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

Pril, R. de

Chapter 2

Conformational diseases: an umbrella for various neurological disorders with an impaired ubiquitin-proteasome system

Remko de Pril, David F. Fischer and Fred W. van Leeuwen

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Abstract

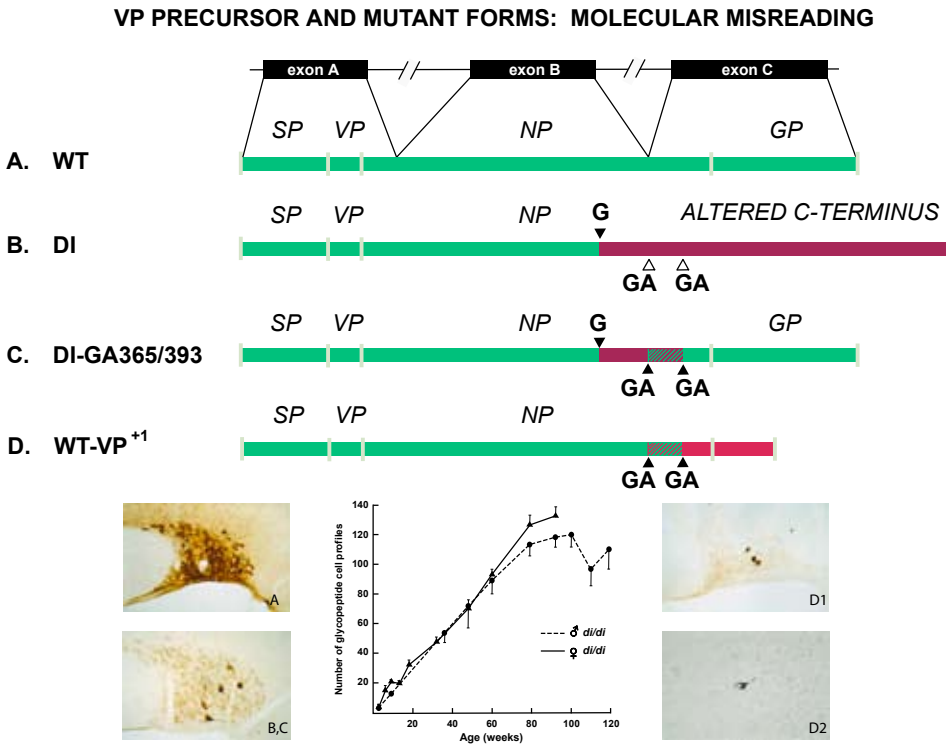
It is increasingly appreciated that failures in the ubiquitin-proteasome system play a pivotal role in the neuropathogenesis of many neurological disorders. This system, involved in protein quality control, should degrade misfolded proteins, but apparently during neuropathogenesis, it is unable to cope with a number of proteins that, by themselves, can consequently accumulate. Ubiquitin is essential for ATP- dependent protein degradation by the proteasome. Ubiquitin^{+1} (UBB^{+1}) is generated by a dinucleotide deletion (ΔGU) in UBB mRNA. The aberrant protein has a 19 amino acid extension and has lost the ability to ubiquitinate. Instead of targeting proteins for degradation, it has acquired a dual substrate-inhibitor function; ubiquitinated UBB^{+1} is a substrate for proteasomal degradation, but can at higher concentrations inhibit, proteasomal degradation. Furthermore, UBB^{+1} protein accumulates in neurons and glial cells in a disease-specific way, and this event is an indication for proteasomal dysfunction. Many neurological and non-neurological conformational diseases have the accumulation of misfolded proteins and of UBB^{+1} in common, and this combined accumulation results in the promotion of insoluble protein deposits and neuronal cell death as shown in a cellular model of Huntington disease.

