The unfolded protein response: a common pathomechanism in tauopathies
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General introduction II

Removing protein aggregates:
The role of proteolysis in neurodegeneration

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Abstract

A common characteristic of neurodegenerative diseases like Alzheimer’s disease, Parkinson’s disease and Huntington’s disease is the accumulation of protein aggregates. This reflects a severe disturbance of protein homeostasis, the proteostasis. Here, we review the involvement of the two major proteolytic machineries, the ubiquitin proteasome system (UPS) and the autophagy/lysosomal system, in the pathogenesis of neurodegenerative diseases. These proteolytic systems cooperate to maintain the proteostasis, as is indicated by intricate cross talk. In addition, the UPS and autophagy are regulated by stress pathways that are activated by disturbed proteostasis, like the unfolded protein response. We will specifically discuss how these proteolytic pathways are affected in neurodegenerative diseases. We will show that there is a differential involvement of the UPS and autophagy in different neurodegenerative disorders. In addition, the proteolytic impairment may be primary or secondary to the pathology. These differences have important implications for the design of therapeutic strategies. The opportunities and caveats of targeting the UPS and autophagy/lysosomal system as a therapeutic strategy in neurodegeneration will be discussed.
Introduction

Correct protein folding is central to all cellular functions, and misfolding may lead to loss of function as well as a toxic gain of function of the protein involved. The maintenance of homeostasis in the folding of proteins is therefore extremely important. The complement of this homeostatic machinery has been termed the “proteostasis network” and is comprised of components that assist in folding as well as factors involved in the recognition and removal of misfolded proteins. The final destination of terminally misfolded proteins is degradation by the ubiquitin proteasome system (UPS) or the autophagy/lysosomal system. Protein misfolding is a common event in any cell, and is dealt with very efficiently under physiological conditions. In case of disturbed proteostasis, the aberrant proteins accumulate and can form aggregates.

Neurons are post-mitotic cells that cannot remove aggregates by cell division and are therefore very dependent on proper functioning of protein quality control. Many neurodegenerative diseases are characterized by the accumulation of aggregates of misfolded proteins in neurons, indicating a severe disturbance of proteostasis in these cells. In this review, we will discuss neurodegenerative diseases categorized by accumulation of common intracellular aggregating proteins: tau, α-synuclein and proteins containing expanded polyglutamine tracts (Table 1). Different types of aggregates preferentially occur in specific brain regions and induce neuronal loss which is reflected in specific clinical symptoms. The aggregates can accumulate at different subcellular locations and result in a toxic gain or loss of function by interfering with cellular processes as diverse as vesicle transport, transcription or mitochondrial function. Over the last decade a common theme for protein folding diseases has emerged. Initially small, oligomeric assemblies are formed that represent the most toxic species, whereas the larger, highly structured aggregates appear a relatively safe way for deposition. The inclusion bodies found in neurons affected by neurodegenerative disease may thus reflect the last resort of failed proteostasis, at the point where proteolysis is incapable to degrade the aberrant proteins. The initiating event that drives aggregation is not always clear and is often multifactorial. In some cases a mutant protein facilitates the aggregation, but more often an age dependent accumulation of wild type proteins takes place. This is likely to be caused by a combination of increased formation of aggregates and a decreased clearance, for example by an age-related decline of proteolytic capacity.
At present, there is still no cure available for neurodegenerative disorders. Symptomatic treatments exist, that may improve a subset of symptoms for limited time, such as levodopa supplementation in Parkinson’s disease (PD) and cholinesterase inhibition in Alzheimer’s disease (AD). These treatments are of limited efficacy and have significant side-effects. Therefore a tremendous research effort is put into disease modifying strategies. The focus of these is on aggregated proteins, due to their key role in the pathogenesis, by prevention of their formation or stimulation of their removal. This review will discuss the proteolytic machineries that remove aggregation prone proteins. Proteolytic mechanisms may also be involved in the generation of fragments that facilitate aggregation (e.g. presenilins, calpains, caspases and matrix metalloproteinases) but this is beyond the scope of this review. We will focus on the UPS and the autophagy/lysosomal system that are the major players in protein quality control, although also other proteases may contribute to the degradation of aberrant proteins.

We will first discuss some basic aspects of the UPS and the autophagy/lysosomal system. Subsequently we will address the crosstalk between these two major proteolytic machineries and the regulation of proteolysis in case of disturbed proteostasis by stress pathways like the unfolded protein response (UPR). Decline of the proteolytic capacity is in many cases a major contributor to the pathogenesis of neurodegenerative diseases. This may be the primary defect that causes the accumulation of aggregates, but may also be secondary to accumulation of aggregates that cause impairment of the proteolytic machinery and initiate a vicious circle. Therefore, in the second part of the review we will discuss how the UPS and the autophagy/lysosomal system are affected in neurodegenerative diseases in view of opportunities for specific therapeutic intervention.

**Proteolysis of misfolded proteins**

*Ubiquitin proteasome system*

The UPS is the major degradational system in the cell that is involved in the degradation of short-lived, misfolded and defective proteins. Proteins that are destined to be degraded by the UPS are first modified with ubiquitin, a 76 amino acid protein used as a molecular tag that marks proteins for a variety of cellular pathways. Some proteins, mostly monomeric and unfolded proteins, can be degraded in an ubiquitin independent manner, as reviewed in. Ubiquitin becomes linked to a substrate via a reversible isopeptide
bond mediated by the sequential action of three distinct ubiquitin binding proteins. First, an ubiquitin activating enzyme (E1) activates monomeric ubiquitin in an ATP dependent manner and forms a high energy thiol-ester linkage with the C-terminal glycine of ubiquitin. Second, the activated ubiquitin is transferred to an ubiquitin conjugating enzyme (E2). Third, an ubiquitin-ligase (E3) cooperates with the E2 and either directly catalyzes or mediates transfer of ubiquitin to the ε-amino group of a Lys (K) residue in the substrate protein. Alternatively, ubiquitination can occur at the N-terminal residue of the substrate protein. Additional ubiquitin proteins can be ligated onto one of seven K residues (K6, K11, K27, K29, K33, K48 and K63) within the attached ubiquitin molecule. In this manner mono- or polyubiquitin chains can be added to proteins which target them to different pathways. Polyubiquitination on K48 and K63 is specifically associated with substrate degradation.

The human genome contains 2 E1 and approximately 40 E2 and ~600 E3 enzymes. E3 ligases are substrate specific and convey a high level of regulation and selectivity to the UPS. The E3 ligases are divided into three subtypes based on their catalytic domain; homologous to E6-associated protein C terminus (HECT), really interesting new gene (RING) and U-box ligases. The HECT domain E3 accepts ubiquitin from an E2 conjugating enzyme and transfers it to the substrate, whereas RING and U-box E3s do not form ubiquitin intermediates but form a scaffold between the E2 and the substrate protein. A number of E3 enzymes and their substrates are fully characterized and based on these studies E3 enzymes are thought to recognize a specific sequence, structural element or post-translational modification (e.g. phosphorylation, glycosylation and acetylation) within the substrate protein. Most HECT E3 ligases function as monomers, whereas most RING and U-box E3 ligases need to form a complex with other proteins to bind their respective E2 enzymes and substrate proteins. Specific E2/E3 combinations attach K48 or K63 polyubiquitin chains to substrate proteins. Analogous to kinases and phosphatases, deubiquitinating enzymes (DUBs) exist that are capable of removing ubiquitin moieties and in this manner regulate ubiquitin dependent targeting. The majority of human DUBs are cysteine proteases, complemented by a small group formed by metallo proteases. The large variety in DUBs indicates they have numerous functions and this relates to the large variety of ubiquitin chain modifications that exist within the cell.

