Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction

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Abstract | Eukaryotic protein degradation by the proteasome and the lysosome is a dynamic and complex process in which ubiquitin has a key regulatory role. The distinctive morphology of the postmitotic neuron creates unique challenges for protein degradation systems with respect to cell-surface protein turnover and substrate delivery to proteolytic machineries that are required for both synaptic plasticity and self-renewal. Moreover, the discovery of ubiquitin-positive protein aggregates in a wide spectrum of neurodegenerative diseases underlines the importance and vulnerability of the degradative system in neurons. In this article, we discuss the molecular mechanism of protein degradation in the neuron with respect to both its function and its dysfunction.

Exergonic
An exergonic reaction is characterized by a negative change of overall Gibbs free energy. It is thermodynamically favourable and can occur spontaneously.

Ubiquitin
An 8.5 kD protein that is ubiquitously expressed in eukaryotic cells. The covalent modification of a protein by ubiquitin is called ubiquitination or ubiquitylation.

Synaptic plasticity
The ability of the synaptic connection between two neurons to change in strength.

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Eukaryotic cells have evolved complex machineries for protein degradation, and the precise regulation of degradation underlies many fundamental cellular processes[1–3]. The first indication that proteins, rather than being stable entities, undergo constant turnover came from isotope-labelling studies in the late 1930s[4]. In the 1950s, protein degradation was shown to be ATP dependent. The result was surprising because the hydrolysis of the peptide bond is exergonic[5]. Today we understand that major proteases in eukaryotic cells are confined to specialized protein complexes (proteasomes) and organelles (lysosomes) to prevent nonspecific proteolysis[2]. The energy expenditure of degradation is related to the selection of substrates and their delivery to the proteolytic machinery[6] (FIG. 1). In many cases, proteins destined for degradation are tagged by ubiquitin[1,7].

If all cells require regulated protein degradation to function properly, why are nerve cells of particular interest? The unique morphology of neurons (with specialized zones for presynaptic neurotransmitter release and postsynaptic receptor activation) and the plasticity of synapses (which is tightly coupled to changes in the synaptic proteome) impose special challenges on the cellular machinery for both protein synthesis and degradation[8]. In this context, we aim to understand how degradative pathways are tailored to facilitate key brain functions such as learning and memory[9,10]. Increasing evidence shows that protein degradation has important roles in both neuronal development and long-term synaptic plasticity (for reviews on developmental aspects, see REFS 10–13). Moreover, many neurodegenerative diseases, such as Alzheimer's (AD), Huntington's (HD) and Parkinson's (PD) diseases, are associated with abnormal protein aggregates, implicating degradative dysfunction[14,15]. In this Review we focus on proteolytic function and dysfunction in differentiated neurons.

Before we delve into neuronal protein degradation, it may be helpful to briefly describe the two major degradative systems in eukaryotic cells: the proteasome and the lysosome. The ubiquitin–proteasome system (UPS) (FIG. 1) is responsible for degrading most intracellular, soluble proteins[1,2], but it can also degrade transmembrane proteins if they are extracted from the membrane into the cytosol[16]. The lysosome (FIG. 2) degrades most membrane and endocytosed proteins[1,17], but it can also digest cytosolic proteins through autophagy[18].

Synaptic plasticity and protein turnover

As early as 1949, Hebb postulated that "some growth process or metabolic change" may underlie activity-dependent modifications of neuronal connectivity[19]. As proteins are the molecular machines that mediate signal transduction, it is no surprise that protein synthesis and degradation are important for plasticity and memory[8]. Memory impairment caused by protein-synthesis inhibitors was first demonstrated in the 1960s and subsequently confirmed by many studies[20]. The repression of translation by anisomycin, cycloheximide or puromycin does not affect learning; rather, it affects the retention of memory when the animal is tested hours or days later.

Alzheimer's disease (AD). The most common type of neurodegenerative dementia. Patients often show impairments in learning and memory. The disease's neuropathology includes neuron loss in the cerebral cortex and in some subcortical regions and the presence of aggregates in the forms of plaques (containing amyloid-β) and neurofibrillary tangles (containing hyperphosphorylated tau).

Parallel findings using inhibitors of protein degradation were recently reported. Retrograde amnesia in rats was observed in one-trial inhibitory avoidance learning when the proteasome inhibitor lactacystin was injected into the CA1 region of the hippocampus at 1, 4 or 7 hours post-training, but not 10 hours[21]. In another study, impairment of fear learning by injection of anisomycin into the mouse hippocampus was rescued by lactacystin co-injection[22]. These findings suggest that protein synthesis and degradation work in an orchestrated manner to regulate synaptic functions that underlie learning and memory[8].

Early studies: the UPS in* Aplysia *neurons. One of the first links between the UPS and synaptic plasticity came from the study of long-term facilitation (LTF) in *Aplysia*, which is similar to long-term potentiation (LTP) in higher animals, at the sensory-to-motor neuron synapse[23]. Here, LTF is induced by the release of the neuromodulator serotonin at the interneuron synapse, which triggers cyclic-AMP-dependent postsynaptic signalling[24] (FIG. 3). A key requirement of LTF is the persistent activation of cAMP-dependent protein kinase A (PKA) after the transient, stimulus-evoked elevation of cAMP levels[23,25,26]. The PKA holoenzyme is composed of a regulatory subunit and a catalytic subunit, and the latter is inhibited when the two are associated. When cAMP binds to the regulatory subunit of PKA, the catalytic subunit is released and enters the nucleus. In the nucleus, the catalytic subunit phosphorylates and activates the transcription factor cAMP-response-element-binding protein (CREB), leading to the expression of immediate-early genes that initiate a cascade of events that contribute to synaptic strengthening. One of the immediate-early genes, *Aplysia* ubiquitin C-terminal hydrolase (*Ap-Uch*), encodes a deubiquitylating enzyme (DUB) that binds to

