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Review

Proteostasis and neurodegeneration: The roles of proteasomal degradation and autophagy ☆

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ABSTRACT

All proteins in a cell continuously turn over, each at its own rate, contributing to a cell's development, differentiation, or aging. Of course, unnecessary protein(s), or those synthesized in excess, that hamper cellular homeostasis should be discarded rapidly. Furthermore, cells that have been subjected to various environmental stresses, *e.g.*, reactive oxygen species (ROS) and UV irradiation, may incur various types of protein damage, which vitiate normal and homeostatic functions in the cell. Thereby, the prompt elimination of impaired proteins is essential for cell viability. This housekeeping is accomplished by two major catabolic routes—proteasomal digestion and autophagy. Strict maintenance of proteostasis is particularly important in non-proliferative cells, especially neurons, and it is plausible that its failure leads to a number of the neurodegenerative diseases becoming prominent in the growing elderly population. This article is part of a Special Issue entitled: Ubiquitin–Proteasome System. Guest Editors: Thomas Sommer and Dieter H. Wolf.

1. Introduction

Proteins within a cell exist in a dynamic state, a balance between synthesis and degradation, in which they turn over continuously and perhaps stochastically. Indeed, the half-life of an individual protein ranges from minutes to months, a ~100,000-fold difference in timespan. Proteolysis plays a central role in protein renewal, which maintains the quality of proteins within the cell by destroying dysfunctional components. Two main sophisticated machineries—the proteasome and autophagy—ensure proteolysis in eukaryotic cells (Fig. 1). The proteasome, in collaboration with a refined ubiquitin system used for tagging target proteins, selectively degrades short-lived regulatory proteins involved in the homeostatic control of the cell, and abnormal proteins with aberrant structures, the excess accumulation of which is usually harmful [1]. By contrast, the lysosomal proteolysis guided by autophagy was initially thought to be a bulk protein degradation system designed to non-selectively engulf cytoplasmic constituents [2]. This latter route was seen to be a way for the cell to secure nutrients while under starvation conditions. However, more recently, surprising data have emerged demonstrating that autophagy, working in tandem with ubiquitin, also contributes to a selective-degradation process [3]. Accordingly, it is plausible that both proteasomal degradation and autophagy are interrelated, using ubiquitin as a common marker of proteins destined for degradation. Proteolysis is indispensable to a wide variety of cellular events because it can impact biological pathways irreversibly and in a spatiotemporal fashion. Proteolysis is also integral to the elaborate quality-control mechanisms that ensure steady-state levels of proteins (proteostasis), and it is becoming clear that the failure of this control leads to a variety of neurodegenerative disorders[4,5]. Indeed, protein misfolding, aggregation, and deposition are common components of many neurodegenerative disorders including Alzheimer's disease (AD) and Parkinson's disease (PD). The accumulation of damaged or abnormally modified proteins may lead to perturbed cellular functions and eventually to neuronal death, ultimately manifesting as neurodegenerative disease.

This highlights the importance of the cellular surveillance system, including the degradative pathways of the proteasome and autophagy. Another important aspect of the cellular surveillance system is the molecular chaperones that refold misfolded proteins, helping to prevent cellular toxicity. However, chaperone function is beyond the scope of this review. Here, we will focus on the consequences of neuronal proteasome and autophagy deficiency leading to neurodegenerative disorder(s), and will discuss several remaining issues that await further clarification.

2. Ubiquitin and neurodegeneration

Ubiquitin tags proteins that are destined for degradation by a post-translational modification in which the ubiquitin is attached through an isopeptide linkage between its terminal carboxyl-residue and a particular lysine-residue on the target protein. Since its discovery around 1980, the biochemical and molecular mechanisms of ubiquitinylation have been clarified through extensive studies [6]. Today, it is established that this posttranslational modification is catalyzed through the coordinated actions of the three types of enzymes: ubiquitin-activating

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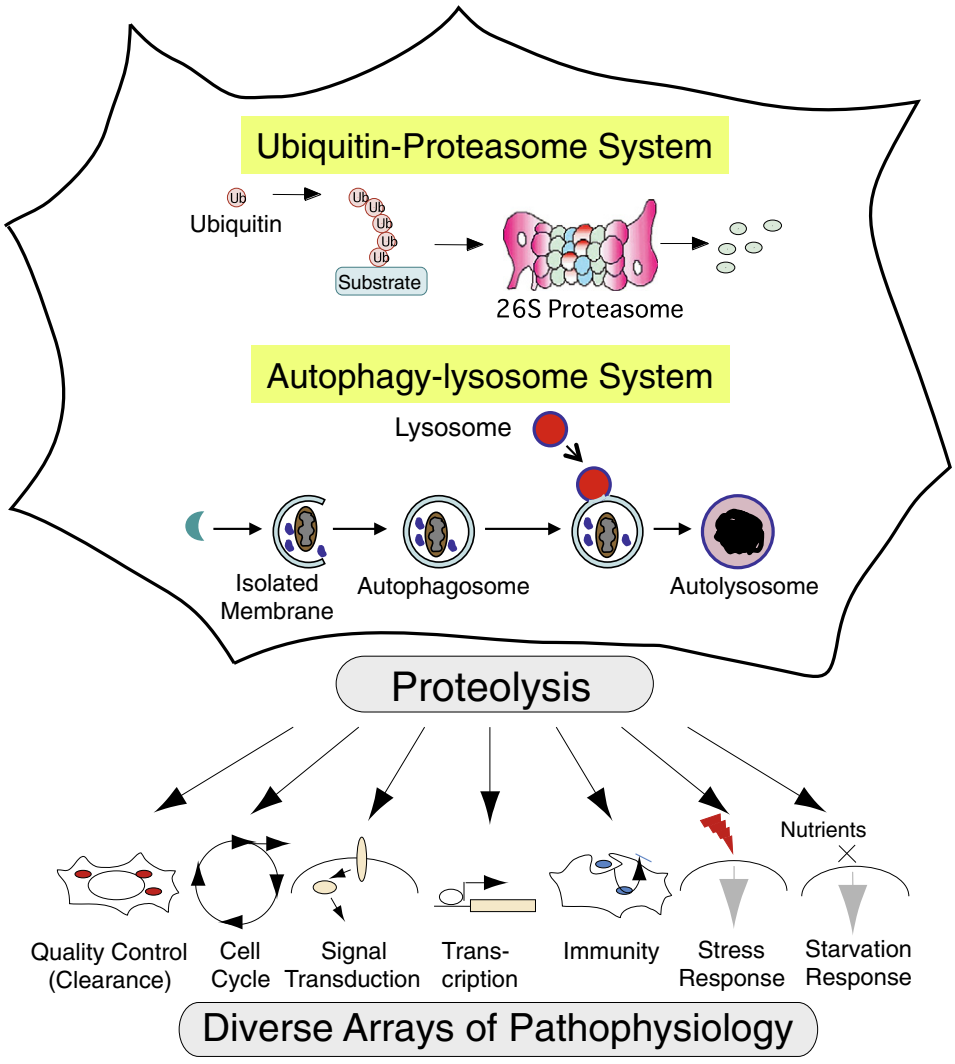