Introduction

Only at the 9th Alzheimer meeting in Philadelphia, 2004, "conformational diseases" were acknowledged as a group of disorders that share a common feature: the accumulation of insoluble protein deposits in the affected cells (Carrell and Lomas, 1997). To this group belong many age-related neurodegenerative disorders, such as tauopathies (e.g. Alzheimer and Pick disease), synucleinopathies (e.g. Lewy body disease) and polyglutamine diseases (e.g. Huntington disease, and several spinocerebellar ataxias) (Ciechanover and Brundin, 2003; Landles and Bates, 2004). In addition, in non-neuronal disorders inclusions are present as well (e.g. alcoholic liver disease, inclusion body myositis and α1-antitrypsin deficiency (Askanas and Engel, 2003; French et al., 2001; Lomas and Carrell, 2002; McPhaul et al., 2002). Although the deposits vary in protein composition, shape and localization, each of these structures (e.g. aggregates, aggresomes or inclusions) is mainly composed of insoluble misfolded proteins (e.g. Huntingtin, α-synuclein or hyperphosphorylated tau), different molecular chaperones (e.g. heat shock proteins) and various components of the ubiquitin-proteasome (UPS) (e.g. E3 ligases such as CHIP, 19S and 20S proteasomal subunits of the 26S proteasome) (McDonough and Patterson, 2003; Sherman and Goldberg, 2001). The presence of these factors suggests that these insoluble proteins are misfolded and have been targeted for degradation, but instead of being properly refolded or proteolytically degraded, they accumulate in insoluble protein deposits (Alves-Rodrigues et al., 1998). The coexistence of heat shock proteins and UPS compounds is expected, since one of the fundamental tasks of the UPS is to degrade damaged or abnormal proteins and to protect cells during stress responses. If the capacity of the UPS is exceeded (e.g. during aging (Keller et al., 2004)), autophagy, essential for the elimination of aggregates, is induced. Autophagy is considered a highly regulated but non-selective pathway by which cytoplasmic constituents are degraded in lysosomes (see (Cuervo, 2004; Nixon, 2005; Ross and Pickart, 2004)). In the past few years excellent reviews on cross-talk between the UPS (Keller et al., 2004; Pickart and Cohen, 2004) and autophagy (Shintani and Klionsky, 2004) in relation to neurodegeneration have appeared (Ciechanover and Brundin, 2003; de Vrij et al., 2004; McNaught, 2004; Ross and Poirier, 2004).

In the present minireview the discovery of an unexpected type of mutation ("molecular misreading") and the contribution of one of the resulting aberrant proteins (UBB^{+1}) to proteasomal dysfunctioning will be discussed in relation to various conformational diseases that have an impaired UPS (Song and Jung, 2004).

Figure 1: Molecular misreading.



Schematic representation of the vasopressin (VP) gene, its precursor protein and its mutant forms. The VP gene was cloned in the early eighties and consists of 429 nucleotides divided over three exons. After splicing, a precursor protein is translated at the endoplasmatic reticulum which is enzymatically processed within granules and subsequently transported to the nerve terminals. In the normal situation (A) a wild-type (wt) VP precursor is synthetized in the supraoptic nucleus (SON) that can be posttranslationally processed and packaged in neurosecretory granules that are subsequently axonally transported towards the neural lobe. The homozygous Brattleboro rat (B) lacks a single G base in exon B by which an out of frame mutant protein is formed with a polylysine tail that cannot be processed properly. As a result a severe diabetes insipidus (DI) develops. The volume of urine formed daily can reach 70% of the body weight. This trait is autosomally recessive and inherited in a simple Mendelian manner. In the SON and the paraventricular nucleus surprisingly an age-dependent increase of solitary neurons with a revertant VP phenotype (C, diagram) was found (van Leeuwen et al., 1989) that appeared to be due to a second mutation (ΔGA) in downstream located GAGAG motifs. As a result the VP (mutant) precursor (i.e. the glycoprotein-containing part) can be processed and the neurosecretory granules can be axonally transported again towards the neural lobe (Evans et al., 1996). As GAGAG motifs are also present in the wt-VP gene of rat and human (D), a similar process can take place and change the normal VP precursor in an aberrant one. This was shown in neurons of the SON of the rat (D1) and the human (D2).

