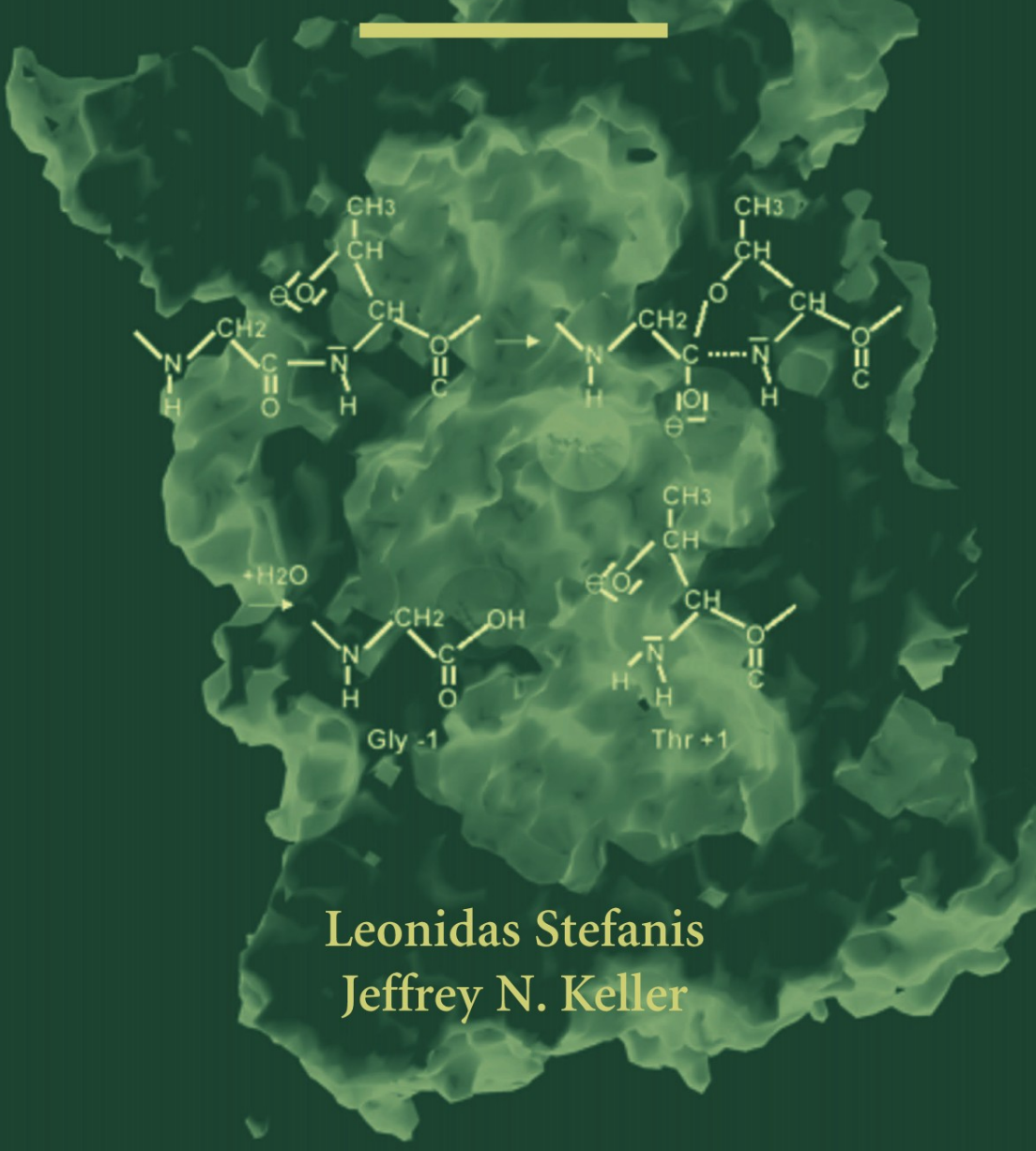
The Proteasome in Neurodegeneration



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PREFACE

In the last 50 years a wealth of information has allowed us to understand the contribution of various regulatory factors that alter mRNA and protein synthesis to a variety of physiological and pathological conditions. However, such regulation is only one of many factors that contribute to the levels of a given protein. One major factor that has been relatively obscure until recently has been the contribution of protein degradation to the regulation of the steady state level of protein expression and protein function. This rapidly evolving field has made a significant mark on the scientific community, as highlighted by the Award of the Nobel Prize in Chemistry for 2004 to Aaron Ciechanover, Avram Hershko and Irwin Rose for their pioneering work on the ubiquitin-proteasome system (UPS) of protein degradation, which is the subject of this volume. In recent years evidence has been accumulating that suggests a role for UPS function in both physiological and pathological settings. In particular, studies have implicated a central role for the UPS in cell cycle regulation, cancer and neurodegeneration. Two points are however worth bearing in mind: First, ubiquitin's function appears to extend far beyond the UPS and protein degradation; second, there are other important systems of intracellular protein degradation, most notably autophagic systems through the lysosomes, and these may also be involved in disease pathophysiology.

In parallel with this general surge of interest in the UPS, two other recent trends have enabled this volume to come into existence. Neurodegenerative diseases, once considered very distinct pathophysiological entities, have come closer together, as common threads between them are increasingly being recognized; and, with advances in genetics and animal modeling, neurobiology of disease is now largely based on facts, and not on hypothetical models that may or may not approximate disease states. This latter trend is highlighted by the inclusion of a new section, that of Neurobiology of Disease, in the Journal of Neuroscience, the Journal of the Society for Neuroscience.

Among the "common threads" holding the different neurodegenerative conditions together is the concept of impaired UPS function. How does the UPS relate to neurodegeneration? As outlined throughout this volume, converging genetic, pathological and biochemical data suggest that impairment of the UPS may underlie a number of neurodegenerative diseases states, or, at the very least, play a contributing role in neuronal dysfunction and death. It is this recurring theme that has galvanized us to create this volume, in which we have sought to present a wide range of information and opinions on the subject, tackling it from different angles. Authors who have been invited to contribute include internationally renown experts in the field. Chapters represent a blend of the authors' own research with thorough reviews of the respective fields. We have elected not to simply present the evidence linking the UPS to specific neurodegenerative disease states. This is indeed done in the last section of the book, where the potential link of the UPS to Parkinson's disease, Alzheimer's disease, Huntington's disease, motor neuron diseases, prion diseases and aging is discussed in a critical fashion. In each one of these chapters, basic aspects of the pathophysiology of each of these conditions are discussed and their relationship to the new concepts related to the UPS is analyzed. In designing this book, we thought that this last and, in a sense, main section of the volume, should be buttressed by a number of other sections that are devoted to the subject of the UPS and neurodegeneration, irrespective of specific disease states, thus providing the wider framework in which to view this relationship. Thus, in total, there are 6 sections in this volume. In the introductory section, the main players in this system are introduced. One of the two chapters here is devoted to the yeast UPS, the study of which has proved invaluable in the deciphering of the structure, components and function of the proteasome. The other chapter is more tailored to the UPS in the context of the mammalian nervous system. We then have a second section devoted to the relationship between protein aggregation, inclusion formation and the UPS. This is a complicated subject, and one that is quite controversial. It is especially important, given the recent evidence that changes in protein conformation may underlie most cases of neuronal degeneration. We have tackled it by providing first a review of the topic that attempts to address the controversies in the field, and then two chapters that elaborate on specific experimental paradigms, which have offered insights into this relationship. The third section deals with the close relationship between the redox system and the UPS. Whereas the first chapter here tackles this relationship, and makes the point that it is reciprocal, and likely to be of great importance in neurodegeneration, the second introduces another player in the mix, inflammation, which may help to mediate interactions between the

two systems. The fourth section delves into the role of UPS in neuronal cell death. This again is a controversial topic, given that inhibition of the UPS has been linked both to neuronal survival and death. The two chapters here deal with these opposing, but not mutually exclusive, views. The fifth section addresses cellular and animal models of UPS dysfunction. Whereas the first chapter here deals with various pharmacological and molecular tools that can be used to model UPS dysfunction, the second is devoted to the *gad* mouse, a unique animal model that links defects in UPS to neurodegeneration.

We hope that this volume represents a comprehensive review of the role of the UPS in neurodegeneration. There are advantages and disadvantages in generating a book on a field that is as new and rapidly evolving as the field of UPS research. An obvious advantage is that the interest factor is high for neuroscientists of various backgrounds, for neurologists with an interest in the pathophysiology of neurodegeneration, and for biologists with an interest in protein degradation. A potential disadvantage is that the data presented here may be outshone by new developments. That is why we have emphasized critical appraisal of the literature, together with cutting-edge new advances. We believe that the concepts outlined here will be of relevance for many years to come, and are intended to generate interest in the nuances of the UPS system, and highlight new avenues for the understanding of the UPS and the generation of UPS-based therapies for the treatment of neurodegenerative diseases.

Jeffrey N. Keller
Leonidas Stefanis

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YEAST PROTEASOME STRUCTURE AND BIOGENESIS

Cordula Enenkel

1. INTRODUCTION

"Aging is an artefact of civilization."
-LEONARD HAYFLICK, *How and Why We Age*

Medicine, especially in the last century, has endeavoured to forestall death to the point where most people in developed societies are living to a greater age than ever before in human history.

In spite of this progress the molecular pathways underlying the aging process and the pathogenesis of neurodegenerative diseases are only gradually being understood. Increasing evidence indicates that accumulation of aberrant or misfolded proteins accompanied by aberrations in the ubiquitin-proteasome system represent unifying events in the pathogenesis of slowly progressive neurodegenerative disorders (1,2).

The regulation of protein turnover by ubiquitylation provides a means to finetune both protein functions and levels and controls basic cellular processes. The target proteins are registered for destruction by ubiquitin conjugation and scavenged by the proteasome, a macromolecular protease vital for all eukaryotic cells.

Proteasomes constitute the basis in the enzyme hierarchy of the ubiquitin-proteasome system. The proteolytically active core particle of the shredding machinery is formed by the 20S proteasome. Association with 19S regulatory 'cap' complexes yields the 26S proteasome. Components of the 19S regulatory complex recognize ubiquitylated protein substrates and inherit substrate de-ubiquitylation. They comprise ATPases which are most likely required for substrate unfolding and translocation into the proteolytic cavity of the 20S proteasome. Arrived in the proteolytic cavity substrates are cleaved into oligopeptides, which are the common end products of proteasomal proteolysis (3).

Besides 19S 'cap'complexes other types of regulatory complexes are found to be associated with 20S proteasomes. In mammalian cells, the 11S activator complex (also called PA28) implicates 20S proteasomes in antigen processing (4). Another recently detected regulator of 20S proteasome activity is PA200, which was reported to be involved in DNA damage repair. Homologous proteins of PA200 apparently exist throughout the kingdom. In yeast, the structural homolog of PA200 is named Blm3 (5).

In spite of the evolutionary distance between yeasts and mammals, the principle cellular structures and pathways such as proteasomes and their biogenesis are conserved from yeast to man. The evolutionary conservation legitimated to study the general pathways in yeast as a model organism of eukaryotic cells (6–9). Even aging and apoptosis are processes which happen in yeast as an unicellular organism (10).

Yeasts teach us, that accurate proteasome assembly and activation is a prerequisite to accomplish the burden of protein aggregation which raises to critical levels in slowly progressive pathological processes induced by environmental, epigenetic and genetic events. However, our knowledge from yeast is not directly applicable to aging processes and pathogenic pathways of inherited and acquired neurodegenerative diseases in human beings.

Here, I attempt to give a compressed overview on proteasome structure and biogenesis in yeast. I indicated where these findings in yeast might be relevant for studies of aging and neurodegeneration of human beings.

2. STRUCTURE AND FUNCTION OF YEAST PROTEASOMES

2.1. The Quaternary Structure of the 20S Proteasome

Eukaryotic proteasomes consist of 14 different subunits. Mammals possess constitutive and immuno 20S proteasomes. Immuno 20S proteasomes differ from constitutive 20S proteasomes by three proteolytically active β subunits, which are expressed upon cytokine induction and replace the constitutively expressed counterparts (4). Yeasts have only constitutive 20S proteasomes.

In 1997, the crystal structure of the yeast 20S proteasome was resolved as the first structure of eukaryotic 20S proteasomes (6) (1FNT, accession number in the protein data base PDB). The quaternary structure of the 20S proteasome, as previously visualized by negative stain electron microscopy, resembles a barrel with dimensions of 15 nm in length and 11 nm in diameter. Four stacked rings form the barrel. Each ring contains seven protein subunits with theoretical masses in the range of 21 to 34 kDa. The subunits fall into two classes, α and β, on the basis of sequence similarity. The α subunits constitute the outer rings and the β subunits the inner rings. Therefore, the particle has a pseudo-seven-fold symmetry axis and consists of two identical moieties (α_{1-7} β_{1-7} β_{1-7} α_{1-7}, C2 space group).

The primary sequences of the α- and β-subunits show low homology. Nevertheless their tertiary structures are similar (Fig. 1A). The α- and β-subunits display a common sandwich fold characterized by two layers of β-sheets, each consisting of five antiparallel β-strands, stacked between two layers of α–helices. Major differences in the tertiary structure of the α- and β-subunits reside in the N-terminal regions. The α subunits possess a helix (H0) at the N-terminus, which seal the central pore of both α rings. Thus, it was believed that unfolded polypeptide chains cannot penetrate closed α rings, consistent with biochemical data suggesting that 20S proteasomes occur as 'latent' enzymes (11).

The crystal structure analysis of the 20S proteasome allowed insight into the catalytic core of the protease. Three inner cavities with a diameter of approximately 5 nm exist. Between the α and β rings two antechambers are situated which are separated by approximately 3-nm-wide β-annuli from the central proteolytic chamber formed by two face to face oriented β rings (6)(Figs. 1, B-C).

2.2. The Proteolytically Active Site Residues and their Liberation by Proprotein Processing

The β1, β2 and β5 subunits harbour N-terminal threonines (Thr) as active site residues, which are exposed to the central chamber (Fig. 1C). These

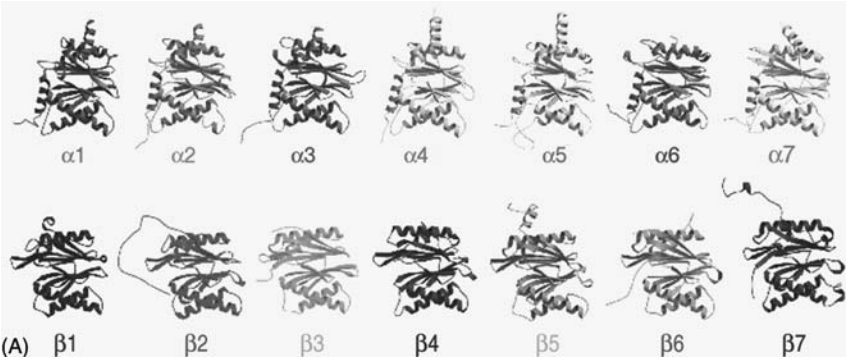

Figure 1. The structure of the yeast 20S proteasome (A) Gallery of ribbon drawings of the seven different α and β subunits. The subunits show the common αββα sandwich fold with two β-sheets (formed by five antiparallel β–strands each) stacked between two layers of α-helices.

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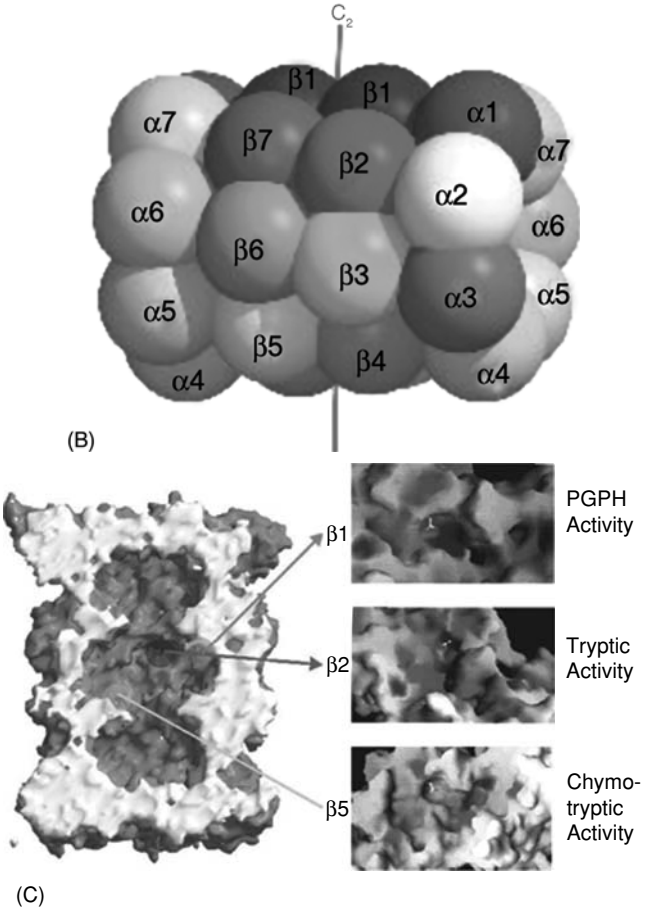

Figure 1. cont'd (B) – (C) Different views of the yeast 20S proteasome showing the subunit arrangement; (B) the calotte model and (C) the surface model. The latter model derives from the 20S proteasome crystallized in the presence of calpain inhibtor I. To gain insight into the proteolytic cavity, the barrel shaped particle was cut along the cylinder axis. The intersections were coloured in white. The three cavities were depicted in blue. The central cavity harbours six active site threonine residues, which are exposed by the β1, β2 and β5 subunits, respectively. The proteolytic active centers are coloured: red, β1; blue, β2; yellow, β5. Cleavage preferences, termed post-glutamyl-splitting, tryptic and chymotrypticlike activity are zoomed and illustrated in surface presentation. The nucleophilc Thr1 is presented by ball and sticks. Basic residues are coloured in blue, acidic residues in red and hydrophobic residues in white. A central pore of the outer rings as entrance for substrate polypeptide chains is gated in the crystal structure conformation due to the tight interactions of the seven α subunit N-terminal regions. The figures were kindly provided by Michael Groll (6,12) and printed with the permission of Nature (*http://www.nature.com*) and ChemBioChem (published by Wiley-VCH). (See color insert.)

β subunits are synthesized as precursor proteins with N-terminal propeptides. During 20S proteasome maturation the active site threonines are liberated by an autocatalytic internal cleavage of the propeptides, which are of different length and unrelated sequence, but with a conserved glycin (Gly) residue pro-

ceeding the active site Thr. Such features classify the proteasome as a member of the N-terminal nucleophile amino-hydrolase family. N-terminal nucleophilic amino-hydrolases are also synthesized as inactive zymogenes and converted into the active form by internal limited proteolysis (7).

In the hydrolysis reaction of peptide bonds the hydroxyl group of the Thr side chain (Thr-Oγ) reacts as nucleophile with the carbonyl group of the substrate's peptide bond, while the amino group Thr-N is the proton acceptor. During autocatalytic propeptide cleavage the Thr-N is still bound to the Gly-Thr peptide bond, thus not accessible as proton acceptor. A water molecule is predicted to mediate the nucleophilic attack of the Thr-Oγ to the carbonyl group of the Gly in the proceeding peptide bond. Highly conserved residues surrounding the active site threonines are further involved in peptide bond hydrolysis. They build up a charge relay system which for example is responsible for the deprotonation of the Thr hydroxyl group resulting in the nucleophilic phenolate ion (13)(Fig. 2).

Not only β1, β2 and β5 subunits are synthesized as precursor proteins, but also β6 and β7. However, β6 and β7 do not produce catalytic sites upon propeptide cleavage during 20S proteasome maturation. The question arises why propeptide processing occurs at non-catalytic β-subunits? Response is retrieved from eukaryotic evolution. Eukaryotic proteasomal subunits arose from gene duplication events. Archaic proteasomes for example from the archaebacterium Thermoplasma acidophilum show a α7β7β7α7 configuration with a single

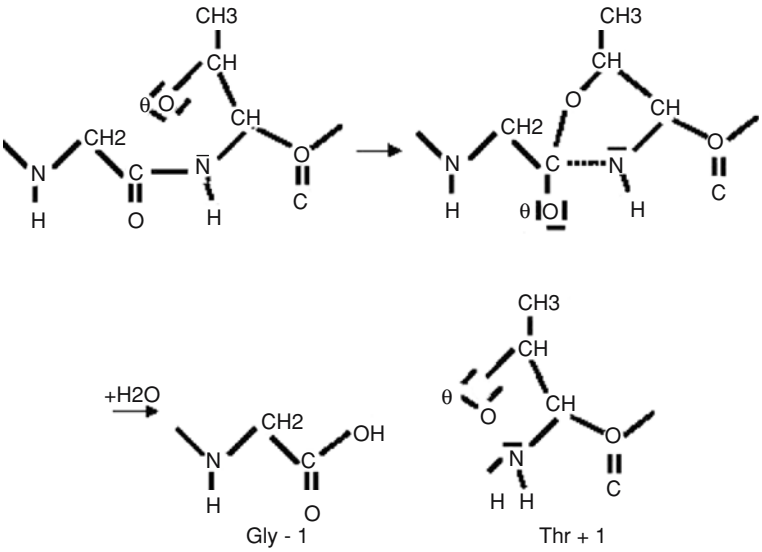

Figure 2. Model for the autocatalytic cleavage of the prosequence of the Gly-1 Thr+1 consensus motif. A charge relay system around the active site Thr is responsible for deprotonation of the Thr hydroxyl group resulting in the nucleophilic phenolate ion (ThrOγ). Addition of ThrOγ to the carbonyl group of Gly-1 is followed by ester bond formation, which is hydrolysed by incorporation of water into the the product. The water molecule mediates the nucleophilic attack of the carbonyl group of the Gly at position -1, while the amino group of Thr at position +1 is the proton acceptor (13).

species of α and β subunits, respectively. All β-subunits are activated by zymogene processing yielding 2 × 7 active site Thr. During evolution the number of active β-subunits from 2 × 7 in prokaryotes was reduced to 2 × 3 in eukaryotes, while gene diversifications took place (14). Thus, eukaryotic propeptides of noncatalytic β-subunits are considered as remnants, which are cleaved off to exspatiate the catalytic chamber of the 20S proteasome. So far, we know that the lack of the β1, β2 and β7 propeptides is compatible with yeast cell survival (15). Mutants in which the β1 and β2 propeptides were deleted produced mature proteasomes with active β5 Thr but N-α acetylated, thus inactive β1 and β2 Thr. Therefore, one function of the propeptides might rely on preventing catalytic site inactivation by protection against co- or posttranslational modifications (16). In contrast to the β1 and β2 propeptides which have only subtle effects on 20S proteasome maturation, the β5 propeptide is indispensable for yeast cell life. This means that yeast mutants expressing β5 without propeptide are not viable. However, viability was regained when the β5 propeptide was expressed as a separate entity (17).

With 75 amino acid residues the β5 propeptide is the longest among the β propeptides. In the presence of active β1 and β2 Thr, the β5 propeptide is processed in two steps. The initial cleavage within the propeptide occurs in trans by an intermolecular mechanism. Already matured subunits in the β ring, most likely β2, execute this initial cut. The final cleavage of the β5 propeptide remnant at Gly-Thr adopts the intramolecular mechanism in cis for autocatalysis as proposed above (18).

These findings provided evidence that the three active sites are not of equivalent importance for eukaryotic 20S proteasome activity. Yeast mutants were created in which the N-terminal threonine of β1, β2 and β5 were replaced by alanine, respectively. The β1β2 Thr1Ala double mutant was viable suggesting a redundancy among the active site centers. In contrast to β1and β2, the β5 Thr1Ala mutation is lethal demonstrating that the β5 active site threonine is vital for 20S proteasome function (17,18). A hierarchy was established with a dominant function of β5 in the autolytic and proteolytic catalysis, and a gradually more important function of β2 compared with β1 (15). Moreover, these data demonstrated that the β5 proprotein is able to self-activate independent of the presence of active β1 Thr and β2 Thr. Intermolecular and intramolecular reactions could be imagined for β5 propeptide self-processing, since both β5 subunits are neighboured in the face to face oriented β rings, thus allowing cooperativity between the pairwise arranged β5 propeptides.

2.3. The Preferred Peptide Bond Cleavage Sites and Inhibition

The peptide cleavage preference of the β5 subunit descends from the archetype proteasome β-subunit which confers chymotrypsin-like activity to ancestral 20S proteasomes (7). Biochemical studies using chromogenic or fluorogenic peptide substrates and specific inhibitors revealed the cleavage preference of eukaryotic proteasomes at the carboxyl group of large hydrophobic amino acid residues. In these assays the endopeptidase hydrolyses the amide bond between the carbonyl group of an amino acid and the amino group of the chromogenic compound, which yields a spectroscopically measurable chromophor. Beside the chymotrypsin-like activity, proteasomes exhibit trypsin-like and post-glutamyl

splitting (peptidyl-glutamyl peptide hydrolyzing) enzyme activities. They produce peptide bond cleavages at the carboxyl-terminal side of basic and acidic amino acid residues, respectively (11).

In the early nineties, yeast mutants were created in which these three proteasome activities were selectively impaired. Complementation studies using these yeast mutants allowed to assign the chymotrypsin-like activity to the β5 subunit (19). The β1 and β2 subunits were found to confer the post-glutamyl splitting (peptidyl-glutamyl peptide hydrolyzing) and the trypsin-like activity, respectively. The genetic data in yeast nicely reconciled previous biochemical studies on proteasomes from different organisms and confirmed that proteasomes are multicatalytic proteases with independent active sites and different specificities. Furthermore, studies with yeast mutants affecting non-catalytic β subunits suggested that non-active site subunits participate in substrate binding or cooperate in regulating the catalytic site activities (20). For example, the C-terminal region of β7 (Pre4) plays an important role in pro-β1 (Pre3) processing and stabilizes a conformation which is required for the post-glutamyl-splitting activity of the β1 subunit, consistent with the previous finding of two complementation groups (*pre3* and *pre4* mutants) which contributed the post-glutamy-splitting activity (21).

The availability of the crystal structure of the yeast 20S proteasome allowed to inspect the catalytic cavity. The distinguishing specificities of the active sites could be realized in the context of the corresponding substrate binding pockets, which mark the preferences for peptide bond cleavage at the carboxy-terminal side of hydrophobic, basic and acidic amino acid residues (Fig. 1C). In the β1 pocket, an arginine residue is suited to balance the charge of acidic residues. An acidic environment in the substrate binding pocket of β2 accounts for the preferential binding of basic residues. The apolar character of the substrate binding pocket of β5 favours hydrophobic residues, which also explains why naturally occuring inhibitors of proteasome activity are preferentially accommodated by the β5 pocket. These inhibitors are widely used in biochemical studies in the mammalian system in order to compromise proteasome function substantially. They are generally accepted tools to relate the turnover of a given protein to the proteasomal degradation machinery (22).

Peptide aldehydes such as Calpain inhibitor I or Carbobenzoxy-Leucyl-Leucyl-Leucinal (MG132) preferentially bind in a reversible manner to the active site Thr of β5, but also bind to β1 and β2 Thr dependent on the inhibitor concentration (see also Fig. 1). They form a hemi-acetal with the Thr-Oγ and are not only attacked by the active site threonine of the proteasome but also by serine and cysteine proteases. More selective and potent than peptide aldehydes are the naturally occurring inhibitors lactacystin and epoxomycin, which are cell permeable and bind in an irreversible manner. Crystal structure analysis of yeast 20S proteasomes with lactacystin shows the inhibitor molecule bound to the β5 pocket, which provides the molecular basis for selectivity (6)(1G65 accession number in the protein database PDB). The most potent and selective proteasome inhibitor is known to be VELCADE™ (bortezomib; PS341), which belongs to the class of peptide boronates. VELCADE™ is the first proteasome inhibitor to be studied in human clinical trials and expected to impede cancer cell proliferation. It blocks proteasomal degradation resulting in an overload of conflicting cellular regulatory signals, which cannot be processed by the cancer cell. The cancer cells commit

suicide, while normal cells are less sensitive to the pro-apoptotic effects of the inhibitors and recover.

Proteasomes both repress and induce apoptosis or programmed cell death. Upon the reception of an apoptotic stimulus proapoptotic molecules were released whose level is initially kept low by proteasomal degradation. However, some proapoptotic molecules escape proteasomal destruction and trigger caspase activation. Recent studies in a mammalian system showed that activated caspases even attack and inactivate proteasomes, so facilitating the execution of the apoptotic program by providing a feed-forward amplification loop (23). With regard to neurodegenerative diseases, the systemic exposure to proteasome inhibitors has to be discerned with care, since proteasome inhibitors induce formation of proteinaceous inclusion bodies. Upon the exposure to proteasome inhibitors animal models recapitulated the features of neurodegenerative disorders. This observation led to the speculation that naturally occurring proteasome inhibitors aggravate slowly progressing neurodegeneration (2).

2.4. Educts and Products

In vivo substrates of proteasomes are short-lived proteins regulating myriads of intracellular processes. Furthermore, aberrantly folded, damaged or malignant proteins are scavenged by proteasomes. Malfolded secretory proteins were found to be retrograde-translocated from the ER into the cytoplasm for final proteasomal degradation. Thus, proteasomes partake in endoplasmic reticulum-associated degradation (ERAD) as initially detected in the yeast system (24). Dysfunctions in ER-associated degradation are connected with multiple diseases, which reveal the importance of proteasome function in ERAD. In contrast to substrate breakdown, proteasomal proteolysis can also be restricted to distinct domains of a given protein, such as known for transcription factors that are released into their active form by limited proteasomal proteolysis (25).

Usually substrates are signalled for destruction by polyubiquitylation, but there is increasing evidence that proteasomes are actually able to degrade non-ubiquitylated proteins, as long as they are adequately unfolded. Beside chromogenic tri- and tetrapeptides commonly used in assays for proteasomal activities longer oligopeptides and small proteins (occasionally denatured) are accepted substrates of 20S proteasomes. A multitude of studies characterized the length distribution of the digestion products, which ranges between 3 and more than 20 amino acids. Preferred cleavage motifs within peptides and the impact of residues proceding and following a given cleavage site were comprehended. The data were interpreted by statistical calculations in order to allow predictions of the digestion pattern of a given protein (26). Exploiting the power of yeast genetics, wild type and mutant 20S proteasomes with inactive β1 and β2 were used to address the peptide cleavage preferences of each catalytic subunit. By soaking permeabilized yeast mutant cells with artificial peptide substrates it could be confirmed that the post-glutamyl splitting and trypsin-like activity of the proteasome is abolished upon β1 and β2 inactivation. Proteolysis of a natural protein by mutant proteasomes with inactive β1 and β2 yielded almost no digestion products with C-terminal acidic and basic amino acids, respectively (27).

Protein substrates enter the 20S proteasome by the α ring pore which in the 26S configuration is most likely gated by the adjacent ATPase ring of the 19S regulatory complex (28). Here, substrates are thought to be fed as unfolded chains from their termini into the catalytic cavity and progressively degraded. However, this mechanism cannot account for proteasome-dependent processing of transcription factor domains from inactive proproteins. Natively disordered substrates were generated and offered to latent 20S proteasomes. The disordered polypeptide chains were cut at internal peptide bonds even when they lacked accessible termini suggesting that substrates themselves are able to promote gating of the α ring pore. Thus, the endoproteolyic machinery of the 20S proteasome may provide a molecular mechanism which allows to access internal folding defects of multidomain proteins without the alliance of 19S regulatory complexes (29).

2.5. The 19S Regulatory Complex

The 19S regulatory complex is required for recognition of the bulk polyubiquitylated substrates. It is composed of about eighteen subunits, which were assigned to two subcomplexes, the base and the lid. With few exceptions proteasomal subunits are encoded by essential genes in yeast. Thus, each subunit plays an important role in the proteolytic scenario. However, the functions of most subunits of the regulatory complex are still unknown (30)(Fig. 3).

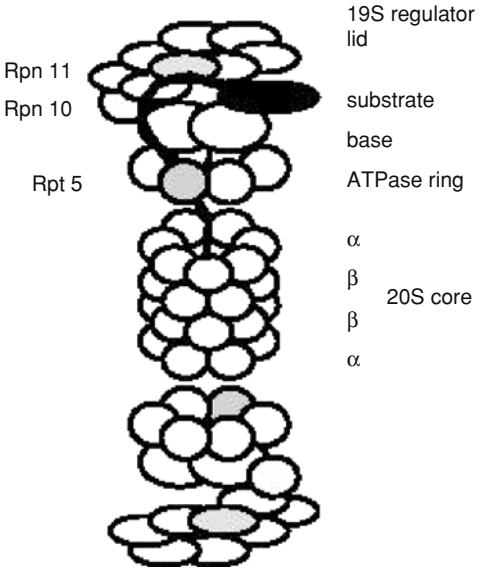

Figure 3. The 26S proteasome is formed by the 20S core particle capped by two regulatory 19S complexes. Each 19S regulatory cap complex is composed of 12 Rpn and 6 Rpt ATPase subunits. The Rpt ATPases, Rpn1 and Rpn2 form the base complex, the remaining Rpn subunits constitute the lid complex. Rpt5 (32) and Rpn10 (34) are involved in binding the polyubiquitylated substrate. Rpn11 exhibits a cryptic ubiquitin isopeptidase activity, which couples substrate deubiquitylation and degradation (35,36). Rpt5, Rpn10 and Rpn11 are shaded in grey. The substrate is coloured in black.

The base complex consists of two high molecular mass proteins, named Rpn1 and Rpn2, and six ATPases, numbered from Rpt1 to Rpt6, which belong to the family of triple A-ATPases (ATPases associated with multiple functions). These ATPases form six-membered rings which are attached to one or two α rings of the 20S proteasome in the 26S configuration. The ATPases confer ATP-dependence to proteasomal proteolysis, though it is still not proven which step of proteasomal degradation consumes energy. 19S regulatory and 20S core particles were reported to be only stably associated in the presence of ATP, resulting in the formation of the 26S proteasome. In contrast to chaperone complexes, which assist protein folding, the proteasomal ATPase module presumably consumes energy for substrate unfolding and promoting the denatured polypeptide chain through the α ring channel into the proteolytic cavity. The base complex was shown to assume primal chaperone functions in substrate refolding and protecting against proteasomal degradation (31).

Each ATPase of the base complex seems to be specialized in a separate function in proteasomal proteolysis. The ATPase Rpt2 plays a key role in opening the substrate channel in the α ring pore, as deduced from studies in yeast using combinations of *rpt2* and mutants affecting the N-termini of distinct α subunits (28). The ATPase Rpt5 provides specific contacts with the polyubiquitin chain of the substrates. This interaction was modulated by ATP hydrolysis suggesting that recognition of the ubiquitin signal is an ATP-dependent event (32).

In order to deliver substrates for final degradation several polyubiquitin chain binding proteins transiently interact with the 19S regulatory complex (33). One of the first discovered polyubiquitin-binding proteins, which is associated with the 26S proteasome, is Rpn10 (34). However, Rpn10 is not essential in yeast. The dispensability of Rpn10 reflects the redundancy of polyubiquitin chain binding proteins (33). Furthermore, Rpn10 was proposed to assist in connecting base and lid complexes, though 26S proteasomes form stable entities in the absence of Rpn10, which illustrates that lid and base connections are mediated by several subunits (30).

20S proteasomes are not able to degrade polyubiquitylated substrates. The polyubiquitin signal must be cleaved off before translocation into the proteolytic cavity. A cryptic metallo-isopeptidase activity is exhibited by Rpn11, a subunit belonging to the lid complex, which couples substrate deubiquitylation and degradation. Thus, one role of the lid complex can be envisioned in recycling ubiquitin moieties from target substrates (35,36).

3. BIOGENESIS OF YEAST PROTEASOMES

The question is how 2×14 different subunits incorporate into the right position of such a complex structure as the eukaryotic 20S proteasome. 20S proteasomes with low complexity exist in archaeons and eubacteria, which harbour one or two different α and β subunits. Their subunits could be expressed as recombinant proteins in E. coli. This approach allowed to identify assembly intermediates and finally the reconstitution of the mature particle. Based on these studies two models arose which describe the formation of hemi- or half-proteasome precursor complexes starting from early assembly intermediates. One model proposes

a seven-membered α ring which serves as template upon which the β proproteins are assembled. The other model suggests that seven dimers of α - and pro-β subunits oligomerize into hemi- or half proteasome precursor complexes (7).

In yeast cells, the formation of early assembly intermediates is assisted by an additional factor, the maturation factor Ump1. The name of the *UMP1* gene product originates from a complementation screen of *ump1* mutants defective in ubiquitin-proteasome mediated proteolysis. The *ump1* mutant accumulated poly-ubiquitylated proteins and ceased to grow upon stress such as elevated temperatures or environmental poisoning which induce the synthesis of aberrantly folded protein. The severe effects of absent Ump1 were partly compensated by increased proteasome expression. However, this compensatory mechanism is not sufficient for cell survival under stress conditions (21).

Ump1 is a 17 kDa protein with an intriguing similarity with the proteasome inhibitor contrapsin, which once suggested that Ump1 inhibits premature proteasome activation. However, in yeast cells lacking Ump1 the maturation of 20S proteasomes seems to be less efficient, which is reflected by slowed kinetic rates of pro-β1, β2 and β5 processing (21). Until now it is unknown at which stage Ump1 attends proteasome assembly and whether half-proteasome precursor complexes result from α ring or α pro-β dimer formations. In order to identify precursor complexes from yeast Ump1 was functionally tagged with IgG binding domains of protein A from Staphylococcus aureus. The tag provided a means to stabilize early assembly intermediates and made their isolation feasible from yeast lysates by one step affinity chromatography on IgG sepharose. The subunit composition of Ump1-associated precursor complexes was analyzed by two-dimensional gel electrophoresis. All seven α subunits and at least five of the seven β subunits, namely pro-β1, pro-β2, β3, β4 and pro-β5, were identified (37). In yeast, these precursor complexes sediment as 15S particles in glycerol gradient ultracentrifugation and appear to represent a population of stable assembly intermediates. In mammalian cells a similar population of assembly intermediates was already detected (38,39).

The pro-β6 and pro-β7 subunits seem to be incorporated late into the precursor complex finally yielding structures which were symbolized as hemi- or half-proteasomes. Presumably, two half-proteasomes instantaneously dimerize into the so called preholoproteasome, a very short-lived late assembly intermediate (17). The interactions between both approximating β rings are not only mediated by the propeptides and Ump1. Also, the prominent C-terminal extension of the β7 subunit seems to facilitate the formation of preholoproteasomes, since its deletion causes the accumulation of Ump1-associated precursor complexes. It protrudes into a cleft between the β1 and β2 subunits in the opposing β ring and functions like a clamp between the two halfs of the proteasome suggesting that it stabilizes the mature 20S proteasome (40).

In the preholoproteasome, propeptide processing and Ump1 degradation takes place. Pulse chase experiments followed the conversion of pro-β subunits into mature β subunits concomitantly with the turnover of Ump1. According to these experiments the maturation process is completed after 30 min, which corresponds to one-third of the time span of the yeast cell cycle (21).

The mechanism of active site generation accompanied by Ump1 degradation is still obscure. In yeast, a high molecular mass protein named Blm3 was

found to be associated with the preholoproteasome. Blm3 appears to delay propeptide processing in preholoproteasomes. Thus, we proposed that Blm3 coordinates late steps in 20S proteasome maturation (41). The question which remains is what triggers the final conversion of proteolytically inactive precursor complexes into mature proteasomes. Is it the meeting of pro-β subunits at the halfproteasome interface as originally postulated by Mark Hochstrasser? Based on his studies in yeast he proposed the first model on eukaryotic proteasome maturation (17). Until today, his model stands the tests with slight modifications by the addition of Ump1 (Fig. 4). The function of the β subunit propeptides is obviously not restricted to keeping active-β subunits in a dormant state, until the catalytic chamber of the proteasome is sealed. Yeast genetic data point to a special role of β propeptides in chaperoning β subunit incorporation, especially in association with Ump1 (21).

Genetic studies in yeast revealed that the deletion of Ump1 suppresses the lethality of a missing β5 propeptide. How can such a suppressor effect be explained? The β5 propeptide and Ump1 mutually induce conformational or positional changes upon dimerization of half proteasomes. Ump1 could function as a conductor in half-proteasome dimerization and finally trigger β5 propeptide processing. Thereby, Ump1 becomes enclosed in the proteolytic cavity and the first substrate of the nascent 20S proteasome (21).

In yeast, a predominant fraction of proteasomes resides in the nucleus as meanwhile shown by a global localization study of the yeast proteome (42). Each

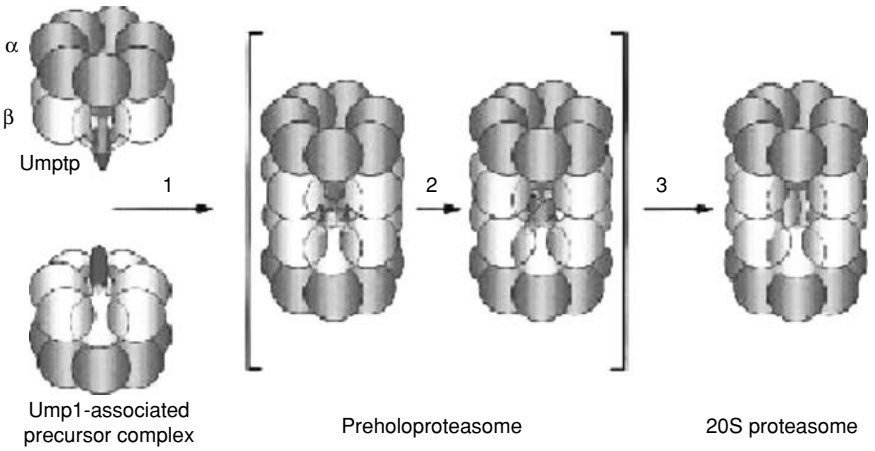

Figure 4. Model for yeast 20S proteasome assembly and maturation as originally proposed by Chen and Hochstrasser in 1996 (17) and modified by addition of the maturation factor Ump1 (21). Ump1-associated precursor complexes (~ 15 S complex) are symbolized as half-assembled proteasomes, which are composed of a ring of seven α-subunits and a ring of seven β-subunits, of which five contain N-terminal propeptides. Two half-proteasomes join to build a short-lived intermediate, namely the preholoproteasome. Upon the meeting of the two β?subunit rings, conformational changes trigger the autocatalytic propeptide processing, which are most likely conducted by the entrapped Ump1. The catalytic chamber of the nascent 20S proteasome is finally exspaciated by Ump1 degradation. The figure was kindly provided by Jürgen Dohmen (21) and printed with the permission of Cell (published by Elsevier; see color insert.)

proteasomal subunit was chromosomally replaced by fusion to the green fluorescent protein. The cells as monitored by direct fluorescence microscopy show the characteristic fluorescence in the nucleus and around the nuclear membrane (43). The micrographs are accessible in the yeast genome database (SGD). With regard to the nuclear population of proteasomes, an interesting question was, whether proteasomes are imported into the nucleus as whole mature particles or as inactive subcomplexes. The latter seems to be the case, since we found precursor complexes containing Ump1 and unprocessed β subunits in nuclear extracts of yeast cells. Yeast mutants deficient in nuclear import pathways exist which were exploited to find the respective import receptor. The classical nuclear import receptor karyopherin αβ turned out to be responsible for nuclear import of proteasomal components, since mutations in karyopherin αβ caused mislocalizations of proteasomal components to the cytoplasm (37,44). Multiple classical nuclear localization signals, which are prerequisites to recognize a cargo protein by karyopherin αβ, are present in proteasomal subcomplexes. The nuclear localization signal of Rpn2, a subunit of the base complex, was found to be essential for efficient nuclear import of base complexes and cell survival under stress conditions (45).

Based on our observations, we came to the conclusion that proteasomes are imported into the nucleus as inactive subcomplexes. We proposed that the maturation of nuclear 20S proteasomes and the final assembly with 19S regulatory complexes occurs in the nucleus (37). Our model of proteasome biogenesis in budding yeast was further supported by findings in fission yeast suggesting that lid and base complexes are independently imported into the nucleus, where they are successively assembled into 26S proteasomes (46).

Whether our model on nuclear proteasome biogenesis in yeast is applicable to higher eukaryotic cells awaits future work. At least, the nuclear localization signals of proteasomal subunits and the respective receptor karyopherin αβ are conserved from yeast to man. However, in mammalian cells, an alternative mechanism was postulated. The nuclear envelope disintegrates during mitosis, once leading to the conclusion that proteasomes are mainly taken up into the nucleus upon the reassembly of nuclear membranes (47). A comparable mechanism can be excluded in yeast due to the closed mitosis. Proteins destined to the nucleus have to pass the nuclear membrane across nuclear pores by a signal- and receptor-mediated process (48).

3.1. Benefits of Studies in Yeast for Neurodegenerative Diseases

Until now, we are far away from understanding how proteasome biogenesis is regulated in different compartments. To elucidate the structure, the assembly and activation of 26S proteasomes in different compartments will be a challenge in the future. The understanding of disfunctions in proteasome activation may be a prerequisite to improve the design of a new generation of drugs that cure neurodegenerative diseases. Combined therapies selective for several decisive events that characterize these disorders may prove beneficial in lowering the burden of aberrant proteinaceous inclusions inside affected neurons and in boosting the ubiquitin-proteasome system. One problem that investigators face is distinguishing primary causes from secondary consequences. The impairment of

the ubiquitin-proteasome system at the root of neurodegeneration is still a hypothesis, which is difficult to prove in vivo, since appropriate tools are missing. There are promising attempts made, especially by the group of Susan Lindquist, who studies pathological consequences of protein misfolding by using yeast as a readily manipulable organism. For example, the yeast system provides the opportunity to dissect molecular pathways underlying normal alpha-synuclein biology and the pathogenic consequences of its misfolding. Nucleated polymerization processes and recruitment of alpha-synuclein previously associated with membranes to cytoplasmic inclusions were observed in yeast comparable to aging neurons in Parkinson's disease. Small changes in the quality control balance in which the ubiquitin-proteasome system is suggested to be a key player could produce toxic gain of alpha-synuclein function concomitantly with loss of normal function (49,50). Furthermore, I will put studies in yeast to your attention which addressed the effects of amyloidogenic proteins on the de novo formation of prion-like aggregates in yeast. The data strongly supported the hypothesis of cross-seeding in the spontaneous initiation of prion states and prion-like aggregates (51), which are linked with Parkinson's and Alzheimer's disease.

The invasion of yeast in the research field of neurodegenerative diseases maybe reflects the unfortunate lack of adequate animal models. As a model eukaryotic organism yeast is wellcome to complement studies in the mammalian system. In near future, we will benefit from advances in new technologies allowing the real-time monitoring of ubiquitination in living mammalian cells (52). Exploiting the advantages of fluorescence live-cell imaging and fluorescence resonance energy transfer recent studies in mammalian cells verified in vivo that expression of aggregation-prone proteins falter the ubiquitin-proteasome system action. Proteasomes sequestered irreversibly with protein aggregates and failed to degrade aggregation-prone proteins in vivo despite ubiquitylation (53).

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2

UBIQUITIN-PROTEASOME SYSTEM IN THE CENTRAL NERVOUS SYSTEM

Qunxing Ding and Jeffrey N. Keller

1. PROTEIN DEGRADATION

In order to maintain cellular homeostasis all cells must continually degrade proteins, with proteolysis occurring in a manner that is both highly specific and highly regulated. The proteins to be degraded by intracellular proteolytic pathways include short-lived, long-lived, misfolded, and damaged proteins (2–4). The targeting of each of these different types of proteins for proteolysis is generally achieved by the presence of a targeting motif. Established targeting motifs include a single amino acid residue (i.e. the N-end rule) (5), an amino acid sequence (i.e. PEST sequences) (6), or exposure of a hydrophobic domain (7). Post translational modifications such as phosphorylation and oxidation are also known to increase the targeting of proteins for degradation, with the resulting increase in protein turnover believed in part to be mediated by alterations in the tertiary protein structure. These modifications in protein structure likely promote the exposure of amino acid sequences and/or hydrophobic domains necessary for

protein targeting (7–9). Proteins can also be modified by ubiquitin (Ub) or Ub-like proteins, which appear to dramatically alter protein turnover (10,11). The specificity of protein degradation is necessary to prevent aberrant or unwanted proteolysis, and without such regulation cells would be unlikely to survive for any prolonged period due to the inability to maintain basic aspects of cell homeostasis. Similarly, without effective proteolytic pathways cells would rapidly accumulate unwanted and potentially toxic proteins.

The two principle intracellular proteolytic pathways are the proteasomal and lysosomal system. Proteasome-mediated protein degradation consists of an ATP-dependent (26S) and ATP-independent (20S) form of proteolysis. Meanwhile, thelysosomal proteolytic pathway can also be manifest in several forms including the endosomal-lysosomal pathway and macroautophagy. Together, the proteasomal and lysosomal pathways account for more than 90% of intracellular proteolysis (12,13).

The focus of this book is the proteasomal proteolytic pathway, which can be fully distinguished from lysosomal proteolysis based on several important features. Proteasome-mediated protein degradation occurs at neutral pH, does not require intracellular compartmentalization, occurs within a specialized protein complex, preferentially degrades short-lived proteins, and breaks down proteins to generate peptides not individual amino acids (14,15). Increasing evidence suggests that the proteasome plays an important role in a wide variety cellular processes including inflammation, proliferation, cytoskeletal regulation, and cell signaling (16,17). Numerous studies now also demonstrate a role for the proteasome in a wide range of neurophysiological as well as neuropathological processes, highlighting the significance for understanding the basis and regulation of proteasome-mediated protein degradation in the central nervous system (CNS).

2. THE UBIQUITIN SYSTEM

Ub plays a critical role in 26S proteasome-mediated protein degradation, targeting proteins to be degraded by the 26S proteasome in an ATP-dependent manner (18,19). The Ub protein is small (76 amino acids) and is present in all eukaryotic cells. In addition to its well established role in targeting proteins for degradation, a number of studies are now indicate that Ub may have a role in cellular events other than proteolysis. One of the unique aspects of Ub is that it is encoded and expressed as multimeric repeats (polyubiquitin) and also as single Ub encoding sequences (20,21). Interestingly, the single Ub encoding sequences can be fused in frame with a carboxyterminal extension protein (CEP). In humans there are two different ribosomal proteins L40 and S27a that can be fused to individual monomeric Ub encoding genes (22,23). Each of these Ub-fusions appears to play a critical role in ribosome biogenesis. These data suggest the potential existence of an important link between protein synthesis and protein degradation (23). The multimeric Ub products are modified post-translationally by cleavage events that generate monomeric Ub, while Ub-fusion proteins can yield monomeric Ub following cleavage by carboxyterminal Ub hydrolases (24,25).

There are now a number of Ub-like proteins that may have functions similar to Ub. Some of the best examples of these proteins include small ubiquitin-like modifier (SUMO) (10) and neural precursor cell-expressed developmentally down-regulated (NEDD8) (26). While each of these Ub-like proteins attaches to protein substrates via interactions through their carboxyl termini (like Ub), it appears that these Ub-like proteins are unable to form chains or higher order structures. Ub-like proteins are known to colocalize with Ub inclusions, and possibly modulate the degradation of Ub-modified proteins (11). These data suggest a role for Ub-like proteins in regulating Ub-mediated proteolysis and highlight the importance of developing a greater understanding these proteins play in both physiological and pathological processes (26).

The linkage of Ub to target proteins is mediated by isopeptide bonds between the C-terminal glycine residue of Ub and the amino group of lysine residues on target proteins (27). Following placement of the initial Ub onto the target protein, the establishment of a polyubiquitin chain can be rapidly achieved via the sequential addition of mono-Ub to the lysine residue of substrate bound Ub. The placement of Ub onto protein substrate, and development of polyubiquitin chain, requires a number of specific proteins to work together in a coordinated and complex manner (28). In the first step, the E1 enzyme activates Ub in an ATP-dependent reaction that produces a high-energy E1-thiol-ester Ub intermediate, that is then rapidly transferred to a subsequent enzyme termed E2 (Figure 1). The E2 enzymes catalyze the covalent attachment of Ub to target proteins, or the transfer of activated Ub to an E3 molecule in order to form an E3-Ub intermediate (Figure 1). The E3 enzymes are protein ligases, and are

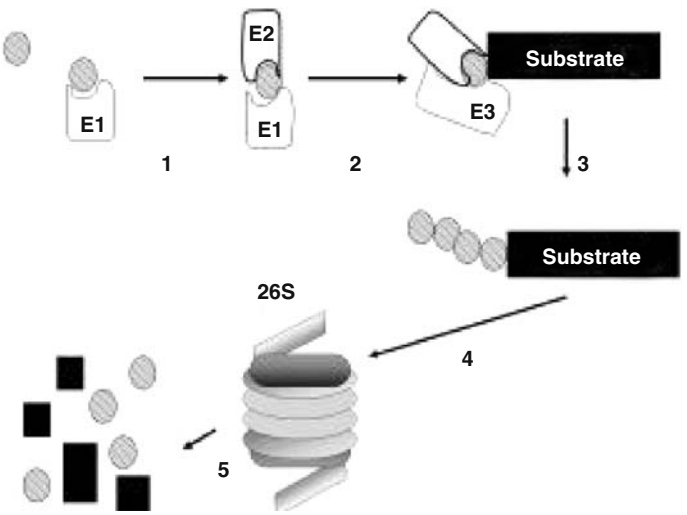

Figure 1. Ubiquitination of target proteins. (1) The E1 recognizes Ub (ovals) and transfers Ub to E2 (1) the E3 recognizes E2-Ub and then conjugates Ub to substrate protein (3) the process continues to add up poly-Ub chain on the substrate protein (4) poly-Ub-substrate is recognized by 26S proteasome and degraded (5) Substrate protein is cleaved into short peptides and Ub is released for recycling.

responsible for the transfer of Ub to the specific protein substrates. Most of the E3 proteins can be placed into two categories: homologous to the E6-AP-C terminus (HECT) (28,29) or the really interesting new gene (RING) (28,30). The HECT E3 proteins accept Ub from E2 enzymes by forming an additional high energy thiolester bond between an active site cysteine and Ub, with the Ub subsequently transferred to the ligase bound substrate. In contrast, the RING E3 ligases serve primarily as a bridge to bring the E2-Ub complex and the protein substrate into closer proximity.

The specificity and complexity of protein ubiquitination becomes evident when looking at the number of genes expressed for each class of enzymes involved in the process. In humans there are two isoforms of E1, more than 50 E2, at least 1000 E3 proteins (31). The large number of E3 ligases is believed to contribute to the specificity and selectivity of protein ubiquitination, with individual E3 ligases exhibiting cell type specific expression and highly specific protein substrate selectivity. Mutation of the Ub pathway, in particular mutation of E3 ligase, may play a particularly important role in neurodegenerative events that selectively affect individual neuron populations.

3. UBIQUITIN AND THE PROTEASOME

It is important to point out that Ub-mediated protein degradation by the proteasome is the result of the 26S proteasome complex and not the 20S proteasome. The 26S proteasome complex has cap-like structure that contains several specialized proteins which aid in the recognition and recruitment of ubiquitinated proteins (Figure 1). It is likely that the increased hydrophobicity conferred by the polyubiquitin chain is what causes the proteins to be recognized by the 26S proteasome. In such a scenario, Ub would serve as a more important targeting mechanism for proteins that have a well preserved tertiary structure, or as a modifier for proteins that are intended to have an extremely short half-life. The 20S proteasome complex, which is several times more abundant than the 26S proteasome complex, degrades a vast array of proteins in an Ub- and ATP-independent manner. In particular the 20S proteasome is responsible for degrading most mildly oxidized proteins. Recent studies have confirmed that oxidized protein degradation by the proteasome is Ub-conjugation independent (32).

4. PROTEASOME AND THE BRAIN

The proteasome is a large intracellular protease composed of multiple subunits that exists in the cytosol and nucleus, and is well conserved from yeast to mammals in both structure and function. The proteasome was first observed in 1968 by J. R. Harries (33), and soon after a number of laboratories reported similar results. Recent studies indicate that in archaea and some bacteria (*actinomycetales*) there is a 20S proteasome possessing four stacked rings instead of the two stacked rings found in the *E.coli* (34), suggesting the proteasome has undergone some evolutionary change but is present even in the most ancient life forms. During the 1970-80s ATP/ubiquitin dependent proteolysis was documented in a cell-free lysate system from rabbit reticulocytes (35), although at that time ubiquitin dependent proteolysis

had not yet been identified as being mediated by the proteasome. During 1970-1980s the proteasome was termed as "multicatalytic proteinase complex", "macroprotease", "prosome", or "macropain" (36,37). Subsequent research identified the proteasome as the protease responsible for ubiquitin dependent protein degradation, indicating the presence of the so-called ubiquitin/proteasome pathway (UPP) (38,39). After two decades research in this area, it is clear now that proteasome has many different structural isoforms and is involved in a number of diverse tasks, including antigen presentation, stress response, cell proliferation and apoptosis.

The CNS is a highly complex system composed of both mitotic cells (astrocytes, microglia) and postmitotic cells (neurons). The functions of UPP in the CNS are not as defined as compared to other systems, such as the immune system. Studies in Alzheimer's disease (AD) and other age-related neurodegenerative disorders have provided evidence that the function of the proteasome is impaired and may contribute to both neuropathology and neuron death (40-42). The dysfunction of the proteasome may also lead to the dysfunction of specific organelles including mitochondria, and potentially generate crosstalk with the lysosome system (15,43,44). Developing a better understanding of the proteasome system in the CNS is likely to aid in the development of therapeutic interventions for neurodegenerative disorders as well as normal brain aging.

5. THE 20S PROTEASOME

The proteasome is a large multicatalytic protease (~700 kDa) that comprises up to 1% of total cellular protein content. The barrel-shaped core of the proteasome is known as the 20S proteasome, and consists of 28 individual α- and β-subunits (18). The 20S proteasome subunits are arranged within four stacked rings, with each ring consisting of either 7α or 7β subunits. The β subunits comprise the two inner rings of the 20S proteasome, with the outer rings comprised of α-subunits. The apparent diameter of 20S proteasome is approximately 11nm × 15nm. The β subunits are responsible for mediating all of the proteolytic activities of the proteasome, while the α-subunits function in stabilizing the 20S proteasome complex (Figure 2). There are three special β subunits PSMB8, PSMB9 and PSMB10 that are not present in the regular 20S proteasome, which are called inducible subunits (Table 1). The induction of these subunits usually occurs with inflammatory factors such as interferon gamma (INF γ) (45). Following their expression, inducible subunits replace other β subunits PSMB5, PSMB6, and PSMB7 to form so called "immunoproteasome". The inducible immunoproteasome subunits are enriched at the endoplasmic reticulum, where they play an important role in generation of MHC class I molecules (46).

The immunoproteasome has been intensively studied for its role in MHC I antigen processing (47), with the functions of the immunoproteasome affected by many factors. For instance, virus infection elevates the level of immunoproteasome (48), but studies indicated that the incorporation of inducible subunits into the proteasome complex may be interrupted by the activities of virus (49). The elevation in immunoproteasome expression after virus infection is INF γ-dependent, with INF α and other cytokines have no effects (50). Alcohol inhibits the induction of immunoproteasome by IFN-γ, and attenuates the

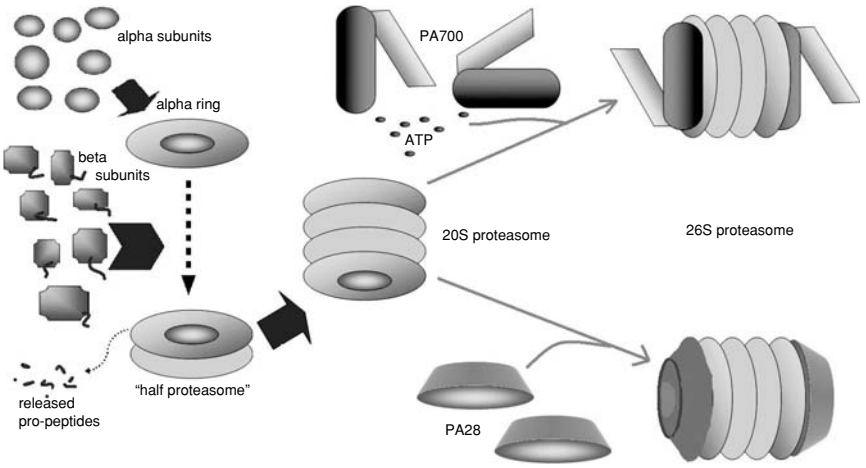

Figure 2. The biogenesis and structure of 20S and 26S proteasome complexes. The α subunits (ovals) form single α ring and then the β subunits (squares, the curved line indicate the propeptides) add up to form dual ring ("half proteasome"). Two of such "half proteasome" form 20S proteasome. Two types of 26S proteasomes exist in the cells: PA700 complex adds to 20S proteasome requiring ATP, while PA28 complex adds to 20S proteasome independent of ATP.

catalytic activities of proteasome (51). Interestingly, INF γ-deficient mice have similar basal expression of inducible subunits as compare to normal mice, suggesting that INF γ does not affect the constitutive expression of these subunits (50). The interaction of viral protein with proteasome subunits have been reported, and may interfere with host anti-viral defenses and also contribute to mechanisms of cell transformation (46). Interestingly, the inducible subunits PSMB8 and PSMB9 are increased in mouse model of Huntington's disease (HD), with neuron preferentially exhibiting increased immunoproteasome, suggesting the involvement of immunoproteasome in neurodegeneration (52,53).

An additional 20S proteasome –like protease exist in the mitochondria, and is referred as Lon (54). Lon is encoded by nuclear gene, but is located in the matrix of mitochondria. Lon is necessary to maintain mitochondria homeostasis (55). Studies indicated that the activities and expression are increased correlatively with the biogenesis of mitochondria (56). Different from 20S proteasome, the catalytic activities of Lon are ATP-dependent, and conserved from archae to human. Besides the hydrolysis of proteins and peptides, Lon also binds single stranded DNA, especially in the TG-rich region (57), suggesting a role of Lon in mitochondrial DNA replication and/or mitochondria gene expression. (54) The expression of Lon gene might be regulated by different factors. For example, the expression of Lon declined in aging mice (58), and may be enhanced by hypoxia or ischemia (59). Interestingly, a bacterial Lon protein has chaperone-like activity (60).

Presently 7 α subunit genes and 10 β subunit genes for the 20S proteasome have been identified in the human genome (Table 1). At least 2 additional β subunits have been described in zebrafish (*Danio rerio*, PSMB11, PSMB12) but have not yet been identified in human (61). PSMA2 and PSMA6 have 2 functional gene copies, while PSMA6 and PSMA7 each has 1 pseudogene copy. The

Table 1. 20S proteasome subunits.

Symbol	GeneID	locus	Alternative symbols	Size**
α subunits				
PSMA1	5682	11p15.1	NU,HC2, PROS30	263 a.a.
PSMA2	5683	7p13	HC3,PSC2	225 a.a.
PSMA3	5684	14q23	HC8,PSC3	248 a.a.
PSMA3P*		14q23.1		
PSMA4	5685	15q24.1	HC9, HsT17706	261 a.a.
PSMA5	5686	1p13	9534, PSC5, ZETA	241 a.a.
PSMA6	5687	14q13	IOTA; p27K;PROS27	246 a.a.
PSMA6'		13q32.2	LOC121906	
PSMA6P		Yq11.21		
PSMA7	5688	20q13.33	C6; HSPC; RC6-1; XAPC7	248 a.a.
PSMA7P		9q22.33		
β subunits				
PSMB1	5689	6q27	HC5	241 a.a.
PSMB2	5690	1p34.2	HC7-I	201 a.a.
PSMB3	5691	17q12	9540, HC10-II	205 a.a.
PSMB3P		2q35		
PSMB3P		12q13.13		
PSMB4	5692	1q21	HN3, HsN3, PROS26	264 a.a.
PSMB5	5693	14q11.2	LMPX, MB1, X	263 a.a.
PSMB6	5694	17p13	DELTA, LMPY, Y	239 a.a.
PSMB7	5695	9q34.11-12	9544, Z	277 a.a.
PSMB8	5696	6p21.3	LMP7, RING10, D6S216	276 a.a.
PSMB9	5698	6p21.3	LMP2, RING12	219 a.a.
PSMB10	5699	16q22.1	LMP10, MECL1	273 a.a.
PSMB11	64279	(*Danio rerio*)		217 a.a.
PSMB12	64280	(*Danio rerio*)		281 a.a.

*The mark' indicates the functional isoforms of the gene while the letter "P" indicates the pseudogene.
**Indicates the longest known amino acid sequence.

PSMB3 gene has 2 pseudogene copies. It is not yet clear why and how these extra gene copies developed, although most pseudogenes are believed to result from genomic evolution (23,62). Interestingly, one of the PSMB3 pseudogenes is in the same location as functional PSMB3 on chromosome 14, while another PSMB3 pseudogene is located on chromosome 2. The inducible subunits PSMB8

and PSMB9 are very close to each other on chromosome 6, with the inducible immune subunit PSMB10 located on chromosome 16. For all α subunits, there is an approximately 30% shared identity in amino acid sequence while the β subunits appear to be much more diverse. Most α subunits possess a conserved motif in their N-termini that is essential for proteasome assembly (63). Another conserved motif is RP×G where R, P and G refer to arginine, proline and glycine, respectively. This motif is found in the contact region among α subunits, although its function is still unclear (61). Some α subunits contain a functional nuclear localization signal that may regulate their nuclear localization (61). Some β subunits have an N-terminus pro-peptide that may work as an internal chaperone to ensure proper folding (64) or to prevent premature activation (65). These pro-peptides are removed during proteasome assembly, in order to expose a threonine that is essential for catalytic activity of most β subunits (66). The functional genes of β subunits are located on different chromosomes (Table 1), and it appears unlikely that their promoter regions share any significant homology. Additionally the activity of the promoters for β subunits is apparently unique, and the exon/intron organization of each β subunit gene lacks any apparent uniformity (67). It's interesting to speculate that cells could coordinate the expression of proteasome subunits in order to fulfill a specific need during normal physiological conditions or in response to stress. For instance, in LMP2 (PSMB9) knock out mice, the expression of PSMA4, PSMB1, PSMB3 are increased while the expression of PSMB5 and PSMB8 are decreased in the brain (Ding and Keller unpublished observation). In the mouse model of HD, the expression of PSMA3, PSMB1, PSMB3 and PSMB6 are selectively up-regulated in the brain. Lastly many proteasome subunit genes change their expression with age (Ding and Keller unpublished observation).

As a multi-subunit complex, the proteasome needs to be assembled from many individual proteins (Figure 2). *In vitro* studies indicate that the α subunits can form 7-member rings (α1-7) by themselves. To form β rings, the presence of α rings is required. In addition, the α rings can associate in pairs without β rings (66). The N-termini conserved sequences of α subunits are important for the assembly of α rings, with deletion or mutation in these regions preventing the formation of α rings (63,66). For β subunits the pro-peptides in the N-termini are essential to ring assembly (Figure 2). Yeast studies have shown that without a proper pro-peptide the β subunit can not incorporate into a 20S complex and the cell is unable to survive (68). Interestingly, when full length β subunits are expressed in *E. coli* they accumulated as inactive monomers, while expression of β subunit without a propeptide forming aggregates possessing peptidase activity (66). Some studies have shown that phosphorylation might be involved in the incorporation of β subunits into 20S proteasome (69), but the details of this process are not clear. Other reports indicated that during assembly of the 20S proteasome, coupled α and β rings (α1-7β1-7) might form an intermediate form of mature 20S proteasome (70). One maturation factor, UMP1 was identified originally in yeast, which is necessary in the assembly of the 20S proteasome (70,71). The mammalian homolog of UMP1 is referred as proteassemblin (72), which is regarded as a chaperone, interacting with standard β subunits and inducible β subunits selectively to assemble either the standard proteasome or the immunoproteasome (73). Data indicated that the inter-

action occurring between the C-termini of β subunits and proteassemblin aid in the formation of four-ring-complex from two-ring-complex (half proteasome) (73). In addition, HSC73 is another chaperone that specifically involved in the formation of immunoproteasome (74). HSC73 appears to aid in holding two half-proteasomes together and Hsp90 co-precipitates with the pro-proteasome suggesting a role for Hsp90 in the assembly of proteasome (74,75). In summary, it is presently accepted that 20S proteasome biogenesis occurs as followed: α subunits form the α-rings and the β subunits then associate with one α-ring (Figure 2). The pro-peptides of β subunits are then cleaved to form a complete β ring on α ring, resulting in the formation of a "half proteasome": α1-7β1-7. Finally two of such "half proteasome" associate together to form the regular 20S proteasome complex: α1-7β1-7β1-7α1-7 (Figure 2) (76). The 20S proteasome complex is far more abundant than the 26S complex, with both 20S and 26S proteasomes outnumbered by developing proteasomes and free subunits.

The 20S proteasome has three principle peptidase activities: chymotrypsin-like activity (cleavage after big hydrophobic residues), trypsin-like activity (cleavage after basic residues), and caspase-like activity (postglutamyl activity, cleavage after acidic residues) (18). These activities are all executed within the inner chamber of the β subunit ring. Purified 20S proteasome can degrade many peptides in ubiquitin- and ATP-independent manner (18,76). The free 20S proteasome particles are present in the cells (69), and some reports indicated that the 20S proteasome degrades oxidized, misfolded proteins, and peptides *in vivo* (77). For example, oxidized hemoglobin is rapidly degraded after ATP depletion in reticulocytes (78), and IKκB α is selectively degraded by 20S proteasome (79). In fresh extracts 20S proteasome is resistant to heat (up to 55˚C), fatty acids, and denaturing agents such as guanidine and SDS (80). Interestingly these treatments have been demonstrated to even enhance the catalytic activities of 20S proteasome(80). Since these treatments would be expected to induce the conformational change of proteins leading to unfolding or denaturing, it is possible that these treatments might aid the entry of misfolded proteins into the 20S proteasome complex. In the living cells, oxidative stressors like H2O2 treatment do not significantly change the activity of 20S proteasome while the function of 26S proteasome is dramatically decreased (see below).

As mentioned above, 20S proteasome complexes are relatively stable, resistant to certain level of heat, detergent as SDS, and oxidative stress. Research indicates that most proteasome complexes may last throughout cell cycle, and in post-mitotic cells like neurons a 20S proteasome complex might last for years. When necessary, it is presumed that proteasome complexes are degraded by lysosome system (81), while additional evidence suggests that caspases may degrade proteasome subunits especially during apoptosis (82).

6. THE 26S PROTEASOME

The 26S proteasome is the principle mechanism for the degradation of ubiquitinated proteins. Generally the term of 26S proteasome refers to a 20S proteasome associated with PA28 or PA700 activator (18). The PA700 complex is a V-shaped complex responsible for the recognizing, binding and unfolding the

ubiquitinated proteins and then delivering them to the hydrolytic sites of 20S (Figure 1). PA700 activator, which is also termed the 19S complex, can combine with 20S proteasome core on one or both α rings in the presence of ATP and greatly enhances the proteasome hydrolysis activity. PA700 can be dissociated from the 20S proteasome core under ATP depletion, and it appears that in the cell PA700 continuously shuttles on and off 20S proteasome complex in response to the environmental stress. PA28 is an alternative cap for proteasome, and is a bell-shaped complex, which binds the 20S proteasome core independent of ATP (Figure 2). Apparently PA28 has a weaker association with 20S proteasome than PA700, and may be released from 20S proteasome readily following exposure to low level ionic conditions. PA28 has been found as a free complex, but the potential function of the free PA28 complex is not clear (83,84). Other factors that have been demonstrated to interact with the proteasome include protein kinases, (85) isopeptidases, (86) heat shock proteins (HSP) and EF-1α (87).

PA700 has 6 subunits with ATPase activities, and 15 subunits that lack ATPase activities (Table 2 & Table 3). Four of the ATPase subunits (PSMC1, PSMC2, PSMC4, PSMC5) form a tetramer ring as the core of PA700 (88), but the functions of non-ATPase subunits are not yet clear. Mutation of these subunits leads to the accumulation of ubiquitinated proteins (89), suggesting that these subunits play a role in recognizing, binding and/or delivering ubiquitinated proteins to 20S proteasome core. Besides ATP the association of PA700 to 20S proteasome is regulated by a 300 KD modulator. Interestingly this modulator contains two ATPase subunits of PA700 (90). These data raise the possibility that regulator subunits may have multiple functions, potentially even proteasome

Table 2. 26S proteasome regulatory PA700 subunits (ATPases).

GDB symbol	GeneID	locus	Alternative symbols	Size**
PA700 ATPase subunits				
PSMC1	5700	14q32.11	P26S4, S4, p56	440aa
PSMC2	5701	7q22.1-3	MSS1, S7	433aa
PSMC2'*		3q22.1	LOC402142	
PSMC3	5702	11p12-13	TBP1	491aa
PSMC3P		9p22.1		
PSMC4	5704	19q13.11-13	MIP224, S6, TBP7	418aa
PSMC5	5705	17q23-25	S8, SUG1, TBP10, TRIP1,p45	406aa
PSMC6	5706	14q22.1	CADP44, P44, SUG2, p42	389aa
PSMC6P		8q11.23		
PSMC6P		12q14.3		

*The mark' indicates the functional isoforms of the gene, while the letter "P" indicates the pseudogene.
**Indicates the longest known amino acid sequence.

Table 3. 26S proteasome regulatory PA700 subunits (non-ATPases).

Symbol	GeneID	locus	Alternative symbols	Size**
PSMD1	5707	2q37.1	P112, S1	953aa
PSMD2	5708	3q27.3	P97, S2, TRAP2	908aa
PSMD2P*		1q43	LOC266783	
PSMD3	5709	17q21.2	P58, RPN3, S3	534aa
PSMD4	5710	1q21.3	AF, ASF, MCB1, S5A, Rpn10, pUB-R5	377aa
PSMD4P		10q23.33		
PSMD5	5711	9q34.11	S5B	504aa
PSMD6	5712		S10	389aa
PSMD7	5713	16q23-24	MOV34, P40, S12	326aa
PSMD7P		17q24.2	LOC280637 HIP6, HYPF, S14, p13,	
PSMD8	5714	19q13.13	Nin1p	257aa
PSMD8P		chromsomel	LOC276721	
PSMD9	5715	12q24.31-32	p27	223aa
PSMD10	5716	Xq22.3	p28	226aa
PSMD10P1		3q28	LOC280644	
PSMD10P2		20q13.13		
PSMD11	5717	17q12	S9, p44.5	422aa
PSMD12	5718	17q24.3	p55	456aa
PSMD12P		3p14	LOC317753	
PSMD13	5719	11p15.5	HSPC027, p40.5	376aa
PSMD14	10213	2q24.3	PAD1, POH1, rpn11	310aa
PSMD15	54035	21q22.13	PSMD4P (pseudogene)	

*The letter "P" indicates the pseudogene.
**Indicates the longest known amino acid sequence.

independent functions. PA28 has four subunits (PSME1, PSME2, PSME3, PSME4) that are homologous (Table 4), and may form a hetero-heptametrical complex (91). Interestingly, PA28 α (PSME1) is capable of forming a hexameric ring composed only PA28 α. (91) Presently it is not clear what roles the PSMA3 and PSMA 4 subunits play in protein degradation. PA28 is γ-interferon inducible, required for the antigen processing, and is necessary for the assembly of immunoproteasome (92).

The 21 genes of the PA700 subunits are located on different human chromosomes (Table 2 and Table 3). The 6 ATPase subunits belongs to the same ATPase

family (AAA), with a second functional copy of PSMC2 gene located on a different chromosome, and another two of these ATPase subunits (PSMC3 and PMSC6) having pseudogenes. PSMC6 actually has 2 copies of pseudogenes (Table 2). Pseudogenes exist in non-ATPase subunits as well, including PSMD2, PSMD4, PSMD7, PSMD8, PSMD10 and PSMD12 genes (Table 3). Interestingly, these pseudogenes are located on different chromosomes, with even double pseudogenes present on separate chromosomes. For example, the functional PSMD10 are located on chromosome X while two of its pseudogenes located on chromosome 3 and chromosome 20 (Table 3). With the high preservation of the proteasome system from bacteria to mammals, it would not be surprising if more pseudogenes and functional copies of proteasomal genes are found in the human genome, demonstrating the evolutionary specialization of the proteasome.

The homology among ATPase subunits is significantly higher than that of non-ATPase subunits, although the highest homology is in PA28 subunits (PSME1, PSME2, PSME3, and PSME4) (Table 4). PSME1 and PSME2 genes are composed of 11 exons each, consistent with gene duplication during vertebrate evolution. The intron/exon organization of these genes is highly conserved, with the PSME2 lacking the exon encoding the lysine and glutamic acid-rich KEKE motif. These two genes are closely linked on 14q11.2, within 30~40 kb(93). In fact, this locus is very close (within 1MB) to one of β subunits of 20S proteasome, PSMB5 (Table 4) (94).

Table 4. 26S proteasome regulatory PA28 subunits.

Symbol	GeneID	locus	Alternative symbols	Size**
PSME1	5720	14q11.2	PA28A, PA28α, REGα	249aa
PSME2	5721	14q11.2	PA28B, PA28β, REGβ	239aa
PSME2'*	389312	5q21.1	LOC389312	239aa
PSME2P1		5q21		
PSME2P2		13q13	LOC338099	
PSME2P3		4p14	LOC338096	
PSME2P4		10p12	LOC338098	
PSME2P5		4q32	LOC338095	
PSME2P6		8p21	LOC338097	
PSME3	10197	17q21	Ki, PA28γ, PA28G, REGγ	267aa
PSME4	23198	2p16.3	PA200	1798aa
others				
PSMF1	9491	20p13	PI31	271aa
p44S10	9861	3p21.1	KIAA0107, SGA-13M,p42A	389aa

*The mark' indicates the functional isoforms of the gene, and letter "P" indicates the pseudogene.
**Indicates the longest known amino acid sequence.

Currently it's not clear whether the linkage of proteasome genes indicates a functional coordination of gene expression (Table 5). It's worthy to mention that in yeast the proteasomal genes are dispersed amongst almost all chromosomes and their expression is both constitutive and possibly correlated. It will be interesting in future studies to elucidate the transcriptional patterns of proteasome subunit expression in different paradigms, to determine if these genes exhibit coordinated expression. With microarray technology it was found that the expression of most of the proteasome subunit genes is tightly coordinated upon initiation of transcription (95). Other studies indicate that under certain situations such as DNA damage, up-regulation of proteasome genes is mediated by a single transcriptional factor (RPN4) (96). RPN4 is a transcriptional activator that promotes the expression of most proteasomal subunit genes in yeast. RPN4 is degraded by the proteasome, thus forming an auto-regulatory circuit. Interestingly, RPN4 is degraded by proteasome in at least two ways, ubiquitin-dependent one and ubiquitin-independent one (97).