Fig. 1. Intracellular proteolysis is broadly classified into two distinct pathways consisting of the ubiquitin–proteasome and autophagy–lysosome, which are linked to a variety of physiological and pathological mechanisms in eukaryotic cells.

(E1), -conjugating (E2), and -ligating (E3) enzymes. Spectacularly, ubiquitin can itself be the target of another ubiquitin molecule, with multiple rounds of repeated ubiquitinylation leading to the formation of a so-called polyubiquitin chain. Ubiquitin possesses seven lysine residues, including those at positions 48 and 63, and an N-terminal methionine residue, all of which can nucleate polymer chains [7,8]. While the biological roles of polyubiquitin remain largely obscure, it has been shown that the K48-linked ubiquitin-polymer chain is the primary degradation signal for the proteasome, while the K63- and Met-linked linear polyubiquitin chains do not typically function as proteasomal-degradation signals. Instead, the latter participate in other processes, *e.g.*, transcriptional regulation, signal transduction, DNA repair, and membrane trafficking. Moreover, K63-linked polyubiquitinylation may also serve as a marker for autophagy, although this proposal remains under debate.

Ubiquitin is encoded by two distinct types of genes consisting of mono- and poly-ubiquitin genes. The numbers of poly-ubiquitin genes and their degree of tandem ubiquitin polymer differ by species. Surprisingly, in what appears to be a universal feature of eukaryotic species examined to date, at least two monoubiquitin genes are fused with ribosomal protein genes. The precise biological significance of this fusion of two proteins with apparently opposite functions remains unknown. In yeast, the polyubiquitin gene is indispensable under thermal stress, but not under normal growth conditions [9]. In other words, the free ubiquitin levels are adequate under normal conditions, but supplies must be rapidly increased for substrate conjugation under stress conditions. As excess ubiquitin levels are harmful, ubiquitin homeostasis is tightly controlled. In yeast, it has been shown that a balance is maintained between a deubiquitinylating enzyme and its inhibitor [10]. In mice, ubiquitin is encoded by two constitutively expressed monoubiquitin (Uba) genes and two polyubiquitin genes, Ubb and Ubc, that are stress inducible. Whereas targeted disruption of Ubb results in male and female infertility due to the failure of meiotic progression [11], Ubc-deficient mice die *in utero* between embryonic days 12.5 and 14.5, stressing the importance of homeostatic cellular ubiquitin levels [12].

In the late 1980s, ubiquitin was found to be a component of paired helical filaments (tau proteins) in Alzheimer's disease (AD) [13]. It remains true that an abnormal enrichment of ubiquitin in an inclusion body is a hallmark of various neurodegenerative disorders. The major molecules associated with various diseases include α-synuclein with Parkinson's disease (PD) and Lewy body dementia, polyglutamine tracts with Huntington's disease, and TDP-43 (TAR-DNA binding protein of 43 kDa) with frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS) [14]. To date, the significance of finding ubiquitin and/or ubiquitin polymer(s) in inclusion bodies remains to be determined. Nonetheless, there are numerous clinical reports demonstrating that ubiquitin is a diagnostic feature of most

neurodegenerative disorders, including AD and PD. While the characteristics of ubiquitin (*i.e.*, number and/or species of polymer chain) in the inclusion bodies of a variety of diseases remains unknown, a number of other details of the ubiquitin–proteasome system (UPS) and/or autophagy in these neurodegenerative disorders have been worked out. Indeed, emerging evidence implies that both UPS and autophagy dysfunctions would lead to an accumulation of polyubiquitinylated proteins, concomitant aggregation into inclusion bodies, and ultimate neural cell death.