The classical signal to target proteins for selective degradation by the proteasome is ubiquitination with a chain of at least four K48 linkages. The 26S proteasome complex
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is responsible for ubiquitin dependent degradation and is found throughout the cytoplasm and the nucleus of all eukaryotic cells. It consists of the 20S core proteasome flanked by one or two 19S regulatory subunits. The 20S core is a cylindrical stack of four heptameric rings composed of heteromeric alpha (α) subunits (outer rings) and beta (β) subunits (inner rings). Together, these rings form a proteolytic chamber into which unfolded peptides enter and are degraded.\textsuperscript{173,172} Three of the proteasome β subunits in the 20S core are catalytically active. Together these subunits, β1, -2 and -5, convey peptidyl-glutamyl peptide hydrolyzing (PGPH), trypsin- and chymotrypsin like activity to the proteasome.\textsuperscript{173} Under the influence of cytokines (e.g. γIFN mediated NF-κB signalling)\textsuperscript{174} the constitutive β subunits can be replaced by the inducible β subunits β1i, -2i and -5i, which slightly alters proteasome catalytic activity. The 19S regulatory particles function

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{A schematic overview of the ubiquitin proteasome system. Ubiquitination commences when an E1 enzyme activates ubiquitin (Ub) in an ATP dependent manner (1) and transfers it to an E2 enzyme (2). The E2 enzyme directly transfers Ub to an E3 (3) or forms a complex with the E3 (4), leading to ubiquitination of the target protein (5). A chain of 4 K48 conjugated ubiquitin molecules signals degradation by the proteasome. Deubiquitinating enzymes can remove the ubiquitin molecules, thereby preventing degradation of the target protein (6). The 19S regulatory cap of the 26S proteasome also has deubiquitiating activity and removes and releases ubiquitin into the cytoplasm (7). Signalling via the NF-κB route leads to expression of the inducible proteasome, in which the catalytically active β subunits (β1, β2 and β5) are replaced by the inducible β subunits (β1i, β2i and β5i, (8)). Different regulatory subunits may associate with the 20S proteasome, e.g. the REG/PA28 subunit that is induced via NF-κB signalling (9).}
\end{figure}
in the recognition of substrate proteins and facilitate their degradation by the 20S core particle. The 19S regulatory particle consists of a lid and a base, the latter is docked on top of the entrance to the 20S proteolytic chamber. The lid component recognizes the K48 polyubiquitin tag on the substrate protein and contains DUB activity which removes and recycles ubiquitin. The base component contains ATPase activity and is involved in substrate unfolding. In addition, it interacts with the 20S core α-subunits to facilitate entry of ubiquitin tagged proteins into the proteolytic chamber. Other regulatory particles can interact with the 20S proteasome and modulate its activity. For example the 11S proteasome activator family (REG/PA28) is induced by γIFN mediated NF-κB signalling. This particle activates peptidase activity of the 20S proteasome but does not promote the degradation of intact proteins as it lacks ATPase activity. However, it has been suggested that a 19S–20S–11S hybrid proteasome could enhance proteolysis. Figure 1 gives a schematic overview of the UPS.

**Autophagy**

Autophagy comprises degradational pathways that ultimately deliver their targets to the lysosome. The three main autophagic systems are microautophagy, chaperone mediated autophagy (CMA) and macroautophagy. Microautophagy involves the direct uptake of cytosolic content, by invagination of the lysosomal membrane. CMA targets proteins with a specific consensus motif recognized by a heat-shock cognate protein, which aids in the direct and selective transport into the lysosome. Macroautophagy involves the formation of a double- or multi-membrane structure, the autophagosome, which engulfs the cellular material targeted for degradation (proteins, cytosolic debris and organelles), and subsequently fuses with the lysosome. In this review we will mainly focus on macroautophagy and therefore we will simply refer to it as autophagy.

Autophagy is a complex and strongly regulated process that involves several autophagy related genes and proteins (**ATG** and **Atg**, respectively). To date, the number of identified Atg proteins has grown to over 30 in yeast. The autophagy process is evolutionary conserved and many human Atg orthologues have been identified. At least 15 Atg proteins form the core machinery of autophagy and these Atg proteins cooperate in complexes that are involved in the sequential steps in the formation and maturation of the autophagosomes; the initiation, elongation and fusion with the lysosome. The
mechanistic details of the autophagic process are described in recent reviews\textsuperscript{186,181} and are illustrated in Figure 2.

A straightforward marker of autophagic activity is currently not available and changes in levels of Atg proteins should be interpreted with caution.\textsuperscript{187} A commonly used marker is microtubule associated protein 1 light chain 3 (LC3). LC3 is the mammalian homologue of Atg8 and is an essential protein for the formation and maturation of autophagosomes. Cytosolic LC3-I is converted to membrane associated LC3-II by the ubiquitin-like conjugation of LC3 to phosphatidylethanolamine (PE). In this process, Atg7 exhibits the ubiquitin-activating E1-like activity and Atg3 the ubiquitin-conjugating E2-like activity.\textsuperscript{188} LC3-II decorates both the inner and outer membrane of the forming autophagosome and the relocalization of LC3-II to membranous structures can be used as a marker for autophagy. LC3-I and LC3-II can be distinguished on Western blot and an increased LC3-II level is often interpreted as increased autophagy. However, this simple interpretation is complicated by the degradation of LC3-II itself during the autophagic process and is therefore dependent on autophagic flux. In conclusion, LC3-II levels can give an indication of steady-state levels of autophagy but autophagic flux measurement

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{A schematic overview of the autophagy/lysosomal system. Formation of the phagophore membrane is initiated by the Vps15/Vps34/Beclin-1 complex (1) and involves the sequential action of several Atg proteins (2). The Atg5/12/16 complex (3) and LC3-II (4) are involved in autophagosome formation and decorate the membrane (5). Atg5/12/16 is associated with the outer membrane, whereas LC3-II is found on both the inner and outer membrane. The target to be degraded is engulfed (6) leading to the formation of an autophagosome (7). Fusion of the autophagosome with a lysosome, mediated via LAMP2A and Rab7, leads to the formation of an autophagolysosome (8) and subsequent degradation of the engulfed material (9). Signalling via mTOR negatively regulates autophagy (10) as does interaction of Beclin-1 with the anti-apoptotic protein Bcl2 (11).}
\end{figure}
will have to be performed in the presence of lysosomal fusion/degradation inhibitors (reviewed in \(^{187}\)).

Basal autophagic activity was considered to mainly regulate the turnover of long-lived cytoplasmic proteins.\(^{189,190}\) More recently, autophagy has been implicated in the degradation of polyubiquitinated protein species. Two different autophagy deficient mouse models (Atg5\(^{-/-}\) and Atg7\(^{-/-}\)) show prominent accumulation of ubiquitin positive protein aggregates in neurons, accompanied by neurodegeneration.\(^{191,192}\) These findings suggest that basal autophagy assists in the clearance of aggregation prone and ubiquitinated protein species in neurons. Apart from the targeting consensus sequence involved in CMA, the recognition of substrate and targeting to the autophagy/lysosomal system is not well understood. LC3-II has been implicated to be involved in the recognition of cargo for the autophagosome.\(^{181}\) In addition, it has been suggested that ubiquitin K63 linkage represents a signal that targets proteins to the autophagic machinery, but the exact mechanism is not known (\(^{193}\), see also below).

Autophagy is increased by perturbations in cellular homeostasis.\(^{194}\) A well described mechanism for the induction of autophagy involves the mammalian target of rapamycin (mTOR) \(^{195}\) that integrates several signalling pathways. Inhibition of mTOR activity initiates a signal cascade that activates autophagy \(^{196}\), which aims to aid in the restoration of homeostasis. Another important factor that regulates autophagy in response to cellular stress is Beclin-1 (the mammalian homologue for Atg6). Beclin-1 forms a complex with vacuolar protein sorting (Vps) protein 34 and Vps15, which activates autophagy.\(^{197}\) The Beclin-1 complex is further modulated by its binding partners, including Atg14, UV radiation resistance-associated gene (UVRAG) and activating molecule in Beclin1-regulated autophagy (Ambra) 1.\(^{198,199}\) Beclin-1 can also interact with the anti-apoptotic protein Bcl2. The interaction of Beclin-1 with Vps34 and Bcl2 is mutually exclusive, and therefore increased levels of Bcl2 lead to decreased Vps34 binding and consequently to decreased autophagy.\(^{200}\) The binding of Beclin-1 to either Bcl2 or Vps34 is therefore well positioned to relay the cellular stress response between the protective autophagy response and cell death.\(^{200}\) After completion of autophagosome formation, fusion with the lysosome takes place in which the lysosomal-associated membrane protein (Lamp) 2A and the small GTP-ase Rab7 are involved.\(^{201}\) This step enables the actual degradation of the material that was sequestered in the autophagosome.
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**Lysosomal degradation**

Lysosomes are acidic membrane-surrounded compartments containing over 50 hydrolytic enzymes that have an acid pH optimum in the range of 4.6-5.0. These organelles are responsible for the intracellular breakdown of various macromolecules including proteins and peptides. The macromolecules present in the lysosome are derived from the extracellular space through endocytosis or phagocytosis, or from the cytoplasm through autophagy, as previously described. Therefore, the lysosomes represent another means by which misfolded or aggregated proteins can be degraded, besides the UPS. Indeed, lysosomes have been shown to degrade α-synuclein, tau and huntingtin involved in PD, AD and Huntington’s disease (HD), respectively.