Table 5. Gene clusters of subunits in human proteasome system.

Gene	**Locus**	**Gene**	**Locus**
PSMB4	1q21	PSMB8	6p21.3
PSMD4	1q21.3	PSMB9	6p21.3
Gene	**Locus**	**Gene**	**Locus**
PSMA7P	9q22.33	PSMC3	11p12-13
PSMD5	9q34.11	PSMA1	11p15.1
PSMB7	9q34.11-12	PSMD13	11p15.5
Gene	**Locus**	**Gene**	**Locus**
PSMB5	14q11.2	PSMC6	14q22.1
PSME1	14q11.2	PSMA3	14q23
PSME2	14q11.2	PSMA3P	14q23.1
PSMA6	14q13		
Gene	**Locus**	**Gene**	**Locus**
PSMB3	17q12	PSMC4	19q13.11-13
PSMD11	17q12	PSMD8	19q13.13
Gene	**Locus**	**Gene**	**Locus**
PSME3	17q21	PSMF1	20p13
PSMD3	17q21.2	PSMD10P2	20q13.13
PSMC5	17q23-25	PSMA7	20q13.33
PSMD7P	17q24.2		
PSMD12	17q24.3		

As mentioned above, a 300KD modulator of 26S proteasome has been reported, which can enhance the function of PA700 on proteasome without affecting the activity of the 20S proteasome (90). Another regulator is PI31, which is believed to be a natural inhibitor of proteasome, is associated with the nuclear envelope/endoplasmic reticulum membrane (98). Recent research indicate that PI31 might act as a modulator of proteasome-induced MHC class I antigen processing (98). Over-expression of PI31 in mouse embryonic cells selectively interferes with the maturation of immunoproteasome precursor complexes, decreased the surface MHC class I levels on IFN γ-treated mouse embryonic cells (98). PAN (proteasome-activating nucleotidase) is a homolog of mammalian 19S complex, expressed in archaeal cells (99). PAN has a molecular weight of ~560kD, possessing high homology to the ATPase subunits in PA700. Besides ATP, PAN can utilize CTP, TTP, GTP,UTP and even ITP to enhance the catalytic activity of proteasome(99). Reports indicate that PAN has chaperone activity to reduce aggregation of denatured proteins and may enhance protein refolding (99). Although ATP is not required, the presence of ATP can increase the efficiency of protein folding by PAN (100). PAN does not promote the degradation of small peptides. Other proteins are also involved in regulating proteasome proteolysis including tripeptidyl peptidase II (TPP II) which plays a critical role in cleaving proteasomal produced peptides into shorter peptides that can then be degraded by aminopeptidases (101).

7. PROTEASOME MEDIATED PROTEIN DEGREDATION

Studies indicated that about one-third of newly synthesized proteins have structural errors, and these proteins need to be removed eventually by the proteasome (102-104). Denatured proteins and otherwise misfolded proteins are degraded by proteasome as well. This proteolytic process is strictly regulated. As mentioned above, Ub and Ub-like proteins (SUMO, NEDD8) are the most popular markers for destruction. In fact, the E3 group of Ub ligases is largely responsible for the recognization of proteins with destruction signals, and the E3 may be activated by structural modification such as phosphorylation or allosteric transition. Environmental and intracellular signals can also trigger the degradation of specific proteins (105). The adaptive cellular immune system in mammals is highly dependent on peptides generation, which are made by the proteasome from viruses and other intracellular pathogens. CD8+ T cells in the adaptive immune system first detect the foreign peptides, and then a clonally restricted receptor is expressed to recognize peptides with 8- to 11-residue, nestling in the groove of major histocompatibility complex class (MHC) I molecules (106). The newly synthesized class I molecules carry viral peptides to the surface of infected cells, where they are recognized by non-self-reactive T cells specific for the given peptide-class 1 complex. Activated T cells then deliver a cocktail of immune effector molecules that is capable of interfering with viral replication either by brute force (killing the virally infected cell) or by subtle pathway (reprogramming the virally infected cell to disfavor viral replication) (107). Recognizing a single peptide-class I complex on the surface of a target cell thus provides the most efficient approach for regulating T-cell function.

It is uncertain what fraction of the rapidly degraded pool of proteins is short-lived proteins, and what fraction represents defective proteins. A very small fraction of proteasome-generated peptides are presented by MHC class I molecules to T cells. It is important to note that peptides are subject to further trimming by endoplasmic reticulum associated aminopeptidases (108). The relative contributions of errors in folding, translation, and transcription to the defective protein pool are also unclear. Data show that a virus nuclear antigen of Epstein-Barr virus (EBV) has an amino-terminal sequence that disfavors proteasome degradation and also reduces translation of its own message. Together, these features reduce the generation of EBNA1 peptides, enabling cells harboring EBV to escape immune surveillance (109). Other studies showed that ribosome can initiate translation aberrantly, generating unintended translation products that contribute to defective protein pool (110-112).

The proteasome can recognize and degrade a class of substrates that do not require ubiquitin modification (97). Ornithine decarboxylase (ODC) is one of such substrates. ODC catalyzes the initial step in polyamine biosynthesis and is regulated by end products spermidine and spermine, through the regulatory protein antizyme 1 (AZ1) (113). Excess polyamines induce the expression of AZ1, which binds the ODC monomer, dissociating the active ODC homodimer and thereby inhibiting its activity (114). AZ1 binding exposes a C-terminal degradation signal in the ODC protein, resulting the degradation of ODC independent of ubiquitination (115). Further studies indicate that the degradation of ODC can process independent of mammalian AZ1, with the degradation signal present in five amino acids on the C-terminal and Cys441 of ODC (116). Other examples of ubiquitin independent 26S proteasome degradation include p21 and RPN4 (117). The protein p21 is a cyclin-dependent kinase inhibitor and RPN4 is a transcriptional activator of genes encoding subunits of the proteasome. Interestingly RPN4 protein is short-lived and interacts with the Rpn2 subunit of the base of the 19S regulatory particle (117).

8. THE PLASTICITY OF PROTEASOME IN THE CNS

Oxidative stress decreases the proteasome peptidase activities in a rapid manner. Treatment with diamide, a potent oxidant, decreased 20S core proteasome activities, de-ubiquitinating activity, and 26S proteasome activities (118). It is suggested that in the CNS the proteasome is progressively inhibited by small accumulations of oxidized and cross-linked proteins, and the impaired proteasome system then promotes further accumulation of oxidized and aggregated proteins. Because the proteasome is composed of multiple proteases, the individual activities of proteasome may be altered differently following oxidative stress. Ethanol administration, which is regarded as a form of oxidative stress, decreases the chymotrypsin-like activity and the trypsin-like activity by 35% to 40%, without affecting the caspase like activity significantly (119). Aged animals have decreased proteasome activity, with the individual peptidase activities differently affected during aging (120).

During the development of glaucoma, the protein levels of proteasome α subunits increase ~3 folds as determined by Western Blot (121). After the injection

of lipopolysaccharide (122), the inducible subunit LMP7 (PSMB9) shown increased protein level in kidney, heart and lung but not brain (123). Interestingly, those organs (kidney, heart and lung) had decreased weights 3 days after LPS injection (123). After global ischemia the expression of 26S complex subunit PSMD1 was elevated at 12 hours in the dentate gyrus (124). After 24 hours, PSMD1 increased its expression significantly in both the CA1 and dentate gyrus compared with control animals. This alteration in proteasome expression was also associated with the change of transcriptional factor (SEF-2) (124).

Data from our laboratory demonstrate that neural proteasome expression is increased in response to oxidative stress (15) and following the expression of proteins with polyglutamine extension (53). These changes in proteasome expression (increased immunoproteasome expression) were associated with a preservation of proteasome function. However, following an additional stressor (heat stress) the proteasome was unable to increase its activity in neural cells with increased immunoproteasome expression (53). These data suggest that proteasome plasticity in the CNS may have beneficial effects in the short-term, but the long-term effects may be deterious, based on the fact that the immunoproteasome appears unable to respond to subsequent stressors.

Proteasome plasticity is a relative new concept, and may explain some of the current controversies associated with the role of the proteasome in neurodegenerative disorders. In AD, HD, and Parkinson's disease (PD), neurodegeneration likely requires decades. It is unlikely that the proteasome contributes to neurodegeneration in these disorders by undergoing permanent and dramatic decreases in function. Far more likely, in each of these conditions there is a short-term proteasome inhibition that is followed by intracellular changes that allow the cells to recover proteasome-mediated protein degradation in the short-term. Changes in proteasome expression, proteasome complex function, and proteasome localization are very likely to play a direct role in mediating these beneficial short-term adaptations. However, the long-term and cumulative effects of proteasome alterations may ultimately result in cytotoxicity and neurodegeneration.

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3

PROTEIN AGGREGATION AND THE UPS: A TWO-WAY STREET

Kostas Vekrellis and Leonidas Stefanis

1. INTRODUCTION

Neurodegeneration in many cases is associated with protein misfolding, assumption of oligomeric forms, frank aggregation and inclusion formation. These altered protein conformations are widely thought to be instrumental in neurodegeneration. In this chapter we will first briefly introduce the concepts underlying the assumption of such aberrant conformations, generally labeled as "protein aggregation", and then examine how such configurations may impact the ubiquitin proteasome system (UPS). Specific neurodegenerative diseases associated with protein aggregation and their link to the UPS will be discussed in later chapters in section 6.

2. PROTEIN AGGREGATION IN NEURODEGENERATION

2.1. Commonality of Aggregated Protein Conformations in Neurodegenerative Diseases

Many neurodegenerative diseases and to some extent physiological ageing are characterized by conformational changes and aggregation of specific proteins, resulting in intra or extra neuronal accumulation of "amyloid fibrils". Mutations in such "aggregate-prone" proteins cause inherited forms of disease, as is the case in Alzheimer's, Parkinson's, Huntington's and prion diseases. A common feature of neurodegenerative disorders characterized by protein aggregation is that the aggregate-prone proteins, despite the fact that they are unrelated in size or amino-acid sequence, are detergent-insoluble, and have high β-sheet content and a cross beta structure. These common biochemical features suggest the possibility of a conserved mechanism of pathogenesis in these otherwise phenotypically diverse disorders. The commonality of such aggregate-prone proteins is further supported by the finding that a single antibody can recognize a common conformational epitope displayed by aggregated Aβ, α-synuclein and polyglutamine-containing peptides (1). Incubation of cell cultures with this antibody blocks the toxicity of these protein oligomers, further indicating that a common mechanism of toxicity may operate in these diseases.

2.2. The Process of Protein Aggregation

The presumed sequence of events leading from the monomeric form of an aggregation-prone protein to its aggregation into insoluble fibrils and the eventual formation of intracellular inclusions or extracellular deposits is depicted in Figure 1. It should be noted that there is no definitive proof that such an ordered sequence of events occurs in vivo, but the available evidence suggests that the steps depicted in Figure 1 occur in most cases. The first step in the process is

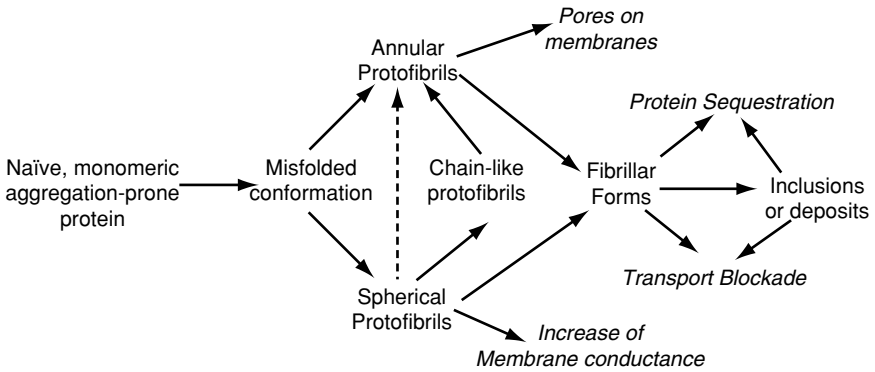

Figure 1. Pathway of protein aggregation: The sequential events leading to protein aggregation are depicted. The dotted arrow indicates that spherical protofibrils can be converted directly to annular protofibrils, or via the formation of chain-like protofibrils. We have indicated in italics possible effects on cellular homeostasis along the pathway of protein aggregation.

thought to be the assumption of a misfolded protein conformation. This essentially means an increase of the β-sheet conformation of the protein and the exposure of hydrophobic domains. Circular dichroism spectroscopy has shown a transition from totally random structure to predominantly β-sheet in solution of Aβ and α-synuclein (2). These β-sheets tend to self-aggregate, forming intermolecular bonds that aggregate the proteins or peptides, leading to the assumption of oligomeric forms, which represent dimers, trimers and multimers of the native protein linked together by covalent bonds. It is thought that such multimers may consist of hundreds of monomeric proteins. Oligomers, when initially formed, are soluble. Such soluble oligomeric species are also termed protofibrils. They are prone to form frankly fibrillar structures, which are insoluble. Twisted fibrils represent the building blocks on which extracellular deposits or intracellular inclusions are created. Such deposits and inclusions are the visible outcome of this process, which can be identified by imunohistochemistry against particular aggregation-prone proteins, or by dyes such as Thioflavin-S and Congo Red, which bind avidly to fibrillar conformations.

Key events in the pathway are thought to be the generation of protofibrillar forms and their conversion to frank fibrils. Spillantini et al., (3) using immunoelectron microscopy, proposed a model in which α-synuclein molecules assemble first to form protofibrils, two of which could associate to produce a variable twisted mature fibril. Intermediate protofibrils disappear as fibrils appear (4-6). The transition from protofibrils to fibrils is thought to be sudden, unpredictable and accelerated by the addition of seed fibrils (4). The fibril morphology strongly suggests that the transition involves the association and winding of protofibrils possibly accompanied by a conformational change. Atomic force microscopy (AFM) of the aggregation of Aβ and α-synuclein has been used to characterize the species in the aggregation process. Protofibrils assessed by this method appear as a mixture of morphologies including spheroid, annular pore-like and chain–like structures (7). In the case of α-synuclein, AFM studies show two distinct forms of α-synuclein protofibrils: rapidly formed spherical protofibrils and annular protofibrils which are produced on prolonged incubation of the spheres (8). It has been demonstrated that fibril formation is a nucleation dependent process resembling in some ways crystallization. Specifically, for Aβ, supersaturated solutions of the peptide are metastable but fibril formation can be seeded by addition of small amount of preformed amyloid fibrils (9). The simplest version of a nucleation dependent polymerization process involves two stable states, the monomeric protein and the fibril. Once the critical concentration of monomer is reached, an equilibrium between monomer and fibril is rapidly established. An alternative mechanism by which effective fibril concentration can be reached is non specific molecular crowding (10,11). This hypothesis suggests that in a crowded cell environment such as the neuronal cytoplasm the volume accessible to any given protein is decreased and protein equilibrium is driven to the lower volume species such as oligomers/fibrils. Significant increases in the level of molecular crowding during ageing could result from a reduction in cell volume and perhaps the inhibition of protein degradation. In this regard proteasomal dysfunction, which has been reported for a number of neurodegenerative diseases, can significantly increase the level of crowding.

Other extrinsic factors have been reported to influence the generation of aggregated species. For example, post-translational modifications of α-synuclein, such as oxidation, nitration or phosporylation have been reported to influence its ability to aggregate in purified solutions or in a cellular context. In particular, oxidation and nitration lead to enhanced rates of fibril and inclusion formation (12-14). Phosphorylation at serine 129 leads to enhanced aggregation, whereas the opposite effect is observed with phosphorylation at multiple tyrosine residues by p72syk (Syk) (15-17). Of particular interest to Parkinson's disease, dopamine and its metabolites were found in a blind screen to accelerate the formation of α-synuclein oligomers, but to also inhibit their conversion to mature fibrils, thus leading to a net marked increase of α-synuclein oligomeric forms (18-20) Heat shock proteins, such as HSP70, on the other hand, have been reported to decrease the rate of α-synuclein oligomer formation (21). HSP70 and HSP40 also inhibited the formation of annular and spherical oligomers of a mutant Huntingtin fragment (22). It is clear therefore that the overall cellular context may determine whether an aggregate-prone protein will follow the pathway of aggregation and which species in this pathway will be favored.

2.3. A Search for the Toxic Species: The Protofibril Hypothesis

As the extracellular or intracellular deposits of neurodegeneration-related proteins are?the obvious, dramatic hallmarks of the aggregation process, for a long time the idea had been that such end-products of the aggregation process or, at the very least, the fibrillar forms prior to their incorporation in the deposits, may be the toxic species (23). The possibility that oligomeric species and not the fibril itself could be pathogenic arose when oligomers rich in? β-sheet structure termed protofibrils where found to be discrete intermediates in the fibrillization of Aβ and α-synuclein in vitro (24,25). A role for soluble oligomers in neurodegenerative diseases is further supported by the following observations: there is no correlation between fibrillar deposits at autopsy and the clinical severity of Alzheimer's or Parkinson's disease (24), transgenic mouse models of these conditions have disease-like phenotypes before fibrillar deposits can be detected (25) and non-fibrillar Aβ oligomers are toxic in cell culture and induce neuronal dysfunction in vivo (26-29). The protofibril hypothesis is also supported by biophysical studies of mutant variants of Aβ and α-synuclein linked to autosomal dominant forms of disease. The Arctic mutation within the Aβ? peptide has an increased propensity to form protofibrils as compared to wild type Aβ suggesting that this mutation predisposes individuals to early onset Alzheimer's Disease due to the formation of relatively long-lived toxic protofibrils (30,31). The A30P and A53T mutations of α-synuclein associated with familial forms of Parkinson's Disease both promote protofibril formation relative to wild type α-synuclein (32,33). The A30P mutation was also shown to delay the formation of amyloid fibrils relative to the wild type protein, suggesting that α-synuclein protofibrils rather than fibrils may be the pathogenic species (19)

The study mentioned earlier, where dopamine or its metabolites accelerated α-synuclein oligomerization, but impeded fibrillization, is also consistent with this idea. An elegant recent study provides further support for the idea that

prefibrillar forms are the toxic species. Arrastre et al. (34) performed a quantitative longitudinal immunofluorescence analysis of single live striatal neurons following the overexpression of truncated forms of mutant Huntingtin fused to GFP, and observed that neurons that did not form inclusions died earlier that those that did. This implies that fibrillar inclusions are protective, rather than toxic.

2.4. What are the Targets of the Toxic Effects?

Although the toxic oligomer hypothesis has gained considerable ground in recent years, it is not proven, and, as such, it is still possible that frank fibrils and deposits may also exert toxic effects. What could these effects be? The major ideas have been that the inclusions either act as physical barriers to information flow in the cell, or that they sequester vital cellular components (23). Such vital components include the monomeric forms of the aggregation-prone proteins. There is more evidence to support this idea in the case of Huntington's Disease, where the depletion of Huntingtin in animal models has been shown to have dramatic consequences on neuronal viability (35). The idea of the physical barrier may make more sense when inclusions, or large aggregates on their way to develop into inclusions, occur at the level of the neuronal processes. Gunawardena et al. (36), demonstrated that in a fly model of Huntington's disease pathogenic polyglutamine proteins exerted their toxic effect on neurons by simultaneously sequestering soluble motor proteins from other critical pathways and by blocking axonal and vesicular transport by forming inclusions on narrow axons. The importance in transport of neurotrophic factors is also evident in Alzheimer's disease, where one of the earliest detectable signs of disease is the loss of synapses and retrograde degeneration of neurons, accompanied the decay of intracellular traffic (37). In addition, excess of APP proteins containing the toxic Aβ region perturbed axonal transport pathways and caused neuronal cell death (38).

The toxic effects of protofibrils have been the focus of intense research recently. The similarity of annular protofibrils to a class of pore-forming bacterial toxins (39), suggests that inappropriate membrane permeabilization might be the cause of cell dysfunction and cell death in neurodegeneration (40). This constitutes the "amyloid pore" hypothesis (41). Consistent with this idea, protofibrils may reside in either the cytosolic or extracellular compartments, and cytosolic protofibrils are toxic when applied externally to cells (26,42). These data further point to the plasma membrane as a potential primary target of annular protofibrils. Indeed, there are several reports of membrane perturbations caused by amyloids like Aβ, but it is not clear whether these effects are specific to soluble oligomers. According to the amyloid pore hypothesis the Aβ peptide of AD permeabilizes phospholipid membranes and forms channels in excised neuronal membranes and in cells in culture (43,44). Other amyloid proteins form pores or channels, including a fragment of the prion protein, a polyglutamine peptide, lysozyme, and calcitonin (45-48). Protofibrils of wild type α-synuclein bind and permeabilize acidic phospholipid vesicles (40). Protofibrillar A30P and A53T and mouse variant were each found to have greater permeabilizing activities per mole than wild type human α-synuclein (20,33). The leakage of vesicular contents

induced by protofibrillar amyloids could damage cells by (a) disrupting membrane potentials and ion gradients, (b) causing loss of vital intracellular ions such as K^+ and Mg^{2+}, (c) allowing influx of toxic ions such as Ca^{2+}, (d) running down energy stores by forcing ion pumps to work harder, (e) disrupting mitochondrial membrane potential and initiating apoptosis by allowing cytochrome c to leak out of mitochondria, and (f) allowing toxic enzymes and other factors to leak out of lysosomes and peroxisomes.

The circumstantial evidence supporting the amyloid pore hypothesis is strong. However, the above studies are essentially all performed in test tubes, with synthetic membranes, and it has not been shown that pore formation occurs by exposure to amyloid-prone proteins in a neuronal cell context or in vivo. Therefore, it remains to be seen if the amyloid pore hypothesis applies to neurodegenerative disease states.

In a recent study, Kayed and coworkers (1), using pure populations of various synthetic oligomers (Aβ, α-synuclein, islet amyloid polypeptide (IAPP), polyglutamine or prion fragments oligomers), suggested that all amyloid oligomers, regardless of sequence, exert their toxic effects not via pore formation but rather via an increase in the lipid bilayer conductance. This would be expected to lead to an increased permeability of the plasma membrane, independent of pore formation. The conductance increase was shown to be dependent on the concentration of spherical oligomers and could be reversed by the addition of a pan-oligomeric antibody. The effect on membrane conductance was not observed by low molecular weight species or by mature fibrils.

3. RELATIONSHIP OF PROTEIN AGGREGATION TO THE UPS

Recent evidence clearly suggests that there is a reciprocal relationship between protein aggregation and the UPS, meaning that one can influence the other (Figure 2). This raises the chicken and egg dilemma (49). Even in cases in which the role of the UPS is not primary, it may serve as an important

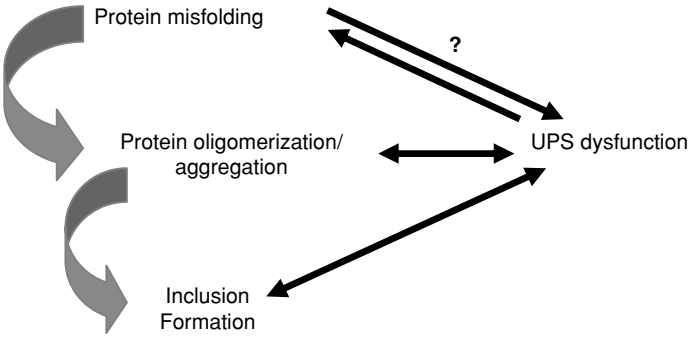

Figure 2. Convoluted relationships: Reciprocal interaction between the UPS and the protein aggregation pathways. It appears that protein misfolding in the absence of oligomerization does not lead to UPS dysfunction (Bennett et al (2005)), although this still awaits further experimental verification, hence the question mark.

feed-forward amplification loop. In this chapter more emphasis will be placed on the effects of protein aggregation on the UPS. Effects of proteasomal dysfunction on protein aggregation and inclusion formation will be discussed in more detail in chapters 4 and 5.

3.1 Effects of Protein Aggregation on the UPS

Although there is no complete agreement on the subject, numerous studies now support the idea that protein aggregation can lead to UPS dysfunction or, at the very least, to compensatory alterations of this system. Such studies are mentioned in more detail in the chapters in Section 6 dealing with particular disease states associated with specific protein misfolding. What is not clear is the nature of the interaction between aggregated proteins and the UPS.

3.1.1 Which Protein Conformations Along the Pathway of Aggregation Impact the UPS?

The particular forms of aggregated proteins that interact with the UPS are unknown. However, some clues are beginning to emerge from recent studies. A landmark study in the field has been the one by Bence et al. (50), from Ron Kopito's lab. These investigators used accumulation of GFP^u, a modified derivative of GFP (Green Fluorescent Protein) that contains a short sequence that targets it to the proteasome for degradation, as an index of UPS dysfunction. They demonstrated that expression of two aggregation-prone proteins, the ΔF508 mutant of cystic fibrosis conductance regulator (CFTR) and an N-terminal fragment of Huntingtin with an expanded polyglutamine repeat (Q103), in Human Embruonic Kidney (HEK) cells was associated with GFPu accumulation. ΔF508 CFTR misfolds and aggregates in the ER, and is retrotranslocated to the cytoplasm, where, depending on its level of expression, its inefficient breakdown by the proteasome may lead to the formation of visible inclusions that have been termed "aggresomes" (51). HEK cells that harbored inclusions generated by ΔF508 or Q103 overexpression showed increased GFPu fluorescence compared to those that demonstrated a diffuse immunostaining pattern (50). Although the authors did not address this point directly, these results seem to indicate that the last step in the aggregation process, i.e. the well formed inclusions, are the ones associated with UPS impairment. How could this occur? Clearly well formed inclusions are unlikely to interact with the proteasome as substrates. They may however act as "sinks" in which proteasomes are recruited during the increasingly futile attempt to degrade the misfolded proteins. This may eventually lead to the depletion of the proteasome and of other UPS components from their usual site of action and subsequent UPS dysfunction. Consistent with this idea, Jana et al. (52) showed that expression of N-terminal Huntingtin with expanded polyglutamine repeats in cultured neuronal cells and in transgenic animals led to the incorporation of the 20S proteasome in inclusions. Similar findings of localization of proteasomal components to inclusions have been reported by many other investigators in various cellular and animal studies, and also in human post-mortem specimens (for example, (53–56). Jana et al. (52) in addition nicely show that this

shift in localization is coupled with a decrease of proteasomal activity in the soluble fraction and an increase in the insoluble fraction of the neuronal cells. Levels and turnover of the proteasomal substrate p53 increased in the cells as a result of this shift. This suggests that in this model it is not the enzymatic function of the proteasome that is suffering, but rather that the problem is due to sequestration within the inclusions.

Apart from the proteasome, other components of the UPS, such as ubiquitin itself or ubiquitin-binding proteins, also associate with inclusions in neurodegenerative diseases (57,58). It is therefore possible that it is the sequestration of such proteins, in addition to that of the proteasome, within inclusions, that leads to UPS dysfunction. Interestingly, ataxin-3, a protein linked in its mutated, polyglutamine-containing form to the neurodegenerative disease Spinocerebellar Ataxia type 3 (SCA3), is itself such an ubiquitin-binding protein (58–61), and it has been proposed that its depletion may lead to cellular dysfunction (58). There is considerable debate about whether actual depletion of such proteins from their normal site of action occurs, with arguments offered both for (58,62) and against such an effect (63). It may be that these effects are highly dependent on the cellular context, and therefore ideally this issue should be resolved in experiments in neuronal cells, or, even better, in vivo.

Although at first glance the compelling data of Bence et al. (50) implicate defined inclusions in UPS dysfunction, this is not necessarily the whole story. It should be kept in mind that the same cells that harbor inclusions likely have a higher burden of the earlier, less developed forms of aggregated proteins. In this case, the presence of the inclusions might also be a marker of the increased burden of more soluble aggregated proteins. In fact, studies of some polyglutamine disease protein-induced inclusions suggest that such structures may be highly dynamic, in that many components associate transiently with the inclusions (62,64,65). Data to support this idea come from the study of Arrastre et al. (34) mentioned earlier. Two days after transfection of striatal neurons with a fluorescent-tagged polyglutamine-containing protein, diffuse GFP intensity was higher in the neurons harboring inclusions compared to those that did not. Assuming that part of that increased GFP fluorescence reflects misfolded Huntingtin not incorporated in the inclusions, such species could be the ones responsible for UPS dysfunction. The same group, using the same longitudinal single cell analysis, has now preliminary findings showing that in fact UPS function appears to be inhibited before inclusions are formed, when the diffuse intracellular levels of mutant huntingtin are high, and that actually the initial at least formation of the inclusion is associated with a relative normalization of proteasomal function (66).

In fact, a more recent follow-up study from Ron Kopito's lab has added further weight to the idea that it is not inclusions per se, but rather less mature forms of aggregate intermediates that are associated with UPS impairment. In this study, even cells with diffuse accumulation of mutant polyglutamine-containing proteins, without visible inclusions by immunocytochemistry, showed GFPu accumulation, indicative of UPS impairment (63). It should be noted however that the accumulation of GFPu in these diffusely immunolabled cells appeared to be of lesser magnitude compared to cells showing inclusions, although a direct comparison was not performed. Bennet et al. (63) also expressed a mutant form of the von

Hippel Lindau protein, which misfolds but does not aggregate, and did not detect an increase in GFPu fluorescence. This provides an additional important insight, that protein misfolding alone, without further progression in the aggregation pathway, does not appear to lead to UPS impairment.

These data in conjunction suggest to us the possibility of a two-hit model: first, relatively soluble oligomeric forms of aggregated proteins impact the UPS, likely by a direct interaction (see below); this is followed by the gradual formation of the inclusions, which initially may absorb the more soluble aggregated forms and actually improve UPS dysfunction. However, as soluble aggregates continue to accumulate in the cell, and a dynamic equilibrium is established between them and the frank inclusions, this initial improvement is abrogated. Finally, at later stages in inclusion formation, depletion of UPS components, including the proteasome, occurs, impacting further the already compromised UPS function.

3.1.2 Are the Effects of Protein Aggregation on the UPS Direct or Indirect?

How are the effects of aggregated proteins potentially mediated at the level of the UPS and in particular the proteasome? One idea is that misfolded/aggregate-prone proteins that are degradation substrates of this system at the same time "clogg" or "choke" its limited capacity and thus lead to its dysfunction. This need not be a complete arrest of the pathway. Even a slowing down of the process may have significant effects on the accumulation of UPS substrates. It should be kept in mind that more fully aggregated forms appear to resist proteasomal degradation (67), and to be degraded in large part through autophagy (68). It is therefore the less aggregated misfolded intermediates that are likely to still function as UPS substrates and to have a direct effect on the UPS.

Data in support of the "clogging" idea and of a direct effect of aggregated proteins on the UPS come from the study of Holmberg et al. (69). These investigators observed by live cell imaging a stable interaction between the proteasome and truncated Huntingtin with expanded polyglutamine repeats or a protein consisting solely of polyglutamine repeats. Furthermore, such proteins were aberrantly and slowly degraded by the proteasome both *in vitro* and in a cellular context. In contrast, Bennett et al. (63) found that exposure *in vitro* of isolated proteasomes to gradually fibrillizing Huntingtin did not lead to impaired proteasomal function, as measured by degradation of model substrates. These discrepancies are difficult to resolve at this point, but suggest to us that at least in certain settings "clogging" of the proteasome by ubiquitinylated, aggregated substrates may occur.

There is also the potential for indirect effects of protein misfolding/aggregation on the UPS. In fact, the study by Bennett et al. (63) has elegantly shown that in their model in non-neuronal HEK293 cells, effects of aggregated Huntingtin on the UPS are to a certain extent indirect. They used constructs and reporters that were selective for nuclear or non-nuclear compartments, and were thus able to show that protein aggregation affects UPS function in the *trans* compartment. The magnitude of this effect seemed less than that observed in the *cis* compartment, suggesting to us that both direct and indirect effects may be operative. Additional support for indirect effects is provided by Sanchez et al. (70).

These investigators also found proteasomal dysfunction, measured by an enzymatic assay, in response to overexpression of truncated mutant Huntingtin in HeLa cells. Congo Red inhibited protein aggregation and toxicity by its action on soluble oligomeric forms of mutant Huntingtin. This effect apparently targeted the now more soluble forms to the proteasome, leading to an interaction of mutant Huntingtin with the proteasome only in the presence of Congo Red. This interaction was associated in fact with improvement of the activity of the proteasome. In this case therefore an increased direct interaction with the proteasome is associated with an improvement, not a deterioration of proteasomal function. An early effect of mutant Huntingtin overexpression in this model is energy depletion, which may impact proteasomal function (70). In another study, caspase activation was shown to be associated with cleavage and inactivation of proteasomal subunits and resultant proteasomal dysfunction (70,71). Sanchez et al. (70) found that the general caspase inhibitor zVAD-FMK not only prevented death, but also ameliorated proteasomal dysfunction, suggesting that such dysfunction was a direct or indirect effect of caspase activation. It appears therefore that oligomeric mutant Huntingtin in this system exerted its effects on the UPS indirectly.

3.1.3. The Paradigm of α-Synuclein

It is worth mentioning here studies performed with various forms of α-synuclein, a molecule linked to Parkinson's disease (PD) by its prominent localization in an aggregated conformation within the characteristic Lewy bodies and by the presence of mutations in rare families with autosomal dominant PD (see also chapter on Parkinson's disease by Mark Cookson). We and others have shown that expression in neuronal cell systems of mutant forms of α-synuclein is associated with a modest UPS inhibition (72–74). In our initial study, we found no evidence for frank aggregation of α-synuclein, as assessed by immunostaining or fractionated Western immunoblotting (72). The immunostaining pictures shown by Tanaka et al. (73), although not commented upon, also appeared to show a diffuse pattern, with no visible aggregates. This suggests that the form of α-synuclein linked to proteasomal dysfunction is not the frankly aggregated one, but rather the soluble monomeric form, or soluble oligomeric forms. The monomeric form of α-synuclein has a natively unfolded structure and, as such, could perhaps have a direct adverse effect on proteasomal function. In vitro studies show that α-synuclein, especially the oligomeric-aggregated conformation, can directly inhibit proteasomal function (75–77). In conjunction, these data suggest that the inhibition of proteasomal function observed in the cellular systems with mutant α-synuclein overexpression may be due to soluble oligomeric forms. Using a special delipidation protocol, we have recently detected such soluble oligomeric forms in extracts of PC12 cells expressing mutant, but not wild type α-synuclein, adding further support to this idea (Figure 3). Further research however is needed to show an association of α-synuclein with the 20S or the 26S proteasome in a cellular context, and to determine the form of α-synuclein involved (monomeric versus oligomeric). A note of caution is in order here: Although initial studies

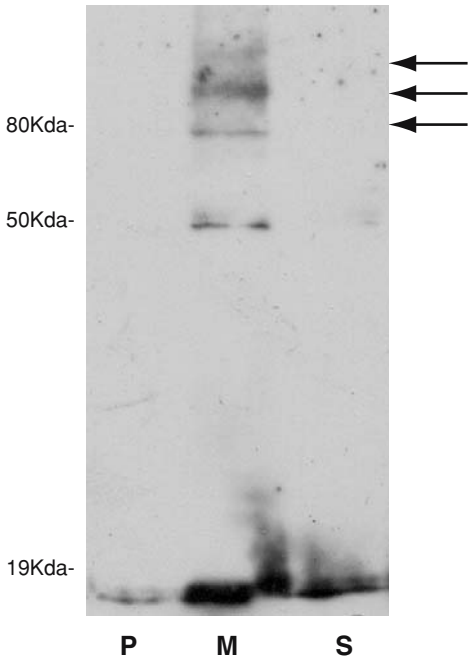

Figure 3. S50 cytosolic lysates of PC12 cells stably expressing either empty vector (P), mutant A53T α-synuclein (M), or wild type α-synuclein (S) were exposed to a delipidation protocol and then run on an SDS/PAGE gel. The monomeric form of α-synuclein is apparent at the bottom of the gel. The arrows indicate oligomeric forms of α-synuclein, that are apparent only in the cells expressing A53T α-synuclein, which are the ones that demonstrate proteasomal dysfunction.

identified α-synuclein as a substrate of the UPS (78–80), more recent studies have shown that the main route of degradation of both monomeric and oligomeric α-synuclein may be through the lysosomes (81–83), and that mutant α-synucleins may inhibit lysosomal function by acting directly at the level of the lysosomal membrane (72,82) It is conceivable therefore that at least part of the effects of mutant α-synucleins on proteasomal function are indirect, for example through the increasingly recognized cross-talk between the pathways of lysosomal and UPS-dependent degradation. Alternatively, soluble oligomeric forms of α-synuclein may have an affinity for certain proteasomal components independent of their nature as proteasomal substrates.

In support of this idea, Lindersson et al. (76) showed that in purified cystosolic systems α-synuclein soluble oligomers bind specifically and stochiometrically to the 20S proteasome, and inhibit its chymotrypsin-like 20S proteolytic activity. They suggested that this may reflect binding of α-synuclein oligomers to the outer surface of the β5 subunit. Binding of α-synuclein has also been reported to S6'/Rpt5/TBP1/PSMC3, a subunit of the 19S proteasome (75–77)

3.1.4 Which Components of the UPS are Affected?

The exact mechanism through which aggregated proteins inhibit the UPS determines also the site of UPS involvement. If the sequestration theory is correct, then various components of the quality control system and the UPS upstream of the proteasome, such as ubiquitin, ubiquitin-binding proteins or Heat Shock Proteins (HSPs), or the proteasome itself, may be sequestered. If misfolded, but still soluble proteins interact directly with the proteasome they could exert inhibitory effects at multiple levels. These levels mirror the normal process of targeting, unfolding and processing of proteasomal substrates that are described in the Introductory chapters.

A first point where soluble oligomeric/aggregated proteins may interfere with the UPS is when such proteins are targeted with ubiquitin molecules. An excess of such substrates may lead to depletion of ubiquitin or related cofactors (point A, Figure 5). As the substrate approaches the proteasome, it is recognized by components of the 19S, such as Rpn10/S5a and Rpt5/S6', to which it binds. It is theoretically possible that "aggregated" proteins bind too strongly or are not readily released from these subunits, with the end result of "clogging" or slowing down of the process (point B), although experimental support for this idea is lacking. The substrate then needs to be deubiquitinated by Rpn11/POH1 (point C) in order to be further processed. Potential inability of the "aggregated" substrate to

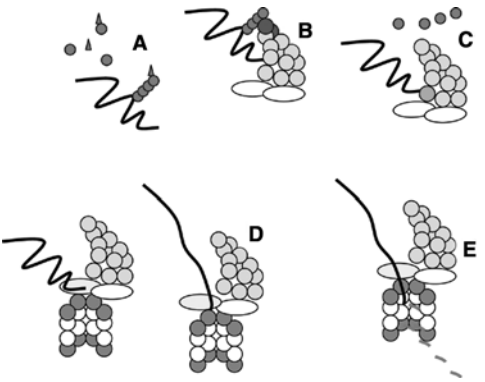

Figure 4. Hypothetical model of potential sites of direct involvement of the UPS with oligomeric/aggregated proteins as these are engaged in the UPS degradation pathway. In A, the aggregated protein substrate, depicted by a curved line, is targeted by ubiquitin moieties, depicted as red spheres. Ubiquitin-binding proteins are depicted by brown triangles. It is possible that an excess of aggregated proteins may lead to depletion of such UPS components at this stage. In B, the aggregated poly-ubiquitinated protein is recognized by elements of the 19S proteasome, depicted in blue. Binding of the substrate to the deubiquitinating component of the 19S, Rpn11/POH1, depicted in green, leads to its deubiquitination (C). Unfolding and threading of the substrate through ATPase subunits at the base of the 19S proteasome (depicted by oval shapes) (D) leads to its entry into the barrel-shaped 20S proteasome and degradation into small peptides, depicted as red curved lines (E). In all these stages (B-E), "clogging" of the pathway may occur due to the nature of the aggregated conformation of the substrate. It should be noted that this depiction is schematic, and that in fact many of these functions – recognition, deubiquitination, threading, degradation, may be performed simultaneously; (See color insert.)

disengage itself from the deubiquitinating enzyme may again lead to clogging of this process. At this point, this idea is also conjectural. Following deubiquitination, the substrate attaches to the ATPases at the base of the 19S proteasome, where it is unfolded and threaded through the pore of the ATPase into the cylindrical pore of the 20S proteasome. Navon and Goldberg (84) have presented data that show that attachment of a large peptide to a proteasomal substrate, a situation akin to what may happen in the case of oligomerization, impedes the unfolding necessary for the threading of the substrate through the pore, and the result is that normal substrates of this system are denied access to the ATPase (point D). Even safely within the proteolytic pore though, "aggregated" substrates may still act as troublemakers. Goldberg and colleagues (85) have recently shown that polyglutamine repeats contained within proteins cannot be digested within the proteasomal enzymatic core. This may cause problems with trafficking within this limited space, as well as the release of the undigested polyglutamine repeat, again acting as a break for the degradation of other physiological substrates (point E).

In all these cases, it should be stressed that even delays in the processing of the "aggregated" proteins, due to their conformation, may have profound effects on the clearance of other physiological substrates.

3.2 Effects of UPS Modulation on Protein Aggregation

As exemplified by the studies summarized in chapter 5 in this volume, it is clear that proteasomal inhibition alone can induce protein misfolding and aggregation in neuronal cells, in that it induces a heat shock response, presumably as misfolded proteins accumulate, it increases levels of detergent-insoluble polyubiquitinated proteins and it induces macroscopic inclusions that contain fibrillar forms of α-synuclein. A process that can in part account for these effects is molecular crowding, as mentioned above. In multiple settings, it has also been shown that pharmacological inhibitors of the proteasome can increase inclusions formed upon overexpression of misfolded proteins (for example, (51,52,55). Interfering with the UPS pathway upstream of the proteasome, at the level of E2 conjugating enzymes, or of E3 ligases, can also modulate inclusion formation. In particular, inhibiting ubiquitination leads to a dramatic decrease of inclusion formation (86-88), suggesting that ubiquitination of proteins is a major factor predisposing to aggregation. It is possible that polyubiquitin chains enable the seeding of inclusions through cross-linking, or that the polyubiquitination of substrates renders them more aggregated and thus prone to form inclusions.

4. CONCLUDING REMARKS

The link between protein aggregation and the UPS in the context of neurodegenerative diseases is still a matter of intense debate. Many issues, especially regarding the nature of the interaction between aggregated proteins and the UPS, remain unresolved. Further experiments, especially in an in vivo context, will be needed to address these issues. Such studies may shed light on the importance of the UPS in neurodegeneration.

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4

THE IMPACT OF INCLUSION FORMATION ON CELL SURVIVAL

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1. INTRODUCTION

Intracellular inclusions are found in many neurodegenerative disorders and are useful pathologic features for diagnostic purposes. Yet, their relevance to the life and death of the neurons in which they reside has been the subject of controversy until recently when technological advances enabled research to address their pathophysiological role. The limitations inherent in studying the cell biological consequences of inclusions in human brain samples have stimulated investigations in experimental systems including inclusions produced in cellular models.

A well defined cellular inclusion that forms in the context of over-expressing certain proteins, particularly in conjunction with proteasomal impairment, is the aggresome. These structures are multi-ubiquitinated cytoplasmic aggregates generated experimentally in cultured cells by over-expressing various proteins including parkin (1), synphilin-1 (2,3), huntingtin (4), cystic fibrosis transmembrane

conductance regulator, presenilin 1 (5), prion protein (6), and the androgen receptor (7), as well as by inhibition of proteasomal activity. These inclusions are characterized by their localization at the centrosome and by the redistribution of intermediate filaments. The function of aggresomes has been postulated to be disposition of misfolded or otherwise damaged proteins that accumulate in cells due to various insults including oxidative stress and proteasomal impairment. Their formation requires an energy-dependent intracellular transport system, since the intermediate state of aggresomes or micro-aggregates that form in the peripheral cytoplasm are transported to the centrosome through the microtubular cytoskeleton mediated by dynein/dynactin complexes (8).

2. PARALLELS BETWEEN THE AGGRESOME AND LEWY BODIES

Several similarities have been pointed out between aggresomes and Lewy bodies (LB), the hallmark pathologic features of Parkinson's disease and of dementia with LBs. In cellular models, the aggresomes that result from the overexpression of parkin or synphilin-1, for example, accompanied with proteasomal inhibition, have both morphologic and immunocytochemical characteristics of LBs, including the core and halo organization and the presence of vimentin, γ-tubulin, α-synuclein, synphilin-1, proteasome subunits, and chaperones (1,9). Additionally, dysfunction of the ubiquitin-proteasome pathway is generally involved in the pathogenesis of PD (10). For example, LBs are rich in ubiquitin and proteasome subunits (11,12), proteasomal activity and expression levels of certain of its components are reduced in the parkinsonian nigra (10,13–15) and α-synuclein, which is an abundant component of LBs (16) and is mutated in rare inherited types of PD (17), is degraded through the ubiquitin-proteasome pathway (18,19). Further, postmortem studies with Parkinson affected brains have shown that LBs contain centrosome components including γ-tubulin, pericentrin, PA700, and P28 (20). Finally, biochemical fractionation of different size α-synuclein aggregates from transfected COS-7 cells and their electon microscopic analysis have revealed that large juxtanuclear inclusions are filled with amyloid-like α-synuclein fibrils, whereas smaller aggregates contain non-fibrillar spherical structures (21). Interestingly, time course analysis from these studies suggest a sequential appearance of aggregates with different sizes and morphological features, with the smaller spherical ones forming first followed by the larger fibrillar ones appearing last. Parallels can be drawn between these observations in transfected cells and the hypothesized role of different shape α-synuclein aggregates formed in vitro (22). The smaller spherical aggregates could represent the cellular equivalents of protofibrils, which are presumed to be toxic, whereas the fibrillar large juxta-nuclear inclusions may represent the equivalent of Lewy bodies. Among various inclusions found in neurodegenerative disorders, the LB resembles an aggresome the most based on its morphologic appearance and its molecular components.

The presence of LBs in the cytoplasm but not necessarily in the juxtanuclear region, as well as the presence of multiple Lewy bodies in some neurons, does not nullify the view that these inclusions are formed through a process akin to that of aggresomes. The difference in the microtubular organization between

neuronal and non-neuronal cells can underlie the morphological differences between LBs in human brains and aggresomes formed experimentally in cultured cells. Microtubules are nucleated at the centrosome in both cell types. But unlike non-neuronal cells, mirotubules are quickly released from the centrosome of neuronal cells through the microtubule-severing protein katanin (23–25). Further, the protein ninenin which typically associates with the centrosome in many cell types and recaptures minus-ends of microtubules after their release, is also present in non-centrosomal locales in cultured neurons where it may impede the recapture of microtubules after their release from the centrosome (26). Thus, poorly anchored microtubules in the neuronal cells may not allow coalesced aggregates to migrate fully to the centrosome and assume a juxta-nuclear localization. The result can be large inclusions dispersed in the cytoplasm as well as multiple inclusions.

3. CHARACTERISTICS OF AGGRESOMES FORMED BY α-SYNUCLEIN AND SYNPHILIN-1

Aggresomes can form efficiently in 293 cells upon the over-expression of synphilin-1 (9,27). This protein, originally isolated by virtue of its interaction with α-synuclein (28), is a presynaptic molecule associated with synaptic vesicles (29) and is present in LBs, especially in the core region (30). Co-expression of α-synuclein and synphilin-1 in cellular models gives rise to eosinophilic cytoplasmic inclusions (28), and over-expression of synphilin-1 alone can also produce inclusions in cultured cells (31). Synphilin-1 is polyubiquitinated and degraded by the proteasome (2).

Treatment of 293 cells stably transfected with FLAG-tagged synphilin-1 (Synph-293 cells (2)) with a proteasome inhibitor results in the development of perinuclear round structures immunoreactive to FLAG-synphilin-1 (Figure 1). These inclusions have all the characteristics of aggresomes. First, vimentin immunoreactivity, which distributes diffusely throughout the cytoplasm under basal conditions, localizes to the halo of synphilin-1 positive perinuclear inclusions upon proteasome inhibition. Second, γ-tubulin, which is a normal component of the centrosome (20,32) and is only weakly immunoreactive in untreated

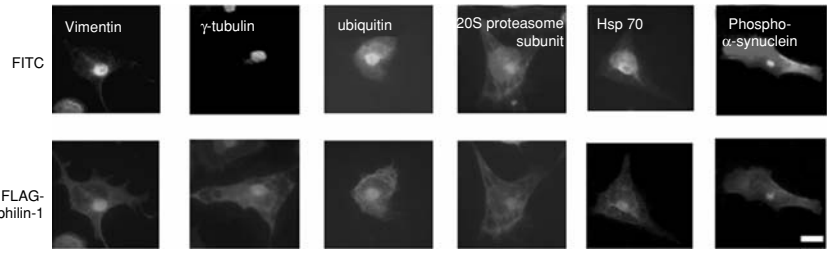

Figure 1. Characterization of aggresomes in Synph-293 cells in the presence of MG132 (9). In addition to FLAG-synphilin-1 staining (lower panels), vimentin, γ-tubulin, ubiquitin, 20S proteasome subunit, Hsp70, and S129 phosphorylated α-synuclein were visualized with fluorescein. Scale bar 10 μm.

cells, acquires an intense signal upon MG132 challenge. Third, synphilin-1 aggregates are immunoreactive to antibodies against ubiquitin, 20S proteasome subunit and to Hsp70, all components of LBs (12,33,34). In particular, the immunoreactivity to Hsp70 localizes intensely to the halo of the aggregates, whereas that of ubiquitin and 20S proteasome subunit distributes homogeneously in both the core and halo. Finally, an antibody raised against phosphorylated α-synuclein at serine 129 immunostains these inclusions, similar to LBs (35). Therefore, these cytoplasmic inclusions represent aggresomes based on morphological and molecular characteristics, including the round structure with a core and halo and the juxta-nuclear localization, as well as based on the presence of centrosome components (36). In addition, similarities between aggresomes and LBs are revealed by the presence of several constituents in both structures including serine 129-phosphorylated α-synuclein (35).

As expected from the known properties of aggresomes, those formed in Synph-293 cells require an intact microtubular transport system. Counting the cells under a fluorescent microscope reveals that only about 7% of Synph-293 cells have synphilin-1 positive aggresomes at basal conditions, but this percentage jumps to 48% in the presence of MG132 (Figure. 2). Treatment with microtubule destabilizing agents, vinblastin or nocodazole, reduces the number of aggregate containing cells, consistent with the properties of aggresomes (37).

4. EVIDENCE FOR THE CYTOPROTECTIVE EFFECT OF AGGRESOMES

Whether such large juxta-nuclear inclusions in cellular models contribute to neuronal death or protect cells from the toxic effects of misfolded proteins is a

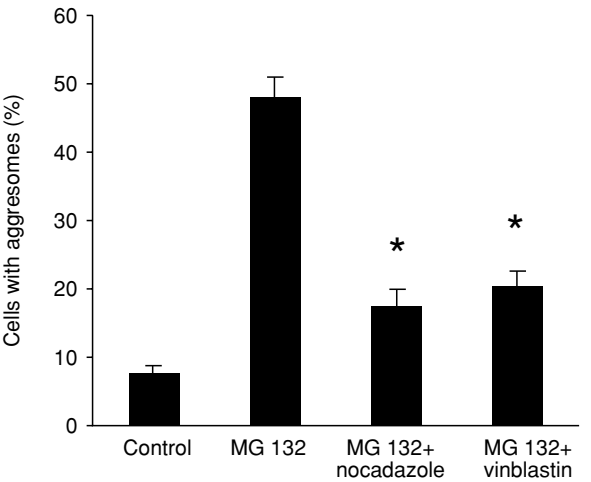

Figure 2. Effect of microtubule destabilization on aggresome formation (9). Synph-293 cells were treated as indicated, and immunostained with anti-FLAG antibody. Three hundred cells were counted from ten randomly selected fields. Values represent means±S.E.M. (n=3) * ANOVA $p < 0.01$ compared to MG132 treatment alone.

question that can have relevance to similar large inclusions in neural diseases such as the LB. Experimentally, FLAG-synphilin-1 positive aggresomes co-localizing with ubiquitin are detected in both non-apoptotic and apoptotic cells identified by condensed DAPI stained nuclei. Similarly, vimentin immunoreactive aggresomes are observed in some apoptotic and non-apoptotic cells. Thus, aggresomes can be detected even in relatively late stages of apoptosis. However, such qualitative assessments cannot establish a correlation between aggresomes and apoptotic nuclei. Some aggresome bearing cells are healthy looking, while some aggresome negative cells are clearly apoptotic.

Quantitative analyses indicate that aggresomes formed by α-synuclein and synphilin-1 over-expression are indeed cytoprotective (9). Synph-293 cells challenged with different α-synuclein isoforms demonstrate various degrees of apoptosis and inclusion formation. In these studies, EGFP is co-transfected in order to distinguish transfected cells from non-transfected ones. Green fluorescent positive cells are evaluated for apoptosis by DAPI staining and for aggresome content by FLAG-synphilin-1 immunoreactivity. Under these conditions, two pathogenic mutants of α-synuclein, A53T and A30P, exacerbate cell death more than their wild-type counterpart (Figure. 3). Toxicity due to α-synuclein mutants increases significantly by 2.0- to 2.5-fold compared to mock control cells. On the other hand, aggresome formation in α-synuclein transfected cells is only minimally higher than in empty vector transfected cells. The number of aggresome bearing cells with A30P expression, for example, is only 1.3 times more than in mock transfected cells. These results suggest that aggresome formation does not predictably correlate with apoptotic cell death.

A connection, if any, between aggresome formation and cell death can also be examined by assessing these two indices in cells treated with the caspase inhibitor z-VAD-fmk. Proteasome inhibition in cells co-expressing wild-type α-synuclein and synphilin-1 causes 19% of the cells to assume apoptotic

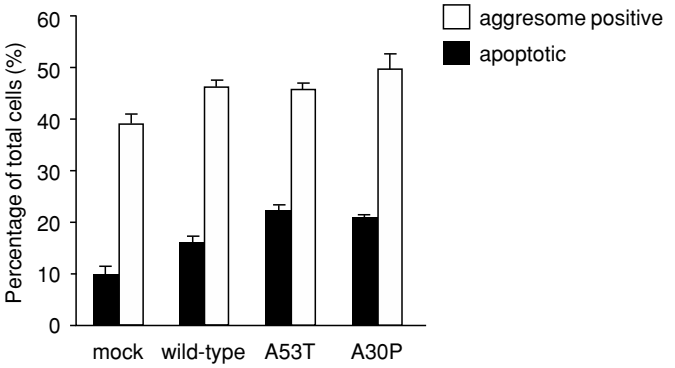

Figure 3. Effects of wild-type and mutant α-synuclein in Synph-293 cells (9). After transiently co-transfecting with α-synuclein and EGFP, and treating with MG132, cells were immunostained for FLAG and with DAPI. Two hundred EGFP positive cells were evaluated for apoptosis (closed bar) and for aggresome formation (open bar) under a fluorescent microscope. Values represent means±S.E.M. of three independent experiments.

morphology. As expected, this percentage diminishes significantly down to 10% in the presence of z-VAD-fmk (Figure. 4*A*). However, aggresome-containing cells, which represent 50% in the presence of MG132 alone, are not affected by the caspase inhibitor (Figure. 4*B*). Therefore, cell death due to the co-expression of α-synuclein and synphilin-1 is not linked to aggresome formation.

The role of aggresomes in cell death can also be studied by evaluating their presence in apoptotic vs non-apoptotic cells. Thus, we have individually classified Synph-293 cells transfected with α-synuclein isoforms, based on apoptotic morphology and aggresome content (Figure. 5). This approach revealed that, among the pool of apoptotic cells, many more cells are aggresome negative compared to aggresome positive (Figure. 5*A*). For example, only about 2% of A53T α-synuclein transfected apoptotic cells have aggresomes, while 20% do not. In addition, the increase in α-synuclein-induced cell death is exclusively among aggresome negative cells (Figure. 5*A*). The co-expression of α-synuclein and synphilin-1 increases apoptosis of aggresome negative cells from 8% to 20% of total cells, whereas the rate of aggresome formation in apoptotic cells remains constant at about 2%. On the other hand, among the pool of non-apoptotic cells overexpressing α-synuclein isoforms, aggresome positive cells are slightly more common than aggresome negative ones (Figure. 5*B*). Under the same conditions, around 40% of total cells have aggresomes but are non-apoptotic. All these observations indicate vulnerability of aggresome negative cells.

Additionally, immunostaining of Synph-293 cells transfected with wild-type α-synuclein for activated caspase-3 reveals that an inclusion-containing cell does not necessarily manifest caspase-3 activation, while an inclusion-negative cell can have activated caspase-3 immunoreactivity (Figure. 6). This result suggests, once again, that inclusions formed by synphilin-1 and α-synuclein are not cytotoxic.

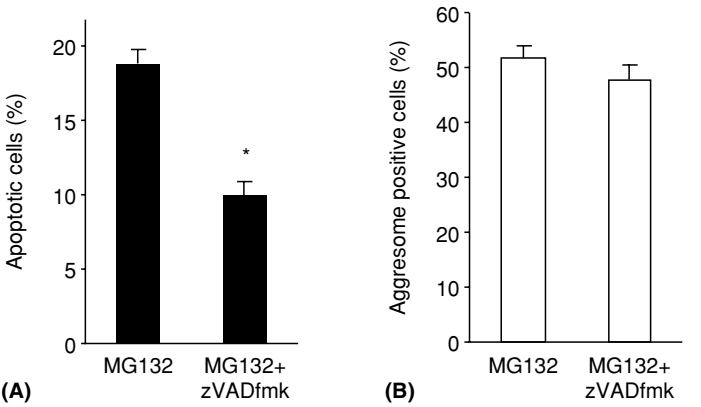

Figure 4. Caspase inhibition reduces apoptosis but not aggresome count (9). Synph-293 cells transiently transfected with wild-type α-synuclein and EGFP were treated as indicated. After immunocytochemistry for FLAG and DAPI staining, transfected cells expressing EGFP were evaluated for apoptosis (*A*) and aggresome formation (*B*). Values represent means±S.E.M. of three independent experiments. *$p < 0.01$ (student t-test) compared to cell death without the caspase inhibitor.

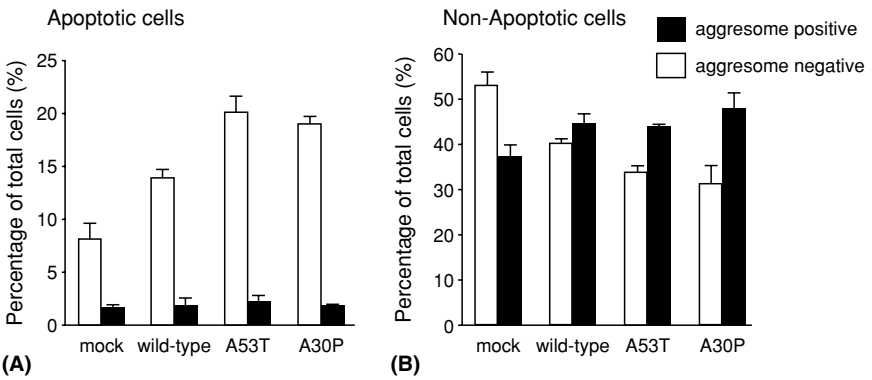

Figure 5. Quantification of aggresome content in apoptotic and non-apoptotic cells (9). Synph-293 cells were transiently co-transfected with the indicated isoforms of α-synuclein in addition to EGFP, and incubated in the presence of MG132 for 12 hr. After immunocytochemistry for FLAG and DAPI staining, transfected cells expressing EGFP were evaluated for aggresome formation in apoptotic (*A*) and non-apoptotic (*B*) cells. Values represent means±S.E.M. of three independent experiments.

5. PROTECTIVE EFFECT OF INCLUSIONS IN NEURODEGENERATIVE DISEASES

The above observations indicate that aggresomes render cells resistant to death induced by the combined over-expression of α-synuclein and synphilin-1 accompanied by proteasome impairment. Therefore, aggresomes appear to have a protective function. This inference is derived in a cellular model with the intrinsic heterogeneity of forming aggresomes in some but not all cells, analogous to

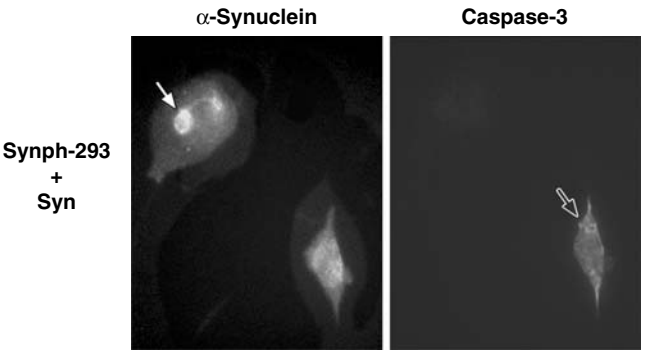

Figure 6. Synph-293 cells transfected with α-synuclein were immunostained for cleaved caspase-3 (rabbit polyclonal antibody 1:50) (right) and for α-synuclein (left), followed by visualization under a fluorescence confocal microscope (Zeiss, LSM 510). A cell without an inclusion but showing caspase-3 activation (open arrow) is seen in the same field as a cell with an inclusion (solid arrow) but no caspase-3 activation.

the presence of inclusions such as LBs in a subpopulation of neurons in affected brain regions in neurodegenerative disorders. The methodological approach of categorizing each cell for these two phenotypic features and quantifying them allows assessment of inclusion formation not only in surviving cells but in dying ones as well. Such an approach, thus, minimizes the bias of assessing only residual surviving cells. Another study observed a significantly reduced number of inclusions but increased susceptibility to staurosporine-induced death in cells expressing R621C mutant synphilin-1, compared with wild-type synphilin-1, when subjected to proteasomal inhibition. This C to T transition in position 1861 of the coding sequence leading to an amino acid substitution from arginine to cysteine in position 621 has been found in two individuals with sporadic PD but not in 702 chromosomes of healthy controls. The authors take their finding to argue that the formation of intracellular inclusions may be beneficial to cells and that a mutation in synphilin-1 that reduces this ability to form inclusions may sensitize neurons to cellular stress (38).

Although the aggresome, and by analogy LBs, have been viewed by some as harmful structures by interfering with metabolic activity or cellular transport due to their space occupying nature (37), accumulating data described in this chapter suggest that they likely represent an active protective response against elevated concentrations of unwanted proteins, particularly since aggresome formation requires an active intracellular transport along the microtubular cytoskeleton (Figure. 2) (8,39). Furthermore, the microtubule-associated deacetylase HDAC6 recruits misfolded proteins to dynein motors for transport to aggresomes (40). Cells deficient in HDAC6 fail to clear misfolded protein aggregates from the cytoplasm, cannot form aggresomes, and are hypersensitive to the accumulation of misfolded proteins (40). Thus, aggresomes are presumed to form as a cellular defense mechanism against elevated concentrations of unwanted proteins (37).

The dissociation between inclusion body formation and cell death in the context of synphilin-1 and α-synuclein over-expression is consistent with previous reports about other pathogenic proteins in neurodegenerative diseases. For example, mutant androgen receptor (AR), the protein responsible for X-linked spinobulbar muscular atrophy, forms insoluble aggregates and is toxic to cultured cells. Mutant AR also forms aggresomes. Molecular and pharmacological interventions used to disrupt aggresome formation have revealed their cytoprotective function. Interestingly, aggresome-forming proteins have an accelerated rate of turnover, and this turnover is slowed by inhibition of aggresome formation. In addition, aggresome-forming proteins can become membrane-bound and associate with lysosomal structures. Together, these findings suggest that aggresomes serve as cytoplasmic recruitment centers to facilitate degradation of toxic proteins (7). Another example is poly-glutamine expanded huntingtin. The exposure of mutant huntingtin-transfected striatal neurons to conditions that suppress the formation of inclusions results in an increase in mutant huntingtin-induced death (41,42). In clonal striatal cells transfected with huntingtin fragments, the caspase inhibitor Z-VAD-FMK significantly increases cell survival but does not diminish nuclear and cytoplasmic inclusions. In contrast, Z-DEVD-FMK significantly reduces nuclear and cytoplasmic inclusions but does not increase survival (42). More recently, direct visualization using a robotic microscope has confirmed that

neurons containing huntingtin inclusions survive better and have lower concentrations of the protein elsewhere in the neuron (43).

Chronically elevated concentrations of proteins such as α-synuclein conceivably exceed the saturation limit of aggresomes. Consequently, misfolded proteins accumulate in the cytoplasm presumably not only as monomers but also as micro-aggregates considered as aggresome intermediates (4). The accumulation of such aggresome precursors upon microtubule disruption reportedly correlates with increased cytotoxicity (7). The protofibrillar oligomeric form of α-synuclein seen in vitro (44) and suggested in cultured cells (21) may represent intermediate aggresomes. Thus, in the cytoplasm of cells that cannot form an inclusion, potentially toxic species of α-synuclein protofibrils could accumulate. On the other hand, in cells that can successfully develop such inclusions, deleterious early aggregates could presumably be sequestered.

6. CONCLUSIONS

Large cytoplasmic inclusions formed under conditions of over-expressing certain proteins and impaired proteasomal function appear to be a commmon cell biological phenomenon recognized in the context of various proteins, many of which are involved in neurodegenerative disorders. Several lines of evidence suggest that these aggresomes are protective rather than toxic. Morphological and biochemical assessments also suggest similarities between aggresomes and Lewy bodies. Since the predominance of apoptotic cells lack aggresomes, an analogy can be extended to the role of LBs in neuronal cells: those that can generate LBs survive preferentially while those that do not form LBs die. Thus, LBs might represent a survival strategy mounted by neurons under the stress of accumulating damaged and misfolded proteins in order to clear the cytoplasm from these damaging molecules. The severe neuronal loss associated with early disease onset in the LB negative autosomal recessive juvenile parkinsonism (45) supports this view.

7. ACKNOWLEDGMENTS

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5

INCLUSION FORMATION AND DISOLUTION FOLLOWING PROTEASOMAL INHIBITION IN NEURONAL CELLS

Leonidas Stefanis and Hardy J. Rideout

1. INTRODUCTION

A number of neurodegenerative diseases are characterized by the presence of proteinacious inclusions present in both neuronal and glial cells, including Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), multiple system atrophy (MSA), Huntington's disease (HD), and others. A great deal of effort has been devoted to the study of how such inclusions are formed and what, if any, relationship exists between their formation and disease progression. Characterization of the components and structure of the inclusions, as well as elucidation of the mechanism of their formation may yield insights into the pathogenesis of each specific disease.

A common element that is emerging among these disparate diseases is the potential involvement of the ubiquitin-proteasome system (UPS). Evidence for involvement of the UPS in these diseases is presented throughout this volume. Such evidence suggests that UPS dysfunction may act as an initiating or potentiating

event in inclusion formation. In this chapter we will address the issue of the formation and dissolution of inclusions in model neuronal systems in which the UPS is inhibited. Specific attention will be paid to the model of pharmacological inhibition of the proteasome in cultured primary neurons used as a model for neurodegenerative diseases.

2. INCLUSION FORMATION AND COMPOSITION IN NEURONAL CELLS FOLLOWING PROTEASOMAL INHIBITION

We and others have applied selective pharmacological proteasomal inhibitors to cultures of neuronal cells in an attempt to model UPS dysfunction. These inhibitors target the 20S catalytic proteolytic activities of the proteasome. Such an approach is based on the fact that in various neurodegenerative conditions the enzymatic activity of the proteasome, measured in post-mortem tissue samples, is inhibited, as reviewed in other chapters in this volume. The idea that this late step in the UPS is dysfunctional is reinforced by the fact that proteinacious inclusions in these diseases contain polyubiquitinated proteins, something that would be expected only if the whole process of ubiquitination and attachment to substrates were intact, but the system were failing at its last step, the actual recognition and degradation within the proteasome.

It should be noted that such an approach has been criticized by some as rather crude, because it disrupts the whole UPS, leading to generalized cellular defects. However, this is what appears to occur in at least some neurodegenerative diseases, where assays that have been performed point to such global deficits of proteasomal function in specific neuronal populations. Furthermore, as will be evident from this chapter and from chapter 9, where the consequences of proteasomal inhibition on neuronal survival are analyzed, application of pharmacological proteasomal inhibitors leads to activation of specific cellular pathways that can be manipulated selectively.

When we initiated these studies in neuronal cells, it was already known by pioneering studies, mainly by Wozcik (1) and Johnston (2), that application of pharmacological proteasomal inhibitors to non-neuronal proliferating cells can lead to the formation of proteinacious inclusions. In addition, Johnston et al. had shown that overexpression of proteins that misfold in the endoplasmic reticulum, such as the mutant cystic fibrosis protein, leads to the formation of similar inclusions, and that their formation was considerably enhanced in the presence of proteasomal inhibition. Further characterization of such inclusions showed that they are formed by a microtubule-dependent transport of small aggregates to the microtubule organizing center (MTOC), located in the perinuclear region (2). Such inclusions have been termed “aggresomes”.

Our initial studies were performed in a neuronal cell line, PC12 cells, which are derived from rat pheochromocytoma. These cells have the unique ability to differentiate into a neuronal phenotype upon application of physiologic concentrations of the neurotrophic factor NGF. Furthermore, they have a dopaminergic phenotype, something especially important in models of PD, which characteristically presents with a dopaminergic deficit. We applied two different selective pharmacological inhibitors of the proteasome, lactacystin and

PSI, to these cultures, both in the undifferentiated and the differentiated state. Because our intent was to study the effects of proteasomal inhibition on neuronal cells in isolation, we used PC12 cells that have not been genetically manipulated to express misfolded proteins. We found that both proteasomal inhibitors caused the accumulation of cytoplasmic proteinacious inclusions, identified by ubiquitin immunostaining (3). Such inclusions were defined by a circumscribed, usually spherical, focal accumulation of ubiquitin immunostaining that did not encompass the whole cytoplasmic volume. The large majority of the inclusions were unique in a given cell, but occasionally two inclusions in a single cell could be perceived. No nuclear inclusions were seen. The proportion of cells that contained such cytoplasmic inclusions was modest, almost always less than 10%. There was quite a large variability in the percentage of cells with inclusions across experiments. Although the precise factors accounting for this variability are unknown, certainly one of them appeared to be the specific batch of cells utilized. Inclusions were seen both in the undifferentiated and the neuronally differentiated state, providing the first evidence that proteasomal inhibition alone could lead to the formation of cytoplasmic inclusions in a neuronal cell context.

In order to further characterize the biochemical composition of proteins present within such inclusions, we performed Western immunoblotting of detergent-soluble and detergent-insoluble fractions of PC12 cells treated with proteasomal inhibitors. There was a marked accumulation of high molecular weight species of polyubiquitinated protein in the detergent-insoluble fraction. A substantial amount of immunoreactive material did not leave the stacking gel, further attesting to its relative insolubility. Therefore, like in neurodegenerative diseases such as diffuse Lewy body disease, inclusions in this model are composed largely of aggregated polyubiquitinated proteins (3).

Because of the evidence strongly linking UPS dysfunction with PD in particular (see chapters 12 and 13 in this volume), it was especially critical to establish whether α-synuclein, a main component of the Lewy bodies (LBs) characteristic of PD, was present within the inclusions we had observed in PC12 cells following proteasomal inhibition. We found that α-synuclein was indeed occasionally present within such inclusions, but only about 10% of ubiquitinated inclusions contained α-synuclein. This may be due to the low levels of expression of α-synuclein in this cell type. In any case, these studies established that proteasomal inhibition could lead, in neuronal cells, to the formation of inclusions that contain both basic elements of Lewy bodies, α-synuclein and polyubiquitinated proteins (3).

Following these initial studies, we turned our attention to primary neuronal cell culture systems. The basic reason for this was that we believed that it was important to study the repertoire of changes induced by proteasomal inhibition in primary neurons, which should approximate more closely the disease situation. Other reasons included the relative lack of consistency in detecting inclusions, and the relative paucity of α-synuclein-positive inclusions in the PC12 cell model. We have used extensively for this purpose a primary neuronal cell culture system of embryonic rodent cortical neurons, derived from E18 rat or E16 mouse cortices. In these experiments, we have used, like in the PC12 cell model, lactacystin and PSI, but also epoxomicin, an even more selective proteasomal inhibitor. All these

agents led to consistent and reproducible ubiquitinated cytoplasmic inclusion formation, with the same characteristics at the fluorescent microscope level as the PC12 cell inclusions (4). Using an in situ detergent extraction method, we showed that ubiquitin within these inclusions consisted of insoluble material, which, on Western immunoblotting, like in PC12 cells, corresponded to polyubiquitinated proteins. Furthermore, the inclusions stained positive for α-synuclein to a much greater degree than in the PC12 cell model, such that about 60% of inclusions were α-synuclein-positive. The inclusions formed over a period of 18–36 hours, and were not detected at subsequent time points in the few remaining viable neurons. At no point in time did the neurons harboring inclusions exceed 15% of the total cell population. We have examined in some detail the antigenic properties of these inclusions. We have found that they contain the heat shock protein HSP-70, the intermediate filament early neurofilament-like protein α-internexin, and β-tubulin, thus sharing many antigenic features with Lewy bodies (4). Also common between proteasomal inhibition-induced inclusions and Lewy bodies is the presence of Parkin, a protein whose dysfunction is linked to autosomal recessive and, possibly, sporadic PD, as analyzed in chapter 14 (see Figure 1A), and the deubiquitinating enzyme UCH-L1 (see Figure 1B), which is discussed in detail in chapter 11. In addition, we have detected the presence within such inclusions of p53 (5), cyclin D1 and cyclin E (6), all proteins that are degraded by the proteasome in this primary neuronal cell culture system. Whether these proteins accumulate in LBs is unclear. It has to be stressed that there is some selectivity in the accumulation of proteins in proteasomal inhibitor-induced inclusions, in that proteins such as ERK-2 or p27 are not detected within them. In our studies, we have also failed to detect immunoreactivity for γ-tubulin, a marker of the microtubule organizing center, within inclusions.

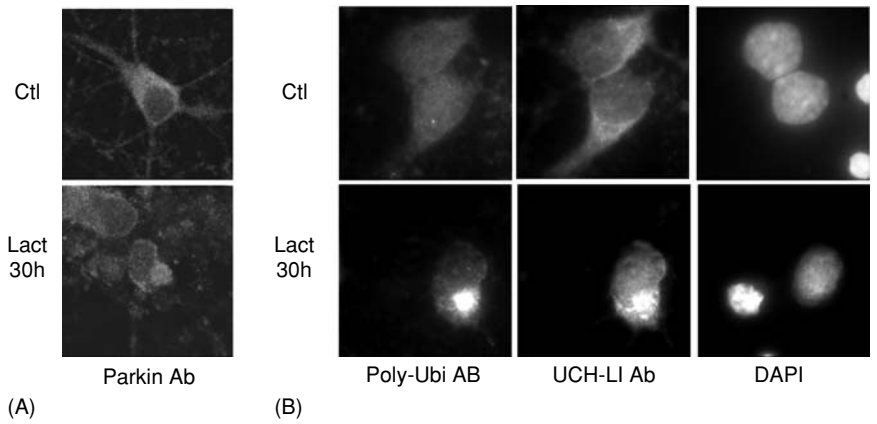

Figure 1. Co-localization of Parkin and UCH-L1 in Inclusions Induced by Proteasome Inhibition in Cultured Cortical Neurons. Primary cortical neurons were cultured from embryonic rat brain, exposed to the pharmacological inhibitor of the proteasome lactacystin (lact), fixed and immunostained for Parkin (A) or poly-ubiquitin (B; left panel) together with UCH-L1 (B; middle panel). Inclusions containing Parkin and UCH-L1 are clearly visible in lactacystin-treated neurons (A, B; lower panels).

A central feature of inclusions in neurodegenerative diseases is the deposition of amyloid-like fibrils. In the case of PD-associated LBs, these fibrils are made up of aggregated α-synculein, and "radiate" out from the core of the inclusion, as seen in ultrastructural studies. Histochemical techniques, such as labeling with Thioflavin S, can also be used to detect the presence of fibrillar material within the inclusions. We have used this labeling technique in our culture system, and have found that a substantial proportion, about 60%, of ubiquitinated inclusions in cortical neurons stain positive for Thioflavin S (4,7). We have however not been able to convincingly identify fibrillar inclusions by electron microscopy in preliminary studies in this model. It may be that the relatively low percentage of cells that contain such inclusions is the limiting factor, and that more extensive studies will identify such inclusions. Another possibility is that, despite the positive Thioflavin S staining, which indicates the presence of fibrillar elements, these structures lack the full organization of bona fide fibrillar inclusions. It is possible that cellular mechanisms that are operative in this system (see below) act to dissolve the inclusions before they assume a full fibrillar ultrastructure.

Other laboratories have used similar approaches in modeling proteasomal inhibition in neuronal cells, both in culture and in vivo. McNaught et al. (8) first demonstrated ubiquitin/α-synuclein-positive cytoplasmic inclusions in cultured embryonic ventral midbrain neurons following lactacystin treatment. Subsequent studies by the same group showed in more convincing photomicrographs that such inclusions were present within dopaminergic neurons, and appeared to show features of aggresomes, in that they were positive for γ-tubulin (9). Fornai et al. (10), like us, identified ubiquitin-α-synuclein-positive cytoplasmic inclusions in PC12 cells following treatment with proteasomal inhibitors. They also identified inclusions at the ultrastructural level. However, in their study, the photomicrographs presented appear to show diffuse, rather than focal accumulation of immunoreactivity. Furthermore, their electron microscopy studies appear to identify "whorled", "multilamellar" lysosomal-related structures (see below), and not inclusions, which should not have delimiting membranes.

McNaught and colleagues were also the first to show that intranigral infusion of lactacystin led to α-synuclein-positive inclusions in vivo in rats (11). They have further extended these studies in which they have used systemic injections of PSI or epoxomicin (12). They have found that such treatment leads to the formation of eosinophilic inclusions similar to LBs in the substantia nigra pars compacta and locus coeruleus. Such inclusions were positive for α-synuclein, ubiquitin, γ-tubulin and Thioflavin-S. Despite the lack of some controls in the photomicrographs shown, this is a provocative report that awaits replication. Fornai et al. in their previously mentioned study also used intrastriatal infusion of lactacystin and epoxomicin and observed, using immunohistochemistry and electron microscopy, "inclusions" in the SNpc similar to the ones seen in PC12 cells (10).