In addition, it has been proposed that aggregated/misfolded proteins are specifically delivered to inclusion bodies by dynein-dependent retrograde transport on microtubules to the centrosome, and that this pericentrosomal cytoplasmic structure is called an aggresome [15]. Interestingly, the aggregated proteins are ubiquitinylated. Therefore, it is likely that misfolded proteins accumulate in aggresomes when the capacity of the intracellular protein degradation machinery is exceeded. However, the exact relationship between the aggresomes and the inclusion bodies of a variety of diseases still remains to be determined.

3. Proteasome and neurodegeneration

3.1. Basic mechanisms of the proteasome

The 26S proteasome is a sophisticated 2.5-MDa complex containing a catalytic core particle (CP, alias the 20S proteasome) and two terminal 19S regulatory particles (RP), which associate with both termini of the central CP in opposite orientations [16–18]. The CP forms a barrel-shaped particle of ~730 kDa consisting of four heptameric rings, which result from the axial stacking of two outer α-rings and two inner β-rings. These two rings are comprised of seven structurally related α and β subunits, positioned in an $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ orientation. The CP contains three β-type subunits of inner β-rings that contain catalytically active threonine residues at their N termini and show N-terminal nucleophile (Ntn) hydrolase activity that cleaves peptide bonds. The β1, β2, and β5 subunits are associated with caspase-like, trypsin-like, and chymotrypsin-like activities, respectively, that cleave peptide bonds at post-acidic, -basic, and -hydrophobic amino-acid residues, respectively. Two pairs of these three active sites reside within a chamber formed by the two abutting β-rings facing the interior. The crystal structures of the CP reveal that the center of the α-ring is almost completely closed, preventing substrate proteins from penetrating into the inner cavity of the β-ring where the proteolytically active sites are located [19,20]. Indeed, the N-termini of the α subunits form a physical barrier that prevents substrate protein(s) from accessing the active sites in the β-ring, indicating that the CP is latent in cells. Thus, a substrate is able to access the active sites only after passing through a narrow opening in the closed gate at the center of the α-rings.

The RP consists of a set of distinct integral subunits that can be subclassified into two groups: a regulatory particle of six triple-ATPase (Rpt) subunits and a regulatory particle of thirteen non-ATPase (Rpn) subunits. A pair of symmetrically disposed RPs of ~930 kDa is attached to both ends of the central CP at opposite orientations, forming the so-called 26S holo-proteasome as the likely functional unit. The RP recognizes polyubiquitinylated proteins, deubiquitinylates them in order to recycle the ubiquitin moieties, and then unfolds and translocates the target proteins into the interior of the CP for destruction. Moreover, the RP is also involved in opening the α-ring in the closed gate of the CP. Thus, the RP essentially contributes to the four critical steps that occur prior to the destruction of target proteins by the CP.

Intriguingly, a recent single particle analysis using cryo-EM revealed that two ubiquitin receptors, Rpn10 and Rpn13, are located at a distant site on the apical part of the RP complex near the periphery [21]. This orientation is rational because both of these subunits serve as ubiquitin (Ub) receptors; Rpn10 and Rpn13 collaboratively function as integral ubiquitin receptors that efficiently trap substrates with at least 4 ubiquitin polymer chains, required for the initial stage of degradation. Very recently, several groups independently delineated the complete subunit architecture of the RP, and provided insight into the sequence of events prior to the degradation of polyubiquitinylated substrates [22–25]. Obviously, another major function of the RP is to deubiquitinylate captured substrates, a process in which the metalloisopeptidase, Rpn11, helps to recycle the ubiquitin. Importantly, the Rpn11 deubiquitinylating enzyme (DUB) cleaves the polyubiquitin chain at a site proximal to the substrate, coupled with substrate degradation. Rpn11 was found to be located near the substrate entry position in the ATPase ring, elucidating how the polyubiquitin chain is cleaved just before the substrate is incorporated into the Rpt complex channel. It is interesting that the other two DUBs, Uch37 and USP14/UBP6, are physically associated with the RP *via* the Rpn13 and Rpn1 subunits, respectively, but their roles remain enigmatic though they cleave ubiquitin distal to the substrate. The ATPase ring is composed of six homologous AAA-ATPase subunits (Rpt1–Rpt6) forming a crown-like shape. The ATPase ring serves the following three functions [26]: 1) promotes substrate unfolding after the Rpt ring pulls substrates into the central pore; 2) opens the channel in the closed α-ring; and 3) translocates the substrates into the CP, thus ensuring their destruction. In this mechanism, it is worth noting that the energy generated by the Rpt ring has sufficient force to promote the unfolding of substrate structure domains. ATP is only used to unfold the target proteins, but is not used to drive any other processes mediated by the RP or the net proteolytic functions of the CP.

3.2. Neurodegeneration and aging

In general, animal models of neurodegenerative disorders are useful to study disease-causing mechanisms and pharmacologic interventions. A decade ago, to demonstrate the idea that proteasomal impairment causes neurodegeneration, McNaught's group reported the establishment of an animal model for PD using proteasome inhibitors, such as lactacystin, epoxomicin, and PSI (Z-Ile-Glu(OtBu)-Ala-Leu-CHO) [27]. When adult rats were treated with proteasome inhibitors, they developed progressive characteristics of Parkinsonism, including bradykinesia, rigidity and tremor, and dopaminergic cell death in the substantia nigra pars compacta. The behavioral abnormalities were alleviated following treatment with a dopamine agonist, and intracytoplasmic α-synuclein- and ubiquitin-containing inclusions resembling Lewy bodies were observed within the degenerating neurons, supporting the validity of this PD rat model and causing great excitement in the PD community. Unfortunately, however, this animal model subsequently became controversial because some groups could only partially replicate previous findings reported by McNaught and colleagues, while several other laboratories could not reproduce the studies, indicating that systemic proteasome inhibition is not a reliable PD model [reviewed in 28 and see references therein]. However, proteasome inhibitor studies are still conducted today, underscoring the imperative role of the proteasome in neurodegeneration.

