Ubiquitin-Dependent Proteolysis in Neurons

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Various studies identified the ubiquitin-proteasome system as the prime suspect in causing neurodegenerative diseases. The present review summarizes our current knowledge about the expression, regulation, and functions of this major protein degradation pathway in the brain, with particular reference to the pathogenesis of associated neurological diseases.

Ubiquitination of proteins and their degradation within the proteasome emerged as the major proteolytic mechanism used by mammalian cells to regulate cytosolic and nuclear protein levels. The ubiquitin-proteasome pathway eliminates a variety of normal and abnormal, often short-lived proteins, indicating the central role of the ubiquitin-proteasome system (UPS) in most cellular functions. Posttranslational protein modification by ubiquitin (ubiquitination or ubiquitylation) may be compared with another mechanism of protein modification: phosphorylation. Both mechanisms regulate each other; enzymes involved in the ubiquitination process or proteins destined to be degraded need in part to be phosphorylated. Alternatively, degradation of phosphatases through the UPS indirectly determines protein phosphorylation of various targets.

Several recent observations reveal the functional versatility of the UPS. For example, proteasomal degradation of precursors is required to yield biologically active transcription factors like NF-κB. Second, ubiquitination not only allows proteins to be directed to the proteasome, but ubiquitin is also used to label membrane proteins or luminal components of the endoplasmic reticulum for degradation in lysosomes. On the other hand, the proteasome is involved in degradation of nonubiquitinated proteins in an ATP-independent fashion. Unrelated to proteolysis, monoubiquitination has been found to be involved in endosomal transport and transcriptional regulation. Hence the UPS plays an important role in a variety of basic cellular functions beyond those that aim at targeting and destruction of misfolded, mutated, or otherwise damaged proteins.

Ubiquitination is accomplished by a series of enzymatic steps that are required for covalent attachment of multiple ubiquitin molecules to the protein designated for degradation (Fig. 1). This process includes the ATP-dependent activation of ubiquitin by the ubiquitin-activating enzyme (E1), transfer of ubiquitin to a member of the ubiquitin-conjugating enzyme (E2) family, and isopeptide linkage of the 76-amino acid residue ubiquitin to a lysine residue of the target protein, which is then catalyzed by an E3 ubiquitin ligase (19). Differences among E2s, which are characterized by a conserved core domain, apparently determine the specificity of their interactions with E3s and their subcellular location. E3s confer specificity in the ubiquitination process. They express particular domains called HECT domains or RING fingers. HECT domains represent the ~350-amino-acid-residue carboxy-terminalsequence exhibiting a conserved cysteine for intermediate binding of ubiquitin, whereas RING fingers consist of eight metal-binding residues coordinating two zinc ions and serve as a docking site for E2s.

Proteins labeled with ubiquitin chains of four or more ubiquitin molecules are recognized by the 26S proteasome. The 26S proteasome is a hollow, barrel-shaped particle of four stackerings (each with seven subunits). It is located in the cytoplasm and in the nucleus of eukaryotic cells. The 20S core enzyme, attached at both sides to 11S (PA28) or 19S (PA700) cap complexes consisting of multiple proteins that participate in recognition and unfolding of the polyubiquitinated proteins. The 11S caps act as activators of the proteasome, probably in response to binding signaling intermediates such as B-Raf. The target protein is then unfolded and cleaved by proteolytic scissors from the carboxy-terminal end of large hydrophobic, basic, acidic residues, with the three catalytic activities buried in the inner β-rings of the core. Monomeric ubiquitin is recycled through ubiquitin carboxy-terminal hydrolase, and the degradation products (short peptide fragments and amino acids) are reused for protein synthesis.

Deubiquitination emerged as an important and tightly regulated process similar to the removal of phosphate groups by phosphatases. Thiol proteases of the ubiquitin carboxy-terminal hydrolase and ubiquitin-specific processing enzyme families are required for removal of ubiquitin from carboxy-terminal fusion proteins or from multibiquitin chains, respectively. They are likely to play a crucial role in separation of ubiquitin from the target protein before it is introduced into the proteasome cylinder, thereby facilitating proteasomal function and preventing the accumulation of ubiquitin chains. Recent evidence points to an important function of ubiquitin carboxy-terminal hydrolase as an immediate-early gene that is required for long-term facilitation in Aplysia neurons (7). This enzyme presumably leads to increased degradation of substrates that normally inhibit long-term memory formation.

Relevance of ubiquitination for neuronal development

The distribution of ubiquitin as well as ubiquitin-conjugating, -ligating, and -hydrolizing enzymes in the brain had not been elucidated until recently. For many years, however, neuroscientists used antibodies against the neuronal marker protein PGP9.5 to label central and peripheral neuron populations as well as the diffuse neuroendocrine system. The anti-
gen was identified as a highly conserved ubiquitin carboxy-terminal hydrolase and is estimated to comprise 1–2% of the total soluble brain protein.