These studies in conjunction show that pharmacological proteasomal inhibition in neuronal cell lines, in cultured primary neurons and in the nervous system in vivo can lead to the formation of cytoplasmic inclusions that share many features with LB inclusions. Convincing evidence for ultrastructural features of LBs in these models however is lacking at this point. Further refinements

of the dosage, route and timing of administration of the drugs may be needed to establish whether proteasomal inhibition alone can lead to bona fide LBs.

3. MECHANISMS OF INCLUSION FORMATION AND ELABORATION FOLLOWING PROTEASOMAL INHIBITION

We have so far discussed the question of the nature and composition of the inclusions that are formed following proteasomal inhibition in neuronal cells. Another question that is perhaps more interesting is the mechanism through which these inclusions are formed. This is a more general question that pertains to inclusions in neurodegenerative diseases. In our model of proteasome dysfunction we have provided evidence that their formation is not merely a passive event due to the build-up of ubiquitinated proteins that aggregate and form an inclusion. The process is an ordered one, requiring, not surprisingly, ubiquitination, but also new or ongoing transcription. Primary cultures of cortical neurons treated with the pharmacological proteasome inhibitor lactacystin, together with the transcriptional inhibitor actinomycin D, did not form ubiquitinated inclusions, despite still accumulating detergent-insoluble high molecular weight ubiquitinated proteins, compared to neurons treated with lactacystin alone (4). We virally over-expressed a dominant negative isoform of the E2 ubiquitin-conjugating enzyme Cdc34, and found a similar reduction in lactacystin-induced inclusion formation compared to mock-infected neurons or neurons expressing wild-type Cdc34 (4). This finding is in agreement with those of Saudou and colleagues (13) who, using the same dominant negative Cdc34, reported a decrease in the formation of nuclear inclusions induced by expression of truncated huntingtin containing an expanded poly-glutamine tract. This suggests that inclusion formation induced by general proteasome inhibition and by mutant huntingtin expression requires the ubiquitination of Cdc34-associated substrate proteins. Alternatively, it is possible that inclusion formation induced by mutant huntingtin, or other expanded poly-glutamine containing proteins, is mediated indirectly through a feed-forward inhibition of the proteasome induced by aggregated huntingtin. One potential mechanism for this is suggested by recent work of Kopito and colleagues showing inhibition of proteasome function in cells harboring inclusions (14). It is also possible that the effect of the dominant negative E2 expression on inclusion formation induced by each "insult" is due to a sequestration of either ubiquitin itself or certain E3 ubiquitin ligases; both of which could prevent aggregation of ubiquitinated proteins, by different mechanisms. Regardless of the mechanism of the inhibition of inclusion formation, these findings indicate that ubiquitination per se is a critical factor in inclusion formation. This is supported by data indicating that Parkin E3 ligase activity is required for inclusion formation (e.g. (15)). In addition, the fact that inclusion formation was blocked by inhibition of transcription in our studies suggests that it is an active process of the neuron rather than simply the gradual build-up of ubiquitinated, but not yet degraded, proteins. The specific gene products required for inclusion formation are not known, but could include chaperones, or accessory proteins such as the ubiquitin binding protein p62 (16–18). Another possibility is that these inclusions represent aggresomes, and that therefore the

microtubule system may need novel or ongoing transcriptional activity of some of its elements in order to form such inclusions. However, as noted above, in our system, and in contrast to reports by others ((9), see also chapter 4), we have not detected the MTOC marker γ-tubulin within the inclusions. Attempts to use microtubule destabilisers, which ought to inhibit aggresome formation, in this system, have been inconclusive, due to the severe toxicity associated with these reagents in primary neuronal cell cultures.

Given the importance of α-synuclein aggregation, as well as its localization within LBs (see chapters 3 and 13), we examined the potential role α-synuclein plays in the formation and the development of the fibrillar structure of these inclusions. In our initial study we had not detected, in proteasome inhibitor-treated neurons, α-synuclein aggregation, as defined by increased α-synuclein-immunoreactive species in detergent-insoluble material or by the presence of oligomers (4). In a follow-up study, however, using a method of in situ extraction, we have clearly identified, using two different antibodies, α-synuclein oligomers specifically in the remaining detergent-insoluble fraction of proteasomal inhibitor-treated neurons (7). The detergent extraction procedure that we have employed effectively solubilized *cytoplasmic* α-synuclein, but not α-synuclein present within inclusions. Thus, the appearance of higher molecular weight α-synuclein species within the same detergent-resistant fractions suggested to us that the oligomerization of α-synuclein might occur once the inclusions had begun to form, and therefore may occur, so to speak, after the fact, after the initial formation of such inclusions. To examine this idea in a more mechanistic fashion, we employed cultures from α-synuclein null mice. We found that neurons derived from such mice formed inclusions at the same rate as those from wild-type mice following proteasomal inhibition (7), indicating that the presence of α-synuclein is not required for the initial formation of inclusions following inhibition of the proteasome. In addition, the absence of α-synuclein did not alter the relative solubility of the ubiquitinated inclusions. Importantly however, we found that the inclusions formed in α-synuclein-deficient neurons lacked any fibrillar components, shown by the absence of Thioflavin S staining ((7); see Figure 2). The fact that inclusions were still able to form in the absence of α-synuclein, and in particular in the absence of oligomeric protofibrillar α-synuclein, raises the possibility that the formation of these species may occur at a secondary stage of the elaboration of such inclusions in neurodegenerative diseases, and that it may not be required for the initial events leading to their formation. A potential mechanism for the secondary oligomerization and fibrillarization of α-synuclein, once this is found within the inclusions, is the phenomenon of "molecular crowding", whereby it is the increased concentration of α-synculein, as well as that of other proteins found within the inclusions, that influences oligomer formation (19,20). Related to the above idea, a recent report has demonstrated α-synuclein immunoreactivity within inclusions from HD patients and mouse transgenic models of HD (21), which could also contribute to the fibrillar nature of these inclusions, possibly in concert with huntingtin. Consistent with this, another report demonstrated that over-expression of wild-type α-synuclein (which could mimic the "molecular crowding" phenomenon) increased the formation of huntingtin inclusions compared to huntingtin alone (22).

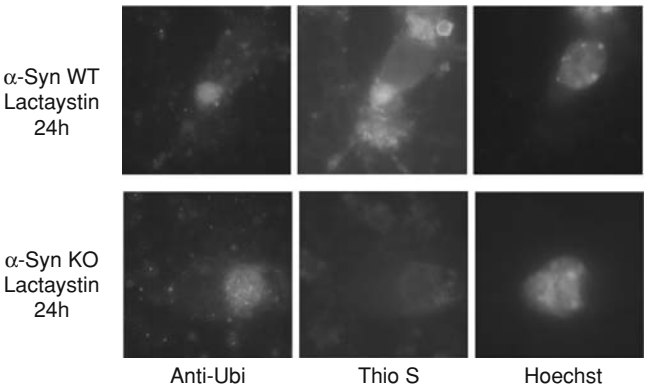

Figure 2. The Absence of α-Synuclein Alters the Structure of Poly-Ubiquitinated Inclusions in Cultured Neurons Following Inhibition of the Proteasome. Primary cortical neurons were cultured from embryonic wild-type or α-synuclein knock out mice, and exposed to the pharmacological inhibitor of the proteasome lactacystin. The cultures were fixed and immunostained for poly-ubiquitin (left panels) and counterstained for amyloid-like fibrils with the histochemical fluorescent stain Thioflavin S (middle panels). Ubiquitinated, insoluble, inclusions are observed at the same frequency in both wild-type and α-synuclein knock out cultures, however the inclusions present in neurons deficient in α-synuclein lack amyloid-like fibrils (compare Thioflavin S staining between top and bottom panel).

Few other studies have examined mechanisms of inclusion formation in neuronal cells following proteasomal inhibition. Fornai et al. (10) presented very interesting data suggesting that dopaminergic metabolism may contribute to such inclusion formation. They showed, in both models mentioned above, that treatment with α-methyltyrosine, an inhibitor of dopamine synthesis, led to a dramatic decrease of inclusion formation following application of proteasomal inhibitors to PC12 cells or to rat striatum. Furthermore, co-application of pargyline, which blocks dopamine metabolism and therefore increases dopamine levels, or of L-DOPA, the dopamine precursor, with the proteasomal inhibitors, enhanced inclusion formation. Notwithstanding the reservations mentioned above about the characterization of these inclusions, these provocative data suggest a possible explanation for the relatively selective pattern of inclusion formation in dopaminergic neurons in PD.

4. THE FATE OF INCLUSIONS IN CULTURED NEURONS

Equally important as the question of how and why inclusions are formed, is the question of their fate within the cell, or neuron, once they are formed. Once formed, are inclusions permanent structures? Does their ultrastructure change? If inclusions are, in fact, removed from the cell, what is the mechanism for their removal? This is a relatively new area of research, but recent data suggest that the autophagic/lysosomal system, the other major cellular system devoted to the removal of proteins, may be involved. Collectively, this system is comprised of three distinct pathways responsible for the degradation of specific cellular components:

macroautophagy, chaperone-mediated autophagy, and microautophagy (reviewed in detail elsewhere; (23)). In macroautophagy, a double-membraned vesicular structure derived from the endoplasmic reticulum, known as the autophagosome, engulfs cytoplasmic material including organelles to be degraded. The autophagosome fuses with a primary lysosome, which then acidifies, and the contents are subsequently degraded via the activity of a number of enzymes, including members of the cathepsin family. The mature acidic vesicle is known as the autophagolysosome. Since this system also mediates the removal of defective organelles such as mitochondria (macroautophagy), it is therefore possible to imagine that it is capable of removing large intracellular structures such as inclusions (see also (24)for review).

The first demonstration of the removal of inclusions, in the form of aggregates of over-expressed GFP/polyglutamine fusion proteins, came from Ravikumar and colleagues (25). In this work, the removal of the inclusions was enhanced or suppressed by pharmacological agents that stimulated or inhibited, respectively, the autophagic system at various points. We asked if a similar removal pathway existed for inclusions formed in primary cortical neurons exposed to pharmacological inhibitors of the proteasome. Using a similar approach of modulating activity of the autophagic system with pharmacological agents, we found that stimulation of autophagy with rapamycin led to the increased removal of ubiquitinated inclusions (26). Conversely, inhibition of autophagy at two different points in the pathway with 3-methyladenine (3-MA), which blocks formation of autophagosomes by blocking the activity of phosphatidylinositol 3-kinase (27), or bafilomycin A-1, an inhibitor of the vacuolar ATPase, which blocks the acidification of lysosomes (28), resulted in more neurons harboring ubiquitinated inclusions. In another model of inclusion formation, that of PMP22-positive aggresome formation in cultured Schwann cells, aggresomes induced by proteasome inhibition were removed during a wash-out period in a mechanism that was sensitive to 3-MA (29).

We further examined the time course of the formation and removal of these inclusions together with markers of lysosomal activation at the single cell level. We found that at the initial phase of inclusion formation during the first 24–36h following exposure to inhibitors of the proteasome there was little or no co-localization between ubiquitinated inclusions and the lysosomal enzyme cathepsin D (26). However at later time points, fewer ubiquitinated inclusions were observed, but increased numbers of cells were found with smaller punctate ubiquitin aggregates throughout the cytoplasm. These smaller ubiquitin aggregates co-localized partially with cathepsin D at these later time points. These results suggested two possibilities: either the neurons harboring inclusions and small aggregates represented two different populations, or the inclusions break down and form small aggregates. To resolve this question, we examined the percentage of neurons showing such smaller aggregates following concomitant application of the autophagy modulators. We found that in the case of autophagy inhibitors, where, as mentioned, inclusions increased, aggregates decreased, whereas in the case of rapamycin, where inclusions decreased, aggregates increased. In conjunction, these findings indicate that the larger inclusions are breaking down into smaller ubiquitinated aggregates through the process of macroautophagy (26). It is still possible however that some cells, perhaps those that have a greater degree of

autophagic activity, may form small aggregates without going through the formation of full-fledged inclusions.

Lee and colleagues (30) examined the clearance of aggregates and inclusion bodies in cells over-expressing wild-type human α-synuclein, and found that the lysosomal system mediated the removal of α-synuclein aggregates but not mature fibrillar inclusions. The discrepancy with our findings with respect to the removal of inclusions may be due to the initial factor leading to α-synuclein aggregation and inclusion formation; namely, over-expression of wild-type protein compared to direct inhibition of proteasome degradation. Alternatively, the clearance of inclusions may also be related to their ultrastructure. It is possible that mature fibrillar inclusions may be resistant to lysosomal-mediated removal, whereas inclusions that have not yet acquired a fibrillar structure may be sensitive to such degradation. In our studies of inclusion removal in cortical neurons treated with proteasome inhibitors, we did not directly compare the removal of Thioflavin S-positive versus Thioflavin S-negative inclusions. In any case, recent data in other systems suggest that inclusions formed in models of polyglutamine diseases are not static, but dynamic structures that change over time (31–33). It is likely that in our system as well the fate of inclusions depends on a delicate balance between the forces that drive inclusion formation (ubiquitination, novel or ongoing transcriptional activity) and elaboration into fibrillar structures (α-synuclein) and those that drive inclusion dissolution (activation of macroautophagy). This balance may shift back and forth during the evolution of an inclusion.

There is evidence of activation of the lysosomal system in various neurodegenerative diseases and in animal or cellular models of these diseases. For example, examination of post-mortem PD brain tissue revealed evidence of autophagic neuronal degeneration in neuromelanin-containing neurons of the SNpc (34), and cell culture models of dopamine neuronal death have reported the activation of the autophagic system following exposure to methamphetamine (35). It is possible then that there is a degree of "cross-talk" between the two major degradative systems within the cell, the proteasome and autophagy, such that under conditions in which proteasome-mediated degradation is impaired, lysosomal-mediated degradation (e.g. macroautophagy, microautophagy or chaperone-mediated autophagy) becomes activated. Likewise, in situations in which inclusion bodies form, macroautophagy may be activated in an attempt by the cell to remove the inclusion or aggregate.

5. PROTEASOME INHIBITION, INCLUSIONS AND NEURONAL DEATH

In addition to formation of cytoplasmic inclusions, inhibition of the proteasome in cultured neurons and neuronal cell lines leads to cell death; with the mechanism of death activated often depending on the cell type, dose, time of exposure or other factors. The subject of neuronal death in response to proteasomal inhibition is addressed in more detail in chapter 9. Here, we will comment briefly on the relationship of inclusion formation to neuronal death. Interestingly, on a qualitative level, the phenomena of inclusion formation and neuronal death appear to be dissociated in our models of pharmacological inhibition of the

roteasome. In each cell type we have examined (with the exception of cultured midbrain dopamine neurons, see below), inclusions were observed exclusively in viable cells based on nuclear morphological criteria (3,4), suggesting that distinct pathways regulate the formation of inclusions and cell death following proteasome inhibition. This is further reinforced by the fact that, in most cases, enhancement of survival does not lead to a decrease of inclusion formation. In fact, in some cases the opposite occurs. For example, in neurons that are deficient in the tumor suppressor p53 we have found that apoptotic death elicited by proteasome inhibition is decreased, however inclusions are formed at a greater rate (5). Similarly, in cultured dopaminergic PC12 cells, increased inclusion formation was observed in conditions in which cell death was prevented by caspase inhibition or elevation of intracellular cyclic AMP (3). We have also found that post-mitotic primary cortical neurons activate elements of the cell cycle following treatment with pharmacological inhibitors of the proteasome ((6); and reviewed in greater detail in chapter 9). However, pharmacological or molecular blockade of this activation did not impact formation of ubiquitinated inclusions, despite providing significant protection from apoptotic cell death. The case of actinomycin D represents an exception. This agent significantly diminished both apoptotic death and inclusion formation (4). Given the dissociation observed with the other strategies, it is likely that there are different sets of genes involved in neuronal death and in inclusion formation, and that actinomycin D impacts both sets.

Further evidence for dissociation between inclusion formation and neuronal death is provided by studies in which we have primarily attempted to manipulate the processes related to the inclusions. Eliminating the fibrillar component of the inclusions in α-synuclein null neurons had no effect on survival following proteasomal inhibition (7). This finding further indicates that oligomeric, fibrillar α-synuclein, when present within the inclusions, may not be toxic to the neurons. Furthermore, the autophagy modulators mentioned above, despite having a significant impact on inclusions, did not influence survival. This is in contrast to the study by Ravikumar et al., in which autophagy inhibitors enhanced death and, conversely, rapamycin ameliorated survival following cellular overexpression of mutant huntingtin (25). These investigators have recently extended these findings to an in vivo setting (36). It may be that the crucial effect of rapamycin in these models is not mediated at the level of the clearance of inclusions, but rather at the earlier stages of protofibrillar huntingtin.

Our studies with the autophagy inhibitor 3-methyladenine in particular revealed something quite interesting: when this agent was applied, we were able to detect for the first time numerous cortical neurons with formed inclusions that were undergoing apoptosis (26). This suggests that the reason that there is a dissociation at the single cell level between apoptosis and inclusion formation in this model may be that, prior to or concomitantly with apoptosis, autophagy is activated and removes the inclusions.

Running against the general trend of dissociation of neuronal death and inclusions in our model, we observed that neurons treated with the dominant negative Cdc34, which, as mentioned above, eliminated inclusions, also led to diminished death (4). As with actinomycin D, it is likely that this agent disrupts these processes through different mechanisms. It is known for example that components

of the cell cycle may be ubiquitinated via Cdc34, and therefore inhibition of this process with our dominant negative strategy may affect neuronal death in this model, where we have shown that aberrant activation of cell cycle components is essential for death.

In a more recent study, we have examined neuronal death and inclusion formation in phenotypically defined midbrain dopamine neurons cultured from embryonic rat. Interestingly, while pharmacological inhibition in these neurons also leads to neuronal apoptotic death and inclusion formation, the two phenomena appear to be *less* distinct for several reasons. First, in these cultures, tyrosine hydroxylase (TH) positive neurons comprise approximately 5% of the total neuronal population, with the bulk of non-dopaminergic neurons being GABAergic in phenotype (37). Application of pharmacological inhibitors of the proteasome induced inclusion formation in both dopaminergic and non-dopaminergic neurons (38), whereas neuronal death was preferentially seen only in TH-positive dopamine neurons. This selective vulnerability had been reported indirectly previously on the basis of a selective defect in dopamine uptake compared to uptake of labeled GABA in these cultures exposed to proteasome inhibitors (8,9). Secondly, in contrast to our previous findings in the cortical neuronal system, in which inclusions were observed only in viable neurons, both apoptotic and non-apoptotic dopamine neurons were detected with ubiquitin or α-synuclein positive inclusions (although apoptotic neurons containing inclusions were significantly less frequent) suggesting that the presence of inclusions in these cells may be related to survival in a different way than in non-dopaminergic neurons; a notion supported by the observation that the dopamine synthesis enzyme TH was also present within the cytoplasmic inclusions. However, pharmacological inhibition of cell cycle activation or of caspases, that diminished dopaminergic neuron apoptosis, did not affect inclusion formation. Actinomycin D again prevented apoptosis and inclusion formation. Therefore, despite some differences, there are substantial similarities in the mechanisms of proteasomal inhibition-induced inclusion formation and death in cortical and ventral midbrain dopaminergic neurons. It may be that the mechanism of autophagy is activated in a delayed fashion in dopaminergic neurons, resulting in the concomitant presence of apoptosis and inclusions in a single cell.

The relationship between inclusions and neuronal death in neurodegenerative diseases is a subject of debate. Evidence for a toxic or protective function of inclusions has been presented over the past few years. The balance in general seems to be shifting in favor of the latter possibility. Recent work by Arrasate and colleagues (39), employing a system to follow inclusion formation and neuronal survival in individual neurons expressing GFP-tagged mutant huntingtin suggests that the presence of inclusions in this setting may be protective. The reduction in diffuse mutant huntingtin (htt) throughout the neuron accompanied by the formation of htt-containing inclusions was associated with reduced neuronal death (39). This is consistent with earlier work by Saudou (13) in which reduced inclusion formation was associated with the potentiation of neuronal death induced by mutant htt expression. Mouradian et al. present in this volume their important work (chapter 4), where they show that HEK293 cell death induced by α-synuclein is mitigated when the cells are able to form inclusions. Our studies

generally suggest that in the model of acute neuronal proteasomal inhibition inclusions are irrelevant to survival, since these processes appear to occur largely independently of each other. This may be because potential protective or toxic effects of the inclusions are masked by the rapid activation of cell death pathways that occurs following acute proteasomal inhibition.

6. CONCLUSIONS

Figure 3 shows the pathway of inclusion formation and removal based on the model of proteasome dysfunction in neurons using pharmacological inhibitors of the proteasome. The initial event of inhibition of proteasome-mediated protein degradation (point A in Figure 3) can be caused by a number of factors; including the direct damage of one or several of the catalytic subunits in the 20S core (oxidative damage, or inhibition by naturally occurring proteasome inhibitors), impairment of the recognition of ubiquitinated substrates and association with the 19S regulatory particle, the inability to remove and de-polymerize the poly-ubiquitin chain into free ubiquitin, depletion of cellular ATP, or due to the effects of endogenous or pathogenic proteins/aggregates that negatively

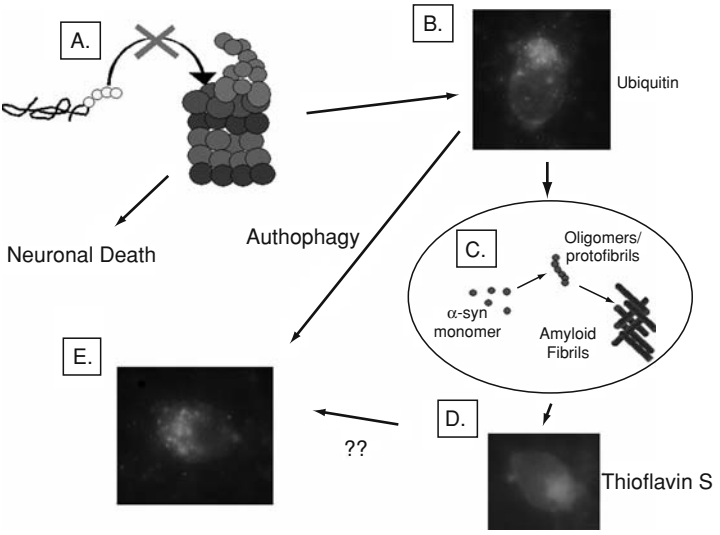

Figure 3. Schematic of the Pathway of Inclusion Formation and Removal in Neurons Treated With Proteasome Inhibitors. The interruption of the degradation of poly-ubiquitinated protein substrates (A) leads to the formation of inclusions. An example of a cytoplasmic ubiquitin-positive inclusion in cortical neuron cultures exposed to lactacystin is shown (B; anti-ubiquitin). Parallel signaling events following proteasome inhibition can lead directly to neuronal death, independently of inclusion formation. In some cases, the ubiquitinated inclusions are disassembled by the neuron in a process involving macroautophagy, resulting in smaller punctate ubiquitin aggregates throughout the cytoplasm, which corresponds temporally with neuronal death (E; anti-ubiquitin). Proteasome inhibition also causes the oligomerization/fibrillization of α-synuclein (C) within the same detergent-insoluble fraction as the ubiquitinated inclusions, which leads to the amyloid-like structure (D; Thioflavin S). Whether such more fibrillar inclusions can be degraded by autophagy is not known.

regulate proteasome function. The accumulated poly-ubiquitinated proteins, coupled with an as yet unknown transcriptional activation (see (4)), aggregate into detergent-insoluble inclusions (point B in Figure 3). We have also shown that parallel pathways are activated in primary cortical neurons exposed to proteasome inhibition that lead, independently, to inclusion formation and neuronal death. Blockade of either neuronal death, or inclusion formation, does not generally impact the other pathway elicited in this model system.

Ultrastructurally, the inclusions induced by inhibition of the proteasome, as well as by over-expression of α-synuclein, resemble LB's from PD or DLBD in that they are comprised, in part, of amyloid-like fibrils (7,26,40). In our model of neuronal proteasome dysfunction, we have shown that the presence of α-synuclein, presumably in an oligomeric/fibrillar conformation, is necessary for the deposition of amyloid fibrils within the inclusions (point C & D in Figure 3; and (7)). Interestingly, the inclusions formed by inhibition of the proteasome and by expression of proteins containing expanded poly-glutamine repeats can be removed or disassembled by the neuron, in a process involving macroautophagy ((25,26); point E in Figure 3). What remains to be determined is the mechanism regulating this removal. Is it simply an up-regulation of lysosomal activity in response to deficient proteasome function? Does the structure of the inclusion itself signal the cell to remove it? For example, are mature fibrillar inclusions more or less resistant to autophagic disassembly compared to inclusions that do not possess amyloid-like fibrils?

The pathways described here are derived largely from models employing acute pharmacological inhibition of the proteasome, although similar elements of inclusion formation have been observed in models of misfolded protein overexpression. The neurodegenerative disease condition, on the other hand, follows a gradual course over many years leading to neuronal death and inclusion body formation. While the data accumulated thus far provide important clues concerning the formation and removal of neuronal inclusions, their validity as models of neuronal proteasome dysfunction should be confirmed using animal models that will perhaps more accurately reflect a chronic condition.

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6

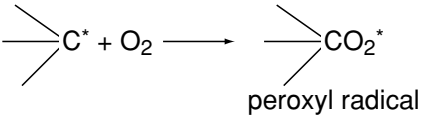
THE PROTEASOME: SOURCE AND A TARGET OF OXIDATIVE STRESS?

Barry Halliwell

1. INTRODUCTION: SOME BASICS

1.1. Oxidations by Oxygen

We are surrounded by oxygen: essential for life, but poisonous (1). Oxygen is a biradical, containing two unpaired electrons. Fortunately, the parallel spin of these two electrons makes it difficult for oxygen to react directly with non–radicals. As a result, direct oxidation of most biomolecules by O_2 is slow and the human body does not spontaneously combust. However oxygen does react fast with other free radicals, such as carbon–centered radicals (1)



Thus once a radical reaction has started, O_2 easily propagates it. The classic example of this is lipid peroxidation, in which a reactive radical ($R^{\bullet}$) abstracts a hydrogen atom from a polyunsaturated fatty acid residue (LH), leaving a carbon–centered radical ($L^{\bullet}$)

$$LH + R^{\bullet} \rightarrow L^{\bullet} + RH$$

which then reacts fast with O_2

$$L^{\bullet} + O_2 \rightarrow LO_2^{\bullet}$$

to generate a peroxyl ($LO_2^{\bullet}$) radical that propagates the reaction by abstracting hydrogen from previously–unmolested fatty acid residues

$$LO_2^{\bullet} + LH \rightarrow \underset{\text{lipid peroxide}}{LO_2H} + L^{\bullet}$$

The great majority of the O_2 taken up by humans is reduced to water in mitochondria, a 4–electron process

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

Chemically, it is impossible to add four electrons to O_2 at once; it must be done stepwise (1)

$$O_2 + 1e^- \rightarrow O_2^{\bullet -} \text{ (superoxide radical)}$$

$$O_2^{\bullet -} + 1e^- + 2H^+ \rightarrow H_2O_2 \text{ (hydrogen peroxide)}$$

$$H_2O_2 + 1e^- \rightarrow OH^- \text{ (hydroxyl ion)} + OH^{\bullet} \text{ (hydroxyl radical)}$$

$$OH^{\bullet} + 1e^- + H^+ \rightarrow H_2O$$

$$OH^- + H^+ \rightarrow H_2O$$

Reduction of O_2 to H_2O is catalyzed by mitochondrial complex IV, cytochrome oxidase. This multi–enzyme complex also has the unenviable task of sequestering partially–reduced intermediates on its active sites until reduction to H_2O is completed, preventing their release to cause havoc in the rest of the cell (1).

The first reduction step, oxygen to superoxide, is fairly easy and many systems with a reduction potential of –0.16V or less can achieve it. Thus some $O_2^{\bullet -}$ is produced "free" in mitochondria by escape of electrons onto O_2 from electron carriers (2). This superoxide is largely or entirely removed by mitochondrial superoxide dismutase enzymes, especially manganese–containing SOD, MnSOD. This enzyme is essential to survival, as revealed by the observation that transgenic mice lacking MnSOD usually die soon after birth with a series of defects including neurodegeneration (3,4). Several biomolecules autoxidize to make $O_2^{\bullet -}$, including dopamine, adrenalin, tetrahydropteridines and thiols (1). Reaction starts slowly because direct reaction of non–radicals, such as these molecules, with O_2 is disfavored. Traces of transition metals (iron, copper, manganese etc) can catalyze one–electron transfers to promote autoxidation reactions (1).

Superoxide, as a pure chemical, is not very reactive: it does not attack DNA, lipids, or most proteins, for example (1). So why is its removal, particularly in mitochondria, so essential? Superoxide can attack certain iron–sulphur proteins, including the Krebs cycle enzyme aconitase, inactivating them and releasing iron (5). Superoxide also mobilizes iron from the storage protein ferritin (6).

$$\text{Ferritin } (Fe^{3+})_n + O_2^{\bullet -} \rightarrow \text{Ferritin } (Fe^{3+})_{n-1} + Fe^{2+}_{(aq)} + O_2$$

Superoxide can protonate

$$O_2^{\bullet -} + H^+ \rightleftarrows HO_2^{\bullet}$$

hydroperoxyl radical

Hydroperoxyl radical, the simplest peroxyl radical ($LO_2^{\bullet}$, where L=H), is much more reactive (1) than $O_2^{\bullet -}$. The pKa for $HO_2^{\bullet}$ is 4.8, which means that little $HO_2^{\bullet}$ is present in $O_2^{\bullet -}$ –generating systems at pH 7.4. Nevertheless, more will be present at low pH. Superoxide cannot cross most biological membranes, whereas $HO_2^{\bullet}$ should be able to. Like other peroxyl radicals, it can attack unsaturated fatty acid residues and propagate lipid peroxidation.

$$LH + HO_2^{\bullet} \rightarrow L^{\bullet} + H_2O_2$$

1.2. Nitric oxide and Peroxynitrite

Superoxide can react very fast with other free radicals, including nitric oxide ($NO^{\bullet}$).

$$O_2^{\bullet -} + NO^{\bullet} \rightarrow ONOO^- \text{ (rate constant} > 10^9\ M^{-1}s^{-1})$$

Reaction of $O_2^{\bullet -}$ with $NO^{\bullet}$ produces peroxynitrite (7), $ONOO^-$.Its protonated form, peroxynitrous acid (ONOOH) is a powerful oxidizing, hydroxylating and nitrating species that reacts with many biomolecules including DNA, proteins and lipids to cause damage (7).

1.3. Hydrogen Peroxide

Non–enzymic or SOD–catalyzed dismutation of $O_2^{\bullet -}$ generates H_2O_2

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

H_2O_2 is also produced directly by several enzymes, such as glycolate– and monoamine oxidases (1,8). Like $O_2^{\bullet -}$ and $NO^{\bullet}$, H_2O_2 is not highly reactive. Its levels are kept low by catalase, glutathione peroxidase and peroxiredoxin enzymes, the latter two being of greater importance in the brain than is catalase (1). However, H_2O_2 is not eliminated entirely *in vivo* because it plays an important role as an intracellular and extracellular signal transduction molecule (9). Facilitating this role are its poor reactivity, which allows H_2O_2 to survive to reach its cellular targets (unless intercepted by enzymes on the way), its ability to pass through membranes, probably using the aquaporin channels (10) and the peculiar properties of peroxiredoxins, in that their activity is modulated by their H_2O_2 substrate (11).

The reactivity of H_2O_2 can be increased in three ways. First, some peroxidase enzymes can use H_2O_2 to generate reactive products (1). In neutrophils, myeloperoxidase (MPO) oxidizes chloride ions to the reactive oxidizing and chlorinating agent hypochlorous acid, aiding the killing of micro–organisms (1).

$$H^+ + Cl^- + H_2O_2 \xrightarrow{MPO} H_2O + HOCl$$

MPO levels in the brain are low, unless traumatic injury causes an influx of neutrophils. However, it has been suggested that MPO becomes expressed in the brain in AD (12).

Second, UV light can split H_2O_2 into hydroxyl radical by homolytic fission of the O–O bond (1).

$$H_2O_2 \xrightarrow{UV} 2OH^{\bullet}$$

Third, and most important to the nervous system (1,13-15), H_2O_2 can react with several metal ions to generate $OH^{\bullet}$. The best studied are iron and copper ions, but some chromium, vanadium and nickel species are also effective (1). Interestingly, Mn^{2+} is not effective in forming $OH^{\bullet}$ from H_2O_2 (16).

$$\underset{(Cu^+)}{Fe^{2+} + H_2O_2} \rightarrow \begin{pmatrix} \text{Intermediate} \\ \text{Oxo} - \text{iron} \\ \text{or oxo} = \text{copper} \\ \text{complex}(Cu^{2+}) \end{pmatrix} \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

The ligands to the metal ions influence the rate of the reaction with H_2O_2 and the amount of $OH^{\bullet}$ generated (1).

Hydroxyl radical is indiscriminately–reactive: it can attack whatever biomolecules are next to it whenever it is formed. Its diffusion distance is essentially zero (1). By contrast, H_2O_2 can pass through intracellular and plasma membranes, causing little if any damage. If H_2O_2 meets a strategically–placed peroxidase enzyme or transition metal ion, it can cause site–specific damage (1,8,13-15). For example, H_2O_2 does not react with DNA. However, cells treated with H_2O_2 show oxidative DNA damage. This is because the H_2O_2 penetrates to the nucleus and reacts with iron (or possibly copper) ions bound to DNA, and the resulting $OH^{\bullet}$ causes instant oxidative DNA damage (1,17).

Transition metal ions also promote other free radical reactions (including autoxidations, as we mentioned above): indeed metalloenzymes such as peroxidases, hydroxylases, and oxygenases harness and expand this power for useful metabolic purposes. Iron and copper ions can decompose not only H_2O_2, but also lipid peroxides (1)

$$LOOH + Fe^{2+}\,(Cu^+) \rightarrow \underset{\text{alkoxyl radical}}{LO^{\bullet}} + Fe^{3+}\,(Cu^{2+}) + OH^-$$

$$LOOH + Fe^{3+}\,(Cu^{2+}) \rightarrow \underset{\text{peroxyl radical}}{LOO^{\bullet}} + Fe^{2+}\,(Cu^+) + H^+$$

Thus controlling metal ion availability is an important feature limiting oxidative damage rates in the human body (1,13), and this is especially important in the brain (13-15).

1.4. Oxidative Stress

The term oxidative stress essentially refers to a serious imbalance between production of reactive species and antioxidant defense. Sies (18) defined it as *a disturbance in the pro-oxidant–antioxidant balance in favor of the former, leading to potential damage*.

Oxidative stress can result from:

1. *Diminished levels of antioxidants,* for example, mutations affecting the activities of antioxidant defense enzymes such as MnSOD, or glutathione peroxidase, or toxins that deplete antioxidant defenses. For example, many xenobiotics are metabolized by conjugation with GSH; high doses can deplete GSH and cause oxidative stress even if the xenobiotic is not itself a generator of reactive species (1). Deficiencies in dietary minerals (e.g. Zn^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Se) and/or antioxidants can also cause oxidative stress.

and/or

2. *Increased production of reactive species,* for example, by exposure of cells or organisms to elevated levels of O_2 or to other toxins that are themselves reactive species (e.g. $NO_2^{\bullet}$) or are metabolized to generate reactive species, or excessive activation of 'natural' systems producing such species (e.g. microglial cells in neurodegenerative diseases) (19).

1.5. Oxidative Damage

This can be defined as *the biomolecular damage caused by direct attack of reactive species during oxidative stress* (20). Oxidative damage levels depend on

- the extent of oxidative stress
- the rate of repair of oxidatively–damaged biomolecules

For example, various reactive species can chlorinate, nitrate and oxidize amino acid residues on proteins. Attack of several reactive oxygen species on proteins, especially $OH^{\bullet}$, can oxidize amino acid residues into products that contain carbonyl groups. Hence assays detecting such groups are frequently used as "generic" assays of oxidative protein damage (reviewed in ref. 20). Carbonyls can also be generated because lipid peroxides can break down to cytotoxic unsaturated aldehydes such as 4–hydroxynonenal (HNE). These can react covalently with proteins, especially with –SH groups, to "attach" carbonyls to the protein.

Consequences of oxidative stress can include:

1. *Adaptation* of the cell or organism by upregulation of defense systems, which may

- completely protect against damage
- partially protect against damage
- 'overprotect' (e.g. the cells is then resistant to higher levels of oxidative stress imposed subsequently). Defense systems include not only antioxidants but also chaperones, heme oxygenase etc

2. *Injury*: This involves damage (*oxidative damage*) to any or all molecular targets: lipids, DNA, protein and carbohydrate. Oxidative damage can also occur during adaptation and may sometimes be the mechanism triggering it (point 1 above). Not all damage caused by oxidative stress is oxidative damage: damage to biomolecules can result from oxidative stress-related changes in ion levels (e.g. Ca^{2+}) leading to activation of proteases, for example.
3. *Cell death*: The cell may

- recover from the oxidative damage by repairing it or replacing the damaged molecules, or survive with persistent oxidative damage or oxidative damage, especially to DNA, may trigger cell death, by apoptosis or necrosis.

2. THE ROLE OF THE PROTEASOME

In non–dividing cells, such as the great majority of neurons in the adult brain, the protein content of cells is approximately constant. Since protein synthesis is continuous, it must be matched by an equal rate of protein degradation. Cellular proteins can be degraded by the lysosomal system, but a system of greater importance to the normal functioning of nervous system is the proteasome, which is described in detail elsewhere in this volume. The ubiquitin–proteasome system is essential to the development and maintenance of neuronal morphology (21-23). It also plays a role in axonal degeneration after nerve injury (23).

I first became interested in this system in 1998 (24), when we realized that accumulation of abnormal proteins may be a feature of all neurodegenerative diseases, and that the accumulation of oxidized proteins observed by us (25-27) and others (15,28) in these diseases could be due not only to increased oxidative damage but also to failure to clear damaged proteins (21,24,29). In mammalian cells, oxidized proteins appear to be largely removed by the 20S proteasome (21,22,30,31), an exception being removal of oxidized aconitase by the mitochondrial Lon protease (32). Surprisingly perhaps, ubiquitination does not appear to be required for degradation of oxidized proteins (30,33), except in a few special cases. One of these is iron regulatory protein 2 (IRP2), which plays a role in regulation of cellular iron metabolism and is especially important in the brain; knockout of the gene causes iron deposition and neuronal damage in mice (34). Oxidized IRP2 is recognized by an E3 ubiquitin–protein ligase (35). So how does the 20S proteasome recognize oxidized proteins? The answer is not clear; one suggestion is that oxidation increases surface hydrophobicity, but more studies to investigate the mechanisms by which this could trigger recognition are needed (30,31).

Levels of oxidized proteins in brain tend to increase with age (36,37), consistent with several reports that proteasome activity decreases with age (22,38-40). Lon protease activity also decreases with age (41). Some animal studies suggest that levels of brain protein carbonyls are positively correlated with cognitive impairment (36,37,42). Such a correlation does not itself prove a cause–consequence relationship, although in gerbils *tert*–butyl–α–phenylnitrone decreased carbonyl levels and also improved cognitive function (42). Caloric restriction had a similar effect in rats (43). A relationship of protein damage to neuronal dysfunction is likely, because the oxidized proteins include enzymes

essential to neuronal energy metabolism (28). Protein carbonyls can also be generated by binding of glucose to proteins followed by oxidation, a process often called glycoxidation (44).

Increased levels of nitrated proteins have been observed in nervous tissues from subjects with AD, PD, HD or ALS (45-52), although some earlier studies may need re–evaluation because of methodological artifacts (53,54). Again, the usual assumption is that this rise in levels is caused by generation of more reactive nitrogen species (RNS) such as $ONOO^-$ (although nitration is not a specific marker of $ONOO^-$, rather of RNS generally) (48,55), but defects in clearance of nitrated proteins could also contribute. How nitrated proteins are removed *in vivo* is uncertain; "denitrase" enzymes have been described but proteasomal degradation of nitrated proteins may also be important (56,57). How the proteasome might recognize them is unclear. Their degradation by the proteasome would presumably eventually result in release of free nitrotyrosine. Indeed, elevated free nitrotyrosine levels have been reported in ALS (58). The role of ubiquitination in degrading nitrated proteins is uncertain; in bovine aortic endothelial cells degradation of nitrated transferrin receptor did involve ubiquitination (59), although isolated 20S proteasome without the ubiquitin system is able to degrade nitrated CuZnSOD (57).

It has therefore been proposed that there is a specific critical threshold of proteasome function needed for neurons to function adequately, and that as activity declines with age this threshold can be crossed, at an age depending on how active the proteasome was initially (13,21,29,38,60,61). There are variations in proteasome activity between individuals; whether any of this has a genetic basis remains to be explored. If the proteasome is damaged or downregulated early in life, perhaps by exposure to neurotoxins, this threshold could be crossed at an earlier age. Although neurodegenerative diseases can originate in many ways, it is possible that impaired proteasome function plays a key role in all of them (Figure. 1). Indeed, levels of the proteasome are decreased in AD (62), PD (63-65), after cerebral ischemia–reperfusion (66,67) or intermittent hypoxia (68) and possibly in prion diseases (61,69). In transgenic mice expressing ALS–associated mutant CuZnSOD, proteasome activity in the spinal cord was decreased (70).

Proteasomal dysfunction could be due to defects in the ubiquitin system or a lack of ATP for ubiquitination. It could also involve either or both of the following:

1. ***Direct inactivation of the proteasome*** by various reactive species (71-73). Indeed, Glockzin *et al* (74) suggested that $NO^{\bullet}$–induced apoptosis in RAW264.7 macrophages involves proteasomal inhibition. How sensitive is the proteasome as a direct target of oxidative damage? High levels of HOCl and $ONOO^-$ rapidly inactivate the protease activities of the isolated proteasome (73,75,76) under certain conditions, as can hydroxyl radical (77,78). Some studies have reported activation of protease activity on exposure to reactive species, or both activation and deactivation depending on the concentration of the reactive species (73,76,79,80). One point worth making is that many papers use small fluorogenic substrates to measure proteasome function; their hydrolysis is independent of ubiquitination i.e. they would not detect impairments of protein turnover at the pre–proteasome level) and, more relevant to this section, the levels measured can be affected by

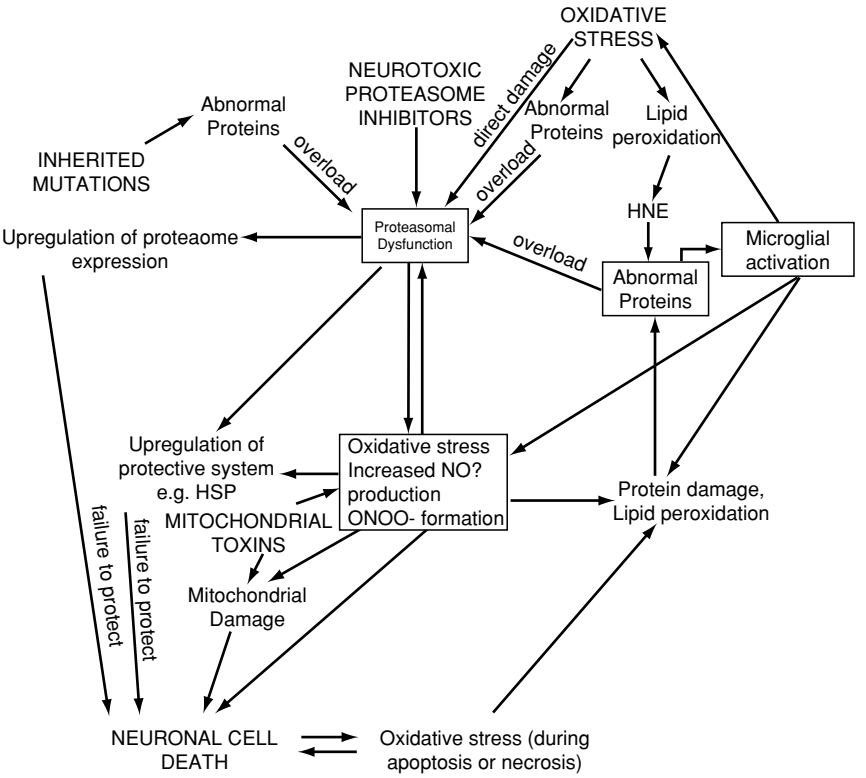


Figure 1. Interplay of mitochondria, oxidative damage and the proteasome in neurodegeneration. Low–level proteasome inhibition can cause transient neuroprotection (e.g. by induction of heat shock proteins, HSP)

the presence of detergents that can activate latent hydrolytic activities, e.g. by "opening up" the proteasome. Indeed, the "activations" by reactive species may be due to damage to the structure, exposing the catalytic sites; which in fact would not be good for the cell! We found (unpublished) that low levels ($\leq 100\mu M$) of HNE had no effect on the isolated proteasome. Similarly treatment of NT2 or SK–N–MC cell lines with HOCl led to no significant fall in proteasome activity even at 3h, and indeed a slight (but nonsignificant) trend to a rise (81). Lipid peroxides (80) dopamine oxidation products (82), aldehydes such as glyoxal (78), and isoketals (83) reactive products generated during lipid peroxidation, are also potential inhibitors. The 20S proteasome appears less sensitive to oxidative damage than the 26S proteasome (75,78,84), suggesting that the regulatory complexes may be more important targets. Oxidative stress, if not too intense, can also upregulate the expression of genes encoding proteasome subunits (59,85-87).

In general, my overall impression from the literature is that the 20S proteasome is not very sensitive to direct inactivation by oxidative stress. However, the importance of its rapid inactivation by isoketals (83) needs more study, since

this pathway of lipid peroxidation is very prominent in Alzheimer's disease (88,89). It is also possible that ROS generated in mitochondria after inhibition of the electron transport chain can affect the proteasome, in part by decreasing ATP levels (90). In neuroblastoma cells treated with rotenone there was a decrease in proteasome activity associated with its modification by acrolein, suggesting also some direct damage (91). By contrast, loss of viability in primary rat neurons induced by another complex I inhibitor, MPP^+, was accompanied by *increased* proteasome activity (92). Hence one should be cautious about generalizing to the *in vivo* situation from experiments on a single cell line, especially as cell culture conditions can have profound effects on cell behaviour (93), including proteasome activities (94).

In addition, reactive species might affect other steps in the ubiquitin–proteasome system. It has been proposed that E1 and E2 enzymes are reversibly inhibited by oxidized glutathione, i.e. their activities could be impaired by oxidative stress–dependent falls in cellular GSH/GSSG ratios (95,96). This occurs because GSSG can react with protein –SH groups essential to catalytic function, converting them to form mixed disulphides, a process often called S–glutathionylation. More work to explore the physiological relevance of this mechanism is required. It could be particularly relevant to PD, where GSH levels in the substantia nigra are significantly depleted accompanied by rises in the levels of cysteinyl–dopamine conjugates, indicative of dopamine oxidation (97,98). Inhibition of the proteasome by dopamine in PC12 cells (82) might be due to its modification by reactive quinone and semiquinone products of dopamine oxidation. The phenolic "antioxidants" BO–653 and probucol were reported to decrease the gene expression and levels of the proteasome in human endothelial cells (99), suggesting that many more agents than we currently suspect may act to modulate proteasome function.

2. Overload of the system by abnormal proteins. Although research is often focused on abnormal proteins arising from mutated genes or aberrant mRNA splicing, abnormal proteins can arise by a variety of mechanisms (Table 1) and many are degraded by the proteasome system. The presence of such proteins may "overload" the system, either if they are degraded more slowly than usual and "clog up" the system (13,100) or if the cell "senses" that they are abnormal and turns them over faster, in either case requiring a greater total amount of "proteasomal time". For example, the mutant α–synucleins associated with some cases of familial PD appear to be degraded more slowly than normal α–synucleins (101). Their expression in PC12 or in neuroblastoma cells led to decreased proteasomal activity (102,103), as did expression of mutant CuZnSOD enzymes associated with ALS (81,104,105). Parkin is also degraded by the proteasome (106,107), the mutant parkins associated with juvenile PD apparently abnormally slowly (107). The LI66P mutant DJ–1 protein associated with a few familial PD cases is also degraded by the proteasome (108). Paired helical filament tau has been reported to block proteasome function and may account for decreased activity in AD (109); another possible "blocker" is Alzheimer's–associated variant ubiquitin (110) UBB^{+1}. Yet another scenario is that abnormal proteins bind to the cap structures and interfere with feeding of the ubiquitinated protein into the proteasome core and/or with the binding of ubiquitinated proteins (111). In neural SH–SY5Y cells, expanded polyglutamine proteins did not markedly

decrease proteasome function, but they did significantly impair the cells' ability to upregulate proteasome levels in response to thermal stress, illustrating yet another potential mechanism (112).

Overload of the proteasome can also be caused by the presence of increased levels of oxidized and nitrated proteins. For example, isoketal–modified, HNE–modified (including HNE–modified β-amyloid) and possibly acrolein–modified (91) proteins, can decrease proteasome function by entering and getting "stuck" because they cannot be rapidly degraded (109,113-115). For example, in NT–2 or SK–N–MC cells treated with HNE, HNE became associated with the proteasome (81),either by direct binding of HNE to proteasomal subunits and/or by association of other HNE–modified proteins with the proteasome.

3. DYSREGULATION OF THE PROTEASOME, OXIDATIVE STRESS AND CELL DEATH

Various effects of adding proteasome inhibitors have been described on a wide range of cells. They include causing neurite outgrowth (that was how the widely–used inhibitor lactacystin was discovered) (116), *protection* of cells against apoptosis (e.g. by preventing upregulation of NF–κB (117,118) or by upregulating heat shock proteins (86,119) and interference with cell division (120,121). Cyclins involved in regulation of cell division are degraded by the proteasome, and so its inhibition dysregulates the cycle (116,122). Upregulation of NFκB can be prevented because iκB is degraded through the proteasome (118). These variations in published results may be due to the use of different cell types (e.g. dividing versus non–dividing), inhibitors of different types applied at different concentrations achieving various degrees of inhibition, or different routes leading to cell death (122). Nevertheless, proteasomal inhibition causes apoptosis in several neurons or cell lines, including cultured cerebellar granule cells (121),

Table 1. Processes generating abnormal proteins that might contribute to aggregation.

Gene mutations
Aberrant splicing of mRNA
Faulty post–translational modification
Oxidation of amino acid residues by reactive oxygen species
Nitration and/or oxidation of amino acid residues by reactive nitrogen species
Halogenation and/or oxidation of amino acid residues by reactive chlorine or bromine species
Glycation/glycoxidation
Spontaneous deamination
Modification by acrolein/HNE/other aldehydes/isoketals and other end–products of lipid
peroxidation

neonatal mouse sympathetic neurons (123), NT–2 (124), SK–N–MC (124) and PC12 cells (both naive and neuronally–differentiated) (103) and in rat oligodendrocytes (125). It also caused cell death and activation of poly (ADP–ribose) polymerase (PARP1) in the PC6 cell line; inhibition of this enzyme decreased cell death (126). We (127) found that high levels of proteasome inhibitors are cytotoxic to primary mouse cortical neurons, but low levels tended to prolong cell viability in culture, associated with increased levels of heat shock proteins and a range of changes in gene expression including upregulation of the expression of genes encoding proteasome subunits. The rise in HSPs may be triggered by the early stages of accumulation of abnormal proteins, and will maintain survival only if the chaperone activities can cope with the amounts of abnormal protein present (Figure. 1). It is important in such studies to follow the cells for as long a period as possible; what appear initially to be neuroprotection might switch to accelerated cell death as proteins continue to pile up. In some cases HSPs might even facilitate protein aggregation (128).

In NT–2 and SK–N–MC cell lines, apoptosis induced by lactacystin or epoxomicin was slowed by adding NOS inhibitors. Production of extra $NO^{\bullet}$ was demonstrated, due to a rise in nNOS levels (124), presumably because this protein is normally turned over by the proteasome (128). There was also a rise in markers of oxidative damage and of protein nitration (124). Exactly why proteasomal inhibition causes oxidative stress is uncertain, but oxidative stress increases intracellular Ca^{2+} levels (1), which would activate the accumulated nNOS and raise cellular levels of $NO^{\bullet}$. This can react with $O_2^{\bullet-}$ to form $ONOO^-$, promoting protein nitration. Similar studies were reported in SH–SY5Y neuroblastoma cells over–expressing an ALS–related mutant CuZnSOD; the cell death induced by adding lactacystin could be ameliorated by the nNOS inhibitor 7–nitroindazole (130). The toxic effects of proteasome inhibitors were aggravated if cells were over–expressing mutant CuZnSOD or parkin (107). Interestingly, interference with the ubiquitination process also caused increased $NO^{\bullet}$ production and protein nitration, as well as decreased proteasome activity (131). In other words, interference with cellular protein clearance mechanisms at several points may be able to cause oxidative and nitrative stress, impair cell function and increase sensitivity to neurotoxins such as HNE, mitochondrial complex I inhibitors and neurotoxic metal ions such as Cd^{2+} (107,131). Some of the reactive oxygen species may originate from the mitochondria (132), others from activation of NADPH oxidases (133). In liver cells, formation of protein aggregates and cell death induced by lactacystin were decreased by lowering O_2 levels, consistent with a role of reactive oxygen species in damage induced by proteasome inhibition (134).

Proteasome inhibition in NT–2 and SK–N–MC cells provoked the formation of protein aggregates in the cell cytoplasm; among the constituents were α–tubulin, ubiquitin, CuZnSOD, α–synuclein and 68K neurofilaments (104). Nitrotyrosine was also present, and aggregate formation was decreased by NOS inhibitors, consistent with suggestions that nitration may facilitate aggregate formation (50,104,135). Similarly, introducing a mutant proteasome subunit that decreased chymotrypsin–like activity hypersensitized mouse neuronal cells to oxidative stress, and such stress resulted in protein aggregate formation (136). Of course, $NO^{\bullet}$ may not be the only mediator of death upregulated by proteasome

inhibition; activations of PARP (126) and of COX–2 (with subsequent increased prostaglandin production) (127,137) may also be important, depending on the cell type used and its growth conditions (see the caveat above) (93, 94).

4. IMPLICATIONS

If the hypothesis that proteasomal dysfunction, involving increased RNS production, is a major contributor to neurodegeneration is correct, several conclusions follow.

1. Selective inhibitors of nNOS may have a general therapeutic role in the neurodegenerative diseases. Of course, other sources of $NO^{\bullet}$, such as increases in iNOS (e.g. related to the inflammatory component of AD) may also be important. iNOS expression via NFκB would be expected to be decreased by proteasome inhibition, since iκB would not be destroyed. However, in human epithelial kidney cells it was reported that proteasome inhibitors had the overall effect of increasing iNOS levels, by blocking its degradation (138). eNOS expression was upregulated by proteasome inhibitors in bovine pulmonary artery endothelial cells (139). The relevance of these effects to the nervous system is unclear as yet.
2. Agents that upregulate proteasome function, whether by relieving blockage or increasing transcription of the relevant genes, should be neuroprotective. We observed that overexpression of the antiapoptotic protein bcl–2 increases proteasome activity in cells (140) and it also delays cell death associated with the presence of mutant proteins (141). The latter has been observed *in vivo* and in cell culture (141,142). Of course, these data do not prove that the bcl–2 is protecting by raising proteasome activity since this protein has multiple cellular effects.
3. Because of their ability to block proliferation, cause apoptosis, and downregulate NFκB (which can decrease production of iNOS and pro–inflammatory cytokines), proteasome inhibitors are being extensively investigated for the treatment of cancer and chronic inflammatory diseases (67,143). They have also been proposed for use in stroke (67), and indeed they can attenuate damage by suppressing inflammation and phagocyte recruitment (67,118,144). In rat cortical neurons lactacystin blocked the cytotoxicity of β–amyloid (145). However, these studies were conducted over short time–windows, and it is important to check that an initial protective effect is not followed by delayed neurotoxicity. Another area of interest is the possible use of proteasome inhibitors to protect against axonal degeneration (23).

However, when considering the therapeutic use of proteasomal inhibitors for the treatment of cancer or inflammatory disease, it is *essential* to ensure that the agents used do *not* cross the blood–brain barrier (29,146). Indeed, infusion of lactacystin into the substantia nigra pars compacta of rats caused degeneration and behavioural abnormalities (147). In a similar study (148) damage was selective for striatal dopamine cells and could be slowed by decreasing dopamine synthesis using a tyrosine hydroxylase inhibitor, or worsened by injecting L–DOPA or pargyline (to inhibit monoamine oxidase and raise dopamine levels). Treatment with proteasome inhibitors capable of crossing the blood–brain barrier caused adult rats to develop a progressive parkinsonian syndrome (149).

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7

INFLAMMATION AS A MEDIATOR OF OXIDATIVE STRESS AND UPS DYSFUNCTION

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1. INTRODUCTION

Neuroinflammation is viewed as a process that occurs in the CNS and that involves primarily glial responses (1). It does not reproduce the classical characteristics of peripheral inflammation. The term neuroinflammation, apparently not used prior to 1995, is associated with chronic CNS inflammation. It is now considered to be an innate immune response in the brain and is implicated in many chronic unremitting neurodegenerative disorders associated with activated glial cells (1). The complex interactions and feedback loops between glia and neuronal cells make it difficult to establish simple linear cause and effect cascades in these disorders. In this chapter we discuss two of the mechanisms, oxidative stress and UPS dysfunction, that can mediate the detrimental aspect of neuroinflammation.

2. WHAT IS INFLAMMATION?

Inflammation is the body's natural response to a variety of insults ranging from infection by bacteria or viruses to injury by chemical or physical agents. Inflammation is a double-edged sword that can benefit or harm the host: it is an extremely important survival tool in the body's defense system, but prolonged or unregulated inflammation can cease to be a beneficial event contributing to the pathology of many diseases. For example, chronic peripheral inflammation is known to be a major cause of asthma, chronic hepatitis, lupus and rheumatoid arthritis. More recently, chronic inflammation of the CNS has been implicated in various neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS). In addition, acute CNS inflammation has been implicated as a secondary injury mechanism following ischemia and stroke.

Inflammation was already recognized in ancient Egyptian times as described in the Smith Papyrus *circa* 1650 BC (Figure 1). Later on, the Roman Cornelius Celsius (*circa* AD 25) was the first to define inflammation as a process characterized by four cardinal signs readily visible on the body surface: **heat, redness, swelling and pain** (Figure 2). A fifth cardinal sign of inflammation, **loss of function,** was added by the famous 19th century German pathologist Rudolf Virchow (Figure 2). Following pro-inflammatory events, tissues release chemical signals of infection or injury/damage including vasoactive and chemotactic mediators that contribute to the five cardinal signs of inflammation. Heat and redness at the site of injury are caused by a rise in blood flow, swelling by increased vascular permeability, pain by stimulation of nerve endings and loss of function by destruction of the tissue.

The three major functions of inflammation are (1) to eliminate the source of the insult to prevent its spread, (2) to prepare the injured site for repair and (3) to restore tissue homeostasis. While the inflammatory response is crucial for containing infection and delivering cellular and humoral components of the body's defense systems to the site of injury or infection, an excessive or over-long period of inflammation can be problematical. Resolution of inflammation (anti-inflammatory response) is an active process controlled by

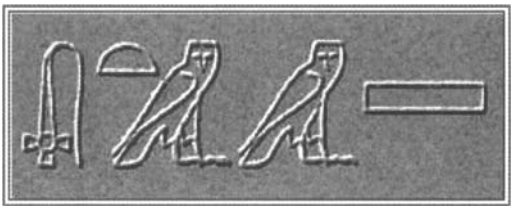

Figure 1. The hieroglyph reads from right to left. The bar is a phonetic "sh"; the two falcons are each a phonetic "m" and the half circle (a loaf of bread) is phonetically "t". The far left item is a flaming brazier with smoke curling up and down - something hot. All together the word sounded something like "shememet" and was the earliest description of inflammation, [from: http://rdh.c.home.att.net/pdf/inflamm.pdf].

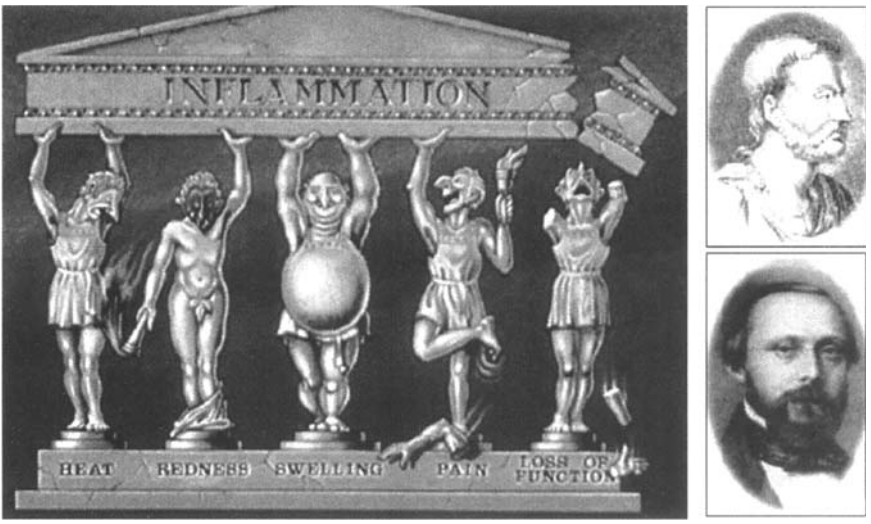

Figure 2. *Left*: CARDINAL SIGNS OF INFLAMMATION: heat, redness, swelling, pain and loss of function. Reproduced from (2) with permission from Macmillan Magazines Ltd. *Right:* Cornelius Celsius (top) and Rudolf Virchow (bottom), [from: http://rdh.c.home.att.net/pdf/inflamm.pdf].

endogenous mediators that suppress pro-inflammatory gene expression and cell trafficking and induce inflammatory-cell apoptosis and phagocytosis. An optimal balance between pro- and anti-inflammatory responses is required to prevent the highly detrimental effects of extensive, prolonged or unregulated inflammation.

3. INFLAMMATION IN THE CNS

There is abundant evidence that an inflammatory reaction is mounted within the CNS following trauma, stroke, infection and seizure, all of which can augment brain damage. The brain was long considered to be an immunologically privileged site, particularly because of the blood brain barrier (BBB) and the lack of a lymphatic system. However, more recently it has been shown that the brain mounts an inflammatory response, as noted from the occurrence of edema, activation of resident macrophages (microglia), local invasion of circulating immune cells and production of cytokines and other immune factors. Brain inflammation is also often associated with astrocyte activation and proliferation [reviewed in (3)].

3.1. Cell Types

Many cell types involved in the CNS response to injury can directly or indirectly affect neuronal survival (Figure 3). Neurons are rarely replaced once lost and are the cells that primarily determine CNS function and survival. Glia cells play a particularly important role in the CNS response to injury.

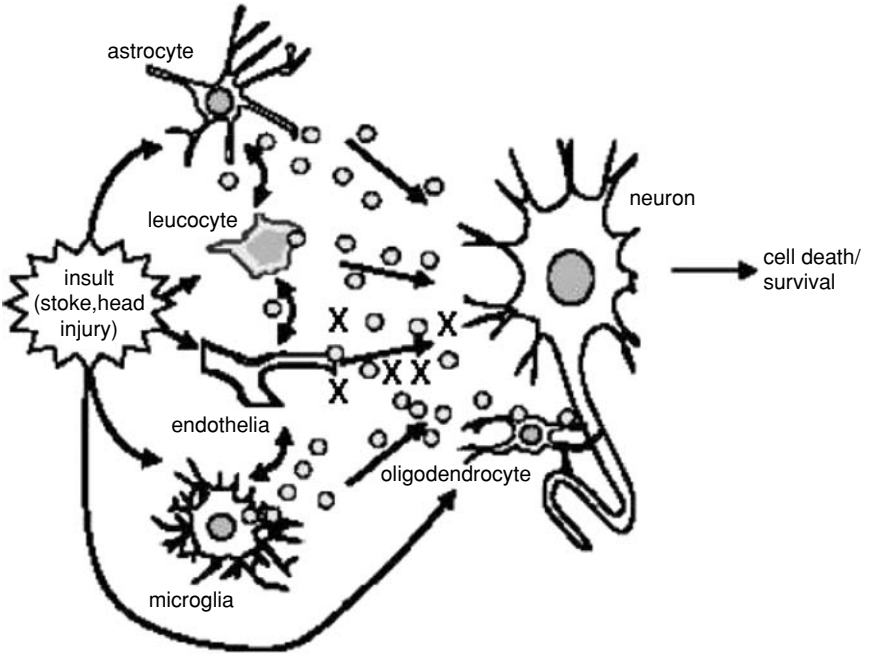

Figure 3. Overview of cells involved in cytokine release and action in neurodegeneration. Many cell types can release and respond to cytokines (small circles) and other inflammatory mediators, such as prostanoids (small crosses). These agents produce direct or indirect effects on neurons contributing to or limiting neuronal cell death in response to different insults. Reproduced with permission from (3).

The primary glial cells implicated in inflammation are microglia, which are derived from precursors of hematopoietic lineage. They reside in the brain and are activated in response to infection, inflammation and injury. Microglia are important phagocytic cells and release pro-inflammatory and neurotoxic factors including cytokines such as TNFα and IL1β, free radicals such as nitric oxide and superoxide, fatty acid metabolites such as eicosanoids and neurotoxins such as quinolinic acid (4).

In an attempt to enhance neuronal survival astrocytes, which are the most abundant cells in the CNS, can release neuroprotective molecules such as neutrophins. However, activated astrocytes can also produce inflammatory and potentially neurotoxic molecules such as particular types of cytokines, including TNFα and β, as well as nitric oxide (5).

Oligodendrocytes, the myelinating glia of the brain, also produce inflammatory molecules and respond to pro-inflammatory stimuli but their contribution to inflammation is less defined (6).

3.2. Vasculature

Under certain injurious conditions, it is well documented that the blood brain barrier is disrupted increasing its permeability to immune cells, such as

leucocytes and macrophages. These white blood cells can invade the brain parenchyma and release neurotoxins, activate local inflammatory processes, or, in the case of macrophages, phagocytose cells or cell debris. Inflammatory mediators such as cytokines can also escape from the blood vessels through leaky junctions or can exert their effects by binding to specific receptors on the blood vessel walls (Figure 4). In addition, vascular endothelial cells may secrete inflammatory molecules, such as nitric oxide and prostaglandins [reviewed in (3)].

3.3. Inflammatory Mediators

A variety of soluble inflammatory mediators are released during inflammation. Most of the primary mediators of inflammation identified in peripheral tissues are also expressed during CNS damage and have been implicated in CNS inflammation and disease. The soluble factors that mediate these responses fall into four main categories: (1) inflammatory lipid metabolites such as platelet activating factor (PAF) and the numerous derivatives of arachidonic acid (prostaglandins,

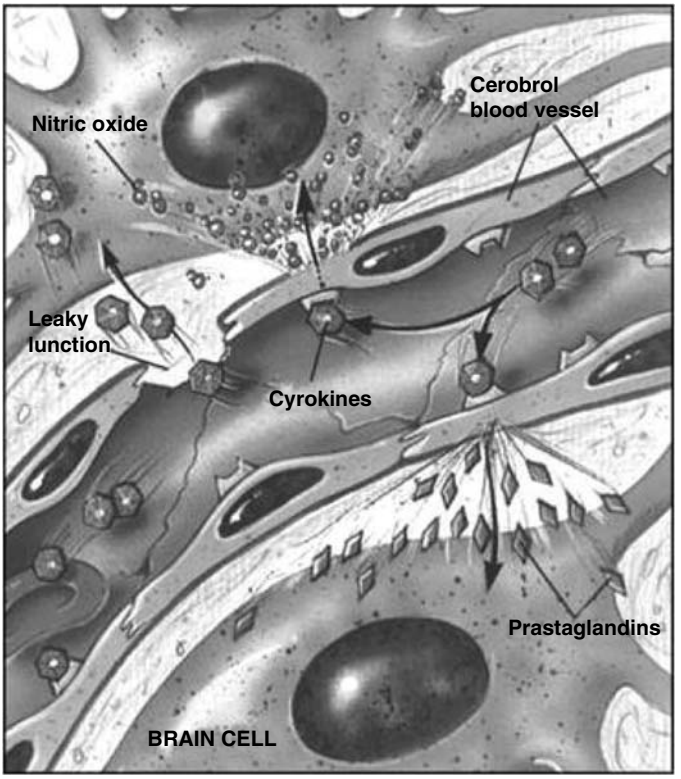

Figure 4. Cytokines cannot cross the blood brain barrier but they slip across leaky junctions. They can also bind to receptors on endothelial cells and trigger the production of molecules such as nitric oxide and prostaglandins. These molecules then directly affect neurons and glia. Illustration by Lydia Kibiuk,

leukotrienes, lipoxins), which are generated from cellular phospholipids; (2) three cascades of soluble proteases and substrates (clotting, complement, and kinins), which generate numerous pro-inflammatory peptides; (3) free radicals such as superoxide and nitric oxide, a potent endogenous vasodilator whose role in the inflammatory process has only recently begun to be explored; and (4) cytokines, proteins that serve as signaling chemicals and that control the direction, amplitude and duration of the inflammatory response. Cytokines are involved in extensive networks that involve synergistic as well as antagonistic interactions and exhibit both negative and positive regulatory effects on various target cells (7).

3.4. Triggers of CNS Inflammation

Crucial to the activation of inflammatory responses in a tissue is the sensing by host defense mechanisms of a noxious or foreign agent or an injurious process (Figure 5). The ability to distinguish foreign from self and abnormal from

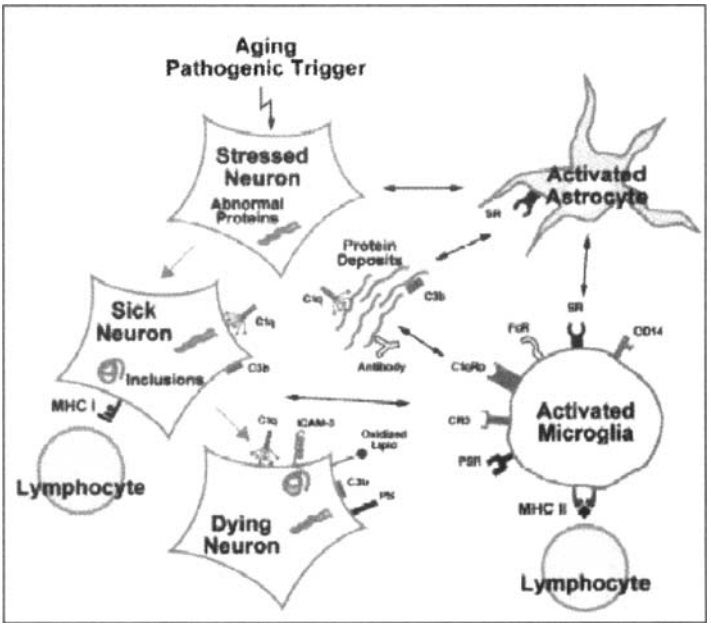

Figure 5. Some Components of the CNS Inflammatory Response. Accumulation of abnormal proteins in cells or extracellular spaces elicits stress responses and can result in the progressive dysfunction of neurons. Interactions indicated by arrows involve a large number of soluble factors. Cytokines and other inflammatory mediators are released by astrocytes and microglia to orchestrate defense mechanisms and initiate the removal or sequestration of the pathogenic triggers. Abnormal proteins and dysfunctional neurons are tagged by complement proteins (C3b or C1q) or by antibodies for recognition and phagocytosis by glial cells. Dysfunctional neurons may also be phagocytosed if they display intercellular adhesion molecule-3 (ICAM-3), phosphatidyl serine (PS), or oxidized lipids on their cell surface. Receptors on glial cells recognize these tags and initiate inflammatory responses. C1qRp, phagocytosis receptor; CR3, complement receptor 3; FcR, Fc receptor; PSR, phosphatidyl serine receptor; SR, scavenger receptor. Reproduced from (8) with permission from Elsevier.

normal is one of the most important aspects of inflammation in the CNS and elsewhere.

Inflammation in the CNS may result from (1) the formation of intracellular or extracellular protein aggregates, known to be associated with many neurodegenerative disorders, (2) accumulation of other abnormally modified cellular components, (3) molecules released from or associated with injured neurons or synapses and (4) deregulation of inflammatory control mechanisms such as in aging, which is associated with glial activation and increased production of inflammatory mediators [reviewed in (8)]. The inflammatory responses induced by these events aim to remove the pathogenic trigger.

In conclusion, inflammatory processes occur in the CNS through mechanisms that may differ from systemic inflammation and with distinct cellular effects. There are multiple aspects of neuroinflammation, all working simultaneously. The neuroinflammatory response to pro-inflammatory stimuli includes immune cell proliferation, glia activation, release of cytokines and induction of tissue repair enzymes that together limit cellular damage and help regenerate the CNS. However, these same inflammatory reactions are often the primary cause of tissue damage in both acute and chronic CNS pathology. The capacity for the same inflammatory response to both heal and harm the CNS makes it difficult to assess the significance and potential protective mechanisms of neuroinflammation (9).

4. FORMS OF INFLAMMATION

Inflammation can be classified based (1) on its duration, as acute versus chronic inflammation and (2) on the molecular pathways that are activated, as pro-versus anti-inflammatory responses.

4.1. Acute and Chronic Inflammation

The acute and chronic forms of inflammation are distinguished not only by the time course of the inflammatory response, but also by histopathological characteristics.

Acute inflammation lasts only for a few days. It can be caused by physical damage, chemical substances, micro-organisms or other agents. The inflammatory response includes changes in blood flow and blood vessel permeability as well as escape of cells from blood vessels into tissues. The changes are essentially the same independently of the cause and site of occurrence.

Chronic inflammation lasts weeks, months, or even indefinitely. The extended time course of chronic inflammation is provoked by persistence of the causative stimulus in the tissue. The inflammatory process inevitably causes tissue damage, which is accompanied by simultaneous attempts at healing and repair. The exact nature, extent and time course of chronic inflammation is variable and depends on the balance between the causative agent and the attempts of the body to remove it. Chronic inflammation may develop in the following ways: (1) as a progression from acute inflammation if the original stimulus persists, (2) after repeated episodes of acute inflammation, and (3) *de novo* if the causative agent produces only a mild acute response.

There are often no sharp boundaries in space or time among acute inflammation, chronic inflammation and repair processes. Acute inflammation, chronic inflammation and repair follow one another in some form or another in most inflammatory lesions.

4.2. Pro- and Anti-Inflammatory Responses

Inflammation includes the sequential activation of pro- and anti-inflammatory pathways. One of the best characterized pro-inflammatory responses is mounted by activation of the transcription factor NFκB, which regulates expression of pro-inflammatory mediators such as cytokines (TNFα and IL1β), adhesion molecules, chemokines, growth factors and the inducible enzymes cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS) [reviewed in (10)]. The aim of the acute pro-inflammatory response is to neutralize the noxious or foreign agent or the injurious process and remove it before it spreads to other parts of the body.

Relatively little is known about the anti-inflammatory mechanisms that resolve and "switch-off" acute inflammation. The resolution of acute inflammation is not due to a simple catabolism of pro-inflammatory mediators. The anti-inflammatory response is actively coordinated by its own endogenous "pro-resolving and anti-inflammatory" mediators. Notably, NFκB activation is also required to initiate the anti-inflammatory response and thus resolve inflammation [reviewed in (10)]. In the anti-inflammatory phase of inflammation NFκB promotes apoptosis and expression of anti-inflammatory mediators such as TGFβ1 and cyclopentenone prostaglandins as well as anti-inflammatory genes such as Bax and p53 (10).

Most of the currently available drugs to treat inflammation, including steroidal and non-steroidal anti-inflammatory drugs, are not really "anti-inflammatory" in the real sense of the word. They don't stimulate or trigger the anti-inflammatory response. Instead they halt the pro-inflammatory response. This may not be the most effective strategy to treat inflammatory conditions, since the innate pro-inflammatory response is a beneficial defensive event. If the injurious process is not completely eliminated and/or the anti-inflammatory activation is prevented this can lead to chronic inflammation. Detailed characterization of the anti-inflammatory biochemical pathways that resolve inflammation may offer novel and more effective strategies to treat inflammatory disorders and produce fewer side effects [reviewed in (11)].

5. INFLAMMATION-DEPENDENT OXIDATIVE STRESS IN THE CNS

All aerobic organisms are susceptible to oxidative stress simply because the reactive oxygen species (ROS), superoxide and hydrogen peroxide, are produced by mitochondria during respiration. The exact amount of ROS produced is considered to be about 2% of the total oxygen consumed during respiration, but it may vary depending on several parameters. Brain is considered abnormally sensitive to oxidative damage and in fact early studies demonstrating the ease of peroxidation of brain membranes supported this notion (12).

Figure 6 presents in simplified form the rationale of why brain is considered to be susceptible to oxidative stress (12). Brain is enriched in the more easily peroxidizable fatty acids (20:4 and 22:6), consumes an excessive fraction (20%) of the total oxygen consumption for its relatively small weight (2%) and is not particularly enriched in antioxidant defenses. In fact, brain is low in catalase activity containing about 10% of liver catalase. Additionally, human brain has higher levels of iron (Fe) in certain regions and in general has high levels of ascorbate. Thus, if tissue organizational disruption occurs, the Fe/ascorbate mixture is expected to be an abnormally potent pro-oxidant for brain membranes (12).

5.1. Oxidative Stress and Glia

Free radicals and ROS (radicals derived from oxygen) are atoms or groups of atoms that are highly reactive with other cellular molecules because they contain unpaired electrons. As ROS and other free radicals react with cellular molecules, they lead to injury and may even cause cell death. ROS and other free radicals may also trigger activation of various proteins that in turn activate the inflammatory response. The concept of ROS and free radical toxicity actually has its roots in inflammation biology (12). The secretion of reactive oxygen and nitrogen free radical species by "inflammatory" cells is a major mechanism for attacking a noxious or foreign agent or an injurious process (Figure 7). For example, in response to several factors including pro-inflammatory cytokines, glia (microglia and astrocytes) are capable of producing large amounts of nitric oxide due to their increased expression of inducible nitric oxide synthase (iNOS).