As an alternative approach to using proteasome inhibitors to uncover the *in vivo* role of the proteasome, mutant mice deficient in various proteasomal genes have been generated. However, the ablation of most proteasomal genes leads to embryonic lethality. Exceptions include the immune-related proteasomal subunits, for example, the triple deletion of three immunosubunits, β1i, β2i, and β5i [29], and the deletion of the thymoproteasome subunit β5t [30]. Accordingly, the establishment of a conditional knockout (cKO) mouse is necessary; John Mayer's group made the first cKO mouse of the proteasomal ATPase subunit Rpt2/Psmc1 using the Cre/loxP system ($Psmc1^{fl/fl}$) [31]. In this model, Rpt2 could be specifically inactivated in dopaminergic neurons within the substantia nigra. The specific impairment of proteasomal activity in neurons of the nigrostriatal pathway led to intraneuronal ubiquitin- and α-synuclein-positive Lewy-like inclusions and extensive neurodegeneration. The authors claimed that the observed Lewy-like inclusions resembled human pale bodies, which are thought to be the early Lewy body form observed in patients with PD.

Thus, the depletion of proteasomes in mouse dopaminergic neurons can result in neurodegeneration and Lewy-like inclusions resembling human PD, providing direct evidence for the involvement of neuronal proteasomal dysfunction in PD pathology. Furthermore, mice heterozygous for Psmc1(+/−) function were neuropathologically and biochemically indistinguishable from wild-type mice. Thus it is plausible that defective function of the proteasome may contribute to the pathology of PD. Interestingly, an age-related accumulation of intraneuronal lysine 48-specific polyubiquitin-positive granular staining was found in the wild-type and heterozygous Psmc1(+/−) mouse brain, although both mice were neuropathologically and biochemically indistinguishable each other [32].

In another recent example, cKO mice in which another ATPase subunit of the 26S proteasome, Rpt3/Psmc4, was deleted in a motor neuron-specific manner revealed locomotor dysfunction accompanied by progressive motor neuron loss and gliosis [33]. Moreover, diverse amyotrophic lateral sclerosis (ALS)-linked proteins, including TDP-43, fused in sarcoma (FUS), ubiquilin 2, and optineurin, were mislocalized or accumulated in motor neurons, together with other typical ALS hallmarks such as basophilic inclusion bodies, emphasizing the role of the proteasome to maintain motor neuron homeostasis. Taken together, these two genetic analyses in mice indicate that the proteasome has a tight causal link to neurodegenerative diseases in general.

It is a widely accepted concept that age is a major risk factor for sporadic and age-related neurodegenerative diseases. However, the links between age-dependent proteotoxicity and cellular protein degradation systems, *e.g.*, the proteasome, remain poorly understood because of the difficulty of evaluating proteasome functions *in vivo*. Several synthetic peptide substrates with fluorescence probes are used to assay proteasomal activity *in vitro*; however, the resulting data are presumably not directly related to the *in vivo* situation. Recently, a genetic gain-of-function screen in the fruit fly revealed that 26S proteasomal activity and abundance attenuate with age, and that this loss of activity is associated with the impaired assembly of the CP and RP into the 26S proteasome [34]. In fact, Rpn11, which encodes a deubiquitinylating subunit of the RP that recycles ubiquitin tags from the target proteins, was identified as a suppressor of expanded polyglutamine-induced progressive neurodegeneration. Intriguingly Rpn11 overexpression suppressed the age-related reduction of the 26S proteasomal activity, resulting in the extension of fly lifespans and the suppression of age-dependent accumulation of ubiquitinylated proteins. By contrast, Rpn11 loss of function led to the early onset of reduced 26S proteasomal activity and the premature age-dependent accumulation of ubiquitinylated proteins. Rpn11 deficiency also caused a shorter lifespan and an enhanced neurodegenerative phenotype. These results suggest that maintaining the 26S proteasome with age could extend lifespan and suppress the age-related progression of neurodegenerative diseases.

In genetic analyses using *Caenorhabditis elegans*, it was reported that Rpn6/rpn-6 of the proteasomal RP subunit determines longevity under proteotoxic stress conditions [35]. The forced re-investment of resources from the germ line to the soma in *C. elegans* results in an elevated somatic proteasomal activity, the clearance of damaged proteins, and increased longevity. Interestingly, this activity is associated with the increased expression of Rpn6 by the FOXO transcription factor DAF-16. The ectopic expression of Rpn6 is sufficient to confer proteotoxic stress resistance and extend lifespan, implying that Rpn6 is a candidate to correct deficiencies in age-related protein homeostasis disorders. Recently, it was also reported that human embryonic stem cells (hESCs) exhibit high proteasomal activity that is correlated with increased levels of Rpn6 and a corresponding increased assembly of the 26S proteasome [36]. Ectopic expression of Rpn6 is sufficient to increase proteasomal assembly and activity. FOXO4, known as an insulin/insulin-like growth factor-I (IGF-I)-responsive transcription factor associated with long lifespan in invertebrates, regulates proteasomal activity by modulating the expression of Rpn6 in hESCs. Proteasomal inhibition in hESCs affects the expression of pluripotency markers and the levels of specific germ layer markers. Taken together, these findings suggest a novel regulation of proteostasis in hESCs that links longevity and stress resistance in invertebrates to hESC function and identity. The molecular mechanism of the Rpn6 subunit and why it would be solely responsible for proteasomal function are unknown. Intriguingly, the crystal structure of Rpn6 and its integration into the cryo-EM density map of the 26S holocomplex reveal that Rpn6 has a pivotal role in stabilizing an otherwise weak interaction between the CP and the RP, functioning as a molecular clamp holding the core and regulatory subcomplexes together, a possible explanation for the key role of Rpn6 in the homeostatic stability of the 26S proteasome [37].

