscr and pcDNA3-SP-Aß42 expression also did not result in accumulation of the reporter (not shown).

Chapter 6

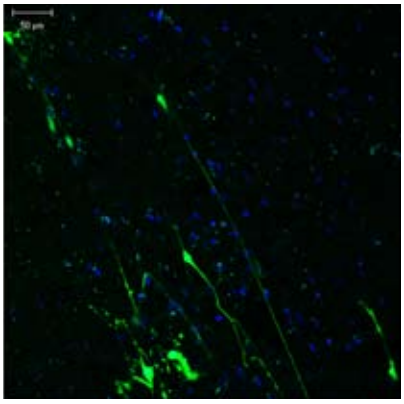

Fig.2 Successful transduction of human post mortem cortex culture with LV-GFP. Within a maximum of 8 h postmortem delay, 250 µm slices of frontotemporal cortex were made with a tissue chopper and put into culture free-floating in medium, which consisted of Neurobasal A medium (Gibco), complemented with 0,1 mg/ml vitamin C, B27 supplement (Gibco), 10 U/ml penicillin and 10 µg /ml streptomycin (Gibco). Lentiviral vectors were applied by dropping a 20 µl drop of medium containing 10^6 transducing viral particles on top of a dry slice, which was replenished with medium after five minutes. At several different times after transduction, slices were fixed for 1 h in 4% formalin, after which they were stained free-floating as described previously for cortex slices of mice (Chapter 4).

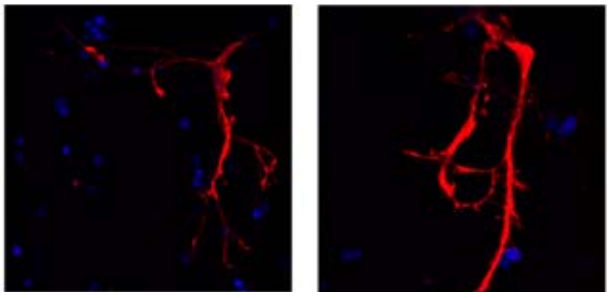

Fig.3. Transduction of human post mortem cortex cultures with LV-UBB^{+1} results in accumulation of UBB^{+1} in large cells. Ubi3 staining for UBB^{+1} (red), TO-PRO staining for nuclei (blue).

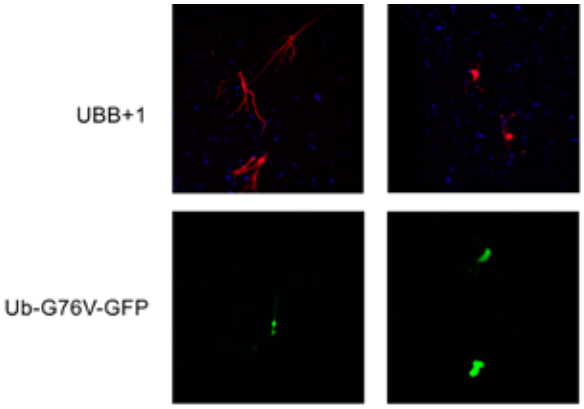

Fig.4 Proteasome activity is functional in one pilot experiment of human post mortem cortex cultures. UBB^{+1} accumulation seems to be highly toxic in these cells. Human post mortem cortex cultures were transduced with LV-UBB^{+1} or LV-Ub^{G76V}-GFP and subsequently treated with 1 µM epoxomicin. Ub^{G76V}-GFP (green) accumulated in 17 cells total in 2 slices, as opposed to 3 cells in the control situation. The amount of UBB^{+1} accumulating cells (red) decreased after epoxomicin treatment an they had a less healthy appearance than in the control situation.

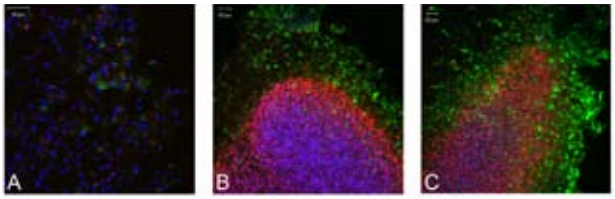

Fig.5 No concentration-dependent difference between cell types in vulnerability to proteasome inhibition. Transgenic Ub^{G76V}-GFP mouse cortex cultures were treated with epoxomicin in concentrations of **A** 100 nM, **B** 500 nM, **C** 1 µM. Cultures were stained for the neuronal marker NeuN (red), TO-PRO (blue) and GFP (green).