Glia are relatively resistant to high levels of nitric oxide but neurons are much more susceptible. The response of glia to cellular damage is quite complex. It includes increased expression of genes involved in nitric oxide and cytokine syntheses as well as the release of superoxide generated by NADPH-oxidase, and the release of hydrogen peroxide. The latter can be altered by peroxidases to form hypochlorous acid (HOCl), which is highly cytotoxic. In addition, the strong

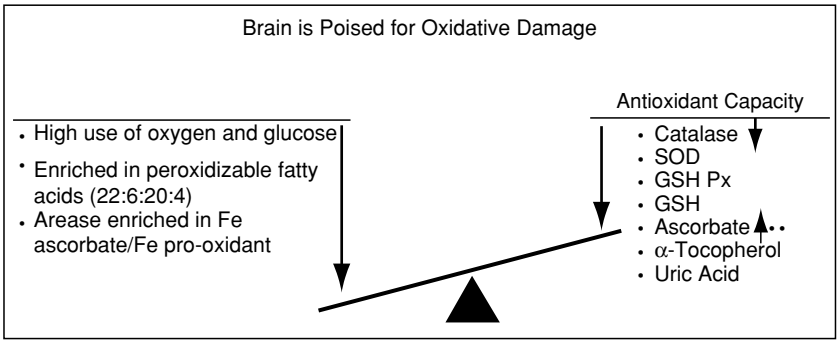

Figure 6. Summary of why the brain is poised to undergo oxidative damage. Under normal conditions, oxidative stress is held in check; however, specific insults such as a stroke or general aging will induce oxidative damage. *Brain has 10-20% of liver and heart; **Brain 1.1mM, plasma 62.4μM. Reproduced with permission from (12).

oxidant peroxynitrite (ONOO) can be formed by the interaction of nitric oxide with superoxide (13). The large amounts of ROS and other free radicals acutely produced by activated glia as well as their chronic release lead to neuronal injury and may even cause cell death.

5.2. Oxidative Stress and Cyclooxygenases

Free radicals are also produced by cyclooxygenases (Figure 8), the enzymes that catalyze the rate-limiting step in the biosynthesis of prostaglandins (PG), prostacyclins and thromboxane A2, from their precursor arachidonic acid [reviewed in (14)].

Cyclooxygenases are bifunctional proteins that catalyze the cyclooxygenation of arachidonic acid to PGG2 followed by the hydroperoxidation of PGG2 to PGH2 (15). Specific reductases, isomerases and synthases then convert PGH2 to other PGs and thromboxane A2. There are at least three distinct cyclooxygenase isoenzymes, but most studies up to date have focused on COX-1 and COX-2 [reviewed in (16)]. The brain expresses COX-1 and COX-2 under normal physiological conditions but COX-2 levels are dynamically regulated by pro-inflammatory signals and by physiological neuronal plasticity involving, for example, NMDA receptor activation (17). In neurons, COX-2 has mostly a perinuclear location. It is also present in dendritic arborizations and spines of excitatory neurons in the cerebral cortex, hippocampus and amygdala, suggesting that COX-2 products play a role in post-synaptic signaling (18). COX-2 up-regulation following CNS injury is not restricted to neurons [reviewed in (9)], since COX-2 induction is also apparent in astrocytes. Microglia express predominantly COX-1.

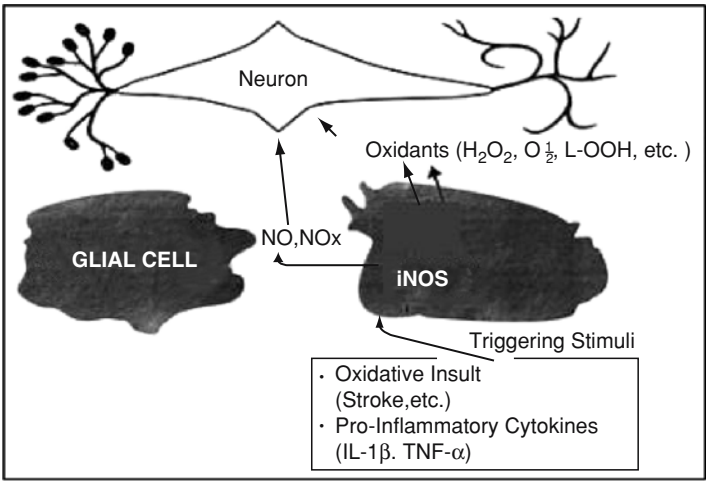

Figure 7. Illustration presenting the basic concepts of neuroinflammation where glia cells are activated to produce oxidants and nitric oxide (NO) and its oxidation products, which are toxic to neurons. Glia are activated by various triggering stimuli resulting in the induction of iNOS and other genes. Reproduced with permission from (12).

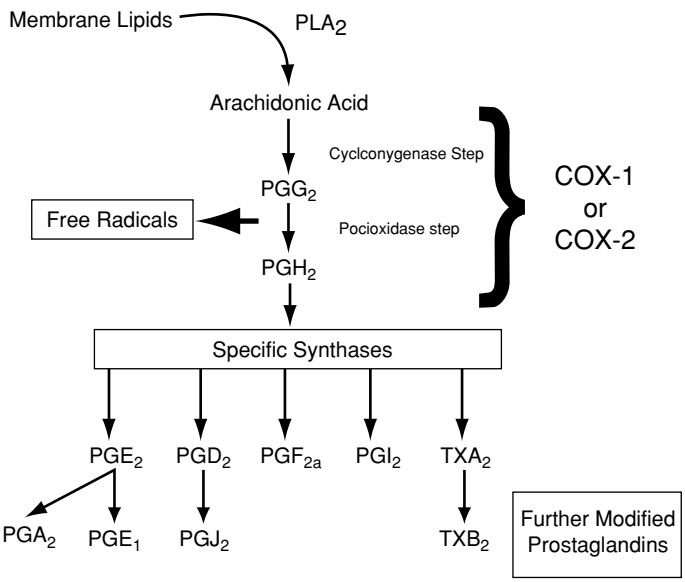

Figure 8. The activity of cyclooxygenases generates the precursors for the prostaglandin family. Membrane lipids are broken down by phospholipase A2 (PLA2) into arachidonic acid (AA). Subsequently, the COX enzyme (either COX-1 or COX-2), first via the cyclooxygenase activity of the enzyme, converts AA to prostaglandin G2 (PGG2) and then via the perioxidase step converts PGG2 to Prostaglandin H2 (PGH2). The perioxidase step results in the production of free radicals. PGH2 can then be converted by tissue-specific synthases to various prostaglandins including prostaglandin E2 (PGE2), prostaglandin D2 (PGD2), prostaglandin F2α (PGF2α), prostacyclin (PGI2) and thromboxane A2 (TXA2). These products can further be modified (e.g., PGA2, PGE1, PGJ2, TXB2, and others products not shown in this figure). Reproduced from (9) with permission from Elsevier.

The peroxidase activity of cyclooxygenases, which converts PGG2 to PGH2, is the enzymatic step that also produces free radicals including superoxide (Figure 8). It is unlikely that up-regulation of COX-2 alone produces enough free radicals to account for the degree of oxidative damage associated with inflammation. However, COX-2 up-regulation by pro-inflammatory signals may be one of the several pro-oxidant mechanisms that cause significant cumulative neuronal damage associated with inflammation (9).

Cyclooxygenases are also able to oxidize dopamine to dopamine quinone via their peroxidase activity (Figure 9). These enzymes will readily utilize dopamine as an electron donor to support their peroxidase activity generating an electron-deficient dopamine quinone as a byproduct. Dopamine quinone can then covalently bind to the sulfhydryl groups of cysteine residues on proteins. If the covalently modified cysteine is located at or near the protein active site, the binding of dopamine quinone will cause inactivation of protein function. If these protein functions are essential for cell viability, their inactivation may account for quinone-induced cytotoxicity [reviewed in (19)].

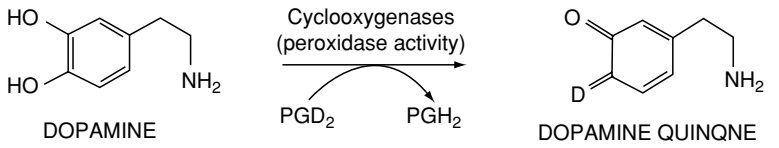

Figure 9. Oxidation of dopamine by the peroxidase activity of cyclooxygenases. Reproduced from (19) with permission from Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

5.3. Oxidative Stress and Prostaglandins of the J2 Series

The pro-oxidant effects of some of the prostaglandins constitute another source of oxidative stress associated with neuroinflammation. Prostaglandins (PGs) are a family of structurally related molecules produced in response to numerous extrinsic and intrinsic stimuli and are involved in a wide variety of physiological and pathophysiological responses. The coupling of PGH2 synthesis, the precursor of all prostaglandins, with the respective downstream enzymes that produce the different types of prostaglandins, is intricately orchestrated in a cell specific fashion [reviewed in (20)]. PGD2 is the major prostanoid made in the mammalian CNS. It is produced by a PGD2 synthase, which is an enzyme that carries out the isomerization of PGH2 to PGD2 (21) (Figure 10). The brain form of PGD synthase has dual function acting as an enzyme and also as a transporter of its product throughout the brain (22). Furthermore, brain PGD synthase is co-localized with COX-2 as, for example, in brain meningeal cells providing evidence for its function as a PGD2-producing enzyme (23).

PGD2 readily undergoes *in vivo* and *in vitro* non-enzymatic dehydration to generate the biologically active cyclopentenone prostaglandins of the J2 series, which include PGJ2, Δ12-PGJ2 and 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2) (24) (Figure 10). Unlike most other classes of eicosanoids, prostaglandins of the J2 series contain a cyclopentenone ring with α,β-unsaturated carbonyl groups, making them susceptible to Michael addition reactions with free sulfhydryl groups of cysteines in glutathione and cellular proteins (reviewed in (25)). Furthermore, Δ12-PGJ2 was found to bind irreversibly to synthetic polymer-supported thiols that mimic thiol-containing proteins, suggesting that the binding of these cyclopentenones to proteins is irreversible (26).

Prostaglandins of the J2 series induce oxidative stress by causing a decrease in glutathione, glutathione peroxidase activity, mitochondrial membrane potential and production of protein-bound lipid peroxidation products, such as acrolein and 4-hydroxy-2-nonenal (27). These effects suggest that prostaglandins of the J2 series are either a source of markedly increased ROS generation or modulators of ROS sensitivity (28).

5.4. Oxidative Stress and Lipoxygenases

Another source of oxidative stress associated with arachidonic acid signaling in the CNS is the lypoxygenase (LOX) pathway. Although eicosanoid

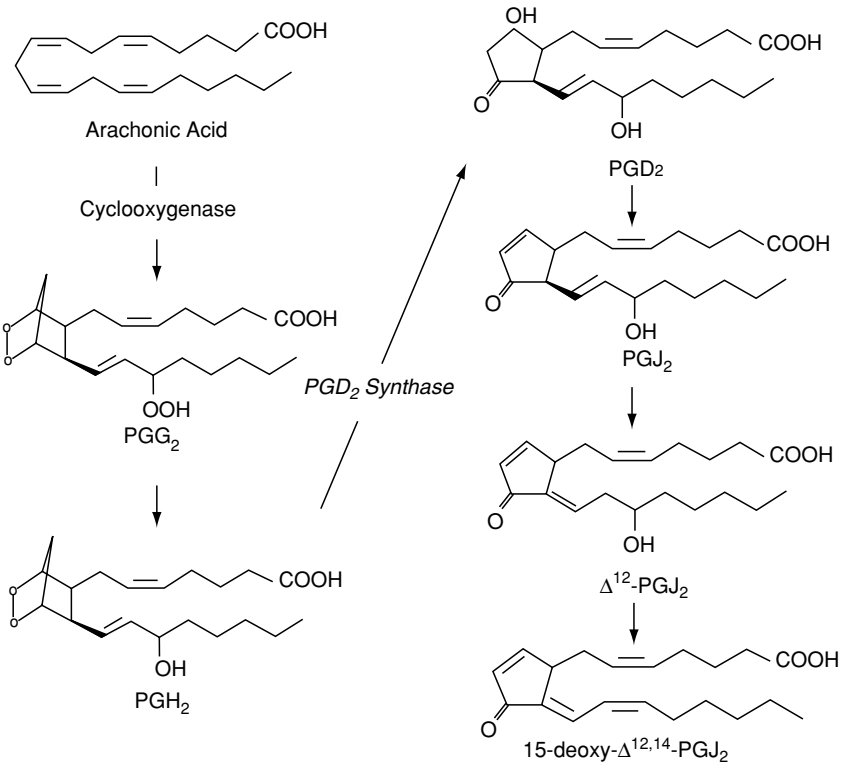

Figure 10. Synthesis pathway of cyclopentenone prostaglandins of the J2 series. Reproduced with permission from (29).

synthesis in the CNS involves cyclooxygenase as well as lypoxygenase pathways, much less is known about the contribution of the latter to oxidative stress induced by neuroinflammation. LOXs are a family of monomeric non-heme, non-sulfur iron dioxygenases, which catalyze the conversion of polyunsaturated fatty acids into conjugated hydroperoxides [reviewed in (30)], (Figure 11). When arachidonic acid is the substrate, different LOX isozymes can add a hydroperoxy group (OOH) at carbons 5, 12 or 15, and are thus designated 5-, 12- or 15-LOXs. These LOXs generate 5-, 12- and 15-hydroperoxyeicosatetraenoic acids (5-, 12- and 15-HPETE), respectively. Some LOXs, such as 12/15-LOX, can form two HPETE compounds at the same time (31). Mammalian cells can reduce the lipid hydroperoxides (LOOH) to the supposedly less toxic hydroxides (LOH), in a reaction requiring glutathione (GSH) and catalyzed by glutathione peroxidase:

$$LOOH + 2GSH \rightarrow LOH + GSSG + H_2O$$

Depletion of intracellular GSH might thus enhance hydroperoxide-induced cell death [reviewed in (30)]. HPETE are able to uncouple mitochondria within hours. 15-LOX activity can dioxygenate mitochondrial membranes,

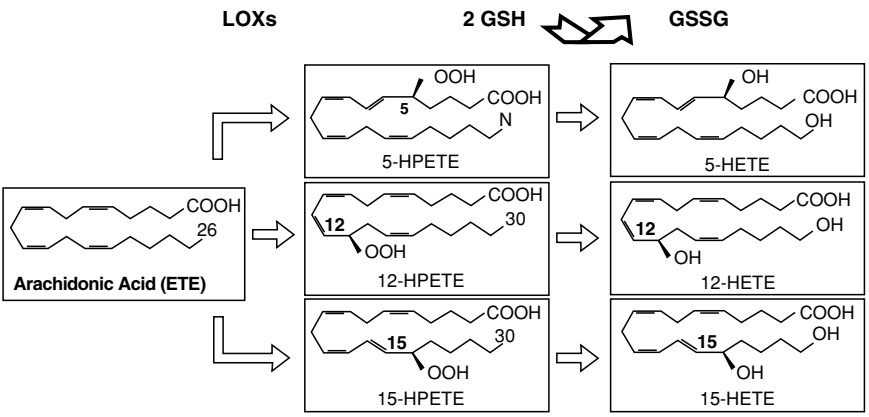

Figure 11. Hydroperoxides generated by different lipoxygenases (LOXs) with arachidonic acid as substrate. The hydroperoxides can be reduced to the corresponding hydroxides by cellular glutathione peroxidases, which concomitantly convert reduced glutathione (GSH) to its oxidized form (GSSG). Reproduced from (30) with permission from Macmillan Publishers Ltd.

leading to formation of pore-like structures observable by electron microscopy also in membranes of endoplasmic reticulum, thus initiating programmed organelle disruption [reviewed in (30)].

The predominant LOX in the brain is 12-LOX and its mRNA was detected in neurons, oligodendrocytes and astrocytes [reviewed in (32)]. 12-LOX is involved in neuronal death pathways such as kainic acid excitotoxicity, βamyloid peptide- and prion peptide-induced apoptosis, and oxidative glutamate toxicity [reviewed in (33)]. Furthermore, studies with rat mesencephalic cultures support a role for arachidonic acid and its lipoxygenase metabolites in the toxicity induced by GSH-depletion (34).

A model of "Redox Stress Sensor" was developed by Finazzi-Agro and colleagues (Figure 12). In this model LOX activity generates toxic intermediates (such as hydroperoxides and superoxide anions), which lead to apoptosis by increasing membrane fluidity and permeability, intracellular calcium concentration, mitochondrial uncoupling and cytochrome c release. The equilibrium between LOX and glutathione (GSH) might interfere with the intracellular level of ROS. This situation is further complicated by the ability of LOX to induce directly the events triggered by the toxic intermediates. Collectively, the balance between these elements and the status of the membranes will finely tune the sensitivity of the cell to resist to redox stresses or to die (30).

In conclusion, neuroinflammation can trigger oxidative stress by at least two different mechanisms: (1) production of high levels of ROS by activated glia such as microglia and astrocytes and (2) arachidonic acid signaling through the activation of cyclooxygenase and lypoxygenase pathways. We do not discuss the P450 arachidonic acid metabolic pathway because not much is known about its role in neurodegeneration. As more roles and interactions for arachidonic acid

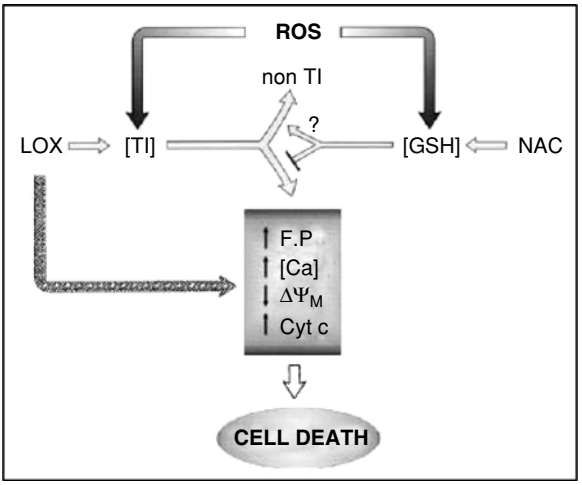

Figure 12. The 'Redox Stress Sensor' model, whereby lipoxygenase (LOX) activity generates toxic intermediates (TI), which lead to apoptosis by *(i)* increasing membrane fluidity (F) and permeability (P), *(ii)* elevating intracellular calcium concentration ([Ca]i), *(iii)* decreasing mitochondrial membrane potential ($\Delta\Psi$m) and *(iv)* increasing cytochrome c (Cyt c) release. The intracellular level of glutathione (GSH), which can be regulated by N-acetylcysteine (NAC), counteracts the effect of LOX activity, also by possibly promoting detoxification of TI to non-toxic species. The equilibrium between LOX and glutathione (GSH) might interfere with the intracellular level of reactive oxygen species (ROS). This picture is further complicated by the ability of LOX to induce directly the events triggered by the toxic intermediates. Reproduced from (30) with permission from Macmillan Publishers Ltd.

products are identified it is clear that this polyunsaturated fatty acid will be the subject of future biomedical research (28).

6. INFLAMMATION AND UPS DYSFUNCTION

A wide variety of neurodegenerative disorders are associated with the accumulation of ubiquitinated proteins in neuronal inclusions as well as with signs of inflammation [reviewed in (35)]. The relationship between these two events and their role in neurodegeneration is not well defined. The inability of some neurons to degrade ubiquitinated proteins may result from a functional failure of the UPS or from structural changes in the protein substrates rendering them inaccessible to the degradation component [reviewed in (36)]. Disruption of the UPS can result from damaging events, such as oxidative stress (reviewed in (37)) and production of neurotoxic molecules, from mutations or from an aging-induced decrease in proteasome function [reviewed in (38)]. A dysfunctional UPS may then cause proteins that are normally turned over by this pathway to aggregate and form inclusions. The role of the inclusions in the progression of the disease has yet to be elucidated (39). Inclusions may develop as a cellular attempt to compartmentalize accumulated proteins and prevent the obstruction of normal

cell function. On the other hand, inclusions may confer cytotoxic effects that contribute to cellular damage and neurodegeneration.

6.1. Inflammation and Ubiquitinated Protein Aggregates

One of the mechanisms by which the abnormal accumulation and aggregation of ubiquitinated proteins may mediate neurodegeneration is by triggering a neuroinflammatory response. Accordingly, agents that elicit accumulation and aggregation of ubiquitinated proteins in neuronal cell cultures, including proteasome inhibitors and pro-oxidant agents such as cadmium and the cyclopentenone prostaglandin J2, also increase the expression and activity of the pro-inflammatory cyclooxygenase COX-2 (40-43). These results clearly demonstrate that a neuroinflammatory response can be triggered *in vitro* by the intracellular accumulation and aggregation of ubiquitinated proteins. The effect of these agents on neuroinflammation under *in vivo* conditions has yet to be determined.

6.2. Inflammation-dependent Oxidative Stress and UPS Dysfunction

It is clear that pro-inflammatory responses trigger oxidative stress by different means as discussed above in section 5. Products of oxidative stress can in turn affect UPS activity by mechanisms described in other chapters of this book.

6.3. Inflammation and UCH-L1 Activity

Some of the products of inflammation, such as the neurotoxic PGD2 and its metabolites PGJ2, Δ12-PGJ2 and 15d-PGJ2, directly affect the activity of components of the UPS leading to a raise in the levels of ubiquitinated proteins in neuronal cells (27).

Although one study reported the binding of biotinylated 15d-PGJ2 to the proteasome (44), PGD2 and its metabolites appear to not affect directly the activities of the 26S or 20S proteasomes (42;45). Instead, Δ12-PGJ2 was found to inhibit the ubiquitin isopeptidase activity of RKO cell lysates, a colorectal cancer cell line (45). More importantly, Δ12-PGJ2 inhibits the activity of the ubiquitin C-terminal hydrolase UCH–L1 (42). The effect of Δ12-PGJ2 on UCH-L1 was unique, since four other PGs tested, namely A1, D2, E2 and J2, did not affect the activities of UCH-L1. PGJ2 is unstable and can be dehydrated to the more stable Δ12-PGJ2, which is a more active product (25). Δ12-PGJ2 contains a cyclopentenone ring with two α,β-unsaturated carbonyl groups while PGJ2 contains only one (Figure 13).

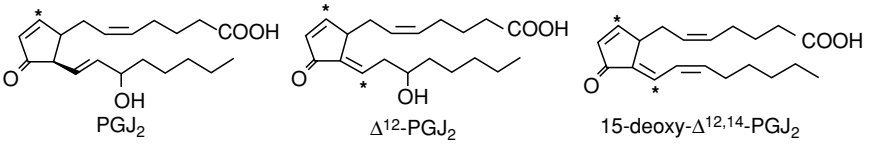

Figure 13. Chemical structure of PGJ2, Δ12-PGJ2 and 15d-PGJ2. *Asterisks* (*) depict the electrophilic carbons, one in PGJ2 and two in Δ12-PGJ2 and 15d-PGJ2.

These carbonyl groups make Δ12-PGJ2 susceptible to Michael addition reactions with free sulfhydryl groups of cysteines [reviewed in (25)]. Since UCHs contain active site cysteines, it is possible that Δ12-PGJ2 may undergo Michael addition reactions with free sulfhydryl groups on these residues, thus inhibiting UCH activity. In fact, 15d-PGJ2, a metabolite of Δ12-PGJ2, was shown to induce cysteine-targeted oxidation/thyolation of several proteins including UCH-L1 (46).

UCH-L1 is reported to comprise 1-2% of the total soluble protein in brain (47) and is a neuronal specific UCH (48). Inhibition of UCH-L1 is relevant to neuronal pathology as a missense mutation in the *uch-l1* gene, which results in a decrease in UCH activity, was identified in a German family with PD (49). UCH-L1 was also found to exhibit E3 ligase activity (50). Interestingly, a polymorphic UCH-L1 variant (S18Y) with reduced E3 ligase activity is associated with decreased PD risk (50). More recently, UCH-L1 was found to be down-regulated in AD and PD brains and to be a major target of oxidative stress (51), being altered by carbonyl formation as well as methionine and cysteine oxidation (52). In addition, an in frame deletion of exons 7 and 8 in the *uch-l1* gene causes a "dying back" type of axonal degeneration in the *gad* mouse with *g*racile *a*xonal *d*ystrophy (53). In this mouse model of neurodegeneration, discussed in another chapter of this book, ubiquitinated proteins accumulate retrogradely along sensory and motor neurons. Together, these studies indicate that UCH-L1 impairment induced by genetic mutations or pathological conditions such as neuroinflammation, is an important contributor not only to the accumulation and aggregation of ubiquitinated proteins but also to neuronal cell death, both occurring in most types of neurodegenerative diseases.

6.4. Inflammation and Ubiquitin Ligases

E3 ubiquitin ligases are the critical components that provide specificity to the ubiquitin conjugation system as they interact directly and specifically with the substrates. E3-promoted ubiquitination is involved in many biological processes, such as receptor down-regulation, signal transduction, protein processing or translocation, protein-protein interaction, gene transcription and proteasome-mediated protein degradation. Searches in gene databases reveal that there are hundreds of E3s and they are divided into two main groups: the HECT (homology to the E6-associated protein carboxyl terminus) domain-containing E3s and the RING (really interesting new gene) domain-containing E3s. E3 ubiquitin ligases are involved in many aspects of neuroinflammation. For example, LPS- or IL1-activation of nuclear factor κB (NFκB) requires ubiquitination by specific E3 ligases and subsequent degradation of its inhibitor IκB. Notably, mutations in E3 ubiquitin ligases are linked to neurological disorders such as the HECT domain E3 ligase E6-AP in Angelman syndrome and the RING-finger domain E3 ligase parkin in PD. Since new E3 ligases are emerging so rapidly it is impossible to discuss each and every E3 and its potential role in neuroinflammation. Instead, we would like to direct the reader to an excellent review recently published on the subject of ubiquitin ligases and the immune response where inflammation is also discussed (54).

6.5. Inflammation Regulator with De-ubiquitinating and Ubiquitin Ligase Properties

The zinc-finger protein A20 is a regulator of inflammation and cell survival by preventing NFκB activation and apoptosis. A20-deficient mice exhibit chronic inflammation and cell death because of failure to inhibit NFκB transcriptional activity (55). A20 was recently shown to be a de-ubiquitinating enzyme (56). The amino-terminal domain of A20, which is a de-ubiquitinating enzyme of the OTU (ovarian tumor) family, removes K63-linked ubiquitin chains from the receptor interacting protein (RIP), an essential mediator of the proximal TNF receptor 1 signaling complex (57). The carboxyl-terminal domain of A20 is composed of seven C2/C2 zinc fingers and functions as a ubiquitin ligase by polyubiquitinating RIP with K48-linked ubiquitin chains, thereby targeting RIP for proteasomal degradation (57). A20 does not have a global effect on ubiquitinated cellular proteins, which indicates that its activity is target-specific (56).

6.6. "Pseudo" Anti-Inflammatory Properties of Proteasome Inhibitors

Proteasome inhibitors have been suggested to be anti-inflammatory drugs [reviewed in (58)]. However, proteasome inhibitors are not true anti-inflammatory agents since they do not trigger the resolution of inflammation. They only halt inflammation when administered during the initiation phase of the pro-inflammatory response most likely by preventing NFκB activation. When administered after the onset of inflammation, the proteasome inhibitors prevent its resolution and, in fact, significantly exacerbate the pro-inflammatory response (10). This occurs most likely because proteasome inhibitors prevent NFκB activation, which is also required for triggering the anti-inflammatory response (10). The two proteasome inhibitors currently under clinical evaluation, Velcade (PS-341) for multiple myeloma and PS-519 for inflammatory events associated with acute stroke do not cross the blood brain barrier (BBB) and therefore are not bioavailable to the CNS unless the blood brain barrier (BBB) is dismantled (58).

The purpose of most currently available drugs to treat inflammatory diseases is to halt the host's response to injury. It is thought that failure to adequately resolve acute inflammation may lead to the development of chronic inflammation. To interrupt the pro-inflammatory response, therefore, may not be the best strategy to treat inflammatory diseases. A more efficient strategy would be to target the mechanisms responsible for the anti-inflammatory response. Once the anti-inflammatory mediators are well characterized, attempting to mimic their action should promote a more effective resolution of inflammation (11).

6.7. Anti-Inflammatory Properties of PPARγ Agonists

The J2 series of prostaglandins were once considered to be inactive degradation products of PGD2, the major brain prostaglandin. However, they are currently recognized as regulators of diverse processes including inflammation [reviewed in (59)]. These cyclopentenone prostaglandins enter the cells by an active transport system (60). Once inside the cell, an additional transport mechanism

allows these PGs to enter the nucleus where they regulate gene transcription (Figure 14). Alternatively, PGD2 that enters cells by an anionic transporter can be metabolized to prostaglandins of the J2 series in the cytoplasm. Once inside the cells, the cyclopentenone prostaglandins enter the nucleus and regulate gene transcription through different transcription pathways. For example, 15d-PGJ2, one of the cyclopentenones of the J2 series, seems to be an endogenous agonist for the transcription factor peroxisome proliferator-activated receptor γ (PPARγ). Prostaglandins of the J2 series were also shown to regulate gene expression through PPARγ-independent transcription including activation of the p38MAPK and JNK pathways (43;61) and inhibition of the NFκB pathway (62;63).

Due to its complexity, the role of 15d-PGJ2 in inflammation is the subject of intense *in vivo* and *in vitro* research [reviewed in (59)]. On the one hand, 15d-PGJ2 has emerged as a key anti-inflammatory agent. Through PPARγ-dependent and independent pathways it inhibits the production of pro-inflammatory mediators such as iNOS, TNFα and IL1β, suppresses microglia and astrocyte activation and induces apoptosis (64-66). On the other hand, 15d-PGJ2

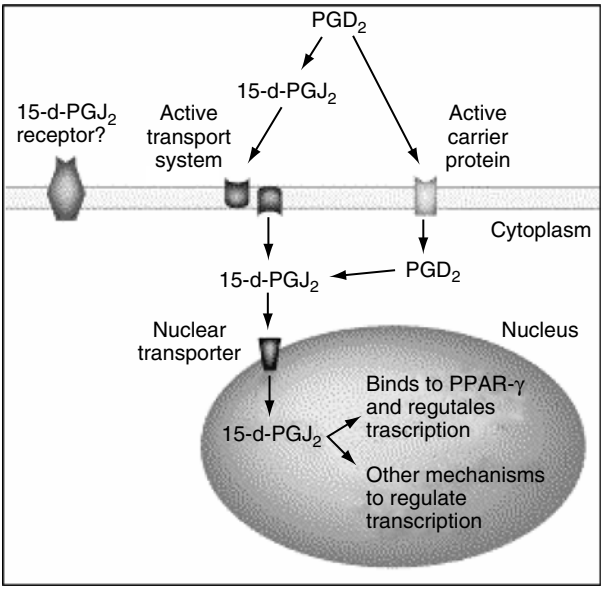

Figure 14. Possible modes of action of 15d-PGJ2 in the cell. Prostaglandin D_2 (PGD_2) is broken down into 15d-PGJ2, which could gain entry to the cell by an active transport system, and then enter the nucleus by a nuclear transporter, as described for other cyclopentanone prostaglandins. Also, 15d-PGJ2 could bind to an undiscovered cytoplasmic receptor. Alternatively, PGD_2 could gain entry into the cell by means of an anionic carrier protein and then be metabolized in the cytoplasm to 15d-PGJ2, which could then gain entry to the nucleus by a nuclear transporter protein. Once inside the nucleus, 15d-PGJ2 can bind to and activate peroxisome proliferator-activated receptor-γ (PPARγ) to regulate gene transcription. There are also other mechanisms by which 15d-PGJ2 mediates transcription in a PPARγ-independent manner. Reproduced from (59) with permission from Elsevier. * It is likely that other prostaglandins of the J2 series act through similar mechanisms.

is a pro-inflammatory agent. It stimulates the production of pro-inflammatory mediators such as IL8, the expression of COX-2 and activates MAPK (67;68). A better understanding of the mechanisms that mediate the effects of 15d-PGJ2 and other prostaglandins of the J2 series will most likely lead to new approaches for the treatment of inflammatory disorders.

PPARγ activation in the brain by 15d-PGJ2 limited the deleterious effects (fever) of LPS-induced acute inflammation in rats (69). More importantly, a PPARγ agonist (pioglitazone) was capable of clinically stabilizing chronic inflammation in a patient with secondary progressive multiple sclerosis (70). In addition, some of the non-steroidal anti-inflammatory drugs (NSAIDS) that inhibit cyclooxygenases, in particular indomethacin and ibuprofen, were found to activate PPARγ (71). This NSAID-PPARγ link may be responsible for the decreased risk of AD observed in NSAID-treated patients, since only the epidemiological studies that used a PPARγ-activating NSAID were reported to have a benefit (64).

Together, these studies support the notion that PPARγ agonists may have potential for treatment of neurodegenerative disorders associated with chronic inflammation. However, it is essential to keep in mind that prostaglandins of the J2 series affect other transcription factors besides PPARγ. Activation of these other pathways together with their pro-oxidant and UPS disrupting effects render these cyclopentenone prostaglandins extremely neurotoxic and capable of inducing neuronal cell death (42;72;73). Additional research to accurately and comprehensively define the mechanisms mediating the effects of these prostaglandins is pivotal to the development of novel therapeutic strategies to prevent or treat chronic neuroinflammatory diseases.

6.8. Physiological Relevance of Prostaglandins of the J2 Series in the CNS

Numerous studies demonstrate that prostaglandins are formed in regions of the brain and spinal cord in response to different types of challenges [reviewed in (74)]. Physiological concentrations of PGs in body fluids are found to be in the pico-nanomolar range (75). However, their levels rise considerably under pathological conditions such as hyperthermia, infection and inflammation, reaching the micromolar range at the site of damage (76;77).

Synthesis of cyclopentenone prostaglandins, such as PGJ2, was found to increase in the late phases of inflammation and to be associated with its resolution (78). For example, in carrageenin-induced pleurisy in rats, the induction of COX-2 expression following pro-inflammatory stimuli was found to be biphasic occurring two hours after the pro-inflammatory insult and again 48h after the insult. The second COX-2 surge was significantly greater (~350%) than the first one and coincided with the anti-inflammatory phase meant to resolve inflammation. While COX-2 specific inhibitors (NS398) or COX-1/COX-2 inhibitors (indomethacin) prevented inflammation at two hours they significantly exacerbated inflammation at 48h (78). Most importantly, the exacerbation effect of these NSAIDs corresponded to a decrease in PGD2 and 15d-PGJ2 and was overturned by replacement of these PGs (78).

The notion that 15d-PGJ2 is an endogenous PPARγ agonist was questioned because 15d-PGJ2 could not be detected during adipocyte differentiation or throughout LPS-administration to humans or in the synovial fluid of patients suffering from arthritis (79). It is possible that 15d-PGJ2 cannot be detected in biological fluids because it has a short half-life and binds avidly to free sulfhydryl groups and thus most of it could be bound to intracellular proteins. Attempts to measure 15d-PGJ2 endogenous levels should focus on assessing intracellular 15d-PGJ2/protein complexes (11). Interestingly, the levels of 15d-PGJ2 were found to be elevated in spinal cord motor neurons of ALS patients (72). It is thus likely that PGJ2 and its metabolites are produced in the CNS. Their concentrations may be increased in response to pro-inflammatory stimuli, particularly during the resolution phase of the inflammatory response.

6.9. Model for the interaction between Inflammation and UPS Dysfunction in Neurodegeneration

It is possible that neurodegenerative factors, such as genetic make-up, aging or environmental toxins contribute to the development of initial cellular lesions containing ubiquitinated proteins. These neuronal inclusions, which are hallmarks of neurodegeneration, may themselves lead to neuronal damage. The neurodegenerative process, however, could be exacerbated by stimulation of a pro-inflammatory response that would contribute to a more rapid decline in neuronal survival.

In neurodegenerative disorders characterized by neuronal inclusions containing ubiquitinated proteins, a disruption of the UPS may act in conjunction with COX-2 to exacerbate the neurodegenerative process. Some of the COX-2 metabolic products such as prostaglandins of the J2 series may, in turn, perturb the UPS. This toxic positive feedback may create a self-destructive mechanism that contributes to the neurodegenerative process (35).

Our mechanistic model for the interaction between neuroinflammation and UPS dysfunction in neurodegeneration is depicted in Figure 15. We propose that injurious stimuli that affect protein structure, such as oxidative stress, neurotoxic compounds or mutations may lead to UPS impairment. The consequent accumulation of ubiquitinated proteins may then trigger the production of pro-inflammatory mediators, such as COX-2.

Products of COX-2, including ROS and neurotoxic prostaglandins, such as cyclopentenone prostaglandins of the J2 series, would then contribute to neuronal damage. A positive feedback (+) between the COX-2 products and impairment of the UPS could exacerbate neuronal damage to a point of no return, thus leading to neurodegeneration.

7. INFLAMMATION AND NEURODEGENERATIVE DISORDERS

Neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD) and amytrophic lateral sclerosis (ALS), found to be associated with the accumulation of ubiquitinated proteins in neuronal inclusions also exhibit signs of inflammation. For example, neurofibrillary tangle

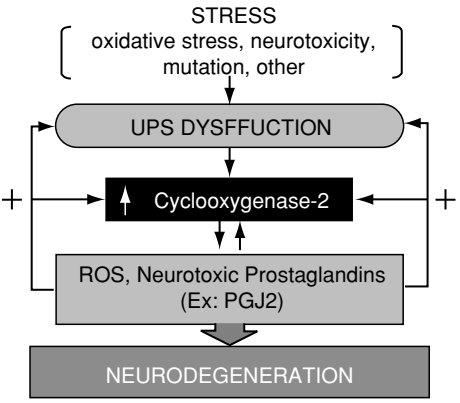

Figure 15. Scheme linking disruption of the UPS and inflammation to neurodegeneration (see text for explanation). This scheme depicts only one of the putative pathways of neurodegeneration and does not exclude other mechanisms leading to the development of neurodegenerative disorders associated with the accumulation of ubiquitinated proteins in neuronal inclusions. Reproduced from (35) with permission from Elsevier.

(NFT)-containing and damaged neurons in brains of Down's syndrome and AD patients exhibit high expression of COX-2 (80-82). Furthermore, up-regulation of COX-2 was found to precede the appearance of NFT-containing neurons and neurodegeneration in patients with Fukuyama-type congenital muscular dystrophy, a neurodegenerative disorder transmitted through autosomal recessive inheritance (83). When compared to control brains, the substantia nigra in parkinsonian brains contain higher levels of PGE2, which is symptomatic of an increased COX activity (84;85). Moreover, a marked increase in COX-2 levels was detected in the spinal cord of ALS patients (86).

Animal models of AD, ALS and PD corroborate the relationship between COX-2 activity and neurodegeneration. Transgenic mice carrying mutations in amyloid precursor protein as well as presenilin and that develop AD-like beta-amyloid deposits, show a significant increase in astrocyte COX-2 levels (87). In addition, transgenic mice overexpressing neuronal COX-2 and producing elevated levels of prostaglandins in the brain developed an age-dependent AD-like memory dysfunction (88). Notably, prostaglandins were shown to act as neurotoxins by increasing the levels of ubiquitin-conjugates and β-amyloid production in differentiated neuroblastoma PC12 cells (89).

A transgenic mouse model of ALS expressing the superoxide dismutase mutation G93A in the spinal cord also exhibits COX-2 up-regulation (90). Moreover, COX-2 specific inhibitors confer neuroprotection in an MPTP-mouse model of PD (91). Recent studies demonstrated that JNK-mediated induction of COX-2 is indispensable for MPTP-induced neurodegeneration in a PD mouse model (92). Collectively these studies support the notion that the spatial and temporal association of COX-2 with neuropathological changes correlates with neurodegeneration in these diseases [reviewed in (8;16)]. Inflammatory processes may also be involved in the pathogenesis of polyglutamine disorders as inflammatory

genes, such as IL1β, were found to be up-regulated in spinocerebellar ataxia type 3 brains (93).

In conclusion, these findings strongly support the notion that for many neurodegenerative diseases for which the root cause is unknown, neuroinflammation may play a key role. However, it is unclear if these diseases are caused by inflammation or if inflammation reflects an attempt to remove and repair neuronal damage caused by cellular injury. Regardless of which comes first, it is clear that inflammatory pathways involving cyclooxygenases and subsequent generation of prostaglandins are potential targets for treatment to halt the progression of neurodegeneration associated with inflammation.

8. CONCLUSION

Neuroinflammation has both beneficial and deleterious effects on the CNS. Nevertheless, there is little doubt that neuroinflammation is a major contributor to diverse, acute and chronic neurodegenerative disorders. Anti-inflammatory targets identified as strategies to treat acute inflammatory conditions in the CNS may turn out to also be effective for chronic neurodegenerative conditions.

A key question that remains unanswered is whether the different forms of neurodegenerative disorders share a common mechanism, i.e. neuroinflammation (3). If so, how can the enormously varied etiology, presentation and time course of these devastating disorders be explained? For example, while head injury is a rapid, accidental event that affects mostly young individuals, PD is characterized by damage to specific brain regions resulting in motor disturbances and chronic degeneration. This variety of disease manifestations could be correlated to the primary brain region affected by the injurious event, its severity and duration.

Much more needs to be learned about the functions of inflammation in the normal and diseased CNS. The challenge resides in dissecting the dual nature of neuroinflammation as it has both positive and negative effects differing spatially and temporally in the CNS. For example, additional knowledge is required to understand how and when cyclooxygenase or lipoxygenase inhibition may be beneficial or deleterious for patients suffering from inflammatory and degenerative neuropathologies. Arachidonic signaling through the cyclooxygenase and lipoxygenase pathways yields an enormous variety of products, some of them with pro-survival others with pro-death effects. Rather than inhibit cyclooxygenases, it may be more effective to target different prostaglandin synthases as their products can play different roles in recovery or degeneration. In addition, although there is a plethora of drugs that halt the pro-inflammatory response almost none target the anti-inflammatory response which resolves inflammation. The latter therapeutic approach entails the development of agents that mimic the effects of pro-resolving mediators and will most likely produce more effective strategies to treat neuroinflammation and the ensuing neurodegeneration with a lower burden of side effects.

9. ACKNOWLEDGMENTS

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8

ROLE OF THE UBIQUITIN PROTEASOME SYSTEM DURING NEURONAL CELL DEATH

Nadia Canu and Pietro Calissano

1. INTRODUCTION

In recent years much effort has been devoted to understanding the nature of neuronal cell death and the proteolytic systems involved in neurodegenerative diseases. Although the precise mechanism of cell death in neurodegenerative disorders is not known, PCD (programmed cell death) has been implicated (1). Two major kinds of PCD have been proposed (2,3). Type I PCD, or classical apoptosis, is a tightly regulated process morphologically characterized by loss of cell volume, chromatin condensation, cell blebbing, neurite retraction and nuclear fragmentation. At the biochemical level a family of cytoplasmic proteases termed the caspases contributes to the execution phase of this process (4). Type II PCD, or autophagic cell death, on the other hand, is morphologically characterized by proliferation of the autophagosomal-lysosomal system and early destruction of the cytoplasm (5, 6, 3). Although biochemically and morphologically different, these two types of PCD may co-exist and account for the complex

anatomo-pathological pictures characteristic of a range of neurodegenerative diseases. These diseases are characterized by death of specific neuronal populations and by progressive accumulation of "lethal" aggregates mainly formed by a single protein such as amyloid-beta and tau protein in Alzheimer disease, prion protein in scrapie and CJD disease or α-synuclein in Parkinson's disease.

Proteinaceous deposits in such neurodegenerative diseases tend to be ubiquitinated, and constitute a visible hallmark of ubiquitin-proteasome system (UPS) impairment. There is a complex interrelationship between the deposition of aggregates, the function of the UPS and neuronal cell death. We have investigated the role of the ubiquitin-proteasome system during PCD with the aim of testing the hypothesis that its altered functions may mimic those occurring in neurodegenerative diseases. We have utilized an "in vitro" paradigm of neuronal death mimicking the process of an *in vivo* deafferentation of a neuronal population taking place either during embryogenesis or in various neurological diseases. Thus, cultured cerebellar granule cells (CGCs) undergo massive cell death when the depolarizing potassium concentration normally employed for their culturing is reduced from 25 mM to 5.0 mM. This manipulation, experimentally compared to the surgical disconnection of the nerve afferents to cerebellar granule neurons (7) activates an internal program of PCD in which biochemical and morphological elements of apoptosis and autophagy intersect and influence each other (8, 9, 10, 11). In this model, the UPS appears to be involved in channelling neurons to death through two pathways: 1) by acting as a master proteolytic system that orders caspase activation; 2) by subsequently undergoing a loss of function which, in turn, contributes to secondary damage likely due to the accumulation of pro-apoptotic molecules.

2. PROTEASOME INHIBITORS DELAY CELL DEATH

The first evidence of an active role of the UPS in PCD came from the studies of Schwartz and colleagues describing an increase in polyubiquitin and proteasome subunit gene expression during the intersegmentation of muscles and the morphogenesis of some neuronal populations in larvae of the hawk moth Manduca sexta (12). Numerous subsequent studies employing different model systems revealed that the involvement of the UPS in PCD is not always accompanied by a consistent increase in the expression of its protein components (13, 14, 15). More recently, the role of the UPS in PCD has been analyzed by the use of pharmacological inhibitors of this proteolytic system. It should be noted that the interpretation of the results may be difficult since fine tuning of this proteolytic system is apparently required to ensure cell survival, as widely underlined in the subsequent chapter of Lang-Rollin and Stefanis in this volume.

A number of studies have found that pharmacological proteasome inhibition leads to an inhibition or delay of neuronal death. For instance, proteasome inhibitors prolonged the survival of NGF-deprived sympathetic neurons (16), prevented thymocyte apoptosis induced by glucocorticoids (17), blocked MPP or rotenone-induced dopaminergic neuronal death (18), and reduced infarct volume in a rat model of focal cerebral ischemia (19). In all these cases arrest of cell death was associated with the inhibition of apoptotic markers such as the perturbation

of mitochondrial membrane, caspase activation and DNA laddering. We have confirmed a neuroprotective effect of proteasomal inhibition in CGC cultures induced to undergo apoptosis in the presence of several highly selective proteasome inhibitors such as lactacystin and epoxomicin (10, 20). The observation that the neuroprotective effect was more evident when the drugs were added within 1 hour after apoptosis induction indicated that proteasomes play a regulatory key function in the very early phase of apoptosis, as also reported in other models (16).

Consistent with this finding, we also found that proteasome activities were slightly increased during the early phase of apoptosis. Sawada et al. (18) also found increased proteasomal activities early on after application of MPP+ or rotenone to ventral midbrain cultures.

To answer the question of whether the neuroprotective effects of proteasome inhibitors were correlated with the inhibition of classical markers of apoptosis, we determined the activity of caspase-3 in CGCs deprived of potassium. These drugs were able to prevent caspase-3 activity and pro-caspase 3 activation, suggesting that proteasomes control the activation of caspase(s) (Figure 1). Accordingly, events such as calpain/caspase-mediated cleavage of tau (21, 10), Reactive Oxygen Species (ROS) production, DNA laddering and the onset of a deficit of the antioxidant system normally occurring downstream of caspase activation were also prevented (22, 20) (Figure 1). Activation of caspases in CGCs occurs mainly through the intrinsic pathway, in which mitochondrial perturbation causes cytochrome c release in the cytosol to form the apoptosome, the promoter of the caspase cascade. We found that proteasome inhibitors were able to interfere with cytochrome c release (23) (Figure 1), suggesting that they may control proteins involved in the organization of the mitochondrial pore through which efflux of mitochondrial proteins occurs.

3. PROTEASOME-DEPENDENT DEGRADATION DURING THE EARLY PHASES OF APOPTOSIS

Given that activation and involvement of proteasomes have been described as an integral parts of the initiation phase of apoptosis, both in neuronal and non neuronal settings (16, 24, 10, 20, 25, 18, 26), three major questions arise:

3.1 How does the Proteasome Mediate the Early Steps of Neuronal Apoptosis?

The logical answer to this question is that the proteasome may be responsible for the degradation of some critical substrates that mediate neuronal survival. Thus, when the proteasome is activated early on in apoptosis, it accelerates the degradation of such pro-survival factors, leading to their relative depletion and engagement of the apoptotic pathway. Which are these potential pro-survival proteasomal substrates? In non-neuronal paradigms of apoptosis different targets of proteasome degradation have been identified. They include, amongst others, transcription factors that regulate genes coding for polypeptides involved in cell proliferation and survival (c-Fos; NFkB, AP-1, ODC), and proteins, like IAPs, that normally repress caspases (27, 28, 29, 30, 25).

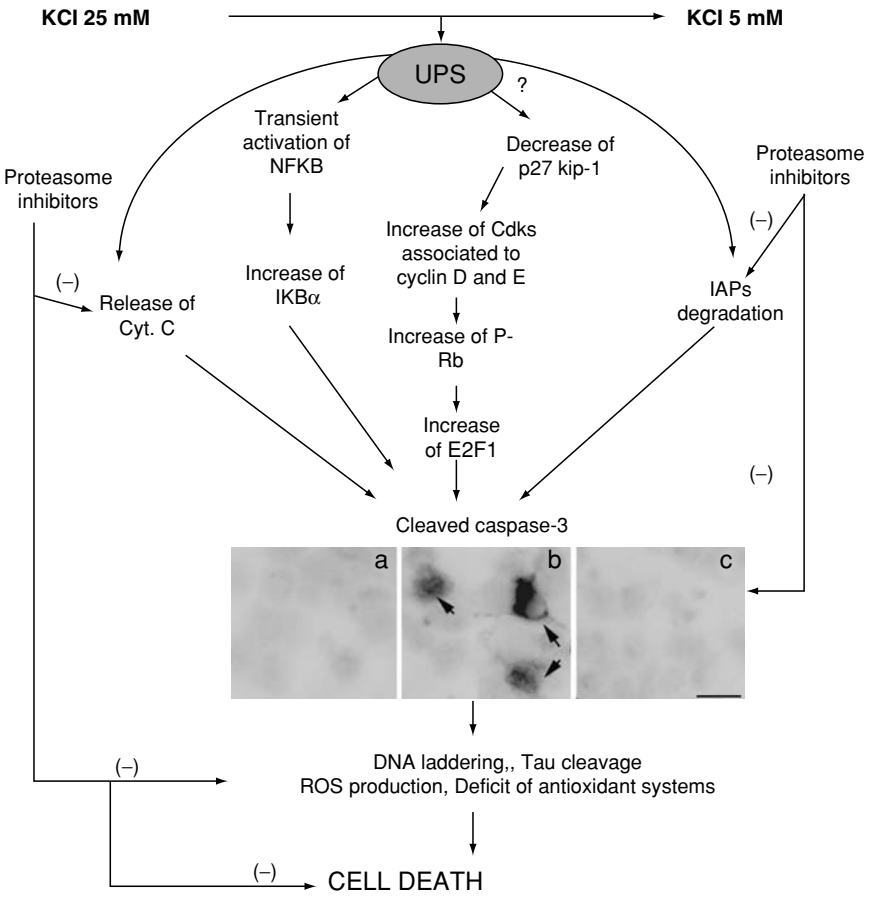

Figure 1. This drawing depicts the series of known or hypothetical events occurring in cerebellar granule cells following the apoptotic trigger. The black arrows point out the established findings underlying the role of UPS in the sequence of events leading to caspase activation, and subsequently DNA laddering, tau cleavage, ROS production, and antioxidant system failure. Gray arrows indicate the hypothesized events linked to UPS activation during the course of apoptosis and (?) indicates that the direct or indirect involvement of UPS in the decrease of p27 during CGCs apoptosis has not been investigated. The panel visible in the central part of the figure shows an immunofluorescence analysis of cleaved, activated caspase-3 (black) in control cells (a) or in potassium-deprived cells, in the absence (b) or presence (c) of the UPS inhibitor lactacystin. Notice the presence of 3 apoptotic neurons in which the staining of activated caspase 3 is clearly visible (b) and that in the presence of lactacystin such activation does not occur (c); (-): inhibition; Bar: 7 μ

As mentioned, in CGCs, the proteasome-mediated step(s) that promote(s) apoptosis occur(s) before mitochondrial changes and caspase activation. We had previously reported that ROS are involved in the release of cytochrome c in this model (31). This raised the possibility that the proteasome may down regulate the anti-oxidant system constituted by catalase, SOD, and GSH/GSSG, and thus promote apoptosis. We have indeed found that the antioxidant system increased

after proteasome inhibition before eventually declining, suggesting that the UPS may participate directly or indirectly in its turnover (20). Whether these molecules are normally degraded by the UPS or whether they acquire the ability to be UPS substrates during neuronal apoptosis remains to be established. In this regard, it must be pointed out that catalase, the major effector in the defense of aerobic cells against oxidative stress, is degraded by proteasomes in a phosphorylation-dependent fashion catalyzed by c-Abl and Arg tyrosine kinases (32). By contrast, a direct link between UPS and SOD has been demonstrated only for mutant Cu/Zn SOD whose level increases in the presence of proteasome inhibitors (33).

Other important constituents degraded by the UPS during apoptosis are IAPs (inhibitor of apoptosis proteins). IAPs are a family of proteins containing one or three BIRs (baculovirus IAP repeat) and a RING finger domain that confers ubiquitin protease ligase (E3) activity. IAPs are endowed with many functions, including the ability to bind to activated caspases and to inhibit their activity, most likely by ubiquitinating them and targeting them to the proteasomes (34). It has been reported that in thymocytes IAPs undergo autoubiquitination and degradation by the proteasome in response to apoptotic stimuli, an event critical for commitment to cell death (25).

To determine how proteasome inhibitors prevent CGC death, we examined whether proteasomes degraded these anti-apoptotic proteins and found that proteasome inhibitors, but not caspase inhibitors, stabilized and increased the levels of IAPs (Nadia Canu, unpublished observation). This finding suggests that IAPs are targeted to proteasome for degradation during the CGC apoptosis, thus favouring caspase activity as reported in other settings (25), and likely increasing the amount of pro-apoptotic IAP substrates (see also article by Lang-Rollin and Stefanis in this volume). It should be noted however that such an increase of IAPs would not be expected to lead to inhibition of cytochrome c release, as IAPs generally function downstream of the mitochondrial checkpoint.

Studies carried out in the same experimental model suggest that proteasomes could also be operative at other levels. A peculiar case is that of NF-kB. The processing of this transcription factor from an inactive precursor to an active form involves the degradation of part of the precursor and the release from the inhibitory effect of IkBα and β. Both these functions are mediated by the proteasome (35, 36). Thus, this transcription factor has been found to be activated by the UPS in the early phase of apoptosis (37), presumably via limited proteolysis of the NFkB precursor protein (35). The role of NF-kB in cell death in the nervous system is controversial. It is clear that it can be activated both following pro-apoptotic (38, 39) and pro-survival signals (40). In most settings, inhibiting such activation appears to be associated with pro-death effects (40, 41, 42), but the converse has also been observed (43, 39). It appears that cell type, intensity, type and duration of the death stimulus and duration and temporal relationship of NF-kB inhibition to the death stimulus are important variables (43, 39). If, as in some models (43, 39), NF-kB acts as a death mediator when activated early on in apoptosis, it is possible that the inhibition of its induction by proteasomal inhibitors may account in part for their early protective activity in potassium-deprived CGCs, although this has not been specifically tested.

Proteasomes might also have a crucial role in the pathway involving p27kip that, in certain non-neuronal settings is a proteasome substrate (44). Interestingly, Padmanabhan et al. (45) have reported that in KCl deprivation-evoked death of CGCs a significant decrease in the level of this cyclin inhibitor occurs. Moreover, an enhanced activity of cyclin D1 and E-associated kinases and, more importantly, a transient increase in phosphorylation of Rb (a known substrate of these cyclins, which gets inactivated by phosphorylation), were reported. Consequently, it has been postulated that the Rb function of binding and repressing the transcriptional activity of E2F would be lost, a hypothesis reinforced by the finding that E2F-1 induces and modulates CGC death (46). The changes reported above were prevented by the cyclin-dependent kinase inhibitor flavopiridol but not by caspase inhibitors. It would therefore be interesting to ascertain whether proteasome inhibitors in this system mediate their protective effects through p27kip increase.

Altogether these findings suggest that the events depicted in Figure 1 play a role in the mediation of apoptosis following potassium deprivation in CGCs.

3.2. How is the Proteasome Activated During PCD?

An attempt to answer this question was carried out by Kroesen et al. (26). They reported that B cell receptor activation initiates an apoptotic program characterized by sphingolipid-dependent activation of proteasomes and degradation of IAPs that were prevented by an ISP-1/myriocin, a potent inhibitor of ceramide formation. Proteasome activation occurred likely via sphingolipid-dependent phosphorylation of its C8 and C9 subunit (47). Whether a similar mechanism governs proteasome activation during neuronal apoptosis remains to be established.

3.3. What Specific Signal(s) Channel any Potential Proteasome Substrate Toward Degradation During Apoptosis?

As far as this question is concerned, a particular case is the degradation of IAPs. These inhibitors of death must be inactivated in cells that are doomed to die. Studies in Drosophila demonstrated that this is accomplished by proteins such as Reaper, Hid and Grim. It has been reported that Reaper, a small 65aa protein that is specifically expressed in cell undergoing apoptosis induces apoptosis by specifically stimulating the auto-ubiquitination and degradation of IAPs (48). In mammalian cells a similar role might be played by mitochondrial proteins such as Diablo/Smac and the serine protease HrtA2/Omi (49).

4. PROTEASOME CHANGES DURING THE LATE PHASES OF APOPTOSIS

After such early involvement we found that the proteasome undergoes a series of changes resulting in its inactivation. Before apoptosis is triggered in CGC cells, proteasomes were present in the nuclei as well as in the cytoplasm (50, 51). Upon chromatin condensation, nuclear proteasomes were found mainly in the cytosol. The movement of the UPS is likely due to its involvement in cell

shape changes, since proteasomes have been found in apoptotic bodies and cytoplasmic vesicles (52). Other studies indicate an active role of the UPS through the regulation of ezirin turnover. Ezirin is a cytoskeletal protein involved in anchoring actin to the cell membrane. It is thus involved in the control of cell blebbing, rounding-up, and overall cellular size. Whether the movement of proteasomes to the cytosol in CGCs is correlated to a UPS-mediated rearrangement of the cytoskeleton and to the organization of the apoptotic bodies has not been specifically addressed.

At the biochemical level one of the most impressive proteasome changes is the progressive decline of its function at late stages of apoptosis. Indeed, we found that proteasomes became part of a generalized cellular failure that affects the major activities of the apoptotic neurons after caspase activation. Cell extracts from apoptotic CGCs showed a decrease in proteasome chymotrypsin-like, trypsin and post-acidic-like activities, correlating with the degree of apoptosis observed, similar to findings reported for dexamethasone-induced apoptosis of thymocytes (53). These changes were observed at the time of the execution phase and were prevented by the general caspase inhibitor z-VAD-fmk, suggesting that caspase-dependent proteolysis inactivates proteasome functions (10). It has been previously reported that the impairment of the chymotrypsin-like activity of the proteasome caused an accumulation of ubiquitinated proteasome substrates (54, 55). Therefore, we asked whether the progressive failure of proteasome activity, during PCD, was accompanied by changes in protein ubiquitination. We found that proteasome failure during CGC apoptosis occurred with concomitant increase in the amount of high-molecular mass ubiquitin conjugates, as detected by immunofluorescence analysis (Figure 2) and Western immunoblot (10). Two apoptotic neurons filled with ubiquitin-conjugated proteins are clearly shown in Figure 2.

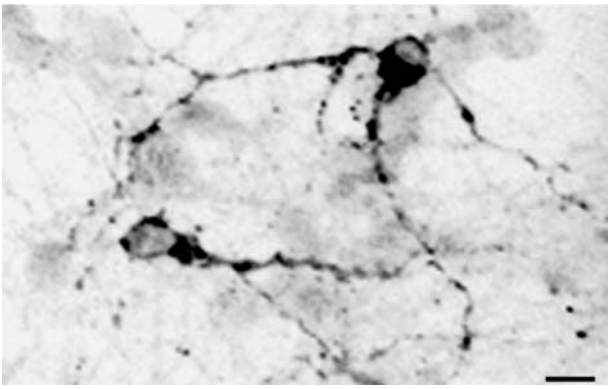

Figure 2. Ubiquitin immunostaining in cerebellar granule neurons undergoing apoptosis analyzed by confocal microscopy. Apoptosis in CGCs was induced by potassium and serum withdrawal. Immunostaining of ubiquitin was performed 6 hr after induction, using a polyclonal anti-ubiquitin antibody. Notice that two apoptotic neurons are filled with ubiquitinated proteins (black) both in the soma and along the neurites, while healthy neurons are barely stained with anti-ubiquitin antibodies. Bar: 7 μ

Interestingly, this event was abolished by caspase inhibitors, confirming that impairment of proteasome function occurs downstream of caspase activation, a conclusion also supported by data from other "in vitro" cellular systems exposed to diverse apoptogenic stimuli (56, 57).

What could be the functional consequences of proteasomal inhibition at the late phases of apoptosis? This subject is dealt in more detail by Lang-Rollin and Stefanis in the subsequent chapter, where the significance of the up regulation of particular potentially deleterious proteins is analyzed. In the case of CGC apoptosis, after the initial increase of NF-kB activity, there is a decrease, mediated largely by the prolonged stability of its endogenous inhibitor IkBα, as its proteasomal degradation is hindered (58, 37). The importance of an elevated level of IkBα in CGCs has been suggested by the findings that over-expression of a stabilized form of this protein renders CGCs more vulnerable to apoptotic stimuli (37) and that during proteasomal inhibition-induced cell death the amount of this inhibitor increases (59, 60). These data suggest that at these late phases NF-kB may act as a survival factor and that inhibiting its activation via proteasomal impairment may accelerate death.

It must also be considered that the accumulation of unique or multiple unspecified and ubiquitinated proteins inside the cells, usually in organized structures referred to as aggresomes, can act as possible apoptotic triggers. Indeed, it has been reported that protein aggregation causes impairment of the UPS and therefore depletes cells of this pivotal and vital proteolytic system, thus causing cellular deregulation and death (61). In this regard, although some findings support a protective role for some of these inclusions, as in the case of cytoprotection exerted by aggresomes formed by alpha-synuclein and synphilin-1 (62, see also chapter 4 in this volume) other studies suggest that some neuronal populations are highly vulnerable to the accumulation of ubiquitinated proteins under the form of aggregates, as found in many neurodegenerative diseases (61, 63, 64). Therefore, we can envisage a vicious circle that starts when accumulated proteins block the interior of proteasomes, thereby reducing their activity and eventually adding noxious inputs to neurons already committed to death. For a more detailed discussion of the topic of protein aggregation and how it influences survival, refer to section 2, chapters 3-5 of this volume.

5. COMPONENTS OF THE PROTEASOME SYSTEM DEGRADED BY CASPASES

Since induction of apoptosis in CGCs resulted in diminished activity of the UPS that was prevented by caspase inhibitors, we asked whether the proteasome itself was a victim of caspase-dependent attack. Interestingly, we found that the amount of the α-2-α7 subunits (10) and the β1-β7 subunits (Canu, unpublished observation) of the core 20S proteasome did not change during CGC apoptosis. By contrast, the amount of the ATPase subunit S6 of the 19S regulator of the 26S proteasome, which is necessary for ATP-dependent proteolysis by the proteasome, was reduced in a caspase-dependent manner during apoptosis of CGCs (Figure. 3). This finding is in line with results recently reported for Jurkat cells treated with etoposide or actinomycin and for PCD in flies (57, 65). In these

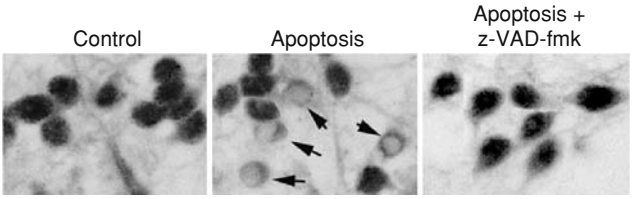

Figure 3. Immunofluorescence analysis of the ATPase subunit S6 of the 19S regulator of UPS. Notice the presence of 4 apoptotic neurons (arrows) in which the immunostaining of this subunit is strongly reduced while in the presence of the caspases inhibitor z-VAD-fmk the staining is comparable to control.

cases proteasomal subunits, in particular S6, S1 and S5a of 19 S, are cleaved by caspases both in vitro and in vivo under different apoptotic stimuli (57, 65). It has been speculated that caspase-mediated cleavage of these subunits may affect the structural integrity and activity of the proteasome, interfering either with the stabilization of the interaction of the lid and base of the 19S subunit (66, 67) or with the recognition and interaction with multiubiquitin chains in intact proteasomes (68, 69, 70, 71).

Another mechanism contributing to impairment of proteasome activity in CGCs is the finding that histone H2A is deubiquitinated (Canu et al. unpublished observation), suggesting that depletion of free ubiquitin takes place in our experimental model as already reported in other systems (72). The availability of free ubiquitin for the ubiquitination reaction is guaranteed by the synergistic activities of different deubiquitinating (DUB) enzymes. We measured these activities in cell extracts of CGCs undergoing apoptosis using as substrate a mixture of (Ub)4, (Ub)3, and (Ub)2 oligomers that are converted by the action of DUB enzymes into monomeric ubiquitin UB1 (73). These activities were markedly and progressively impaired in CGCs undergoing apoptosis, and more importantly, their loss was prevented by a caspase inhibitor (Figure. 4).

DUB enzymes are encoded by two gene families: the UCH family (ubiquitin C-terminal hydrolases, with molecular weight of ~30 kDa, hydrolyzing small C-terminal derivatives) and the UBP family (ubiquitin-specific processing proteases, with molecular weight of ~110 kDa, hydrolyzing large derivatives of ubiquitin). UCHL-1 is one of the most abundant enzymes in the brain, comprising up to 2% of total brain proteins (74). A partial loss of UCHL-1 activity, due to a missense mutation, has been implicated in proteasome failure and aggregation of ubiquitinated proteins in familial cases of Parkinson's disease (74). For more details on UCH-L1 and its role in UPS dysfunction and neurodegeneration, see the chapter by Kwon and Wada in this volume. We found that the amount of this enzyme does not change during CGC apoptosis (Nadia Canu, unpublished observation), suggesting that other caspase-mediated modifications of UCHL-1 may account for an impairment of its activity, or, more likely, that other DUB enzymes are responsible for this deficit. This latter conclusion is supported by the demonstration that isopeptidase T, an enzyme belonging to the UBP family, is cleaved by caspase-3, with loss of function, both in vitro and in vivo (65).

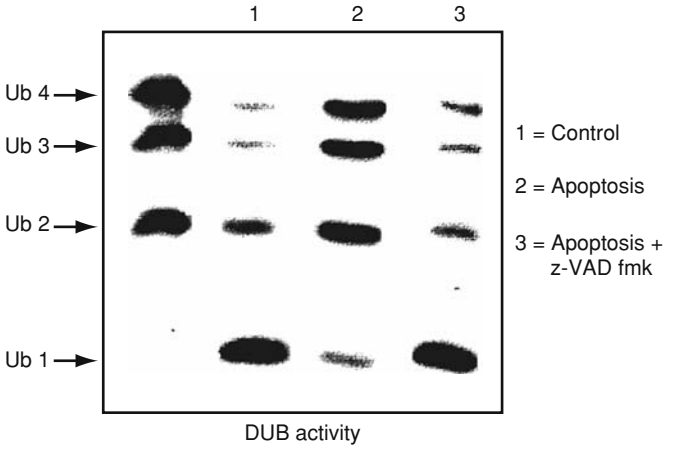

Figure 4. Deubiquitinating activity in granule neurons undergoing apoptosis. Cultures at 6 DIV were induced to undergo apoptosis in the absence or in the presence of the general caspase-inhibitor z-VAD-fmk for12 hr. At the time indicated after the induction of apoptosis, 5 fg of supernatants were incubated with 1 fg of multi-ubiquitin chains, substrates for DUB enzymes at 22°C for 10 min, and immunoblotted with anti-ubiquitin antibody (10).

How may a decrease in DUB activity impair proteasome function? It has been demonstrated that DUB enzymes remove ubiquitin from various cellular adducts, thus playing an important role both in the editing of the ubiquitination state of proteins as well as in the recycling of ubiquitin (73). The recycling of free ubiquitin from poly-ubiquitin remnants is required for the continued activity of the UPS. In fact, insufficiently disassembled polyUb chains bind avidly to, and inhibit, the 26 S proteasome complex, presumably via competition with polyubiquitinated substrates (75, 76). It is also possible that the proteasome efficacy is additionally hampered by post-translational modifications of the proteasome and /or by the generation, during apoptosis, of cross-linked or aggregated proteins. These polypeptides, which are poor substrates for proteolysis and may physically impede proteasome entry by blocking the catalytic site, may be generated as a consequence of the disruption of intracellular sulfhydryl homeostasis (77). The finding that the level of heat shock proteins, which operate in the trafficking of misfolded proteins on their way to the proteasome, are increased during CGC apoptosis (78) supports the hypothesis that the generation of misfolded and eventually aggregated proteins or structures may occur during CGC apoptosis.

6. APOPTOSIS, AUTOPHAGY AND PROTEASOME INHIBITION

In the presence of proteasome inhibitors, CGCs deprived of KCl did not manifest classical hallmarks of apoptosis and remained alive for 12-15 h (10). At later time points, the neuroprotective effect was less evident and at longer time points it was no longer detectable. This situation likely reflects the block of a pivotal proteolytic system as well as the inability of proteasome inhibitors to counteract

autophagy, a caspase-independent mechanism of cell death that, recently, has been reported to occur in this paradigm of neuronal death (11).

Apoptosis and autophagic degeneration are two morphologically and biochemically distinct modes of programmed cell death described in embryogenesis and tissue renewal in adults. A substantial body of evidence has revealed the simultaneous presence of apoptosis and autophagic elements in neurodegenerative disorders (79, 1). However, it is not clear yet whether autophagy is an attempt to protect the cell from apoptosis, or to hasten cellular demise. Data from diverse in vitro models of neuronal death support this latter hypothesis.

Autophagy not only co-exists with apoptosis but may precede and influence it in a process that is induced by apoptotic stimuli (80, 81). In CGCs undergoing apoptosis, activation of autophagy appears to occur very early after the apoptotic stimuli, before any classical hallmark of apoptosis is manifested, although it is more evident in neurons displaying nuclear condensation. We have found that autophagy controls the release of cytochrome C, caspase activation and, more importantly, mediates a caspase-independent process of cell death. Block of autophagy rescues CGCs from apoptosis (11). Similar results have been reported in newly isolated sympathetic neurons deprived of NGF or treated with cytosine arabinoside (80) as well as in PC12 cells deprived of serum or in delayed neuronal death occurring in the CA1 pyramidal layer of the gerbil hippocampus after brief forebrain ischemia (82). These findings suggest that autophagy is likely a mode to initiate apoptosis in different settings.

What is the role of the proteasome system in autophagic cell death? To answer this question we have visualized autophagic vesicles with a specific marker in CGCs undergoing apoptosis in the presence of proteasome inhibitors and found that these inhibitors not only do not cause disappearance of autophagic vesicles (Figure. 5), but that the autophagosome content is quite different in the two experimental settings.

Thus autophagosomes of CGCs treated with proteasome inhibitors contain ubiquitinated neurofilaments suggesting that additional and different mechanisms operate to induce formation of autophagosomes in CGCs undergoing apoptosis in the presence of proteasome inhibitors. Moreover, preliminary

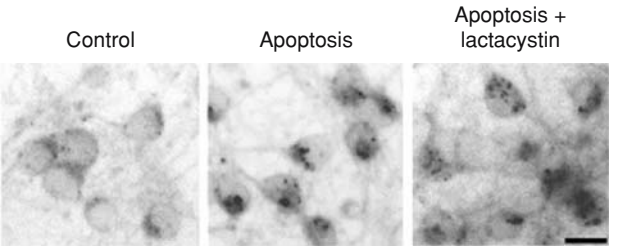

Figure 5. Staining of autophagosomes performed with monodansyl cadaverine, a specific marker of these vescicles, in CGCs undergoing apoptosis in the absence or in the presence of lactacystin. Notice that this proteasome inhibitor does not abolish the autophagosome proliferation that occurs during apoptosis. Bar: 7 μ

experiments indicate that the number of autophagomes per cell section is greater in apoptotic cells treated with proteasome inhibitors compared to apoptotic cells exposed only to potassium deprivation (Canu, unpublished observation), suggesting that pharmacological proteasome inhibition induces autophagy. Whether this additional proliferation of autophagosomal-lysosomal system represents an attempt to override the proteasome deficit, as suggested by Lang-Rollin and Stefanis in this volume, remains to be investigated.

7. CONCLUSIONS.

In CGCs undergoing apoptosis, proteasomes seem to play a crucial, double role. During the commitment phase, proteasomes are activated and are

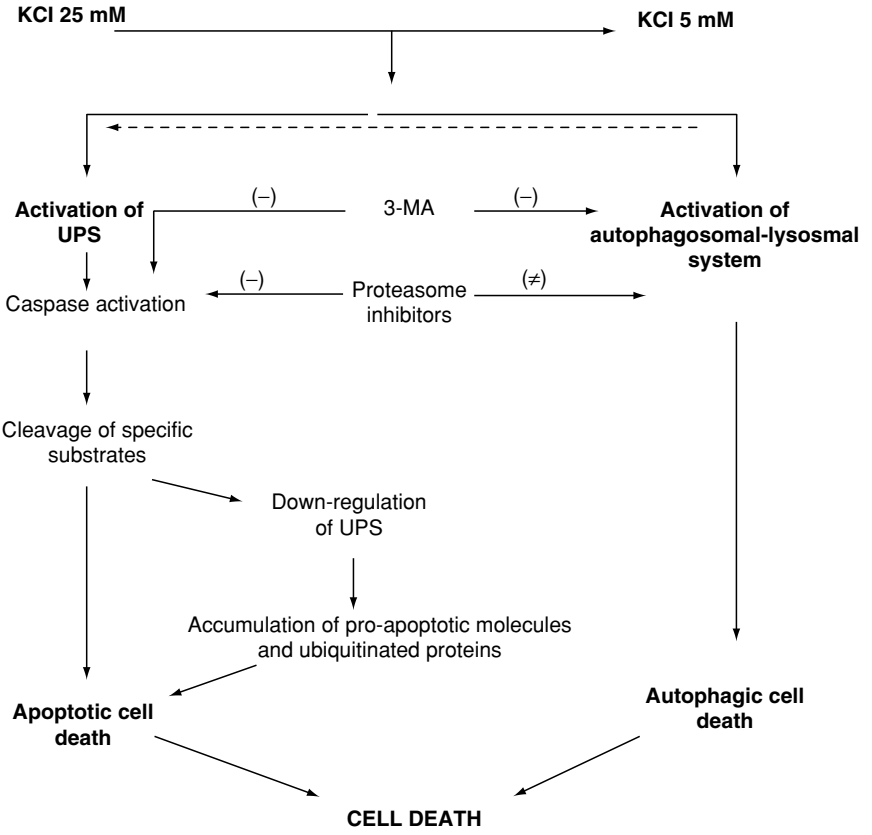

Figure 6. Schematic diagram of the proteolytic pathways activated during apoptosis of cerebellar granule cells. Although it is not established whether the two pathways involving UPS and or the autophagosomal-lysosomal system operate in series or in parallel it is hypothesized that (dashed arrows) the autophagosomal system operates upstream. UPS activation causes apoptotic cell death both directly via caspase activation and indirectly due to its caspase-mediated progressive failure. Notice that proteasome inhibitors, despite blocking caspase activation (-) (see Fig. 1), do not affect the autophagolysosomal system proliferation induced by KCl deprivation (≠). On the contrary, 3-MA (3-methyladenine) not only inhibits this latter proteolytic system, but it also blocks caspase activation.

responsible for caspase activation but subsequently become victims of their targets. Such a double effect is noxious to the cells because, on the one hand it causes activation of the caspase cascade and, on the other, in the subsequent declining phase, it causes accumulation of pro-apoptotic molecules and ubiquitinated proteins. During such cellular events, however, another major player enters the scene, i.e., the autophagosomal-lysosomal system. Although a precise causal relationship between these two degradation systems in cell death processes is still unclear, the data obtained in CGCs undergoing apoptosis show that inhibition of proteasomes does not block this latter proteolytic system. Whether these two systems operate in parallel with overlapping steps or, more likely, in series, remains to be established (see Figure. 6). In view of the growing amount of data regarding the extremely noxious consequences of blocking either proteasomes or the autophagosomal system, it seems clear that any attempt to interfere with their physiological function in order to arrest or slow down the neuronal loss in neurodegenerative diseases appears extremely dangerous. Future studies could, however, bring to light new drugs endowed with more specific mechanism of action.

8. ACKNOWLEDGMENTS

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9

PATHWAYS OF NEURONAL CELL DEATH INDUCED BY PROTEASOMAL INHIBITION

Isabelle Lang-Rollin and Leonidas Stefanis

1. INTRODUCTION

Neuronal cell death occurs normally in the developing nervous system. A term that has been used to describe this death is Programmed Cell Death (PCD). The predominant morphological form of neuronal PCD is that of apoptosis, although other morphologies do occur. Neuronal cell death also occurs during various disease states in the nervous system, either following acute, subacute insults, such as trauma or stroke, or more chronic insults, such as those that take place during neurodegenerative diseases. There is substantial evidence that in animal models that mimic such conditions, as well as in diseased human tissue, elements of PCD, either apoptotic or non-apoptotic, are activated. The classical morphological features of apoptosis correspond to a defined biochemical pathway, whose signature event is the activation of the cysteine proteases caspases. Other morphologies of neuronal cell death, such as autophagy and necrosis, may also be elicited following the application of injurious stimuli to the nervous

system. Despite differences at the morphological level, these pathways may share common biochemical elements with apoptotic pathways. In addition, following a given stimulus, neuronal populations may respond with a wide range of morphologies and biochemical events, depending in part on the chronicity and intensity of the damage inflicted (reviewed in 1).

How is the proteasome related to these pathways? Can proteasomal dysfunction, which is thought to occur in various neurodegenerative diseases, lead to neuronal cell death? If so, what are the mechanisms? We and others have attempted to answer these questions by applying selective pharmacological proteasomal inhibitors to neuronal cells. Although the specificity of such proteasomal inhibitors is not absolute, the fact that similar findings are observed with application of a variety of agents suggests that these effects are indeed due to proteasomal inhibition and not to other pharmacological actions.