We obtained a similar age-related scenario by generating PAC1-conditional knockout mice [38]. Previously, we identified a set of proteasome-dedicated assembling chaperones that assist in the assembly of the CP and the RP proteasomal complexes [39,40]. One of them is the proteasomal assembly chaperone 1 (PAC1) that assists in the formation of the α-ring of the CP complex. To elucidate the biological significance of PAC1-mediated proteasomal assembly *in vivo*, we generated PAC1-null and PAC1-cKO mice. PAC1-null mice, which systemically lacked functional PAC1, exhibited early embryonic lethality with death around E6.0. This result indicated that efficient PAC1-mediated proteasome biogenesis is needed to develop beyond the blastocyst stage, and thus is essential for mouse development. Subsequently, we made mice in which PAC1 was ablated only in the central nervous system (CNS). These animals displayed a large decrease in the levels of both 20S and 26S proteasomes, which resulted in the severe impairment of CNS development. Taken together, the study clearly demonstrated that PAC1 is essential for mammalian development, both during rapid embryonic growth and during the explosive neuronal cell proliferation after birth.

4. Autophagy and neurodegeneration

4.1. Basic mechanisms of autophagy

Autophagy is a bulk degradative-recycling system coupled with the lysosome, which contains a large set of hydrolytic enzymes for all macromolecules such as proteins, carbohydrates, lipids, and nucleic acids. The major autophagic pathway is called macroautophagy (hereafter referred to simply as autophagy); however, there are other mechanistically different types of autophagy, subcategorized as microautophagy and chaperone-mediated autophagy, whose physiological roles remain mostly unknown [41]. On the other hand, starvation-induced adaptive autophagy and basal or constitutive autophagies have functionally distinct roles, though they share the same molecular players, *i.e.*, Atg (autophagy-related gene) proteins [3,42]. The principal role of the former type is to supply nutrients to cells under energy crisis and is dramatically induced in response to various stimuli, including starvation (nutritional step-down) and humoral/trophic factors (*e.g.*, glucagon). Indeed, in response to nutritional stress, an isolation membrane appears promptly in the cytosol, where it gradually elongates to sequestrate large amounts of cytoplasmic constituents. Thus, autophagosomes surround cytoplasmic components including organelles at random to adapt for starvation. Subsequently, the edges of the membrane fuse together to form double-membrane structures termed autophagosomes. An autophagosome rapidly fuses with the lysosome, and their contents, together with the inner membrane, are degraded by a variety of lysosomal digestive hydrolases. Actually, autophagy-deficient mice produce fewer amino acids during the neonate starvation period and die within 1 day after birth [43]. Analogously, autophagy is essential for the preimplantation development of mouse embryos under circumstances leading to energy deprivation [44]. Adaptive autophagy is generally characterized as a non-selective process.

In addition to the importance of starvation-induced (*i.e.*, adaptive) autophagy, growing lines of evidence point to the importance of a basal autophagy that is constitutively active at low levels even under

nutrient-rich conditions, and to its key role in the global turnover of cellular components and quality control of cytoplasmic materials including organelles [41,42]. This type of autophagy is characterized as a selective process, and is referred to by various means, depending on the degradative target(s). For example, terms for the selective disposal of excess and damaged organelles include mitophagy, pexophagy, and reticulophagy; for the selective disposal of ribosomes, ribophagy; for the selective removal of lipid droplets, macrolipophagy; for the selective elimination of pathogens, xenophagy, and innate immunity; and, for the selective clearance of aggregate-prone proteins, aggrephagy. In addition, autophagy is responsible for the global turnover of long-lived, but not short-lived, proteins, and for MHC class II presentation of endogenous proteins (adaptive immunity). Recent studies have focused on the mechanisms and pathophysiological roles of selective autophagy.

4.2. Neurodegeneration and aging

The brain appears to be a specially protected tissue where nutrients (*e.g.*, amino acids, glucose, and ketone bodies) are kept in constant supply at the expense of other organs, even under severe starvation conditions. Consequently, autophagy does not need to operate in the brain in response to nutritional stress, although this phenomenon is controversial, because there is a report showing the induction of neural cell autophagy by short-term fasting [45]. However, morphological analyses revealed that the accumulation of numerous or irregularly-shaped autophagic vacuoles is a common feature of many inherited neurodegenerative diseases [42]. Indeed, the accumulation of aggregated proteins in the surviving neurons detected that postmortem is a pathological hallmark of diseases like AD, Huntington's disease (HD), and PD. However, the physiological and pathological significance of these inclusion bodies remain under debate (see below).