Ubiquitin levels are high during neuronal development, with strong staining in the dendritic compartment of early postnatal pyramidal neurons in the cortex and hippocampus as well as in Purkinje cells of the cerebellum. Ubiquitin mRNA is elevated after axotomy in the adult, suggesting an enhanced requirement for ubiquitin during axonal regeneration. Hence, increased protein ubiquitination appears to be particularly prominent during neuronal process formation and remodeling.

Recent studies addressed the function of ubiquitination during neuronal development. One of the most widely used in vitro models of neuronal differentiation is the pheochromocytoma PC12 cell line, which extends processes and acquires various neuron-like characteristics in response to treatment with nerve growth factor (NGF). NGF-mediated neurite outgrowth of PC12 cells is accompanied by 1) increased levels of high-molecular-weight ubiquitin conjugates in PC12 nuclei, 2) decreased levels of free ubiquitin in cytoplasmic and nuclear extracts, 3) upregulation of at least four E2 activities, and 4) enhanced capacities for ubiquitination (16). However, proteasomal degradation rates are not elevated compared with control cells (12). These findings suggest that, upon neuronal differentiation, proteins with nuclear functions during proliferation are ubiquitinated at an enhanced rate and targeted for degradation. Nevertheless, it is also possible that ubiquitinated proteins act as positive regulators of neurite outgrowth.

To further dissect the role of the UPS in neuronal differentiation, inhibitors of a specific pathway involving E2-14kD and E3/ubiquitin ligase that targets cyclin B for degradation at the end of mitosis. This ligase is kept active in association with CDH1, a substrate-specific subunit of APC. Both APC and CDH1 are detected in nuclei of postmitotic neurons in all brain regions, indicating important functions beyond the control of cell cycle progression. Other ubiquitin ligases have primarily been found in their mutated form, causing rare, inheritable neurological diseases. Prominent examples include MID1, a microtubule-binding protein that, if mutated, causes X-linked Opitz G/BBB syndrome (17). Opitz syndrome is characterized by ventral midline defects and is associated with mental retardation and various other abnormalities. Mutated MID1 causes accumulation of the catalytic subunit of protein phosphatase 2A, resulting in hypophosphorylation of microtubule-associated proteins. Another congenital disorder, Angelman syndrome, is caused by mutations in the E6-AP gene, which encodes a HECT ubiquitin ligase. Affected children exhibit movement disturbances, are unable to speak, and are mentally retarded. Finally, the ubiquitin ligase parkin has been extensively studied in the brain because of its involvement in autosomal recessive Parkinson’s disease (see below).

**Regulation and function of the proteasome in neurons**

Proteasomal subunits are encoded by a considerable number of genes that are differentially expressed between organs i.e., proteasomes from the brain are different from those found in the liver or other organs.
in the spleen (11). Within the brain, immunoreactivity against the 20S proteasome particle is heterogeneously located in all areas, with predominant staining of large motor neuron cell bodies (10). Dendritic and axonal processes, including their synaptic terminals, contain proteasomes, indicating a functional role of localized protein degradation in various aspects of neuronal morphology.

Cytosolic proteasomal activity accounts for most of the total activity, although nuclei exhibit strong immunoreactivity for proteasomal subunits in the brain. Some subunit mRNAs are predominantly synthesized in specific areas. C2 mRNA is highly expressed in the cerebellum, whereas PA28β is mainly found in the striatum and brain stem, in addition to its strong synthesis in the cerebellum (4). PA28α and the α-subunit C6 mRNA are developmentally regulated in the rat midbrain, with highest expression in the early postnatal phase (5). C2 mRNA is significantly decreased contralateral to an ischemic area, with highest expression in the early postnatal phase (5). C2 mRNA synthesis in the cerebellum (4). PA28α and the α-subunit C6 mRNA are developmentally regulated in the rat midbrain, with highest expression in the early postnatal phase (5). C2 mRNA is significantly decreased contralateral to an ischemic area, with highest expression in the early postnatal phase (5).

The role of the proteasome in the nervous system was initially studied in neuronal cell lines by using specific proteasome inhibitors. During the early period of treatment with membrane-permeable and irreversible inhibitors like lactacystin, cytoplasmic processes are extended, reflecting early stages of neuronal differentiation. Neuroblastoma cells acquire a bipolar morphology and become multipolar later on. Similar observations have been made in PC12 cells (12, 13), suggesting that in tumor cells proteasomal inhibition shifts the balance of cell cycle-associated proteins required for proliferation and proteins necessary for differentiation to the latter. Inhibition of other proteases like calpain does not promote neurite outgrowth, indicating the specificity of the lactacystin response. Interestingly, the effects of proteasome inhibition on neurite outgrowth do not require ERK or phosphatidylinositol 3-kinase, although ERK activities are induced in PC12 cells treated with lactacystin or acetyl-Leu-Leu-norleucinal. Instead, proteasome inhibition may utilize stress-activated protein kinase to induce differentiation. Hence, regulating protein levels via modification of proteasomal activity may play an important role in neuronal development.