The basic premise of such experiments is that dysfunction or inhibition of the proteasome leads to an increase in the levels of protein substrates that are normally degraded by the proteasome. Such proteins therefore accumulate in the cell. The particular proteins that are degraded through the proteasome in neurons are largely unknown, and may differ from those in other cell types. The accumulation of specific proteins in neurons may have deleterious consequences on cell function and viability. In addition, the proteasome is involved in the processing of a limited number of proteins to active forms, a case in point being the transcription factor NF-kB. Disruption of this process through proteasomal inhibition may also have important effects. We will review here studies that have investigated the effects of proteasomal inhibition on neuronal cell viability and function, placing particular emphasis on studies performed in primary neurons, because of the potential cell specificity of such effects.

Although initial reports suggested that the toxicity associated with the use of pharmacological inhibitors of the proteasome was confined to cycling cells, it soon became clear that post-mitotic neuronal cells were also prone to the toxic effects of these agents. With few exceptions, the death induced by proteasomal inhibition of cultured neurons is apoptotic by morphological and biochemical criteria. This is the case of cultured cortical, mouse sympathetic and cerebellar granule neurons (2-6) (see Figure 1 for an example of apoptosis induced by application of the pharmacological proteasomal inhibitor lactacystin to mouse sympathetic neurons). However, despite manifest neuronal death, no morphological apoptosis of cultured postnatal dopaminergic neurons occurred following proteasomal inhibition in experiments reported by Petrucelli et al. (7). In addition, we have found that, in contrast to mouse, rat sympathetic neurons largely undergo a non-apoptotic form of death in response to proteasomal inhibitors (Isabelle Lang-Rollin, Hardy J. Rideout and Leonidas Stefanis, unpublished observations). It appears therefore that the form of death (apoptotic versus non-apoptotic) may depend on the particular neuronal cell context. In vivo experiments show apoptotic features in the cortex of adult rats following intracerebroventricular administration of the pharmacological proteasome inhibitor PSI (8) as well as in the substantia nigra of adult rats following intrastriatal or systemic injection of proteasomal inhibitors (9, 10). The molecular mechanisms responsible for neuronal death in these settings are just beginning to be elucidated.

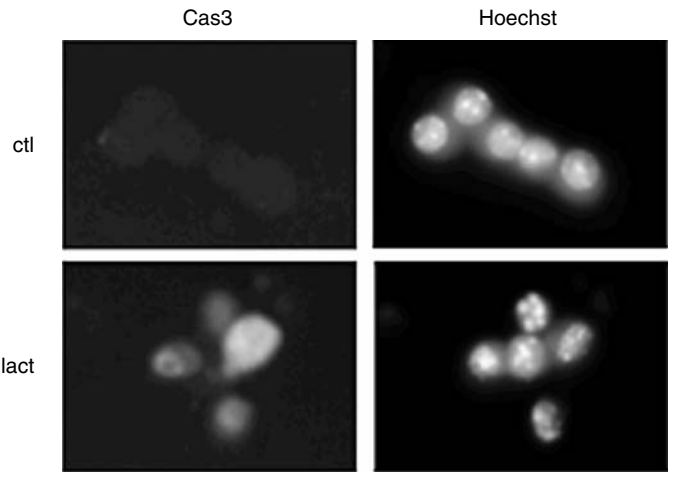

Figure 1. Cultured post-natal mouse sympathetic neurons were exposed to either no additives (ctl) or to 10 μM lactacystin (lact). 24 hrs later, they were immunostained with an antibody specific for the activated form of caspase 3 (cas3) and then labeled with the nuclear dye Hoechst.

2. MECHANISMS OF PROTEASOMAL INHIBITION-INDUCED DEATH

2.1 Accumulation of Rapidly Turning over Proteins

A major class of proteins that are degraded by the proteasome are rapidly turning over proteins. These are proteins whose levels need to be tightly regulated in order to ensure cellular homeostasis. Such proteins include those involved in cell cycle control. A very precise mechanism of control exists in cycling cells in order to ensure smooth progression through the cell cycle. Proteolysis plays a major role in ensuring that proteins that are involved in one stage of the cycle are downregulated at the following step, to allow for the process to continue. If the proteasome is inhibited in cycling cells, invariably cell cycle arrest ensues (11, 12). This appears to be due, to a large extent, to the upregulation of p27, a Cyclin-dependent kinase (Cdk) inhibitor that arrests the cycle at G1/S by inhibiting cyclin D1 and cyclin E-related kinases Cdk2, 4 and 6 (13). It has recently become quite clear that cell cycle-related proteins are also present in post-mitotic neurons. What is then the effect of proteasomal inhibition on the levels of these proteins within neuronal cells? One earlier study had reported an increase in the levels of cyclin D1 in cultured cerebellar granule cells following application of lactacystin, one of the most specific proteasomal inhibitors available (14). We have confirmed this finding in cultured embryonic rat cortical neurons, and have shown that the increase in cyclin D1 levels is paralleled by nuclear translocation. There was also nuclear translocation of cyclin E, although a clear induction of total cyclin E levels was not observed. Remarkably, there was no increase in the levels of p27. This is consistent with the fact that p27 changes from a very rapidly turning over protein in cycling cells to one with a long half-life in differentiated cells. While none

of the other cell cycle-related proteins at the level of the G1/S phase of the cycle, such as Cdk 2, 4, or 6, showed any significant regulation, 4 to 8 hrs after application of lactacystin to cultured cortical neurons there was phosphorylation of Retinoblastoma Protein (pRb), a critical event at the G1/S transition (15). Thus, whereas in cycling cells proteasomal inhibition leads to cell cycle arrest, in primary neurons it leads to cell cycle progression, at least to the point of the G1/S transition. This might be explained by the absence of proteasomal-dependent p27 regulation in neurons.

Are these changes in cell cycle control related to the death that occurs following proteasomal inhibition? To answer this question, we used the specific pharmacological Cdk inhibitor flavopiridol. This agent provided sustained survival of lactacystin or epoxomicin (an even more specific proteasomal inhibitor)-treated cultured cortical neurons. Furthermore, it inhibited the induction of pRb phosphorylation, without affecting the upregulation of cyclin D1 levels. These results suggested the sequence of events depicted in Figure 2 following proteasomal inhibition of cultured cortical neurons. These findings were confirmed through the molecular inhibition of components of the G1/S phase of the cycle using viral delivery systems. Expression of the Cdk inhibitors p27 or p16, or of dominant negative (DN) forms of Cdk2, 4, or 6 was protective against

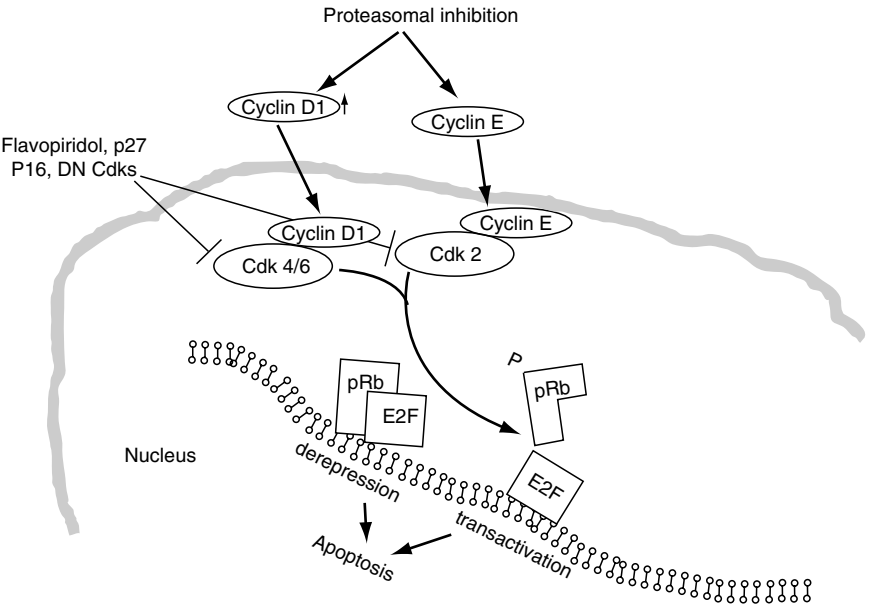

Figure 2. According to this model inhibition of the proteasome leads to translocation of cyclin D1 and E to the nucleus, where they form an active complex with their corresponding Cdks. This complex leads to phosphorylation of pRb and release from its complex with E2F. Derepression of the E2F/pRb complex and E2F transactivation then induce pro-apoptotic genes. The pharmacological Cdk inhibitor flavopiridol as well as molecular Cdk inhibitors prevent activation of the cyclin/Cdk complexes and protect against cell death.

proteasomal inhibition-induced death. Similar results were achieved through expression of a constitutively active form of pRb, lacking a number of phosphorylation sites (15). These findings indicate that Cdk activation at the G1/S transition is necessary for the propagation of death in proteasomal inhibitor-treated neurons. Consequences of pRb phosphorylation include release of pRb from its binding partner E2F, derepression of the E2F/pRb complex, and E2F transactivation (16). Whether derepression or transactivation of E2F is the critical downstream pathway resulting from G1/S activation in this setting remains to be elucidated. In a more recent study, we have extended our findings to dopaminergic neurons in embryonic rat ventral midbrain cultures; these neurons are also protected by flavopiridol against epoxomicin- or lactacystin-induced apoptosis, suggesting that aberrant activation of Cdks is also involved in the death of these neurons, which is critical for PD pathogenesis, following proteasomal inhibition (6).

Another molecule that has a short half-life and is regulated by the proteasome in cycling cells is p53 (17). Presumably, its levels need to be tightly regulated, because in the case of genotoxic stress it enables DNA repair or growth arrest (18). On the other hand, excess levels of p53 may lead to cell death in a number of cell types, including primary neurons (18-20). Previous studies in non-neuronal cells had given contradictory results. Although most found an upregulation of p53 following application of proteasomal inhibitors, in many cases p53 played no role in cell death (21-23). Whether in a neuronal context p53 is a rapidly turning over protein, whether it is regulated by the proteasome, and whether it is required for proteasomal inhibition-induced death had not been studied up till recently. We have now reported that the levels of p53 increase dramatically in rat or mouse cultured cortical neurons following 4-8 hrs of proteasomal inhibition. This is accompanied by nuclear translocation of p53 (24). To test whether the induction of p53 is necessary for death induced by proteasomal inhibition, we treated cortical neuron cultures derived from wild type or knock-out p53 mice with lactacystin or PSI. There was substantial delay, but not complete inhibition of apoptotic death in neurons that did not express p53. Furthermore, inhibition of Cdks by Flavopiridol did not affect p53 induction, and lack of p53 did not impair pRb phosphorylation, implying that p53 and Cdk activation were activated in parallel in response to proteasomal inhibition (24) (Figure 3). In this

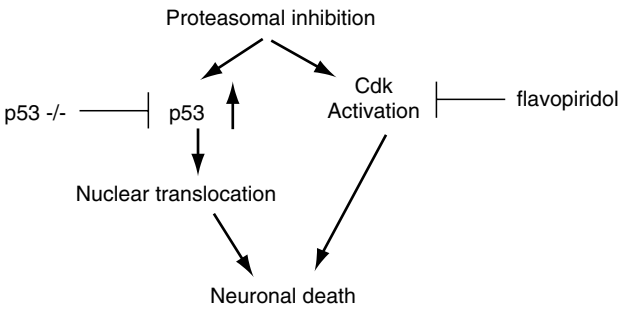

Figure 3. Proteasomal inhibition leads to parallel activation of the p53 and Cdk-dependent pathways of apoptosis.

respect, this death pathway is similar to that induced in cortical neurons following DNA damage (25).

Cell cycle-related proteins and p53 are only some of the proteins that are normally rapidly degraded by the proteasome. It remains to be seen which other such proteins exist in neuronal cells, and whether their upregulation following proteasomal inhibition could also have deleterious consequences and lead to cell death. Potential candidates include proteins that influence the jun kinase pathway and the subsequent phosphorylation and activation of the transcription factor c-jun, a critical event in many apoptotic pathways. A prime candidate amongst these proteins is POSH, a scaffold protein that mediates activation of JNK apoptotic cascade (26, 27). POSH has a ring finger domain with putative E3 ubiquitin ligase activity. The absence of this domain leads to much higher levels of POSH upon transient transfection, suggesting that POSH is acting as a ligase for its own degradation. Consistent with this idea, levels of overexpressed POSH are regulated by proteasomes (26). Whether endogenous POSH degradation occurs via the UPS in primary neurons is not known. In studies in neuronal cell lines, activation of c-jun terminal kinase (JNK) and phosphorylation of c-jun have been observed following the application of proteasomal inhibitors (28, 29). Furthermore, dominant negative inhibition of JNK abrogated proteasomal inhibition-induced death in this setting (29). In our system of primary cortical neurons, however, preliminary results show that application of a pharmacological inhibitor of this pathway did not modify survival (Isabelle Lang-Rollin, Hardy Rideout and Leonidas Stefanis, unpublished results).

It should be noted that the experiments mentioned address only the scenario of acute proteasomal inhibition. Rapidly turning over proteins have a number of other mechanisms, via feedback and feedforward inhibition, that ensure relative stability of their levels. It is possible that in conditions of more chronic proteasomal inhibition, as may occur in neurodegenerative diseases, such rapidly turning over proteins are not that dramatically upregulated, and henceforth may not be that critical for death induction.

2.2. Accumulation of Misfolded Proteins

Another class of proteins that are degraded by the proteasome are misfolded proteins. Whereas in the past it was thought that misfolded proteins are only generated under stress conditions, it is now clear that many proteins in the non-stressed cell, when generated within the endoplasmic reticulum (ER), have an unfolded conformation. The unfolded protein response within the ER and the molecular chaperones within the cytoplasm cooperate in refolding such proteins (30). There is now evidence that molecular chaperones also participate in the presentation of such proteins to the proteasome, and thus facilitate their degradation. Thus, a continuous balance exists between the generation of misfolded proteins and their refolding or degradation (for reviews see 31, 32). This balance can be perturbed when there is excess production of misfolded proteins (e.g. under conditions of heat stress, or because of a genetic defect that leads to the misfolding of a particular protein), or when the degradation system is defective, as is the case when there is proteasomal dysfunction. In these cases,

misfolded proteins accumulate in the cell with potential deleterious consequences (33).

Consistent with the importance of protein misfolding in the setting of proteasomal inhibition, neuroblastoma cell lines stably expressing the chaperone HSP40 were partially protected against proteasomal inhibition-induced death (34). We have found a dramatic induction of the inducible form of HSP70 in a subpopulation of cultured cortical neurons following proteasomal inhibition. This presumably reflects a response to the increase in protein misfolding (35). Interestingly, dopaminergic neurons in ventral midbrain cultures fail to induce HSP70 in response to proteasomal inhibition, whereas other cells in the culture show a marked induction. This may reflect a relative inability of these specific neurons to mount this beneficial refolding response. The fact that such dopaminergic neurons do form inclusions and undergo apoptosis in this setting indicates that they are exposed to proteolytic stress. It appears therefore that these specific neurons may be unable to mount this beneficial refolding response, and this may account in part for their selective vulnerability (6). In accordance with the results of Ding and Keller (34), we have also detected a partial survival effect with overexpression of HSP70 in proteasomal inhibitor-treated primary cortical neurons (Hardy Rideout and Leonidas Stefanis, unpublished observations). In conjunction, these results suggest that the accumulation of misfolded proteins may have deleterious consequences to neurons exposed to proteasomal inhibition, and that enhancement of the chaperone response of protein refolding may be beneficial. It is possible that in conditions of more long-standing proteasomal inhibition this accumulation of misfolded proteins may play an even more important role. Further studies are needed, however, because HSP70 and other molecular chaperones also have direct effects on components of the apoptotic pathway that may be independent of their chaperone function (36).

2.3. Accumulation of Free Radicals/Oxidized-Nitrosylated Proteins

Reactive oxygen species and nitrogen species that are generated during various settings, including normal cellular homeostasis, may lead to oxidation and nitration/nitrosylation of intracellular proteins. Only a limited number of such proteins can be enzymatically repaired, and the bulk of them need to be degraded in order to prevent accumulation. There is substantial evidence that the proteasome is involved in the degradation of such proteins (see for example 37). Dysfunction of the UPS would be expected then to lead to accumulation of such modified proteins, with potential deleterious consequences. Further details on the subject are provided in chapter 6 in this volume, which is devoted to the relationship between oxidative/nitrative stress and the UPS.

The question we will address here is whether in a neuronal cell setting UPS dysfunction indeed leads to accumulation of oxidatively-nitratively modified proteins, and whether this has consequences on cell viability. Lee et al. (38) indeed observed accumulation of protein carbonyls and 3-nitrotyrosine, indicative of oxidative and nitrative attack respectively, following proteaosmal inhibition of neuroblastoma cells. Furthermore, blocking nitric oxide signaling ameliorated survival, indicating that at least the reactive nitrogen species played

a role in neurotoxicity in this model. Similar results were observed when UPS function was inhibited at the level of ubiquitination (39). Several other studies have demonstrated that proteasomal inhibition could exacerbate toxicity of oxidative stressors (34, 40-42). Not always is it clear however if the increase in toxicity is solely due to the impairment of degradation of oxidatively damaged proteins or if proteasomal inhibition in itself leads to oxidative injury. In a model using primary mesencephalic cell cultures epoxomicin did not have any effect on the direct increase of ROS, but led to an elevation of oxidized protein and increased neuronal vulnerability to normally subtoxic levels of several complex-I inhibitors which induce oxidative stress. This increase in toxicity was partially abrogated by antioxidants suggesting that in this model proteasomal inhibition reduced the cellular capacity to detoxify oxidized protein (42). In contrast, in a study by Kikuchi et al. (41) epoxomicin directly led to a significant increase of ROS and consequent neuronal death in mesencephalic neurons. Significant changes in mitochondrial function were seen following application of the proteasomal inhibitor, which could account for the generation of ROS. Interestingly, while most studies support the idea that dysfunction of the proteasome and oxidative injury potentially exert cumulative effects, proteasome inhibition has also been reported to increase SOD and catalase activities, as well as the GSSG/GSH ratio and thus protected cerebellar granule cells from oxidative stress-induced neuronal death (43, see also chapter 9 in this volume). In another study, low level chronic proteasomal inhibition induced protein oxidation in neuroblastoma cells, but these cells actually were more resistant to oxidative insults (44). An explanation for these discrepant results might be that, depending on the severity and the time course of the insult in different neuronal populations, pro- and anti-oxidant proteins may be differentially regulated in the face of proteasomal inhibition. Proteasomal inhibition could thus tilt the balance between antioxidative defenses and oxidative stress-related changes in opposing ways. (For a schematic representation of possible effects of proteasomal inhibition on the oxidative status of a cell see figure 4).

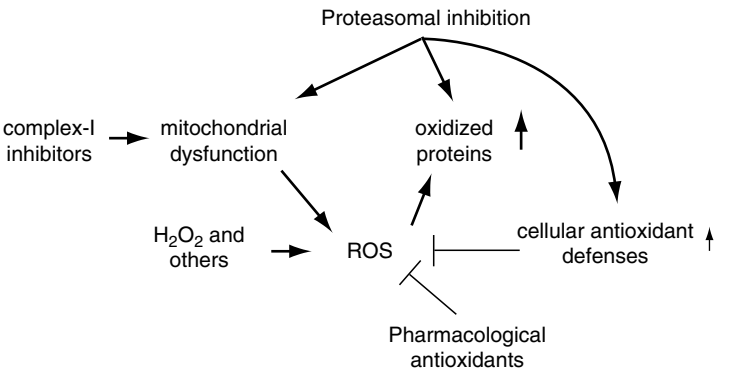

Figure 4. Proteasomal inhibition leads to differential effects on the oxidative status of a cell.

2.4. Elements of the Core Apoptotic Pathway

Emphasis has been placed on transcriptional and translational regulation of the core elements of the apoptotic pathway, and there is little known about their mechanisms of degradation. Bcl-2 family members are critical for apoptosis. They can be either pro- or anti-apoptotic, and the balance between them may determine death or survival (45). The pro-apoptotic proteins of the bcl-2 family Bax and Bim have been shown to be ubiquitinated and degraded by the proteasome in non-neuronal cells (46-50), but few similar studies have been performed in a neuronal cell context. One recent study reported that Bax was upregulated following epoxomicin treatment of rat ventral midbrain cultures (41), but we found no upregulation of Bax following application of proteasomal inhibitors to mouse sympathetic neurons, using both immunoblotting and immunocytochemistry (51). Bim, which is a BH3-only member of the bcl-2 family, was upregulated in our model, suggesting that it is normally degraded by the proteasome in primary neurons, and that it could play a role in proteasomal inhibition-induced neuronal death (51, Figure 5). Of particular interest was the apparent upregulation of the more toxic isoform Bim-S. It should be noted that Bim is known to be transcriptionally regulated in other models, such as NGF deprivation from sympathetic neurons (52, 53), and therefore the possibility exists that the upregulation at the protein level that we have observed is not direct, but rather due to the regulation of transcription factors that in turn regulate Bim levels. Experiments in the PC12 cell line have also shown an upregulation of Bim, but no change of Bax, following proteasomal inhibition (54).

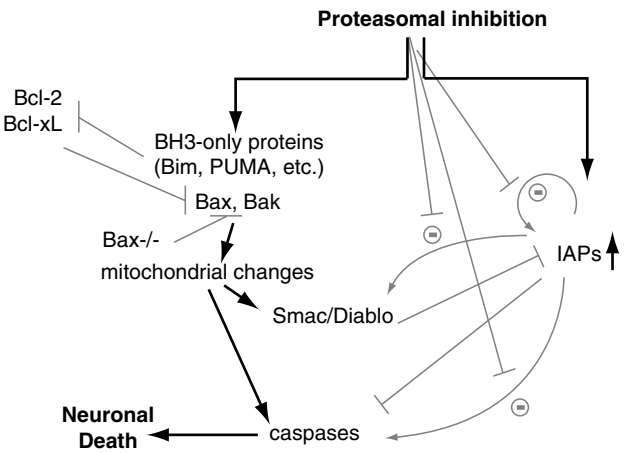

Figure 5. Model of regulation of elements of the core apoptotic pathway when the proteasome is inhibited. Bcl-2 family members might be upregulated and initiate the intrinsic pathway of apoptosis which includes mitochondrial release of Smac/Diablo and activation of caspases. On the other hand IAPs, which inactivate caspases through binding, may also be upregulated by proteasomal inhibition. IAPs however also act as E3-ligases, involved in the ubiquitination of smac/diablo, caspases and themselves, thus favoring proteasomal degradation of these target proteins (curved gray arrows). The net effect of these simplified interactions depends on the cellular context and extent of proteasomal dysfunction.

A class of proteins that has been studied in more detail in this regard is the family of IAPs (Inhibitors of Apoptosis Proteins). IAPs act as endogenous caspase inhibitors, by binding to particular caspases (Figure 5). They have recently been found to be E3 ligases, facilitating the ubiquitination and degradation of other apoptosis-related proteins, including themselves (55). A prime target for ubiquitination by IAPs appears to be Smac/Diablo, a pro-apoptotic factor that acts as an IAP inhibitor (56). Caspase 3 may also be ubiquitinated by IAPs (57, 58). Therefore, the prediction would be that following proteasomal inhibition there would be an accumulation of IAPs, but also of their pro-apoptotic substrates. The degree of proteasomal inhibition and the cellular context would determine whether the net effect would be that of arrest or propagation of apoptosis.

Whether IAPs or their substrates accumulate in neurons following proteasomal inhibition has not been formally examined. In a recent study, self-ubiquitination and degradation of overexpressed XIAP, another member of the IAP family, occurred in neuroblastoma cells, abrogating its survival-promoting effects. In contrast, in primary sympathetic neurons XIAP overexpression was protective, implying that the process of its degradation was much less active in this primary neuron setting (59). This again underscores the importance of the cellular context in determining the role of the proteasome in the degradation of particular substrates (see figure 5 for a model of potential interactions of pro- and antiapoptotic molecules under conditions of proteasomal inhibition).

Regardless of whether particular elements of the apoptotic pathway are regulated by the proteasome, it is clear that proteasomal inhibition of sufficient severity in many types of cultured neurons leads to activation of the core apoptotic pathway and to the morphological features of apoptosis. We and others have shown that proteasomal inhibition of cultured rat cortical neurons, mouse sympathetic neurons or cerebellar granule cells leads to cytochrome c release and loss of mitochondrial transmembrane potential (5, 2, 51, 60, 4). Overexpression of bcl-xL or bcl-2, or deletion of Bax prevents the mitochondrial changes and cell death (5, 51). In PC12 cells we were able to detect a conformational change of Bax following proteasomal inhibition, which was inhibited by bcl-2 or bcl-xL overexpression (54). Proteasomal inhibition of neurons leads to caspase 3 activation, and death is blocked by general caspase inhibitors, which do not prevent cytochrome c release (5, 2, 51). In PC12 cells a dominant negative form of caspase 9 blocked death induced by proteasomal inhibition (54). It appears therefore that in these cases there is participation of the "intrinsic" pathway of apoptosis. Interestingly, as in other models of neuronal cell death, there is requirement for novel or ongoing transcription (5, 2, 60, 6), which may be the consequence of Cdk and p53 activation, and occurs upstream of the mitochondrial alterations.

2.5. Autophagy

Autophagy, and in particular macroautophagy, is defined morphologically by the accumulation of autophagosomes, double membrane structures that engulf components of the cytoplasm, including intracellular organelles. Such autophagosomes fuse with mature lysosomes, leading to the degradation of their contents. Such morphological features are observed in neurons undergoing PCD

in the developing nervous system and are characteristic of type II PCD (61). There is debate as to whether the process of autophagy is actually responsible for the demise of the cells through autodigestion, or if it may be irrelevant to the death, or if it may even serve a protective function.

We found that application of proteasomal inhibitors to primary cortical neurons induced macroautophagy, as well as a general activation of the lysosomal pathway (62). Similar results were reported in a more chronic model of proteasomal inhibition in neuroblastoma cells by Ding et al. (40). In our study, ultrastructural evidence of macroautophagy was observed in neurons that did not show features of apoptosis. Co-application of the survival-promoting agent flavopiridol, however, led to a substantial decrease of lysosomal activation, suggesting that, at least in part, in our experiments macroautophagy was activated secondarily following induction of apoptosis (Hardy J. Rideout and Leonidas Stefanis, unpublished observations). In this setting therefore it appears that neuronal cell death shares features of both apoptotic and autophagic cell death. In another model, that of stable overexpression of mutant A53T α-synuclein in PC12 cells, cell death was associated with macroautophagy, but not apoptosis. An accompanying feature in these lines was proteasomal inhibition (63). Although it is not clear that the relatively modest proteasomal inhibition is responsible for death in this setting, such findings reinforce the idea that autophagic cell death may also be a response of neuronal cells to proteasomal inhibition in certain settings.

3. DIFFERENTIAL SENSITIVITY OF GROUPS OF NEURONS

A number of interesting studies suggest that certain types of neurons may be more vulnerable to proteasomal inhibition. An attempt has been made to correlate this selective vulnerability with the specific neuronal subtypes that degenerate in particular neurodegenerative diseases. Cultured motor neurons of the anterior horn were more sensitive to cell death compared to other neurons in spinal cord cultures (64). In two studies, dopaminergic neurons of embryonic or post-natal rat ventral midbrain cultures were more sensitive to lactacystin- or MG132-induced death compared to GABAergic neurons (65, 7). An in vivo study confirmed selective vulnerability of dopaminergic neurons in the substantia nigra following striatal microinjections of a proteasomal inhibitor. Selective toxicity in this model was dependent on the endogenous dopamine content (9). Another recent study reported remarkable selectivity of neuronal death induced by systemic administration of pharmacological proteasomal inhibitors. Such treatment led to degeneration in areas of the nervous system that are affected in Parkinson's Disease, such as the locus coeruleus, the substantia nigra pars compacta and other brainstem nuclei, whereas striatal neurons were not affected (10). However, another group reported that cultured ventral midbrain dopaminergic neurons were actually more resistant to epoxomicin-induced death compared to other neurons in the cultures (41). This was attributed to the high levels of tetrahydrobiopterin in dopaminergic neurons, levels which provide protection against free radical generation. It was unclear whether the discrepancy between these studies reflects differences in culture techniques or other methodological issues, or whether epoxomicin, which has a somewhat different pharmacological profile,

exerts divergent effects. In our own recent study (6), we have found selective apoptosis of embryonic rat dopaminergic neurons in ventral midbrain cultures in response to either lactacystin or epoxomicin, and we therefore believe that the latter possibility is unlikely. It has to be noted however that micromolar concentrations of lactacystin, for example, are eventually toxic to most cell types, neuronal or non-neuronal, and that therefore sensitivity to proteasomal inhibition cannot be the only determinant of selective neurodegeneration. A factor may be the ability of certain neuronal populations to adjust to the insult of proteasomal inhibition by generating new proteasomes or individual subunits or with other unknown mechanisms, such that they may even show higher enzymatic proteasomal activity following proteasomal inhibition. This phenomenon was observed in the study of McNaught et al. (10) in areas such as the cerebellum that did not show neuronal degeneration following systemic proteasomal inhibition.

4. PROTEASOMAL INHIBITION AND PCD: A COMPLICATED RELATIONSHIP

The reader who has gone through both the previous chapter and the current one may be somewhat baffled. Is proteasomal inhibition deleterious, as suggested by all the data presented in this chapter, or is it beneficial, as suggested by the seminal work of Sadoul et al. (66), Canu et al. (67) and other studies referenced in the preceding chapter? There is no doubt that one important variable is the intensity and timing of the inhibition. As demonstrated first by Lin et al. (68), pro-survival effects occur with low concentrations, whereas pro-death effects occur with high concentrations of pharmacological proteasomal inhibitors, when applied to cultured prostate carcinoma cells. A survey of some in vivo experiments further reinforces this idea. For example, Sawada et al. (69) injected nanomolar concentrations of lactacystin in mouse substantia nigra and observed protective effects on MPP-induced neurodegeneration of dopaminergic neurons, whereas McNaught et al. (70) used micromolar concentrations of the same agent and observed selective toxic effects on rat substantia nigra dopaminergic neurons. Notwithstanding the species difference between these studies, the more than 15-fold difference in dosage is quite likely to account for the discrepant findings. Another variable that we believe is important is the particular cell type that is exposed to proteasomal inhibition, as detailed also in the preceding section, and even the particular species. A prime example is the case of cultured sympathetic neurons, where a striking species difference exists. A dose of 1 μM lactacystin is, as reported by Sadoul et al. (66) and confirmed by us, protective against NGF deprivation-induced death over a period of 48 hrs, whereas it induces apoptosis within 24 hrs in similarly cultured mouse sympathetic neurons (51 and Isabelle Lang-Rollin, Hardy J. Rideout and Leonidas Stefanis, unpublished observations). Presumably, such differences may be explained by the "arsenal" of proteins in a particular neuronal type that are normally targeted for proteasomal degradation. Another variable is also the timing of proteasomal inhibition. Transient, reversible inhibition is more likely to lead to survival-promoting effects due to the interruption of proteasomal activation that occurs at least in some models of neuronal apoptosis soon after the offending insult, as reviewed in the

preceding chapter. The converse is true for sustained, irreversible proteasomal inhibition.

How are then proteasomal function and PCD interrelated during the evolution of neurodegenerative diseases? A hypothetical model is shown in Figure 6. Given the amount of data suggesting proteasomal dysfunction in various such neurodegenerative conditions (see section 6 in this volume), and the likely triggers of protein aggregation (see chapter 3 in this volume) or oxidative/nitrative stress (see chapter 6), it is reasonable to assume that proteasomal dysfunction occurs prior to engagement of apoptotic pathways. Once these pathways are engaged, either through proteasomal dysfunction or other parallel, converging events, they may require, depending on the neuronal subtype and the exact pathogenesis, proteasomal function in order to be fully activated. In the face of proteasomal inhibition such pathways may be partially blocked, and the neuron may undergo an alternative form of PCD. This is demonstrated nicely in the recent study by Canu et al. (71), where potassium-deprived CGCs treated with proteasomal inhibitors show features of autophagy, but not apoptosis. In our own experiments, application of lactacystin, which protected against NGF deprivation-induced death of sympathetic neurons, eventually led to a non-apoptotic form of death in this neuronal cell culture system, even in the presence of NGF (Isabelle Lang-Rollin, Hardy J. Rideout and Leonidas Stefanis, unpublished results). Such findings may help explain why in many cases no clear evidence of classical apoptosis can be obtained in such neurodegenerative conditions. In the case that the apoptotic pathway is fully engaged and leads to caspase activation, there will be, as detailed in the preceding chapter, further proteasomal dysfunction, leading to a feedforward amplification loop.

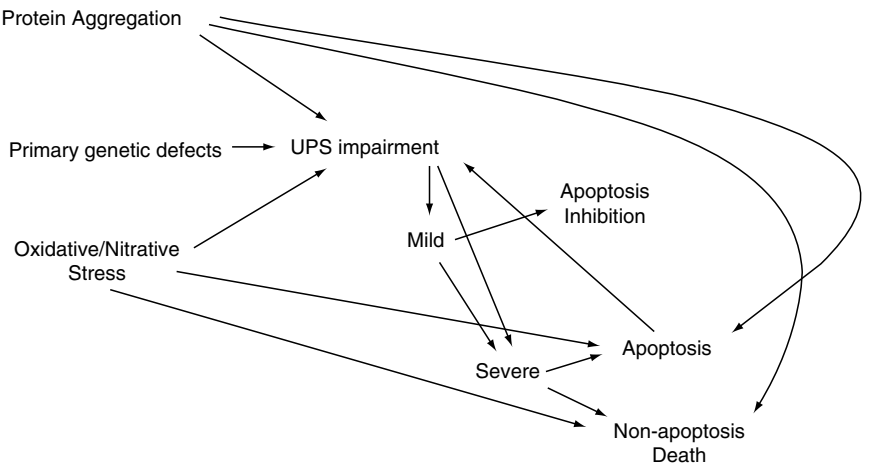

Figure 6. Schematic diagram of potential involvement of the UPS in neuronal cell death in neurodegenerative conditions.

5. CONCLUSIONS

Acute proteasomal inhibition of sufficient severity in cultured primary neurons leads to the upregulation of a number of potentially deleterious proteins, such as p53 or those related to cell cycle control. Accumulation of misfolded or oxidized proteins may also have detrimental effects. Such changes lead in most cases to the activation of the "intrinsic" apoptotic pathway with participation of the mitochondria. Specific pro-apoptotic components of this intrinsic pathway, such as BH3-only members of the bcl-2 family, may also accumulate and play a role in neuronal death. The question of whether similar pathways are involved in vivo in the setting of long-standing inhibition of the proteasome, such as may occur in certain neurodegenerative conditions, awaits the generation of appropriate animal models.

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10

PHARMACOLOGICAL AND MOLECULAR MODELS OF PROTEASOMAL DYSFUNCTION

Thomas Schmidt-Glenewinkel and Maria Figuierdo-Pereira

1. INTRODUCTION

The genome of eukaryotic organisms encodes a large and complex repertoire of proteins – e.g. at least 25,000 proteins in humans, based on the number of protein encoding genes in the human genome, but the actual number of proteins is certainly larger because of splice variants (1). The expression levels of these individual proteins is not only regulated at the transcriptional and translational level but also controlled by degradation. Two major pathways exist in eukaryotes which allow for the controlled degradation of proteins. The initial discovery of the lysosome by deDuve and colleagues (2,3) revealed for the first time the existence of an intracellular machinery which degrades a wide range of proteins. However, subsequent investigations clearly demonstrated that the lysosome lacks the exquisite specificity, tight temporal control and ATP–dependency to account for the experimental observed turnover of proteins. With the discovery of the ubiquitin-proteasome system (UPS) an intracellular mechanism was identified which degrades both short- and long-lived proteins with high specificity and selectivity [for a review, see (4)]. This pathway is also responsible for the

removal of misfolded and denatured proteins as well as for the degradation of the large amount of aberrant proteins formed during protein synthesis.

While the details of the principle pathway of protein degradation through the UPS is discussed in other chapters, it is important to emphasize that protein degradation is a dynamic, highly regulated process which achieves its specificity through a system of E3-ubiquitin ligases and a variety of ancillary proteins. Data from the human genome reveal the existence of several hundred E3-ubiqutin ligases. The interaction between these ligases and their protein substrates is frequently not constitutive but involves either the activation of the respective E3-ubiquitin ligase by post-translational modification or by modification of the substrate to allow ubiquitination to occur. In addition, ancillary proteins like molecular chaperones frequently play a role as recognition elements [for an excellent review see (5)].

Based on the large body of published data it is clear that the UPS not only turns over normal and aberrant proteins, but is part of an intricate regulatory system which is involved in many essential cellular processes – e.g. long term memory, modulation of receptors and ion-channels, circadian rhythms, and regulation of the cell cycle to name only a few. This key position of the UPS raises the question about the consequences for the cell or organism if this pathway fails. Current evidence indicates that in certain human diseases like Angelman's syndrome, Liddie's syndrome, cystic fibrosis or infection with human papilloma virus the interaction of certain key proteins with the UPS is changed (6).

There are only a few known natural occurring mutations in this pathway – e.g. in the human parkin gene which encodes an E3-ubiquitin ligase and the UCH-L1 gene that encodes a C-terminal ubiquitin hydrolase (7). In addition, there are no known natural occurring mutations in the 26S proteasome. However, we have tantalizing hints that in several neurological diseases proteasome function is impaired although the nature of this dysfunction is not understood. One of the main disease characteristics in these neurological disorders are protein deposits, designated neurofibrillary tangles (Alzheimer's disease), Lewy bodies (Parkinson's disease), Bunina bodies (Amyotrophic lateral sclerosis) and nuclear inclusions in CAG expansions as found in Huntington's disease, spinobulbar muscular atrophy and spinocerebellar ataxias. Whether these protein deposits are pathogenic or represent a coping mechanism of the neuron to prolong survival is a hotly debated question (8). It is clear however, that the proteins which form the protein deposits escape somehow the normal degradation process. While a more detailed analysis of these neurological disorders relevant to the UPS is presented in other chapters of this book, the subsequent discussion will focus on the more general question of the consequences of proteasome dysfunction in the nervous system. The principal experimental approach to address this issue rests on the use of proteasome inhibitors and of site-specific mutations, both of which will be discussed below.

2. INHIBITORS OF THE PROTEASOME

The available proteasome inhibitors and their properties will be briefly discussed because they are important not only as probes for the study of the UPS

but also as potential therapeutic agents for human disease. A detailed report of proteasome inhibitors is available in three excellent reviews (9-11).

The three peptidase activities associated with the proteasome belong to the class of N-terminal nucleophilic hydrolases which, in the case of the proteasome, use the side chains of their N-terminal threonine residues for the hydrolysis of the peptide bond. The principle reaction scheme is outlined in Figure 1.

The proteolytic activities of the proteasome are associated with three different β subunits. The β1 subunit contains a caspase-like activity (peptidyl-glutamyl-peptide-hydrolase) while a trypsin-like activity is associated with the β2 subunit. The β5 subunit has chymotrypsin-like activity. In addition, the β1 subunit has limited branched-chain amino acid activity while both a limited branch-chain and small neutral amino acid activity has been observed for the β5 subunit (11). The combined enzymatic activities of the proteasome hydrolyze the substrate protein to small peptides with an average length of seven amino acids.

It should be noted however, that the enzymatic activities of the proteasome are not entirely independent of each other. Inhibition of the chymotrypsin-like activity or its inactivation by mutations will reduce the proteolytic activity of the proteasome to a large extend while inactivation of the trypsin-like or caspase-like sites does not affect overall proteolysis (9).

Pharmacological inhibitors of the proteasome can be categorized according to their reactive groups. Figure 2 offers a brief overview of the proteasome inhibitors with the reactive group encircled. Most of the proteasome inhibitors are not highly specific as they also inhibit other cellular proteases. In most cases the chymotrypsin-like activity is inhibited but at least one of the other two main peptidase activities is also affected. A brief discussion of the individual classes of proteasome inhibitors is presented below.

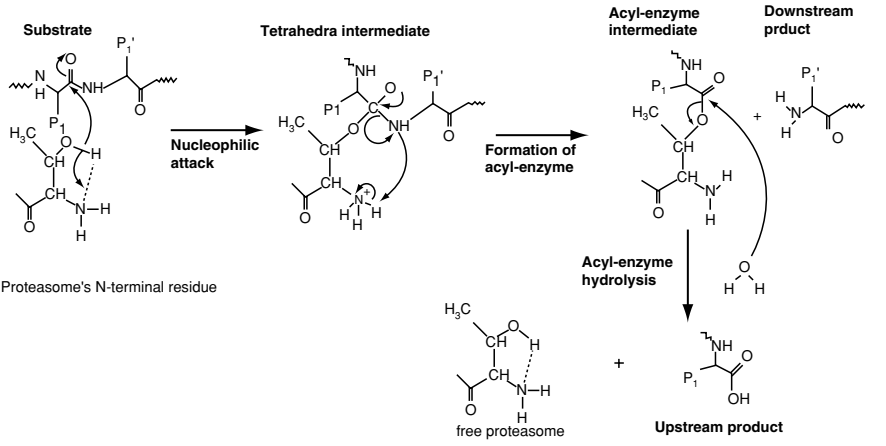

Figure 1. The catalytic mechanism of the proteasome. Reproduced from (9) with permission from Elsevier.

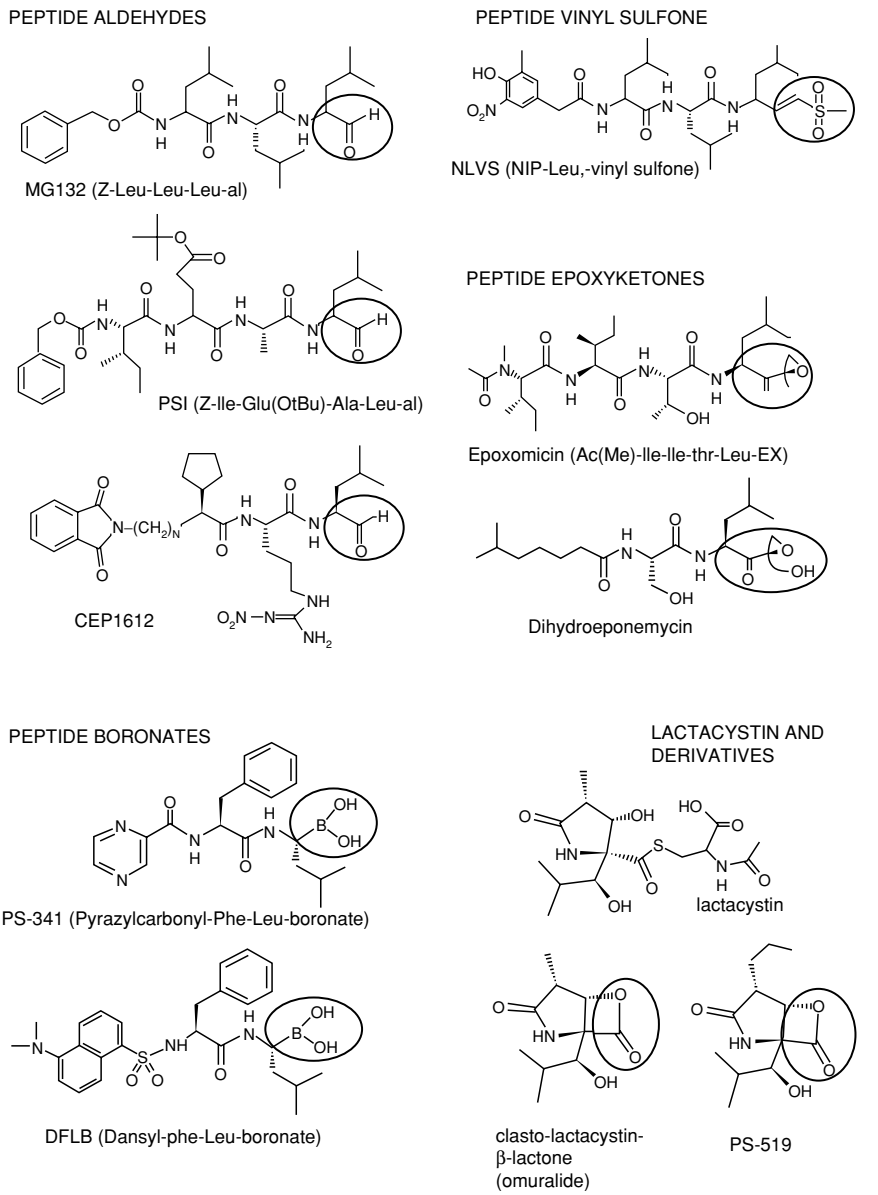

Figure 2. Major classes of proteasome inhibitors. The pharmacore is encircled. Reproduced from (9) with permission from Elsevier.

2.1. Peptide Aldehydes

Peptide aldehydes are reversible inhibitors mostly of the chymotrypsin-like activity of the proteasome. They enter the cell easily because of their lipophilic properties. Inactivation occurs by oxidation of the aldehyde group to the corresponding acid or by transport via the multi-drug resistant system carrier. Peptide aldehydes are the least specific of the proteasome inhibitors because they also inhibit cellular cysteine and serine proteases in general.

2.2. Peptide Boronates

When compared with peptide aldehydes, peptide boronates are more selective, more potent and only slowly reversible inhibitors of the proteasome. Inhibition of thiol-proteases is greatly reduced by peptide boronates because of the weak interaction of boron with sulfur. They are also weaker inhibitors of serine proteases – e.g. 1,000 fold in the case of PS-341 (Pyrazylcarbonyl-Phe-Leu-boronate, Velcade, Bortezomib) which has been demonstrated in clinical trials to be effective against multiple myelomas. A more detailed mechanistic study revealed that PS-341, at least in tissue culture, also inhibits ubiquitin-thiolesterification and reduces levels of free ubiquitin. This observation was extended to two peptide aldehyde inhibitors, MG132 (Benzyloxycarbonyl-Leu-Leu-Leu-al) and ALLN (N-acetyl-Leu-Leu-Nleu-al) as well as lactacystin (12).

2.3. Peptide Vinyl Sulfones

Peptide vinyl sulfones are irreversible inhibitors of proteasome activity. NLVS (NIP-Leu-Leu-Leu-vinyl sulfone) reacts preferably with the chymotrypsin-like active site, while NIP-Leu-Leu-Asn-vinyl sulfone reacts at comparable rates with all three catalytic active sites of the proteasome. This class of inhibitors does not inhibit serine proteases but can inhibit cysteine proteases depending on the peptide sequence of the inhibitor.

2.4. Epoxyketones

Epoxyketones were originally discovered in a screen for antitumor drugs in mice. Subsequently it was discovered that they exert their biological effects by inhibition of proteasome activity. Epoxyketones are the most selective of the proteasome inhibitors known so far. Their specificity regarding the three enzymatic activities of the proteasome varies. Epoxomicin inhibits the chymotrypsin-like activity while its analog eponomycin also reacts with the caspase-like activity at a similar rate.

2.5. Lactacystin and Derivatives

Lactacystin is a non-peptide inhibitor of the proteasome. It modifies the β5 subunit of the proteasome, blocking almost irreversibly ($t_{1/2} \sim 20$ hours) its activity. The other active sites of the proteasome are also blocked but at a much

slower rate and reversibly. Lactacystin is not directly cell permeable but is converted in tissue culture at neutral pH into the β-lactone which is cell permeable. The β-lactone is hydrolyzed rapidly at neutral pH.

2.6. Natural Occurring Proteasome Inhibitors

In addition to the earlier mentioned epoxomicin and lactacystin several other compounds are know to occur in nature which inhibit proteasome activity. TMC-95A is a cyclic peptide compound found in *Apiospora montagnei*. It inhibits all three proteolytic activities of the proteasome by inserting itself into the active site without modifying the threonines in the catalytic sites.

A fungal compound gliotoxin was found to inhibit the chymotrypsin-like activity of the proteasome but the mechanism of action is uncertain. It requires the presence of the disulfide bond in the toxin for activity.

Epigallocatechin-3gallate (Figure 3) was characterized from green tea and is most likely attacked by the threonine in the catalytic centers of the proteasome resulting in the acylation of the threonine.

The occurrence of natural occurring proteasome inhibitors has to be considered as a possible causative factor for the etiology of neurological diseases. In a recent paper it was reported that the systemic injection of epoxomycin or the peptide aldehyde PSI [Benzyloxycarbonyl-Ile-Glu(gamma-*t*-butyl)-Ala-Leu-al] over a two week period caused a progressive model of Parkinson's disease (13).

2.7. UCH Inhibitors and Ubistatins

A high-throughput screen for inhibitors of ubiquitin C-terminal hydrolases (UCH), identified a class of isatin O-acyl oximes that selectively inhibits UCH-L1, a protein that is highly abundant in the brain and that is linked to PD. Three examples of this class of inhibitors (#s 30, 50 and 51) are shown in Figure 4. Inhibition of UCH-L1 by these compounds is reversible, competitive and active site directed and the drugs are cell permeable (14). Notably, these agents were found to be proproliferative when applied to a lung tumor cell line that expresses UCH-L1.

A chemical genetic screen to discover molecules that arrest the cell cycle in *Xenopus* extracts resulted in the identification of a novel class of inhibitors known

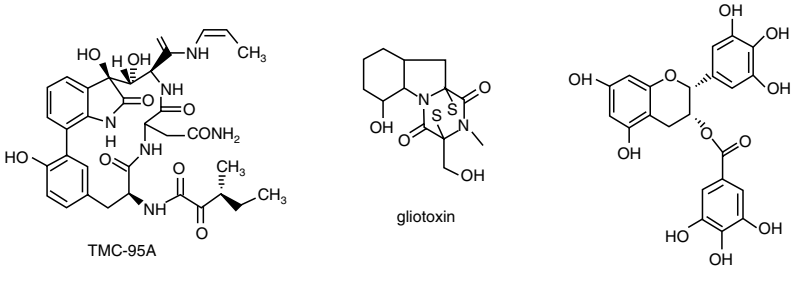

Figure 3. Natural product inhibitors of the proteasome. Reproduced from (9) with permission from Elsevier.

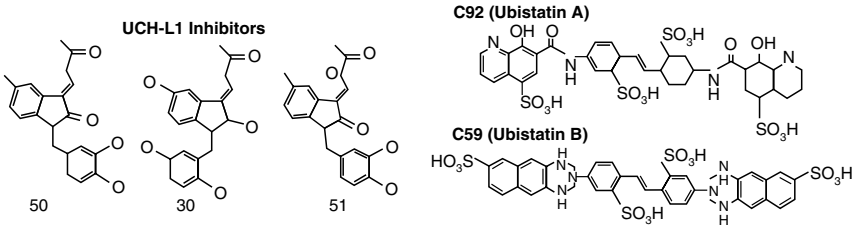

Figure 4. Left: Chemical structure of UCH-L1 inhibitors reproduced from (14) with permission from Elsevier. Right: Chemical structure of ubistatins reproduced from (15) with permission from AAAS.

as ubistatins (15). These molecules (Figure 4) target the ubiquitin-ubiquitin interface of K48-linked polyubiquitin chains disrupting their binding to the ubiquitin-chain receptors of the proteasome. Due to their negative charge these molecules are not cell permeable, but following microinjection into mammalian cells they efficiently inhibited the degradation of polyubiquitinated proteins by the proteasome.

2.8. Genetic Activators of Protein Degradation by the UPS

Although the focus of this section is on inhibitors of the UPS we want to discuss a very interesting chemical genetic approach to selectively increase the degradation of UPS substrates. This strategy enables the design and synthesis of molecules that will specifically bind to selected proteins *in vivo* and target them for degradation by the UPS. (16). PROteolysis TArgeting Chimeric molecule S (PROTACS) are heterobifunctional molecules that comprise a ligand for the target protein, a linker moiety and a ligand for an E3 ubiquitin ligase (Figure 5).

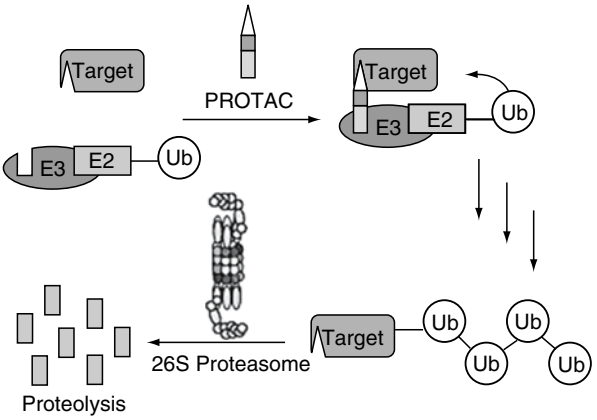

Figure 5. Targeted proteolysis using a PROTAC molecule. Ub = ubiquitin, target = target protein, E3 = E3 ubiquitin ligase complex, and E2 = E2 ubiquitin transfer enzyme. Reproduced from (16) with permission from ACS.

PROTACS serve as bridges that connect a particular E3 ligase to the target protein, promoting its E3-dependent ubiquitination and degradation by the proteasome. The advantages of this strategy are: (1) its selectivity based on the unique site of the protein substrate chosen to be recognized by an E3 ligase and (2) the ability to render it tissue specific because some E3 ligases are expressed in a tissue-specific manner. By generating "chemical knockouts" of selective proteins, PROTACS allow for the control of protein function within cells. Moreover, PROTACS could be used as drugs to remove toxic or disease-causing proteins once their cell permeability is improved.

3. PHARMACOLOGICAL MODELS OF PROTEASOME DYSFUNCTION

Pharmacological inhibitors have been used to unravel the complexity of the biological pathways in which the proteasome is involved. In the nervous system a linkage of the UPS to a large number of neurodegenerative diseases has been suggested because of the presence of proteinacious inclusion bodies which are the hallmark of these diseases. The use of proteasome inhibitors aiming at demonstrating proteasome dysfunction as a causative factor in the etiology of these diseases, will be discussed in other chapters of this book.

3.1. Proteasome Inhibitors in Tissue Culture

The study of proteasome activity in tissue culture cells is now greatly facilitated by the availability of a suitable reporter construct, involving the green fluorescent protein (GFP) (17). This was accomplished by fusing a CL1-degron sequence to the carboxyl terminal of GFP. Inhibition of proteasome activity leads to an increase of the reporter protein which is detectable by monitoring the fluorescence of the cells.

Acute inhibition of the proteasome with high levels of proteasome inhibitors abolishing all or most of the proteasome activity in primary neurons or neuronal cell lines will lead inevitably to the death of cells. Preceding cell death accumulation of ubiquitinated proteins and heat-shock proteins is detectable. Proteasome inhibitors also cause a transient and combined up-regulation of all mammalian 26S proteasome subunit mRNAs. This increase in transcription of the proteasome mRNAs results in up-regulation of all the proteasome subunits with a concomitant *de novo* assembly of proteasomes (18). Notably, pre-treatment of neo-cortical neuronal cultures with sub-toxic concentrations of proteasome inhibitors reduced neuronal susceptibility to oxidative stress (19). This cytoprotective result was attributed to up-regulation of core proteasome subunits and the ensuing increase in proteasome activity rather than to up-regulation of heat shock proteins (19).

Chronic administration of low concentrations of proteasome inhibitors that do not induce neuronal death was shown to increase the levels of protein oxidation, protein insolubility and DNA as well as RNA oxidation (20,21). RNA appeared to be more affected than DNA and both the 18S and 28S ribosomal RNA levels were significantly decreased following proteasome inhibition. Furthermore, chronic low-level proteasome inhibition seems to activate the

lysosomal system manifested by an increase in macroautophagy. A microarray analysis of neuronal cells subjected to chronic proteasome inhibition revealed a limited (less than 0.8%) alteration in gene expression some of the genes being relevant to aging, AD and PD. However, the vast majority of genes altered by chronic proteasome inhibition have not been characterized yet, suggesting that impaired proteasome function affects neuronal homeostasis by still uncharacterized mechanisms (22).

It is important to keep in mind that pharmacological inhibitors of the proteasome should be used with caution in cell studies in which proteins are expressed from a heterologous promoter. As expected, the proteasome inhibitors MG132, ALLN and lactacystin induced the accumulation of transfected parkin or α-synuclein driven by the cytomegalovirus (CMV) promoter in PC12 cells. However, the high levels of parkin or α-synuclein detected upon treatment with proteasome inhibitors was found to be associated with increased protein synthesis rather than degradation (23). These data suggest that the proteasome inhibitors increase CMV-driven transcription in a non-specific manner. Proteasome inhibitors may stabilize transcription and translation factors or activate transcription pathways regulating the CMV promoter (23).

3.2. The Use of Proteasome Inhibitors in Animal Models

The effect of proteasome inhibitors on the brain of intact animals has recently been explored. Initial studies with the administration of the tea polyphenol epigallocatechin-3gallate into the stomach of male and female mice, demonstrated a wide organ distribution including the brain (24). Acute systemic administration of PSI [Z-Ile-Glu(OtBu)-Ala-Leu-al] to ovariectomized adult female rats increased progesterone receptor levels in the preoptic area and hippocampus but not in the frontal cortex (25). Due to the acute nature of these two studies, no behavioral or chronic effects of the proteasome inhibitors on the CNS were discussed.

Seven days after striatal microinfusions of the proteasome inhibitors lactacystin or epoxomicin into rats, the nigrostriatal dopaminergic neurons exhibited selective neurotoxicity leading to retrograde apoptosis and the development of ubiquitin positive inclusions (26). Neurotoxicity induced by the proteasome inhibitors was prevented by inhibition of dopamine synthesis and was enhanced by drugs promoting dopamine synthesis. These studies demonstrate a close correlation between proteasome inhibition and dopamine synthesis that selectively damages nigrostriatal dopaminergic neurons. No behavioral changes were observed in animals microinfused with proteasome inhibitors alone. This was attributed to the inability to induce a 90-95% loss of nigrostriatal dopaminergic neurons, which is required to produce the abnormal turning behavior.

In a long term study, rats were systemically injected with the proteasome inhibitors PSI and epoxomicin, over a two week period. Two weeks later the animals showed signs of progressive parkinsonism with bradykinesia, tremor, rigidity and abnormal postures. Six weeks after the end of proteasome inhibitor injections, postmortem analysis showed dopaminergic cell death and inflammation in the *substantia nigra pars compacta*. Neurodegeneration was also observed

in the locus coerulus, dorsal motor nucleus of the vagus and the nucleus basalis of Meynert. Histology of the neurodegenerative sites detected inclusions resembling Lewy bodies (13).

The studies described above demonstrate that the proteasome inhibitors epigallocatechin-3gallate, PSI and epoxomicin cross the blood brain barrier. However, not all proteasome inhibitors have this property. For instance, intravenous administration of the peptide boronate PS-341 resulted in a rapid and widespread distribution of the drug with highest levels found in the liver and gastrointestinal tract and the lowest in the skin and muscle. No apparent penetration of the CNS was reported (27). Since proteasome inhibitors differ in their chemical and biophysical properties, variations in lipophilicity, solubility, potency and ability to cross the blood brain barrier are to be expected.

4. GENETIC MODELS OF PROTEASOME DYSFUNCTION

Proteasome inhibitors may exert nonspecific effects as bound molecules or through interactions with other cellular proteins. For example, proteasome inhibitors were shown to increase transcription driven by the CMV promoter in a non-specific manner (23). An alternative approach to investigating the outcome of proteasome impairment is to genetically manipulate proteasome function. The use of this approach in tissue culture and *in vivo* animal models is discussed below.

4.1. Effect of Proteasome Mutants in Tissue Culture

RNA interference was used to determine the function of 26S proteasome subunits (28) and the effects of reduced expression of individual subunits of the 26S proteasome in *Drosophila* S2 cells (29). Cells deficient in any of several 26S proteasome subunits from the 19S regulatory particle or the 20S proteasome, exhibited increased apoptosis, decreased cell proliferation and accumulation of ubiquitinated proteins (29). Like in cells treated with proteasome inhibitors, post-transcriptional silencing of many of the 26S proteasome subunits promoted increased expression of non-targeted subunits and disrupted proteasome assembly. Notably, cells deficient in certain proteasome subunits were more resistant to toxic concentrations of proteasome inhibitors, suggesting an adaptation to conditions of UPS impairment.

In chicken DT40 cells the conditional knockdown of the proteasome subunit β2, which has the trypsin-like activity, arrested cell-cycle and enhanced the accumulation of polyubiquitinated proteins as well as the expression of molecular chaperones HSP40 and HSP70 (30). In this cell line, expression of the β2 subunit is under the control of a tetracycline-repressible promoter in a β2-nullizygous genetic background so that treatment with doxycycline inhibits β2 expression.

Partial impairment of the proteasome chymotrypsin-like activity was accomplished in mouse neuronal HT4 cells by overexpressing a proteasome β5 subunit with a mutation in the active site (31). The N-terminal threonine to alanine active site substitution was not lethal under homeostatic conditions. However, this single amino acid substitution significantly hypersensitized the cells

to oxidative stress triggering the accumulation and aggregation of ubiquitinated proteins including synuclein, as well as cell death. These results demonstrate that this genetic manipulation of proteasome activity involving a single amino acid substitution causes the formation of protein aggregates in stressed neuronal cells independently of the occurrence of mutations in other cellular proteins. These results support the notion that proteasome disruption may be central to the development of familial as well as sporadic cases of neurodegeneration.

In human senescent fibroblasts, subunits of the 20S proteasome and 19S regulatory particle were found to be down-regulated. The decrease in subunit levels is consistent with a decline in proteasomal peptidase activities and increased levels of both oxidized and ubiquitinated proteins in the senescent cells (32). Overexpression of the proteasome β5 subunit in human senescent fibroblasts and a variety of other human cell lines, elevated the expression of other proteasome subunits (33). This genetic manipulation resulted in increases in the three catalytic activities of the proteasome. Notably, β5 overexpression enhanced cell survival upon treatment with various oxidants and delayed senescence. The increased survival rate under conditions of oxidative stress was attributed to a high rate of protein degradation in the cells overexpressing the β5 subunit. These results single out proteasome activity as playing a pivotal role in cellular senescence and survival.

4.2. Genetic Manipulation of Proteasome Activity in Animal Models

To investigate changes in UPS activity in *in vivo* paradigms, several transgenic mice were developed expressing a variety of reporter proteins such as ubiquitin-luciferase (34) and GFP with a constitutively active degradation signal (35). These reporter proteins are rapidly degraded under homeostatic conditions and stabilized in a time- and dose-dependent manner in response to proteasome inhibitors. A hexahistidine-tagged ubiquitin-GFP transgenic mouse was developed to facilitate the analysis of *in vivo* ubiquitination events (36). Cleavage of the hexahistidine-tagged ubiquitin-GFP protein by endogenous enzymes produces epitope tagged-ubiquitin detectable as a monomer or conjugated to other proteins. A variation of the hexahistidine-tagged ubiquitin-GFP transgenic mouse was generated by expressing a dominant negative K48R mutant form of ubiquitin as hexahistidine-tagged K48R mutant ubiquitin-GFP (37). The latter transgenic mice provide a tool for investigating the outcome of interference with ubiquitination, since K48R mutant ubiquitin is unable to support the formation of K48-linked polyubiquitin chains.

In spite of the existence of excellent mammalian models of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Amyotrophic lateral sclerosis, none address the effect of constitutive proteasome impairment. One line of transgenic mice was developed to address the role of the immunoproteasome in antigen presentation (38). In these transgenic mice expression of the immunoproteasome LMP2 subunit was knocked-out. LMP2 is not a constitutive component of the 20S proteasome as its expression is induced by agents such as γ-interferon. LMP2 knock-out mice exhibit impaired inflammatory responses manifested by reductions in the numbers of CD8+ lymphocyte

and influenza nucleoprotein-specific cytotoxic T lymphocyte precursors (38). Besides displaying increased body weight, the LMP2 knock-out mice were found to have a higher degree of motor function indicating that specific proteasome subunits may play a role in regulating brain function (39). Notably, the non-obese diabetic (NOD) mouse, a spontaneous model of Type 1 diabetes, was found to exhibit a phenotypic LMP2 down-regulation that parallels the incidence of diabetes in these mice (40). No correlation between this phonotype and brain function in the NOD mouse was discussed.

Drosophila is an attractive *in vivo* model to study neurodegeneration due to a combination of its short life span (~60 days), easy genetic manipulation, rapid screen for mutations and a biological complexity that is, in many ways, comparable to that of mammals. Moreover, critical mechanisms in *Drosophila* neurodegeneration are thought to be regulated similarly in humans. Two *Drosophila* lines expressing temperature-sensitive dominant negative missense mutants of the 20S proteasome subunits β2 and β6 were recently identified (reviewed in (41). When raised at permissive temperatures (22-25˚C) heterozygous flies had no abnormal phenotypes but died as undifferentiated pupae at the restrictive temperature (29˚C). It was proposed that the mutant proteasome subunits that are incorporated into the quaternary structure of the 20S proteasome act as "poison subunits" disrupting the function and stability of the entire complex.

A *Drosophila* mutant strain exhibiting a deletion of the Rpn10 subunit of the 19S regulatory particle of the 26S proteasome displayed accumulation of ubiquitinated proteins and larval-pupal polyphasic lethality (42). Rpn10 is known to be one of the 19S subunits that binds polyubiquitinated proteins with a high affinity. When compared to the wild type, the mutant strain also exhibited a significantly higher accumulation of fully assembled 26S proteasomes and the absence of free subunits or partially assembled 26S proteasomes (42). These findings indicate that expression of proteasomal genes are subjected to a negative feedback that regulates their expression in cells accordingly to temporal and/or spatial demands.

Several proteasome subunits were shown to undergo caspase-dependent proteolysis resulting in a decline in proteasome activity in *Drosophila* and human cell lines undergoing apoptosis (43). These data suggest that caspase-dependent proteolysis regulates proteasome activity. Regulation of proteasomal activity can also occur via glycosylation as shown in *Drosophila* and mammalian cells [reviewed in (44)]. Increased glycosylation of 19S regulatory particles was found to parallel a decrease in proteasome activity (44). Studies examining the levels of proteasome glycosylation under different physiological settings that change proteasome activity, may define new roles for glycosylation in aging, starvation, neurodegenerative disease and other forms of cellular stress.

Overall these studies support the notion that alterations or reductions in the activity of the UPS may play a critical role in the etiology of neurodegeneration. Given the current interest in the UPS, the genetic manipulation of proteasome activity in animal models has the potential for opening the doors to new discoveries in the field of neurodegeneration with therapeutic relevance to devastating disorders such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis and Huntington's disease, just to name a few.

5. PHARMACOLOGICAL AND MOLECULAR PROTEASOME DYSFUNCTION DURING AXONAL GUIDANCE AND AT THE SYNAPSE

Increasing evidence links the UPS to the mechanism of axonal guidance, synapse formation and synapse modulation in both invertebrates and vertebrates (for a review see (45). In *Drosophila* the *bendless* mutation caused failure of the giant fiber axon to make an appropriate turn and to establish connection to its motor neuron target (46). The *bendless* gene was later identified as an E2-ubiquitin ligase (47). In *Xenopus* it was demonstrated that the growth cone of retinal neurons in culture contains ubiquitin, the E1-ubiquitin activating enzyme as well as proteasomes (48). When the growth cone encountered the guidance molecules netrin and LPA (L-α-lysophosphatidyl acid) the levels of ubiquitin protein conjugates doubled. Inhibition of the proteasome with ALLN (N-Acetyl-Leu-Leu-NorLeu-al) or lactacystin prevented the netrin-dependent growth cone turning and the growth cone collapse normally induced by LPA.

The essential role of the proteasome was also demonstrated in axonal regeneration (49). When axons from rat dorsal root ganglia were severed in culture, the necessary growth cone regeneration was greatly reduced in the presence of lactacystin and ALLN. Axotomy was carried-out 200-300μm away from the cell body eliminating the possibility that protein synthesis in the cell body contributed to growth cone regeneration within the experimental time period of 30min. These results indicate that intra-axonal protein synthesis and degradation is required for growth cone formation.

Studies on the *Drosophila* neuromuscular junction analyzed the role of UPS in regulating the local concentrations of functionally important proteins at the synapse (50). In less than an hour, proteasome inhibition with lactacystin or epoxomicin caused specific synaptic accumulation of DUNC-13, a protein involved in the regulation of vesicle priming at the synapse. The same was accomplished by disrupting proteasome activity using the temperature-sensitive mutant of the β6 subunit of the 20S proteasome. In agreement with these observations, a pharmacological inhibition of the proteasome with lactacystin or epoxomicin significantly increased the efficiency of the synaptic transmission. A transcriptional contribution to this effect could be ruled out because the motor axon was cut 30min after application of the inhibitors prior to the recording. The same authors used a special reporter construct composed of a thermolabile dihydrofolate reductase degron fused to GFP, to investigate the activity of the proteasome at the synapse. Two constructs were prepared differing only in their N-terminal amino acid, which was either methionine or arginine. According to the N-end rule (51), methionine should stabilize the reporter construct while arginine should destabilize it. Both constructs are stable at 18˚C but undergo a conformational change at 35˚C making them substrates for the UPS. Both reporters M-DHFR$_{ts}$-EGFP and R-DHFR$_{ts}$-EGFP expressed well in the nervous system. Based on fluorescent measurements in synaptic boutons, activation by a 35˚C heat shock for 30-min reduced the levels of R-DHFR$_{ts}$-EGFP by more than 50% while M-DHFR$_{ts}$-EGFP was decreased by less than 3%. These results were confirmed by western blotting indicating that UPS-specific protein degradation occurs *in vivo* at the synapse.

6. CONCLUSION

Proteolysis is an important cellular event involving tightly regulated removal of unwanted proteins and retention of those that are essential. In addition to its function in normal protein degradation, the UPS plays a critical role in the quality control process. It eliminates mutated or abnormally folded proteins by degradation preventing their accumulation as aggregates as those found in neuronal intracellular inclusions detected in many neurological disorders, such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis and Huntington's disease. The protective or damaging effects of these aggregates is discussed in another chapter.

Neither animal models nor cell culture systems are able to reproduce all the patterns of pathologies encountered in human neurodegenerative disorders. However, cell models with impaired proteasome activity exhibit unique features useful to address the mechanistic aspects of neurodegeneration associated with impairment of the UPS. These culture systems permit easy handling, repetitive sampling, direct observation under the microscope and easy access for biochemical analysis. A thorough knowledge of the mechanisms involved in cellular neurodegeneration due to UPS impairment is necessary to precisely identify the critical steps and cellular and subcellular targets in this process. Moreover, since rodents are highly comparable to the human in respect to physiological systems, rodent models with disturbed proteasome function are unique in offering the possibility to understand the *in vivo* relationship between proteasome impairment and neurodegeneration.

One of the major challenges that we are faced with is to single out the UPS as a therapeutic target for preventing neurodegeneration. The challenge rests on developing therapeutic strategies that will enhance degradation of oxidatively-modified and toxic proteins generated by a lifetime's worth of environmental damage without compromising the normal function of the UPS.

7. ACKNOWLEDGMENTS

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11

THE *GAD* MOUSE: A WINDOW INTO UPS-RELATED NEURODEGENERATION AND THE FUNCTION OF THE FUNCTION OF THE DEUBIQUITINATING ENZYME UCH–L1

Jungkee Kwon and Keiji Wada

1. INTRODUCTION

Deubiquitinating enzymes reversibly control the state of protein ubiquitination. Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) is a deubiquitinating enzyme that is exclusively expressed in neuronal cells and in the testis/ovary. UCH-L1 is a constituent of cellular aggregates that are commonly observed in neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD). The UCH-L1 gene reportedly causes familial Parkinson's disease (PD) and is the basis for the gracile axonal dystrophy (*gad*) mouse. These facts suggest that the function of UCH-L1 is closely related to the survival and maintenance of neurons. This chapter summarizes the role of UCH-L1 in the pathogenesis of neurodegenerative diseases, with particular emphasis on the *gad* mouse, which offers unique insights into the relationship of UPS to neurodegeneration in an *in vivo* setting.

2. THE DEUBIQUITINATING ENZYME UCH-L1

The process of ubiquitination and targeting to the proteasome of substrate proteins is reviewed elsewhere in this volume. For the purposes of this chapter, it is important to appreciate that ubiquitination is a dynamic and reversible process. The disassembly of polyubiquitinated chains is carried out by a class of enzymes called deubiquitinating enzymes (DUBs). DUBs cleave ubiquitin (Ub) from proteins and from residual proteasome-associated peptides, and disassemble poly-Ub chains. Several DUBs have been reported and are divided into two classes: Ub carboxyl-terminal hydrolases (UCHs) and Ub-specific processing proteases (UBPs or USPs) (1). Members of both classes are thiol proteases that hydrolyze the isopeptide bond between the substrate and the C-terminal Gly76 of Ub.

UCHs can hydrolyze bonds between Ub and small adducts or unfolded polypeptides *in vitro*. UCHs also can cleave Ub gene products—either tandemly conjugated Ub monomers (UbB, UbC) or Ub fused to small ribosomal proteins (L40, S27a)—very slowly *in vitro* to yield free Ub or ribosomal proteins, respectively. Thus, UCHs are thought to serve dual functions: to salvage Ub that has been trapped by reactions with low molecular weight thiols/amines and to process polyubiquitin or ubiquitinated proteins (Figure 1). In mammals, there are at least four closely related low molecular weight UCH family members, UCH-L1, UCH-L3, UCH37 (UCH-L5) and BAP1. UCH-L1 and UCH-L3 are the major UCHs in mammalian cells. Larsen (1998) showed that UCH-L1 cleaves linear polyubiquitin more efficiently than UCH-L3 (2). In contrast,

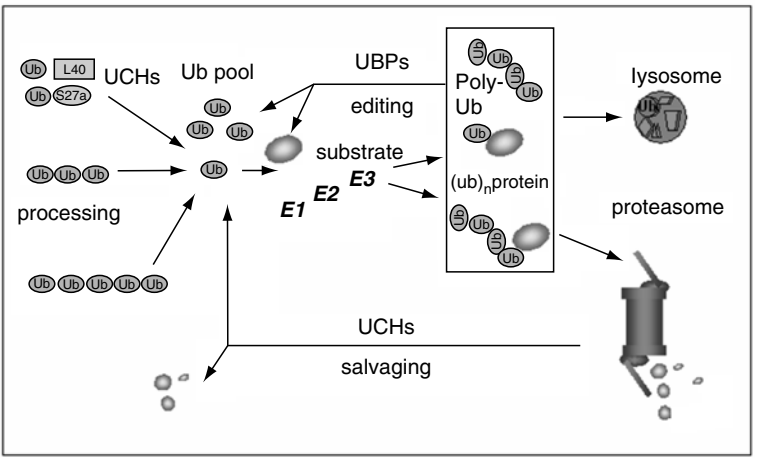

Figure 1. UCH function *in vitro*. UCHs can hydrolyze bonds between Ub and small adducts or unfolded polypeptides *in vitro*. UCHs can cleave Ub gene products very slowly, either tandemly conjugated Ub monomers (UbB, UbC) or Ub fused to small ribosomal proteins (L40, S27a). UBPs can cleave the isopeptide bond between ubiquitins in a poly-Ub chain, as well as the isopeptide bond between Ub and proteasomal substrates.

UCH-L3 appears to preferentially cleave ubiquitin fused to small ribosomal proteins. UBPs are generally larger and are thought to be responsible for removing Ub from larger proteins. UBPs also are involved in the disassembly of poly-Ub chains.

3. UCH-L1 AND PD

PD is characterized by the progressive loss of dopaminergic neurons in the substantia nigra and by the presence of cytoplasmic inclusions called Lewy bodies. Six different genes have been linked to familial forms of Parkinson's disease: α-synuclein, UCHL1, DJ-1, parkin, PINK1 and LRRK2/dardarin (3–12). Certain protein products of these genes are associated with the UPS. The relationship of PD to the UPS is analyzed in detail in chapters 12 and 13. Here we will review only the evidence linking UCH-L1 to PD.

3.1. Structural Alteration of UCH-L1 in PD

UCH-L1 is one of the most abundant proteins in the brain (1–2% of the soluble protein in neurons), where it catalyzes the hydrolysis of C-terminal ubiquityl esters. This activity is presumed to be critical for cytoplasmic protein degradation and results in the recycling of free Ub during the degradation of polyubiquitinated proteins in the proteasome (2,13). As with Ub, UCH-L1 is a constituent of cellular aggregates (e.g., Lewy bodies) that are hallmarks of neurodegenerative diseases such as PD (14). The UCH-L1 gene was first linked to PD upon identification of an autosomal dominant point mutation (I93M) that was found in two siblings of a PD-affected family, and this gene has been implicated in the development of an inherited form of PD (15). Since the I93M mutation decreases the hydrolytic activity of UCH-L1 *in vitro* (to ~55% of wild-type UCH-L1), this form of PD was proposed to result from a partial loss of UCH-L1 hydrolytic function. However, several lines of evidence suggest that simple loss of hydrolytic function does not completely explain the PD phenotype in this family. First, the parents of the affected sibling were unaffected, indicating that this substitution could be a rare polymorphism that manifests as a mutation with incomplete penetrance (16). Second, *gad* mice lacking functional UCH-L1 do not develop a Parkinsonian phenotype (17–22). Neuronal loss in the *gad* mouse occurs in the gracile tract, as opposed to the substantia nigra in PD (18).

Conversely, it has been confirmed that a polymorphism (S18Y) in UCH-L1 gene reduces the risk of developing sporadic PD, especially in early onset cases (16,23–27). The S18Y polymorphism is relatively rare in the European population (allele frequency 14–20%) but is common in Japanese (39–54%) and Chinese (~50%). Protection is dependent on the S18Y allele dosage; that is, homozygotes are at significantly lower risk (relative risk of 0.31) than are heterozygotes (relative risk between 0.55 and 0.81) (16). Additionally, the S18Y polymorphism may have modest protective effects in patients with Huntington's disease (HD) (28). The S18Y variant has greater hydrolase activity than wild-type UCH-L1 (29). The effects of the I93M mutation and S18Y polymorphism on the incidence of PD can therefore be partly explained by altered enzymatic function (15,16,29).

3.2. The Relation between Ubiquitin Ligase Activity of UCH-L1 and PD

The simplest explanation for the genetic association of UCH-L1 variants to PD is that S18Y is protective because its effect on UCH-L1 hydrolytic activity is opposite to that of the I93M mutation. However, this explanation is inconsistent with the predicted location of residue 18 on the protein surface, distal to the active site and the Ub binding site (Figure 2) (30,31). Furthermore, the fact that position 18 is one of only a few residues that are not conserved between human and other mammals (e.g., horse, mouse, and rat have alanine at position 18 (16)) suggests that residue 18 is not involved in the normal biological activity of UCH-L1. Furthermore, human UCH-L1 deletion mutants have not been reported, although polymorphisms and missense mutations in UCH genes have been linked to PD (15,16). This raises the possibility that a gain, rather than a loss of function of UCH-L1 may be linked to PD, and that the S18Y polymorphism may offer protection through an inhibition of such aberrant function. Consistent with this idea, recently Liu at al. reported that UCH-L1, in addition to its function as a DUB, also contains an aberrant ligase activity in its dimeric form (16). The *in vitro* ligase activity of the UCH-L1 dimer offers a simple mechanistic explanation for the fact that the S18Y polymorphism reduces susceptibility to PD (16).