To define experimentally the role of autophagy in the *in vivo* brain, we generated a mouse conditionally deficient in *Atg7*, an essential gene for the induction of autophagy [46]. In these animals, autophagy could be selectively inactivated in the CNS. Mice lacking Atg7 in the CNS exhibited various behavioral defects, including abnormal limb-clasping reflexes and a reduction in coordinated movement, and died within 28 weeks after birth. Histological analyses showed that *Atg7*-deficiency was associated with neuronal loss in the cerebral and cerebellar cortices. Indeed, the loss of specific neurons, *e.g.*, pyramidal neurons in the cerebral cortex and hippocampus, and Purkinje cells in the cerebellum, was clearly observed. Unexpectedly, immunohistological analysis using an anti-ubiquitin antibody revealed an abundant accumulation of polyubiquitinylated proteins in the *Atg7*-deficient neurons. The polyubiquitinylated proteins appeared as inclusion bodies, whose size and number increased with aging, but the *Atg7*-deficient neurons had functionally intact proteasomes, the loss of which would cause abnormal ubiquitin-mediated proteolysis. This finding provided the first direct evidence to show that ubiquitin is used by both the proteasomal and the autophagy degradative pathways as a discriminating tag flagging proteins for selective elimination. At the same time, Hara et al. reported that almost all of these phenotypes were observed in mice that had neural-specific deficiencies in *Atg5*, another autophagy-essential gene [47]. Thus, many of the critical phenotypes detected in neural-specific autophagy-deficient mice resemble those of patients with neurodegenerative disorders. Considered together, these studies stress the importance of autophagy in neurodegeneration by demonstrating that suppressing autophagy through the genetic deletion of Atg7 or Atg5 in the CNS causes neurodegenerative disease in mice.

Furthermore, the specific ablation of Atg7 in Purkinje cells initially causes cell-autonomous, progressive dystrophy (manifested by axonal swellings) and degeneration of axon terminals, highlighting the indispensable role of autophagy in neurodegeneration followed by cell-autonomous Purkinje cell death and mouse behavioral deficits [48]. However, whereas the motor neuron-specific knockout of Rpt3, which impacts the proteasome, led to severe malfunction, surprisingly, the motor neuron-specific knockout of Atg7, which impacts autophagy, resulted in only subtle cytosolic accumulation of ubiquitin and p62, with no TDP-43 or FUS pathologies or motor dysfunctions [33]. These data suggested that proteasomal degradation, not autophagy, fundamentally governs the development of ALS mimic neurodegenerative disease at least under the experimental setting. This finding is in sharp contrast to autophagy deficiency in Purkinje cells as mentioned above. Accordingly, it is plausible that the contributions of proteasomal degradation and autophagy to the homeostatic control of proteostasis differ among individual neurons.

While accumulation of ubiquitinylated proteins and cell death was noted in autophagy-deficient neurons, the same phenotypes were not observed in growing cells, *e.g.*, mouse embryonic fibroblasts (MEFs) and astroglia, irrespective of autophagy deficiency. Thus, it seems that autophagy is not required in rapidly dividing cells, at least with respect to their proliferation [3]. These results might also reflect the difference in autophagic activity among cell types. It is possible that the cell division cycle results in a dilution of ubiquitinylated proteins in autophagy-deficient MEFs, thus preventing their accumulation. Accordingly, autophagy is critical to non-dividing quiescent cells, such as neurons, but not to growing cells with adequate nutrient supplies.

Intriguingly, genetic studies in *C. elegans* have shown that some of the autophagy-related genes are required for the lifespan extension induced by the inhibition of insulin/IGF-like signaling and calorie restriction, although not all autophagy-related genes are longevity-promoting. Likewise, autophagy is also required for lifespan extension induced by the activation of sirtuins (higher eukaryote homologs of the yeast NAD^+-dependent deacetylase Sir2) and by silencing of TOR [reviewed in 42,49 and see references therein]. Indeed, genetic studies in *C. elegans* have shown that some of the autophagy-related genes are required for the lifespan extension by the silencing of TOR and the longevity-promoting effects are associated with caloric restriction followed Sirtuin 1 (eukaryotic NAD^+-dependent deacetylase) activation and suppression of insulin/IGF-1 signaling. These results indicate that autophagy is a common downstream effector in various life-prolonging signaling pathways. Thinking mechanistically, how much does autophagy contribute to the overall longevity effects? How can autophagy prolong lifespan? Presumably, its role in removing misfolded or aggregation-prone/unfolded proteins and damaged organelles (*e.g.*, mitochondria) could be important. Moreover, autophagy may reduce inflammatory cytokine secretion and spontaneous tumor formation, which may also account for its longevity-promoting effects. Additionally, normal and pathological aging are associated with a reduced autophagic potential. Thus, the age-dependent onset of neurodegenerative diseases most likely correlates with the age-dependent decline of autophagic activity.

5. The disposal of misfolded proteins and defective mitochondria with respect to neurodegeneration

Over the last decade, studies on neurodegenerative diseases have revealed a common mechanism underlying disease pathogenesis. Namely, the adoption of abnormal conformations by certain proteins leads to the formation of misfolded protein oligomers, which subsequently develop into large protein aggregates. Then, the intracytoplasmic protein aggregates entangle other denatured proteins and lipids to form disease-specific inclusion bodies [2,42]. However, the mechanisms underlying inclusion body formation in neurons are vague, and little is known about the pathophysiological significance (Fig. 2). The landmark molecule in this context is p62, which harbors various protein-interacting domains, such as the N-terminal self-oligomerizing domain and the C-terminal ubiquitin-binding domain (UBA). p62 also interacts with LC3 (*via* LRS, LC3-recognition sequence), Keap1, and various signal transduction proteins, including TRAF6 and aPKC [50]. Quite interestingly, p62 is degraded *via* autophagy and is often used as

