intermediate referred to as the "half-mer" precursor complex.[101] During β-ring assembly in human cells, release of PAC3 (and perhaps with PAC4) is coupled to $\beta3$ incorporation, which is consistent with the observation that Pba3–Pba4 was selectively copurified with $\beta2$ but not with other β subunits in yeast, indicating a conserved mechanism in the roles of PAC3–PAC4 and Pba3–Pba4 during proteasome assembly.

Propeptides and the tails of 20S proteasome β subunits facilitate proteasome assembly; these types of domains are called 'intramolecular chaperones'.[107] The N-terminal propeptides and C-terminal tails of β subunits play pivotal roles in proteasome assembly through specific interactions with *cis* and *trans* β-rings in yeast and humans. For example, the propeptide from $\beta2$ influences cooperative proteasome assembly.[108] The $\beta5$ propeptide facilitates the incorporation of this subunit and is essential for yeast viability.[109] On the other hand, the $\beta5$ propeptide does not appear to be required for incorporation of $\beta5$ but rather it is used for $\beta6$ recruitment in human cells.[110] The propeptides of $\beta1$ and $\beta2$ are dispensable for cell viability in yeast, although mutants lacking these two propeptides displayed subtle defects in proteasome biogenesis. Thus, the role(s) of these propeptides remains obscure. In human cells, loss of the $\beta2$ propeptide eliminated $\beta3$ recruitment and was thus fatal to the cells. Of note, the C-terminal tail of $\beta2$, which wraps around $\beta3$ within the same β-ring, is also essential for proteasome biogenesis both in yeast and human cells.

Interestingly, the amino-acid sequences of the human β-subunit propeptides are considerably different from those of their yeast counterparts, unlike the mature β subunits, which are well conserved between yeast and humans. Such differences are also found in the extrinsic proteasome assembly chaperones, such as PAC1-4 and Ump1 (i.e., 5–20% identity), as discussed previously.[98] Why the chaperones have diverged during evolution is unknown; nonetheless, their basic functions and tertiary structures are highly conserved.

Unexpectedly, intermediates resulting from siRNA-mediated knockdown of each β subunit accumulated as two major and minor bands, in which the composition of each major and minor band in terms of α and β subunits was identical.[110] PA28 was associated with the slow-migrating minor bands, different from PAC1 and Hsp90α, which were detected only in the major bands. Hsc70 was observed in both the major and minor bands. Neither Hsp90α nor Hsc70 was detected in the α-ring. At present, it is unknown whether these conventional chaperones really have any roles in proteasome biogenesis or whether they are merely associated with the intermediates as experimental artifacts.

8.1.3 *Role of another chaperone Ump1 and dimerization of half-proteasomes.* Ump1 was identified in mutant yeast defective for ubiquitin-mediated proteolysis and is the first identified extrinsic assembly factor for 20S proteasomes.[111] Ump1 specifically associates with the assembly intermediates of 20S proteasomes and appears to enter the assembly pathway after association of $\beta2$, $\beta3$ and $\beta4$ in yeast. Upon dimerization of the half-proteasomes, Ump1 is encapsulated and degraded within the newly formed 20S proteasome like PAC1 and PAC2. Loss of Ump1 caused accumulation of assembly intermediates as well as half-proteasomes with unprocessed β subunits, indicating that Ump1 coordinates the processing of β subunits and dimerization of half-proteasomes in yeast.[111] On the other hand, Ump1 is also thought to function as an assembly checkpoint factor that inhibits dimerization of half-proteasomes until a full set of β subunits have been recruited to the α-ring.[101]

The human ortholog of Ump1 (hUmp1, Proteassemblin, or POMP) was identified using homology searches.[112),113] hUmp1 is included in precursor proteasomes with unprocessed β subunits and is degraded upon completion of proteasome assembly with a similar half-life to that of PAC1–PAC2.[110] Interestingly, knockdown of hUmp1 expression inhibited $\beta5$ recruitment, and resulted in the accumulation of α-rings with no β subunits. Moreover, hUmp1 can bind to the α-ring in the absence of β subunits and incorporation of hUmp1 is coupled with $\beta2$ binding, suggesting that hUmp1 is incorporated into proteasome precursors earlier than yeast Ump1. Therefore, hUmp1 is required for the initiation of β-ring formation, differing from the reported role of yeast Ump1. In the final step of β-ring assembly, the C-terminal tail of $\beta7$ is inserted into a groove between $\beta1$ and $\beta2$ in the opposite half-mer precursor, which triggers dimerization of the half-proteasomes in both yeast and humans.[68),101] Correct dimerization of half-proteasomes is followed by removal of the β propeptides and degradation of Ump1 and PAC1–PAC2 (for details, see Ref. 98.) (Fig. 4).