Proteins that are polyubiquitinated via linkage to Ub Lys48 are destined for proteasomal degradation, whereas those linked to Ub Lys63 are stable. The

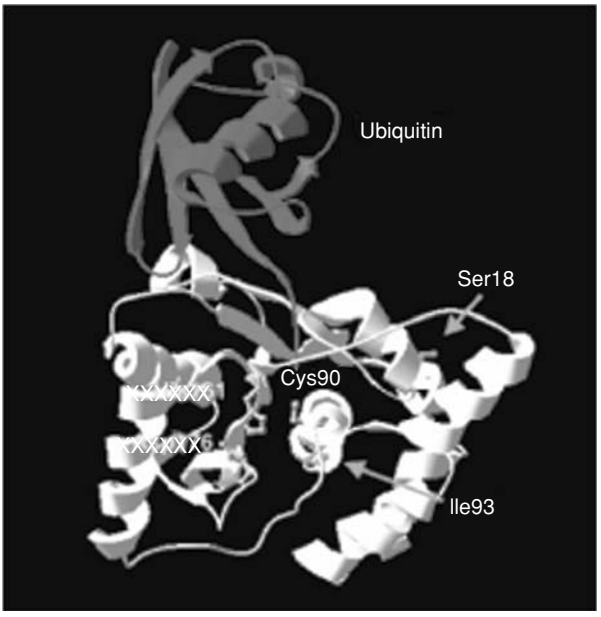

Figure 2. A structural model for UCH-L1 and the location of several key amino acids. Residue 93 is proximal to the active site nucleophile (C90), whereas S18 is distant from both the active site and the Ub binding site. (from Liu *et al.*, 2002).

protective variant S18Y-UCH-L1 exhibits diminished dimerization and ligase activities relative to wild-type UCH-L1. Therefore, S18Y-UCH-L1 is predicted to accelerate the degradation of protein substrates, such as α-synuclein, by making less stable species conjugated to Ub Lys 63. The genetic association of UCH-L1 to PD could be explained by the effects on the concentration of α-synuclein and potentially other substrates (16). However, it is unclear whether UCH-L1 has ubiquityl ligase activity *in vivo*.

Recently, UCH-L1 was found to accumulate in the sarkosyl-insoluble fraction of brains from AD patients, suggesting that the accumulation of insoluble UCH-L1 may also contribute to the pathogenesis of AD.

3.3. Oxidative Modifications and Reduction of UCH-L1 Hydrolase Activity in PD

Oxidative stress is another important factor that has been implicated in the pathogenesis of a number of age-related neurodegenerative diseases, including PD and AD. UCH-L1 is a member of the papain-like cysteine protease family, having conserved Cys and His residues within the active site. Certain cysteine proteases are known targets for covalent modification during cellular oxidative stress (32), and several lines of evidence implicate this phenomenon in sporadic PD (33). One of the endogenous factors that is toxic to neurons during oxidative stress is 4-hydroxynonenal (HNE), an aldehyde product of fatty acid peroxidation (34). HNE can induce neuronal death and is thought to form covalent cross-links with proteins via Michael addition to Cys, His, and Lys residues, thus altering the function of cysteine proteases. HNE-modified proteins have been detected in substantia nigra neurons and in Lewy bodies in sporadic PD (35).

Nishikawa (2003) showed that HNE directly modifies and inactivates UCH-L1 *in vitro* (Figure 3) (29). In this study, HNE covalently modified UCH-L1 and reduced its hydrolase activity by 40–80%. Moreover, excess N-acetyl-L-cysteine (NAC, a competitive cysteine analog) prevented both HNE modification and the decrease in UCH-L1 hydrolase activity, thus confirming that human UCH-L1 was modified by HNE. Oxidative alterations of proteins by reactive oxygen species have been implicated in the progression of aging and age-related neurodegenerative disorders such as AD (36). Choi (2004) demonstrated that UCH-L1 is a major target of oxidative damage in the brain of PD and AD patients (37). The observed oxidative modifications might cause irreversible alterations in the conformation and/or enzymatic activity of UCH-L1 and have deleterious effects on neuronal function and survival.

4. UCH-L1 AND THE *GAD* MOUSE

The *gad* mouse is an autosomal recessive spontaneous mutant that was identified in 1984 (38). It is the first mammalian model with a defect in the UPS to exhibit a neurological phenotype (18). These mice have an intragenic deletion of *Uchl1* gene including exons 7 and 8 and do not express UCH-L1 protein, making them comparable to a *Uchl1* null mutant (Figure 4A, B).

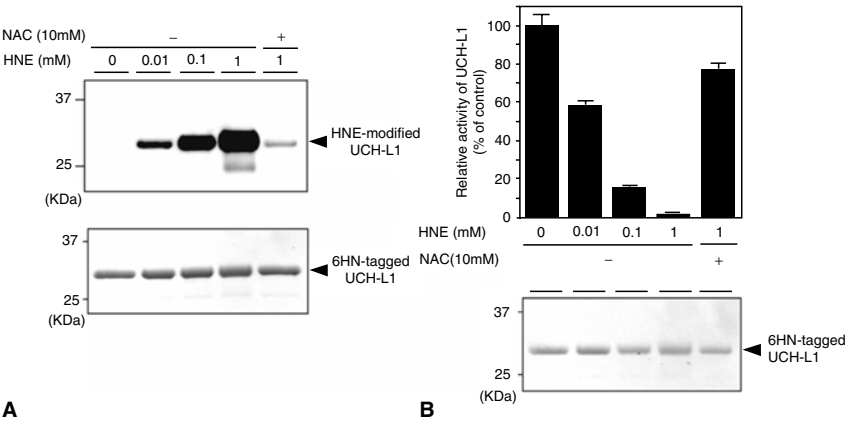

Figure 3. Effect of HNE and NAC on UCH-L1 hydrolase activity. (A) Modification of wild-type UCH-L1 with HNE was visualized by immunoblotting with an HNE adduct-specific antibody. HNE-modified UCH-L1 from the same sample was resolved by SDS-PAGE and stained with Coomassie brilliant blue (lower panel). (B) Inactivation of UCH-L1 hydrolase activity by HNE and rescue with NAC. The residual activity of HNE-modified UCH-L1 hydrolase activity by HNE and rescue with NAC. The residual activity of HNE-modified UCH-L1 prepared from the same sample as in (A) was measured by a hydrolase assay. (from Nishikawa *et al.*, 2003).

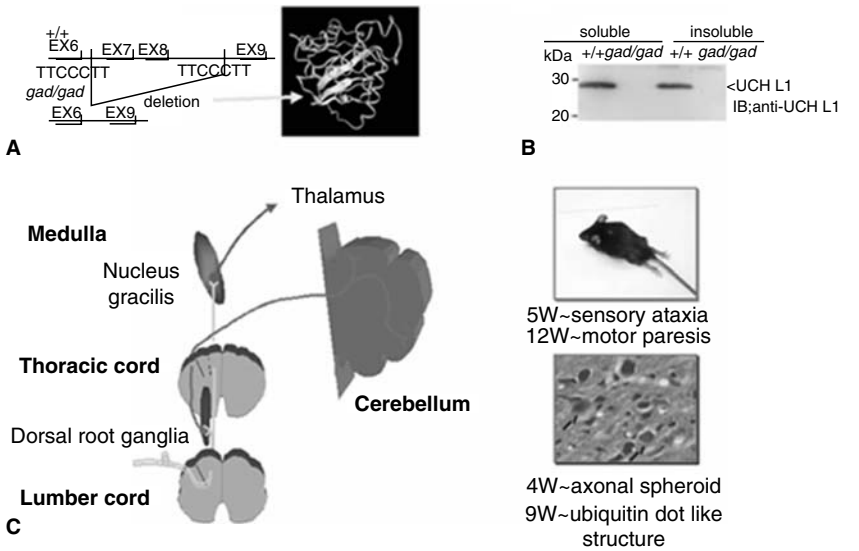

Figure 4. Characterization of the *gad* mouse. (A) The *gad* mouse has a deletion of exons 7 and 8 of *Uchll*, encompassed by a tandem repeat. The secondary structures of the peptides deleted in the *gad* mouse are depicted with an arrow. (B) Both the soluble and insoluble fractions from wild-type and *gad* mice were subjected to SDS-PAGE and immunoblotted with anti-UCH-L1. (C) Pathological features of the *gad* mouse. Dorsal root ganglion neurons project to the nucleus gracilis in the medulla oblongata. Degeneration starts at the synapse in the nucleus gracilis and extends to the gracile tract, resulting in gracile axonal dystrophy (gad). Sensory ataxia is noted at ~5 weeks with crossing of the hind limbs when the tail is pulled up. Characteristic pathological features include axonal swellings called spheroids (right lower) and punctuate Ub-rich inclusions.

4.1. The *Gad* Mouse as a Model of Neurodegenerative Disease

Analyses of *gad* mice have revealed the first example of a Ub pathway enzyme involved in neurodegeneration. The *gad* mouse exhibits severe sensory ataxia at early stages of the neurodegenerative process, followed by motor paresis at later stages. The primary defect in the *gad* mouse is axonal degeneration in the gracile tract, which results in sensory ataxia (38). Pathologically, the *gad* mouse displays dying-back type of axonal degeneration of gracile tract with the formation of spheroid bodies in nerve terminals (19,20,39) (Figure 4C). These pathological changes associate with brain ageing and neurodegenerative disease with progressive accumulation of ubiquitinated protein conjugates (40). Most interestingly, *gad* mice develop accumulation of amyloid precursor protein (APP) in ubiquitin-positive deposits along the sensory and motor nervous systems (41).

Saigoh (1999) positionally cloned the corresponding lesion and identified a deletion in a genomic fragment within *Uchl1* that includes exons 7 and 8 (18) (Figure 4A). Given such a substantial deletion, the protein encoded by the *gad* allele most likely lacks the core structure of UCH-L1 and is thus unstable. Immunoblotting using a polyclonal antibody to UCH-L1 failed to detect UCH-L1 in either soluble or insoluble brain lysates from the *gad* mouse (Figure. 4B). Thus, the *gad* mouse is equivalent to a *Uchl1* null mutant. UCH-L1 dysfunction in the *gad* mouse appears to interfere with Ub recycling, which may result in the accumulation of abnormal proteins that would normally be degraded by the UPS.

4.2. UCH-L1 and Ubiquitin Stabilization

UCH-L1 is a small but abundant protein in neurons. Although the role of UCH-L1 *in vivo* remains unclear, its abundance and neuron-specific expression suggest a role in neuronal function. Although it has been established that deletion of *Uchl1* in mice causes gracile axonal dystrophy, Osaka (2003) recently reported a novel *in vivo* role for UCH-L1 in Ub homeostasis, namely that UCH-L1 functions as a Ub carrier protein (42). These data show that UCH-L1 is associated with Ub in neurons and suggest that this association is important for the maintenance of mono-Ub levels in these cells.

As shown in Figure 5, selected gel filtration chromatography fractions from wild-type and *gad* mouse brain lysates were subjected to SDS-PAGE and immunoblotted with anti-UCH-L1 or anti-Ub. Wild-type mono-Ub eluted in the range of ~10–50 kDa, overlapping significantly with the elution of UCH-L1, whereas the *gad* mouse mono-Ub eluted exclusively at ~10–14 kDa, corresponding to free Ub (Figure 5A). In addition, the levels of free mono-Ub in various brain areas of *gad* mouse by radioimmunoassay were also reduced by ~20–30% (Figure 5B). These data suggest that the absence of UCH-L1 reduces the level of mono-Ub in neurons, which in turn causes a reduction in the overall level of mono-Ub in the nervous system.

In contrast, we found that UCH-L1 overexpression increases the level of Ub in the nervous system *in vitro* as well as *in vivo*. Mouse embryonic fibroblasts (MEFs), which do not express UCH-L1, were infected with adenovirus encoding UCH-L1 protein (adeno-*Uchl1*). In these infected MEFs, Ub and UCH-L1

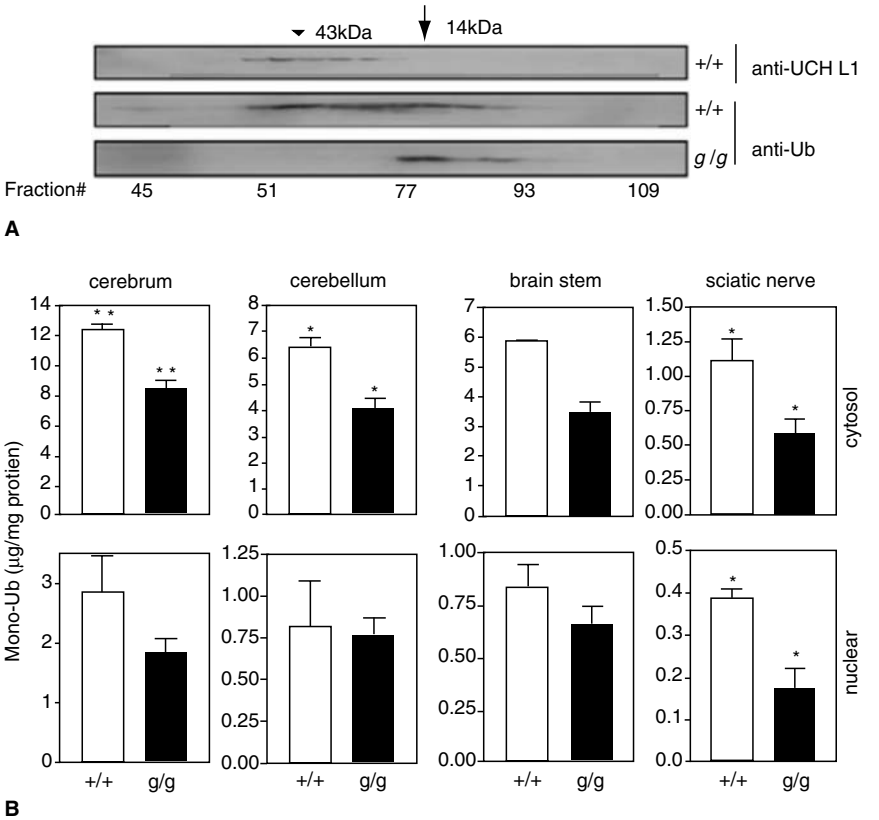

Figure 5. Loss of UCH-L1 decreases the levels of mono-Ub in *gad* mice. (A) Selected gel filtration chromatography fractions of brain lysates from wild-type and *gad* mice were subjected to SDS-PAGE and immunoblotted with anti-UCH-L1 or anti-Ub. (B) The level of free mono-Ub in cytosolic and nuclear fractions was measured by radioimmunoassay in various brain structures from wild-type and *gad* mice. Scale bars, 10um. (from Osaka *et al.*, 2003).

immunoreactivities completely overlapped, suggesting that Ub is recruited to sites of UCH-L1 localization (Figure 6A). Moreover, transgenic mice overexpressing UCH-L1 showed increased levels of Ub in the nervous system (Figure 6B).

As shown in Figure 7, pulse-chase labeling of MEFs suggests that UCH-L1 affects Ub metabolism and extends Ub half-life by inhibiting its degradation. Because an inhibitor of lysosomal function extended Ub half-life and partially diminished the effect of UCH-L1, UCH-L1 probably prevents Ub degradation in lysosomes. These results suggest that UCH-L1 is linked to the endosome-lysosomal pathway. Actually, Ub itself contains all the necessary signals for both targeting and degradation of monoubiquitinated proteins in the endosomal-lysosomal pathway, and several Ub residues are critical for these functions (43,44). These data suggest that the functional basis of UCH-L1 binding to Ub is to suppress Ub degradation in lysosomes.

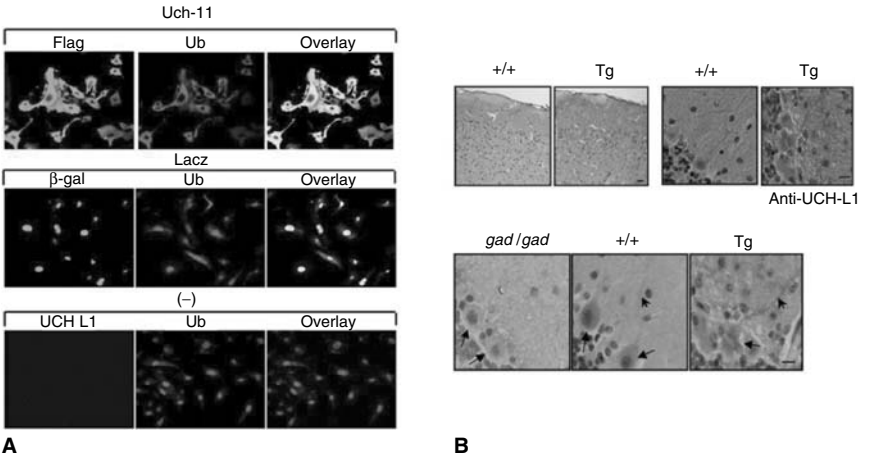

Figure 6. Overexpressed UCH-L1 colocalizes with Ub and increases Ub levels *in vitro* and *in vivo*. (A) Primary mouse embryonic fibroblasts (MEFs) were infected with adenovirus expressing UCH-L1 epitope tagged with either FLAG or β-gal. Antibodies to FLAG and β-gal were used to immunostain exogenous UCH-L1 or β-gal, respectively. MEFs were also labeled with polyclonal anti-Ub. Ub and UCH-L1immunoreactivities completely colocalized in MEFs infected with adeno-*uchl1* but not in cells infected with β-gal or cells that were not infected. (B) UCH-L1 overexpression increases the level of Ub in the mouse nervous system. Immunostaining with an anti-UCH-L1 in cerebral cortex (left) and cerebellum (right) from UCH-L1 transgenic and wild-type mice (upper panel) and with polyclonal anti-Ub in cerebellar sections from *gad*, wild-type and UCH-L1 transgenic mice (lower panel). Ub immunoreactivity increased in transgenic mice overexpressing UCH-L1. Scale bars 20 μM (from Osaka *et al.*, 2003; see color insert).

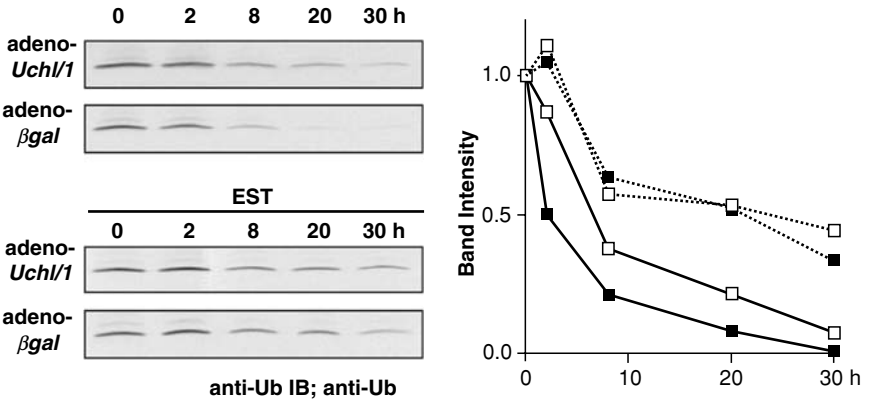

Figure 7. Analysis of UCH-L1 affinity for Ub. Adeno-*Uchl1*-transfected or adeno-*β-gal*-transfected MEFs were labeled with [^{35}S]-Met. Autoradiograms of anti-Ub immunoprecipitates pulse-chased at the indicated times in the absence (upper left panels) or presence of EST (2,3-*trans*-epoxysuccinyl-L-leucylamide-3-methyl butane ethyl ester; lower left panels) are shown. Relative band intensities are quantified and mean values of two independent experiments are shown (right). From Osaka *et al.*, 2003.

Loss of functional UCH-L1 could lead to inadequate ubiquitination via a decrease in free Ub. In the *gad* mouse, an initial pathological lesion begins at the nerve terminals of axons in the dorsal root ganglia. Ub undergoes anterograde transport over long distances via slow axonal transport to nerve terminals (45). A decrease in Ub and the consequent inadequate ubiquitination of proteins may lead to increased levels of proteins that should undergo Ub-dependent degradation, resulting in the accumulation of such proteins within spheroids that are observed in *gad* mice (39). However, as with α-synuclein, the loss of UCH-L1 function does not appear to cause PD. Actually, I93M-UCH-L1 was shown to enhance Ub immunoreactivity to a level similar to that of wild-type UCH-L1 in transfected cells (42). Moreover, nigrostriatal dopaminergic pathology has not been observed in either heterozygous or homozygous *gad* mice (42). Therefore, an unknown 'gain-of-toxic-function' mechanism may underlie PD in patients carrying the I93M-UCH L1 mutation (Figure 8).

Recent pathological observations of *gad* mice have exhibited a pathological accumulation of γ and β-synuclein in the spheroids that appear in the gracile nucleus from the earliest stages. Such accumulation which might affect axonal transport from ganglia, leading to the dying-back type of degeneration of axons

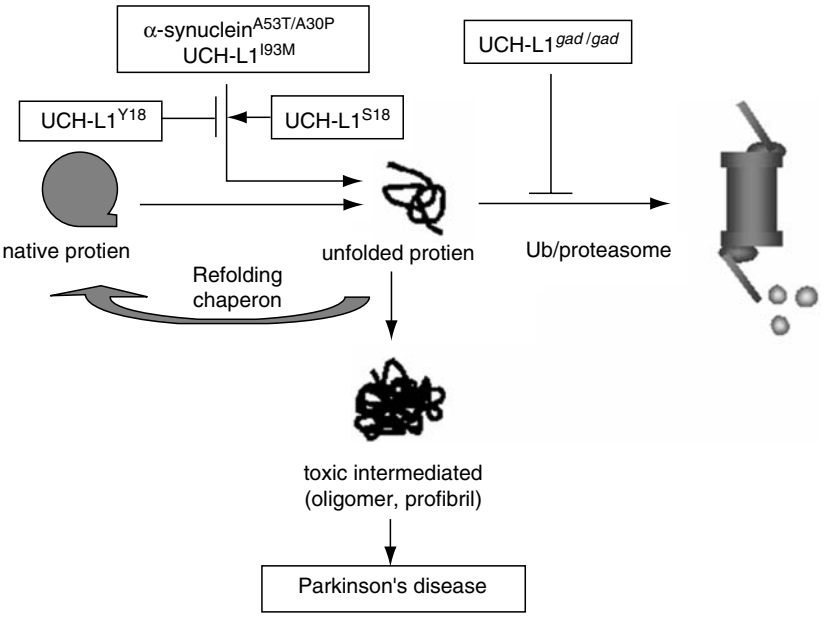

Figure 8. The role of UCH-L1 in neurodegeneration in PD. Unfolded proteins are refolded by molecular chaperones or degraded by the ubiquitin/proteasome pathway. Genetic defects such as A53T/A30P mutations in α-synuclein and 193M in UCH-L1 may result in a gain-of-toxic-function that leads to PD. A S18Y polymorphic variant of UCH-L1 has been associated with a decreased risk for PD. However, loss of UCH-L1 function in the *gad* mouse does not appear to cause PD. Thus, the accumulation of a toxic intermediate (oligomer or protofibril) is proposed to precede and promote neurodegeneration.

(46). The alteration of many pathways in *gad* mice has been indicated by microarray analyses (47) and by proteomic studies, which showed increased protein oxidation in *gad* mouse brains, providing novel insights into the pathophysiology of neuronal degeneration in this mouse model (48). Mi *et al.* recently reported that the slow Wallerian degeneration gene, Wld^s, inhibits axonal spheroid pathology in *gad* mice (49). These results raise the possibility that Wld^s may have an effect on several CNS disorders characterized by axonal spheroids.

5. NEW FUNCTIONS FOR UCH-L1

UCH-L1 is abundant in neurons as well as in testis (3). Although it has relatively weak hydrolase activity, UCH-L1, as mentioned above, exhibits dimerization-dependant ubiquityl ligase activity, at least *in vitro*. In addition, it has been suggested that UCH-L1 associates with mono-Ub and prolongs Ub half-life in neurons (42).

Recent analyses of UCH-L1 function in *gad* mice suggest that these mice are resistant to cryptorchid-induced testicular germ cell apoptosis (50). This observation is consistent with a previous report that the loss of UCH-L1 function suppresses ischemia-induced retinal cell apoptosis in *gad* mice (51). The expression of both antiapoptotic and prosurvival proteins such as Bcl-2, Bcl-xL, XIAP, pCREB and BDNF is significantly elevated in *gad* mice following apoptotic stress (50,51). Furthermore, UCH-L1 and UCH-L3, the two predominant UCHs, have reciprocal functions in testicular germ cells during cryptorchid-induced apoptosis (50). This is surprising, since both isozymes have 52% amino acid identity and share significant structural similarity. Together, these results suggest that UCH-L1 is involved in an apoptosis-inducing pathway that promotes neuronal and testicular cell death. These data are in accordance with a number of studies which have linked inhibition of the UPS with suppression of apoptosis, as detailed in chapter 8.

On the other hand, studies in *Aplysia* suggest a role for UCH-L1 in long-term facilitation. *Aplysia* UCH (Ap-uch) is induced by stimuli that promote long-term facilitation but not stimuli that promote short-term facilitation (52). Biochemical analyses show that Ap-uch associates with the proteasome and that this association increases the rate of degradation by the proteasome (52). These studies in *Aplysia* suggest that UCH-L1 may play a role in synaptic plasticity as well as other neuronal functions.

6. CONCLUSION

The role of UCH-L1 in neuronal function is becoming more apparent with the use of refined cellular and molecular approaches and several *in vitro* and *in vivo* model systems. Although the I93M-UCH-L1 mutant and S18Y-UCH-L1 variant are clearly involved in the pathogenesis of PD, our understanding of the underlying molecular mechanisms of UCH-L1 function is still limited. Further investigation of the relationship between neurodegeneration and UCH-L1 modifications should help to elucidate the mechanisms of pathogenesis in PD and AD and may suggest novel targets for therapeutic intervention.

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12

PARKINSON'S DISEASE AND RELATED DISORDERS

Mark Cookson

1. INTRODUCTION

Parkinson's disease (PD) is a relatively common adult neurodegenerative disease that manifests itself clinically as a movement disorder. This includes the famous tremor described by James Parkinson, but also several other characteristics including rigidity or stiffness of the limbs and trunk, bradykinesia (slowness of motor movements) and postural instability or impaired balance and coordination. The underlying pathological event that produces these features is loss of neurons in the brainstem that are responsible for the initiation or cessation of movement. A great deal of attention has been placed on neurons of the substantia nigra *pars compacta* that project to the striatum and use dopamine as a neurotransmitter (Figure 1). These neurons are progressively lost in PD and other parkinsonian disorders, but they are not the only neurons to be affected and the patterns of neuronal damage in PD are somewhat more complicated. The other critical pathological event is the formation of Lewy bodies and Lewy neurites, which are characteristic inclusion bodies that contain protein and lipids and help define PD as a protein aggregation disease.

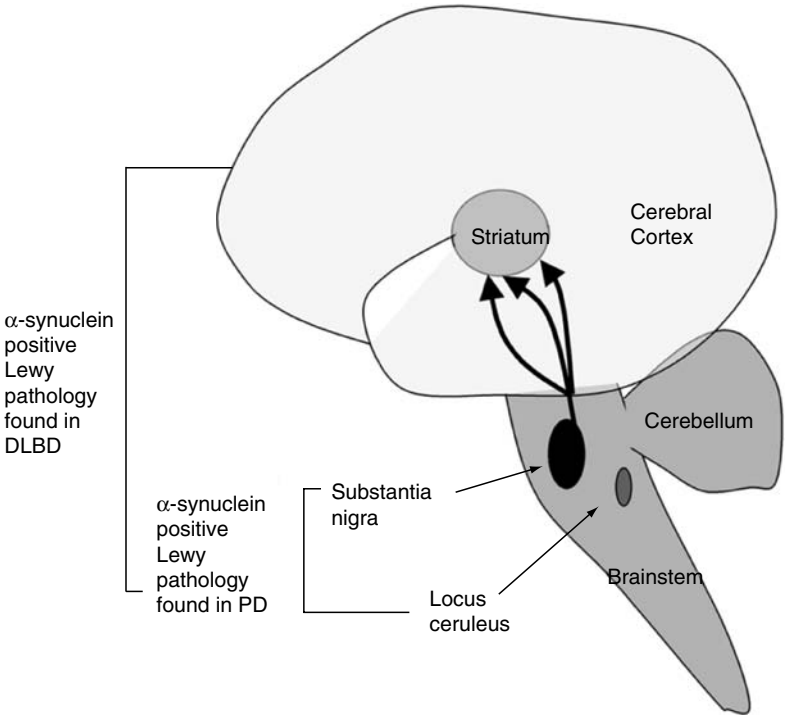

Figure 1. A crude diagram of the major neuronal groups affected in Parkinson's disease and related disorders. PD = Parkinson's disease. DLBD = diffuse Lewy body disease.

Over many years, there have been many attempts to understand the fundamental process that "drives" neuronal loss in PD. As will be discussed later, the involvement of oxidative stress and mitochondrial dysfunction have attracted a great deal of attention, as toxins that inhibit mitochondrial complex I activity and also produce free radicals can be used to model aspects of the disease, particularly loss of neurons from the substantia nigra. More recently, the possibility that disruption of the ubiquitin-proteasome system (UPS) may also make a contribution has attracted attention. The best evidence for a direct role of the UPS in PD is the observation that mutations in parkin, an E3 protein-ubiquitin ligase, are associated with recessive parkinsonism. As this will be discussed in detail in a subsequent chapter, here I will describe the body of other evidence implicating dysfunction in the UPS in typical PD.

Aside from parkin, most of the rest of the evidence about UPS dysfunction in PD hinges around a small protein, α-synuclein, which is associated with both genetic and sporadic PD. The strongest evidence for an association of α-synuclein with sporadic, typical PD is the observation that α-synuclein forms the building blocks of Lewy bodies and other pathologies seen in the *post-mortem* brain (1). α-Synuclein is a very sensitive marker of these proteina-

ceous inclusions and can be used to "stage" the pathology in PD (2). Mutations in the gene encoding α-synuclein cause a disease that includes such Lewy pathology in several brain regions (3). Furthermore, having multiple copies of the same gene with the wild type sequence produces a similar disease in a dose-dependent manner (4,5).

In this chapter, I will concentrate largely on the evidence linking UPS dysfunction and α-synuclein. The reason for this is that, of all the genes associated with PD, α-synuclein is the one that most obviously also links to the sporadic disorder. The most logical way to present these studies from different laboratories is not in order of discovery, but to start from the most basic *in vitro* observations, and then to work through model systems and into the human diseases. Briefly, I will discuss some other observations that may or may not constitute evidence for proteasomal dysfunction in this disease.

2. α-SYNUCLEIN INHIBITS PROTEASOME FUNCTION *IN VITRO*

α-Synuclein is an unusual, although not unique, protein in that it has a natural tendency to self-aggregate (reviewed in 6). As this property is enhanced by mutations that change amino acids and by increasing concentration of α-synuclein, it is thought that aggregation is the underlying reason why this protein becomes toxic. As the pathology in cases with α-synuclein mutations overlaps with typical, idiopathic PD, it is assumed that α-synuclein aggregation is also important in sporadic disease. The sequence motifs within the protein that lead to this property are relatively well established, as is the major pathway for aggregation (6). The aggregation of α-synuclein involves a nucleation event and the initial formation of smaller oligomeric species that subsequently form larger aggregates called fibrils (Figure 2). Lewy bodies contain fibrillar α-synuclein and thus are linked to relatively late stages of protein aggregation.

One might reasonably assume that any property of α-synuclein that is enhanced by aggregation might contribute to toxicity of this protein seen in PD or experimental models. There have been several recent studies suggesting that recombinant α-synuclein can have an inhibitory effect on net turnover of the proteasome in entirely *in vitro* systems (7,8). In most of these cases, it is aggregated protein that has the strongest inhibitory effect. For example, monomeric α-synuclein inhibits the proteasome at high micromolar concentrations but α-synuclein that has been pre-aggregated to form fibrils is inhibitory in nanomolar amounts (7). Similarly, oligomeric α-synuclein is also an effective proteasome inhibitor (8). These results imply that when α-synuclein starts to aggregate it can prevent proteasomal turnover of other substrates.

Critically, these studies show that α-synuclein alone is *sufficient* to induce proteasome inhibition and the mechanism does not have to involve other cellular components (see below). What is the exact mechanism? There is suggestive evidence that aggregated α-synuclein binds to S6' (also known as Rpt5), a component of the 19S regulatory cap that is involved in the control of substrate entry into the catalytic core of the proteasome. This is consistent with an earlier report of binding of α-synuclein to the rat homologue of the same protein (9).

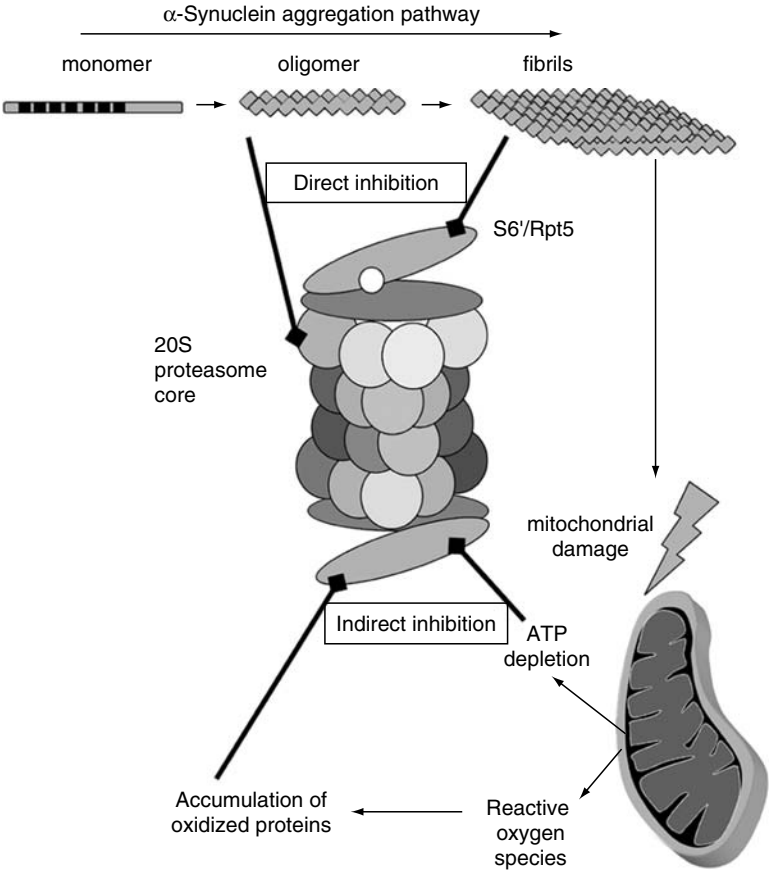

Figure 2. Direct and indirect mechanisms for α-synuclein-mediated inhibition of the proteasome.

In contrast, α-synuclein filaments can bind to purified 20S proteasome particles implying an interaction with the core of the proteasome as well as with the cap. These two sites of action, assuming both are equivalent, suggest that α-synuclein will have effects on several types of proteasome-mediated protein degradation. This is consistent with the observation by Snyder *et al.* (7), that both ubiquitin-dependant and ubiquitin-independent proteasome degradation are impaired by aggregated α-synuclein.

Very recently, Snyder *et al.* have shown that β-synuclein can antagonize the effects of α-synuclein on proteasomal inhibition (10). β-Synuclein is a homologue of α-synuclein that is relatively similar for most of its sequence but is resistant to aggregation. Adding β-synuclein to α-synuclein slows aggregation of the latter, making it a candidate for a natural neuroprotective protein (reviewed in 11). Showing that β-synuclein antagonizes α-synuclein effects on the proteasome strengthens the argument that aggregation is key to the detrimental effects of α-synuclein.

Therefore, aggregated α-synuclein binds to one or more components of the proteasome and inhibits its activity. A minimalist hypothesis would be that α-synuclein is a proteasome substrate and, when aggregated, becomes resistant to degradation thus blocking proteasome function in a competitive mode. There is data that α-synuclein can be degraded in an ubiquitin-independent manner *in vitro* (12-15), supporting this concept. However, as discussed below, data from intact cells is somewhat confusing over whether the majority of α-synuclein is degraded by the proteasome. The possible interaction with S6' might additionally indicate a non-competitive mode of inhibition. Making the distinction between different sites of binding and mode of inhibition will be important in the future for understanding which UPS-mediated protein degradation pathways are most critical for the development of PD and other diseases.

3. α-SYNUCLEIN AND THE PROTEASOME IN CELLULAR AND ANIMAL MODELS

Increasing the complexity of the systems, several groups have also reported that α-synuclein affects proteasome function in intact cells. Two dominant mutations, A30P (16) and A53T (17), have been shown to produce small decreases in the enzymatic activity of the proteasome assessed using fluorogenic substrates, and in both of these studies wild type α-synuclein had no measurable effect. We subsequently confirmed these observations using the artificial GFPu reporter construct (18), which demonstrates that both mutations slow overall proteasome-mediated protein turnover (19). The effects are often quite modest in stably transfected cell lines. Inducible expression of A30P α-synuclein produces a 20-25% suppression of all three protease activities of the proteasome, whilst stable expression of A53T α-synuclein lowers chymotrypsin-like activity by 25-35% (17). Although small, it is reasonable to expect that low level chronic inhibition might produce important effects on the cell. There is an additional mutation in α-synuclein, E46K (20), that has not yet been tested for its ability to inhibit the proteasome. However, as described above for the *in vitro* studies, aggregation appears to be responsible for the inhibitory actions of α-synuclein on proteasome function, and not the presence of the mutation *per se*. Overexpresion of wild type α-synuclein has been shown to inhibit the trypsin-like activity of the proteasome (21). Although not directly tested, one assumes that the expression levels were high enough to promote aggregation.

Generally, the overexpresion of α-synuclein (especially mutant forms) increases the sensitivity of transfected cells to proteasome inhibition (16,19,22). Whether this is supportive of the concept that proteasome inhibition mediates all or part of the toxicity associated with α-synuclein is not completely clear. Although it is tempting to speculate that the altered sensitivity in these cells results from a chronic effect of decreased proteasome activity, proteasome inhibitors can increase sensitivity to several toxins (see below) and therefore specificity has yet to be fully determined.

At the time of writing there has been only one study of the direct effects of forced over-expression of α-synuclein in the intact CNS (23). In this study, all three proteasomal enzyme activities were similar in wild type mice and in mice

transgenic for A30P mutant α-synuclein. The model includes neuritic pathology and astrogliosis without loss of nigral neurons (24). These authors also examined the steady state levels of well-characterized proteasome substrates and the expression patterns of protein components of the proteasome (23). Such *in vivo* data might suggest that many of the observations from *in vitro* systems have little or no relevance to the *in vivo* situation. However, one difficulty in comparing the different models is that it is hard to assess the aggregation state of α-synuclein in these situations. As Martin-Clemente *et al*. have pointed out, it is not merely the presence of α-synuclein that causes damage, as the protein probably needs to aggregate as well (23). The aggregation is most often and most obviously seen in human brain. Recently, we have seen increased expression of this α-synuclein in blood samples from patients with increased gene dosage but only saw aggregation and deposition into insoluble fractions in brain samples from patients with the same mutation (25). If the *in vitro* results were correct, then proteasome inhibition would only be expected to occur after α-synuclein is aggregated. There are animal models of both transgenic (26,27) and toxin-induced (28) α-synuclein aggregation, and hence it will be of interest to see if proteasome inhibition occurs in these models. There is already some evidence that toxins that induce α-synuclein aggregation also produce proteasome inhibition in cellular models (see below).

As discussed for *in vitro* data, there is some confusion about whether α-synuclein is a proteasome substrate in intact cells. Whilst early reports suggested that α-synuclein degradation is sensitive to the proteasome inhibitor MG132 (29), the concentrations used were rather high and subsequent efforts failed to replicate this finding (19,30). Instead, most of the pool of α-synuclein within the cell is sensitive to lysosomal inhibitors (31) and is degraded by chaperone-mediated autophagy (32), a process that is also inhibited by mutant forms of α-synuclein. Some studies have argued that both autophagy and proteasomal degradation of α-synuclein can occur in some cell types (33). Further complicating the picture, some promoters used to drive over-expression in transfected cell lines may also be sensitive to proteasome inhibitors (34), thus giving potential false positives of apparent increases in protein when only steady state levels, not protein turnover, are measured. Synphilin, a proposed α-synuclein interacting protein, is a more convincing proteasome substrate (35,36).

4. PROTEASOME FUNCTION IN HUMAN LEWY BODY DISEASES

There is also evidence of proteasome dysfunction in human diseases that prominently include α-synuclein pathology. McNaught and Jenner were the first to demonstrate that proteasome activity is lower in the substantia nigra of *post-mortem* tissue taken from PD patients compared to controls (37). A difficulty with this study is that tissue from the substantia nigra was used. One can argue that this is the most relevant tissue as it is the region of the brain that is most prominently affected in PD. However, because of loss of neurons and the presence of reactive gliosis, the cellular composition changes between control and disease. We don't know if the activity of the proteasome varies between neurons and glia and thus it is difficult to control for this potential confound.

In an attempt to address this problem, McNaught and colleagues have shown that the protein expression of several individual proteasome subunits is decreased in diseased neurons. Using immunocytochemical staining, they found lower staining for α-subunits of the proteasome core in neurons from PD patients compared to controls. Intriguingly, nigral neurons have lower amounts of two proteasome activators (PA28 and PA100) compared to other brain regions (38). The implication is that the substantia nigra, where there is both α–synuclein aggregation and cell loss, is particularly vulnerable to proteasome inhibition, an idea that has been tested by several groups and is discussed below.

In Diffuse Lewy body disease (DLBD), α-synuclein-positive Lewy pathology is found throughout the brainstem and in several areas of the cortex (Figure 1). Measurements of proteasome function in DLBD reveal that, like PD, activity is decreased in the substantia nigra, but not in those cortical areas that are affected by pathology (39). These same areas showed decreased proteasome function in Alzheimer's disease samples. Other studies of PD have also failed to show decreased proteasome function in areas not affected by neuronal loss in this disease (40). Therefore, decreases in proteasome function are not tightly correlated to the presence of Lewy body pathology, but are correlated to areas vulnerable to neuronal loss in several diseases including PD. One possibility is that proteasome inhibition might be related to cell death rather than a direct consequence of α-synuclein accumulation. For example, it has been shown recently that activation of apoptotic cascades can decrease proteasome activity (41).

It is worth noting that α-synuclein is not the only aggregating protein that has the ability to inhibit UPS function. For example, mutant huntingtin, an expanded polyglutamine protein associated with Huntington's disease (HD), also inhibits net proteasomal function in transfected cell lines (18). There is evidence for decreased proteasome function in *post-mortem* samples from HD brains compared to controls (42). Similarly, mutant forms of Cu/Zn superoxide dismutase associated with amyotrophic lateral sclerosis (ALS) can inhibit proteasome activity in cell lines (43). Some of this data will be discussed in other chapters of this book. The main point is that proteasome inhibition is likely to be a common property of aggregating proteins and is probably not specific to α-synuclein and disorders like PD. Whether there are subtleties in the types of inhibition produced by different proteins has not yet been addressed and would be an interesting concept to study in the future.

5. PROTEASOME INHIBITION AND SELECTIVE NEURONAL LOSS

These considerations show that whilst α-synuclein in an aggregated form can cause decreased proteasome function, this is not a unique property of this protein and could be related to relatively downstream events that occur long after aggregation. Therefore it is critical to consider whether decreased proteasome activity is a *necessary* part of the process that leads to cell loss in PD. Several *in vitro* studies have shown that dopaminergic neurons from cultured from the midbrain are more sensitive than non-dopaminergic cells in the same preparations. For example, in our laboratory tyrosine-hydroxylase positive neurons were more sensitive to proteasome inhibitors in cultures made from the postnatal

mouse ventral midbrain (19). Similar results have been reported in other studies (34,44) although the opposite result, *i.e.* that dopaminergic neurons embryonic rat midbrain were less sensitive than non-dopaminergic cells, have been reported in one study (45).

Several recent studies have addressed the question of whether the neurons affected in PD really are more vulnerable to proteasomal inhibition *in vivo*. Rats exposed to the relatively specific proteasome inhibitor lactacystin by intranigral injection show loss of nigral neurons with inclusion body formation as well as slowed movement which may relate to some of the clinical symptoms of PD patients (46). The structurally unrelated inhibitor epoximicin damages the nigra relatively specifically and produces α-synuclein and ubiquitin-positive fibrillar inclusions in rats when infused into the striatum (47). More recently, intraperitoneal injections of the lipophilic proteasome inhibitors epoximicin or PSI have been used to provide a picture of neuronal loss that is claimed to be representative of the complex patterns of cellular damage seen in PD (48). It is interesting that cell bodies of neurons in the striatum (for HD) and spinal cord (for ALS) were not affected. Although we are awaiting replication of this result, if it is robust it would indicate that dopaminergic neurons of the substantia nigra truly are more vulnerable than other cells of the brain to proteasome dysfunction.

An interesting observation in the studies of McNaught and colleagues (48) is that exposure to proteasome inhibition in many brain regions does not produce lasting decreases in proteasome activity. In this chronic paradigm, proteasome activity rebounds to about twice that seen basally in many brain areas after two weeks of treatment with proteasome inhibitors (48). In contrast, in the lower brainstem and ventral midbrain, proteasome activity did not return to normal and remained below control levels. Again, this strengthens the view that the substantia nigra and related areas in the lower brainstem have a decreased capacity to withstand chronic, low-grade proteasome inhibition.

A more complex view of the same process is that proteasome inhibition may increase sensitivity to other toxic damage. Proteasome inhibitors have been shown to act synergistically with mitochondrial complex I inhibitors (MPP^+ and rotenone) in cultured cells (49), although some studies have suggested that proteasome inhibitors do not add to MPP^+ toxicity but ameliorate it (50). The possibility that proteasomal and mitochondrial stressors have additive effects may have a parallel in HD, where proteasome inhibition occurs in several brain regions but cell loss only occurs in regions where decreases in proteasome activity and mitochondrial complex II activity occur in tandem with decreased BDNF levels (42). Proteasome inhibition also increases cellular sensitivity to oxidative toxins including 6-hydroxydopamine (51), unfolded protein stress and depletion of glutathione (44). We have shown that proteasome inhibitors exacerbate the toxicity of mutant α-synuclein *in vitro* (19). Therefore, proteasome inhibitors generally have an additive effect with other stressful stimuli. I have never been clear whether this tells us much about PD; perhaps any two toxic stimuli can add together. However, it does indicate that if UPS dysfunction is present along with other types of stress (oxidative damage, mitochondrial dysfunction), then both might be important even if neither is really enough to kill the cell.

Adding another layer of complexity, these different pathways that might contribute to cell damage have been shown to be interactive. Thus, mitochondrial abnormalities can result from the application of proteasome inhibitors (52-54). Furthermore, several studies show that the proteasome is sensitive to mitochondrial toxins MPP^+ (21,55) and rotenone (56). Recent data shows that chronic infusion of MPTP into the rat brain produces proteasome inhibition (57). These effects might be mediated by ATP depletion, as the proteasome is heavily ATP dependent, but are also possibly mediated by oxidative stress (58). Dopamine is reported to be required for the toxicity of proteasome inhibitors to the substantia nigra in vivo (47). Some of the possible direct and indirect pathways that might result in proteasome inhibition are summarized in Figure 2.

The summary of the above evidence is that nigral neurons appear to have a lower capacity to survive proteasome inhibition, perhaps related to their lower starting proteasome activities. However, the interpretation of this data is complicated by the fact that dopaminergic nigral neurons have also been shown to be sensitive to a number of additional stresses, dopamine and mitochondrial damage being the most commonly used, which may interact with UPS dysfunction within the intact cell. Therefore, it is difficult to genuinely separate these different pathways and assign primacy to any of them.

6. OTHER EVIDENCE

One piece of evidence often quoted to support the concept that dysregulation of the UPS is important in PD is the observation that Lewy bodies are positive for ubiquitin, the small protein that labels targets for proteasomal destruction. Unfortunately, it is not entirely clear whether this is evidence of a primary deficit in the UPS or is a more generalized response to protein aggregation. Some pathologists would argue that ubiquitin accumulation in neurodegenerative diseases reflects a secondary effect that represents an attempted protective response of the cell (59). Supporting this contention, detailed temporal studies of an animal model of α-synuclein deposition reveal that ubiquitin addition occurs after deposition of α-synuclein into insoluble fractions. Not all inclusion bodies were ubiquitin positive, arguing that ubiquitin is not required for inclusion body formation. Aggregated α-synuclein tends to be mono- or di-ubiquitylated (39,60) rather than polyubiquitylated.

Lewy bodies have been shown to contain a number of components of the UPS. Furthermore, these inclusions are also positive for proteins involved in the unfolded protein response, including pancreatic protein disulfide isomerase (61). Such components can also been seen in experimental models of inclusion body formation, such as chronic methamphetamine toxicity (62), mitochondrial cybrids (63) or exposure to proteasome inhibitors (64). Whether inclusion formation is beneficial or detrimental to neuronal cell survival is controversial. There have been suggestions that by sequestering relatively soluble toxic proteins into one area of the cell, a neuron may be attempting to minimize α-synuclein toxicity (65). Supporting this idea, Lewy bodies contain a number of markers that are also characteristic of aggresomes (66), a cellular response to the presence of misfolded proteins where aggregates are centralized to the perinuclear area by

an active, transport-dependent process (67,68). However, it is also possible that inclusion bodies are detrimental to neuronal function, as the presence of fibrillar protein aggregates may interfere with processes such as intracellular transport or sequester other critical cellular components away from their site of action (69). Chapters 3, 4 and 5 in this volume discuss in more detail the role of inclusions in cell survival and death.

Another suggestive piece of evidence for a central role of UPS failure in PD is the report that an I93M mutation in an ubiquitin hydrolase, UCHL1, is associated with familial PD (70). Although no other mutations have been found, association studies showing a protective effect of a common variant of the same gene (S18Y) on the risk of developing sporadic PD have been convincingly replicated (71). The difficulty with the UCHL1 story is that the initial family with the I93M mutation is rather small and it is hard to be certain that this is truly a causal mutation. This controversy has been eloquently discussed by Healy and colleagues, who argue that the role of UCHL1 should be considered with great caution (72). Having given this *caveat*, it is interesting that one report suggests that UCHL1 may have a direct effect on α-synuclein protein levels (73). The subject of UCH-L1 and its possible relationship to PD is discussed in more detail in Chapter 11 of this volume.

Finally, two additional genes for recessive Parkinsonism have both been reported to protect cells against damaging effects of proteasome inhibition. Knockdown of DJ-1 increases sensitivity to MG132 in one study (74), although we have not yet been able to replicate this result (M.R. Cookson, unpublished observations). PINK1 can prevent MG132-induced decreases in mitochondrial function, an effect that is specific to the wild type protein and lost with recessive mutations (52).

7. CONCLUSIONS

The evidence discussed in this chapter suggests that the proteasome probably has a role to play in diseases including PD that are characterized by α-synuclein aggregation and pathology. What is not clear is whether the UPS plays a truly central role. On this more important issue, evidence is fragmentary. Whilst it is possible to argue that dopaminergic neurons of the substantia nigra, and other important neuronal groups affected in PD, are selectively vulnerable to UPS dysfunction, the same arguments have been made for other affected neuronal groups in different neurological diseases. Perhaps the most critical experiment, to establish whether equivalent chronic inhibition of the proteasome throughout the CNS leads to PD-like symptoms (48) has been attempted with positive results, although the portability of this observation to other laboratories has not yet been established.

One might argue that this is a churlish objection: if the UPS is involved at any level in this disease then it represents a drug target for the future. I would add to this that if the UPS is critical for the development of multiple neurological diseases the one might be able to find therapeutic opportunities for several of them. As some of these diseases (ALS, for example) represent relatively small markets for pharmaceutical development, drugs that are useful in many of them

might be economically viable. To my mind, the question comes down to when UPS dysfunction occurs in the disease. If it is a relatively early event, prior to the time that neurons are committed to becoming dysfunctional and eventually to cell death, then this is a good target. However, if it were an epiphenomenon or an event that occurs after the cell is committed to cell death, then the UPS would not be the most attractive target. Careful studies of the timing of UPS dysfunction in PD, probably using animal or cellular models, as well as more detailed descriptions of the type of damage will be required before making this decision.

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13

UBIQUITINATION BY PARKIN – IMPLICATIONS IN PARKINSON'S DISEASE

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1. INTRODUCTION

The ubiquitin-proteasome system (UPS) is critical in maintaining cellular protein levels by targeting unwanted proteins for degradation in the cell. Disruptions in the UPS, due to mutations in the proteins involved in the process, or to a cellular state that burdens the UPS due to excessive clearance of unwanted proteins, termed "proteolytic stress", have been recently implicated in the etiology of numerous neurodegenerative diseases, including Parkinson's disease (PD) (1,2).

Parkinson's disease, first described by James Parkinson in 1817, is a movement disorder that affects 1 to 2% of individuals over the age of 60 (3,4). Clinically characterized by bradykinesia (slowness of movement), tremors, rigidity and postural instability, PD greatly shortens life, while causing disability during life. The most consistent risk factor for PD is age; the increasing age of the general population increases the prevalence of PD (5). The striking pathological features of PD include progressive loss of dopaminergic neurons

from the substantia nigra pars compacta and other brain regions, along with the presence of neuronal processes (Lewy neurites) and protein inclusions (Lewy bodies) (6).The distinct pattern of cell loss correlates with a loss of striatal dopamine and loss of the dopamine transporter, resulting in movement dysfunctions (7).

Compelling evidence for protein mishandling in the pathogenesis of PD has come from the presence of ubiquitin-positive Lewy bodies in neurons of diseased patients, as well as the identification of disease-causing mutations in an E3 ubiquitin ligase, *parkin* (8). Lewy bodies are spherical, 8-30 μm in diameter, intracytoplasmic eosinophilic inclusions that have an intense core with a peripheral halo of radiating fibrils (9). While they have classically been a defining feature of PD, Lewy bodies have also been reported in other neurological disorders, including dementia with Lewy bodies (DLB), the Lewy body variant of Alzheimer's Disease, and Down's syndrome (10). Furthermore, recent studies suggest that not all forms of PD, especially those associated with mutations in *parkin*, have Lewy bodies (11). Although they have been described for almost a century now, the mechanisms by which these inclusions form and their role in neurodegenerative diseases remains unknown. Initially thought to be the cytotoxic factor responsible for the death of dopaminergic neurons in PD, there is growing evidence that these Lewy bodies may not be deleterious to cells, but may in fact be neuroprotective by sequestering toxic proteins, including abnormal/misfolded proteins that could cause proteolytic stress (10). Recent evidence highlighting a potential role for lysine-63 (K63) ubiquitin linkage in the formation of Lewy body-like inclusions further ties the ubiquitin system to PD (12).

While the molecular mechanisms leading to the onset of PD remains elusive, the observed pathological and genetic factors indicate that disruptions in the UPS may be a potential contributor to the degenerative process. Hence an insight into the mechanisms by which the cellular homeostasis of critical proteins goes awry in the cell and the role of parkin and the UPS in this process will be important in understanding the pathways underlying the pathogenesis of PD. In this chapter we review the current literature on the role of parkin in the pathogenesis of PD, and the growing link between the ubiquitin system and neurodegeneration.

2. PARKIN IN THE GENETICS OF PARKINSON'S DISEASE

Until about a decade ago, PD was thought to have little or no genetic component. However, the identification of several genes for monogenically inherited forms of the disease, although rare, and accounting for only 5-10% of the cases, has accelerated the study of molecular pathways leading to PD (13). Mutations in at least five genes have been strongly linked to autosomal dominant or recessive forms of PD, including *α-synuclein*, *parkin*, *DJ-1*, *PTEN-induced kinase 1* (*PINK1*), and *Leucine-rich repeat kinase 2* (*LRRK2*) (14-19). So far only two of these genes – *α-synuclein* and *parkin* – have been connected to UPS dysfunction.

Immunohistochemical data show that α-synuclein aggregates may be a principal component of Lewy bodies in idiopathic and genetic forms of PD (20,21). Thus if Lewy bodies are structures that segregate unwanted and potentially toxic proteins, then α-synuclein accumulates in Lewy bodies either because it is insufficiently degraded due to UPS dysfunction or mutations in α-synuclein

result in misfolding or excessive production leading to proteolytic stress (22). These observations and implications to PD are discussed in detail in Chapter 12. Parkin, on the other hand, is more directly related to the UPS. Parkin functions as an E2-dependent E3 ubiquitin ligase, catalyzing the addition of ubiquitin molecules to specific substrate molecules (23-25). Mutations in *parkin* have been linked to an autosomal recessive form of juvenile parkinsonism (AR-JP), a distinct clinical entity from sporadic PD in spite of overlapping clinical symptoms (15,26).

An early onset form of PD, AR-JP is characterized by the selective loss of dopaminergic neurons in the substantia nigra and locus coerulus, usually with the absence of Lewy bodies (11,27,28). While sporadic PD and AR-JP have common characteristics such as dystonia, sufficient response to levodopa, lack of dementia and classic parkinsonism symptoms, the two forms can be separated based on sleep benefit for parkinsonian symptoms, female predominance, retropulsion, dystonia of the feet, hyperreflexia and pathological findings (26). Some PET studies have shown similar patterns of metabolism between sporadic and parkin-liked PD patients (29,30). However, Portman et al performed PET studies on AR-JP patients with mutations in the *parkin* gene and noted a marked reduction in fluorodopa uptake in the caudate nucleus and cerebellum, indicating a different nigrostriatal dopaminergic pattern from sporadic PD patients and a distinct pathophysiology for AR-JP (31). The same study concluded that the cerebral energy metabolism in AR-JP patients was comparable to sporadic cases. Another PET study corroborates the difference in dopaminergic dysfunction between sporadic and parkin-linked PD patients, suggesting a more severe synaptic disruption for parkin-linked patients (32). The heterogeneity in clinical symptoms and neuropathology of parkin-associated PD cases is further complicated by marked variation observed in the age of onset (33,34).

Linkage analysis of several Japanese families with ARJP localized the causative gene of this most common form of familial PD to a locus on chromosome 6, *PARK 2* (35). Since the initial identification of large deletions in *parkin* associated with ARJP, a multitude of mutations have been identified, including deletions of single or multiple exons, duplications or triplications of exons, frame shift mutations, and point mutations (36). Numerous *parkin* mutations occur as homozygous or compound heterozygous mutations; however, in spite of extensive screening, several published cases appear to have only one of the alleles mutated (37,38). While this could reflect the insensitivity of the genetic analyses, especially with a locus as large and complex as *PARK2*, it is also likely that haploinsufficiency of parkin due to loss of one copy of the gene constitutes a risk factor for the onset of PD. Such heterozygous mutations may lead to disease when coupled with environmental conditions, such as oxidative or nitrosative stress, or may be a toxic gain-of-function mutant or have a dominant negative effect (39-41).

3. THE PARKIN PROTEIN

Parkin, a 465 amino acid protein, is a RING-type E3 ubiquitin ligase and is composed of three parts – an N-terminal ubiquitin-like domain, a C-terminal RING-finger box (with two RING finger domains separated by an In-Between-Region), and a linker SH2-like domain (15,23-25). E3 ubiquitin ligases confer substrate specificity to the UPS while acting as scaffolding proteins. They interact with

a cognate E2 and a specific substrate to facilitate the transfer of activated ubiquitin from E2 to the substrate (42). Parkin appears to use both UbcH7 and UbcH8 as its E2 ubiquitin conjugating enzyme, in addition to the ER-associated E2s, Ubc6 and Ubc7 (23,24,43).

The speculation that parkin's loss of function as an E3 ligase results in the accumulation of a substrate that is not properly targeted for degradation by the UPS, leading to cellular dysfunction and ultimately the selective susceptibility of dopaminergic neurons, initiated the quest for parkin substrates. Parkin catalyzes its own ubiquitination, although it is unclear if auto-ubiquitination of parkin is the mechanism by which parkin is degraded in the cell (23,25,44,45). In addition, parkin catalyzes the ubiquitination of several substrates – the synaptic vesicle-associated septins CDCrel-1 and CDCrel-2; the α-synuclein interacting protein, synphilin-1; the putative G protein-coupled parkin-associated endothelin-like receptor (Pael-R); the microtubule proteins, α- and β-tubulin; the cell cycle protein, cyclin E; the p38 subunit of the aminoacyl-tRNA synthetase complex; the vesicle-docking protein synaptotagmin XI; and the dopamine transporter, DAT (23,43,46-54). Another putative substrate, an O-glycosylated form of α-synuclein, has been identified but its relevance in parkin-associated PD is questionable since the data has not been reproduced by other labs (51). None of these substrates appear to accumulate in parkin-null mice, thus it is unclear whether any of them are involved in the pathogenesis of PD due to parkin mutations (55-58). Since E3s regulate the turnover of the extensive and diverse target proteins in the cell, they are thought to associate with at most one or two critical substrates. The large number of identified putative parkin substrates is somewhat surprising and awaits further clarification, but the unique specificities may be attributed to the variety of distinct complexes and proteins that parkin interacts with in the cell.

Primarily localized to the cytoplasm, parkin is widely distributed in different regions of human and rodent brains (59,60). Two independent studies in rat brain reported the localization of parkin to the synapse and, along with the reported synaptic vesicle-associated proteins as parkin substrates, add a new dimension to its function. While one study described parkin immunoreactivity around synaptic vesicles in the pre-synaptic axons of rat brain, another study reported parkin association with synaptic plasma membrane and co-localization of parkin with lipid rafts in post-synaptic densities (61,62). Parkin also functions in a multi-protein ubiquitin ligase complex with components of the SCF-ubiquitin ligase complex, hSel-10 and Cullin-1, to act as a scaffold to bring all the necessary components of the UPS together (49). It can interact with Hsp70 and proteasome, thus linking parkin, the UPS and chaperone systems in the turnover of proteins (52,63). Further, parkin's E3 ligase activity has been shown to be modulated by interaction with CHIP, an E3/E4 ubiquitin ligase, as well by post-translational modifications, such as S-nitrosylation and phosphorylation (39,63,64).

4. PARKIN IN PD

The lack of Lewy body pathology in parkin-associated PD is widely accepted, except in one autopsy case (11,28,37,65,66). However, the presence of proteinaceous aggregates in the form of neurofibrillary tangles or α-synuclein- and

ubiquitin-positive inclusions in neuropils have been reported in some cases (11,65,67). Although somewhat controversial, some groups have shown that Lewy bodies in sporadic PD patients stain positive for parkin, suggesting that Lewy bodies could isolate parkin from its normal function in the cell, implicating a role for functional parkin in the formation of these characteristic protein inclusions (68,69). This observation is challenged by a report that does not observe co-localization of parkin with α-synuclein in Lewy bodies in α-synucleinopathies, using different antibodies to parkin (60).

The lack of Lewy bodies in parkin-linked PD could mean that parkin function may be necessary for the formation of Lewy bodies or that the mechanism of neurodegeneration in parkin-related AR-JP is different from idiopathic PD. This has led to the speculation that Lewy bodies may in fact be a normal defense mechanism in the cell, resulting in the sequestration of poorly degraded cytotoxic proteins, and thus delay the onset of neuronal degeneration. Further, the formation of inclusions in a cell is a concentration-dependent self-driven process, and represents a normal cellular response when the capacity of the UPS is exceeded by the need to eliminate aggregation-prone misfolded and normal proteins (70). This phenomenon is further described in Part 2 of this book. In the case of parkin-related PD, a loss-of-function mutation results in a disruption of parkin's E3 ubiquitin ligase activity, affecting the UPS and leading to the accumulation of ubiquitinated and non-ubiquitinated substrate(s) in the cytoplasm that may be selectively lethal to dopaminergic neurons. Thus the absence of Lewy bodies may lead to rapid neurodegeneration and early onset PD in the form of ARJP.

There is evidence in cell cultures that diminished cellular proteasomal activity results in an accumulation of over expressed parkin in large non-toxic cytoplasmic aggresome-like structures (71,72). Compartmentalization into these structures would preclude parkin from its normal enzymatic activity, resulting in the accumulation of substrates and cell death by the mechanism described above. It is therefore plausible that the impaired proteasome function observed in the substantia nigra in idiopathic PD could impair parkin clearance and induce its aggregation into Lewy bodies (73). Proteasome dysfunction in the cell may be caused by, among other reasons, protein aggregation and the normal aging process (74-76). Thus even a subtle compromise in the function of the UPS could result in the selective vulnerability of neurons by numerous mechanisms, leading to the degeneration observed in PD.

The study of parkin's role as a protective factor against cellular stresses has yielded some interesting findings. In cell lines and primary neuronal cultures, wild type parkin overexpression protected the cells from toxicity associated with proteasome inhibition – an effect that was not observed with the parkin mutant, R42P (77). Thus parkin may protect by modifying the substrate(s) that accumulate under these conditions in the cell. Parkin has also been shown to be protective in other models of cell death, such as ER stress, ceramide-induced mitochondrial-dependent cell death, kainate-induced toxicity, and α-synuclein- and Pael-R-induced toxicity in *Drosophila* (25,49,78,79). Parkin's potential to be protective under multiple stress conditions makes its manipulation in the cell a good potential target for therapeutics in PD.

Since loss of parkin function is associated with onset of disease, several animal models deficient in the parkin protein have been created (56-58,80-82). While none of the models developed robust parkinsonian phenotypes, a *Drosophila* transgenic model demonstrates locomotor defects and reduced lifespan with degenerating muscle and mitochondrial pathology (80). Two mouse models suggest a role for parkin in the nigral dopaminergic system, since parkin-null mice have reduced levels of dopamine transporter, DAT, and increased extracellular dopamine in the striatum, resulting in disrupted synaptic function (56,58). This is further supported by the identification of DAT as a substrate for parkin and the localization of parkin to the synapse (54,61,62); thus a loss of function mutation in *parkin* could result in an accumulation of DAT, causing a disruption of the dopaminergic pathway. Two-dimensional gel electrophoresis followed by mass spectrometry resulted in the identification of several mitochondrial-related proteins that were downregulated in one parkin-null mouse model (83). These mice had dysfunctional mitochondria and increased oxidative stress, that could result in increased toxicity, especially in the presence of elevated extracellular dopamine. One of the parkin-null *Drosophila* models also shows an increased sensitivity to oxidative stress (82). One of the parkin-null models showed loss of catecholaminergic neurons in the locus coeruleus, a commonly degenerating brain region in PD patients, with a concomitant loss of norepinephrine in specific brain regions (57).

Parkin-associated PD has been potentially linked to the more common sporadic form in the recent study on S-nitrosylation of parkin under conditions of oxidative and nitrosative stress in the cell (39,84). S-nitrosylation of parkin inhibits both its E3 ligase activity as well as its ability to protect against cellular stresses, suggesting a role for NO modification in proteasomal dysfunction observed in PD. This finding along with another report suggesting that reactive oxygen species inactivates parkin provides a potential explanation for the pathogenicity of heterozygous parkin mutations (85). Thus, increased oxidative stress conditions may be further augmented by reduced levels of parkin, resulting in a vicious cycle of parkin inactivation. More recently, parkin has been shown to interact with and be modulated by bcl-2-associated athanogene 5 (BAG5) (86). Following injury, BAG5 inhibits the activity of parkin and Hsp70 and specifically sensitizes dopaminergic neurons to death (86). Age- and mutation-mediated differences in extractability of parkin from brain tissue and the increased propensity of some parkin mutants to form inclusion-like structures in cells raises important questions about the availability of the protein for its normal function in the cell, supporting a haploinsufficiency model for some *parkin* mutations (60,87). Thus loss of parkin function – by mutation, S-nitrosylation or modulation by proteins such as BAG5 – contributes to dopamine-specific neurodegeneration through a mechanism that is yet to be elucidated (Figure 1).

The mild phenotypes observed in parkin knockout mice and the lack of accumulation of known substrates in these mice suggests that there may be an alternate mechanism by which mutations in *parkin* cause disease (56-58). There is increasing evidence to hypothesize a second, non-proteasome-related, ubiquitin ligase function for parkin in the cell that may be involved in the pathogenesis of PD. Poly-ubiquitin chains via Lysine-48 (K48) signal proteasomal proteolysis,

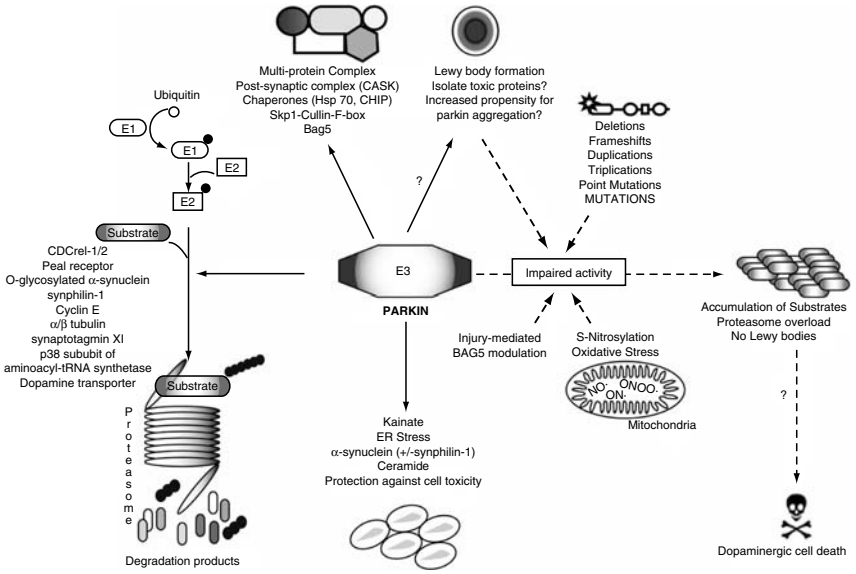

Figure 1. Model for Parkin-associated PD related to the UPS. Normally (solid lines), parkin functions as an E3 ubiquitin ligase and targets substrates to the proteasome for degradation. In addition, parkin protects from cell toxicity induced by various agents and interacts with several multi-protein complexes. However, in the diseased state (dashed lines), parkin's function as an E3 ligase may be attenuated due to various combinations of factors, including mutations and abnormal environmental conditions like oxidative/nitrosative stress and injury, causing UPS dysfunction that leads to the accumulation of toxic substrates in the cell, leading to neurodegeneration. Proteasomal stress could further potentiate parkin aggregation resulting in the formation of Lewy bodies and sequestering parkin away from its normal function.

while K63-linked ubiquitin chains signals distinct processes, such as DNA repair, downstream cell signaling and endocytosis (88). It has been demonstrated that parkin can mediate K63-linked poly-ubiquitin chains, in addition to the classically known K48 chains (12,89). Parkin promotes the formation of Lewy body-like protein inclusions with α-synuclein and synphilin-1 that are predominantly K63-ubiquitinated (12). While this recent data expands the scope of ubiquitination-mediated pathogenesis, the relevance of this unique dual-function property of parkin in protein inclusion formation and PD needs to be further explored.

5. CONCLUDING REMARKS

Although the failure of the UPS has been implicated in both sporadic and familial forms of PD, it is unclear as to whether it is involved in the initiation or progression (or both) of the disease. It is hypothesized that mutations in parkin impair its normal ubiquitination activity, leading to an accumulation of proteins that overload the UPS and result in the selective death of neurons in the substantia nigra by a mechanism that is largely unknown. The paucity of Lewy bodies and therefore a lack of normal cellular defenses against excess levels of toxic proteins may manifest as early onset and severe neurodegeneration in parkin-associated

PD. The pathogenic connection between the ubiquitination pathway and parkin-related PD has been established – the thrust should now be on understanding the mechanisms that cause and contribute to the development of disease.

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14

THE UPS IN NEURODEGENERATIVE DISEASES AND AGING
HUNTINGTON'S DISEASE

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1. CLINICAL AND NEUROPATHOLOGICAL HALLMARKS OF HUNTINGTON'S DISEASE

Huntington's disease (HD) is a devastating autosomal dominant neurodegenerative disorder caused by a CAG triplet-repeat expansion coding for a polyglutamine (polyQ) sequence in the N-terminal region of the huntingtin (htt) protein (1). The causing mutation is fully penetrating but age of onset and clinical manifestations may vary considerably amongst mutation carriers. Typically, patients suffer from motor dysfunction, cognitive decline and psychological disturbances striking at about age 40 and lasting over 10 to 15 years until death. The substantial variation in age of onset, severity of symptoms and interval to death depends, in part, on the length of the polyglutamine stretch (2). Normal population has 36 or fewer CAG repeats, while individuals with 40 or more repeats develop HD. In most patients, the number of CAG repeats is about 40–50, leading to disease onset in mid-life. By contrast, individuals with very long polyglutamine stretches – in excess of 70 glutamine residues – develop a juvenile form of the disease that progresses more rapidly.

Reported prevalence of living affected cases varies considerably amongst different geographic regions mainly due to founder effects. The prevalence of the condition in Caucasian populations may reach as high as 10 per 100,000. Because many gene carriers are yet to develop symptoms, their prevalence is more than twice that of symptomatic cases. The number of people at 50% and 25% or higher risk of HD is 5 and 11 times the disease prevalence, respectively (3).

Motor manifestations of HD begin with clumsiness, hyperreflexia and eye movement disturbances but the most prominent abnormality is chorea (involuntary jerky movements), although the rare cases under age 20 show pathological rigidity instead (2). Psychiatric symptoms, particularly irritability and depression, are frequent and often precede the onset of motor disturbance. Progressive dementia is also more severe in juvenile cases while relatively mild in patients with onset after age 60.

Individuals with HD typically show marked specific neuronal loss and gliosis in a defined region of the basal ganglia, namely, the striatum (caudate nucleus and putamen) as well as in the neocortex (2,4). In the striatum, the most sensitive cells are the GABAergic medium-sized spiny neurons that project to globus pallidus and substantia nigra. Despite remarkable striatal and neocortical atrophy in post-mortem brain of advanced cases of HD, psychiatric and motor symptoms often precede detectable neuronal loss in HD, and many neurological syndromes proceed without obvious cell death (5).

At least, eight other autosomal dominant neurological diseases are also caused by a polyQ expansion mutation in their respective proteins. These CAG triplet-repeat disorders include spinal and bulbar muscular atrophy, several forms of spino-cerebellar ataxia, and dentatorubral and pallidolusyan atrophy (6). All these diseases share an interesting commonality; the presence of intraneuronal aggregates containing the expanded polyQ in the affected areas of the brain (7,8).

2. POSSIBLE PATHOGENIC MECHANISMS IN HUNTINGTON'S DISEASE

Here will focus on the possible pathogenic mechanisms responsible for HD, and in the following heading will do so more deeply in one of them, namely, the postulated role of alterations in the ubiquitin proteasome system (UPS). Since all CAG triplet-repeat disorders are supposed to share a similar molecular pathogenic mechanism, many of the postulated theories and clues regarding pathogenesis will be common for HD and the rest of the CAG triplet-repeat disorders. We will focus here on the evidence pertinent to HD but most clues regarding other CAG triplet-repeat disorders (covered in a previous chapter) may very well apply also to HD pathogenesis.

Apart from the postulated alteration in the UPS that will be presented in detail below, there are many theories about the pathogenesis of HD (9). Here we will summarize some of these non-UPS related possible mechanisms such as conformational toxicity though amyloid-like protofibril formation (10,11), alterations in gene expression (12) including those leading to trophic factor deregulation (13), altered intracellular vesicular transport and endocytosis (9), mitochondrial dys-

function and impaired energy metabolism, synaptic transmission and electrophysiological abnormalities (14–16), and activation of apoptotic pathways (17). Some of these abnormalities are thought to be consequence of sequestration of key proteins in the intraneuronal inclusions, while others might be consequence of interactions of mutant huntingtin with certain proteins independently of the aggregation state.

3. THE UPS IN HUNTINGTON'S DISEASE

The notion that alterations in the UPS may play a role in Huntington's disease and other neurodegenerative diseases arises from the observation that in most of these diseases aberrant proteinaceous deposits can be detected with anti-ubiquitin and anti-proteasome antibodies inside the affected neurons (18,19). The inhibition of the UPS might be a direct consequence of microaggregation of the corresponding aggregate-prone proteins such as α-synuclein, tau, or in the case of HD, mutant huntingtin. These proteins are susceptible to adopt an aberrant self-assembling conformation that may lead to the formation of aggregates. Chaperones and the UPS counteract this phenomenon but, if the aggregate is finally built-up, it can sequester different components of the UPS, thus resulting in reduced UPS activity. Increased aggregation would lead to a further decline in UPS function, thus leading to a positive feedback mechanism of progression. In the end, this will result in inefficient removal of key regulatory proteins and in the accumulation of other abnormal proteins that will cause cellular deregulation and, eventually, cell death.

Testing this hypothesis has been a major challenge due to the complexity of this machinery for regulated proteolysis and to the scarcity of techniques to test it. As deeply described in a previous chapter, the UPS requires the coordinated action of many enzymes and protein complexes. These include the E1 ubiquitin activating enzyme, twenty to forty different E2 conjugating enzymes, and hundreds of different E3 ubiquitin ligases. The E2 conjugating enzymes transfer one molecule of activated ubiquitin to a specific E3 ubiquitin ligase that, finally, binds the ubiquitin molecule to a lysine residue in a given substrate protein (20). Additional ubiquitin molecules are attached to the first one, thus generating the polyubiquitin chain that is recognized by the 19S regulatory complexes located at one or both sides of the 20S proteolytic core of the proteasome (21). The 19S complexes unfold the polyubiquitinated substrate protein and facilitate its entrance into the 20S proteasome where it is degraded into small peptides. Previously, polyubiquitin chains are cleaved from the substrate protein and subsequently recycled into monomeric ubiquitin by different deubiquitinating enzymes (DUBs). Most of these basic components of the UPS are depicted in Figure 1.

