Mutant ubiquitin and the proteasome in Alzheimer's disease

de Vrij, F.M.S.

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CHAPTER I

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

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

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

1 Introduction 12

2 Alzheimer’s disease & protein quality control 13

3 The ubiquitin proteasome system 16
   3.1 Ubiquitin 16
   3.2 Ub-like proteins 18
   3.3 Monoubiquitination 21
   3.4 Ubiquitination machinery 21
   3.5 Deubiquitinating enzymes 24
   3.6 Proteasomes 25
   3.7 Ub-independent degradation 28

4 AD pathogenesis and the UPS 30
   4.1 Tau 31
   4.2 Amyloid β 33
   4.3 Mutant Ubiquitin 36
   4.4 Oxidative stress 37
   4.5 ApoE 38
   4.6 UPS and aging 39
   4.7 UPS, learning and memory 40

5 Concluding remarks 42

Acknowledgements 44
Alzheimer's disease (AD) is the most common cause of dementia, and affects more than twenty million people worldwide. About 25 to 30 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.
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 BA-4, B MC1 tau, C Ub (DAKO), D UBB⁺¹ (ub2).
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
<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Implications</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>Ubiquitinating enzyme</td>
<td>Decreased levels and activity in AD</td>
<td>(Lopez Salo et al., 2000)</td>
</tr>
<tr>
<td>E2-25K</td>
<td>Ub conjugating enzyme</td>
<td>Mediates AB toxicity in vitro; Ubiquititates UBB+1 and huntingtin</td>
<td>(Song et al., 2003)</td>
</tr>
<tr>
<td>E2 enzymes</td>
<td>Ub conjugating enzyme</td>
<td>Downregulated in AD or aging</td>
<td>(Loring et al., 2001; Lu et al., 2004)</td>
</tr>
<tr>
<td>CHIP</td>
<td>Ub ligase</td>
<td>Serves as E3 enzyme for phosphorylated tau</td>
<td>(Petrucelli et al., 2004; Shimura et al., 2004)</td>
</tr>
<tr>
<td>UCH-L1</td>
<td>Deubiquitinating enzyme and dimerised UB ligase activity</td>
<td>Downregulated in AD brain; Accumulates in subpopulation of tangles; Oxidatively modified in AD brain</td>
<td>(Pasinetti, 2001); (Lowe et al., 1990); (Castegnna et al., 2002)</td>
</tr>
<tr>
<td>UBB+1</td>
<td>Unknown, translated from mutant mRNA</td>
<td>Accumulates in AD (also in other tauopathies and HD). Both substrate and inhibitor of the proteasome</td>
<td>(Lindsten et al., 2002; Van Leeuwen et al., 1998b)</td>
</tr>
<tr>
<td>20S β subunits</td>
<td>Protein degradation</td>
<td>All three proteolytic activities are decreased in AD affected brain areas</td>
<td>(Keller et al., 2000a)</td>
</tr>
<tr>
<td>20S α5 subunit</td>
<td>Confining proteolytic chamber</td>
<td>Downregulated in AD brain</td>
<td>(Loring et al., 2001)</td>
</tr>
<tr>
<td>S6b</td>
<td>19S ATPase</td>
<td>Immunoactivity in neurofibrillary tangles</td>
<td>(Fergusson et al., 1996)</td>
</tr>
<tr>
<td>S1</td>
<td>19S non-ATPase</td>
<td>Downregulated in AD brain</td>
<td>(Loring et al., 2001)</td>
</tr>
<tr>
<td>Amyloid β</td>
<td>Unknown, product of APP processing</td>
<td>ABI-42 accumulates in plaques, but is also found intraneuronally. Inhibits proteasomal activity in vitro</td>
<td>(Gregori et al., 1995)</td>
</tr>
<tr>
<td>APP</td>
<td>Unknown, membrane-spanning glycoprotein</td>
<td>C-terminus of APP is degraded by proteasome</td>
<td>(Nunan et al., 2003)</td>
</tr>
<tr>
<td>Presenillin</td>
<td>Essential component of γ-secretase complex</td>
<td>Degraded by proteasome</td>
<td>(Fraser et al., 1998; Kim et al., 1997)</td>
</tr>
<tr>
<td>Pen-2</td>
<td>Essential component of γ-secretase complex</td>
<td>Degraded by proteasome through ERAD, only in absence of functional PS</td>
<td>(Bergman et al., 2004; Crystall et al., 2004)</td>
</tr>
<tr>
<td>Tau</td>
<td>Microtubule associated protein</td>
<td>Accumulates in neurofibrillary tangles as PHF tau, is mono-ubiquitinated. PHF tau inhibits the proteasome in vitro. Normal tau is a proteasome substrate</td>
<td>(Devid et al., 2002; Keck et al., 2003)</td>
</tr>
<tr>
<td>ApoE e4</td>
<td>Lipid transport and cholesterol homeostasis</td>
<td>Gene dosage dependent risk factor for AD. Associated with decreased AB clearance and increased oxidative stress in AD. In macrophages and hepatocytes, apoE is degraded by the proteasome</td>
<td>(Wenner et al., 2001)</td>
</tr>
<tr>
<td>LRP receptor</td>
<td>Receptor for apoE and mediates AB clearance</td>
<td>Cytosolic fragment processed by proteasome</td>
<td>(May et al., 2002)</td>
</tr>
</tbody>
</table>

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 ubiquituous 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.

![Ubiquitin structure](image)

**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 Ub 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.
**Polyubiquitination**

E1 enzyme activates free ubiquitin

Activated Ub is transferred to an E2 conjugating enzyme

E3 ligating enzyme (in this case a RING E3) allows transfer of E2-conjugated Ub to the substrate

ER specific E3’s mediate ubiquitination of misfolded proteins in the ER, that are translocated to the cytosol for ER-associated degradation (ERAD) by the proteasome

Polyubiquitination and/or deubiquitination by deubiquitinating enzymes

Ub-box binds 19S cap of 26S proteasome followed by protein unfolding and translocation to the 20S core

Protein degradation and recycling of Ub

**Antigen presentation**

Normal cytoplasmic or viral proteins can be processed by the immunoproteasome, which generates peptides for MHC1 mediated antigen presentation. The immunoproteasome is induced by IFNγ, causing a subunit switch to α-subunits 1, 2, and 5. The immunoproteasome can be flanked by 1 or 2 11S regulatory particles, which are also induced by IFNγ.

Ub independent degradation

Many oxidized and unfolded proteins are degraded by the 20S proteasome in an Ub independent manner

**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) (Koepl 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 (Iizatakeyama 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), synphilin-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 UBP s (ubiquitin specific proteases). UCHs are small proteins that remove short peptides from the C-terminus of Ub, while UBP s are responsible for cleaving the isopeptide bond linking Ub-Ub or Ub-protein. UBP s 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 (Saighoh 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 (Castegnna 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⁺⁺⁺ processing, would possibly lead to more UBB⁺⁺⁺ 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 archaeabacterial 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 (βi5) (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 histocompatibility 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 MIIIC 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).
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 punctures 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 (PIIF), 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 (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. It 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 Oxygen 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, UCI1-L1 was found to be oxidatively modified in a proteomics study of AD brain (Castegnà 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 PIIF 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 \textit{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\textsuperscript{G74}-GFP mice, that allow monitoring proteasome activity \textit{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 \textit{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$^{\text{GFP}}$-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⁺ may well 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.

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