Discovery of molecular misreading

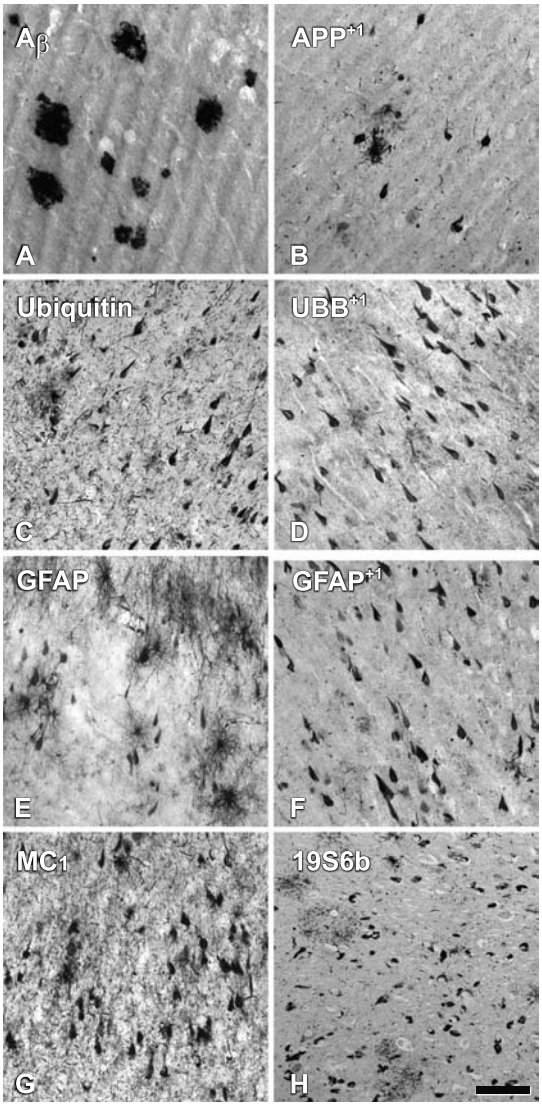
Molecular misreading of genes (i.e. the inaccurate conversion of genomic information into aberrant proteins) was demonstrated in the vasopressin (VP) gene, which is highly expressed in magnocellular neurons of the hypothalamo-neurohypophyseal system. The first occurring knockout ever presented was that of VP, discovered in the city of Brattleboro (Vermont, USA (Valtin, 1982); Figure 1). Gene cloning revealed the mutation; a single G residue deletion in exon B results in a VP precursor with a very sticky polylysine C-terminus which remains in the endoplasmatic reticulum. Antibodies raised against all parts of the VP precursor (e.g. the very C-terminal part of the VP precursor; glycopeptide) showed an intense staining of VP cells (Figure 1A). As a specificity control for these antibodies, the homozygous Brattleboro rat was used. Indeed, many neurons in the magnocellular hypothalamic nuclei showed no reaction (Lu et al., 1982). However, surprisingly, solitary neurons were intensely stained with glycopeptide antibodies (Figure 1B,C). These neurons, which had a revertant VP phenotype, were shown to increase in number with advancing age by the diseased state (diabetes insipidus) of these animals (Evans et al., 1994). Follow-up analysis of the mRNA of these cells revealed dinucleotide deletions (ΔGA) located downstream of the G deletion, in GAGAG motifs. Consequently, at these points the normal reading frame was restored.

Subsequently, the question was raised whether this mutation is a peculiarity of the Brattleboro rat or a more general phenomenon. The wild-type VP genes of rat and human have the same GAGAG motifs. Indeed, antibodies directed against the predicted VP sequences in the -2 or +1 reading frame resulted in an intense staining of magnocellular neurons (Figure 1, D1 and D2; (Evans et al., 1996)). Thus, a similar dinucleotide deletion (ΔGA) is likely to occur in wild-type sequences and is not restricted to homozygous Brattleboro rats. It is important to realize that in these cases an abnormal precursor protein is created out of a normal one. These abnormal +1 proteins are potentially functionally different (Figure 1D).