The postulated impairment of the UPS by mutant htt might occur at different levels of the system (Figure 1). The self-aggregating properties of mutant htt led to suggest a choking of the proteolytic chamber of the 20S proteasome by polyQ oligomers that could form inside the 20S barrel. On the other hand, soluble oligomers can associate to form ordered fibrillar structures that, in turn, are gathered into inclusion bodies that often contain numerous other proteins like

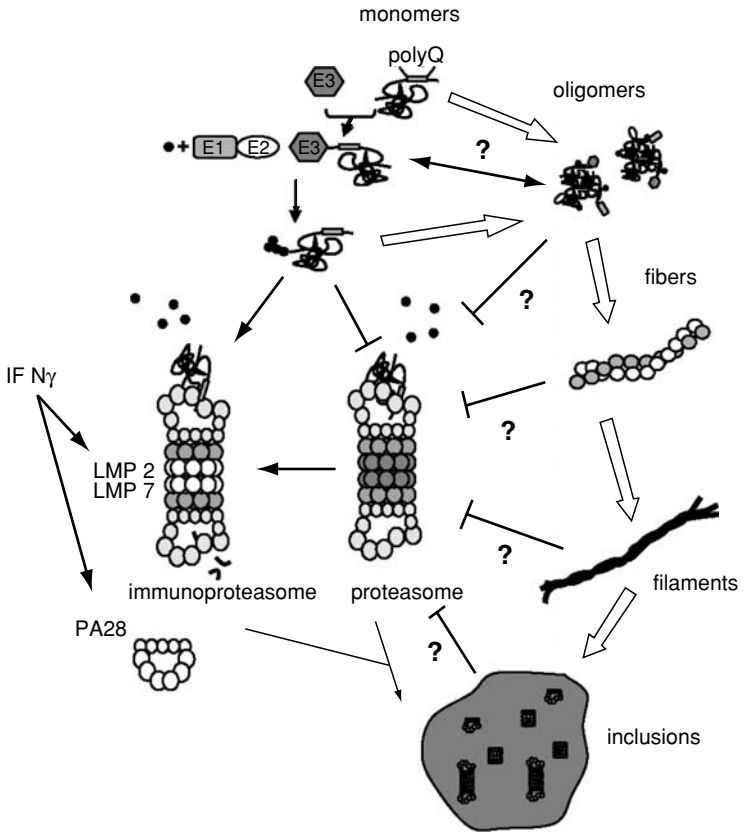

Figure 1. Involvement of the UPS system in Huntington's disease progression: Schematic illustration of possible alterations on the UPS caused by polyQ expansion in htt protein. The enzymes required for poly-ubiquitylation of a substrate protein (like mutant htt) are represented: E1 is the ubiquitin activating enzyme, E2 are the conjugating enzymes, and E3 are the ubiquitin ligases. N-terminal mutant-htt is labeled with ubiquitin (black circle), but it is not normally processed by the proteasome. The scheme also reflects the possible levels of htt aggregation that might interfere with the UPS function. Thus, N-terminal mutant htt monomers can associate to form globular assemblies. These htt-oligomers might lead to the formation of fibers, filaments or intracellular inclusions that in turn can sequester different components of the UPS. This hypothesis is supported by positive immunoreactivity of the inclusion bodies with antibodies against different UPS components. Finally, the neuron may respond to proteasome impairment or to extracellular signals (like interferon-gamma, IFN-gamma; or other inflammatory molecules) by changing the subunit composition of the catalytic core. More precisely, the inducible catalytic subunits LMP2 and LMP7, and possibly the PA28 complex are increased thus leading to induction of the immunoproteasome. (See color insert.)

ubiquitin, proteasome subunits and chaperones. As an alternative to the choking of the 20S proteolytic core, any of the aberrant conformational states of mutant htt, either soluble or in any of its different levels of aggregation, might interact with the 19S caps thus preventing their normal role in recognition and presentation of ubiquitylated substrates. Apart from these mechanisms that imply a direct

interaction of mutant htt with proteasomes it is also possible that the impairment of the UPS takes place due to depletion in the levels of free ubiquitin as suggested by the sequestration of ubiquitin into the inclusion bodies. In summary, the UPS impairment might happen at different levels including ubiquitin availability and recognition by the 19S caps and not only by direct interaction with the 20S catalytic core of the proteasome.

Testing the UPS hypothesis of neurodegeneration has been difficult due to paucity of available experimental techniques (22,23). Some studies have approached this issue of potential UPS impairment in HD by assaying the proteolysis of small fluorogenic substrates specific for each of the three catalytic activities of the 20S proteasome. This has been done in homogenates from cell models such as cell lines transfected with the mutant forms of huntingtin (24), from mouse models of HD (25) and, more recently, from postmortem human brain tissue from HD patients (26). A drawback of exploring UPS activity by assaying proteolysis of these small fluorogenic substrates is that they are degraded by the 20S proteasome in an ubiquitination independent manner. Therefore, these assays can detect alterations in the catalytic activity of the 20S proteasome, but will fail to detect, alterations at any other level of the UPS such as availability of free ubiquitin, polyubiquitination, recognition by the 19S proteasome, and or unfolding and presentation to the 20S proteasome.

A more integrated analysis of UPS function has been accomplished in cell models transfected to express reporter proteins (such as the green fluorescent protein, GFP) tagged for efficient ubiquitination and subsequent degradation by the proteasome. These proteins have an extremely short half-life, but accumulate when cells are exposed to proteasome inhibitors (27–30). This approach has been used also in cellular models of HD (29) and, since reporter transgenic mice expressing modified GFP have recently been generated (31), it will be soon applied to mouse models (23). It should be noted though that despite the great advantages of the UPS-GFP reporters, they still have some limitations that might preclude the detection of an existing UPS impairment. The reason for this is that each type of UPS-GFP reporter is ubiquitylated by a specific subset of E2s, and of E3s (22). Any of these reporter systems might therefore fail to detect UPS impairments in which other E2s and E3s are involved, or if the defect is in some early recognition step like recognition by a chaperone prior to degradation. The availability of a full array of these reporters will maximize the chances to detect UPS impairments both in cell and mouse models of HD (30).

Let's review now in chronological order the information available in the literature regarding a possible impairment of the UPS in HD. The initial observations supporting the hypothesis were the fact that HD intraneuronal aggregates are stained with ubiquitin antibodies in mouse models (32) and in human tissue (33) and that pharmacological inhibition of the proteasome increased the incidence of aggregates in HD cellular models (34) as well as prevented reversal of aggregates in primary neurons from the conditional mouse model (35).

The first study strongly supporting impairment of the UPS in HD was based on the use of N2A cells with ecdysone inducible expression of exon 1 htt with various polyQ lengths (24). By immunofluorescence studies, Jana and coworkers found that 20S proteasome components were diffusely localized in

cytoplasm and nucleus of cells expressing exon 1 htt with non expanded polyQ. However in cells expressing exon 1 htt with 60Q or 150Q, 20S proteasome components were redistributed to the aggregates. Similar results were obtained by immunohistochemical detection of 20S proteasome components in the brain of the R6/1 mouse model. To test whether this alteration in the distribution of 20S proteasome components results in altered proteasome activity, Jana and coworkers used a fluorogenic peptide substrate to measure the chymotrypsin-like activity in soluble and precipitated fractions of cells expressing exon 1 htt with 150Q. Expression of exon 1 htt with 150Q resulted in decreased activity in the soluble fraction while increasing activity in the precipitated fraction. Whether this clear redistributing of proteasome activity results in a decrease in the overall proteasome activity is difficult to infer from these experiments. The shift of the proteasomal components from the total cellular environment to the aggregates, as well as a comparatively slower degradation of exon 1 htt with longer polyglutamine, was paralleled by reduced degradation of key target proteins that are proteasome substrates, such as p53. This altered proteasomal function was associated with disrupted mitochondrial membrane potential, released cytochrome c from mitochondria into the cytosol and activated caspase-9- and caspase-3-like proteases. In summary, these results allowed the authors to suggest that impaired proteasomal function in a cell model plays an important role in polyglutamine protein-induced cell death.

Shortly afterwards, an elegant study by Bence and coworkers employed a UPS-reporter GFP in a cell model of HD (29). This unstable GFP (GFP^u) it's efficiently degraded by the UPS because it is fused to a short degron sequence termed CL1. HEK-293 cells stably expressing GFP^u were transiently transfected with exon 1 htt constructs with different PolyQs. Although most cells expressing exon 1 htt with 103Q exhibited diffuse cytoplasmic staining for transfected exon 1 htt, 10–20% of cells with the highest level of expression of exon 1 htt with 103Q had a single inclusion body that was correlated with increased GFP^u fluorescence. The UPS impairment in cells with high levels of exon 1 htt with 103Q was confirmed by detection of high molecular weight ubiquitin-conjugates by immunoblotting and correlated with predominant 4n DNA content (indicating arrest in G2 as in cells exposed to proteasome inhibitors).

Our group then tested if, as suggested by above described studies on transfected cells (24,29), the UPS is impaired *in vivo* in a mouse model of HD (25). We used the Tet/HD94 conditional mouse model that expresses exon 1 mutant huntingtin with 94Q under control of the CamKIIα promoter (that drives expression to forebrain neurons) leading to aggregate formation in cortical and striatal neurons (the brain areas affected in HD) (36). We performed enzymatic assays for the three peptidase activities of the proteasome in cortical, striatal and cerebellar extracts from Tet/HD94mice. We found no inhibition of any of the activities. Thus suggesting that if UPS impairment happens in vivo in the mouse model, it is not at the level of the proteasome catalytic core. Intriguingly, the chymotrypsin- and trypsin-like activities increased selectively in the affected and aggregate-containing regions: cortex and striatum. Western blot analysis revealed no difference in total proteasome content while an increase in the interferon-inducible subunits of the immunoproteasome, LMP2 and LMP7, was observed. These subunits confer to

the proteasomes catalytic properties that are optimal for MHC-I peptide presentation (37) and their elevation may explain the selective increase of the chymotrypsin- and trypsin-like activities. Immunohistochemistry in control mouse brain revealed LMP2 and LMP7 are mainly located in neurons. Accordingly, their increase in Tet/HD94 mice predominantly took place in neurons and 5% of the ubiquitin-positive cortical aggregates were also LMP2-positive. Ultrastructural analysis of neurons with high level of immunoproteasome subunits revealed signs of neurodegeneration like nuclear indentation or fragmentation, and dark cell appearance. The neuronal induction of LMP2 and LMP7 and the associated signs of neurodegeneration were also found in HD post-mortem brains.

The correlation between heightened levels of LMP2 and LMP7 and signs of neurodegeneration, suggests that the induction of the immunoproteasome might have pathogenic implications. However, it is also possible that the neurodegeneration is secondary to neuroinflammatory stress and the induction of the immunoproteasome subunits could just be a marker of neurons degenerating by this mechanism. This is supported by the observation that LMP2 and LMP7 induction takes place only after substantial neuropathology has developed in Tet/HD94 brains (25). Further evidence supporting that induction of the immunoproteasome subunits are not a direct consequence of expanded polyglutamine expression but rather of inflammatory processes arises from a different study also from our group (38). In that study we found that LMP2 levels are not altered in striatal cultured neurons from Tet/HD94 mice nor are the proteolytic activities of the proteasome. On the other hand, when the striatal cultures were treated with interferon γ (IFN-γ) during 72 hours, a clear increase in LMP2 levels was observed in control neuronal cultures. Interestingly, this increase was much more pronounced (95% higher) in Tet/HD94 striatal cultures. These results indicate that although expression of mutant htt is not sufficient to induce the changes in proteasome catalytic core observed in HD, it synergizes the changes induced by IFN-γ. Furthermore, immunocytochemical studies revealed that Tet/HD94 striatal neurons expressing high levels of LMP2 subunit showed a preapoptotic appearance. These results suggest that the correlation between neuronal induction of the immunoproteasome and neurodegeneration found in HD brains is secondary to inflammatory processes. In any case, it can not be ruled out that altered proteolytic processing of proteasome substrates (due to the altered proteolytic activities) might also contribute to the toxicity elicited by expanded polyQ in advanced stages of the disease.

There is a discrepancy between the studies reporting decreased proteasome activity with fluorogenic substrates in cells transfected with expanded exon 1 htt (24) or infected with a mutant form of ataxin-3 (39) and our data from primary neuronal cultures from the mouse model (38) and from brain homogenates of adult Tet/HD94 mice (25). The most plausible explanation for this is that the level of expression of the pathogenic protein within the transfected or infected cell is much higher that in the transgenic tissue. The level of expression obtained in the transgenic tissue is supposed to be less artifactual since it is high enough to elicit neuronal neuropathology and symptomatology in adult mice, but low enough not to cause premature and artifactual death of the neurons expressing the pathogenic protein. Since we have performed the studies on aggregate containing extracts

from symptomatic mice, our data probably reflect better the situation in the affected neurons in an HD patient. In this regard, there is another report of transfected cells expressing chimeric proteins with expanded polyQ sequences, in which no inhibition of proteasome activity could be detected with fluorogenic substrates (40). Interestingly, this study was performed in stably transfected SH-SY5Y cells that probably elicit lower levels of expression of the foreign protein (as evidenced by the lack of aggregate formation) in comparison to the ecdysone-conditionally overexpressing N2A transfected cells (24) or the infected primary neurons (39).

A recent study by Seo and co-workers analyzes, with fluorogenic substrates, the chymotrypsin and post-glutamyl activities of the proteasome in post-mortem brain samples from HD patients and normal subjects (26). The chymotrypsin activity was decreased in the striatum of grade 0–1 and of grade 3–4 HD patients and also in the cerebellum of grade 0–1 HD patients. This activity did not change in cortex or substantia nigra of HD patients. The post-glutamyl activity was decreased in the striatum and cerebellum of grade 0–1 and of grade 3–4 HD patients, in the cortex of grade 0–1 HD patients and in the substantia nigra of grade 3–4 HD patients. For this analysis, individual proteasome activity values from post-mortem patients' brain samples were normalized using the atrophy index hat results from the macroscopic evaluation of ventricular size of the same HD patients' brains. Unfortunately, this study lacks measurement on total proteasome content in the brain samples, what is the key for the correct interpretation of the results. Interestingly, the article by Seo and coworkers also mentions their unpublished observation of increased proteasome activities in the striatum and cortex of the R6/2 rapidly progressive mouse model of HD (that expresses exon 1 htt with 150Qs under control of the htt promoter). Why both slowly (Tet/HD94) and rapidly (R6/2) progressive mouse models of HD differ from human HD tissue in proteasome activities as measured with fluorogenic substrates remains a matter of debate. A possible explanation may reside on the fact that both mouse models express a minigene with a very short and toxic form of htt (exon 1 only and with long polyQ stretches) while human subjects have different levels of endoproteolityc cleavage of mutant htt and usually shorter polyQ repeats. Furthermore, it is possible that UPS impairment happens in a very limited number of neurons of the mouse models while other cell types (such as a different neuronal subtype or glia) exhibit increased activities. In such case, the enzymatic assay might not be able to detect the UPS impairment in the reduced number of cells due to a dilution effect of the affected neurons in the context of the tissue homogenate.

Interestingly, since huntingtin is ubiquitously expressed in many cell types throughout the human body, Seo and coworkers also analyzed proteasome activities in skin fibroblasts from HD patients to investigate if the inhibition of proteasome is a general phenomenon (26). Both the chymotrypsin and post-glutamyl activities of the proteasome were reduced in fibroblast from HD patients compared to those from control subjects. In the case of the skin fibroblast, semiquantitaive western blot measurements of proteasome β subunit were performed that rule out a decrease in proteasome content as the cause of decreased proteasome activities. Therefore, the skin fibroblast results, apart from opening the possibility for interesting additional experiments on the mechanism of proteasome inhibition, also strengthen the results obtained on brain homogenates.

In summary, the results of decreased cleavage of proteasome fluorogenic substrates on brain homogenates strongly supports an inhibition of the UPS in HD. However, apart of the above mentioned, this experimental approach has many limitations because 20S proteasome in a tissue homogenate can be in a latent form or in an activated form depending on whether detergents or hydrophobic peptides are present during the procedure (41,42) and depending on the content of each tissue in proteasome activators such as the 19S and PA28 complexes (43). For this reason, additional experiments will be required for a full elucidation on how the 20S catalytic activity is impaired in HD. This might be for instance by direct interaction of htt aggregates with the 20S proteasome particle. This possible mechanism can be tested now that a procedure for isolation of htt filaments from HD mice and patients brain has been described (11). In a similar way to what has been done before with purified paired helical filaments isolated from Alzheimer's disease brains (44).

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15

FRAMESHIFT MUTANT UBIQUITIN IN ALZHEIMER'S DISEASE AND OTHER NEURODEGENERATIVE DISORDERS

Robert Layfield

1. INTRODUCTION

In January 1998, a news summary in the journal *Science* proclaimed "Possible New Cause of Alzheimer's Disease Found". The headline referred to a report by a team led by Fred van Leeuwen, at the Netherlands Institute for Brain Research, that aberrant forms of ubiquitin and amyloid precursor protein, so called +1 proteins, could be detected in the pathological lesions that characterise the Alzheimer's disease brain[1]. The mutant proteins appeared to result from mistakes during protein synthesis, by a novel mechanism termed 'molecular misreading', in which dinucleotide deletions in the corresponding messenger RNAs (mRNAs) occur in the absence of gene mutations, during or after transcription.

In the seven years since these initial observations, efforts to determine the role of the frameshift mutant ubiquitin protein (UBB+1) in the pathogenesis of Alzheimer's disease, as well as other conditions, have led to considerable progress

in understanding the relationship between the ubiquitin-proteasome system, and a range of human neurodegenerative disorders.

2. ALZHEIMER'S DISEASE

Alzheimer's disease is the most common cause of senile dementia in Western populations. Pathologically, Alzheimer's disease is characterised by extracellular deposits of the amyloid beta (Aβ) peptide[2], and intraneuronal inclusions termed neurofibrillary tangles, composed of phosphorylated filamentous forms of the microtubule-associated tau protein[3]. The relationship between these pathologies is poorly defined, and although neurofibrillary pathology (and in particular associated synaptic loss) correlates better with disease symptoms, it is likely that both types of lesion contribute to neuronal cell loss.

Alzheimer's disease is a genetically complex and heterogeneous disorder; no single mode of inheritance accounts for its heritability, and mutations and polymorphisms in multiple genes are involved together with non-genetic (environmental) factors[4]. Rare familial cases of early-onset Alzheimer's disease are caused by highly penetrant mutations transmitted in an autosomal dominant fashion. These include mutations in the genes encoding the amyloid precursor protein, presenilin-1, and presenilin-2[5]; these mutations lead to the increased formation of Aβ42, which accumulates in the amyloid plaques. In contrast, the genetics of late-onset Alzheimer's disease are less well resolved. Although candidate genes on multiple chromosomes have been reported, only the gene encoding apolipoprotein E (*APOE*) has been conclusively linked to late-onset Alzheimer's disease, with the *APOE* ε4 allele showing significant association[4].

3. ALZHEIMER'S DISEASE AND THE UBIQUITIN-PROTEASOME SYSTEM

The first connections between the ubiquitin protein and Alzheimer's disease were made some years prior to the observations of van Leeuwen and his co-workers. In the late 1980s, two research groups independently noted that neurofibrillary tangles and plaque neurites stained intensely with antibodies against the ubiquitin protein[6–8]. The era of ubiquitin immunohistochemistry had begun. Therein followed significant activity in the field of ubiquitin and neurodegeneration, and it soon became clear that immunoreactivity to the ubiquitin protein was not limited to Alzheimer's disease. Inclusions in a range of other neurodegenerative disorders, including (but not limited to) Parkinson's disease, dementia with Lewy bodies, Pick's disease, amyotrophic lateral sclerosis, and Huntington's disease, were subsequently found to stain with antibodies to ubiquitin[9–11]. Outside of the nervous system, ubiquitin immunoreactivity was detected in mallory bodies in alcoholic liver disease, as well as cytoplasmic bodies in muscle[9]. Today, ubiquitin immunohistochemistry still routinely forms part of the standard diagnostic procedure at *post mortem*.

So what is the significance of the presence of the ubiquitin protein in these inclusions, and in particular in Alzheimer's disease neurofibrillary tangles? Since almost all of the cellular functions of ubiquitin are mediated by its post-

translational conjugation to other proteins, it is not surprising that ubiquitin within the neurofibrillary pathology is isopeptide-linked to another protein. In this case, ubiquitin is conjugated to phosphorylated, N-terminally truncated forms of tau[12]. Tau protein is the principal constituent of the paired helical filaments that make up neurofibrillary tangles, and interestingly is also found within inclusions in seemingly unrelated neurodegenerative diseases, for example in Pick's disease, progressive supranuclear palsy, and frontotemporal dementias (disorders with tau pathology are collectively referred to as the 'tauopathies'). In other inclusions, different proteins are the principal constituents, for example Lewy bodies in neurones from Parkinson's disease and dementia with Lewy bodies patients contain the α-synuclein protein (disorders with α-synuclein pathology are collectively referred to as the 'synucleinopathies'). Once again, ubiquitin is covalently linked to α-synuclein in these inclusions[13].

The ubiquitin-conjugated tau protein in Alzheimer's disease paired helical filaments is now known to be modified mainly with single copies of ubiquitin (monoubiquitination), although multi-ubiquitination of tau, with ubiquitins linked *via* lysine 48 (Lys48), is also seen[12]. To what extent the apparent preference for tau monoubiquitination over multi-ubiquitination reflects changes that occur *post mortem* is not known. However, a reasonable interpretation of the attachment of Lys48 linked ubiquitin chains, a signal for 26S proteasomal degradation, to tau in the disease-associated inclusions, is that impairment of (presumably neuroprotective) protein handling, at the level of ubiquitin-mediated proteolysis, is occuring. Indeed, E3 ubiquitin ligases which target tau for ubiquitination have recently been described, suggesting that some form(s) of tau can be a substrate of the ubiquitin-proteasome system[14].

Certainly, in the case of Parkinson's disease and related disorders, genetic evidence clearly links altered function of the ubiquitin-proteasome system and neurodegeneration. For example, loss-of-function mutations in enzymes of the ubiquitin conjugation and deconjugation pathway, parkin and UCH-L1 (PGP9.5), cause juvenile parkinsonism and familial Parkinson's disease, respectively[15–16]. No such mutations in ubiquitin pathway enzymes have been found to date in cases of familial Alzheimer's disease, although in Alzheimer's tissue from certain brain regions, impaired peptidase activity of the proteasome has been noted[17], along with evidence of defective ubiquitylation of cerebral proteins *in vitro*[18]. If the ubiquitin pathology which characterises sporadic cases of Alzheimer's disease really is occurring as a consequence of defective ubiquitin-mediated proteolysis, be that a primary (causative) defect, or as a consequence of the pathological process, then this seems likely to involve a mechanism which does not depend upon germline mutations. Since the accumulation of ubiquitin pathology is, like the risk for Alzheimer's disease, age-dependent, any mechanism to explain this accumulation must also take this observation into account.

4. MOLECULAR MISREADING

In fact the phenomenon of 'molecular misreading' detailed in the landmark *Science* paper was not new to van Leeuwen, who had described this process some years previously in Brattleboro rats. These animals carry single nucleotide

deletions in the vasopressin gene, resulting in a lack of functional vasopressin protein. As the animals age, a proportion of the hypothalamic cells are seen to contain functional vasopressin mRNA and protein[19–20]. This apparent 'correction' of the single nucleotide deletion, was found to occur by the incorporation of a dinucleotide deletion (in this case ΔGA) into a specific sequence repeat (a 'GAGAG motif') of a fraction of the mutant vasopressin mRNAs, during or after transcription, hence the term 'molecular misreading'. The precise mechanism by which the misreading event occurs is not known, but is speculated to involve the co-transcriptional slippage or stuttering of RNA polymerase[21].

In the case of UBB+1, similar misreading events (GT dinucleotide deletions) close to GAGAG motifs also affect a proportion of ubiquitin mRNA transcripts, such that rather than correcting the frameshift caused by a single nucleotide deletion, as is the case in the Brattleboro rats, misreading of the ubiquitin-B gene results in a translated frameshifted protein[1]. This UBB+1 protein has a wild type sequence for the first 75 residues, with the extreme C-terminal glycine 76 residue of ubiquitin replaced with 20 amino acids of nonsense sequence (Figure 1).

The findings of van Leeuwen and co-workers, that molecular misreading can lead to the formation and deposition of the mutant UBB+1 protein in neurofibrillary tangles, neuropil threads, and dystrophic neurites[1], provided for the first time a possible mechanistic link between disruption of ubiquitin-mediated processes, and Alzheimer's disease. Importantly however, UBB+1 immunoreactivity

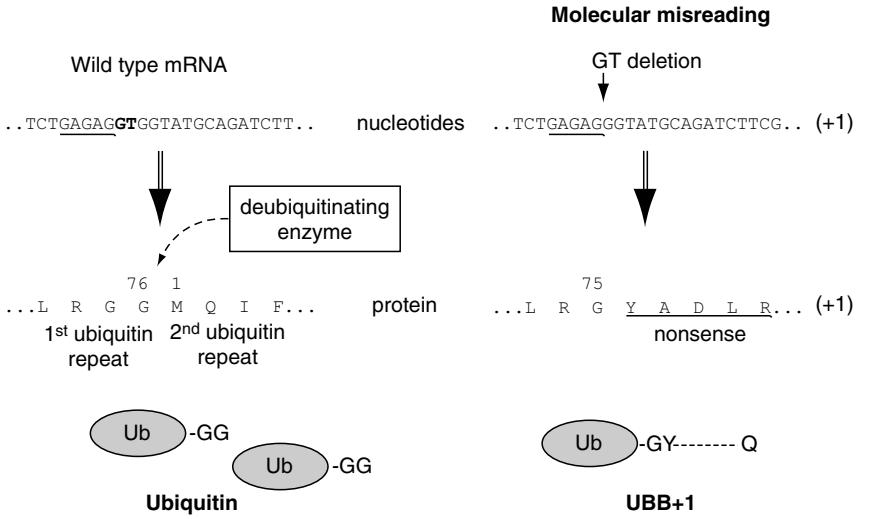

Figure 1. Molecular misreading of the human ubiquitin-B gene. (Left) Ubiquitin is expressed as head-to-tail repeats, which are normally processed to the mature protein by the actions of deubiquitylating enzymes, that cleave after the C-terminal glycine (Gly76) of ubiquitin. (Right) When molecular misreading occurs, a GT dinucleotide deletion close to a 'GAGAG' motif (underlined) results in a frameshift in the translated protein. In the resulting UBB+1 protein, Gly76 of ubiquitin is replaced with 20 residues of nonsense sequence.

was also found in brain lesions of Down's syndrome patients, as well as elderly (but not young) controls[1]. These observations suggested that molecular misreading may not be unique to Alzheimer's disease, and could indeed be an age-dependent event marking the early stages of neurodegeneration. Subsequently UBB+1 immunoreactivity, often co-localised with ubiquitin, has been found to characterise a whole range of other human degenerative disorders (Table 1).

5. CONSEQUENCES OF UBB+1 EXPRESSION

The immunohistochemical observations of van Leeuwen went as far as suggesting that accumulation of (presumably non-functional) UBB+1 protein in the ageing brain could be related to the neurodegenerative process. However, the first indications of how UBB+1 expression might lead to cellular dysfunction came from subsequent studies, which demonstrated that although UBB+1 lacks a functional C-terminus and consequently cannot be activated and conjugated to target proteins, in fact UBB+1 is itself able to serve as a substrate for ubiquitin-conjugation, and can be ubiquitinated by wild type ubiquitin *in vitro* and in transfected human cells[27] (Figure 2).

The resulting polyubiquitinated UBB+1 was found to act as potent inhibitor of 26S proteasomal degradation of a model ubiquitinated substrate *in vitro*[27], presumably through competing for polyubiquitin-binding sites on the proteasome. Significantly, polyubiquitinated UBB+1 is resistant to disassembly by deubiquitylating enzymes such as isopeptidase T, which normally prevents the accumulation of unanchored polymeric ubiquitin chains, and which has an

Table 1. Human disorders associated with UBB+1 immunoreactivity. SCA-3; spinocerebellar ataxia type-3. NFTs; neurofibrillary tangles. 't' indicates disorders characterised by tau pathology and which can be considered as being 'tauopathies'. Note the absence of 'synucleinopathies' from this listing.

Disorder	Pathology stained	Reference
Alzheimer's disease (t)	NFTs, neuropil threads, dystrophic neurites	[1]
Down's syndrome (t)	NFTs, neuropil threads, dystrophic neurites	[1]
Progressive supranuclear palsy (t)	NFTs	[22]
Pick's disease (t)	Pick bodies	[23]
Frontotemporal dementia (t)	Globoid tangles	[23]
Argyrophilic grain disease (t)	Grains	[23]
Huntington's disease, SCA-3	neuronal intranuclear inclusions	[24]
Steatohepatitis, alcoholic liver disease	Mallory bodies (liver)	[25]
Inclusion body myositis	muscle fibre inclusions	[26]

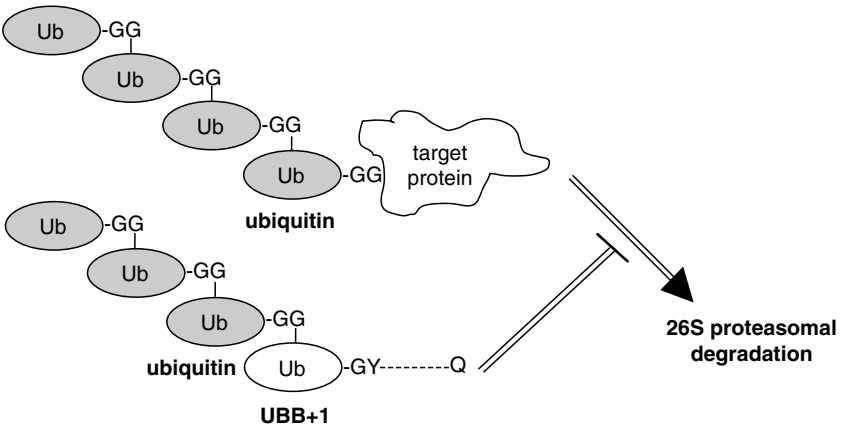

Figure 2. (Top) Wild type ubiquitin can be conjugated to selected target proteins to signal their degradation by the 26S proteasome. (Bottom) UBB+1 lacks a functional C-terminus, and consequently cannot be conjugated to other targets. UBB+1 can however serve as a target of ubiquitination by wild type ubiquitin. The resulting polyubiquitinated UBB+1 can act as a potent competitive inhibitor of proteasomal degradation. Accumulation of polyubiquitinated UBB+1 in neurones, and incorporation into disease-associated lesions, is predicted to have pathological consequences.

absolute requirement for a free C-terminus in the proximal (that at the C-terminal end) ubiquitin of such chains[27]. These observations suggested a possible mechanism by which polyubiquitinated UBB+1 could accumulate with time, for example upon ageing, with deleterious consequences[27].

Thereafter, inhibition of the ubiquitin-proteasome system by UBB+1 was also realised in primary neurones from transgenic mice expressing a green fluorescent reporter carrying a constitutively active proteasomal degradation signal, indicating that the mutant protein is likely to have similar effects on the proteasome *in vivo*[28].

6. MECHANISMS OF NEURODEGENERATION

Other cell-based experiments have demonstrated that expression of UBB+1 is toxic to neuronal cells, resulting in apoptosis by a mechanism now known to involve ubiquitination of both Lys29 and Lys48 of the UBB+1 protein[29–30]. UBB+1 expression also leads to a significant up-regulation of chaperone expression, which may protect against oxidative stress conditions[31]. Since the ubiquitin-proteasome system controls the degradation of key neuronal proteins, including for example presenilins[32] and tau[14], a reasonable hypothesis is that UBB+1 expression and ubiquitination, with subsequent inhibition of 26 proteasome activity in the ageing or diseased brain, could directly contribute to (possibly apoptotic) neuronal cell death and the neurodegenerative process *via* the mismetabolism of such targets[33]. The observation that UBB+1 immunoreactivity is readily detectable in brain tissue from Alzheimer's patients with high levels of pathology, but without prior dementia, would be supportive of a

model in which UBB+1 is in fact involved in the early stages of disease pathogenesis[34].

Of particular note in understanding the mechanism of neurodegeneration in Alzheimer's disease, is a recent study aimed at investigating Aβ-induced neurotoxicity using DNA microarrays[35]. One of the genes found to be up-regulated in neurones exposed to Aβ42 encodes the E2-25K ubiquitin-conjugating (E2) enzyme, which had previously been used to catalyse the ubiquitination of UBB+1 *in vitro*[27]. In fact E2-25K was found to be required not only for Aβ42-induced neurotoxicity and inhibition of proteasome activity, but also neurotoxicity mediated by UBB+1[35]. These intriguing findings suggest a possible direct molecular link between Aβ and dysfunction of the ubiquitin-proteasome system, which is likely to be relevant in Alzheimer's disease pathogenesis, and which clearly merits further investigations.

7. UBB+1 AND HUMAN DISEASE, CAUSE OR EFFECT?

The combined evidence suggests that UBB+1 expression may be directly related to Alzheimer's disease pathogenesis, based on the observations that: the mutant protein is a component of pathological lesions which are hallmarks of the disease[1]; and that UBB+1 has deleterious effects on the ubiquitin-proteasome system *in vitro* and in transfected cells[27, 29–31]. However, a key question which remains is whether UBB+1 expression is causally related to Alzheimer's disease pathogenesis, and indeed of other neurodegenerative disorders, or alternatively arises as a result of the pathological state.

Of interest is the observation that despite the presence of UBB+1 transcripts in brain tissue from a range of neurodegenerative conditions, including synucleinopathies and tauopathies, immunohistochemical evaluation fails to detect the presence of UBB+1 immunoreactivity in the pathological hallmarks of cases of the former[23]. For example, neither Lewy bodies in cases of dementia with Lewy bodies, nor glial cytoplasmic inclusions in cases of multiple systems atrophy, both inclusions which are ubiquitin- and α-synuclein-positive, stain with antibodies to UBB+1[23] (note also the absence of synucleinopathies in Table 1). Since UBB+1 may in fact itself be turned over by the ubiquitin-proteasome system[23], one interpretation of these findings is that UBB+1 accumulates in neurones in certain disorders including tauopathies such as Alzheimer's disease because in these cases the ubiquitin-proteasome system is impaired by a mechanism not involving UBB+1. In this regard, the accumulation of UBB+1 in the diseased brain may simply be acting as a 'reporter' of proteasomal dysfunction (Figure 3). Within this model of the neurodegenerative process, a variety of different mechanisms could account for impairment of proteasome function in the pathological state, for example protein aggregation events[36], or direct inhibition of proteasome function by amyloid[37] or tau fibrils[38].

8. FUTURE STUDIES

The scientific community awaits with great interest clarification of the 'cause or effect' relationship between UBB+1 to the pathogenesis of Alzheimer's

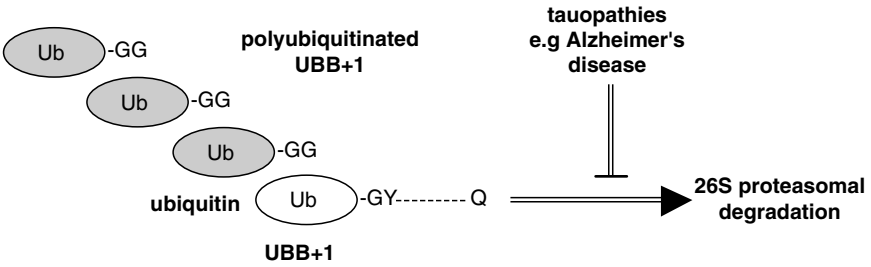

Figure 3. In an alternative model to that presented in Figure 2, UBB+1 may itself be a degradative substrate of the ubiquitin-proteasome system. In certain disorder such as the tauopathies, proteasome inhibition by a mechanism not involving UBB+1 may account for the accumulation of UBB+1 seen. In this model, UBB+1 accumulation serves as a reporter for proteasome dysfunction.

disease, as well as other neurodegenerative disorders. In the future, the generation and phenotypical/pathological characterisation of animal models expressing UBB+1 in the adult mammalian nervous system may go some way to addressing this issue. At the very least, through crosses with other models of neurodegenerative diseases, such animals should offer the opportunity to examine the effects of manipulating the activity of the ubiquitin-proteasome system *in vivo* in a variety of biological and biomedical contexts.

9. CONCLUSIONS

In conclusion, impairment of the ubiquitin-proteasome system appears to be a feature of the Alzheimer's disease brain. Of the different mechanisms that could account for this impairment, inhibition of proteasomal function by ubiquitination of the frameshift mutant UBB+1 protein has the distinction of representing a possible causative mechanism in Alzheimer's disease pathogenesis. However, an alternative possibility, that the accumulation of UBB+1 in the lesions which characterise the Alzheimer's brain simply reflects an impairment of proteasome activity caused by some other mechanism, requires consideration.

Regardless of the precise relationship between UBB+1 and the pathogenesis of Alzheimer's disease, which will no doubt be clarified in the coming years, the seminal observations of van Leuuwen and co-workers made in the late 1990s have further focussed attention on the crucial roles played by the ubiquitin-proteasome system in the nervous system, which will underpin future advances in our understanding of the molecular mechanisms involved in some of the most debilitating human conditions.

10. ACKNOWLEDGMENTS

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16

MOTOR NEURON DISEASE

Heather D. Durham, Edor Kabashi, David M. Taylor, and Jeffrey N. Agar

1. INTRODUCTION

The terms Motor Neuron Disease (MND) and Amyotrophic Lateral Sclerosis (ALS, a.k.a. Lou Gehrig's disease) are often used interchangeably. This chapter, however, will use the term MND in the larger context to include multiple disorders with primarily motor neuronal involvement, focusing on ALS (a.k.a. Lou Gehrig's disease) and Spinal Bulbar Muscular Atrophy (SBMA, a.k.a. Kennedy's disease). In both a familial form of ALS, due to mutations in the gene encoding Cu/Zn-superoxide dismutase (SOD1), and in SBMA there is evidence of involvement of the proteasome in pathogenesis.

Whereas the etiology of SBMA has been established as expansion of CAG repeat sequences in exon 1 of the androgen receptor (AR) gene (1), ALS is a more heterogeneous disorder. ALS, as described by Charcot, is a disease presenting in adulthood as progressive muscle weakness and atrophy (2). Death usually occurs within 3-5 years of clinical presentation. The disease occurs with an

incidence of 2/100,000 population or 3-4/100,000 over 20 yrs old, with a slightly higher affliction of women (male/female ratio of 1.3/1.6:1). Whereas some forms of ALS have a clear pattern of inheritance, 80-90 percent of cases remain of unknown cause, and thus are termed "sporadic". In 1993, the first gene responsible for familial ALS (fALS1) was cloned (3). Mutation of the gene encoding SOD1 on chromosome 21 is responsible for 4-7% of ALS. Over 100 mutations spanning all 5 SOD1 exons have been identified (see http://alsod1.iop.kcl.ac.uk/) and are thought to cause disease through a toxic gain of function. Most are missense mutations causing MND in a dominantly inherited fashion with high penetrance, although a few are truncations. One missense mutation, D90A, exhibits a recessive or dominant pattern depending on genetic background (4). FALS1 is clinically and neuropathologically similar to sporadic ALS.

1.1 Clinical and Neuropathological Features of ALS

The diagnosis of ALS is made in accordance with criteria established by the World Federation of Neurology Research Group on Neuromuscular Diseases (5).The predominant presenting symptom of ALS, muscle weakness, is due to dysfunction and loss of motor neurons in the ventral horn of the spinal cord and brain stem that extend axons to synapse with muscle. Depending upon which motor nuclei are involved, difficulty moving limbs and sustaining posture, speaking, swallowing and breathing may develop. Symptoms progressively spread within a region and to other regions of the neuromuscular system. Muscle fibers are denervated as a result of dysfunction or death of lower motor neurons and may be re-innervated through sprouting of adjacent motor axons. Failure of re-innervation and decreased mobility result in muscle atrophy, giving a wasted appearance to the musculature. There is also involvement of upper motor neurons in the motor cortex that synapse onto lower (brain stem and spinal) motor neurons.

Microscopic findings at autopsy include loss of motor neurons, reactive gliosis (both astrocytes and microglia), and ultrastructural abnormalities of surviving motor neurons including presence of ubiquitinated inclusions, neurofilament-rich hyaline inclusions, proximal axonal swellings filled with accumulations of neurofilaments, and dendritic atrophy (6). Despite the preferential vulnerability of motor neurons in ALS, other brain areas are not completely spared and in some cases may become sufficiently involved to manifest clinically, for example, neurons in the frontal cortex controlling executive (planning) functions. Indeed, certain pools of motor neurons are spared in ALS, in particular those in the oculomotor and abducens nuclei that control eye movements and those in Onuf's nucleus in the sacral spinal cord that control urinary and rectal sphincters.

ALS is a clinical syndrome with multiple underlying causes to which certain pools of motor neurons are particularly vulnerable. An important point is that multiple initiating factors (whether simple or complex genetic traits, environmental factors or a combination) manifest with similar pathology. The cells' ability to survive a disease-related challenge will depend upon protective pathways that can be recruited as well as the presence of other stresses that must be handled. Understanding these properties in motor neurons and the cells with

which they interact provides the basis for design of therapeutic agents to prevent the cascade of events leading to cell death. Two factors contributing to motor neuronal vulnerability are of particular relevance to the UPS and are discussed briefly below: high level glutamatergic, excitotoxic input coupled with low levels of cytoplasmic calcium-binding proteins (7-11), and lack of induction of the heat shock response to stress (12).

2. INVOLVEMENT OF THE UPS IN FALS1

2.1 Involvement of Protein Chaperones and the UPS in Turnover of Mutant SOD1 Proteins

A feature of FALS1 is the presence of ubiquitinated inclusions containing SOD1 in vulnerable motor neurons and surrounding astrocytes (13, 14). Inclusions containing mutant SOD1 also form following gene transfer into motor neurons of dissociated spinal cord cultures (15) and in motor neurons and astrocytes of transgenic mice (14, 16-20). A common property among the mutant proteins is a propensity to aggregate, both *in vitro* and *in vivo*. Almost all wild type SOD1 is soluble in non-ionic detergent when isolated from tissues or cultured cells; however, a significant portion of mutant protein is insoluble in non-ionic detergent and a smaller percentage is even SDS-resistant, appearing as high molecular weight species on SDS-PAGE (14, 20-22). This property manifests in recombinant proteins *in vitro* as spontaneous aggregation and sedimentation upon centrifugation (23-25), decreased thermal stability (26), and polymerization of monomeric mutant SOD1 into highly ordered, fibrillar structures (26-36).

Interestingly, the propensity of mutant SOD1 to form microscopically visible inclusions is variable depending on cell type, both in animal tissues and in cultured cells (15, 20, 37). Inclusions were prominent in motor neurons of dissociated murine spinal cord cells following microinjection of plasmids encoding several different mutant SOD1 proteins, but were exceedingly rare in cultured dorsal root ganglion or hippocampal neurons (15). Inclusions are not prominent in cells cultured from mutant SOD1 transgenic mice (including spinal cord cells and fibroblasts) or transfected cell lines, even though detergent-insoluble protein is present; however, treatment with peptide proteasomal inhibitors results in accumulation of mutant protein (including insoluble fraction) and inclusions in multiple cell types (21, 29, 38-41). Partial reversal of these inclusions occurs upon removal of proteasome inhibitor (37).

Mutant SOD1 can be poly-ubiquitinated *in vitro* (29, 39), indicating that its degradation by the 26S proteasome can proceed in an ATP- and ubiquitin-dependent manner. Oxidatively damaged proteins, including wild type SOD1, are degraded in an ATP- and presumably ubiquitin-independent manner (42-44), indicating that multiple mechanisms of proteasomal degradation exist. Mutant SOD1 proteins also are substrates for heat shock proteins (HSPs) that participate in folding and transport of proteins or targeting abnormal proteins to the proteasome for degradation. By this chaperoning function, the inducible HSPs, and their constitutively expressed analogs, heat shock cognate proteins (HSCs), prevent the interaction of misfolded proteins with inappropriate partners and precipitation into

aggregates: chaperoning activity is reduced in lumbar spinal cord of presymptomatic mutant SOD1 transgenic mice (45); overexpression of HSP70 (45) or a combination of HSP40/HSP70 (46), prevented aggregation of mutant SOD1 and reduced toxicity in cultured cells; although expression of HSP70 alone failed to affect disease in mutant SOD1 transgenic mice (47), treatment with a coinducer of the heat shock response, arimoclomol, did delay disease onset and prolong survival (48); HSP70 colocalizes with mutant SOD1 under various experimental conditions (22, 49); dorfin, a RING-finger type E3 ubiquitin ligase, and CHIP, a co-chaperone of HSC70/HSP70, are present in inclusions and their overexpression reduced levels of mutant SOD1 proteins and their toxicity in cell lines (39, 49-51).

Collectively, these data show that mutant SOD1 proteins are catabolized by the proteasome and that biophysical species with altered solubility are precursors of larger inclusions. Under most circumstances cells are capable of handling mutant SOD1 proteins sufficiently to prevent them from exerting toxicity and/or being sequestered into inclusions. Even motor neurons develop and function well into adulthood. However, under circumstances of increased physiological or environmental stress or compromise of protective mechanisms with aging, the UPS may become overloaded and impaired.

Measurements of total proteasomal activity in various cell lines expressing mutant SOD1 have produced mixed results, showing activity to be decreased (29, 52-54), increased (40, 55) or unchanged (56). Differences in physiological properties of clonal cell lines, including ability to upregulate proteasomes or other protective mechanisms may have contributed to disparate measures of activity. Post-translational modifications to mutant SOD1 also could vary with cell type and the conditions of expression, and affect the rate of catabolism. Biophysical forms of mutant SOD1 could be catabolized with different efficiencies and differentially affect proteasome function. Pulse chase studies in transfected cell lines showed the half-life of several mutant proteins to be less than wild type human SOD1 (21, 57, 58). Either proteasomes efficiently catabolize mutant SOD1 unless their activity is otherwise compromised or, in the pulse chase studies, a portion of mutant protein gradually precipitated into insoluble aggregates and was not represented in the assay.

To examine the effect of mutant SOD1 expression in tissues affected by disease, total and specific proteasomal activities were measured in tissues from transgenic mice expressing either wild type human SOD1 or the mutant $SOD1^{G93A}$ (59). Phenotypic and neuropathological changes have been extensively documented in the B6SJL-TgN($SOD\text{-}1^{G93A}$)1Gur line of transgenic mice established by Gurney (60). The onset of motor dysfunction depends on the particular measure, but once gait difficulties are apparent, the disease progresses rapidly until incapacity at around postnatal day 120. Significant loss of lumbar and cervical motor neurons occurred at 80-90 days of age; no loss of thoracic or cranial motor neurons occurred, although vacuolar changes were documented (61). Early, pre-symptomatic changes include vacuolation of mitochondria in ventral root axons and motor neuronal perikarya as early as 2 weeks of age (62). Such changes become prominent by 45 days (61). Early disruption (at 31 days of age) of the Golgi apparatus has been well documented in a more slowly progressing line of transgenic mice (63). Detergent-insoluble, high molecular weight com-

plexes of SOD1 were detected on Western blots of spinal cord from 30-day-old mice (20, 21, 37), although cytoplasmic inclusions containing mutant SOD1 become apparent later. Inclusions were visible in sections of lumbar spinal cord by immunhistochemistry at day 112 of $SOD1^{G93A}$ mice (19) and were isolated from symptomatic mice using a filter-trap assay (20). ICAM-1 and the immunological markers, IgG and FcγRI, were upregulated in ventral spinal cord of 40-day-old mice (64). Activated microglia (the macrophages of the nervous system) and reactive astrocytes (those strongly expressing glial fibrillary acidic protein) were detected in lumbar spinal cord at 75 days of age, then progressively increased in numbers (12, 64, 65).

Based on these measures of disease-progression, proteasomal enzyme activities were measured at three time points in homogenates of lumbar spinal cord, thoracic spinal cord and liver from SOD^{WT} and SOD^{G93A} mice and their nontransgenic littermates (59): day 45, when microscopic changes are present, but there is no significant alteration in cell type (either motor neuron death or glial activation); day 75, when motor neuron loss and glial activation are beginning, and at symptomatic age (around day 120). Assays of individual 20S/26S proteasome hydrolytic activities were based on generation of the fluorescent species, amino methyl coumarin (AMC) following addition of peptide substrates to samples of tissue homogenates (Suc-LLVY-AMC, Boc-LRR-AMC or Z-LLE-AMC as substrates for chymotrypsin-, trypsin-, and caspase-like activities, respectively). Total (normalized to actin) and specific activities (normalized to level of 20S proteasome core α-subunits) were determined. Both total and specific activities were reduced in lumbar spinal cord of presymptomatic $SOD1^{G93A}$ transgenic mice, a region in which motor neuron degeneration subsequently occurs. Chymotrypsin activity was significantly reduced at day 45, but by day 75 all three hydrolytic activities were about 50% of littermate controls (Figure 1). No significant differences in activities were found in liver of these mice or in thoracic spinal cord, the adjacent region of the spinal cord that is affected much later in the disease. It should be noted that significant reduction in proteasome activities has not been detected in our lab (unpublished data) or others (66) when homogenates of whole spinal cord are assayed, presumably because the focal effect of mutant SOD1 in the lumbar region is diluted by normal activity in the other tissue.

Compounding regional reduction in specific proteasomal activity, the amount of 20Sα proteasomal subunit was reduced by day 75 and markedly diminished in surviving motor neurons within the lumbar spinal cord of symptomatic $SOD1^{G93A}$ mice, although the overall amount of 20Sα proteasome was maintained in the surrounding neuropil (Figure 2) (59). Similar findings were reported recently by Cheroni *et al* (66). This reduction in proteasomes could reflect downregulation of subunits at the level of transcription or translation in failing neurons or increased breakdown. Some proteasomal subunits are substrates for activated caspases during apoptosis (67) and multiple caspases are activated in motor neurons of $SOD1^{G93A}$ mice (68-73). On the other hand, proteasome inhibitors can induce neuronal apoptosis and activation of caspase-3 (74, 75), raising the possibility of a positive feedback cascade being activated and accelerating motor neuron death.

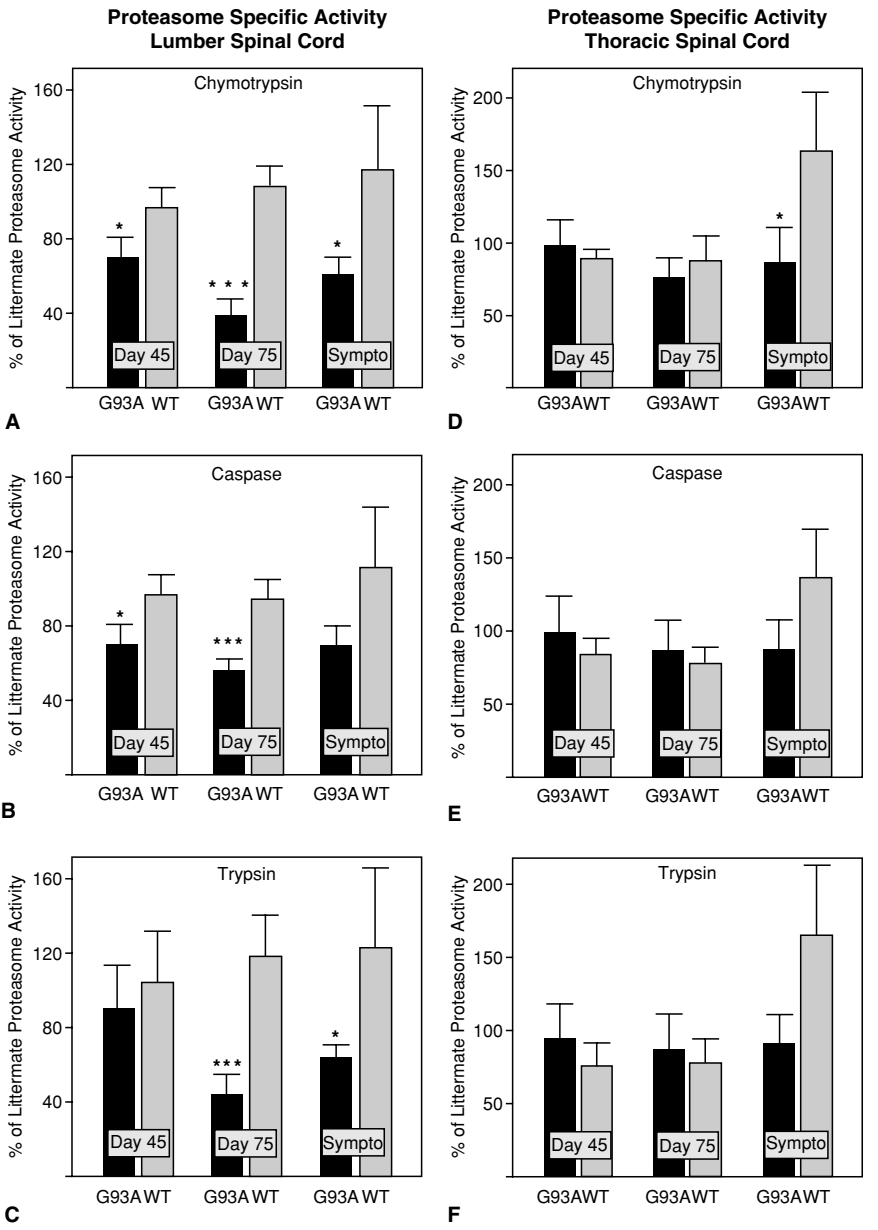

Figure 1. Decrease in specific activities of the proteasome in lumbar spinal cord of SOD1^{G93A} relative to SOD1^{WT} transgenic mice. To compare specific proteasomal activities in tissue homogenates from the two lines of mice, specific activities for tissue from each mouse was calculated by normalizing total activity in nmol/min/mg first to actin then to the levels of 20S proteasome α-subunits. Then specific activities from transgene-expressing mice were expressed as percent of specific activity in littermates. Shown are specific chymotrypsin-(a,d), caspase-(b,e) and trypsin-like (c,f) activities of lumbar (a,b,c) and thoracic (d,e,f) spinal cord homogenates. Shown are means ± SEM. Significant difference between SOD-1^{G93A} and SOD-1^{WT}: * $p<0.05$; ** $p<0.01$; *** $p<0.005$ (from Kabashi et al. (59).

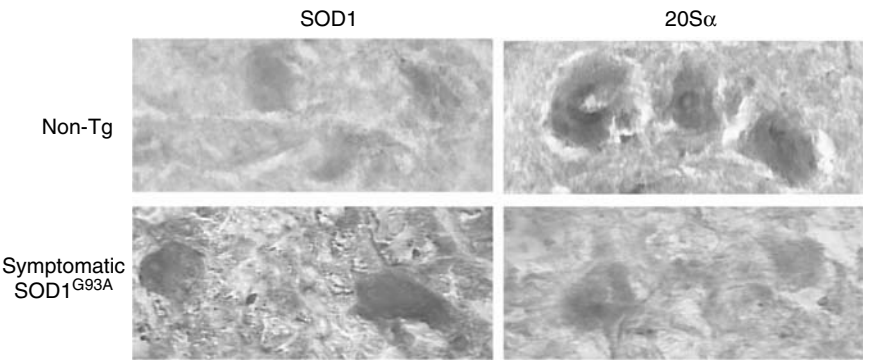

Figure 2. Cellular distribution of 20Sα subunit of the proteasome in the lumbar spinal cord of mutant SOD1 transgenic mice and nontransgenic (Non-Tg) littermates. Twenty μm frozen cross-sections of lumbar spinal cord from symptomatic $SOD1^{G93A}$ mice (approx. 120 days of age), and age-matched Non-Tg littermates (as well as $SOD1^{WT}$ transgenic mice -not shown) were labeled with antibody to SOD1 or α-subunits of the 20S proteasome core. Immunolabeling for the 20S proteasome core was markedly reduced in motor neurons of lumbar spinal cord from symptomatic $SOD1^{G93A}$ transgenic mice relative to the surrounding neuropil and to motor neurons of Non-Tg littermates, despite strong labeling for SOD1. See also Kabashi *et al*. (59).

These data support a role for abrogation of proteasome-mediated proteolysis in the loss of motor neurons in fALS1, at least for the G93A mutant. Proteasomal function was impaired in lumbar spinal cord, the tissue most vulnerable to disease, but early in pathogenesis, before significant change in the cellular composition of the tissue due to motor neuron loss or increase in reactive microglia and astrocytes. Motor neurons comprise only a fraction of the lumbar spinal cord. Thus, approximately 50% reduction in all three proteasomal activities in homogenates of lumbar spinal cord implies a regional impairment of proteasomal function involving multiple cell types. Dysfunction of the proteasome in the surrounding non-neuronal cells could play a significant role in the death of motor neurons. Several studies indicate that death of motor neurons is not cell autonomous, but either requires or is facilitated by the surrounding glia (76-80).

At the time of preparing this chapter, there was little concrete information on the mechanisms underlying abrogation of proteasome function by mutant SOD1, reasons for involvement of particular cells and tissues, or the contribution to pathogenesis of the disease. However, the following sections discuss several possibilities that are being explored experimentally and that illustrate the complexity of the interplay between physiological properties of cells and their response to toxic insult.

2.2 Mechanism Responsible for Inhibition of Proteasomal Activities in the Lumbar Spinal Cord of $SOD1^{G93A}$ Transgenic Mice

The assays of enzyme activity used in the studies described above measure the ability of small exogenous peptide substrates to compete with endogenous substrates in tissue homogenates for cleavage by proteasomal enzymes. Thus,

gradual reduction in specific chymotrypsin-, trypsin- and caspase-like proteasomal activities could reflect:

- *Inhibition of the Proteasome by a Specific Protein including, but not limited to, Mutant SOD1*. That many tissues express high levels of transgenic SOD-^{G93A}, yet reduction in specific proteasome activities was predominant in lumbar spinal cord, argues against direct inhibition of the proteasome by mutant SOD1. However, certain post-translationally modified forms or small oligomers could be inhibitory substrates. The biophysical basis of altered detergent-solubility and polymerization of mutant SOD1 proteins *in vivo* remains to be identified, but is under investigation in our laboratory and others. Studies of recombinant proteins *in vitro* or transfected cultured cells subjected to oxidizing conditions show that dimer destabilization and polymerization of monomers are promoted by disease-causing mutations, demetallation, disulphide reduction, and oxidative modifications (26-36, 81, 82). Full biophysical characterization of mutant and wild type SOD1 proteins from tissues will be required to resolve which states occur under physiological conditions and correlate with disease markers.
- *Nonspecific Inhibition by Increased Amount of Substrate relative to Active Proteasome*. Expression of mutant SOD1 could increase the amount of damaged proteins generated, through direct catalysis of protein modifications (83, 84) or consequential to mitochondrial damage (61, 62, 85-89) or excitotoxicity (see below).
- *Changes in Composition or Post-translational Modification of Proteasome Complexes*. The results cannot be explained fully by reduction in amount of proteasome. Except for the reduction in lumbar motor neurons, levels of 20S α-subunit remained constant in the examined tissues from SOD1^{G93A} transgenic mice. In studies using transfected cell lines, proteasomal subunits were unchanged or increased, not decreased (see above). However, proteasomal activity undergoes an age-related reduction in spinal cord (90) and could become critical in tissues expressing toxic proteins that normally have a high substrate load. Other possibilities include: isoform switching of catalytically active 20S β-subunits (53), altered 20S:26S ratio, association of 20S with 19S or 11S regulatory subunits, or a combination (90); impaired assembly of proteasomal subunits into active complexes, and modification (e.g., oxidation, hyperphosphorylation) of proteasomal subunits that impair enzyme activity, as documented in ischemia-reperfusion injury (91) and ethanol treatment (92). In relation to mutant SOD1, there are few studies that test these hypotheses. In NSC34 cells stably expressing two different mutant SOD1 proteins, the expression of 20S proteasome β5i subunit (LMP7) was substantially reduced relative to cells expressing wild type human SOD1, coincident with a reduction in chymotrypsin-like activity and an increase in the amount of β5 subunit (53). The opposite was reported in spinal cord of symptomatic SOD1^{G93A} transgenic mice -a switch to immunoproteasomes, consistent with a substantial presence of microglia at this stage (66). Preliminary data from our laboratory indicate that a decrease in β5 subunit accompanies the early decrease in chymotrypsin-like activity in lumbar spinal cord of these mice. A comprehensive analysis of proteasome composition and assembly at early stages of pathogenesis is required.

2.3 Relevance of Impaired Proteasome Function to Pathogenesis of FALS1

Impairment of proteasomal activity could simply result in accumulation of mutant SOD1 to a critical mass for oligomerization (21) or exert some enzymatic-based toxicity (83, 84). If proteasome-mediated proteolysis is impaired, not only mutant SOD1, but other substrates would accumulate. The proteasome is the major pathway for degradation of transcription factors and other short-lived regulatory proteins, thus proteasomal inhibition could alter transcription of multiple gene families including those that promote cell death (93). Other consequences of proteasomal inhibition include accumulation of oxidized proteins (94) and disruption of mitochondrial homeostasis (95, 96). Motor neurons in $SOD1^{G93A}$ and $SOD1^{G37R}$ transgenic mice do exhibit mitochondrial abnormalities (61, 62, 85-89), although these changes are not prominent in mice expressing $SOD1^{G85R}$ (16). Impaired mitochondrial function and vacuolation have been attributed to accumulation of mutant SOD1 in the intermembrane space (88, 89, 97), but the involvement of proteasomal dysfunction deserves investigation.

2.4 Relevance of Proteasome Function to the Preferential Vulnerability of Motor Neurons in FALS1

Although cells can survive partial inhibition of proteasomal activities (98), cells with the lowest margin of proteasomal capacity relative to substrate load will be most vulnerable to additional stress. Proteasome levels are normally high in motor neurons relative to the surrounding neuropil (59, 99), although proteasomal activities in spinal cord may be lower than in some other tissues (37). Motor neurons are highly sensitive to peptide proteasomal inhibitors, indicating they have a high endogenous substrate load and would be vulnerable to additional stress (29, 37). However, motor neurons cultured from mutant SOD1 transgenic mice did not show increased sensitivity to treatment with proteasomal inhibitors relative to interneurons or to cultures from nontransgenic mice, as would have been expected (100).

The high level of glutamatergic input to motor neurons is a contributing factor to their vulnerability to toxicity, in part due to the presence of calcium-permeable AMPA receptors (11, 101). Formation of inclusions containing mutant SOD1 and death of cultured motor neurons are reduced by AMPA receptor blockers (11). Rapid cycling of AMPA receptor proteins in and out of the synaptic membrane is a determinant of synaptic strength (102-104). Glutamate receptors are tethered in the synaptic membrane by interaction with postsynaptic density proteins. Ubiquitination and subsequent proteasomal degradation of GluR subunits and anchoring postsynaptic density proteins (PSD including PSD95) are important in this process (105, 106). Inhibiting the proteasome prevents disassembly of PSD proteins (107) and internalization of AMPA receptor subunits (108). Thus, it is possible that compromise of proteasomal function could promote excitotoxic injury.

Although SOD1 functions as a dimer, mutant SOD1 is thought to exert toxicity in its monomeric form. Studies *in vitro* show destabilization of the dimer

interface with mutant SOD1 proteins, promoting monomerization and assembly into highly ordered, fibrillar structures (28, 30, 32, 82, 109). Mutant SOD1 could have a different propensity to aggregate in cells according to conditions that promote these events (e.g., reducing conditions, low pH).

Overexpression of HSP70 or a combination of HSP70/HSP40 reduces aggregation of mutant SOD1 in cells including motor neurons (45, 46, 110), yet motor neurons are impaired in stress-induced upregulation of HSPs. This results from lack of activation of the major heat shock transcription factor, HSF1, subsequent to its binding to heat shock promoter elements (12). Differences in the ability of cells to upregulate proteasomal complexes in response to inhibition could also contribute to preferential vulnerability to expression of mutant or damaged proteins.

3. INVOLVEMENT OF THE UPS IN SBMA

SBMA is an adult onset, slowly progressing MND that may be associated with signs of androgen insensitivity (gynecomastia, reduced fertility). This X-linked disease is caused by increased size of a polymorphic, tandem CAG repeat in the coding region of the AR gene (CAG coding for glutamine or "Q") (1) and thus belongs to the group of trinucleotide repeat diseases. The normal number of CAG repeats is 11-36, but extends up to 68 in AR with disease-associated mutations. Because other "polyQ" diseases, including Huntington's disease, are presented in detail in this volume, discussion of SBMA is brief.

As with other diseases in which repeats are located within the coding region of the gene, SBMA is associated with the formation of nuclear and cytoplasmic inclusions containing a N-terminal proteolytic fragment of the mutant protein (reviewed in 111-113). HSPs are associated with inclusions of polyQAR (114) and overexpression of HSPs suppresses both formation of inclusions and death in cultured cells or transgenic mice (110, 115, 116) most likely by sequestering the monomeric protein, maintaining it in soluble form, preventing interaction with other proteins (e.g., CREB binding protein (117) or transaminase (118)), and facilitating degradation through the proteasome (115, 119). Despite the propensity to aggregate, inclusions are not necessary for pathogenesis (118, 120), but nuclear localization is a requirement for neurotoxicity *in vivo* (115, 121).

Both normal and pathogenic AR proteins are proteasomal substrates. Akt and Mdm2 form a complex with AR and promote phosphorylation-dependent ubiquitylation, resulting in degradation of AR by the proteasome (122). Whereas unmodified polyQ-expanded AR may be degraded efficiently (119, 122, 123), modifications, including intramolecular and intermolecular crosslinking, can impair proteasome function. In a study by Mandrusiak *et al.* (118), transaminase catalyzed crosslinking of N-terminal fragments of AR proteins *in vitro* and when co-expressed in HEK cells stably expressing the fluorescent, proteasome-targeted degron, GFP^{u}-1. Expression of N-terminal AR with an expanded number of repeats resulted in accumulation of GFP fluorescence in a ligand-dependent manner. Relevance of transglutaminase reactions to disease pathogenesis is supported by an increase in N^{ε} (γ-glutamyl) isopeptide

bonds detected histochemically in SBMA transgenic mice, in both brain neurons and in motoneuronal inclusions (118). The authors proposed that the proteasome is unable to properly unfold or degrade the crosslinked polyQ-expanded protein because the next cleavage site is too far from the catalytic core and that consequent blockage of the proteasome would compromise degradation of other key substrates. Polyglutamine sequences cannot be digested by eukaryotic proteasomes, rather fragments are released for hydrolysis by unknown peptidases, a process that may be slower for poly Q-containing peptides and may generate fragments with even higher propensity to aggregate (124); this process could be even slower for crosslinked protein fragments.

Cellular toxicity, formation of nuclear inclusions, and proteasome impairment are all promoted by interaction of polyQ-expanded AR with androgen. Conformational changes induced by ligand binding or release from the HSP90 hormone-binding complex could promote cleavage, transport of N-terminal fragments to the nucleus to exert toxicity, and/or crosslinking (118, 125, 126).

4. COMPARISON OF THE TOXICITY OF MUTANT SOD1 AND POLYQ-EXPANDED AR

The nature of the mutations causing these diseases is quite different. Most disease-causing SOD1 mutations are missense and occur throughout all 5 exons of the gene, whereas AR mutations consist of variable expansion of CAG repeat sequences. However, both proteins become detergent-insoluble and have a propensity to aggregate and form inclusions, although this tendency appears stronger for polyQ-expanded proteins. In native form, both mutant SOD1 and polyQ-expanded AR appear to be metabolized efficiently by the proteasome, yet expression of these proteins can abrogate proteasomal function in cells under certain conditions (see above). This points to the importance of post-translational modifications. In the case of polyQ-expanded AR, proteolytic cleavage generates a more toxic N-terminal polypeptide which can more easily enter the nucleus and appears more prone to crosslinking (118, 125, 126). In the case of mutant SOD1, it is not yet clear which modifications or cellular conditions promote oligomerization *in vivo* and which biophysical form(s) inhibit the proteasome. Interestingly, an increased level of an N-terminally truncated form of SOD1 was found in CSF from FALS1 patients homozygous for the D90A mutation, but its origin and biophysical properties are unknown (127).

Because mutations in SOD1 and AR both cause MND, identifying common mechanisms underlying the vulnerability of motor neurons to toxicity could have therapeutic implications. Because HSPs reduce aggregation of both types of mutant protein (45, 46, 110, 115, 116), the high threshold for activation of the heat shock response may be a contributing factor (12). In cultured motor neurons, toxicity of both mutant SOD1 and polyQ-expanded AR are reduced by treatment with AMPA receptor antagonists and by overexpression of the cytosolic calcium-binding protein calbindin (11) (Tradewell and Durham, unpublished data), indicating even normally non-toxic levels of glutamate receptor activation sensitize motor neurons in a calcium-dependent manner.

5. IMPLICATIONS FOR THERAPY OF MND

If depletion of protein chaperones and inhibition of proteasomes are significant in the pathogenesis of protein folding diseases, would upregulating these pathways be a reasonable therapeutic strategy? On the protein chaperoning side, our studies indicate that coordinate upregulation of multiple HSPs, particularly those that work coordinately such as HSP70 and HSP40, is more likely to be beneficial than a single HSP (Batulan et al., unpublished results). Many drugs known to magnify the heat shock response mediated through the transcription factor HSF1 are not particularly effective in cultured motor neurons or are too toxic (12). However, recent studies show potential for hydroxylamine derivatives (48) and celastrol, a quinone methide triterpene and an active component from Chinese herbal medicine (128) as regulators of the heat shock response in an "as needed" manner. Other components of the UPS (e.g., ubiquitin E3 ligases) that can sequester mutant proteins until they can be degraded or facilitate degradation also are potential targets. Whereas increasing proteasome capacity in compromised cells could be beneficial, global upregulation may have serious drawbacks in some tissues, particularly muscle where the proteasome is key to degrading muscle proteins and activation can result in muscle wasting. Interestingly, a clinical trial of the neurotrophic factor, ciliary neurotrophic factor (CNTF) in ALS was halted because of weight loss. A potential cause is upregulation of components of the UPS by CNTF (129).

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17

THE PARADOXICAL ROLE OF PROTEASOMES IN PRION DISORDERS

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1. INTRODUCTION

The transmission of sheep scrapie to cattle as bovine spongiform encephalopathy (BSE) and onward transmission to humans as variant Creutzfeldt Jakob disease (vCJD) has caused increased awareness and a renewed dread of prion disorders, also referred to as transmissible spongiform encephalopathies (TSE) (1, 2). The word prion is derived from proteinaceous infectious particle, symbolizing the transmissible and pathogenic agent implicated in all prion disorders[3]. Prions are thought to be synonymous with an altered form of a cellular protein, the prion protein or PrP^{C}, that assumes an altered conformation referred to as PrP-scrapie (PrP^{Sc}), and accumulates in TSE affected humans and animals. Although intense research for the past two decades has enhanced our understanding of prion disorders considerably, several important questions remain unanswered. The most important of these is whether PrP^{Sc} is indeed the infectious and pathogenic particle, or merely a surrogate marker of

the disease process. Thus, before any effective therapeutic strategies can be developed, we need a clear understanding of several key issues, including; 1) the precise nature of the causative agent, 2) mechanism by which this agent causes neuronal damage, 3) whether infectious and the pathogenic agents are similar or distinct, and 4) the normal function of PrP^{C}, the underlying agent implicated in these disorders (1-5). Among this confusion, proteasomes have emerged as one of the players implicated in facilitating the neurotoxicity and replication of the pathogenic and the infectious particle. Needless to say, such a role for proteasomes was unexpected given the cellular location of PrP^{C}, which happens to be a membrane bound rather than a cytosolic protein (6-8). Though still enigmatic and highly controversial, several recent studies have clarified the role of proteasomes in prion disorders to some extent (9), and will be the main focus of this review.

2. PRION PROTEIN: THE FRIENDLY FOE

The pathophysiology of prion disorders is both fascinating and perplexing. According to the prion hypothesis, PrP^{Sc} is the main culprit that catalyzes the conversion of host PrP^{C} from a mainly α-helical structure to the pathogenic β-sheet rich PrP^{Sc} form. Unlike PrP^{C}, PrP^{Sc} is insoluble in non-ionic detergents, resists limited digestion by proteinase-K (PK), and oligomerizes into amyloid (1–7, 10). Deposits of PrP^{Sc} in the brain parenchyma are believed to be the principal cause of neuronal death in these disorders (4, 5). However, prion disorders often occur in the absence of detectable PrP^{Sc}, and deposits of PrP^{Sc} have been identified in the absence of disease, raising legitimate doubts about whether PrP^{Sc} is identical with prion.

Like other chronic neurodegenerative disorders, prion disorders affect older individuals, and are invariably fatal. The affected individuals present with clinicopathological syndromes rather than symptoms reflective of individual disorders. Common symptoms include rapidly progressive multifocal dementia with myoclonus, pyramidal and extrapyramidal signs, cerebellar ataxia, cortical blindness, thalamic degeneration, sleep disorders, and a variety of other symptoms that vary with the etiology of the disorder. Sections of prion brains typically show a spongiform change, and hence the name "Transmissible Spongiform Encephalopathies". Some cases present with additional pathological manifestations in the brain parenchyma such as amyloid plaques, astrocytic gliosis, and neurofibrillary tangles, whereas others show a more variable picture (8). Figure 1 exemplifies the spogiosis and neuronal loss associated with PrP^{Sc} deposits from a case of sporadic CJD.

Prion disorders affect both humans and animals, and occur as infectious, inherited, and sporadic disorders. Human prion disorders include Gerstmann Straussler Scheinker disease (GSS), Creutzfeldt Jacob disease (CJD), fatal familial insomnia (FFI), and variant Creutzfeldt Jacob disease (vCJD). Among animals, prion diseases include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in deer and elk, and mink encephalopathy. Prion disease has also been detected in captive wild ruminants, rare zoonotic species, and in domestic cats. Among the human disorders, GSS, CJD, and FFI are inherited as autosomal dominant diseases and manifest in old

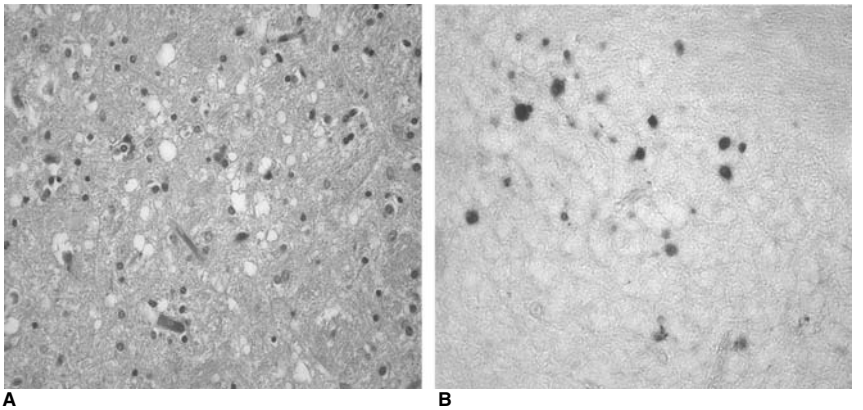

Figure 1. **(A)** Spongiform change in the brain section of a case of sporadic CJD (hematoxylin and eosin stain). **(B)** Immunostaining with anti-PrP antibody 3F4 shows PrP-reactive darkly stained aggregates in the parenchyma.

age. Variant CJD is acquired through infection and manifests at a much younger age (10). In the inherited forms, a point mutation in the prion protein gene is believed to destabilize the protein and promote its structural change to the pathogenic form. In infectious forms such as vCJD, this change is induced by exposure of PrP^{C} to exogenous PrP^{Sc}. In sporadic forms, the transformation of PrP^{C} to PrP^{Sc} is a spontaneous, random event. Following the initial transformation, subsequent conversion of additional PrP^{C} molecules to PrP^{Sc} occurs by an autocatalytic mechanism (3, 5). Two possible mechanisms of prion replication have been proposed; 1) template-mediated conversion, and 2) the nucleation dependent polymerization, depicted diagrammatically in Figure 2 below. In the first model, a molecule of PrP^{Sc} unfolds partially, binds to PrP^{C}, and changes its conformation to a likeness of its own. According to the nucleation polymerization model, PrP^{C} and PrP^{Sc} conformations are in thermodynamic equilibrium. If several monomeric PrP^{Sc} molecules form an ordered 'seed', further accumulation of additional molecules is rapid, eventually forming an aggregate (11). The stimulus that initiates the conversion process in either of the above cases is presently unclear.

It has been established beyond reasonable doubt that PrP^{C} is essential for the development of prion disorders, whether of infectious, inherited, or sporadic etiology. In fact, transgenic mice lacking PrP^{C} are resistant to prion infection even when inoculated intra-cerebrally with PrP^{Sc}, lending support to the notion that PrP^{C} is the main player in these disorders (12, 13). In this regard, PrP^{C} is similar to some of the other proteins that take on a toxic role due to a mutation or a metabolic event such as *huntingtin* and *synuclein* (14, 15), but differs from all known disorders of protein misfolding and aggregation in propagating its own conformation long after the stimulus is removed. Prion disorders, therefore, present a double challenge when it comes to the development of therapeutic strategies;

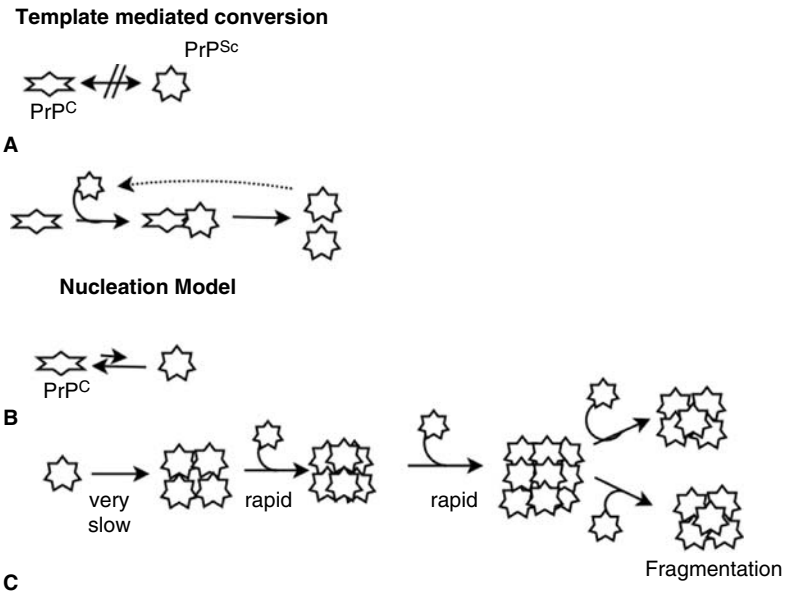

Figure 2. Models of PrP^{Sc} replication. **(A)** The template-mediated conversion model proposes that an interaction between exogenous PrP^{Sc} and host PrP^{C} induces the latter to transform into an exact replica of PrP^{Sc}. **(B and C)** The nucleation polymerization or seeding model proposes that PrP^{C} and PrP^{Sc} are in reversible thermodynamic equilibrium. If the equilibrium shifts towards PrP^{Sc} due to an exogenous or presently undefined stimulus, several monomeric PrP^{Sc} molecules form and ordered seed, on which further accumulation of PrP^{C} occurs rapidly. Fragmentation of this seed increases the number of ordered seeds, and replication continues in an exponential manner. (Modified from ref. 11)

first, effective ways of blocking exposure to exogenous PrP^{Sc} need to be developed, and second, the conversion of endogenous PrP^{C} to additional PrP^{Sc} molecules needs to be blocked. Given the resilience of PrP^{Sc} to sterilization, the former effort has not been very successful so far. As for the latter, there is fragmented information on the mechanism by which PrP^{C} converts to PrP^{Sc}, making it equally difficult to block this process. In an effort to eliminate this 'friendly foe', some scientists are trying to develop PrP^{C} knockout cattle, hoping that these will be resistant to prion infection and make beef a safe food product again. Although this strategy may partly resolve one problem, this is certainly not the cure for all prion disorders.