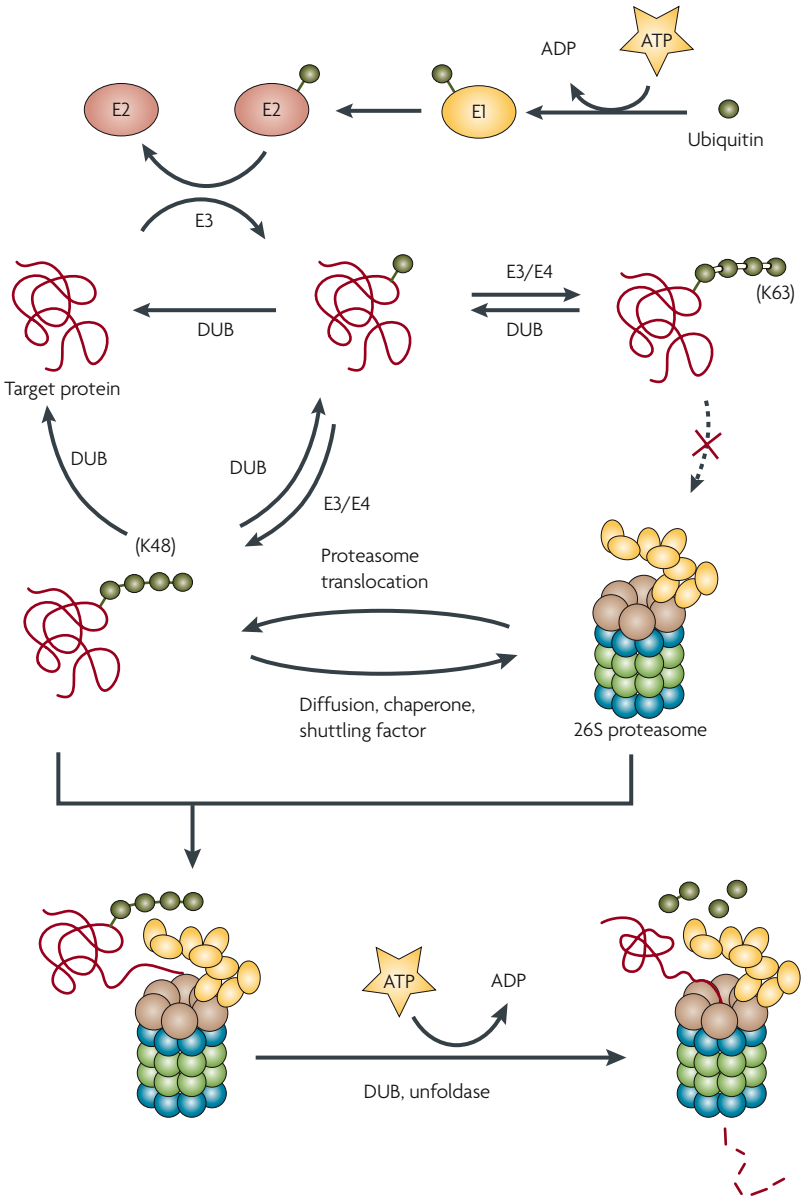

Figure 1 | **The UPS.** Ubiquitin is a small, 76-amino-acid protein. Ubiquitylation is a post-translational modification that forms an isopeptide bond between a lysine residue on the protein and the carboxyl terminus of ubiquitin. The ubiquitylation system consists of four different classes of enzymes: E1–E4. First, ubiquitin is covalently conjugated to the E1 (ubiquitin-activating enzyme) in an ATP-dependent reaction, and then it is transferred to the E2 (ubiquitin-conjugating enzyme). The E3 (ubiquitin–protein ligase) transfers the ubiquitin from the E2 to the substrate protein[3]. After the first ubiquitin has been attached (monoubiquitylation), the E3 can elongate the ubiquitin chain by creating ubiquitin–ubiquitin isopeptide bonds. The E4 enzymes (chain elongation factors) are a subclass of E3-like enzymes that only catalyse chain extension[143]. Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48 and K63), all of which are available and indeed used *in vivo* for chain extension[144]. The significance of complex ubiquitylation patterns is only partially understood[143]: K48 chains (which are the most abundant) lead to degradation of the substrate by the 26S proteasome, whereas monoubiquitylation and K63 chains do not specify degradation but have other biological functions, including marking proteins for endocytosis (see FIG. 2). The E3 is largely responsible for target recognition in this system, through physical interactions with the substrate. The large number of E3 genes in eukaryotic genomes[66] (see TABLE 1) reflects the highly specific nature of substrate recognition in ubiquitin–proteasome system (UPS)-mediated degradation[65]. Several classes of UPS factors are involved in presenting substrates to the proteasome: the ubiquitylating enzymes (E1–E4), deubiquitylating enzymes (DUBs), shuttling factors and chaperones. The organization of these factors can differ for each substrate, and only one potential configuration is represented. The figure shows an example of how a protein's ubiquitylation pattern can be dynamically edited by E3s, E4s and DUBs. When K48-polyubiquitylated, the protein can reach the proteasome by diffusion or with the assistance of chaperones and shuttling factors[102]. In some cases, the proteasome can also move towards the substrate[78]. After substrate–proteasome association, DUB and ATP-dependent unfoldase activities help the substrate to enter the proteolytic lumen of the proteasome[114]. The structure and heterogeneity of proteasomes are illustrated in FIGURE 4.

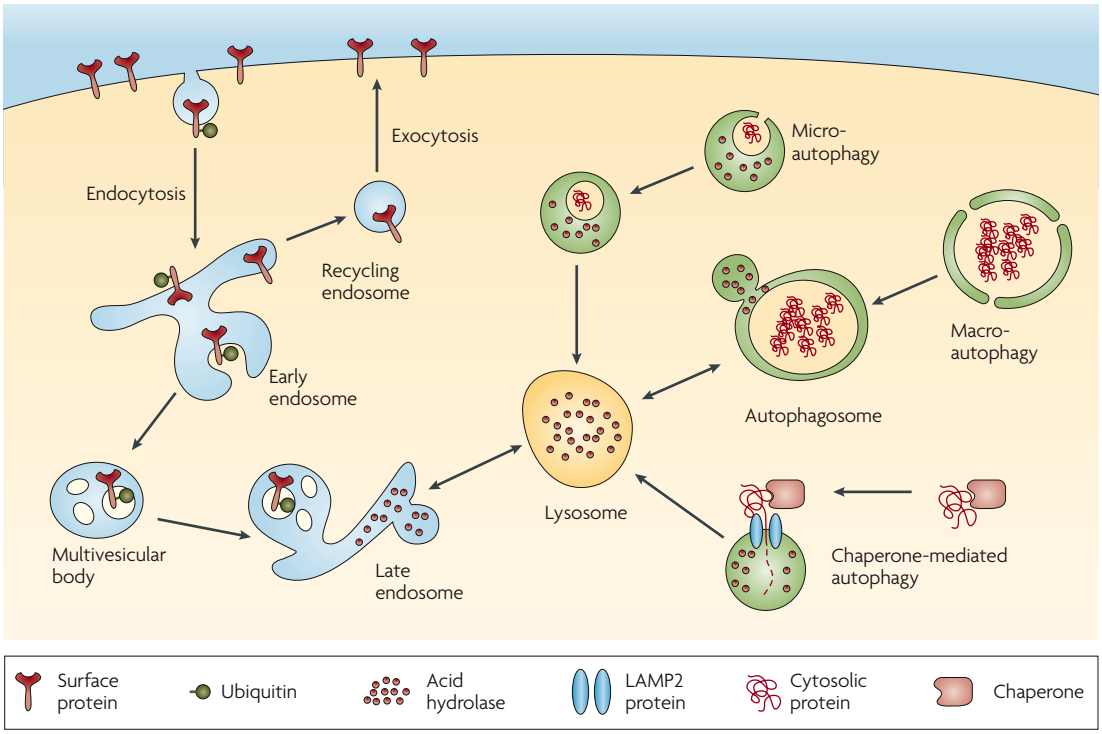

Figure 2 | **Lysosomal pathways.** Lysosomes are organelles that contain acid hydrolases that break down biopolymers and other biomolecules[17]. The primary catabolic route of plasma membrane proteins is endocytosis followed by sorting to late endosomes and lysosomes. Many types of signals can regulate endocytosis and sorting, including monoubiquitylation and K63-polyubquitylation (not shown)[7,145]. Endocytosed membrane proteins first arrive at early endosomes and subsequently get sorted into recycling endosomes (for return to the surface by exocytosis) or multivesicular bodies[146] (for transport to late endosomes or lysosomes). The acid hydrolases in the lumen of lysosomes (pH 4–5) and late endosomes (pH 5–6) are highly active in acidic environments but loose their activities in the cytosol (pH ~7.2). The confinement and the pH-dependence of hydrolases provide dual safeguards[17,147]. Intracellular proteins can enter lysosomes through several autophagic mechanisms[18]. In macroautophagy, large amounts of cytosolic materials or even organelles are surrounded by a double-membrane structure (autophagosome) that fuses with lysosomes. In microautophagy, a small amount of the cytoplasm is internalized through lysosomal invagination. In chaperone-mediated autophagy, proteins that have been unfolded by the chaperone translocate into the lysosome through interactions with lysosome-associated membrane protein 2 (LAMP2)[148].

Parkinson's disease
(PD). A degenerative movement disorder that causes tremor and gait disturbance. The impairment of motor skills is caused by the loss of dopaminergic neurons in the substantia nigra, where deposits of Lewy bodies are found.

Autophagy
The breakdown of a cell's own components by the lysosome.

Retrograde amnesia
A form of amnesia in which the subject is unable to recall events that occurred before the onset of the amnesia or before the injurious event.