The main class of lysosomal proteases responsible for the breakdown of these proteins is the cathepsins. Cathepsins are subdivided into three subgroups based on their active site amino acid. These subgroups are the cysteine (cathepsins B, C, F, H, K, L, O, S, V, U, W and X), the aspartyl (cathepsins D and E) and the serine cathepsins (cathepsins A and G). Cathepsins are expressed in a cell- and tissue-specific manner. In neurons, cathepsins B and L are the most important. Cathepsins cleave their substrate in an unspecific manner and most of the enzymes are endopeptidases. To prevent any unwanted catalytic activity, cathepsins are tightly regulated. First, they are synthesized as inactive zymogens and these inactive enzyme precursors are activated by removal of the N-terminal propeptide by other proteases or by autocatalysis at acidic pH. Another important way by which cathepsin activity is regulated is by interaction with their endogenous protein inhibitors. The cystatins are reversible competitive inhibitors of C1 cysteine proteases and have been classified into three types: the stefins, the cystatins and the kininogens. In conclusion, the activity of the cathepsins is regulated by various mechanisms including regulation of their expression level, zymogen processing and endogenous inhibitors.

**Aging**

The activity of the cellular proteolysis pathways appear to decrease with aging. An increase in the amount of oxidized proteins is found in aging cells and activity of the proteasome decreases with aging in several studies using rat models and in human fibroblasts, T-cells and retinal cells. In addition, the activity of the autophagy/lysosomal system also decreases with aging. The rate of formation of autophagic
vacuoles and their clearance was shown to be reduced in old rat and mouse hepatocytes compared to young hepatocytes. Furthermore, lipofuscin, an undegradable polymeric material, accumulates in the lysosomes of post-mitotic cells (e.g. neurons) with aging and is considered a hallmark of the aging cell. Lipofuscin is thought to inhibit lysosomal degradation and sensitize the lysosomes to oxidative stress. As we will discuss in a later section, impairment of the proteolytic machinery is an important feature of neurodegenerative disorders. The age-related reduction of proteostasis may therefore be an important modulating factor in these age-dependent diseases.

**Regulation**

**Crosstalk**

Accumulation of un- and misfolded proteins is a condition that increases the demand for degradation. These aberrant proteins are recognized by chaperone proteins that prevent aggregation and facilitate refolding. In addition they are involved in targeting misfolded proteins for degradation. Terminally misfolded proteins that arise in either of the major protein synthesizing and folding compartments, the endoplasmic reticulum (ER) and cytosol, can be degraded by the UPS. Misfolded ER proteins targeted to the UPS will first have to be exported from the ER, a process called ER associated degradation (ERAD). The crucial function of protein degradation is illustrated by the crosstalk between the UPS and autophagy. In case of proteasome impairment or of increased demand for degradation during ER stress, autophagy is triggered (ER activated autophagy, EAA), indicating that the two proteolytic pathways used by the ER are tightly coupled. Although in several experimental setups it has been shown that inhibition of proteasome activity leads to increased autophagy, the reverse has not been shown to date. In contrast, inhibition of lysosomal activity was shown to inhibit proteasomal flux, suggesting that the proteasome does not provide a back-up system for the autophagy/lysosomal system. This may relate to a specific function for autophagy in the proteolysis of substrates that are undegradable or inaccessible by the proteasome. The restrained environment of the catalytic core of the proteasome restricts UPS proteolysis to monomeric and unfolded proteins. To facilitate unfolding proteins before feeding them into the catalytic chamber of the proteasome, the assistance of proteins like the AAA-ATPase P97/Valosin containing protein (VCP) is required. For proteins that can not be unfolded or that are aggregated, autophagy provides a degradational pathway. In addition, proteins that are trapped in the
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ER and thus are inaccessible to the UPS, may be disposed of via an autophagic process where parts of the ER are engulfed by an autophagophore: ER-phagy.\textsuperscript{237}

Impaired proteasome function induces the formation of aggresomes \textit{in vitro}. These are ubiquitin positive aggregates that are concentrated at the microtubule organizing center via transport along the microtubules. The formation of aggresomes may facilitate autophagy, by transport of aggregated proteins and proteolytic machinery to the same location in the cell.\textsuperscript{238} It is important to note that aggresomes or aggresome like induced structures (ALIS) are to date only observed \textit{in vitro} in the presence of proteasome inhibitors and that it is not shown that these structures are precursors to the inclusion bodies found in neurodegenerative diseases. However, they may provide insight in the defence mechanisms activated in response to inadequate proteolysis. In support of the validity of aggresomes to model certain aspects of disease related inclusion bodies, a proteomic analysis of aggresomes induced by proteasome inhibition demonstrated overlap with proteins identified in Lewy bodies (LBs).\textsuperscript{239}

An important bridging factor between the UPS and autophagy is p62/SQSTM1. P62 functions as an adaptor between polyubiquitin and LC3, mediating possible crosstalk between the two proteolytic machineries.\textsuperscript{240,241} Isolated p62 can bind K48 chains, but there is evidence that suggests it preferentially binds K63 chains in the cellular environment.\textsuperscript{169,242} K63 linkage has been implicated in targeting of proteins for autophagy.\textsuperscript{193} In agreement with that, large sized p62 positive aggregates can be degraded by autophagy and p62 is required for ALIS removal.\textsuperscript{241} The levels of p62 itself are controlled by autophagy and thus inhibition of autophagy increases p62 levels.\textsuperscript{243,244} This in itself may be central to the function of p62 in sensing proteostatic stress, as increased levels of p62 induce inclusion body formation.\textsuperscript{240,241} As mentioned above, inhibition of autophagy impairs UPS flux. Interestingly, this can be rescued by reduction of p62 levels using siRNA, suggesting that the binding of p62 to polyubiquitinated proteins actively inhibits proteasomal degradation under these conditions.\textsuperscript{233} One of the proteins of which the proteasomal degradation is inhibited by p62 is LC3, a major player in autophagy as discussed previously.\textsuperscript{245} This would suggest that increased p62 binding to aggregated proteins increases autophagy via stabilization of LC3. In this way LC3 may play an important role in p62 mediated proteolytic crosstalk as well. Other adaptor proteins exist that perform a p62 like function, for example the neighbour of Brea1 (NBR1) protein \textsuperscript{246}, but their exact roles are not entirely clear. They may act in a cell type specific manner; however, a function for NBR1
in neurons has not been shown yet. Recent studies have identified autophagy-linked FYVE protein (Alfy) as an important regulator in autophagy that interacts with p62, Atg5 and phosphatidylinositol 3-phosphate (PI3P) in target recognition of ubiquitinated aggregates.\textsuperscript{247} Interestingly, activation of Alfy involves translocation from the nucleus to the cytosolic compartment. In earlier work, Alfy was already shown to be translocated in response to proteasome inhibition.\textsuperscript{248} This is an effect also observed for p62, and the shuttling of Alfy was demonstrated to be dependent on p62.\textsuperscript{249} This indicates that Alfy is also involved in crosstalk between the UPS and autophagy. Another factor

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**Figure 3:** Cross-talk between the UPS and the autophagy/lysosomal system and the role of p62. The UPS and the autophagy/lysosomal system are both involved in degrading ubiquitinated proteins. Under physiological conditions they preferentially degrade K48 or K63 ubiquitinated proteins, respectively. However, under periods of stress, e.g. the UPR or the cytosolic heat shock response, the autophagy/lysosomal system can degrade substrates that were originally targeted to the UPS. During stress, proteins accumulate and could potentially be hazardous if they are not quickly degraded. The ubiquitin binding protein p62 functions as a bridging factor between the UPS and the autophagy/lysosomal system. P62 preferentially binds K63 polyubiquitinated proteins (1) and promotes the formation of aggregates (2) that are subsequently degraded by the autophagy/lysosomal system. P62 can also bind K48 polyubiquitinated proteins (3) and can divert them to the autophagy/lysosomal pathway when they are not effectively cleared by the proteasome. The levels of p62 are regulated by the autophagy/lysosomal system (4) and binding of p62 to K63 polyubiquitinated targets stabilizes LC3, hereby stimulating autophagy (5). In addition, p62/K63 complexes inhibit the proteasomal degradation (6). Inhibition of the proteasome activates autophagy (7), but the reverse has not been demonstrated (8). This indicates that the autophagy/lysosomal system functions as a backup to the UPS and is activated when this system is incapable of coping with the protein load.
that connects the UPS and autophagy is the microtubule associated histone deacetylase (HDAC) 6 that binds to K48 polyubiquitin as well as to dynein motors. It accumulates in aggresomes following proteasome inhibition and it was suggested that it contributes to aggresome formation via microtubule dependent transport. Inactivation of HDAC6 shows that it is required for autophagy, leading to the idea that the interaction with the dynein motor facilitates transport of autophagy components.