Which transcripts have GAGAG motifs?

The next step was to see if other genes do have GAGAG motifs or other simple repeats. The chance to encounter a GAGAG motif is 1:1024, and there are many genomic sequences which have such a potential, error-prone site. We focused on Alzheimer's disease associated genes such as β amyloid precursor protein and ubiquitin B, of which indeed the respective +1 proteins were found to coexist in the hallmarks of all cases of Alzheimer's disease (including sporadic ones) and Down syndrome (van Leeuwen et al., 1998). The proposed dinucleotide deletions were found as well. The existence of these dinucleotide deletions was confirmed independently (van den Hurk et al., 2001). Several +1 proteins appeared to coexist (e.g. Amyloid Precursor Protein, Ubiquitin and GFAP; Figure 2) due to molecular misreading and exon skipping (Hol

Figure 2: Various +1 stainings in comparison to their wild-type form or conventional markers.



Fifty-µm-thick vibratome section of the hippocampus of an Alzheimer patient (AD, #96115, 90 years old) showing Aβ, APP^{+1}, ubiquitin, UBB^{+1}, GFAP and GFAP^{+1}. Abnormal Tau is present in the hallmarks of AD (G, neurofibrillary tangles, neuropil threads and dystrophic neurites) similar to APP^{+1}, Ubiquitin, UBB^{+1} and remarkably with GFAP and GFAP^{+1} (Hol et al., 2003). Note in panel E that GFAP also reacts with astrocytes. In panel H the 19S regulator subunit 6b is present in the same hallmarks indicating its upregulation (patient #88028, paraffin section, (Zouambia et al., 2008)) and proteasomal dysfunction that can be explained by the presence of UBB^{+1} by which the proteasome is clogged up.

et al., 2003). We subsequently focused upon ubiquitin B because of the potentially devastating effects of both a gain or loss of function of the resulting +1 protein in different cellular processes (Ciechanover and Brundin, 2003).

Ubiquitin B and molecular misreading

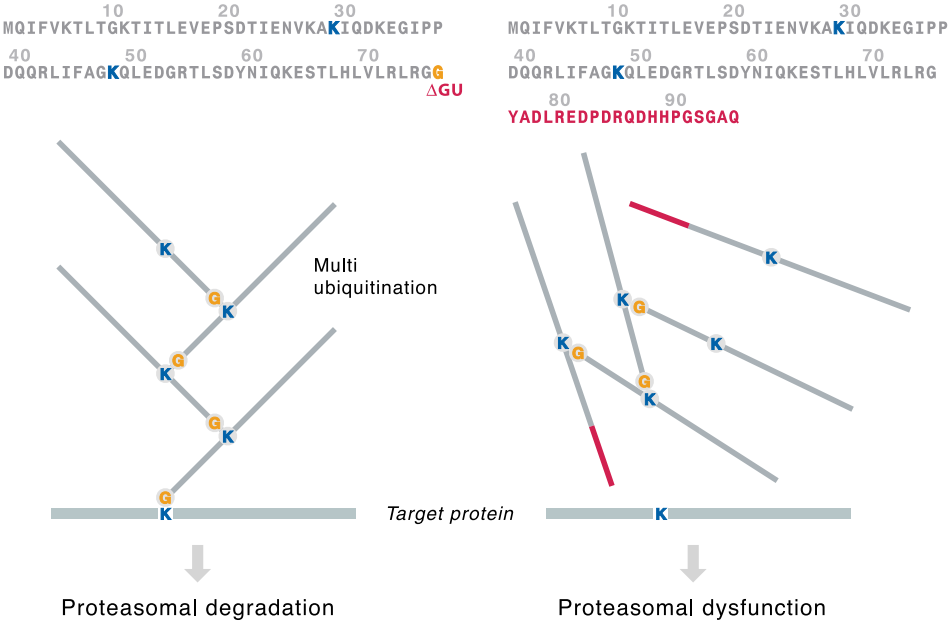
Human ubiquitin B is a protein localized on chromosome 17p11.2 and it is one of the best conserved eukaryotic proteins. From yeast to human, only at three positions does the amino acid sequence show variation in the 76 residues long protein, indicating its functional relevance. Ubiquitin B is synthetized in a three repeat from which 76 amino acids are cleaved off. The ubiquitin molecule harbors a number of lysine moieties, of which #29 and #48 are involved in ATP-dependent multi-ubiquitination, a process that triggers transport to the proteasome, followed by proteolysis. At the very C-terminus of ubiquitin a glycine moiety (#76) is present, which is essential for all biological function of ubiquitin B. A few nucleotides upstream of this C-terminal glycine moiety a GAGAG motif is present. The result of a dinucleotide deletion in this motif is a loss of the C-terminal glycine moiety and a 19 amino acids longer ubiquitin molecule called ubiquitin^{+1} (UBB^{+1}) (Figure 3). It was suggested that UBB^{+1} is unable to ubiquitinate and in fact, might be a substrate for ubiquitination (van Leeuwen et al., 1998), and disturb proteasomal degradation as mentioned in Vogel (Vogel, 1998).