Inhibitory avoidance learning
In this learning paradigm, the animal prevents an aversive stimulus by suppressing a behaviour that is otherwise regularly shown in a particular environment. For instance, a rodent that received an electric foot shock for stepping off a platform learns to stay longer on the platform during test trials.

Long-term facilitation
(LTF). A type of long-lasting enhancement of synaptic transmission that is induced by specific neuronal activities and that was initially described in *Aplysia*. A similar process called long-term potentiation was first described in the mammalian hippocampus.

Aplysia
A marine snail, or sea slug, that has a simple nervous system that makes it a useful model organism for studying synaptic plasticity.

Immediate-early gene
A gene that is activated transiently and rapidly in response to cellular stimuli.

the proteasome and facilitates proteolysis by regenerating monomeric ubiquitin. Ap-Uch promotes the proteasomal degradation of the PKA regulatory subunit, allowing the catalytic subunit to be persistently active. When Ap-Uch is functionally disrupted, LTF is largely impaired[26]. Together, these processes form a feedback control involving both protein degradation and protein synthesis that leads to synaptic strengthening[27] (FIG. 3).

The above-mentioned cAMP–CREB pathway involves the action of the UPS in the soma of the *Aplysia* sensory neuron. It was also shown that synaptic strength can be locally regulated in *Aplysia* by the synaptic UPS. Proteasome inhibition can lead to both presynaptic and postsynaptic changes, including increases in the number of synaptic contacts, the neurite length, and the amplitude of glutamate-evoked postsynaptic potentials[28]. Thus, the UPS can regulate synaptic plasticity by different mechanisms at different subcellular compartments.

UPS in mammalian neurons. Recent studies in mammals suggest that an intricate balance between protein synthesis and degradation underlies long-term plasticity and memory. For example, LTP in rat hippocampal slices can be disrupted by lactacystin[29,30] but is then rescued by anisomycin[29]. Interestingly, anisomycin by itself also disrupts hippocampal LTP[31]. However, how synthesis and degradation are coordinated with respect to synaptic modulation remains unknown.

Like in *Aplysia*, the UPS regulates synaptic transmission at both pre- and postsynaptic terminals in mammals. At the presynapse of cultured hippocampal neurons, for example, the size of the recycling vesicle pool increases by 76% after 2 hours of proteasomal blockade, an effect that is independent of protein synthesis[32]. In hippocampal slices, proteasome inhibition leads to an increase in the frequency of miniature excitatory postsynaptic currents[33]; this increase is largely abolished in transgenic animals that lack SCRAPPER, an E3 enzyme that is localized to the presynaptic membrane. A key substrate of SCRAPPER is RIM1, a Ca^{2+}-dependent vesicle-priming factor. Together, SCRAPPER and RIM1 regulate how vesicle release is affected by presynaptic Ca^{2+} levels[33]. Therefore, at the presynapse the UPS influences the size of the vesicle pool and vesicle release.

The UPS regulates the abundance of the proteins that are involved in the postsynaptic response, including ionotropic glutamate receptors (discussed in a later section) and the proteins that comprise the postsynaptic density (PSD)[10,34]. Chronic blockade of action potentials by tetrodotoxin led to an increase in the levels of certain PSD proteins (SHANK, NR2B and protein phosphatase 1) and a decrease in the levels of some others (PSD95, NR2A and calcium/calmodulin-dependent protein kinase IIα (CaMKIIα))[35]. The blockade of inhibitory neurotransmission by bicuculline induced opposite effects. However, changes in PSD composition were blocked when a proteasome inhibitor was co-applied, demonstrating the importance of proteolysis in restructuring the synapse in response to changes in neural activity.

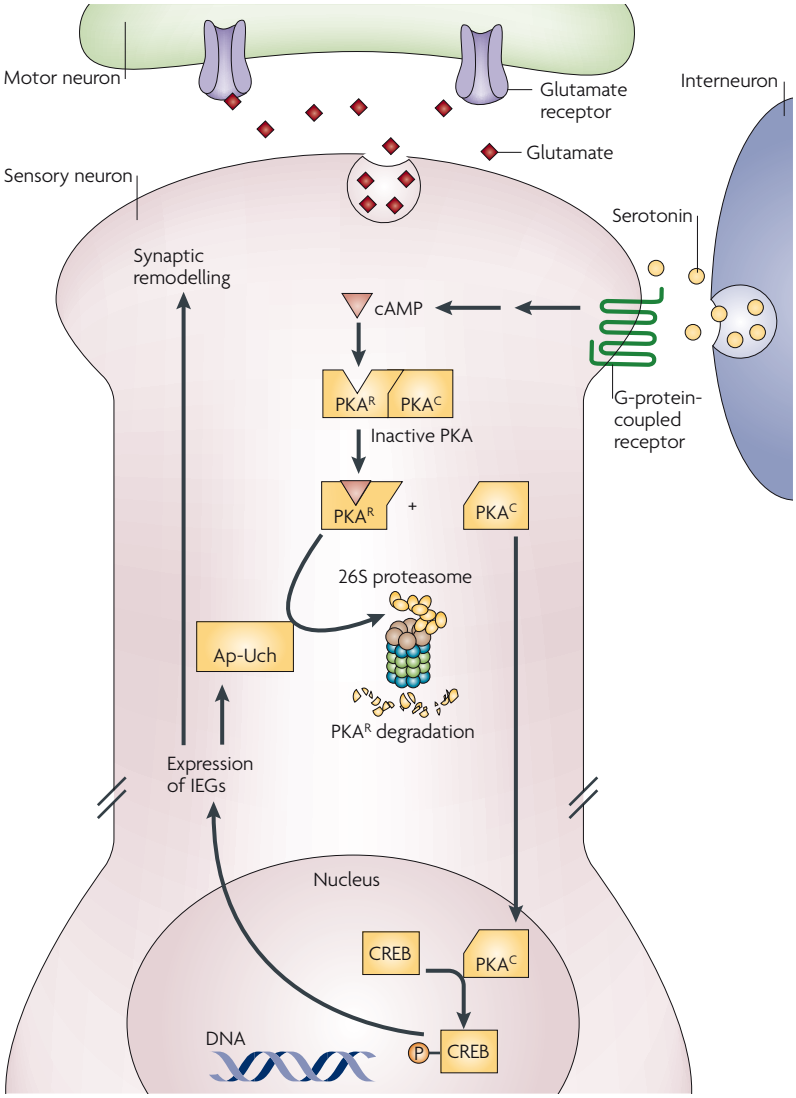

Figure 3 | ***Aplysia* LTF: regulation of PKA by the UPS.** In baseline conditions, the catalytic subunit of protein kinase A (PKA^C) is bound to the regulatory subunit of PKA (PKA^R) and remains inhibited. When serotonin is released, adenylyl cyclase is activated through G-protein-coupled receptors, leading to increased cyclic AMP levels in the postsynaptic cell. The competitive binding of cAMP to PKA^R releases PKA^C, which in turn translocates into the nucleus and phosphorylates cAMP-response-element-binding protein (CREB). Phosphorylated CREB promotes the transcription of immediate-early genes (IEGs), initiating a cascade of cellular processes that leads to synaptic remodelling. One of these IEGs is *Aplysia* ubiquitin C-terminal hydrolase (*Ap-Uch*). Ap-Uch is a deubiquitylating enzyme that binds to the proteasome and enhances the degradation of ubiquitylated substrates, including PKA^R. This positive-feedback control can lead to long-lasting PKA activities that underlie long-term facilitation (LTF)[23,25–27]. UPS, ubiquitin–proteasome system.