In summary, autophagy provides a second line of defence if the UPS is not able to solve the problems. Crosstalk between the two systems is mediated by (possibly specific) ubiquitin linkages and adaptor proteins. The p62 protein is located at a central position to sense disturbances in proteostasis. Figure 3 illustrates the central role of p62 in the crosstalk between the UPS and the autophagy/lysosomal system.

**Stress pathways**

Disturbances in proteostasis activate stress sensing signalling pathways that regulate factors to restore homeostasis. In the cytosol the accumulation of misfolded proteins initiates the heat shock response by titrating chaperones away from heat shock transcription factor 1 (HSF1), which then translocates to the nucleus, where it activates transcription of heat shock responsive genes, including chaperones. One of the major chaperones induced by the heat shock response, Hsp70, influences lysosomal integrity and function, and could thereby affect autophagic flux. This is corroborated by the recent development of a small molecule inhibitor of Hsp70, which disrupts autophagic clearance and enhances aggregate formation.

Accumulation of misfolded proteins in the ER results in activation of the UPR, which comprises a more complex signalling network. The mechanistic details of the UPR are elaborately described in Chapter 1 of this thesis. Another pathway that was reported to be activated in response to ER stress is the ER overload response (EOR). This involves activation of the NF-κB transcription factor, which is one of the major inducers of inducible proteasome subunit expression. This in combination with the different catalytic activity of the inducible proteasome and its reported localization at the ER membrane prompted our lab to study whether the inducible proteasome is regulated by ER stress. We found no evidence for upregulation of the inducible proteasome by ER stress in neuronal cells. We could not detect activation of the EOR in response to ER stress suggesting that this
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Response may be a specific function in cells of the immune system that is not operant in neuronal cells. It appears that regulation takes place at the level of factors that facilitate degradation rather than activation of the proteolytic machinery itself. Our own work ([Chapter 4](#)) indicates that overall proteasomal activity is not increased by activation of the UPR in neuronal cells.\(^{256}\) **Figure 4** gives a schematic overview of the influence of the stress pathways on the cellular proteolytic machineries.

### Proteolysis in neurodegenerative diseases

Several neurodegenerative disorders are characterized by the accumulation of insoluble cytoplasmic or nuclear aggregates, implicating an insufficiency in the capacity of proteolysis. Impairment of the UPS or the autophagy/lysosomal system is therefore an important contributor to disease pathogenesis. This may be primary or secondary

<table>
<thead>
<tr>
<th>Stress response</th>
<th>Mediators</th>
<th>Proteolytic effect</th>
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<tbody>
<tr>
<td>UPR</td>
<td>→ ATF6</td>
<td>BiP and other chaperones → Facilitates unfolding and degradation via ERAD</td>
</tr>
<tr>
<td></td>
<td>→ IRE1</td>
<td>XBP1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XBP1/–</td>
</tr>
<tr>
<td></td>
<td>→ PERK</td>
<td>ATF4</td>
</tr>
<tr>
<td>EOR</td>
<td>→ NF-κB</td>
<td>Chaperones</td>
</tr>
<tr>
<td>HS</td>
<td>→ HSF1</td>
<td>Hsp70</td>
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**Figure 4**: Schematic overview of the effect of stress responses on cellular proteolysis. The UPR influences the cellular proteolytic system by enhancing expression of molecular chaperones (e.g. BiP) and components of the ERAD and autophagy/lysosomal pathway. This is achieved by signalling via ATF6, IRE1 and PERK and the transcription factors XBP and ATF4. Increased XBP1 expression and XBP1 deficiency differentially affect the degradational pathways. An alternative ER stress pathway, the EOR, signals via NF-κB and could lead to increased inducible proteasome subunit expression. In the cytosol, the heat shock (HS) response increases expression of molecular chaperones via the HSF1 transcription factor, leading to increased unfolding and thereby facilitation of degradation. The HSF1 responsive Hsp70 chaperone specifically enhances the autophagy/lysosomal pathway.
**Table 1:** General characteristics of the tauopathies, synucleopathies and polyglutamine expansion disorders. This table lists the different neurodegenerative disorders that are associated with tau, α-synuclein or polyglutamine pathology. These disorders differ in the appearance of the pathology, brain areas affected and/or the association with specific mutations.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Pathology</th>
<th>Affected brain areas</th>
<th>Mutations</th>
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<tbody>
<tr>
<td><strong>Tauopathies</strong></td>
<td></td>
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<tr>
<td>Alzheimer’s disease</td>
<td>Extracellular Aβ deposits NFT, NP, NT</td>
<td>Early involvement transenthorinal region, subsequent progression into limbic areas and neocortex</td>
<td>APP, PS1, PS2</td>
</tr>
<tr>
<td>Pick’s disease</td>
<td>PB, inclusions in glial cells</td>
<td>Frontal and temporal lobes</td>
<td></td>
</tr>
<tr>
<td>Progressive supranuclear palsy</td>
<td>Diffuse cytoplasmic aggregates in neurons, globose type NFTs, inclusions in tuft-shaped astrocytes, coiled bodies in oligodendrocytes</td>
<td>Basal ganglia, brainstem, cerebral cortex, cerebellum, spinal cord</td>
<td></td>
</tr>
<tr>
<td>Corticobasal degeneration</td>
<td>Diffuse cytoplasmic aggregates in neurons, NFT, NT, astrocytic plaques, coiled bodies in oligodendrocytes</td>
<td>Predominantly motor cortex, basal ganglia, diencephalon, brainstem</td>
<td></td>
</tr>
<tr>
<td>Frontotemporal dementia associated with chromosome 17</td>
<td>PB or NFT depending on the MAPT mutation, astrocytic inclusions and coiled bodies</td>
<td>Most severe in frontal lobe (grey and white matter), basal ganglia, substantia nigra and hippocampus</td>
<td>MAPT</td>
</tr>
<tr>
<td><strong>Synucleopathies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>Neuronal LB composed of α-synuclein</td>
<td>Predominantly basal ganglia (substantia nigra), progression into the midbrain and cortex</td>
<td>SNCA, PARK2</td>
</tr>
<tr>
<td>Dementia with Lewy bodies</td>
<td>Neuronal LB composed of α-synuclein</td>
<td>Cerebral cortex, substantia nigra</td>
<td>SNCA</td>
</tr>
<tr>
<td>Multiple system atrophy</td>
<td>Oligodendroglial cytoplasmic inclusion bodies composed of α-synuclein and tau</td>
<td>Predominantly in putamen, caudate nucleus, substantia nigra, locus coeruleus, pontine nuclei, inferior olivary nucleus, Purkinje cells of the cerebellar cortex</td>
<td></td>
</tr>
<tr>
<td><strong>Polyglutamine disorders</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>Neuronal intranuclear and cytoplasmic inclusion bodies composed of huntingtin protein/ polypeptides</td>
<td>Predominantly basal ganglia</td>
<td>HTT</td>
</tr>
<tr>
<td>Spinocerebellar ataxia 1-3, 6-7 and 17</td>
<td>Neuronal intranuclear inclusions of expanded polyglutamine containing proteins/ polypeptides</td>
<td>Predominantly cerebellum</td>
<td>ATXN-1, ATXN-7</td>
</tr>
<tr>
<td>Dentatorubral pallidolusian atrophy</td>
<td>Neuronal and glial intranuclear inclusions</td>
<td>CNS, spinal cord</td>
<td>ATN1</td>
</tr>
<tr>
<td>Spinobulbar muscular atrophy</td>
<td>Neuronal intranuclear inclusions</td>
<td>Lower motor neurons</td>
<td>AR</td>
</tr>
</tbody>
</table>
to disease pathology which has important implications for therapeutic approaches. In the following section we discuss neurodegenerative disorders based on three reactive proteins commonly found in the inclusions; tau, α-synuclein and polyglutamine. Some general characteristics of the aggregates in these diseases are summarized in Table 1.