Towards an effect of ubiquitin^{+1}

The functional relevance of UBB^{+1} has been clarified during the past five years. UBB^{+1} can be degraded at low concentrations (Lindsten et al., 2002), apparently after deubiquitination, but is also refractory to deubiquitination (Lam et al., 2000). Ubiquitinated UBB^{+1} furthermore appears to inhibit the proteasome potently (Lindsten et al., 2002). In case of high concentrations, neuronal cell death by apoptosis was found (De Vrij et al., 2001). Since deubiquitination of UBB^{+1} is a prerequisite for its entrance, subsequent unfolding, substrate recognition, chaperoning and channelling by 19S regulatory subunits to the 20S proteolytic core are possibly disturbed. Consequently, degradation of UBB^{+1} by the β 1, 2 and 5 subunits in the proteolytic core is impaired (Pickart and Cohen, 2004).

The next step to find out whether UBB^{+1} contributes to the neuropathogenesis of Alzheimer's disease, was to generate transgenic mice expressing UBB^{+1} regionally (hippocampus and cerebral cortex). This work has been executed successfully is in progress; we are currently using these transgenic UBB^{+1} mice to explore the downstream effects of UBB^{+1} (e.g. proteomic and behavioral analyses, long-term potentiation (LTP) and transcript and protein analysis of gene products involved in LTP, e.g. CREB; (Hegde, 2004)). Indeed, UBB^{+1} induces expression of heat shock proteins, as shown in human neuroblastoma cells (Hope et al., 2003), and a behavioral

Figure 3: Multiubiquitination of proteasome substrates.



Simplified and schematic representation of how ubiquitin acts via multiubiquitination through a number of enzymatic steps (E_1, E_2 and E_3) (left panel, for details see (Ciechanover and Brundin, 2003)). The lysine moieties at positions 29 and 48 are involved in the multiubiquitination and degradation. At the C-terminus of ubiquitin a GAGAG motif is present. It was shown that adjacent to this motif a dinucleotide deletion (ΔGU) occurs resulting in an extension of 19 amino acids (right panel, for further details see (Gerez et al., 2005)). Due to the dinucleotide deletion, the G_{76} moiety at the C-terminus, essential for binding to a target protein, is not synthetized. Consequently this molecule cannot ubiquitinate. In fact, it has become a substrate for degradation and contains a ubiquitin fusion degradation signal as well. It has been shown that ubiquitinated UBB^{+1} protein inhibits the proteasome (Lindsten et al., 2002) and acts synergistically with polyglutamine repeats with regard to aggregation and cell death in a cellular model of HD (de Pril et al., 2004).