Neurodegeneration and protein aggregates

Protein aggregates — resulting from decreased degradation, not increased synthesis[36] — have been observed in the neuropathology of a large number of seemingly unrelated neurodegenerative diseases, including PD, AD, polyglutamine (PolyQ) expansion diseases and prion-related diseases[14]. Collectively, these diseases are now considered 'proteinopathies' of the nervous system, characterized by deposits of misfolded proteins that are relatively resistant to degradation. Ubiquitylated proteins are generally present in the respective pathological aggregates, which include plaques and tangles (AD), Lewy bodies (PD) and polyQ inclusion bodies[14,37,38]. Moreover, immunostaining using antibodies raised against ubiquitin led to the identification of a prevalent type of late-onset dementia, dementia with Lewy bodies (DLB)[39,40]. Curiously, abnormalities of just four proteins — α-synuclein, amyloid-β, tau and TAR DNA-binding protein (TDP-43) — seem to be associated with ~90% of neurodegenerative dementias[41,42].

The frequent detection of proteasomes and lysosomes around ubiquitin-positive aggregates in post-mortem brains implies that proteins in these aggregates are marked for degradation but not efficiently removed[14,43]. Consistent with this model, characterization of Lewy bodies from DLB and PD patients revealed that the major ubiquitylated protein was α-synuclein and that this protein was K48-ubiquitylated (see FIG. 1)[44]. The ubiquitin-chain length was between one and three ubiquitins; K48 chains of four ubiquitins or longer are required for efficient targeting to the proteasome[45]. Thus, the di- and tri-ubiquitylated α-synuclein species that have been detected seem to be trapped intermediates that have failed to reach their proteolytic destination.

Furthermore, overall proteasome activity in brain tissue decreases with aging, and further loss is observed in degenerative conditions such as AD and PD[46]. Hence, it has been proposed that neurodegeneration might be linked to degradative dysfunction by several mechanisms[14,15]. First, the impairment of degradation might be responsible for the aetiology, and aggregate formation might be a secondary phenomenon. Alternatively, protein aggregation might be toxic and in turn might interfere with the degradation of other proteins[47,48], causing widespread problems. To date, it has been difficult to determine whether aggregates actually cause neurodegeneration[49]; further studies of proteolytic pathways in the brain may provide insights into why aggregates form and how they affect cellular function.

The UPS and PD. Dysfunction of the UPS[15,50,51], the lysosome and autophagy[52,53] have all been implicated in neurological diseases; an interesting example is the discovery of UPS mutations associated with familial PD.

Although most PD cases are idiopathic, the identification of monogenic mutations that are linked to hereditary forms of PD (approximately eight genes to date) provides new insights into the pathogenesis[54,55].

One of the more common types of familial PD is caused by mutations in *PARK2* (REF. 56), which encodes the E3 ubiquitin ligase (see FIG. 1) parkin. Mutations in *PARK2* cause early disease onset, with the loss of dopaminergic neurons in the substantia nigra in the general absence of Lewy bodies[56]. This suggests that UPS dysfunction contributes to the pathogenesis[51]. On the other hand, the genetic association of *UCHL1*, a DUB, with familial and sporadic PD is somewhat controversial, but UCHL1 is found in Lewy bodies[55,57]. *Uchl1* is also mutated in a neurodegenerative ataxia in mice that arises from spontaneous mutations, called gracile axonal dystrophy[58,59]. Affected mice exhibit retrograde accumulation of amyloid-β aggregates, but not α-synuclein aggregates, in the gracile tract axons that control the hindlimbs. This finding implies that deposits of aggregation-prone proteins are a secondary effect of global UPS impairment, because UCHL1 helps to maintain the pool of monomeric ubiquitin[60].

Neuronal proteolysis: demands and challenges

The prevalence of neurodegenerative proteinopathies seems to suggest that neurons are susceptible to failures to clear aberrant proteins. Many of the aggregate proteins are widely expressed in other tissues, however, so what may account for the particular sensitivity of neurons? We will now consider how neuronal characteristics present unique demands and challenges to protein degradation.

Neurons are postmitotic cells that cannot be easily replaced in the adult mammalian brain[61]. They need to remain plastic in neural circuits and need to constantly renew internal components to function properly — capabilities that critically depend on efficient protein degradation[62]. In humans, this means that the proteolytic system has to cope with usually 70–80 years of cumulative stress and insult.

The distinct morphology of the neuron, including large dendritic trees and axons, means that neurons have a much greater surface-area-to-volume ratio than ordinary cells. For example, a typical hippocampal CA3 pyramidal neuron with a somatic diameter of 15 μm has a 100-fold larger surface area than a spherical cell of the same diameter and a 10-fold higher surface-area-to-volume ratio. Arborization creates over 100 dendritic and axonal segments, which are decorated with thousands of dendritic spines and axonal boutons[63]. How does the neuron handle the increased demand of surface-protein turnover? Is each compartment self-sufficient in performing proteolysis or do substrates and degradative machineries need to overcome spatial separations before proteolysis can occur?

Intriguingly, primitive invertebrates like hydra do not undergo senescence and constantly regenerate their neurons[62]. Flies, on the other hand, can exhibit notably relevant neuropathologies when they express pathogenic human polypeptides such as amyloid-β or polyQ proteins[64]. It is remarkable that neurodegenerative conditions develop within the relatively short lifespans of flies in these experimental models. It seems that possession of non-regenerating neurons that are susceptible to proteinopathies is one of the costs of building sophisticated neural networks using postmitotic neurons with complex morphologies. What are the evolutionary adaptations of the degradative system in this potential trade-off? Although there are no simple answers to the above questions, new insights have emerged from studying the molecular mechanism of neuronal protein degradation in normal and diseased states.

UPS-mediated degradation in the neuron

Between the monoubiquitylation of a protein and its entry into the 20S proteasome, several classes of factors are involved: ubiquitylating enzymes, DUBs, shuttling proteins and chaperones (FIG. 1). For each substrate, the specific factor at each stage of the degradative pathway can be different. Given the intricate relationship between these factors, we need to carefully examine their molecular functions to understand their roles in neuronal function and dysfunction. It should be noted that most of our knowledge about UPS mechanisms is derived from non-neuronal cells. Below we highlight neuron-specific data when they are available, but otherwise it is generally assumed that basic UPS features are similar between neurons and other eukaryotic cells.

Ubiquitylating enzymes. E1, E2, E3 and E4 enzymes are different classes of ubiquitylating enzymes that together can attach monoubiquitin or various polyubiquitin chains to target proteins. The transfer of ubiquitin from the E1 to the E2 can occur far away from the substrate, but the E2, the E3 and the substrate need to form a trimeric complex for substrate ubiquitylation to occur (FIG. 1). Given that mammalian genomes encode just a few E1s, dozens of E2s and hundreds of E3s[65] (TABLE 1), it is likely that the specific interaction between E2s and E3s has certain regulatory roles, although this interaction is little studied in the neuron. The E3 ligase can be a single protein or a protein complex with modular features. Examples of modular E3 complexes include the anaphase-promoting complex (APC) and the SKP1–CUL1–F-box protein (SCF) complex[66]. High-throughput methods for identifying E3 substrates are generally unavailable, and ubiquitylation motifs on substrates remain undefined[67]. It is known that one E3 can have multiple substrates[68] and that one substrate can be targeted by multiple E3s[69]. Hence, mapping E3–substrate relationships remains a difficult task in all cell types. In the neuron it is even more challenging because subcellular differences in UPS organization need to be carefully considered. For instance, a recent study in flies showed that an SCF-type E3 complex can be spatially regulated at a specific subset of synapses through its modular composition[70].

The first E3 ligase to be implicated in LTP[71] was UBE3A (also known as E6-AP), the mutation of which causes Angelman syndrome in humans, a disease that is associated with defects in neuronal development. In a transgenic mouse model of the disease, high-frequency

Ionotropic glutamate receptors
Glutamate receptors that exert their effects through the modulation of ion channel activity. In mammals they are classified into three major subtypes according to their agonist: AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, NMDA (*N*-methyl-D-aspartate) receptors and kainate receptors.