From studies using fluorogenic peptide substrates of the proteasome, it became clear that proteasomal activity is inhibited during aging and under various pathological conditions, such as Parkinson's, Lewy bodies, or Alzheimer's disease, as well as ischemia-reperfusion injury (4). Prolonged inhibition of proteasomal activity causes accumulation of a number of proteins, resulting in neuronal vulnerability. These include proteins associated with apoptosis, e.g., cyclin D1, a marker of the G1/S transition of the cell cycle that induces cell death when overexpressed in neuronal lines. Furthermore, proteins relevant for the pathogenesis of neurological diseases are elevated in the presence of proteasome inhibitors, among them proteasome inhibitors prevent early caspase-3 activation and tau cleavage in cerebellar granule neurons undergoing cell death induced by reduced extracellular potassium (2). In this model, however, UPS function is significantly impaired in the later, execution phase of neuronal apoptosis, with accumulation of ubiquitinated proteins and impairment of the proteasomal trypsin- and chymotrypsin-like activities.

Posttranslational modifications of the proteasome are likely to play a role in impaired proteasome function, because decreased activity is not accompanied by reduced total proteasome content (9). Reactive oxygen species have been suggested to play an important role in proteasome inhibition, probably by increasing the level of lipid peroxidation products followed by protein cross-linking. Aggregated proteins may then physically obstruct proteasome entry or block the catalytic sites within the β-rings of the central core. Neurons are probably protected to some degree against high concentrations of reactive oxygen species by increasing levels of heat shock proteins, which aid in folding, trafficking, or disaggregation of proteins on their way to the proteasome. Other mechanisms may include reversible blockade of the proteasome for short periods of time by physiological modulators (like PI31) to promote degradation of oxidized proteins in the lysosomal pathway instead. It should be pointed out, however, that reactive oxygen species (e.g., nitric oxide) serve a number of essential intracellular functions as signaling intermediates.

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and may even enhance proteasomal activity when concentrations are moderately increased.

Involvement of the UPS in inclusion body formation and neurological disease

Neurodegenerative disorders like Alzheimer’s disease and dementia with Lewy bodies account for the cognitive decline of the majority of elderly patients. Both diseases are characterized by the accumulation of intraneuronal inclusions, which may be regarded as neuropathological hallmark of other human chronic neurodegenerative disorders as well. The proteinaceous, insoluble aggregates (“aggresomes”) are found in the neuronal cytoplasm, axoplasm, or neuronal nuclei and may be encapsulated by intermediate filaments, resulting in filamentous deposits. Recent evidence suggests that they are formed through cross-linking of various hydrophobic regions exposed by several mechanisms (e.g., mutation, misfolding, or oxidative damage) and retrogradely transported along microtubules to the centrosome.

The importance of the UPS in inclusion body formation was elucidated when a variety of molecules involved in ubiquitin-mediated protein degradation, among them proteasomal subunits and other components of the UPS, were detected in inclusion bodies. Increasing evidence suggests that proteins forming aggregates either resist or inhibit proteolysis via the UPS. Mutations that impair normal ubiquitination (or deubiquitination) lead to protein accumulation, resulting in proteasomal inhibition (1), and, conversely, inhibition of the proteasome using membrane-permeable and irreversible blockers of the proteasomal catalytic activities results in aggresome formation (Fig. 2).

Neurofibrillary tangles in Alzheimer’s disease or Lewy bodies in Parkinson’s disease represent examples of cytoplasmic inclusions. Nuclear inclusions are observed in expanded polyglutamine protein disorders, e.g., spinocerebellar ataxia type 1. This disease is caused by mutant ataxin-1, which is ubiquitinated like normal ataxin-1 but resists proteasomal degradation. The HECT ubiquitin ligase E6-AP is apparently necessary for inclusion formation, because mice exhibiting expanded polyglutamine ataxin-1 and simultaneously lacking E6-AP have significantly fewer nuclear inclusions (3).

Ubiquitin immunoreactivity is detected within intranuclear inclusions and dystrophic neuronal processes in patients with Huntington’s disease, suggesting that abnormal huntingtin is targeted for proteolysis but resistant to effective removal (4).