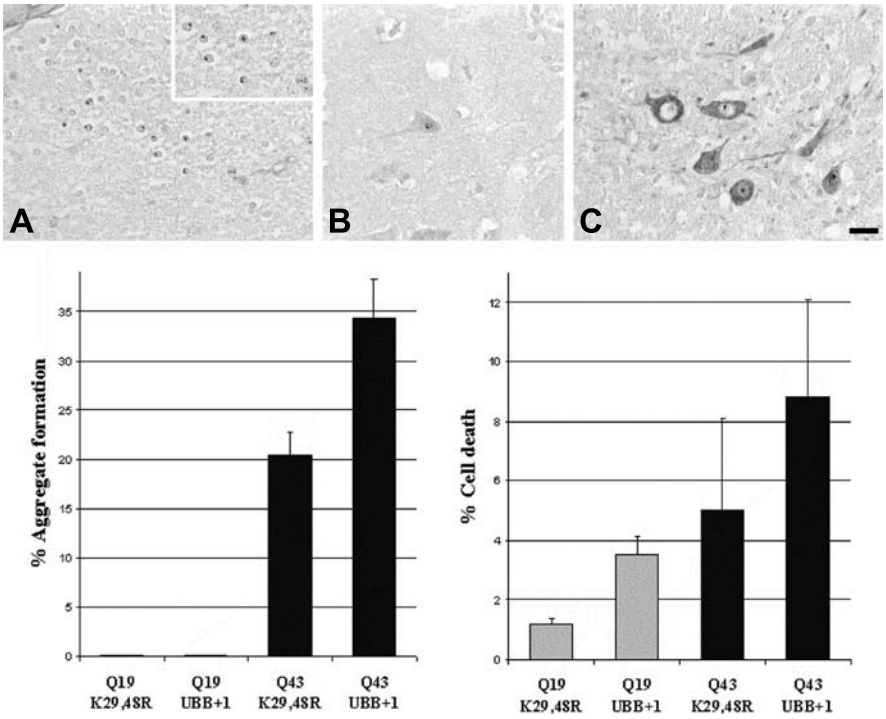
phenotype was found accompanied by other proteomic changes in these mice (Fischer et al., 2009). In this manner UBB^{+1} mouse lines have become available with a life-long genetically encoded inhibition of the proteasome. These unique lines can be used for crossing with other Alzheimer transgenic lines and other mouse models that are currently being used for other types of tauopathies (e.g. frontotemporal dementia) and Huntington disease. UBB^{+1} is present in the hallmarks of other tauopathies (Fischer et al., 2003) and polyglutamine diseases (Figure 4A-C) (de Pril et al., 2004). It was shown in a cellular model for Huntington disease (HD) that UBB^{+1} has a strong synergistic effect on aggregate formation and cell death (Figure 4D) (de Pril et al., 2004). These results are in agreement with the conclusion of Wexler et al. (Wexler et al., 2004), who reported that approximately 40% of the variance remaining in onset age is attributable to genes other than the HD gene and 60% is environmental.

Fischer et al. (Fischer et al., 2003) showed in the rat brain that UBB^{+1} requires the lysine moieties of position 29 and 48 for its proteasomal degradation. It appeared that under normal circumstances in both the rat brain and in young non-demented control patients UBB^{+1} can be degraded rapidly and only accumulates upon proteasomal impairment. In other words, the accumulation of UBB^{+1} in various tauopathies and polyglutamine diseases, but not in synucleinopathies, points to UBB^{+1} as a marker for proteasomal dysfunction (de Pril et al., 2004; Fischer et al., 2003). The latter results indicate a difference between tauopathies and polyglutamine diseases at one side and synucleinopathies at the other one. In synucleinopathies the ubiquitin machinery may be disturbed, as shown in rare forms of Parkinson's disease; the E3 ligase Parkin, the deubiquitinating enzyme ubiquitin carboxy terminal hydrolase UCH-L1, α-synuclein and DJ1 are mutated in familial forms of Parkinson's disease (Ciechanover and Brundin, 2003; Giasson and Lee, 2003). In tauopathies and several polyglutamine diseases, accumulation of UBB^{+1} (possibly following a failure to deubiquitinate; (Lam et al., 2000)) and other substrates of the proteasome (e.g. Aβ, huntingtin) inhibit the proteasome (Lindsten et al., 2002). However, whether or not the ubiquitin machinery or the proteasome itself is impaired, both in tauo- and synucleinopathies, and in polyglutamine diseases, proteasomal dysfunction is the outcome in all these neurological disorders.