Postsynaptic density
An electron-dense structure adjacent to the postsynaptic membrane, visible in electron micrographs, that contains receptor channels, structural proteins and signal transduction proteins.

Polyglutamine (PolyQ) expansion disease
Any genetic disease that results from expansions of CAG trinucleotide repeats, which are translated into lengthened polyQ regions that induce protein aggregation. Human polyQ diseases include HD, Kennedy's disease and spinocerebellar ataxias.

Lewy bodies
Protein aggregates that contain α-synuclein, ubiquitin and other proteins. They were first identified in PD and were later found to be present in other neurodegenerative diseases.

Dementia with Lewy bodies
(DLB). A prevalent type of late-onset dementia that is characterized by the presence of Lewy bodies distributed throughout limbic, paralimbic and neocortical regions.

Hydra
Small, fresh-water predatory animals with a simple body plan and radial symmetry. Hydras are model organisms for studying regeneration and body development.

Chaperone
A protein that assists the folding/unfolding and assembly/disassembly of other macromolecular structures.

Table 1 | **The number of ubiquitylation-related proteins encoded in four eukaryotic genomes**

Class	*Homo sapiens*	*Mus musculus*	*Drosophila melanogaster*	*Saccharomyces cerevisiae*
E1	16	16	11	8
E2	53	61	32	15
E3	527	442	189	68
DUB	74	78	27	20

DUB, deubiquitylating enzyme. Data from REF. 66.

stimulation failed to induce hippocampal LTP[71]. Thus, failure of induction of hippocampal LTP is a plausible cause of the cognitive problems in human patients. Furthermore, UBE3A is localized to dendrites and spines, and its absence leads to reduced spine density and length[72]. The specific synaptic function and substrates of UBE3A remain to be identified.

In addition to *PARK2* (REF. 55) and *UBE3A*[73], an increasing number of E3 genes are now linked to neurogenetic disorders, including *UBR1* (Johanson–Blizzard syndrome)[74], *NHLRC1* (Lafora's disease)[75], and *CUL4B*[76], *BRWD3* (REF. 77) and *HUWE1* (REF. 78) (X-linked mental retardation). At first glance, we might expect to attribute the aetiology to the mutated E3s' substrates, which accumulate with maladaptive effects. To date, however, it seems that none of the disorders mentioned above has been satisfactorily explained by this simple model, even though in the case of parkin (encoded by *PARK2*) no fewer than 12 candidate substrates have been reported[68]. Therefore, it may be important to consider additional models of E3 function.

For example, parkin is reported to possess monoubiquitylation and K63-polyubiquitylation activities[68]. Parkin adds K63-polyubiquitin to misfolded DJ-1 (encoded by *PARK7*), a protein that has been implicated in familial PD, and directs it to inclusion bodies through dynein-dependent mechanisms[79]. Parkin also has the property of autoubiquitylation, which can be upregulated by a kinase called LRRK2 (REF. 80) that is also implicated in familial PD. A long-standing question is why mutations in *PARK2*, a gene that is widely expressed in the brain, can lead to specific defects in dopaminergic neurons. As the precise function of an E3 is dependent on numerous other collaborating factors, it is conceivable that an E3's function differs from one neuron type or one subcellular compartment to another.

UBE3A and parkin are both reported to associate with the proteasome[81–83]. Using a non-biased approach, mass-spectrometric characterization of affinity-purified 26S proteasome from HEK293 cells detected only 2 E3s among ~70 proteasome-interacting proteins: UBE3A and UBE3C[83,84]. This indicates that most E3s in the cell are not appreciably or stably associated with the proteasome. Although parkin was not detected in this study, perhaps because of the purification conditions that were used or because of tissue differences in proteasomal composition, parkin has been reported to bind both the 26S proteasome and the 20S proteasome in several systems, including the brain[81,82,85]. At least four pathogenic missense mutations have been mapped to the ubiquitin-like (Ubl) domain of parkin, which mediates 26S-binding[85]. It is worth noting that it is still unclear whether UBE3A or other pathogenic E3s bind to proteasomes in the brain, as no such studies have been published to date. As both parkin and UBE3A localize to the synapse[72,86], it would be interesting to examine whether their ability to interact with the proteasome has a role in synaptic protein turnover.

DUBs. Close to a hundred DUBs have been identified in the human genome (TABLE 1), most of which belong to two major families: ubiquitin-specific processing proteases (UBPs) and ubiquitin C-terminal hydrolases (UCHs)[87]. UBPs are mainly responsible for cleaving ubiquitin–substrate and ubiquitin–ubiquitin isopeptide bonds, reversing protein ubiquitylation. UCHs, on the other hand, remove small adducts from the ubiquitin to maintain the monomeric pool.

Protein deubiquitylation by DUBs can either antagonize or facilitate substrate presentation to the proteasome (FIG. 1). If DUBs remove the polyubiquitin before the substrate has been recognised by the proteasome, the degradative fate of the substrate is reversed. Less intuitive is the facilitation of degradation by DUBs that associate with the proteasome, although we have mentioned such a DUB before in connection with *Aplysia* LTF: Ap-Uch. After a substrate reaches the proteasome, polyubiquitin is removed from the protein before it enters the 20S complex, to prevent the obstruction of the 20S pore by a forked substrate and the wasteful proteolysis of ubiquitin[88]. The task of deubiquitylation is partially accomplished by one of the ubiquitous 19S subunits with DUB activity, RPN11 (REF. 87). In the mouse brain, two additional DUBs seem to be physically associated with the proteasome: USP14 and UCH37 (REF. 89)

Interestingly, the loss of USP14 has been associated with the *ataxia* (ax^J) mice[90]. The ax^J mice arose from spontaneous mutations and showed severe tremor and hindlimb paralysis. The loss of USP14 results not in protein aggregation but in a ~35% reduction of monomeric ubiquitin in the brain[89], and causes developmental defects in both the PNS and the CNS as well as abnormalities in neurotransmitter release[90].

UCHL1 is a neuron-specific DUB that has drawn much attention, partly because it is one of the most abundant proteins in the brain (1–2% of total protein) and because it helps to maintain monomeric ubiquitin levels[55,60]. It possesses DUB activity in its monomeric form and E3 activity in its dimeric form[91]. Notably, an I93M mutation, which has been tentatively linked

Angelman syndrome
A neurogenetic disease that is caused by defects in the maternal copy of *UBE3A* (owing to genomic imprinting, the maternal copy of *UBE3A* is preferentially expressed in specific brain regions). The disease is characterized by brain growth retardation and jerky movements.

Johanson–Blizzard syndrome
A genetic disease that is characterized by pancreatic dysfunction, malformation and mental retardation. The disease is now linked to *UBR1*, which encodes an E3 ligase that selects substrates on the basis of their N-terminal residues.

Lafora's disease
A fatal genetic disorder that causes seizure, myoclonus and progressive dementia. Most cases are caused by mutations in *EPM2A* (which encodes laforin, a protein phosphatase) or *NHLRC1* (which encodes malin, an E3 ligase).