**Tauopathies**

Tau pathology is observed in several neurodegenerative disorders including AD and frontotemporal lobar degeneration with tau pathology (FTLD-tau). The tauopathies are extensively described in Chapter 1. Here we discuss tau and tau pathology in relation to its proteolysis. Tau is involved in microtubule stabilization and the dynamic phosphorylation and dephosphorylation of tau by kinases and phosphatases regulates its binding to the microtubules. In tauopathies hyper phosphorylated tau (p-tau) accumulates in insoluble cytoplasmic inclusion bodies. Tau isoform composition, cellular and regional distribution and the morphology of the inclusions vary in each disorder. The most prevalent tauopathy is AD, characterised by the intracellular accumulation of p-tau in the form of neurofibrillary tangles (NFTs) and the extracellular accumulation of amyloid beta (Aβ) in senile plaques. Small oligomeric species of tau or tau fragments are thought to be most toxic, whereas the larger aggregates might be protective as they sequester the toxic oligomers from important cellular compartments. The majority of data discussed in this section deals with AD, however data on FTLD-tau are added if available. Tau is degraded by the 26S and the 20S proteasome in *in vitro* degradation assays. The use of proteasome inhibitors in cell and animal models leads to tau accumulation in some studies, whereas in others tau levels do not change or are even decreased. Furthermore, tau is a substrate for degradation by lysosomal proteases (e.g. cathepsin D) *in vitro* and in tissue slices. Perturbation of the autophagy/lysosomal system in an inducible tau model also leads to tau accumulation. Thus tau appears to be a substrate for both proteolytic systems *in vivo*. In a recent study, Dolan et al. demonstrated that a
truncated form of tau, tau∆c, is rapidly degraded by the autophagy/lysosomal system whereas full length tau is preferentially degraded by the UPS.\textsuperscript{293} The tau∆c fragment is generated by caspase cleavage, is highly aggregation prone and is postulated to act as a seed for the formation of aggregates.\textsuperscript{293} This further implicates the autophagy/lysosomal system as a back up system for the UPS. Soluble tau is targeted to the proteasome via ubiquitin dependent and independent mechanisms, whereas aggregation prone and oligomeric aggregates of tau, which can not be cleared by the UPS, are efficiently degraded by the autophagy/lysosomal system. Mass spectrometry studies find K48\textsuperscript{294} and K63\textsuperscript{295,242} polyubiquitin linkages on tau isolated from inclusion bodies. Furthermore, immunohistochemistry shows reactivity to p62\textsuperscript{296-298}, the E3 ubiquitin ligase TNF receptor associated factor (TRAF) 6\textsuperscript{295} and proteasomal subunits\textsuperscript{299,300} in tau positive inclusion bodies. This indicates tau is at some point targeted to the UPS and the autophagy/lysosomal system, but is somehow not degraded.

A disturbance in the autophagy/lysosomal system is implicated in AD pathology. Electron microscopy studies demonstrate that autophagic vacuoles accumulate in the AD brain and are mostly absent from controls.\textsuperscript{301} Interestingly, autophagic vacuoles are already found in non-dystrophic neurites in the AD brain. Their numbers increase exponentially in dystrophic neurites and in cell bodies of neurons containing paired helical filaments (PHF).\textsuperscript{301} This indicates that a disruption of the autophagy/lysosomal pathway is an early and even primary event in AD, which starts before the onset of tau pathology. In addition, granulovacuolar degeneration (GVD\textsuperscript{302}), thought to be a form of disrupted autophagy\textsuperscript{303}, is found in the pyramidal neurons of the AD hippocampus and is also observed in FTLD-tau.\textsuperscript{304} A recent study indicates that GVD bodies in AD are positive for markers of late stage autophagosomes which have not yet matured into autolysosomes.\textsuperscript{305} Furthermore, alterations in the endocytic pathway are observed in AD. Neurons in susceptible brain areas show abnormalities in the size and volume of endosomes.\textsuperscript{306} The endocytic and the autophagy pathway both converge at the level of the lysosome and their accumulation in AD is indicative of a disruption of lysosomal degradation. Strikingly, recent data show that presenilin-1 (PS1) is required for macroautophagy. Mutations in PS1 are responsible for the great majority of familial AD cases. PS1 depletion selectively inhibits the macroautophagic degradation of proteins and this was shown to be caused by the mislocalization of the v-ATPase v01a subunit. This mislocalization causes impairment of lysosome acidification and cathepsin activation. Strikingly, this phenotype was also observed in fibroblasts of AD patients carrying PS1 mutations.\textsuperscript{307} In addition, levels of the
autophagy initiating protein Beclin-1 are decreased in mild cognitive impairment (MCI) and AD brain, further supporting involvement of the autophagy/lysosomal system early in the disease process.\textsuperscript{308} Heterozygous deletion of Beclin-1 in mice decreases neuronal autophagy and results in the disruption of lysosomes. Crossing of Beclin-1 knockout mice with APP mice results in the accumulation of extra- and intraneuronal Aβ and neuronal loss, indicating that also Aβ is normally cleared via the lysosomal route. Interestingly, the loss of Beclin-1 in cell culture leads to an increase in LC3\textsuperscript{309}, probably because this protein is no longer degraded in the autolysosomes. Furthermore, this study shows increased levels of LC3 in the AD brain, which is also observed by our lab in neurons that show an active UPR (Chapter 4).\textsuperscript{256} Decreased levels of Beclin-1 combined with high numbers of accumulated autophagic vesicles indicates that the autophagy route is active in these neurons, but autophagosomes are not sufficiently cleared.

The observation that tau accumulates in ubiquitin positive aggregates that are associated with proteasome subunits\textsuperscript{299,300}, also suggests involvement of the UPS. This is corroborated by several studies that reported a decrease in proteasomal activity in the AD brain.\textsuperscript{310,215} However, these studies all use end-stage AD material, making it difficult to determine whether proteasome inhibition is responsible for or secondary to disease pathology. In one study 20S proteasomes isolated from MCI and AD brain show a decreased capability of degrading oxidized bovine serum albumin (BSA), even though a clear decrease in chymotrypsin-like activity was only observed for proteasomes isolated from AD brain.\textsuperscript{311} Strikingly, PHF-tau co-precipitates with proteasome subunits during immunoprecipitation and proteasome subunits can be co-isolated with PHF from the AD brain.\textsuperscript{310} This indicates that tau that has not formed an inclusion body is capable of interacting with the proteasome and could possibly block degradation of substrate proteins. The formation of inclusion bodies might be a protective mechanism to sequester small tau aggregates until the cell’s degradational capacity is enhanced. One method by which the UPS might be upregulated is by the incorporation of inducible subunits into the 20S proteasome as described in the UPS section. It has been shown that the inducible proteasome is capable of degrading tau more efficiently compared to the constitutive proteasome in vitro.\textsuperscript{284} Enhanced degradation of newly synthesized proteins prevents further accumulation into aggregates. Increased levels of the inducible proteasome subunits are found in the AD hippocampus\textsuperscript{256} and\textsuperscript{312}, primarily in astrocytes and microglia but also in the pyramidal neurons. This indicates that proteasomal degradation is modulated in AD affected areas, most probably due to an inflammatory response.
Several lines of evidence thus implicate dysfunction of the autophagy/lysosomal system and the UPS in tauopathies. Disruption of the autophagy/lysosomal system occurs early in disease pathology whereas impairment of the UPS appears to be a later event when oligomeric species are already formed. The UPR is activated in pre-tangle neurons in the AD brain\textsuperscript{2,3} and data from our group show that UPR activation is associated with pre-inclusion body tau pathology \textit{in general} (Chapter 3).\textsuperscript{256} The UPR preferentially activates the autophagy/lysosomal system during ER stress to degrade accumulated misfolded proteins in the ER Impairment of the autophagy/lysosomal system and activation of the UPR both occur early in disease pathology, before inclusions are formed. Increased activity of the autophagy/lysosomal system, initialized by UPR activation, could lead to a depletion of upstream autophagy related proteins (e.g. Beclin-1) and the accumulation of autophagosomes as insufficient amounts of lysosomes and lysosomal proteases are present to degrade all substrates. Alternatively, a pre-existing primary disruption of lysosomal proteolysis could be exacerbated by UPR mediated activation of autophagy, as the system is overwhelmed by substrates. In both cases the neurons are forced to form insoluble aggregates as a temporary storage of proteins for later degradation. This phenotype is probably enhanced by an age related decline in activity of the autophagy/lysosomal system.\textsuperscript{222,223} Evidence indicates that the UPR activates the major tau kinase glycogen synthase kinase (GSK) 3\(\beta\) and promotes the phosphorylation of tau.\textsuperscript{313,314} Up to a certain point tau phosphorylation and its release from the microtubules into the cytoplasm facilitates transport as steric hindrance of the motor proteins is reduced.\textsuperscript{315} In this environment phosphorylated tau might readily aggregate, form oligomeric aggregates that impair the UPS, and larger aggregates that are not cleared because of the impairment of the autophagy/lysosomal system. Studies done in an inducible tau mouse model indicate that, once formed, the NFTs are difficult targets for the cellular proteolytic systems. The induced expression of tau in this model leads to cognitive deficits and tau accumulation into aggregates. The cognitive deficit is ameliorated when tau expression is inhibited. However, at this stage, the NFTs remain and are not efficiently degraded.\textsuperscript{121}