Molecular misreading in non-neuronal cells

Again using the VP system, we were able to address the question whether molecular misreading occurs outside of neuronal cells. In order to do so we used transgenic mice in which the rat VP gene is expressed ectopically in epithelial structures of secretory organs under the control of the mouse mammary tumor virus long terminal repeat promotor. Indeed, VP was found in the gonadal system (e.g. testis and epididymis). Using antisera specific for frameshifted VP (VP^{+1}), we were able to show that in the principal cells of the caput of the epididymis VP^{+1}, VP and other parts of the VP precursor coexist. This result was supported by specific in situ hybridization

Figure 4: UBB^{+1} accumulates in polyglutamine diseases and enhances aggregate formation and cell death.



UBB^{+1} was found to accumulate in all thirty cases of Huntington disease tested (A, striatum, #4 of (de Pril et al., 2004), having 59 glutamine repeats, insert shows various intranuclear inclusions (blow-up of A, 1.3x) and B, frontal cortex of #5 in (de Pril et al., 2004), having 41 glutamine repeats). The same reaction was found in six cases of spinocerebellar ataxia-3 (C, pons of #36 in (de Pril et al., 2004), having a polyglutamine repeat length 24-70). Note the intense staining in the intranuclear inclusions as well as the cytoplasmic accumulation of UBB^{+1}. Digital recordings of 6-µm-thick paraffin sections were made by the extended-depth program by which different pictures were combined to give one in-focus composite image (Imagepro 5.0, Media Cybernetics, Silver Springs, USA). Bar = 20 µm. D, In a cellular model of HD we found that UBB^{+1} not only increases the aggregate formation of expanded polyglutamine repeats but in addition has a synergistic effect on polyglutamine-induced cell death. Differentiated SH-SY5Y neuroblastoma cells were lentivirally infected with constructs with truncated huntingtin fragments containing 19 or 43 glutamine repeats (HA-Q-GFP) in combination with either UBB$^{+1;K29,48R}$ or UBB^{+1}. Cells were quantified for aggregate formation 4 days after infection (left panel). Note that huntingtin with 19 glutamines does not result in the formation of aggregates. Constructs with 43 glutamines show a marked increase in the number of aggregate forming cells in the presence of UBB^{+1}. Cell-death was assessed by a cell survival assay 4 days after infection (right panel). Polyglutamine-GFP positive cells were analyzed by flow cytometry for the percentage of cell death under different conditions (de Pril et al., 2004). Q19 with UBB$^{+1;K29,48R}$ shows the background percentage of cell death due to infection or culturing. A minor increase in cell death was observed with either expansion of the polyglutamine repeat or co-infection of UBB^{+1} with Q19. A marked increase in cell death was observed after coinfection of UBB^{+1} with the construct with 43 glutamines (n=3; P<0.005; for details see (de Pril et al., 2004)).

(Van Leeuwen et al., 2000). Thus, VP^{+1} is formed in non-neuronal cells (molecular misreading), due to molecular misreading of the transgene. Subsequently, frameshifted ubiquitin (UBB^{+1}) was found in inclusions of various human diseases and suggests a similar mechanism as found in neuronal cells: the Mallory bodies of hepatocytes during cirrhosis in alcoholic liver disease (McPhaul et al., 2002), in hepatocytes of patients with an α1-antitrypsin deficiency (Wu et al., 2002) and in aggregates of inclusion body myositis (Fratta et al., 2004).