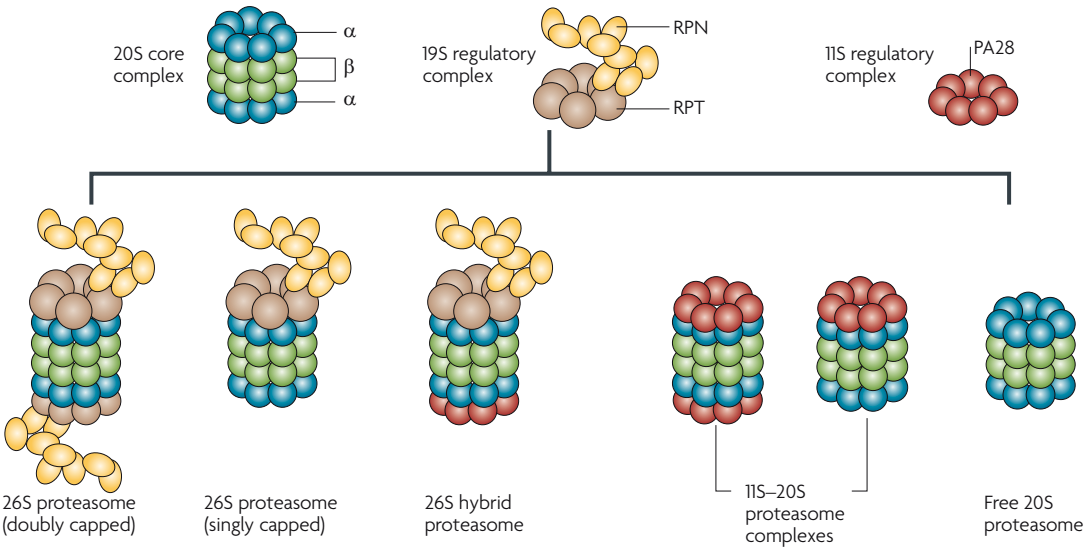

Figure 4 | **Proteasome structure and heterogeneity.** The proteasome is a large, multisubunit protease complex[114]. The 20S core complex is composed of four heptameric rings of α1–7 and β1–7 subunits. The proteolytic activities are conferred by three of the β subunits in the interior of the barrel. The 19S regulatory complex, or PA700, contains a hexameric ring of AAA-ATPase subunits (RPT1–6) and ~12 non-ATPase (RPN) subunits. The 19S complex receives the K48-polyubiquitylated substrate, removes the ubiquitin and, using ATPase activity, unfolds the protein for translocation into the 20S chamber[114]. Another complex that regulates the 20S complex, called 11S, REG or PA28, is a heptameric ring of PA28 subunits (α, β and γ) and is highly abundant in mammalian cells. This complex is thought to activate the 20S complex by widening the pore[115]. The mix and match of 20S complexes, 19S complexes and 11S complexes gives rise to a variety of proteasomes[116]. Owing to experimental difficulties in characterizing proteasome heterogeneity, the nomenclature of proteasome subtypes is not yet standardized in the literature. Different proteasome subtypes and the names that we have used for them in this article are shown. In this article we use '26S proteasome' to denote singly and doubly capped species as well as hybrid proteasomes, because all three subtypes can degrade K48-polyubiquitylated proteins.

to familial PD, promotes its dimerization and the K63-polyubiquitylation of α-synuclein[91]. This may be relevant to α-synuclein accumulation, as K63-polyubiquitin can inhibit proteasomal degradation (FIG. 1).

Another DUB that has been linked to human neurogenetic disorders is ataxin 3 (ATXN3), the polyQ expansion of which causes type 3 spinocerebellar ataxia[92]. It is not clear whether the pathology is due to impaired deubiquitylation, polyQ-induced aggregation or both. Ataxin 3 might also play a part in substrate delivery to the proteasome, as discussed in the following section[93].

Chaperones. Protein folding, protein–protein interaction and protein–membrane association are largely driven thermodynamically. Reversing these processes and, specifically, delivering a polypeptide into the 20S proteolytic chamber is a formidable task. It requires enzymes that expend energy to extract proteins from the native environment and unfold them — these enzymes are known as chaperones. The most apparent need of chaperone activity is at the entrance to the 20S complex, where the unfolding of protein substrates is fulfilled by the six AAA-ATPase subunits of the 19S complex (FIGS 1,4).

The importance of additional chaperones for proteasomal degradation has been demonstrated at the endoplasmic reticulum (ER)[94]. There, newly synthesized proteins that are aberrant or misfolded are removed by a quality-control mechanism termed ER-associated degradation (ERAD). Both lumenal and transmembrane ERAD substrates are first ubiquitylated and then delivered to the cytoplasmic proteasome. A recent study in yeast showed that a misfolded transmembrane ER protein is ubiquitylated on the cytosolic side by the transmembrane E3 Doa10p, assisted by the cytosolic heat shock protein (HSP) complex HSP70–HSP40[16]. It is then extracted from the ER membrane by the cytosolic chaperone complex Cdc48p (a homologue of mammalian VCP/p97). Ufd2p, an E4 enzyme that binds to Cdc48p, elongates the ubiquitin chain for efficient processing by the proteasome.

Mutations in the human *VCP* gene, which encodes a multipurpose AAA-ATPase motor/chaperone, can cause IBMPFD (inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia)[95]. This is accompanied by neuronal inclusions of ubiquitylated TDP-43, a DNA- and RNA-binding protein[96]. The most common mutation of *VCP*, R115H, does not compromise the protein's ATPase activity but does cause ERAD defects and ubiquitin-positive aggregates, indicating a problem in protein turnover[95]. This is consistent with the central role of the VCP chaperone in regulating proteolytic substrates[97]. VCP associates with ubiquitin, several ubiquitin-binding adaptors, the proteasome, UFD2 (an E4) and ataxin 3 (a DUB), as well as with clathrin and ubiquitin-interacting vesicle proteins[98,99]. Thus, it is well positioned to deliver ubiquitylated proteins to both proteasomes and lysosomes[97].

Spinocerebellar ataxia
A class of genetic disorders that are characterized by slowly progressive incoordination of gait and that are often associated with poor coordination of hands, speech and eye movements.

HSP70, in collaboration with the chaperones HSP40 and HSP90, is important for protein-folding quality control. It recognizes unfolded or misfolded proteins and helps them to refold or directs them to proteolysis. It has crucial roles in neurons' defence against oxidative stress and aberrant proteins[100]. The involvement of HSP70 in the UPS is suggested by a set of HSP-interacting proteins that contain UPS-related domains: CHIP (which contains a U-box E3 domain), HSJ1 (which contains a polyubiquitin-binding domain) and BAG1 (which contains a Ubl domain for 26S-binding). These proteins enable misfolded proteins that have been sequestered by HSP70 to be tagged with ubiquitin and presented to the proteasome[101]. Enhancing the expression of HSP70 and HSP90 ameliorates disease symptoms in animal models of several neurodegenerative diseases, such as HD and amyotrophic lateral sclerosis (ALS)[100].

UPS substrate delivery: not a trivial task

Ubiquitylated substrates have to reach the proteasome by diffusion, by factor-assisted shuttling or by proteasome translocation to the substrate, as most E3s do not reside at the proteasome. The 19S complex subunits RPT5 and RPN10 can bind polyubiquitin and might serve as docking sites for substrates[102]. As only a minor fraction of RPN10 is associated with the proteasome at a given time, the exchange between the free pool and the bound pool might serve as a shuttling mechanism[103]. Additional shuttling factors that have been identified in mammals are RAD23 and the ubiquilin family members, which have a polyubiquitin-binding motif and a Ubl domain for 26S-binding[83,104].

Boutons and spines — a compartmentalized UPS. The most-compartmentalized parts of neurons — axonal boutons and dendritic spines — are also the parts with the most-dynamic degradative demands. Little is understood about the localization of various UPS components and how these components are mobilized on-demand to deliver proteolytic substrates to proteasomes in such confined spaces. Recent evidence suggests that spines have developed unique delivery solutions. Following KCl-induced depolarization, proteasomes in the dendritic shaft rapidly translocate into spines and remain anchored to the actin cytoskeleton for more than an hour[105] (FIG. 5). The translocation might facilitate the degradation of multiple synaptic substrates. Several postsynaptic proteins have been reported to be UPS substrates following pharmacological stimulations of synaptic activity, including the PSD scaffolding proteins GRIP1, PSD95, AKAP150 and SHANK[35,106–108]. Learning-induced polyubiquitylation and degradation of SHANK have also been observed in the mouse hippocampus[22]. How are these scaffolding proteins presented to the proteasome within the confines of the densely packed PSD[34]? Proteomic characterization of purified PSD detected the presence of chaperones (HSP40 and HSP70 family members) and the chaperone-associated E3, CHIP[109]. These chaperones might extract ubiquitylated PSD scaffolding proteins and present them to nearby proteasomes, which in turn might explain why proteasomes are recruited into spines in an activity-dependent manner.