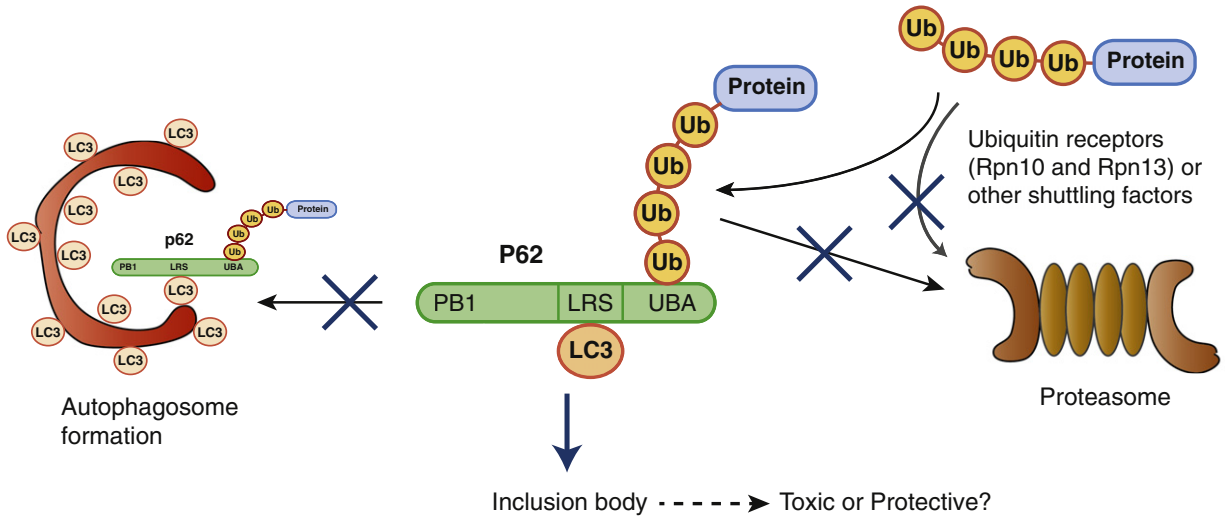

Fig. 2. A controlling model for proteostasis in eukaryotic cells. Note that p62 is involved in the formation of protein aggregation caused by the loss of proteasomal and autophagic degradations. For details, see text.

a typical example of an autophagy-selective substrate [51], but there is a report that p62 is also degraded by the proteasome [52].

Of morphological and pathological interest is the observation that most ubiquitin-containing inclusion bodies clearly stain with an anti-p62 antibody; thus, p62 is also known as sequestosome 1 (SQSTM1). The pivotal role of p62 in the formation of protein aggregates has been confirmed using a p62-null mouse model. The genetic ablation of p62 suppressed the appearance of ubiquitin-positive protein aggregates in neurons with impaired autophagy, indicating that p62 plays an important role in inclusion body formation [51] (Fig. 2). These results imply that polyubiquitinylated proteins might be selectively sequestered into autophagosomes in part *via* p62 [42,51]. Thus, our results indicate that autophagy serves to increase the supply of amino acids under nutrient-poor conditions, and to clear away damaged proteins under nutrient-rich conditions. A number of neuronal pathologists have revealed that protein aggregation (as a dynamic process), especially oligomeric intermediates, is a mediator of toxicity. However, it is currently claimed that the formation of "large" aggregates may reduce this toxicity by virtue of the aggregates having lower surface area to volume ratios and thereby being less "reactive". Whether the formation of inclusion bodies in neurons is protective or toxic is under debate, although emerging evidence suggests that protein aggregation can be a protective mechanism (Fig. 2).

As mentioned above, the 26S proteasome has two integral ubiquitin receptors, Rpn10 and Rpn13, which recognize and trap polyubiquitylated proteins directly. In addition to the intrinsic ubiquitin receptors, there are many shuttling factors, functioning as extrinsic ubiquitin receptors, such as the UBL (ubiquitin-like)-UBA (ubiquitin-associated) domain-containing proteins (*e.g.*, Rad23, Dsk2, and Ddi1) and p62, which can recruit indirectly many substrate proteins to the 26S proteasome for destruction [1] (Fig. 2). Of them, p62 is a unique protein that serves as an adaptor of autophagy and is also responsible for inclusion body formation by the impairment of autophagy. It is well known that proteasome dysfunction can also cause the accumulation of ubiquitin-positive inclusion bodies, but the mechanism(s) of aggregation is poorly understood. It is interesting to question whether p62 is involved in the aggregate formation evoked by proteasomal deficiency. In this regard, using multiple strains of mice carrying targeted genetic deletions, we recently obtained genetic evidence that p62 regulates the formation of protein aggregates caused by deficiencies of the 26S proteasome (Kageyama et al., unpublished results), indicating the generalized principal role of p62 in protein aggregation (Fig. 2).

However, to our surprise, the additional loss of p62 had little effect on neuronal degeneration caused by autophagy-deficiency [51]. Nonetheless, protein aggregation in neurons lacking both Atg7 and p62 was prevented, suggesting that the formation of an inclusion body itself does not induce neuronal vulnerability. So why do autophagy-deficient mice develop neurodegeneration? In-depth morphological analyses of autophagy-deficient neurons revealed abnormalities in various organelles, primarily mitochondria, whose autophagic elimination is termed mitophagy. Considering that autophagy serves to remove mitochondria and that malfunctioning mitochondria are tightly linked to cell death, the excess accumulation of abnormal mitochondria is likely to be another key factor in neuronal death due to the loss of autophagy.