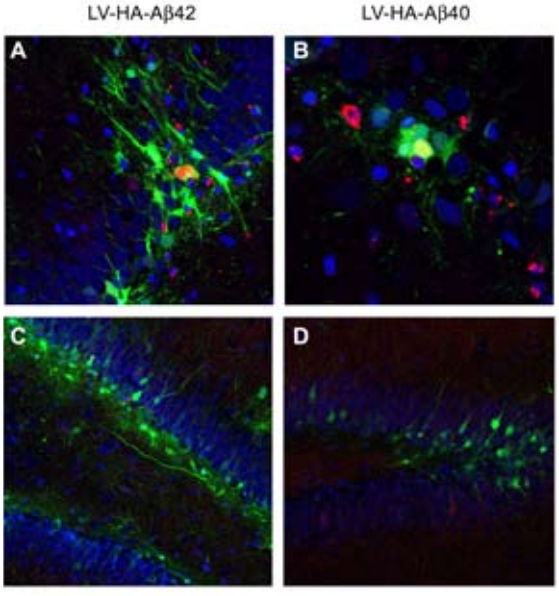

Fig.6 Injection of Aß fusion protein lentiviral vectors in rat hippocampus results in very few positive cells that have altered morphology. Adult Wistar rats were injected with $8x10^5$ TU LV-HA-Ub-Aß1-40 or LV-HA-Ub-Aß1-42 in 0,9 µl saline solution (0,2 µl/min) and coinjected with 0,1 µl with $8x10^5$ TU LV-GFP. After perfusion, 50 µm thick coronal vibratome sections were stained for anti-HA (red), GFP (green) and TO-PRO (blue).

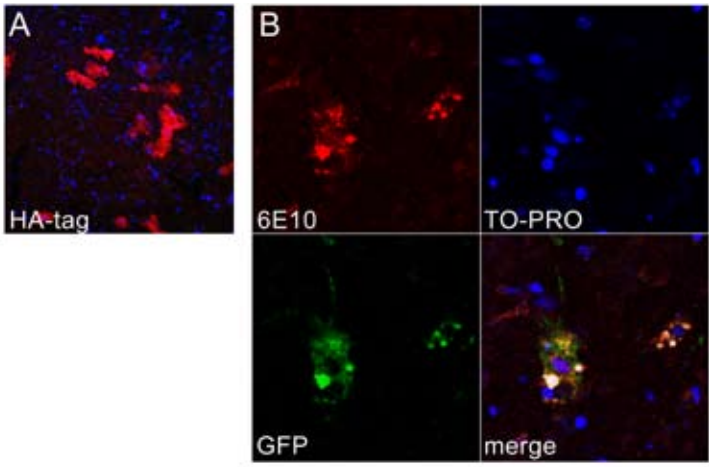

Fig.7 Transduction of organotypical mouse cortex slice cultures with lentiviral vectors encoding Aß fusion proteins. A anti-HA staining of LV-HA-Ub-Aß1-42 transduced cortex slice. **B** 6E10 positive cell in LV-HA-Ub-Aß1-42 transduced slice. Many HA-tag positive cells were observed, in contrast to LV-injections in rat hippocampus. However, only very few Aß positive (6E10) cells were observed, of which some seemed to form aggregates.

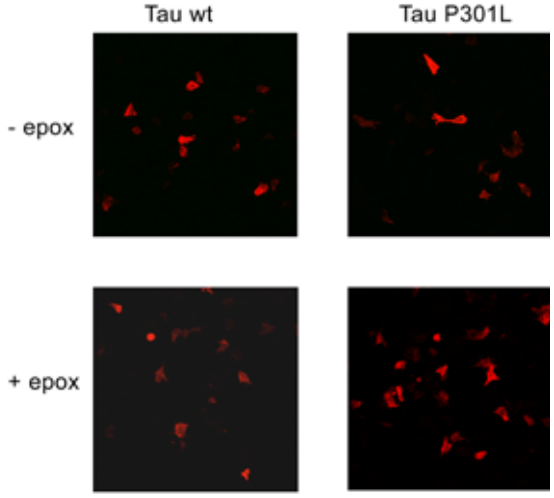

Fig.8 No differences in transduction efficiency or morphology between LVτ and LVτP301L cells. SH-SY5Y cells were transduced with LVτ or LVτP301L and subsequently stained with tau5a6 antibody (Developmental studies Hybridoma Bank, red). Treatment of transduced cells with 100 nM epoxomicin overnight, resulted in a slight increase in immunopositive cells.