Proteasome heterogeneity

As discussed in previous sections, UPS components that are associated with the 26S complex can have important roles in substrate presentation. Additionally, affinity-purified proteasomes from yeast and mammalian cell lines reveal dozens of proteasome-interacting proteins with diverse cellular functions, such as transcription, translation and endocytosis[83,84,110,111]. Most interacting proteins are physically associated with the 26S proteasome through the 19S complex, and are thought to guide proteasome localization or substrate selection. Thus, the 26S proteasome is a dynamic, heterogeneous apparatus with variable composition, and is further regulated by post-translational modifications such as phosphorylation and glycosylation[112,113].

20S, 19S and 11S complexes — different proteasome compositions. In a broader sense, the 19S complex is just one of the regulatory complexes that assist substrate entry into the 20S complex. Additional regulators include the 11S (also known as PA28) activator complex, the COP9 signalosome and PA200, but their functions are little understood[114,115]. Together, the 20S, 19S and 11S complexes can form six subtypes of proteasomes (FIG. 4). In HeLa cytosolic extract, ~40% of the 20S complexes are associated with 19S complexes[116]. The 11S complex is slightly more abundant than the 19S complex, and is associated with ~60% of the 19S-bound 20S complex and ~30% of the 19S-free 20S complex. The relative abundance of these proteasome subtypes (FIG. 4) remains uncharacterized in the brain. Although the 11S complex and the 19S complex might cooperate by as-yet-undefined mechanisms in hybrid proteasomes (11S–20S–19S proteasomes), the function of 11S–20S complexes is somewhat elusive because these complexes do not degrade ubiquitylated proteins[116,117]. Few 19S-independent proteasome substrates have been reported to date[118,119].

Subunits of the 11S complex. In most tissues, the cytosolic 11S complex is a heteroheptamer of PA28α and PA28β subunits, whereas the nuclear form is a PA28γ homoheptamer. However, PA28β expression is low in the brain, and PA28γ is elevated[120]. The reason for this difference is unclear, but it might be related to synaptic plasticity: the transcription of PA28β (encoded by *PSME2*) is further downregulated by EGR1, an immediate-early transcription factor that is induced in LTP[106]. As yeast lacks PA28 and flies have only one PA28 orthologue (which is similar to the mammalian PA28γ), PA28α seems to have co-evolved with the neuronal function of higher animals.

Intriguingly, a mutant form of PA28γ, but not the wild-type form, stimulates the degradation of polyQ oligopeptides by the 20S proteasome *in vitro*[121]. Moreover, the PA28α–PA28β heteroheptamer has been shown to assist protein refolding by HSP40, HSP70 and HSP90 family members[122]. It is possible that the 11S complex serves as a link between proteasomes and

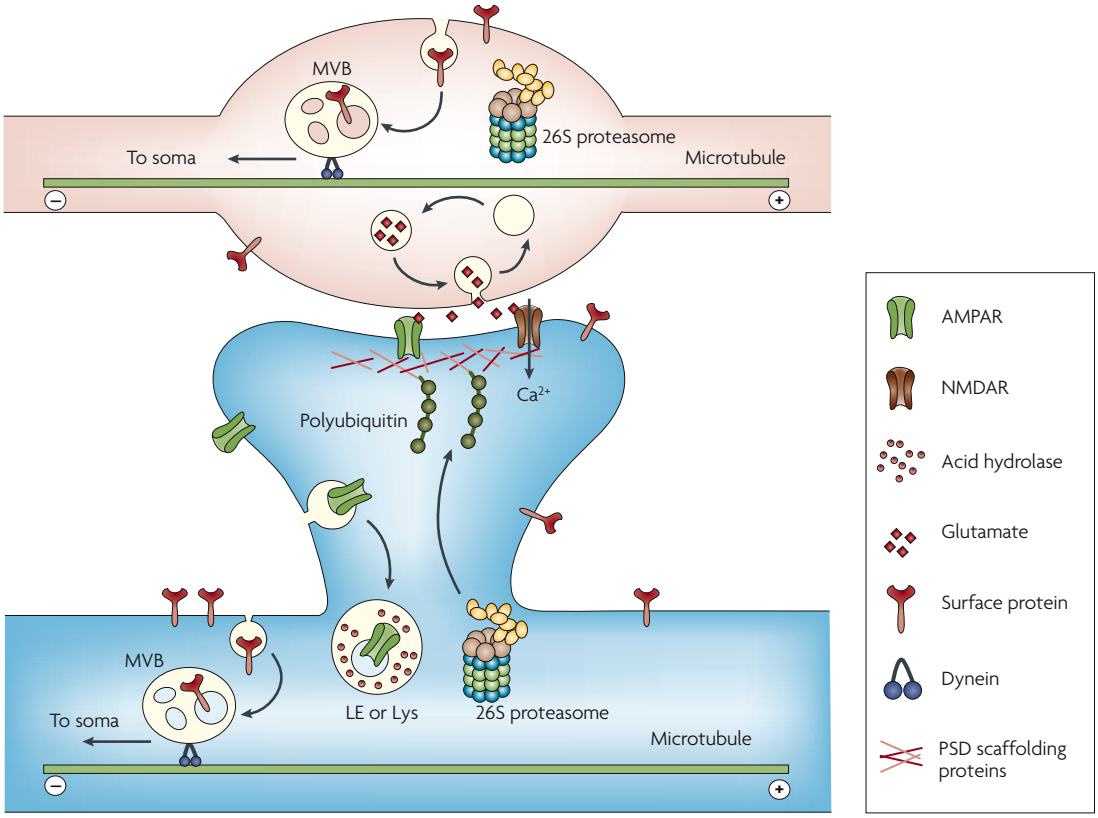

Figure 5 | **Degradation of synaptic proteins.** A schematic representation of multiple synaptic degradative events mentioned in the text. The ubiquitin–proteasome system (UPS) regulates the recycling and the release of synaptic vesicles at the presynaptic terminal (axonal bouton, top). Released glutamate activates AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors) and NMDARs (*N*-methyl-D-aspartate receptors) at the postsynaptic terminal (dendritic spine, bottom). This can trigger the polyubiquitylation of postsynaptic density (PSD) scaffolding proteins and the translocation of 26S proteasomes into spines. The degradation of PSD scaffolding proteins leads to the de-anchoring of AMPARs, which are endocytosed at the lateral zone of the spine. Endocytosed AMPARs that have been marked for degradation are sorted into late endosomes (LEs) or lysosomes (Lys), which contain acid hydrolases. In mature hippocampal neurons, endocytosis occurs along the entire dendrite and at the presynaptic terminal but not in the shaft of axons. In both cases of endocytosis, many internalized proteins are transported in multivesicular bodies (MVBs) to the soma for breakdown. Retrograde vesicular traffic requires dynein motors that travel towards the minus end of the microtubule.

chaperone-mediated clearance of polyQ aggregates, as PA28α, PA28β and PA28γ as well as HSP40, HSP70 and HSP90 family members all co-purify with proteasomes from a HEK293 human cell line[84]. It has also been suggested that the 11S complex, the 20S complex and HSP90 all collaborate in the degradation of oxidatively damaged proteins[123]. Further characterization of this pathway may help to further our understanding of neuroprotective mechanisms.

Lysosomal degradation in neurons

Owing to their high surface-area-to-volume ratio, the degradation of membrane proteins by the endocytic/lysosomal pathway must be especially efficient and tightly regulated in neurons. We will now briefly discuss the unique features of the endosome/lysosome system in neurons, including the dynamic regulation of glutamate receptors and the distribution of lysosomes in neurites, and the effect of genetic mutations in this pathway on protein turnover and neurological disorders.