Recently, the underlying mechanisms of mitophagy have been investigated, with particular emphasis on its relationship to Parkinson's disease (PD), a neurodegenerative disorder associated with the progressive loss of dopaminergic neurons of the substantia nigra pars compacta of the basal ganglia and locus coeruleus [53]. To date, increasing evidence has revealed that mitochondrial failure and the generation of reactive oxygen species (ROS), a by-product of ATP synthesis in the mitochondrial respiratory chain, is tightly linked to the cause of PD [54]. In this scenario, two key molecules, the causative gene products of the two types of autosomal recessive early-onset familial PDs, play a central role. One is Parkin, a ubiquitin–protein ligase E3 [55,56], and the other is PINK1, a mitochondrial-targeted Ser/Thr kinase [57]. The mechanisms through which the dysfunction of PINK1 and/or Parkin causes PD have only recently become clear: Parkin-mediated ubiquitinylation and PINK1 phosphorylation are key events in disease pathogenesis. First, PINK1 monitors mitochondrial membrane potential (integrity) and recruits and activates cytosolic parkin to depolarized mitochondria [58–62]. Next, Parkin ubiquitinylated mitochondrial outer membrane protein(s), thus inducing clearance by proteasomal degradation and/or mitophagy [53]. Specifically, PINK1 monitors depolarized/damaged mitochondria in two elegant ways. In normal cells bearing healthy mitochondria with a high membrane potential ($\Delta\Psi m$), newly synthesized PINK1 is rapidly degraded by a unique mechanism consisting of $\Delta\Psi m$-dependent import through the TOM translocator of the outer membrane into the TIM translocator in the inner membrane where the PARL processing protease is located [63]. PINK1 is first cleaved by PARL, and then is exhaustively degraded by the 26S proteasome that is attached to the outer mitochondrial membrane. Accordingly, PINK1 is barely detectable in healthy mitochondria. On the other hand, loss of $\Delta\Psi m$ causes the accumulation of PINK1, which is no longer imported into mitochondria and so avoids PARL-mediated proteolysis [64]. Moreover, the

accumulated PINK1 is converted to an active form through autophosphorylation at Ser228 and Ser402 in a low ΔΨm-dependent manner [65]. Thus, PINK1 is regulated quantitatively and qualitatively by mitochondrial membrane integrity. Activated PINK1 on damaged mitochondria recruits cytosolic Parkin to the depolarized organelle. Interestingly, cytosolic Parkin exists in a latent form whose activation is mediated in part by the phosphorylation of its N-terminal Ser65 residue by activated PINK1 [66], and is simultaneously recruited to the outer mitochondrial membrane following full activation. Parkin, whose domains consist of N-terminal UBL, RING0, RING1, IBR, and C-terminal RING2, is a RING-IBR-RING-type ubiquitin-protein ligase (E3), functionally defined as a HECT- and RING-type hybrid E3 enzyme [67], *i.e.*, RING1 binds E2-ubiquitin and transfers it to RING2, which forms a thioester linkage with ubiquitin, in a ΔΨm-dependent fashion [67, Iguchi et al., to be published]. Activated Parkin strongly polyubiquitinylated a set of outer membrane proteins in a K48- and K63-linked polyubiquitin fashion, the signal for proteasomal shredding and/or clearance by autophagy (mitophagy) [53]. Finally, PINK1 accumulation by the decrease of and subsequent recruitment of Parkin to mitochondria is etiologically important because it was largely impeded by PD-linked mutations of PINK1 or Parkin. Our results, together with reports by other groups, strongly suggest that recessive familial PD is caused by dysfunctional quality control for clearing depolarized mitochondria. At the present, we do not know whether the aforementioned pathogenic mechanisms of recessive familial PD can be generalized to the prevalent sporadic PD. However, the clinical symptoms of recessive familial PD caused by dysfunction of PINK1 or Parkin resemble that of idiopathic PD, with the exception of early-onset pathogenesis, and thus it is plausible that there is a common pathogenic mechanism. Accordingly, we believe that our results provide solid insights into the molecular mechanisms of PD pathogenesis, not only for familial forms, but also for the major sporadic form of PD.

6. Concluding remarks

As depicted in Fig. 1, both proteasomal degradation and autophagy perform a remarkable array of physiologically important cellular tasks, and consequently their functional loss may lead to various pathological situations. In this review, we have discussed the interplay between proteostasis and neurodegeneration, focusing on proteasomal degradation and autophagy, both of which display age-dependent decreases in activities and association with neurodegenerative diseases (see a model depicted in Fig. 2). It is generally accepted that deficits of the proteasome are linked to various neurodegenerative disorders accompanied by protein precipitation. While the proteasome fails to degrade large protein aggregates, drug-induced activation of autophagy can effectively remove them [68], indicating that autophagy significantly contributes to the clearance of aggregate-prone proteins independent of the proteasome. Unless the abundance of aggregates surpasses the clearance capacity of the proteasome or autophagy, the activation of those machineries by pharmaceutical agents could be effective in preventing disease progression. For example, it is known that rapamycin, which induces autophagy, has such an effect. However, while inhibitors are relatively easy to find, activators are harder to come by, particularly with respect to the proteasome. Intriguingly, treating cultured cells with a small molecule inhibitor of USP14, a proteasome-associated deubiquitinylating enzyme, enhanced the degradation of several proteasomal substrates that have been implicated in neurodegenerative disease [69]. Enhancement of proteasomal activity through inhibition of USP14 may offer a strategy to reduce the levels of aberrant proteins in cells under proteotoxic stress. Accordingly, activators of the proteasome and/or autophagy may help to design new strategies for the prevention of neurodegenerative diseases.

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