\textbf{Synucleopathies}

The term synucleopathy is used to describe neurodegenerative disorders that are characterised by the accumulation of \(\alpha\)-synuclein positive cytoplasmic inclusion bodies in select populations of neuronal or glial cells.\textsuperscript{269} These disorders include PD, dementia with
Lewy bodies (DLB) and multiple system atrophy (MSA). In PD and DLB, α-synuclein accumulates in neuronal cell bodies in the form of LBs and in dystrophic neurites. MSA shows ubiquitin, tau and α-synuclein positive glial cytoplasmic inclusions associated with neuronal loss and gliosis in specific brain regions. PD is the most extensively studied synucleopathy and is characterised by the degeneration of the dopaminergic neurons of the substantia nigra pars compacta (SNc) with surviving neurons showing accumulations of α-synuclein. In addition to α-synuclein, LBs are immunoreactive for ubiquitin, p62, LC3, LAMP2A and components of the 20S/26S proteasome. Inhibition of proteasome activity in cell and animal models causes the accumulation of α-synuclein positive inclusions resembling LBs. However, as is the case for studies done with tau, these results could not always be repeated in other studies. Furthermore, macroautophagy and CMA play a role in α-synuclein turnover as inhibition of these pathways leads to α-synuclein accumulation in cell culture and α-synuclein is found in lysosomes in vivo. Interestingly, mutations in several genes associated with the cells proteolytic machinery are causative of familial PD, reviewed in.

Mutations found in the α-synuclein gene cause a dominantly inherited form of familial PD. These mutations increase the propensity of α-synuclein to aggregate and form β sheets. Several studies indicate that α-synuclein is capable of binding to and inhibiting the proteasome. As in tauopathies, oligomeric species of α-synuclein appear to be the most toxic. Oligomeric wild type α-synuclein strongly inhibits ubiquitin dependent and independent proteasomal degradation in vitro. Overexpression of A53T mutant α-synuclein in PC12 cells decreases proteasome activity as measured by the cleavage of fluorescent substrates and by the accumulation of ubiquitinated species. In this study, small oligomeric aggregates of A53T α-synuclein co-isolate with the 26S proteasome, supporting the notion that α-synuclein can interact with and inhibit proteasome activity. Treatment with Congo Red or pharmacological inducers of chaperones (both aimed to inhibit the aggregation) ameliorates the inhibitory effect of A53T α-synuclein and almost completely restores proteasome activity. Similar results were obtained when wild type or mutant α-synuclein was co-transfected with GFP-CL1 in neuronal SH-SY5Y cells. Under physiological conditions GFP-CL1 is rapidly degraded by the UPS. Upon inhibition of proteasomal degradation GFP-CL1 accumulates and can be visualized. In this model transient co-expression of wild type α-synuclein and GFP-CL1 causes a dose dependent accumulation of GFP-CL1, indicating α-synuclein is inhibiting GFP-CL1 degradation. This is in line with observations that increased expression of α-synuclein...
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is sufficient to cause familial PD. Accumulation of GFP-CL1 was further exacerbated when A53T or A30P mutant α-synuclein was introduced. Combined these studies suggest that accumulation of α-synuclein, either by overexpression or the presence of mutations, increases its aggregation propensity leading to proteasome binding and inhibition. In addition, a mutation in the ubiquitin carboxyl terminal esterase L1 (UCH-L1) is associated with familial PD. UCH-L1 is a DUB, is highly expressed in the brain and thought to function in maintaining the cellular free ubiquitin pool. In vitro experiments demonstrate that the deubiquitinating activity of mutant UCH-L1 is decreased compared to wild type UCH-L1. Under conditions where less ubiquitin is available, substrates are less efficiently targeted for degradation and the formation of oligomeric species might be favoured. Sporadic PD cases do not show mutations in the disease related genes, but do have α-synuclein positive inclusion bodies and diminished proteasome activity in the SNc. These studies suggest that a disruption in the UPS occurs early in synucleopathies. This disruption might be primary as in UCH-L1 mutation carriers. Alternatively, it may be secondary in familial cases where the increased formation of oligomeric species impairs proteasome function. In sporadic cases both primary and secondary mechanisms are likely to play a role. The autophagy/lysosomal system also functions in degrading α-synuclein and diminished activity of this pathway probably contributes to its accumulation and aggregation. Similar to tauopathies, a decrease in lysosomal markers and the accumulation of autophagosome-like structures is apparent in the PD brain. This indicates an increased burden on the autophagy/lysosomal system that, with age or caused by additional factors, can no longer be maintained. Mutations in the E3 ubiquitin ligase parkin are associated with autosomal recessive juvenile Parkinsonism (AR-JP). These mutations lead to a loss of parkin ubiquitin ligase activity and are thought to cause a defect in the clearance of its substrates. Strikingly, α-synuclein positive inclusion bodies are rarely observed in this type of PD. Parkin is capable of forming K48 and K63 polyubiquitin chains but is thought to preferentially mediate K63 polyubiquitination in vivo. For example, parkin is involved in K63 polyubiquitination of the α-synuclein interacting protein synphillin-1 and enhances the formation of LB like inclusions formed by the co-expression of synphillin-1 and α-synuclein. In addition, parkin associates with functionally impaired and depolarized mitochondria, promotes their ubiquitination and subsequent degradation by the autophagy/lysosomal system. This action was shown to be dependent on p62 and HDAC6, proteins involved in targeting substrates to autophagy.
As indicated above, the inclusion bodies found in neurodegenerative diseases are thought to be protective. Loss of parkin might result in decreased formation of inclusion bodies, leaving oligomeric species to interact with the proteasome. This could explain the early onset of disease in cases carrying familial parkin mutations. Parkin probably also plays a role in sporadic PD as it is known that oxidative stress causes misfolding and aggregation of wild type parkin in neuronal N2A and SH-SY5Y cells, inhibiting its activity. Oxidative and nitrative stress is a prominent feature of PD and this could lead to a gradual decline in parkin activity. In accordance with this, the parkin substrates aminoacyl tRNA synthetase complex-interacting multifunctional protein (AIMP) 2 and fuse-binding protein 1 (FBP-1) are found in LBs in sporadic PD.

Data generated in our lab show that the UPR is activated in melanin containing neurons that show α-synuclein pathology in PD. A recent study demonstrates this is also observed in MSA; where UPR markers are found in α-synuclein containing glial cells. This indicates a tight connection between α-synuclein and UPR activation and Cooper et al. demonstrated that α-synuclein induces ER stress by disrupting ER-Golgi vesicular trafficking. This is in contrast to tauopathies where ER stress is thought to initiate tau hyperphosphorylation and aggregation.

Polyglutamine expansion disorders

Polyglutamine expansion disorders are characterised by the expansion of a CAG trinucleotide repeat in the translated region of a gene which results in the expansion of glutamine residues in the corresponding protein. To date, nine polyglutamine expansion disorders are known, which include dentatorubropallidoluysian atrophy (DRPLA), spinobulbar atrophy (SBMA), spinocerebellar ataxia (SCA) 1–3, 6-7 and 17 and HD, the latter being the most numerous and most extensively studied. Cytoplasmic and/or nuclear inclusion bodies of disease related protein are observed in neurons in specific brain regions in these disorders. Normally the disease related proteins contain polyglutamine expansions that range between 6–35 glutamine residues which are required for proper protein function, however, expansion of these repeats above a certain threshold leads to disease. The onset and severity of the disease depends directly on the length of the polyglutamine tract. The longer this is, the earlier the disease begins and the more severe the symptoms are. For example, the N-terminal huntingtin fragment forms cytoplasmic and/or nuclear inclusion bodies if the glutamine expansion exceeds 35 residues. A variety
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of experiments using proteasome inhibitors indicate that proteasomes are responsible for the normal degradation of wild type polyglutamine proteins and the mutant polyglutamine expanded proteins.\textsuperscript{349,351} Li et al. compared the efficiency of the UPS and the autophagy/lysosomal system in degrading mutant huntingtin in cell and mouse models and observed that inhibiting the UPS led to a greater accumulation of mutant huntingtin than inhibiting autophagy.\textsuperscript{352} This suggests that under normal conditions the UPS is the major mechanism for degrading polyglutamine containing proteins. Early alterations in the autophagy/lysosomal system have been reported in several mutant huntingtin knock-in mice.\textsuperscript{353,354} However, to our knowledge they are not reported to occur in the diseased human brain.