3. THE PRION PUZZLE; OR IS IT?

Unlike other neurodegenerative disorders, prion diseases have baffled the scientific community by their mixed etiology. It is quite intriguing that these diseases are infectious in the absence of detectable nucleic acid(s), and affect mammals as far apart as mice and men (3, 4). The intense focus on prion research by the scientific community for the past decade is not merely driven by fascination. The transmission of sheep scrapie to cattle and subsequently to humans as vCJD

has already taken 155 lives in the United Kingdom, and almost crippled the beef industry (16). The recent spread of chronic wasting disease, a prion disorder of cervids in North America has caused considerable apprehension due to the potentially dangerous risk of transmission to farm animals grazing on overlapping grounds (17). Such an occurrence would contaminate several consumable supplies with infectious prions. Needless to say, the urgency to understand, develop a workable treatment plan, and prevent the spread of these disorders in animals and humans is probably more vital today than it was a decade ago.

Several times it appears that we understand prions completely, only to realize with the next report how this mysterious agent has fooled us again! For example, despite rigorous research by several groups, it is still unclear whether PrP^{Sc} is the only component of the infectious and pathogenic particle. Different hypotheses propose PrP^{Sc} as an integral part of such an agent, partially overlapping with the agent, an upshot of the disease process, or just a reliable surrogate marker of prion disease and infectivity. Other studies implicate normal PrP^{C} itself in prion disease pathogenesis. It appears that in addition to serving as a substrate for PrP^{Sc} replication, PrP^{C} may actively induce neuronal death by activating certain cell death pathways (18). Alternately, loss of PrP^{C} function may contribute to neuronal demise either directly or indirectly (19, 20). In this report, we review the normal biogenesis of PrP^{C}, and conditions under which the aberrant metabolism of PrP^{C} may induce neurotoxicity. More importantly, we discuss where and how the proteasomes are involved in prion disease pathogenesis. Although several pieces of the prion puzzle are together, some critical pieces are still missing. Proteasomal involvement happens to be one of the latter.

4. BIOGENESIS OF PrP^{C}

In order to understand the replication and pathogenic potential of PrP^{Sc}, much attention has been focused on PrP^{C}, the immediate substrate of PrP^{Sc}. At the outset, the biogenesis of PrP^{C} appears quite mundane. PrP^{C} is a glycoprotein linked to the cell surface by a glycosylphosphatidyl inositol (GPI) anchor. Like other secretory proteins, PrP^{C} is synthesized in the endoplasmic reticulum, where the N-terminal signal peptide is cleaved co-translationally, and the C-terminal GPI signal peptide is removed in a transamidation reaction with the concomitant addition of a pre-assembled GPI anchor. Simultaneously or in a subsequent reaction, immature high mannose glycans and the disulfide bond are added, and modified PrP^{C} is transported along the secretory path to the cell surface (Figure 3A). During its transit through the Golgi, N-linked glycans are modified further to achieve their mature highly sialylated form. At the cell surface, PrP^{C} is linked to the outer leaflet of the plasma membrane by the GPI anchor. As part of its normal life cycle, PrP^{C} undergoes recycling from the plasma membrane, and is cleaved by disintegrins ADAM10 and TACE in an endosomal compartment resulting in a C-terminal 18kDa fragment that is transported back to the plasma membrane (21, 22). Under steady state conditions, ~60% of the cell surface PrP^{C} is represented by the C-terminal 18kDa fragment (3, 6, 7). It is pertinent to mention here that the 18kDa fragment is distinct from the PK-digested C-terminal PrP^{Sc} that is ~19-29kDa (3). The amino terminus of the 18kDa

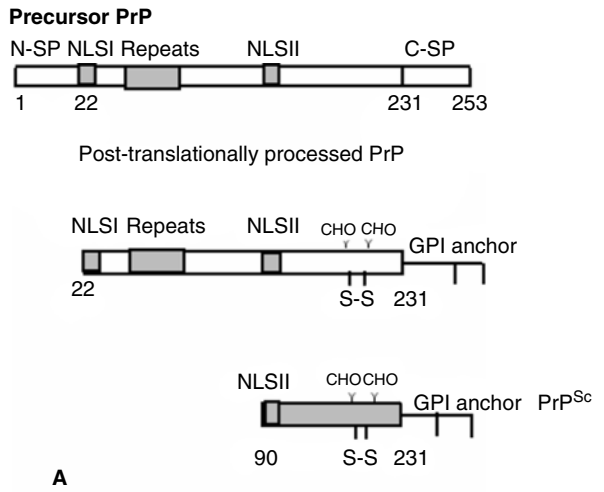

Figure 3. **(A)** Precursor PrP contains two signal sequences; N-terminal signal peptide is cleaved co-translationally, whereas C-terminal signal peptide is removed in a transamidation reaction with the concomitant addition of a GPI anchor. Simultaneously, or in a subsequent reaction, N-linked glycans and a disulfide bond are added to the polypeptide. PrP contains two nuclear localization signals at residues 23-28, and 101-106. **(B)** Transmembrane forms of PrP are inserted in the lipid bilayer through residues 112-136. ^{Ctm}PrP is inserted with the C-terminus facing the ER and the N-terminus in the cytosol. This form is glycosylated, is believed to contain an intact N-signal peptide, and is GPI-linked. ^{Ntm}PrP has the opposite orientation, is not glycosylated, and does not contain the N-signal peptide or the GPI anchor. Cytosolic forms represent PrP molecules that fail to translocate into the ER and thus contain the N-signal peptide, and misfolded retro-translocated molecules that do not contain the N- or C-signal peptides.

fragment is at residues 111/112, and that of PrP^{Sc} fragment is at or near residue 90, and includes the amyloidogenic 106-126 region of PrP (Figure 3A).

Alternate processing of the nascent PrP polypeptide can result in forms that are inserted in the lipid bilayer through the hydrophobic transmembrane domain comprising of residues 112-136 of PrP (23, 24). Transmembrane PrP forms

can assume two different topologies at the ER membrane. The C-transmembrane form (^{Ctm}PrP) is oriented with its C-terminus in the ER lumen, and N-terminus in the cytosol. This form is glycosylated, GPI-linked, and is believed to maintain an uncleaved N-terminal signal peptide in the cytosol 7, 23, 24). The N-transmembrane form has the opposite orientation, with N-terminus of PrP in the ER lumen and C-terminus in the cytosol (^{Ntm}PrP) (23, 24). Information about any post-translational modifications on ^{Ntm}PrP is presently lacking (Figure 3B). Mammalian PrP^C has a half-life of ~6 hours, following which it turns over in the lysosomes. A small but significant proportion of PrP^C is also degraded by the proteasomes under steady state conditions. Transmembrane forms of PrP are believed to degrade exclusively by the proteasomal pathway (7). Some studies report the transport of ^{Ctm}PrP to the Golgi and possible turnover by the lysosomes, whereas others report the generation of a metabolic product of ^{Ctm}PrP at the ER membrane that is transported to the cell surface, and is possibly degraded by the lysosomes (23, 24, 60). More recently, cytosolic forms of PrP have been described that are cytotoxic, and under certain circumstances become infectious if spared by the proteasomal degradation machinery (43, 44). Certain mutant PrP forms are retrotranslocated to the cytosol, where they exert cytotoxicity by presently undefined mechanisms (42–45). Cytosolic forms that fail to translocate into the ER have an intact N-signal peptide, whereas abnormal PrP forms that are retro-translocated from the ER have lost the N-signal peptide (Figure 3B). In addition, two nuclear localization signals are present in post-tranlationally processed PrP between residues 23-28, and 101-106 (Figure 3A).

Several steps in the biogenesis of PrP^C (Figure 4) shed light on the process of PrP^{Sc} replication and accumulation, and the underlying pathogenic process: 1) With the help of cell culture models, it has been established that expression of PrP^C on the plasma membrane is essential to infect cells with PrP^{Sc}. Exogenous PrP^{Sc} is believed to interact with host PrP^C in sphingolipid rich microdomains or in an endocytic compartment, and initiate its conversion to PrP^{Sc} at the plasma membrane or in an endocytic compartment (3, 7). 2) Proteolytic processing of PrP^C to the C-terminal 18kDa fragment is believed to protect the transformation of PrP^C to PrP^{Sc} due to disruption of the amyloidogenic 106-126 region (7). 3) Mutant PrP forms are sequestered in different cellular compartments depending on the point mutation in the PrP gene. Some forms aggregate in the ER, whereas others accumulate in more distal compartments of the secretory path. Some, instead, are retrotranslocated to the cytosol for proteasomal degradation. Each mutant PrP appears to initiate cytotoxicity by a distinct mechanism. For example, mutant forms aggregated in the ER can induce the aggregation of additional mutant or normal PrP^C molecules by the nucleation dependent phenomenon (25, 26). If the C-terminal GPI signal peptide is not removed due to a mutation or misfolding, PrP^C is retained in the ER and degraded by the proteasomal pathway. Other, more specific pathways of neurotoxicity by different PrP forms are described below. 4) The C-transmembrane form and cytosolic forms of PrP are believed to mediate neurotoxicity by presently unknown mechanisms in both inherited and infectious prion disorders. In this regard, it is interesting to note that manipulation of the N-signal peptide can alter the ratio of different topological forms of PrP^C, further linking the processes of biogenesis and toxicity. 5)

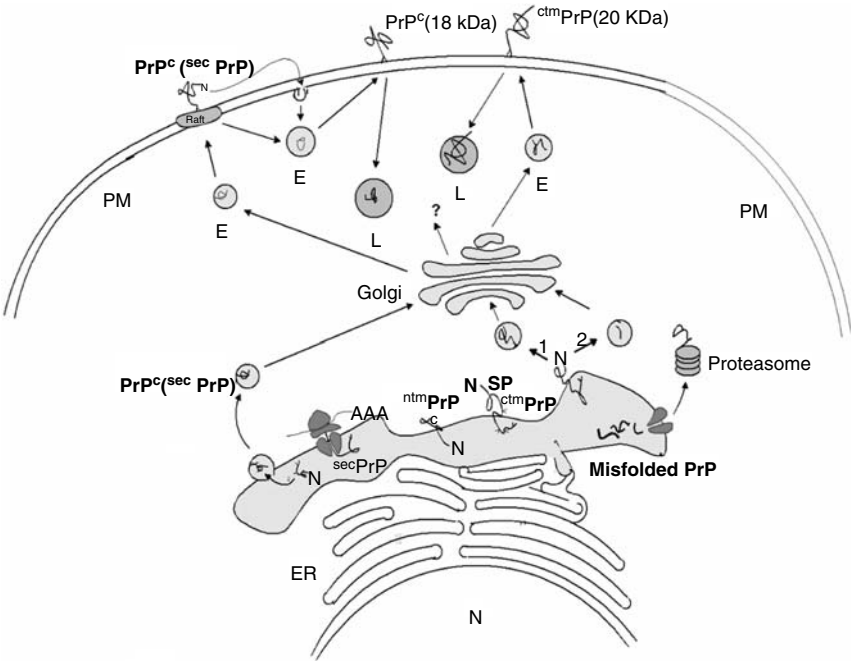

Figure 4. Biogenesis of PrP^C: PrP is synthesized in three distinct topological forms at the ER membrane. SecPrP or PrP^C is translocated fully into the ER lumen and is transported along the secretory path. This form is linked to the plasma membrane by a GPI anchor. During constitutive rounds of recycling through lipid rafts or clathrin-coated pits, PrP^C is truncated, and the C-terminal 18kDa fragment is transported back to the plasma membrane. CtmPrP is either retained in the ER (1), truncated at ~residue 90 and the C-terminal fragment is transported to the plasma membrane (2), or is transported to the Golgi apparatus, beyond which its fate is uncertain. The fate of NtmPrP is unclear. Misfolded PrP forms are retro translocated to the cytosol and degraded by the proteasomes. PM: plasma membrane; E: endosome; L: lysosome; ER: endoplasmic reticulum; N: nucleus.

It has also been reported that over-expression of PrP^C itself can induce cell death by caspase-3 activation, and in stark contrast, another study reports a neuroprotective function of PrP^C since it protects human primary neurons against Bax-mediated cell death (1, 7, 18-21).

In addition, since the normal function of PrP^C is obscure (27), it is difficult to judge whether the disease is caused by accumulated PrP^{Sc}, or by the depletion of PrP^C. Given the abundance of PrP^C on all cells, especially on cells of the central nervous system and its conservation in all mammals through evolution, it is difficult to envision that PrP^C would have no important function to serve. Thus, the disease could result from either gain of function by conversion to PrP^{Sc}, or loss of function of PrP^C. Further investigations are needed to resolve this issue. As our understanding of the biogenesis of normal and mutant PrP improves, the underlying pathways of neurotoxicity in both familial and infectious prion disorders will become evident. Although at this time several diverse pathways of neurotoxicity seem to exist under different

conditions, a final common pathway of neurodegeneration must exist in all prion disorders in order to explain the similar clinical outcome in both animal and human forms of this disease.

5. PRIONS AND THE CELLULAR QUALITY CONTROL

Prion protein is one more protein in the growing list of misfolded and aggregated cellular proteins implicated in neurodegenerative disorders. Under steady state conditions, a certain percentage of proteins usually fail to fold properly following synthesis. This percentage rises if the protein has a mutation that interferes with folding. To avoid accumulation of such misfolded and potentially toxic proteins, cells have developed sophisticated mechanisms to ensure correct folding and targeting of polypeptides, and expend a considerable amount of basal metabolic energy in eliminating improperly folded proteins. Several checkpoints have been developed along the secretory path to ensure the highly selective nature of this process, beginning with the ER. Only proteins with a mature, transport-competent secondary structure are allowed to exit the ER. The rest are sequestered in the ER, and await degradation by the proteasomes (6, 28). However, when considered in the context of prion protein, the ER quality control has unusual implications. Misfolded PrP in the ER can not only initiate cytotoxicity, but can mediate the co-aggregation of additional, normal PrP molecules on the aggregated 'seed', thereby amplifying the toxic signal (25). Thus, the various mechanisms by which the ER quality control copes with and exonerates itself of misfolded or aggregated prion protein molecules has serious ramifications for the disease process.

Unlike PrP^{C}, PrP with point mutations in the coding region follows diverse routes of synthesis and turnover. More than thirty different mutations in PrP have been described, all of which segregate with the familial prion disorders GSS, CJD, and FFI (Figure 5). These disorders are classified based primarily on the phenotypic presentation (5).

Unlike other misfolded proteins, mutant PrP is instrumental in mediating cytotoxicity by either undergoing a transformation to PrP^{Sc}, or by alternative, poorly understood pathways. Depending on the location of a specific mutation in the PrP coding region, distinct post-translational anomalies are conferred on mutant PrP, altering its transport and processing in distinct ways. The important

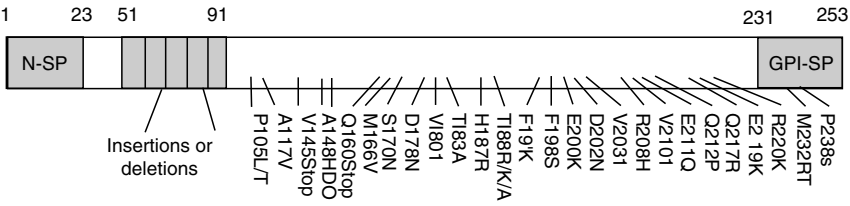

Figure 5. A diagrammatic representation of pathogenic mutations in the human prion protein gene. Some mutations have been identified even in the C-signal peptide of PrP that is absent in the mature protein.

determinants governing PrP folding, transport, and turnover include: 1) Cleavage of the N- and C-signal peptides (26), 2) addition of the GPI anchor, 3) glycosylation at one or both N-glycan sites (29), 4) C-terminal domain of PrP which is eliminated in certain stop codon mutations (30), and 5) the folding state of mature PrP. Within the ER, cellular chaperones assist in folding, prevent the aggregation of abnormal PrP forms, and attempt to refold denatured molecules. If attempts at refolding fail, most of the misfolded PrP forms are retrotranslocated for degradation by cytosolic proteasomes. Often abnormal PrP forms are isolated in inclusions or aggresomes that are perinuclear structures surrounded by a vimentin cage, and located at the centriole (31). Aggresomes are also noted if PrP^{C} expressing cells are exposed to cyclosporin-A in the absence of proteasomal inhibitors, and in disease associated PrP102L and PrP105L mutations if proteasomal function is inhibited (32). PrP forms sequestered in these structures often acquire partial protease-resistance, similar to PrP^{Sc}. Although aggresomes are portrayed as the cellular defense mechanism to protect the cell from harmful effects of misfolded proteins, often these aggregates themselves prove to be toxic. In long-lived cells like neurons, accumulation of such misfolded proteins over time may initiate neuronal death by overwhelming or poisoning the proteasomal system, or by triggering the cellular stress response. An ER stress response and caspase 12 activation has been reported in mouse neuroblastoma cells infected with PrP^{Sc} (33). Thus, proper functioning of the molecular chaperones and the proteasomal degradation pathway play a particularly important role in prion disorders.

6. HOW ARE PROTEASOMES IMPLICATED IN PRION DISORDERS?

The proteasomes are mostly involved in the degradation of cytosolic and nuclear proteins, not membrane linked glycoproteins like PrP. The involvement of proteasomes in the turnover of PrP was first noted with the report that the mutant prion protein in GSS associated with the Y145stop mutation is retrotranslocated to the cytosol and degraded by the proteasomes. In this specific case, inhibition of proteasomal function led to the accumulation of mutant PrP145stop in the nucleus (34). Following this report, PrP160stop was also shown to accumulate in the nuclei of transfected cells (35). A detailed analyses of the signals responsible for the transport of mutant or truncated PrP to the nucleus instead of its normal transport to the cell surface showed that the N-terminal region of PrP contains two nuclear localization signals (NLS). The N-terminal fragments of PrP including residues 1-114 and 1-180 are retrotranslocated from the ER and transported to the nuclei of transfected cells. PrP 1-180 localizes to the nucleus even in the absence of proteasomal inhibitors. On the other hand, N-terminal fragments 1-190 and 1-200 accumulate in the ER. Thus, the presence of one or two glycans masks the NLS and prevents the transport of C-terminally truncated PrP forms to the nucleus (36). Onward transport along the secretory path of course is subject to proper folding and addition of the GPI anchor (Figure 6). Since a variety of N-terminal fragments are generated during the normal and aberrant processing of PrP^{C} and mutant PrP forms, nuclear accumulation of these fragments could be potentially hazardous to cell health. Within the nucleus, PrP has been shown to bind nucleic acids. In a recent

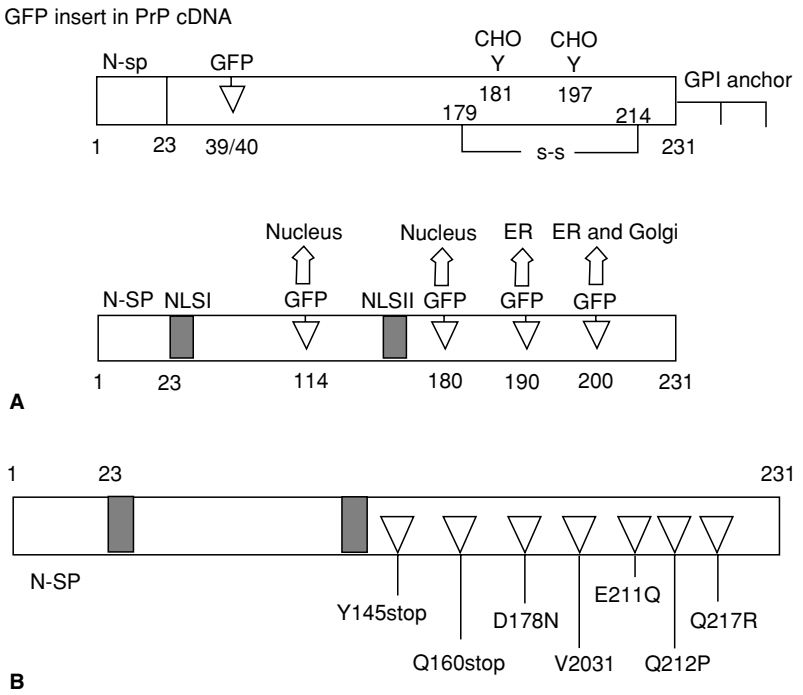

Figure 6. **(A)** To track PrP^{C} through the cell, a green fluorescent protein tag was inserted between residues 39 and 40 of PrP. GFP-tagged PrP^{C} is processed normally, and is linked to the plasma membrane by the GPI anchor. Truncation of PrP at residue 114 or 180 leads to the accumulation of these N-terminal fragments in the nucleus of transfected cells. Inclusion of one or two N-glycans by extending the N-terminal fragment to residues 190 or 200 results in the accumulation of these forms in the ER. **(B)** Disease associated mutant PrP forms identified so far that are degraded by the proteasomal pathway.

report, PrP^{Sc} with all its glycans has been shown to accumulate in the nucleus and bind to chromatin, possibly influencing transcription in infected cells (37). However, PrP aggregates have not been observed in the nuclei of cells in sections prepared from prion-infected brains. Although this may appear as a major caveat, one must keep in mind that prion brains represent the ultimate outcome of past events, not the underlying processes that lead to that outcome.

Similarly, disease-associated mutant PrP forms accumulate in distinct cellular compartments based on the particular post-translational abnormality conferred by the mutation. Aberrantly glycosylated forms are retained within the ER and degraded by the proteasomes. Examples include CJD associated with T183A, where abnormal forms are not transported out of the ER (38, 39). In the case of CJD E200K, abnormally glycosylated PrP exits the ER, but accumulates in the lysosomal compartment (28). In some cases aberrantly glycosylated PrP has been reported to assume a PrP^{Sc}-like conformation. Mutant PrP in GSS Q217R is diverted to the lysosomes where it assumes a partially protease-resistant conformation (40). The GPI anchor, in addition to its mundane function as a

mere anchor, plays a critical role in promoting PrP folding and transport (41). Although a deficiency of the anchor by itself does not cause a significant disruption in the normal processing and transport of PrP, its absence due to persistence of the GPI signal peptide disrupts PrP transport. PrP with the uncleaved GPI signal peptide is retained in the ER in association with the chaperone BiP, and is targeted for proteasomal degradation. Although the efforts at refolding this form are ultimately futile, the association with BiP keeps this abnormal PrP in a relatively soluble state in the ER until its turnover by the proteasomes (26). This is in contrast to the observations reported on CJD and FFI associated with 178N, and in CJD associated with PrP mutations at codons 203, 211, and 212, where retrotranslocated mutant PrP accumulates in the cytosol of COS and neuroblastoma cells in aggresome-like structures (31, 42-44). In addition, interference with disulfide bond formation or glycan addition to normal PrP induces its transformation to a PrP^{Sc}-like form, suggesting that a reducing and deglycosylating environment in the cytosol may be conducive to PrP aggregation (45).

Thus, a decline in proteasomal function in advanced age could initiate or enhance toxicity by normal or mutant PrP through several potential mechanisms: 1) in the cytosol, PrP could form aggregates or cause direct cytoxicity, 2) in the

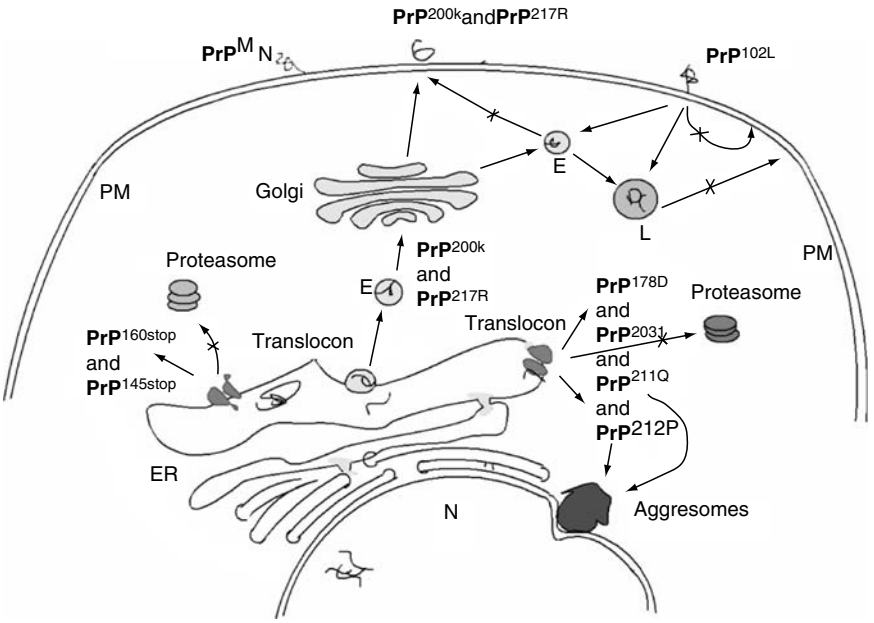

Figure 7. Procesing of mutant PrP forms: Inhibition of proteasomal function results in the accumulation of mutant PrP 178D, 203I, 211Q, and 212P in aggresome-like structures in the cytosol, and of PrP145stop and 160stop in the nucleus. PrP32 with an intact GPI signal peptide is retained in the ER. PrP217R and PrP200K are targeted mainly to the lysosomes, whereas PrP102L shows inefficient recycling from the PM. Several other mutant PrP forms acquire resistance to PI-PLC cleavage at the plasma membrane. PM: plasma membrane; E: endosome; L: lysosome; AG: aggresome; T: translocon; ER: endoplasmic reticulum; N: nucleus.

lysosomes, low pH could induce a change in its conformation to PrP^{Sc}, with consequent cellular toxicity, 3) in the nucleus, binding of PrP to nucleic acids could induce or suppress the transcription of a variety of genes that may initiate cell death by different mechanisms. Efficient proteasomal function, on the other hand, may prove to be the cure all for at least a subset of these disorders.

7. PROTEASOMES AND CYTOSOLIC PRP

By far, the report by Ma and Lindquist caught the most attention by demonstrating that suboptimal proteasome function is the key event in both inherited and infectious prion disorders. These authors reported that transgenic mice expressing PrP in the cytosol developed a prion-like neurodegenerative disorder. There was severe loss of cerebellar granule neurons and gliosis in these mice. Furthermore, the study went on to show that even small amounts of PrP in the cytosol are toxic. If the translocation of PrP to the cytosol is rapid, some of the PrP molecules transform to the PrP^{Sc} conformation, resulting in the generation of infectious PrP^{Sc} molecules (42-44). While several aspects of this study are ingenious and informative, others have limitations. For example, the toxic nature of cytosolic PrP explains the cytotoxicity observed in certain cell lines expressing mutant PrP forms that are subject to ERAD. Such a situation may arise in vivo when proteasomal function deteriorates with age. Alternately, intracellular aggregates of PrP^{Sc} may inhibit proteasomal function, as reported for other proteins that have the propensity to aggregate (46, 50). In both instances, compromised proteasomal function would affect cell health. It has also been suggested that some mutant forms of PrP accumulate in the cytosol due to impaired translocation into the ER either due to inefficient function of N-signal peptide of PrP, or lack of the C-terminal domain that is necessary for import into the ER (29, 47). There has been a suggestion that some of the results reported by Ma et al. (41-43) are due to an artifactual upregulation of PrP mRNA due to the high level of PrP expressed in these cells, and the use of proteasomal inhibitors**.** Based on this study, neither PrP^{C} nor any of the mutant PrP forms are retro-translocated from the ER (48). However, evidence from several groups indicates the accumulation of normal or mutant PrP in the cytosol in the absence or presence of proteasomal inhibitors, leaving little doubt that PrP is subject to retro-translocation from the ER under certain conditions. A clear example is PrP with the 145stop mutation. This mutant PrP is synthesized in two forms, a signal peptide containing form of 15.5kDa, and a signal peptide cleaved form of 14kDa. Pulse chase analysis of newly synthesized protein shows a precursor-product relationship between the 15.5 and 14kDa for, indicating that the signal peptide is cleaved from the 15.5kDa form following the chase. More importantly, the 14kDa form accumulates if a similar evaluation is carried out in the presence of proteasomal inhibitors, indicating that PrP145stop is retro-translocated from the ER, where the signal peptide is cleaved (33). Similarly, the N-signal peptide is cleaved in the PrP1-180 form before it accumulates in the nucleus, implying that it was first translocated into the ER. Although the N-signal peptide may be cleaved within the translocon as mentioned by Range et al. (46), the C-signal peptidase is localized to the ER lumen, and PrP with an uncleaved C-signal peptide is retained within the ER.

Thus, if the C-signal peptide has been removed from cytosolic PrP, it is a strong indication of retro translocation rather than a failure to achieve ER tranlocation. A careful analysis of different parameters is required to resolve this issue. Regardless of whether PrP fails to undergo translocation or is retro-translocated from the ER, these forms may add to the burden of cytosolic misfolded proteins and affect proteasomal function, or cause impaired cellular function by interacting with the ER membrane through the uncleaved N-SP (29).

As to the propagation of infectious PrP^{Sc} in the cytosol and further dissemination to other cells, though attractive, this model has several caveats. Although the authors demonstrated a PrP^{Sc}-like characteristic of cytosolic PrP, this species is not infectious. In the case of infectious prion disorders, it is difficult to envision how exogenous PrP^{Sc} would gain access to the cytosol, induce the conformational change of additional PrP^{C} molecules, and spread to neighboring cells. Moreover, the deposits of PrP^{Sc} observed in prion brains constitute fully glycosylated forms that are GPI linked and disulfide bonded. These modifications are highly unlikely to persist if PrP^{Sc} is generated in the cytosol.

A different viewpoint suggests that cytosolic PrP is in fact protective for neuronal cells. When expressed in human primary neurons, PrP interacts with Bax and prevents against Bax-mediated apoptosis (20, 49). Perhaps the apparently controversial findings of PrP retro-translocation and the protective vs the cytostoxic role of cytosolic PrP can be explained by the complex environment to which PrP is exposed in the cytosol. For example, cytoplasmic chaperones influence the folding and processing of newly synthesized rhodopsin by undergoing crenellation and inserting in the ER membrane. In the case of CFTR, Hsp70 targets aberrant forms for ubiquitination and proteasomal degradation with the help

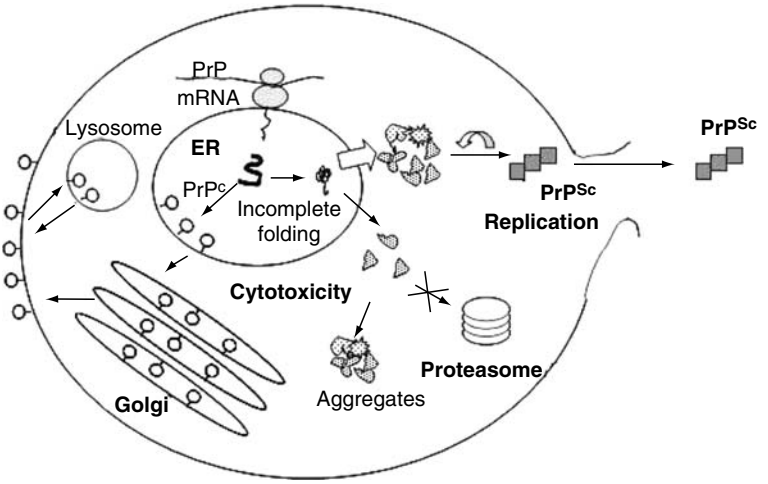

Figure 8. Model representing cytotoxicity and replication by cytosolic PrP: Failure to translocate into the ER or retro translocation from the ER to the cytosol of even small amounts of PrP causes cytotoxicity. If the accumulation of PrP in the cytosol is rapid, it transforms into PrP^{Sc} and induces the conformational change in additional PrP molecules, thus initiating the infectious process. (Adapted from Ma et al, (Ref 42))

of co-chaperone CHIP (51, 52). In addition, with the help of farsenylated DnaJ protein, Hsp70 is believed to facilitate the biogenesis of CFTR (51). A similar interaction of PrP with cytosolic and ER chaperones and the complex but delicate balance between cytosolic and ER chaperones and the proteasomes may very well produce the confusion that is apparent in the field at this time. Future studies aimed at identifying specific steps in the processing and turnover of various PrP forms of will help in resolving this issue.

Despite the controversies, proteasomes are undeniably involved in prion disorders, as demonstrated by the identification of ubiquitin tagged PrP^{Sc} and an overall increase in ubiquitinated proteins in the brains of mice in the terminal stages of prion disease and in prion affected human brains (53, 54). Ubiquitination of PrP^{Sc} in infected mouse brains occurs after the development of protease-resistance, suggesting that aggregates of PrP^{Sc} adversely affect proteasome function, much like the inhibitory effect of other proteins that accumulate in the ER (46, 55). However, one could argue that inhibition of proteasomal function leads to the intracellular accumulation of PrP^{Sc} aggregates, much like the "Sometimes the chicken, sometimes the egg" report (56). It will be informative to know whether proteasomal dysfunction or PrP aggregation occurs first, so that concerted efforts can be directed at inhibiting that phenomenon to prevent neuronal toxicity.

8. PROTEASOMES AND THE CASE OF TRANSMEMBRANE PRP

An equally thought provoking and contentious idea is that neurotoxicity in both inherited and infectious prion disorders is mediated by a transmembrane form of PrP. It has been demonstrated, quite convincingly, that PrP is synthesized in three topological forms and that the C-transmembrane form induces neurotoxicity (23, 24). In certain inherited disorders where mutations in PrP lie within the transmembrane region, mutant PrP integrates into the lipid bilayer through its transmembrane domain, resulting in cellular toxicity. In infectious disorders, it has been proposed that aggregates of PrP^{Sc} upregulate the synthesis of C-transmembrane PrP, thus inducing neuronal death (23, 24). The common denominator in either case is the upregulation of C-transmembrane PrP, which also occurs if proteasomal function is inhibited. Since the first report demonstrating the neurotoxic effect of C-transmembrane PrP in transgenic mice representing a model of inherited PrP with a mutation at codon 117 and an infectious model of mouse scrapie, several investigators have reported data in favor of, and against this unifying model of PrP neurotoxicity. Using cell culture models, Stewart and Harris demonstrated that the amounts of cytosolic and transmembrane PrP are not altered in cells infected with PrP^{Sc}, making these PrP forms unlikely intermediates of neurotoxicity in familial or infectious prion disorders (57-59). Subsequently, other reports were published demonstrating the determinants of PrP topology, and several regions in the PrP sequence were shown to influence the proportion of PrP synthesized in a particular topological conformation. However, the mechanism by which C-transmembrane PrP causes neurotoxicity remains unclear, although it has been suggested that it may translocate into the cytosol and induce toxicity in that locale. A 20kDa C-terminal proteolytic product of ^{Ctm}PrP has been shown to accumulate when proteasomes are inhibited experimentally (Figure 9), or in the presence of PrP102L

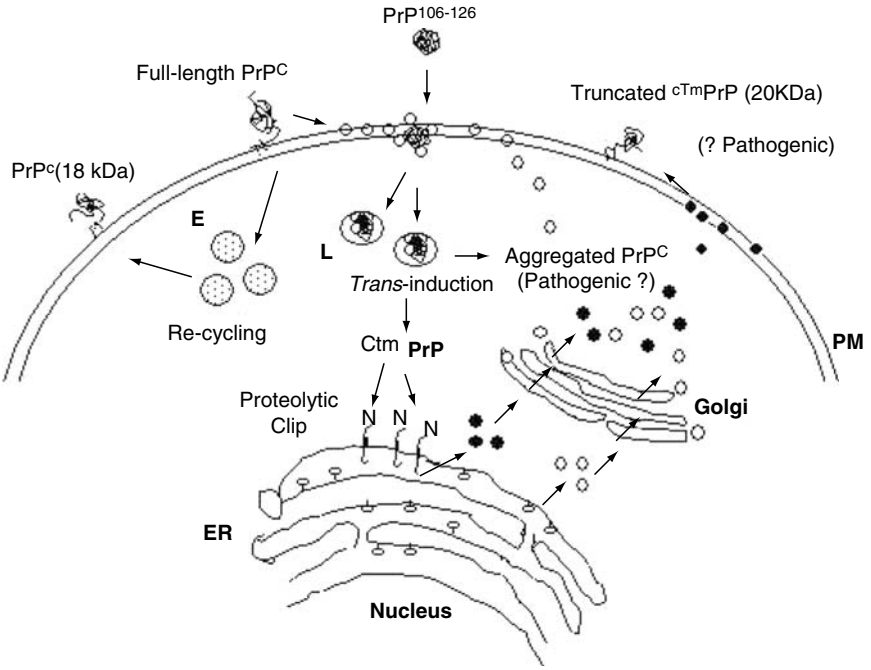

Figure 9. Proposed model of PrP^{C} aggregation and induction of ^{Ctm}PrP: Micro-aggregates of $PrP^{106-126}$ bind to the plasma membrane and initiate the aggregation of PrP^{C}. Aggregated proteins are endocytosed in large vesicular structures and transported to lysosomes. Intracellular aggregates of PrP induce the synthesis of ^{Ctm}PrP through presently unknown *trans*-activating factors. Subsequently, the N-terminus of ^{Ctm}PrP is cleaved by a cytosolic protease, and the C-terminal 20kDa fragment is transported to the cell surface where it is inserted in the plasma membrane by the GPI anchor. PM, plasma membrane; ER, endoplasmic reticulum. (Adapted from Ref 60).

mutation associated with GSS (60). Interestingly, upregulation of the 20kDa fragment and by inference ^{Ctm}PrP is also noted if aggregates of PrP accumulate in the ER or in an endocytic compartment (25, 61), supporting the earlier report that intracellular accumulation of PrP aggregates or exposure of PrP^{C} expressing cells to extra cellular aggregates of PrP^{Sc} up regulates ^{Ctm}PrP. Proteasomes play a significant role in this pathway since ^{Ctm}PrP turns over in the proteasomes, and inhibition of this machinery by experimental means leads to the accumulation of ^{Ctm}PrP and its 20kDa metabolic product. The intracellular signaling pathways that lead to ^{Ctm}PrP upregulation and subsequent cell death have significant implications, and are the focus of ongoing research in several laboratories. Proteasomes are again implicated in this cell death pathway almost by default.

9. CONCLUSIONS

At this point, there are more questions than answers with regard to the pathophysiology of prion disorders. Although it is clear that host PrP^{C} is

required for the development of disease, the precise mechanism by which PrP^{C} is converted to PrP^{Sc} or the biochemical pathways leading to neuronal damage remain unclear. Several triggers of prion-associated neuropathology have been proposed, including toxicity by PrP^{Sc} deposits, inherent toxicity of cytosolic PrP, initiation of pathogenic signals by C-transmembrane PrP, activation of the caspase pathway by PrP^{Sc} or PrP^{C}, interference in transcription by association of PrP^{Sc} with chromatin in the nucleus, and several other equally important mechanisms supported by experimental evidence. In some instances, impaired proteasomal function contributes to the disease process, in others it does not. Converging evidence suggests that prion disorders are not the result of protein aggregation alone, but a combination of aberrant PrP metabolism and a failure of the cellular quality control mechanisms to cope. Thus, mis-metabolism of PrP and its eventual aggregation, and an overwhelmed chaperone and proteasomal response may precipitate these disorders. Such conditions have a greater propensity to occur with advanced age, explaining the late onset of these disorders despite the presence of germ-line mutations in certain instances. Accumulation of misfolded PrP may result in further deterioration of proteasomal function and an autocatalytic accumulation of misfolded forms. Although the cell death pathways activated by the culmination of these events are not entirely clear, research in the development of strategies that optimize or enhance the cellular quality control mechanisms including proteasomal function may help in the prevention of prion disorders

10. ACKNOWLEDGEMENTS

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18

AGING AND THE PROTEASOME

Qunxing Ding and Jeffrey N. Keller

1. INTRODUCTION

Recent studies indicate that proteasome inhibition occurs during, and possibly contributes to, the aging process. In particular, *in vitro* and *in vivo* studies now demonstrate that inhibition of proteasome function is sufficient to induce a variety of pathological events associated with aging. Specifically, alterations in the proteasome proteolytic pathway may contribute to the elevations in protein oxidation, protein aggregation, and neurodegeneration evident in the aging central nervous system (CNS). The focus of this chapter is to discuss what is presently known about the effects of aging on proteasome biology, discuss the mechanisms responsible for altering proteasome function, and lastly to describe the possible role alterations in proteasome biology may play in age-related pathology in the CNS, and discuss the role of proteasome in age-related disorders of the CNS.

2. AGING ALTERS PROTEASOME ACTIVITY

Alterations in proteasome function during normal aging have been described in a wide range of species, ranging from yeast to humans (Table 1), and reported to occur in a wide variety of tissues (Table 1). It is important to point out that even within individual organs a regional specificity, with regards to the severity of proteasome inhibition can occur. This is best illustrated in the CNS where there are clearly brain region susceptibilities with regards to age-related proteasome inhibition(1–4). In addition to these *in vivo* examples of age-related proteasome inhibition, *in vitro* aging is also associated with declines in proteasome function (Table 1), occurring in a diverse range of cell types. The proliferative state of cells also appears to be an important factor regulating age-related impairments in proteasome function. As an example, post-mitotic cells undergo more severe inhibition of proteasome activity as compared to mitotic cells(5–9).

Does aging inhibit all of the individual proteasome peptidase activities equally? It has been demonstrated that post-mitotic cells exhibit a preferential loss of postglutamyl peptidase activity, while mitotic cells undergo a loss in trypsin-like, chymotrypsin-like, and postglutamyl peptidase activities of the proteasome(5–9). In the liver, there is a 50% reduction in proteasomal postglutamyl peptidase activity with no significant differences in either trypsin-like or chymotrypsin-like activity reported(10). In rats there is a loss in chymotrypsin-like proteasome activity throughout the CNS during aging. Decreases in chymotrypsin-like activity are evident within the cortex, hippocampus, and spinal cord of 12-month-old rats(1,11). In contrast, no impairment in chymotrypsin-like activity is evident in either the brain stem or cerebellum.[11] Impairments in the chymotrypsin-like activity of the proteasome are also evident by 12-months of age in the heart, kidney, liver, but not the lung of these aged rats(1,11).

In addition to age-related alterations in basal proteasome activity, it is important to point out that aging has been demonstrated to impair the ability of the proteasome to respond to stress(12,13). The ability of the proteasome to up-regulate its activity in response to environmental or genetic stressors would be

Table 1 Organisms, tissues and cell types exhibiting age-related decreases in proteasome function.

Organisms	**Tissues**	**Cell Types**
Canine[a]	Brain	Epidermal
Fly	Heart	Fibroblast
Humans	Kidney	Lymphocytes
Mouse	Liver	Trabecular meshwork
Rat	Retina	Yeast
	Skeletal muscle	
	Spinal Cord	

[a] J. N. Keller (unpublished observations)

expected to play a pivotal role in determining whether a cell was able to survive the wide variety of stressors it is likely to encounter during aging. In this scenario, the lack of proteasome plasticity would result in an ineffective or inhibited proteasome, which could contribute to cell pathology and cytotoxicity. As mentioned previously, the expression of the proteasome in neural cells is dramatically altered in response to oxidative stress and the expression of proteins with an increased propensity to aggregate(14,15). Together; these studies show an apparent increase in immunoproteasome complex formation. Interestingly, studies in neural cells expressing polyglutamine containing proteins suggest that the immunoproteasome is not capable of increasing activity in response to subsequent stressors(14), and may ultimately be deleterious towards long-term viability.

3. BASIS FOR AGE-RELATED CHANGES IN THE PROTEASOME

So what is the molecular and cellular basis for age-related changes in proteasome function? At the present time it is believed that age-related impairments in proteasome-mediated protein degradation can occur as the result of alterations in protein targeting, excessive cross linking proteasome substrates, compromises in heat shock protein (HSP) capacity, alterations in the intracellular localization of proteasome complexes, alterations in proteasome composition, impairments in proteasome plasticity, and increased oxidative damage to the proteasome complex. Each of these events is discussed in detail below.

Increases in protein hydrophobicity appear to be central mechanism for targeting proteins to be degraded by the 20S or 26S proteasome.[15] In order to efficiently degrade these "marked" proteins they must be rapidly identified, and upon identification be brought together with the proteasome complex in a timely and efficient manner. In most aging tissues it is likely that there may be an overwhelming amount of proteins targeted to the proteasome. Oxidized, misfolded, and damaged proteins are all proteasome substrates, and increases in their formation undoubtedly occur in aging cells. This increase in substrates may override the targeting systems, contributing to inefficiency in proteasome-mediated protein degradation, as some proteins are unable to reach a proteasome complex. The ubiquitin-pathway is known to be negatively affected by oxidative stress(16), may be deleteriously affected by aging. Inefficiencies in the ubiquitin system would also be expected to negatively affect proteasome-mediated protein degradation. Each of these manifestations may lead to a specialized form of proteasome inhibition, namely the inhibition of protein turnover by failure to deliver proteins to the proteasome.

While the mild oxidation of proteins is known to serve as a potent inducer of proteasome mediated proteolysis(17–20), excessive oxidation is known to mediate inhibition of the proteasome. Impairment of proteasome-mediated protein degradation by excessively cross linked proteins is believed to be mediated by the blockage that occurs at the entrance of proteasome complex. This obstruction at the openings between the α- and β-subunits is sufficient to block the entrance of subsequent protein substrates into the proteasome. Cross linking may be achieved by oxidants (ROS) (19,20), or as the result of lipid peroxidation products such as 4-hydroxynonenal (HNE)(21). Increased oxidative damage to

proteins, including increased levels of protein cross linking, is known to occur during normal aging. These data are consistent with a role for increased protein cross linking mediating inhibition of the proteasome during normal aging. Cross linking of proteins is also likely to impair the unfolding of proteins, which is required for their degradation by the proteasome(22). Inhibition of this process could also provide an additional mechanism for impairment of proteasome mediated protein degradation.

Increasing evidence suggests that oxidative damage to the proteasome complex may be a mediator of at least some forms of proteasome inhibition in the CNS. Studies from our laboratory demonstrate that dopamine may support ROS-induced impairment of proteasome function in the CNS(23). Several features of the CNS presumably make it very vulnerable to oxidative stress including the fact that the CNS has a high metabolic rate that may produce a higher level of mitochondrial derived ROS, may undergo age-related decreases in antioxidant levels, and has a high content of readily oxidized lipids that are capable of promoting oxidative stress. Post mitotic cells in the CNS, which survive for decades, are particularly susceptible to an age-related accrual and elevation in oxidative damage. Proteasomes can undergo direct oxidative modification by a variety of mechanisms. For example, peroxynitrite and HNE can be generated in the intraceullular environment and directly interact with the proteasome and inhibit its function(1,24-29). This inhibition is mediated in part by changes in proteasome stability as well as potentially mediated by oxidative modification of the active enzymatic sites. However, because the proteolytic activities of the proteasome face the inner core of the proteasome, it is unlikely that much interaction between oxidants and the actual enzymatic sites occurs. Studies have now demonstrated that oxidative modification of the proteasome occurs in conditions where proteasome inhibition is present(1,11,26). In particular, oxidation of the proteasome is observed during normal aging in the spinal cord and in experimental models of ischemia-reperfusion injury(1,11). It is interesting to point out that within the spinal cord there are detectable levels of proteasome oxidation within 3-month-old rats, which are not detectable in other regions of the CNS, without any apparent loss of proteasome activity(11). These data suggest that increased oxidation of the proteasome does not always result in proteasome inhibition.

The degradation of proteins by the proteasome requires that proteins be unfolded and inserted within the proteasome complex(22). The unfolding of proteins must be mediated by HSP. Studies have demonstrated that increased HSP expression ameliorates oxidative stress-induced proteasome inhibition(30), consistent with HSP playing a critical role in preserving proteasome function during periods of oxidative stress. The identification of which HSP are most important in this process has not been elucidated. Age-related compromises in HSP capacity therefore provide a mechanism by which proteasome-mediated protein degradation may be inhibited, via failure to deliver and/or unfold proteasome substrates.

It is clear that the localization of proteasome complexes can be altered in response to specific stressors(31-34). The localization of the proteasome to either nuclear or synaptic compartments may be particularly important for neuron function and neuron viability. It is important to point out that localized alterations in

proteasome function, through decreases in the number of available of proteasome complexes or decreases in specific activity distinct proteasome populations, may not be readily evident when measuring proteasome function in brain homogenates. In neurons, the loss of proteasome function in the synapse could be particularly deleterious to neuronal signaling, excitotoxicity, and synaptic plasticity. Impairments in nuclear proteasome function could selectively affect the activity of transcription factors, histone function, and chromatin remodeling. Elucidating these localized alterations in proteasome function are critical to accurately understanding the contribution proteasome inhibition may play in aging and age-related disorders of the CNS.

Continual generation of new proteasome complexes is presumably necessary to replace damaged and/or less efficient proteasome complexes. Additionally, a perpetual generation of proteasome complexes allows for the generation of proteasomes with altered composition, and the generation of proteasomes that are more efficient at degrading proteins under stressful conditions. In aging, and age-related disorders of the CNS, proteasome biogenesis may be altered and contribute to the loss of proteasome function. This impairment in biogenesis could result from a loss of proteoassemblin (35–37), reduced levels of molecular chaperones that participate in proteasome biogenesis, alterations in proteasome subunit expression, oxidative modification of proteasome subunits, or oxidative attack on a developing proteasome complex. Additionally, polymorphisms in proteasome subunits may contribute to alterations in proteasome subunit expression. A number of studies now demonstrate a clear association between polymorphisms in proteasome subunits and Graves' disease, ankylosing spondylitis, and insulin-dependent diabetes mellitus(38–42). Studies have shown that LMP2 codon polymorphisms can alter age-related susceptibility to TNF-α induced apoptosis in peripheral blood mononuclear cells(42). LMP2 polymorphisms may also be associated with AD(43). Presumably, these polymorphisms in the LMP2 subunit promote deleterious alterations in proteasome function and may provide an additional means by which proteasome inhibition occurs in aging and age-related disorders of the CNS.

Changes in proteasome composition appear to be an important means by which proteasome function can be specialized in order to address a specific need. Changes in proteasome subunit expression occur in the aging of the retina, fibroblast, muscle, and liver(9,44–48). Cytokine-induced expression of immunoproteasome has been reported in a variety of tissues and cell types that are not part of the immune system(44,49,50). These data raise the possibility that immunoproteasomes may be generated as a means of increasing the turnover of specific proteins in aging, including the degradation of oxidized proteins. Additionally, studies have demonstrated that proteasome subunits exhibit a hierarchical susceptibility to HNE modification(51), which may be important in determining the amount of HNE-induced inactivation that occurs following a variety of stressors. It is interesting to note that formation of immunoproteasome, while allowing for continued proteasome function, may impair the ability of the proteasome to respond to subsequent stressors(14). Aging and age-related diseases of the CNS may promote changes in proteasome composition that in the short term allow for maintenance of proteasome function, but in the long term

promote proteasome inhibition or at least impair the ability of the proteasome to respond to subsequent stressors.

4. EFFECTS OF PROTEASOME INHIBITION WITHIN THE CNS

Numerous studies have now demonstrated that inhibition of the proteasome is sufficient to induce neuron death in primary neuronal cultures, as well as neural cell lines(52–55). A number of the 26S proteasome substrates are involved in the apoptotic pathway(56,57), with the best characterized of these substrates is p53. Normally a very short-lived protein, the expression of p53 is kept at a low level, and thus is unable to induce its pro-apoptotic effects. However, following inhibition of proteasome function the level of p53 would be expected to become elevated(58–61), eventually elevating to the point that it is able to induce its pro-apoptotic pathways. Indeed, p53 has been demonstrated to play a causal role in the apoptosis induced by severe proteasome inhibition(61).

It is important to point out that proteasome inhibition does not appear to induce neuron death in all neuron populations or experimental paradigms. These data raise the possibility that proteasome inhibitor toxicity may be cell type specific, based on the function of the proteasome in a given cell. For example, the proteasome is responsible for some forms of NF*k*B activation, which can have pro-apoptotic or anti-apoptotic effects depending on cell type. As such, proteasome inhibition could have very different effects on cell survival based on the differential role of NF*k*B in these two cell populations. Alternatively, these data could indicate the inadequacy of some neuronal populations to utilize non-proteasomal proteolysis, in order to maintain neuronal homeostasis. In such a scenario, cells able to sufficiently up-regulate lysosomal activity would be expected to exhibit little toxicity in response to the application of proteasome inhibitors. Cell specific susceptibilities to proteasome inhibition may also be due in part to alterations in HSP capacity, with neurons possessing higher levels of HSP capacity being more resistant to proteasome inhibitor toxicity.[30] It is important to keep in mind that the majority of *in vitro* studies are conducted in cultures established from embryonic tissue, or tissue from early postnatal brain. As such, one must take into account the possibility that embryonic tissue may have a different dependence on proteasome activity than established neurons within the mature and developed CNS.

The clearance of oxidized proteins is an important means by which cells are able to prevent the increase in oxidative damage (most notably increased protein oxidation), and thus proteasome-mediated protein degradation is an important "antioxidant"(62–64). In this capacity the proteasome aids in preventing the elevation in oxidative damage and induction of oxidative stress. This "antioxidant" feature of the 20S proteasome is not only important in the aging of the CNS, but also is likely important in numerous age-related disorders of the CNS.

Impairments in 20S proteasome function likely play an important role in the age-related increases in protein oxidation observed in a variety of tissues, including the CNS(44,65–67). It is important to note that during aging protein oxidation does not typically exhibit a gradual and progressive increase, rather during aging there is a very low level increase in protein oxidation that dramatically

increases several fold in late age(19,68–72). Proteasome inhibition may serve an important role as a trigger for the sudden and dramatic spike in protein oxidation observed in very late age. Therefore, early in the aging process there is likely a dynamic cellular environment that helps to prevent large increases in protein oxidation. For example, it is likely that proteasome plasticity and increases in stress response (present in young cells) prevent the accumulation of oxidative damage that could potentially occur as the result of cellular stressors (Figure 1). Over time the ability of these protective pathways to prevent increases in protein oxidation dramatically decrease, with inhibition of proteasome function serving as a mechanism for rapidly and profoundly elevating protein oxidation (Figure 1). Additionally, once the levels of oxidized proteins are increased to a deleterious stage, or allowed to persist in the intracellular space for prolonged periods of time, they may serve as potent inhibitors of proteasome function. In this model, excessively oxidized proteins inhibit the entry of other proteasome substrates, thus causing inhibition of proteasome-mediated protein degradation. Consistent with this model, studies from our laboratory have demonstrated that increased heat shock protein expression ameliorates oxidative stress-induced proteasome inhibition(2).

Recent studies provide direct experimental evidence for proteasome inhibition serving as a mediator of lipofuscin-ceroid,[73] which is one of the most common forms of oxidative damage observed in aged tissues. Interestingly, this increase in lipofuscin-ceroid may be related to impairment in mitochondria turnover and mitochondrial function(73). Because of the importance to mitochondria dysfunction to aging and age-related diseases of the CNS, these data

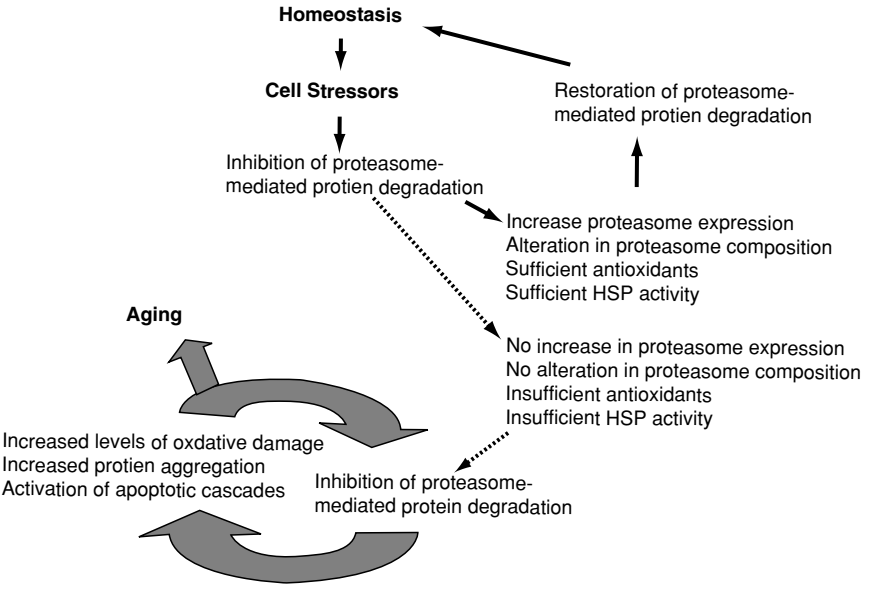

Figure 1. **The proteasome and aging**. Maintaining proteasome function protects against different forms of cell stress, while inhibition of proteasome promotes cellular aging.

indicate a novel mechanism by which proteasome inhibition may contribute to neuropathogenesis. Additionally, our laboratory has demonstrated that inhibition of proteasome function (low-level inhibition) is sufficient to increase autophagy(74), which are observed in the aging CNS as well as several age-related disorders of the CNS. The chronic activation of autophagy is likely deleterious towards neural homeostasis, based on the fact that rapid and large scale degradation of cytoplasmic complexes and organelles cannot be beneficial towards the long term cellular viability(75). Therefore, induction of autophagy may serve as an additional mechanism by which proteasome inhibition contributes to cytotoxicity in the CNS. Lastly, inhibition of proteasome function in neural cells alters gene expression in a manner that is highly relevant to a variety of age-related disorders(76), including modulating the genes involved in regulating beta amyloid metabolism.

A number of studies have suggested a link between DNA repair and the proteasome. For example, the degradation of oxidized histones is mediated by the proteasome(77,78), with additional studies showing that proteasome subunits may play a role in DNA repair(79,80). Data from our laboratory demonstrated that proteasome inhibition is sufficient to induce RNA and DNA oxidation in primary CNS cultures(81). Interestingly, nucleic acid oxidation occurred in neurons and astrocytes, although it was much more severe in neurons as compared to astrocyte cultures. The oxidation of RNA was associated with an alteration in RNA processing(81). These data suggest that there is potential crosstalk between proteasome-mediated protein degradation and the translation/protein synthesis processes. The proteasome is also capable of increasing ROS production(30,73,82,83), which can increase oxidative stress. Studies have shown that both severe and moderate proteasome inhibition are capable of stimulating ROS generation in neural and non-neural cells. In at least 1 study the increase in mitochondrial derived ROS has been reported(73).

Together, these data that there are multiple mechanisms by which proteasome inhibition can contribute to increased oxidative damage, and potentially the induction of oxidative stress. The ability of proteasome inhibition to induce so many disparaging effects should not be considered surprising when one considers the large number of proteasome substrates, and the likelihood that alterations in bulk protein turnover may impact multiple systems. The fact that proteasome inhibition is capable of inducing so many forms of age-related oxidative damage, and the fact that proteasome inhibition appears to be a common occurrence in aging, these data suggest that proteasome inhibition has to seriously be considered as a mechanism for increasing oxidative damage during normal aging. Therefore the proteasome should not only be thought of as a target of ROS, but also be discussed in the context of proteasome inhibition being a potential mediator of oxidative stress. We propose that in healthy cells the activity of the proteasome is compromised for a small period of time (Figure 1), but the presence of antioxidants, heat shock proteins, and proteasome plasticity are sufficient to re-establish proteasome function. The fact that proteasome inhibition is only allowed to persist for a short period, the induction of oxidative stress and activation of apoptotic pathways can be avoided (Figure 1). In older cells it is likely that following cell stress proteasome inhibition is sustained due to the fact that

antioxidant pathways, heat shock protein response, and proteasome plasticity are insufficient (Figure 1). This sustained inhibition of proteasome function allows for elevations in oxidized macromolecules, activation of apoptotic pathways, and increased levels of protein aggregation to occur (Figure 1). Ultimately, the cell stressors induced by proteasome inhibition promote a feed-forward pathway that compounds the amount of proteasome inhibition and induction of pathology that is observed (Figure 1).

5. ROLE OF PROTEASOME INHIBITION AS MEDIATOR OF AGING

Proteasome inhibition occurs in the aging of most cell types and tissue, but does it play any role in mediating aging? Numerous studies suggest that proteasome inhibition may not only occur during normal aging, but may play a direct role in the aging process. As discussed previously, studies have demonstrated that proteasome inhibition is sufficient to induce multiple pathological alterations observed in aging including increased protein oxidation, nucleic acid oxidation, protein aggregation, increased lipofuscin/ceroid, induction of autophagy, and induction of mitochondrial dysfunction. The induction of cellular senescence is also tightly correlated with a loss of proteasome function(6-8,84,85), with proteasome inhibition sufficient to induce multiple aspects of cellular senescence(9,86). Such studies indicate that proteasome inhibition is not only a common feature of cellular and tissue aging, but demonstrate that proteasome inhibition is sufficient to induce age-related pathologies observed in a variety of tissues.

Caloric restriction (CR) is the only manipulation that consistently and reproducibly increases lifespan (average and maximal lifespan) in mammals(20,87). Some studies suggest that CR may blunt age-related impairments in proteasome function(12,48), supporting a potential role for the preservation of proteasome function as a means by which CR increases lifespan. Interestingly, CR is also associated with an amelioration of oxidative damage (including protein oxidation)(20,87,88), raising the possibility that the preservation of proteasome function contributes to the decreased levels of oxidative damage observed in CR tissues. Alternatively, it may be that the decrease in oxidative damage is what promotes the preservation of proteasome function in CR tissues. Clarification of this issue is essential and highlights the importance of determining whether proteasome inhibition necessary for aging. Perhaps even more importantly it remains to be elucidated whether the proteasome plays a role in regulating lifespan. Data from our laboratory demonstrate that the proteasome is essential for yeast aging(89), with decreases in proteasome function decrease lifespan, consistent with the proteasome playing a role in regulating lifespan.

At the present time we believe that the proteasome plays a direct role in regulating aging, with preservation of proteasome function slowing the rate of aging, and inhibition of proteasome function increasing the rate of aging. We believe that the ability of the proteasome to regulate aging is consistent with both the free radical theory or aging and the adaptation model of aging(68,90–92). The free radical theory of aging proposes that aging is the result of cumulative oxidative damage inducing cellular aging, while the adaptation theory of aging suggest that lifespan is regulated by the ability to

successfully adapt to stressors and that the accumulation of adaptations alters cellular function in a manner that ultimately causes aging. In this model the proteasome serves as the trigger for the majority of age-related alterations. In young healthy cells there is considerable proteasome plasticity, allowing the cells to rapidly respond to stressors, and the proteasome providing a barrier of safety from the deleterious effects of cellular stressors. Following exposure to stress, in young healthy cells the proteasome becomes inhibited for a brief period, with proteasome capacity rapidly brought back to basal levels through a host of events including antioxidants, heat shock proteins, and proteasome plasticity. With continual adaptation to stress revolving around the capacity of cells to maintain proteasome function. In aging cells, the ability of the proteasome to regain its full capacity is impaired, thus allowing for the persistence of proteasome inhibition. Sustained proteasome impairment is the result of multiple factors including a decreased antioxidant defense system, reduced HSP capacity, and reduced proteasome plasticity. During the prolonged low-level proteasome inhibition a number of deleterious events occur, promoted by the presence of proteasome inhibition. For example, elevations in oxidative damage and pro-apoptotic pathways occur, thus promoting further inhibition of proteasome function. Once this process is set in motion, a catastrophic feed forward pathway is established, ultimately contributing to cellular aging (Figure 1). Proteasome inhibition thereby serves as a trigger for oxidative stress in the free radical theory of aging, and serves as the switch by which aging is promoted in the adaptation theory of aging. In this model the proteasome is not only affected by aging, but is a central mediator and regulator of aging.

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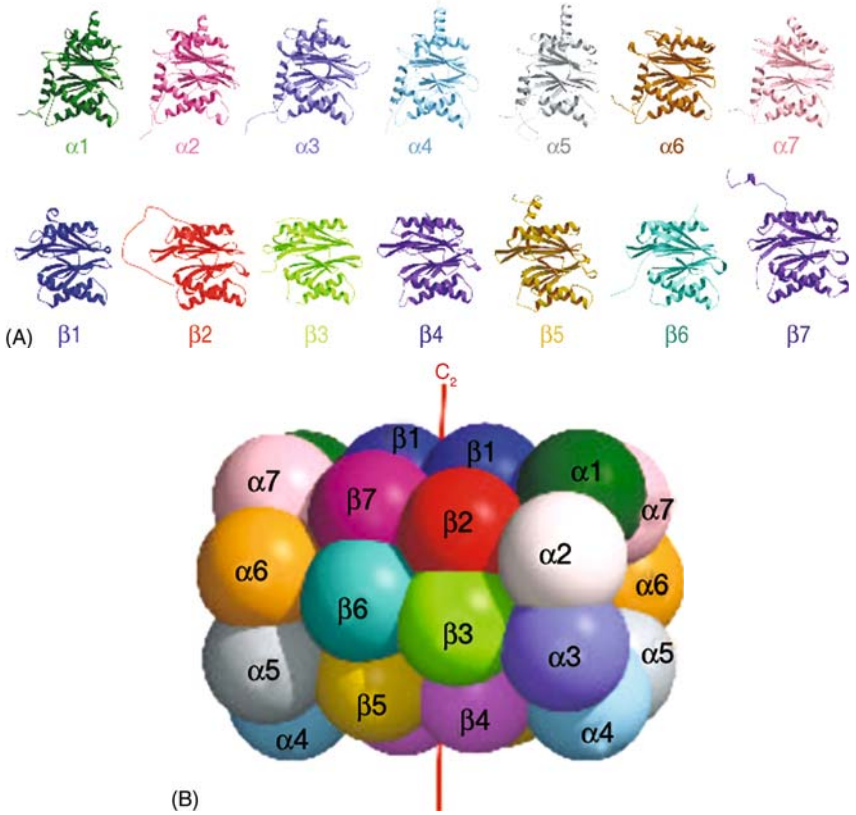

Figure 1.1. The structure of the yeast 20S proteasome (A) Gallery of ribbon drawings of the seven different α and β subunits. The subunits show the common αββα sandwich fold with two β-sheets (formed by five antiparallel β–strands each) stacked between two layers of α-helices. (B) – (C) Different views of the yeast 20S proteasome showing the subunit arrangement; (B) the calotte model and (C) the surface model. The latter model derives from the 20S proteasome crystallized in the presence of calpain inhibtor I. To gain insight into the proteolytic cavity, the barrel shaped particle was cut along the cylinder axis. The intersections were coloured in white. The three cavities were depicted in blue. The central cavity harbours six active site threonine residues, which are exposed by the β1, β2 and β5 subunits, respectively. The proteolytic active centers are coloured: red, β1; blue, β2; yellow, β5. Cleavage preferences, termed post-glutamyl-splitting, tryptic and chymotryptic-like activity are zoomed and illustrated in surface presentation. The nucleophilc Thr1 is presented by ball and sticks. Basic residues are coloured in blue, acidic residues in red and hydrophobic residues in white. A central pore of the outer rings as entrance for substrate polypeptide chains is gated in the crystal structure conformation due to the tight interactions of the seven α subunit N-terminal regions. The figures were kindly provided by Michael Groll (6,12) and printed with the permission of Nature (*http://www.nature.com*) and ChemBioChem (published by Wiley-VCH).

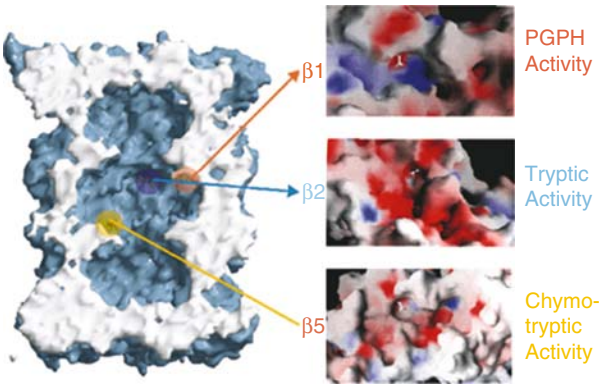

Figure 1.1. cont'd

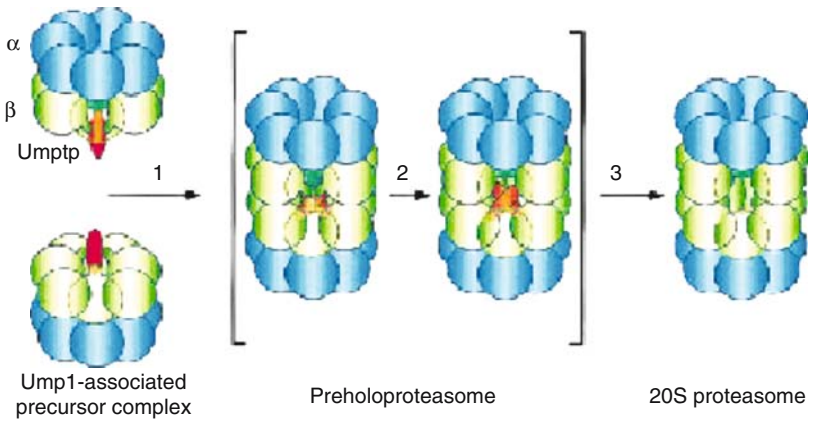

Figure 1.4. Model for yeast 20S proteasome assembly and maturation as originally proposed by Chen and Hochstrasser in 1996 (17) and modified by addition of the maturation factor Ump1 (21). Ump1-associated precursor complexes (~ 15 S complex) are symbolized as half-assembled proteasomes, which are composed of a ring of seven α-subunits and a ring of seven β-subunits, of which five contain N-terminal propeptides. Two half-proteasomes join to build a short-lived intermediate, namely the preholoproteasome. Upon the meeting of the two β?subunit rings, conformational changes trigger the autocatalytic propeptide processing, which are most likely conducted by the entrapped Ump1. The catalytic chamber of the nascent 20S proteasome is finally exspaciated by Ump1 degradation. The figure was kindly provided by Jürgen Dohmen (21) and printed with the permission of Cell (published by Elsevier).

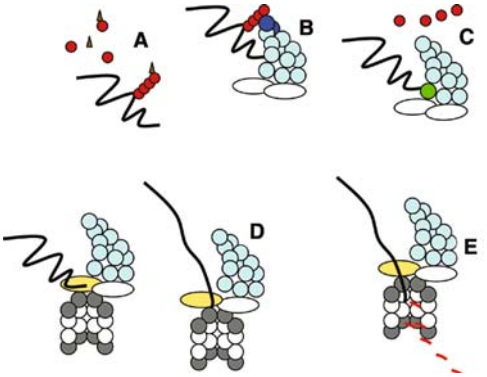

Figure 3.4. Hypothetical model of potential sites of direct involvement of the UPS with oligomeric/aggregated proteins as these are engaged in the UPS degradation pathway. In A, the aggregated protein substrate, depicted by a curved line, is targeted by ubiquitin moieties, depicted as red spheres. Ubiquitin-binding proteins are depicted by brown triangles. It is possible that an excess of aggregated proteins may lead to depletion of such UPS components at this stage. In B, the aggregated poly-ubiquitinated protein is recognized by elements of the 19S proteasome, depicted in blue. Binding of the substrate to the deubiquitinating component of the 19S, Rpn11/POH1, depicted in green, leads to its deubiquitination (C). Unfolding and threading of the substrate through ATPase subunits at the base of the 19S proteasome (depicted by oval shapes) (D) leads to its entry into the barrel-shaped 20S proteasome and degradation into small peptides, depicted as red curved lines (E). In all these stages (B-E), "clogging" of the pathway may occur due to the nature of the aggregated conformation of the substrate. It should be noted that this depiction is schematic, and that in fact many of these functions (recognition, deubiquitination, threading, degradation) may be performed simultaneously.

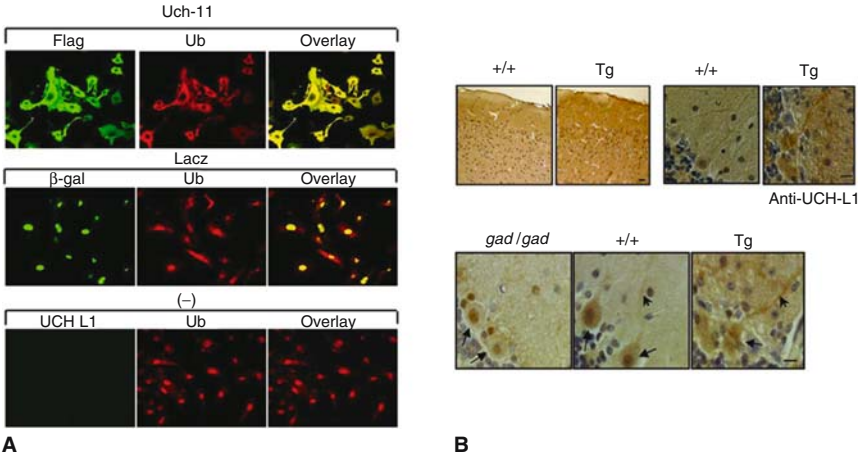

Figure 11.6. Overexpressed UCH-L1 colocalizes with Ub and increases Ub levels *in vitro* and *in vivo*. (A) Primary mouse embryonic fibroblasts (MEFs) were infected with adenovirus expressing UCH-L1 epitope tagged with either FLAG or β-gal. Antibodies to FLAG and β-gal were used to immunostain exogenous UCH-L1 or β-gal, respectively. MEFs were also labeled with polyclonal anti-Ub. Ub and UCH-L1immunoreactivities completely colocalized in MEFs infected with adeno-*uchl1* but not in cells infected with β-gal or cells that were not infected. (B) UCH-L1 overexpression increases the level of Ub in the mouse nervous system. Immunostaining with an anti-UCH-L1 in cerebral cortex (left) and cerebellum (right) from UCH-L1 transgenic and wild-type mice (upper panel) and with polyclonal anti-Ub in cerebellar sections from *gad*, wild-type and UCH-L1 transgenic mice (lower panel). Ub immunoreactivity increased in transgenic mice overexpressing UCH-L1. Scale bars 20 μM (from Osaka *et al.*, 2003).

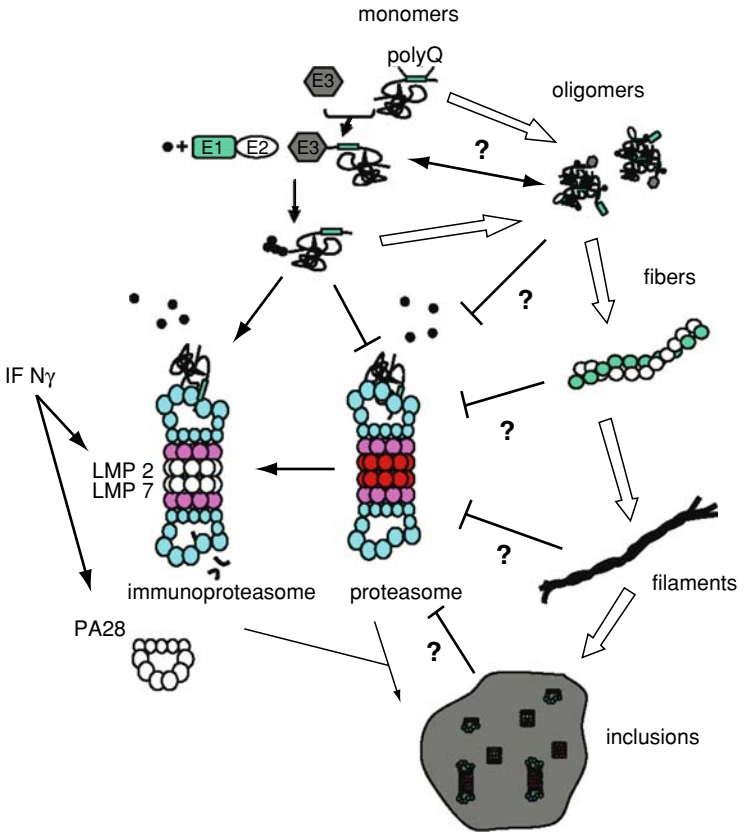

Figure 14.1. Involvement of the UPS system in Huntington's disease progression: Schematic illustration of possible alterations on the UPS caused by polyQ expansion in htt protein. The enzymes required for poly-ubiquitylation of a substrate protein (like mutant htt) are represented: E1 is the ubiquitin activating enzyme, E2 are the conjugating enzymes, and E3 are the ubiquitin ligases. N-terminal mutant-htt is labeled with ubiquitin (black circle), but it is not normally processed by the proteasome. The scheme also reflects the possible levels of htt aggregation that might interfere with the UPS function. Thus, N-terminal mutant htt monomers can associate to form globular assemblies. These htt-oligomers might lead to the formation of fibers, filaments or intracellular inclusions that in turn can sequester different components of the UPS. This hypothesis is supported by positive immunoreactivity of the inclusion bodies with antibodies against different UPS components. Finally, the neuron may respond to proteasome impairment or to extracellular signals (like interferon-gamma, IFN-gamma; or other inflammatory molecules) by changing the subunit composition of the catalytic core. More precisely, the inducible catalytic subunits LMP2 and LMP7, and possibly the PA28 complex are increased thus leading to induction of the immunoproteasome.