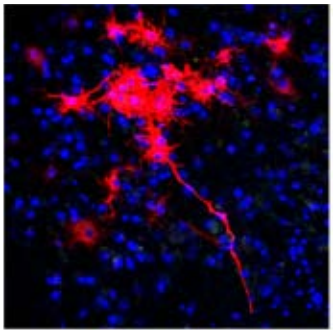

Fig.9 LVτP301L does not lead to proteasome inhibition in Ub^{G76V}-GFP transgenic mouse organotypic cortex slices. Cortex cultures were transduced with LVτP301L and subsequently stained with tau5a6 (red), GFP (green) antibodies and TO-PRO (blue). τP301L expression did not induce accumulation of the GFP reporter and therefore does not seem to inhibit the proteasome.

Abbreviations

Ac-DEVD-CHO	N-Acetyl-Asp-Glu-Val-Asp-al
Aß	amyloid ß
Aßscr	scrambled Aß
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ApoE	apolipoprotein E
APP	amyloid precursor protein
APP	amyloid precursor protein +1
CHIP	carboxyl terminal of Hsc70 interacting protein
CMV	cytomegalovirus
DUB	deubiquitinating enzyme
ER	endoplasmic reticulum
ERAD	ER associated degradation
FCS	fetal calf serum
FTDP-17	frontotemporal dementia and Parkinsonism linked to chromosome 17
GBSS	Gey's balanced salt solution
GFAP	glial fibrillary acidic protein +1
GFP	green fluorescent protein
Gly	glycine
HBSS	Hank's balanced salt solution
IDE	insulin-degrading enzyme
IFNγ	interferon γ
4-HNE	4-hydroxy-2-nonenal
HD	Huntington's disease
LV	lentivirus
Lys	lysine
MEM	minimal essential medium
MHC	major histocompatibility complex
MOI	multiplicity of infection

Nedd8	neural precursor cell-expressed developmentally down-regulated
nfGFP	nonfluorescent GFP
NFT	neurofibrillary tangles
NOS	nitric oxide synthase
PBS	phosphate buffered saline
PGPH	peptidylglutamyl-peptide hydrolyzing
PHF	paired helical filament
PD	Parkinson's disease
PSEN	presenilin
RCA	replication of competent adenovirus
ROS	reactive oxigen species
SCF	Skp1, cullin, F box proteins
SFV	Semliki forest virus
SOD	superoxide dismutase
SP-Aß	signal peptide containing Aß
SUMO	small ubiquitin-like modifier
tBHP	tert-butyl-hydroperoxide
Ub	ubiquitin
UBB^{+1}	ubiquitin-B +1
UBP	ubiquitin specific protease
UCH	ubiquitin c-terminal hydrolase
UFD	ubiquitin fusion degradation
UPR	unfolded protein response
UPS	ubiquitin proteasome system
wtUb	wildtype ubiquitin
Z-L3-VS	carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone
Z-VAD-FMK	Z-Val-Ala-Asp fluoromethyl ketone

Curriculum vitae

Femke Maaike Sophia de Vrij werd geboren op 27 februari 1977 te Rotterdam. Na de gymnasium-β opleiding op het Rotterdamsch Lyceum te Rotterdam, begon zij in 1995 haar studie Medische Biologie aan de universiteit van Utrecht. Bij de afdeling Experimentele Neurologie van het AZU te Utrecht werd een stage gevolgd over de betrokkenheid van corticale motorneuronen bij amytrophe lateraal sclerose onder begeleiding van prof. dr. P.R. Bär en dr. M.G.H. van Westerlaken. Daarna werd een stage gevolgd op het Nederlands Instituut voor Hersenonderzoek te Amsterdam over het ubiquitine proteasome systeem onder begeleiding van dr. E.M. Hol. In december 1999 werd het doctoraal diploma Medische Biologie behaald. In februari 2000 kwam zij in dienst bij het Nederlands Instituut voor Hersenonderzoek als onderzoeker in opleiding, onder begeleiding van prof. dr. D.F. Swaab, prof. dr. J. Verhaagen, dr. F. W. van Leeuwen en dr. E.M. Hol. De resultaten van het onderzoek naar de betrokkenheid van mutant ubiquitine en het proteasoom bij de ziekte van Alzheimer zijn beschreven in dit proefschrift. Sinds augustus 2004 is Femke werkzaam als wetenschappelijk onderzoeker bij de afdeling Klinische Genetica op het ErasmusMC te Rotterdam.

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EN NU IS HET AF, pipo ga je mee dansen?