Polyglutamine inclusions stain positive for K48 and K63 polyubiquitin and components of the 26S proteasome including the 20S core and its 19S regulatory complex.\textsuperscript{355} In addition, reactivity against p62 is found in SCA-6 brain, implicating the autophagy/lysosomal system at least in this polyglutamine disorder.\textsuperscript{356} The presence of proteasomes in inclusion bodies might indicate they are still recruited due to the ubiquitin signal in the inclusion bodies. The eukaryotic proteasome has difficulty in degrading expanded polyglutamine stretches and is thought to release them into the cytoplasm,\textsuperscript{357} possibly to be further degraded by cytoplasmic proteases. Peptides containing numerous glutamines are highly aggregation prone and their accumulation might quickly lead to the formation of small oligomeric aggregates which impair proteasome activity. Filamentous huntingtin, isolated from inclusion bodies, selectively inhibits the 26S but not the 20S proteasome.\textsuperscript{358} Fully formed inclusion bodies fail to inhibit 26S proteasome activity, supporting the notion that impairment of the UPS is an early secondary event in disease pathology and that the formation of inclusion bodies occurs as a protective response. In addition, electron microscopy shows an interaction of huntingtin filaments with the 19S regulatory cap of the 26S proteasome, again indicating that small aggregates can inhibit the proteasome.\textsuperscript{358} Alternatively, it was suggested that polyglutamine fragments remain inside the 20S proteolytic barrel and in this manner impair proteasome function. Activation of the UPR has been reported in several models in which expanded polyglutamine proteins were introduced. In a cell model using mutant androgen receptor (associated with SBMA) the UPR was activated and was shown to modulate toxicity of the expanded glutamine protein.\textsuperscript{359} ER stress and impaired ERAD was described in a PC12 cell model expressing polyglutamine expanded huntingtin fragments and this was caused by sequestration of essential ERAD proteins.\textsuperscript{360} To our knowledge activation of the UPR has not been described in brain material of patients.
Inducing autophagy in polyglutamine disorder models can reduce the toxicity of the expanded polyglutamine protein. Treatment with rapamycin, an agent that enhances autophagy, reduces toxicity in *Drosophila* and mouse models expressing mutant huntingtin. This opens up therapeutic opportunities for patients suffering from these disorders. Interestingly, the autophagy/lysosomal system appears to modulate the severity of the polyglutamine disorders. A recent report indicates that the age of onset of HD is modulated by autophagy. A polymorphism in the *Atg7* gene (V471A) is associated with an earlier disease onset. This indicates that the autophagy/lysosomal system can partially take over proteasome function under these circumstances, but when the activity of this system becomes compromised due to polymorphism or an age related decline, this may lead to further accumulation of polyglutamine fragments.

Table 2 lists the known disturbances in proteostasis that occur in the above described neurodegenerative disorders and categorizes them as being primary or secondary to disease pathology. Even though inclusion bodies might appear similar at the end stage, the pathways leading to these disorders may be quite different and require different therapeutic approaches.

**Therapeutic approaches targeting the UPS and the autophagy/lysosomal system**

Because the proteasome has a very important role in the regulated degradation of proteins, for example in the cell cycle, modulating the activity of the proteasome via the catalytic activity does not appear the method of choice. More selective targeting of the UPS will decrease side effects. The endogenous activators of the proteasome seem a likely target, as they have tissue specific expression. The PA28 γ isoform of the 11S regulator, REGγ, is predominantly expressed in the brain. It is mainly found in the nucleus of neurons and therefore it has been investigated in view of clearing polyglutamine aggregates. Since REGγ inhibits the PGPH activity that is involved in cleavage of Gln-Gln bonds, it was proposed that REGγ may contribute to “clogging” of the proteasome by polyglutamine proteins. However, deletion of REGγ does not affect inclusion body formation or disease parameters, so this approach has been abandoned. Since there are only a few proteasome regulators, this is probably not the way for specific targeting. A better chance of substrate specificity may for example be achieved by activating specific ubiquitin ligases, because these target limited numbers of substrates. At present this
Table 2: Disruption of the UPS and autophagy/lysosomal system in neurodegenerative disorders. A disruption in cellular proteolysis can be primary or secondary to disease pathology. This table lists the known causes for disruption of proteolysis in tauopathies, synucleopathies and polyglutamine expansion disorders. Substrates may accumulate because of a defect in the proteolysis system (primary) or accumulation of substrates can inhibit proteolysis (secondary). Disruption of lysosomal clearance is a primary feature of tauopathies (e.g. AD), whereas for example inhibition of the proteasome by oligomeric species is a secondary effect in disease pathology. In synucleopathies both primary and secondary UPS impairment are involved, the autophagy/lysosomal system is only secondary involved. The polyglutamine inclusions are primarily caused by changes in the substrate proteins. The UPS and autophagy/lysosomal proteolysis are secondarily involved.

<table>
<thead>
<tr>
<th>Primary</th>
<th>Secondary</th>
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<tbody>
<tr>
<td><strong>Tauopathies</strong></td>
<td>Disruption in lysosomes:</td>
</tr>
<tr>
<td>- Abnormal size and volume of endocytic vesicles in the AD brain</td>
<td>Proteasome activity impaired in late stage AD</td>
</tr>
<tr>
<td>- Autophagic vesicles accumulate early in AD disease pathology</td>
<td>Impairment UPS by oligomeric tau species</td>
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<tr>
<td>- GVD granules in AD and FTLD-tau</td>
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<tr>
<td>- Decreased Beclin-1 levels in AD brain</td>
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<tr>
<td>- Increased LC3 levels in Beclin-1− cells and AD brain</td>
<td></td>
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<tr>
<td>- Mutations in PS1 disturb lysosomal acidification and cathepsin activation</td>
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<tr>
<td><strong>Synucleopathies</strong></td>
<td>Mutations in components of the UPS that impair targeting of substrates:</td>
</tr>
<tr>
<td>- Mutations in DUBs (e.g. UCH-L1)</td>
<td>Impairment of the UPS by oligomeric species</td>
</tr>
<tr>
<td>- Diminished activity of E3 ligases (e.g. parkin,271, 338-341, 344)</td>
<td>- Mutations in α-synuclein increase aggregation propensity or inhibit degradation</td>
</tr>
<tr>
<td><strong>Impairment UPS in SNc of sporadic cases 271</strong></td>
<td>- Autophagy/lysosomal system overwhelmed</td>
</tr>
<tr>
<td><strong>Polyglutamine disorders</strong></td>
<td></td>
</tr>
<tr>
<td>- Proteasome inhibition causes nigral degeneration and inclusion bodies in rats 325, 326</td>
<td>Activation UPR 346</td>
</tr>
<tr>
<td></td>
<td>Impairment UPS by oligomeric species of polyglutamine expanded proteins and fragments 356-352, 355, 357, 358</td>
</tr>
<tr>
<td></td>
<td>Autophagy/lysosomal system overwhelmed 361, 364, 370</td>
</tr>
<tr>
<td></td>
<td>Activation UPR 360</td>
</tr>
</tbody>
</table>

AD, Alzheimer’s Disease; GVD, granulovacuolar degeneration; FTLD-tau, frontotemporal lobar degeneration associated with tau positive inclusions; LC3, microtubule associated protein 1 light chain 3; PS, presenillin; UPR, unfolded protein response; DUB, deubiquitinating enzyme; UPS, ubiquitin proteasome system; SNc, substantia nigra pars compacta.
is in particular investigated for cancer therapy, where inhibition of the proteasome is desired.\textsuperscript{374} Activation of the ligase activity, which should be the aim in neurodegenerative disease strategies, is probably pharmacologically more difficult to achieve, but the field may benefit from studies with E3 inhibitors as well as activators.