The unfolded protein response and the UPS

Many newly synthetized proteins are translocated into the lumen of the endoplasmic reticulum (ER), which is the place for their folding and assembly. Alterations in homeostasis by various cellular stressors that prevent protein folding cause an accumulation of misfolded proteins in the ER, which are referred to as types of ER stress. Eukaryotic cells can adapt, for survival, to deal with an accumulation of unfolded proteins in the ER by various signals from the ER lumen to the cytoplasm and the nucleus. This induction system is called unfolded protein response (UPR) and includes the transcriptional induction of UPR target genes (e.g. ER-resident chaperones, such as GRP78/BiP to facilitate protein folding), translational attenuation of global protein synthesis and ER-associated degradation (ERAD). In conformational diseases such as Alzheimer's disease, recent reports indicate that the UPR is involved (Katayama et al., 2004), and a link between ERAD and the UPS has been suggested (Kopito and Sitia, 2000; Kostova and Wolf, 2003). In addition, a proteasome independent ERAD pathway may exist (Donoso et al., 2005). So far no link between UBB^{+1} effects and the UPR activity has been reported.

Challenges for the next decade

- It is evident that the UPS contributes substantially to conformational diseases, such as Alzheimer's disease, during which "it stops delivery" (Miller and Wilson, 2003). This might reflect a primary or secondary event. Of course one should realize that Alzheimer's disease and other related inclusion diseases are multifactorial. The latter aspect needs to be kept in mind when generating transgenic mice. The step towards multiple transgenic mice has been taken (e.g. (Oddo et al., 2004)) and the numerous contributing factors to Alzheimer's disease can be organized in a temporal pattern (e.g. (Konishi et al., 2003; Muchowski and Wacker, 2005; Song and Jung, 2004)). The recently generated UBB^{+1} mouse lines are an obvious candidate for further crossings. Long term potential (LTP) measurements in the hippocampus of these mice and changes in gene and protein levels (e.g. CREB, synaptophysin and AMPA receptors, (Hegde, 2004)) as well as further proteomic analysis (Fischer et al., 2009) are currently under investigation.

- Synaptic plasticity is also known to be affected in Alzheimer's disease (Terry and Katzman, 2001). The UPS and its inhibition by UBB^{+1} are attractive candidates for further research (Ehlers, 2003). The recently developed transgenic UBB^{+1} mice (Fischer et al., 2009) may contribute in this respect.
- Another challenge is trying to substantiate the effects of different cellular stressors (e.g. Aβ, E2-25K/Hip2, UBB^{+1}) by introducing them into cell lines followed by rescue experiments, i.e. silencing the effect of these proteins by means of RNA interference (Novina and Sharp, 2004), as shown by Song et al. (Song and Jung, 2004).
- The presence of UBB^{+1} outside the nervous system, in dividing cells, enabled experiments *in vitro* to assess the contribution of UBB^{+1} to aggresome formation (e.g. (Bardag-Gorce et al., 2003; French et al., 2001)). Indeed, when the capacity of the proteasome is exceeded, aggregate formation followed by lysosomal degradation has become an increasingly attractive subject of research (e.g. (Shintani and Klionsky, 2004)). Other possible mechanisms to cope with insoluble proteins are posttranslational modifications like sumoylation and neddylation (e.g. (Schwartz and Hochstrasser, 2003; Steffan et al., 2004)), the contribution of which to inclusion formation in the various conformational diseases needs to be assessed.
- Relevant essential subunits of the ubiquitin and deubiquination machinery as well as of essential proteasome subunits are now ready for analysis to reveal their contribution to neuropathogenesis (e.g. (Cookson, 2004; Guterman and Glickman, 2004; McDonough and Patterson, 2003; Petrucelli et al., 2004)).
- It is clear that proteasomal activators are badly needed (without side-effects). Proteins as activators of the 20S proteolytic core are known as PA28 and PA200, and are obvious targets for the development of even more potent molecules mobilizing the proteolytic machinery (Rechsteiner and Hill, 2005). Alternatively elucidation of deubiquitinating enzymes (DUBS) by screening RNAi libraries might result in DUBS promoting deubiquitination of ubiquitinated UBB^{+1} (Brummelkamp et al., 2003).

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