Glutamate receptor turnover. The level of neurotransmitter receptors at the synapse is a major determinant of synaptic efficacy. In worms the ubiquitylation of glutamate receptors reduces their synaptic levels, possibly through endocytic mechanisms[124]. In mammals, the ubiquitylation of AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors) and NMDARs (*N*-methyl-D-aspartate receptors) has also been observed[125,126]. Moreover, the turnover of AMPARs at the synapse involves both proteasomal and lysosomal activities. AMPA-induced, clathrin-dependent endocytosis of AMPARs can be blocked by proteasome inhibition and by expression of dominant-negative K48R-mutant ubiquitin[127]. It has been suggested that UPS activity downregulates PSD95 and thereby disrupts the anchoring of AMPARs[107,108]. Endocytic zones are localized laterally to the PSD in the dendritic spine[128] (FIG. 5), and hence the release of AMPARs from the PSD scaffold might indeed be a prerequisite for endocytosis. After endocytosis, AMPARs reside in early endosomes

and can be sorted into recycling endosomes for surface reinsertion or into late endosomes/lysosomes for degradation, depending on their subunit composition and on whether NMDARs were simultaneously activated[129,130]. The complexity of the dynamics[131] of AMPARs' degradation, synthesis, post-translational modification and trafficking is no doubt a key mechanism of synaptic plasticity that remains to be fully understood.

Lysosome localization. In cultured pyramidal neurons, late endosomes and lysosomes are primarily localized in the soma and in the proximal dendrites (those within 20–30 μm of the soma)[132]. Detection of lysosome-associated membrane protein 1 (LAMP1), a late endosome/lysosome marker by immunofluorescence revealed a sparse, punctate distribution of these organelles in distal dendrites, where they might serve as proteolytic sites for internalized AMPARs[129]. Previous studies have shown that a large portion of endocytosed proteins at axons and distal dendrites is pooled into large multivesicular bodies and transported to perisomatic regions for breakdown[132] (FIG. 5). Proteolytic organelles contained in axons and dendrites, where the surface-area-to-volume ratio is high, seem to not sufficiently degrade internalized materials. In developing hippocampal neurons endocytosis occurs at both axonal shafts and presynaptic terminals; on maturation it becomes largely restricted to the latter[132]. This may be an adaptation to reduce the degradative load as the axonal surface increases.

Lysosomal dysfunction. In humans ~50 degenerative diseases have been mapped to mutations in the lysosomal pathway. They are collectively called lysosomal storage diseases (LSDs), because each causes intralysosomal accumulation of unmetabolized substances[133]. LSDs can affect multiple organs, but most affect the nervous system by compromising neuronal function.

Many LSDs are caused by mutations in lysosomal hydrolytic enzymes[134]. Once lysosomal function is impaired, many types of catabolism can be affected, including proteolysis. A curious cellular pathology is ectopic dendritogenesis in pyramidal neurons, with dendrites and spines growing out of swollen axon hillocks. Ectopic dendritogenesis has been found in many LSDs but not in other neurological disorders, and its cause has been proposed to be abnormal glutamate receptor turnover[133]. Other studies have detected protein aggregates in LSD neurons, possibly owing to decreased autophagy as autophagosome–lysosome fusion is impeded[135].

Genes that are involved in protein delivery to lysosomes have also been implicated in neurological disorders. Retrograde vesicular transport in neurites depends on dynein motors moving along microtubules (FIG. 5), and motor neurons with very long axons are especially sensitive to dynein disruption in animal models. Mutations in *DCTN1* (a subunit of dynactin, a dynein-activating complex) have been reported in a familial form of human motor neuron disease[136].

Intracellular proteins can also enter lysosomes independently of vesicular transport by interacting with LAMP2 (FIG. 2). *LAMP2* mutations are found in Danon disease, an LSD that results in myopathy, cardiomyopathy and mental retardation[137]. Both dynactin and LAMP2 also have non-proteolytic functions, and hence the pathogenic contribution of proteolytic dysfunction requires further investigation.

Future directions

Although all proteins are born out of the ribosome through the translation of their mRNAs, their destruction can occur through many different pathways and therefore it is challenging to determine the degradative route of a specific protein. The complexity of the UPS is characterized by many seemingly interchangeable components that might function in a cell- or even a compartment-specific manner, making it difficult to extrapolate data from non-neuronal systems to account for their physiological role in the brain. It is evident that we need to develop new methods to examine UPS function in specific subtypes of neurons and in specific subcellular compartments, and at the same time monitor possible crosstalk with lysosomal/autophagic pathways.

Little is known about how the degradation of specific proteins is triggered by different patterns of neuronal firing combined with different neuromodulators, especially with respect to the UPS. Conceptually, synaptic cues can be relayed through signal transduction pathways to affect the post-translational modification states of the UPS and its substrates. More specifically, increasing evidence from non-neuronal cells suggests that the crosstalk between phosphorylation and ubiquitylation is important[138]. It is conceivable that phosphorylation of the substrate can create a degron that is recognized by an E3, or phosphorylation may activate E3 activity. For example, blocking inhibitory synaptic transmission leads to the phosphorylation of spine-associated Rap guanosine triphosphate activating protein by serum-inducible kinase, resulting in its degradation by the UPS and spine loss[139]. Because the mammalian genome encodes hundreds of kinases and E3s, it is plausible that phosphorylation can serve as a general mechanism for regulating E3–substrate interaction. Another important mechanism may be the spatial separation between the UPS factors and the substrate: substrates or UPS components might translocate in or out of the synapse in an activity-dependent manner[105].

Neurons are faced with the challenge of degrading surface proteins and delivering substrates to proteolytic machineries in compartmentalized spaces. We discussed some evolutionary adaptations that help them to cope with these issues, such as the long-range transport of endocytosed proteins and the translocation of proteasomes into spines. The importance of other adaptations, such as the neuronal expression profile of PA28 subunits, is less clear. Other issues also remain so far unanswered. For instance, existing models suggest that axonal and dendritic surface proteins are degraded by late endosomes/lysosomes that are distributed in the processes as well as in the soma. However, proteasomal degradation of membrane proteins at the ER and at the outer mitochondrial membrane have recently been reported[16,140]. As the ER and mitochondria are both present in distal

Multivesicular body
At the ultrastructural level, a structure that appears as a large vesicle that contains multiple small vesicles. It can serve as the transport intermediate between early and late endosomes.

Degron
A specific part of a protein that is recognized by the degradative machinery as a proteolytic signal.

neurites, the question remains whether internalized membrane proteins can be degraded locally by similar mechanisms.

Although small-molecule drugs that upregulate autophagy, such as rapamycin and lithium, are being investigated for therapeutic benefits in proteinopathies[141], enhancers of brain UPS function have not been clearly demonstrated. Additional therapeutic strategies may involve molecular chaperones — either upregulating the cell's chaperone pathway[100] or administering chemical chaperones, such as trehalose, that enhance the stability of native proteins[142]. Therefore, with continuing advances in our understanding of neuronal protein degradation, the hope is that novel therapeutics can be developed to remove aggregates that impair neuronal function by enhancing proteasomal and/or lysosomal activity.

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DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
α-synuclein | ATXN3 | *BRWD3* | *CUL4B* | Doa10p | *HUWE1* | *NHLRC1* | *PARK2* | *PARK7* | RPN11 | SCRAPPER | tau | TDP-43 | UBE3A | UBE3C | *UBR1* | UCH37 | *UCHL1* | Ufd2p | USP14 | *VCP*

OMIM: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM
AD | ALS | HD | IBMPFD | PD

FURTHER INFORMATION

Erin Schuman's homepage: http://www.its.caltech.edu/~emslab/

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