As indicated before, ubiquitination is a reversible process; DUBs can remove ubiquitin from ubiquitinated polypeptides. An increasing number of distinct classes of DUBs have been determined (for review see \textsuperscript{375}). At least 100 genes encode putative members of the DUB family raising the opportunity to target DUBs to modulate proteasomal degradation more specifically.\textsuperscript{375} A small molecule inhibitor of the proteasome associated DUB USP14 was demonstrated to enhance polyubiquitination and thereby proteasomal degradation.\textsuperscript{376} The target specificity of USP14 is not entirely clear yet, but treatment with the inhibitor, IU1, increases degradation of several proteins involved in neurodegenerative disorders: tau, ataxin-3, and TAR DNA-binding protein (TDP) 43. This cell-permeable inhibitor was able to protect against oxidative stress toxicity via its effect on proteasomal degradation.\textsuperscript{376} Ariclimol is a compound that enhances the heat shock response by stabilizing the activated configuration of HSF-1, which potentially would facilitate removal of misfolded proteins by the UPS. It has been tested in phase I and II clinical trials for amyotrophic lateral sclerosis, but when successful it may also apply to other neurodegenerative diseases with cytosolic inclusions.\textsuperscript{377}

Over the last couple of years a lot of effort was dedicated to employ autophagy in combating intracellular aggregates. Stimulation of autophagy can be accomplished by inhibiting the mTOR pathway, for example by rapamycin. This has been shown to clear disease related aggregates (among which α-synuclein and polyglutamine) in models.\textsuperscript{361} Rapamycin interferes with a broad range of cellular pathways and long-term use causes considerable side-effects. Small molecule screens have identified specific compounds that selectively activate autophagy independent of mTOR and are able to clear disease related aggregates.\textsuperscript{370} In a pragmatic approach, a screen revealed a number of FDA-approved drugs that display autophagy enhancing effects\textsuperscript{378} and both approaches may hold a promise for the future. Activation of the UPR induces autophagy and may present another signalling pathway to enhance autophagic clearance. Pharmacological enhancement of the PERK signalling route is possible using derivatives of the compound salubrinal, which inhibits eIF2α dephosphorylation.\textsuperscript{379} Also interference with one of the other signalling pathways, the ATF6 and IRE1 routes, may be beneficial. However, as indicated above, the exact
signalling pathway of the UPR to autophagy is not fully elucidated yet and more research is required to enable selective targeting.

It is important to balance autophagic activation, because overactivation may negatively affect neurons: enhanced autophagy contributes to the degeneration of neurites. More subtle ways of intervention are subject of investigation. Recently, phosphorylation of LC3 was shown to be an effective way to inhibit adverse effects of autophagy. Another approach may be to enhance targeting of substrates to autophagosomes, as was demonstrated by increasing the acetylation of mutant huntingtin via inhibition of deacetylases. Here it was shown in cell models and C. elegans that acetylation induced clearance of the aggregates via autophagy results in neuroprotection. Deacetylase inhibition at present is not sufficiently specific, and also other protein substrates will be targeted for autophagy. More specific drugs need to be developed before application in clinical trials can take place. It is important to realize that enhancement of autophagy may not be helpful if the problem lies at the level of the lysosomal degradation, in that case the situation could aggravate if autophagy is activated. In AD for example there is an accumulation of autophagosomes, suggesting that the problem lies downstream of autophagy. So restoration of the lysosomal system may therefore represent another therapeutic option. This kind of treatment could try to restore lysosomal function by supplementing endogenous lysosomal activity or by introducing new enzymes derived from soil micro-organisms that can degrade the accumulation prone material. Supplementing endogenous lysosomal activity has been done successfully for several lysosomal storage diseases. Supplementation can be done either by direct injection of the recombinant purified enzyme or by means of gene therapy. However, a specific therapeutic difficulty is that for the treatment of neurodegenerative disease these enzymes need to be delivered to lysosomes in the brain and will have to cross the blood-brain-barrier.

Lysosomal membrane permeabilization (LMP) causes the release of the cathepsins and other hydrolases from the lumen of the lysosome into the cytosol and has been implicated in cell death. Moreover, as a result of this LMP there will be a decrease in the amount of lysosomes in the cell. In experimental PD it was shown that accumulation of autophagosomes was caused by abnormal lysosome permeabilization leading to depletion of the lysosomes. Preventing LMP by stabilization of the lysosomal membrane might therefore represent another therapeutic option. Recently endocannabinoids have been
shown to stabilize the lysosomes against the toxic effect of Aβ. This indicates that more research into the exact causes of the proteostatic disturbances in the different diseases is pivotal for the development of viable therapeutic strategies.

**Figure 5:** Proteostasis under physiological and pathological conditions. A: Under physiological conditions substrates are degraded by the UPS or the autophagy/lysosomal system. Targeting of substrates is accomplished by specific combinations of E2/E3 ligases which facilitate K48 or K63 polyubiquitination, respectively. B: Proteostasis can be disturbed at a number of points within the degradational pathways. Mutations in substrates can hinder their degradation. Mutations in specific E2 or E3 ligases can lead to diminished or disrupted ubiquitination, allowing substrates to escape degradation. Proteostasis is further diminished by an age related decline in proteasomal activity and lysosomal degradation. Eventually, this may lead to the accumulation of oligomeric species and inclusion bodies (disease pathology), which may further inhibit proteolysis. Therapeutic approaches can be used to enhance proteolysis; the specific sites are numbered within the figure. Specific ubiquitination of target proteins can be enhanced (1). Several ubiquitin ligases recognize protein modifications and promoting these modifications can lead to targeting of substrate proteins. Specific drugs can be used to enhance the UPS (2), the formation of autophagosomes ((3), e.g. rapamycin) and their clearance by lysosomes ((4), e.g. lysosomal substitution). Oligomeric inclusion bodies may be preferentially targeted to the autophagy/lysosomal route (5) before inclusion bodies are formed.
Chapter 2

Concluding remarks

In this review we discussed the involvement of the UPS and the autophagy/lysosomal system in the degradation of aberrant proteins in tauopathies, synucleopathies and polyglutamine disorders. Even though the end products in these diseases are remarkably similar (inclusion bodies that are reactive to components of the UPS and the autophagy/lysosomal system) upstream events that lead to their formation can be quite specific. Disturbances in proteostasis can be primary or secondary to disease pathology and this determines the therapeutic approaches that can be used. In Figure 5 we give an overview of the proteostasis network during physiological and disturbed conditions and indicate where therapeutic approaches might be beneficial to restore proper proteostasis.

It is important to note that the use of specific therapeutic approaches depends on whether the disruption of proteolysis is primary or secondary (Table 2). The primary disruption of the autophagy/lysosomal pathway, as is the case in AD, is unlikely to benefit from enhancing autophagy, because the defect is at the level of the lysosome. In this case a more sensible approach may be to target the lysosome directly or to stimulate the UPS. In synucleopathies impairment of the UPS can be primary, caused by mutations in the proteolytic pathway, or secondary due to aggregation promoting or degradation escaping mutations in α-synuclein. Impairment of the autophagy/lysosomal system is secondary in synucleopathies and enhancing this pathway might prove beneficial. Polyglutamine expansion disorders are primarily caused by mutations in the substrate proteins, which enhance their aggregation propensity. In these disorders impairment of the UPS and the autophagy/lysosomal system are secondary to disease pathology and enhancement of one or both systems might decrease toxicity.

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Aims and outlines of this thesis

We previously observed activation of the unfolded protein response (UPR) in Alzheimer’s disease (AD) in neurons in close association with phosphorylated tau (p-tau). This suggests a close association between the UPR and tau pathology in AD. The general aim of the studies described in this thesis is to elucidate the role of UPR in tau pathology and investigate the functional connection between UPR activation and tau phosphorylation.

To support the involvement of the UPR in tau pathology, UPR activation was investigated in other neurodegenerative disorders associated with tau pathology, including PiD, PSP and familial FTDP-17. We found a strong association between UPR activation and early tau pathology in all studied tauopathies. These data demonstrate that UPR activation is strongly connected with the accumulation and aggregation of p-tau in general (Chapter 3).

The accumulation of insoluble aggregates in neurodegenerative disorders suggests a disruption in cellular proteolysis. We hypothesized that the UPR is employed to enhance cellular proteolysis. The effect of the UPR on the ubiquitin proteasome system and the autophagy/lysosomal system was investigated and is described in Chapter 4. Our findings indicate that autophagy is the major degradational pathway following UPR activation in neuronal cells.

Our data indicate that the UPR is an early event in tauopathies that is strongly associated with tau pathology. In Chapter 5 we investigate the connection between the UPR and tau phosphorylation in in vitro and in vivo model systems. Our data indicate UPR activation lies upstream of tau pathology. In Chapter 6 we describe that the UPR increases activity of the major tau kinase glycogen synthase kinase (GSK) 3 via the lysosomal degradation of inactive GSK-3.

Chapter 7 gives a summary and general discussion of the findings presented in this thesis and describes options for therapeutical modulation of the UPR.