Other neurodegenerative disorders reveal similar neuropathological findings, for example, Lewy bodies not only contain lipids and neurofilaments but also ubiquitin and other proteins, which have been identified as the cause of familial forms of Parkinson’s disease including mutations of α-synuclein. The rare Ile93Met mutation in ubiquitin carboxy-terminal hydrolase (UchL1) results in Parkinson’s disease probably by decreasing the activity of L1. It is likely that failure of protein ubiquitination or deubiquitination as well as general proteasome inhibition make catecholaminergic neurons particularly vulnerable to cellular stress and to disturbances in the balance of the redox system.

Mutations in parkin cause an autosomal recessive form of Parkinson’s disease (arPD) (15). Parkin is a member of the CUB (complement C1r/C1s, ubiquitin, and epidermal growth factor) family with two RING fingers at its carboxy-terminal end separated by an in-between RING. Interestingly, parkin is also a ubiquitin-domain protein because a ubiquitin-like sequence is located in the amino terminal of parkin. Substrates of parkin are being identified, e.g., the turnover of cell division control protein (CDCrel-1), a member of the septin GTPases probably involved in dopamine release, is impaired in arPD patients (20). Another substrate, the parkin-associated endothelin receptor-like receptor (Pael-R), has been found to elicit cell death because it becomes unfolded and insoluble when overexpressed, whereas coexpression of intact parkin protected against death. Other substrates of parkin have been identified including the glycosylated form of α-synuclein (α-Sp22), synphilin-1 (6). α-Sp22 is associated with parkin as well as with UbcH7 and accumulates in arPD patients in its nonubiquitinated form, suggesting a common pathogenetic mechanism of parkin- and α-synuclein-associated Parkinson’s disease. It appears that all three components, parkin, synphilin-1, and α-synuclein, are required for the formation of Lewy bodies.

Mutations in E3s like parkin reduce the formation of ubiquitin-positive inclusions, suggesting that segregation of abnormal proteins requires ubiquitination and serves as a natural defense mechanism to protect cellular structures from the cytotoxic effects of abnormal proteins that cannot be recognized and degraded by the proteasome. This hypothesis is further supported by the frequent occurrence of Lewy bodies in late-onset sporadic Parkinson’s disease, whereas they are rarely found in patients with the young-onset familial form of Parkinson’s disease that is often accompanied by severe neurodegeneration. The high number of cases with incidental Lewy bodies and mild neurodegeneration in the elderly probably represents a preclinical stage of Parkinson’s disease that is characterized by a still-intact ability of the neuron to compartmentalize abnormal or cytotoxic proteins as insoluble aggregates in inclusions (Fig. 1). The assumption that the UPS promotes inclusion body formation to reduce the toxicity of misfolded, mutant, or otherwise damaged proteins in their soluble form is corroborated by various studies that clearly demonstrate enhanced toxicity of mutant proteins [ataxin-1 (which cannot self-aggregate) or mutant huntingtin] in neu-

FIGURE 2. Dissociated sympathetic neurons treated with the proteasome inhibitor lactacystin (10 μM) for 24 h. Note the phase-dark cytoplasmic inclusion bodies (arrows) in the large, viable neurons with intact nuclei (as revealed by Hoechst staining). Degenerated neurons exhibiting apoptotic nuclei do not contain inclusions. Bar = 15 μm.
 neuronal cultures under conditions that prevent ubiquitination and inclusion formation (14).

**Conclusions**

Beyond its role as the major intracellular protein degradation machinery, the UPS has been identified as an important regulator of protein activation, endocytosis, and transcriptional regulation. In the nervous system, the physiological significance of this pathway is just beginning to be explored. Dissecting the role of the UPS during neuronal differentiation and neuronal apoptosis in cell culture models has laid the groundwork for our current understanding of ubiquitination and proteasomal protein degradation in the brain. The UPS is clearly involved in the pathogenesis of various neurodegenerative disorders like Parkinson’s disease. Familial forms of Parkinson’s disease are associated with mutations of UPS components or of proteins like α-synuclein that may resist proteasomal degradation if mutated. By segregating these proteins into aggresomes, neurons may have developed a mechanism to prevent the toxicity of their soluble or protofibrillar forms. The high sensitivity of postmitotic, fully differentiated cells to oxidized or misfolded proteins, which need to be eliminated rapidly to maintain neuronal metabolism, makes neurons particularly vulnerable to defects in ubiquitin-mediated protein degradation. Therefore, future therapeutic strategies will have to aim at the repair and possibly augmentation of the neuronal UPS, which will impose a major challenge.

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**References**

11. Noda C, Tanashahi N, Shimbara N, Hendil KB, and Tanaka K. Contribution of constitutive proteasomes, immunoproteasomes, and PA28 to the neuronal cultures under conditions that prevent ubiquitination and inclusion formation (14).

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