8.2 Assembly of immune response proteasomes. Vertebrates encode four additional catalytic β-subunits: IFN-γ–inducible β1i, β2i and β5i and thymus-specific β5t (Fig. 3). These alternative proteasomes play key roles in acquired/adaptive immunity by altering antigen processing as mentioned above. Accumulating evidence has clarified the molecular mechanism of immunoproteasome assembly.[98] Despite the coexistence of both immunoproteasome and standard subunits in some cells, immunoproteasomes are preferentially assembled.[114] The propeptides of the immunosubunits and hUmp1 play key roles in this cooperative assembly.[115] Interestingly, β1i enters the assembly pathway of immunoproteasomes earlier than in the standard proteasome assembly process, resulting in an assembly intermediate containing the α-ring, β1i, β2i, β3 and β4. In this intermediate, incorporation of β2i depends on β1i, and incorporation of β1i is facilitated by β2i. β5i is incorporated preferentially over β5 into the intermediates containing β1i and β2i.[114] This interdependency supports the homogenous formation of immunoproteasomes containing all three inducible subunits. Indeed, β2i processing and incorporation is severely impaired in β1i-deficient cells, and β1i incorporation is partially inhibited in β2i-deficient cells, whereas β5i incorporation, which is dependent on the β5i propeptide but not β5i catalytic activity, is not affected in either of these mutant cell lines.[110] β5i-deficient cells exhibited significantly retarded proteasome assembly and accumulation of proteasome precursors containing unprocessed β1i and β2i. Intriguingly, IFN-γ stimulation increased transcription of hUmp1 and immunosubunit mRNA, but decreased hUmp1 protein levels due to ~4-fold augmentation of hUmp1 protein turnover.[116] This rapid turnover was coupled with the maturation of active immunoproteasomes, indicating that the rate of immunoproteasome generation is four times faster than that of standard proteasomes. The higher affinity of hUmp1 for β5i than for β5 is likely to contribute to the rapid maturation of immunoproteasomes.[116]

How the thymoproteasome, another vertebrate-specific 20S proteasome, is assembled is currently unknown. When β5t was ectopically expressed in a human cell line that does not express immunosubunits, the protein was readily processed and incorporated into the proteasome, suggesting that β5t is preferentially incorporated compared with β5 and that β1i and β2i (i.e., partners of thymoproteasomes) are not required for β5t incorporation.[87] Because the majority of proteasomes in cTECs are thymoproteasomes, it is thought that β5t is preferentially incorporated before β5i in the thymus, suggesting that thymoproteasomes employ a specific assembly mechanism. Indeed, considering the high expressions of β1i and β2i, β5i whose gene and β2i gene are located at the same MHC class II region must be expressed in cTECs. According to the scenario for the immunoproteasome assembly, it is plausible that the propeptide or the extended C-terminal tail of β5t contributes to the assembly of the thymoproteasome as an intramolecular chaperone, but there is no available information at present in support of this assumption.[98]

8.3 Assembly of 19S RP and 26S proteasome. Currently, the assembly mechanism for the 19S RP is poorly understood. The yeast lid complex seems to be subdivided into two clusters: one is made up of Rpn5, Rpn6, Rpn8, Rpn9 and Rpn11, and the other contains Rpn3, Rpn7, Rpn12 and Rpn15. The interaction between Rpn3 and Rpn5 connects these two clusters, implying a hierarchy in the incorporation of Rpn subunits into the lid complex.[117] Recently, it was proposed that the 20S proteasome functions as an assembly factor for the RP due to aberrant RP formation in the presence of defective 20S proteasomes in yeast.[105] It was also proposed that the base and the lid are assembled independently, and then joined together.[118] The base is composed of six related AAA-ATPase subunits and four non-ATPase subunits. Putative chaperones may discriminate and arrange the six homologous ATPase subunits in a defined order, as is observed in the assembly of 20S α-ring. Whether assembly chaperones are required for the assembly of the ATPase ring, the lid, the base, and/or the 19S RP complex requires further studies.

The assembly mechanism of the 26S proteasome is largely not understood. Hsp90 is thought to play a role in both the assembly and maintenance of the lid in yeast.[119] Inactivation of Hsp90 was found to cause disassembly of the lid complex, which was then partially reassembled into the 26S proteasome following reactivation of Hsp90 *in vivo* or by adding Hsp90 and ATP *in vitro*. These findings suggest that the ATP-dependent chaperone activity of Hsp90 contributes to the assembly of the lid and 26S proteasomes. The function of Hsp90 in the assembly of 26S proteasomes, however, remains to be elucidated. Inhibition of proteasome active sites also stabilized 26S proteasomes, suggesting that the interface between the RP and the 20S proteasome changes depending on the activities of the 20S proteasome.[120] Related to this result, whether 26S proteasomes undergo obligatory disassembly and reassembly during protein degradation is currently a point of debate in this field. It was first reported that disassembly of the 26S proteasome and dissociation of the RP into subcomplexes or subunits are induced upon ATP-dependent degradation of a substrate protein in yeast.[121] In contrast, it was more recently reported that mammalian 26S proteasomes can degrade polyubiquitylated proteins without disassembling or the release of any subunits or subcomplexes.[122]

9. Proteasome Interacting Proteins (PIPs)

Recent proteomic analyses have identified auxiliary factors with known and unknown functions that are physically and/or transiently associated with the 26S proteasome.[123)–125] These proteins, referred to as proteasome-interacting proteins (PIPs), can be categorized into two groups (Table 1). The first group contains protein factors that are related to the ubiquitylation system. In this article, I described the association of the deubiquitylating enzymes Usp14 and Uch37 with the base subunits Rpn1 and Rpn2 via Rpn13, respectively. The extrinsic UBL–UBA ubiquitin receptors may also belong to this group. In addition, emerging evidence indicates that many ubiquitin E3 ligases, such as Hul5/KIAA10, E6AP, and Parkin, are transiently associated with the 26S proteasome. Moreover, other E3s such as Ubr1, APC, Ufd4 and SCF^{CDC4} as well as some E2 enzymes are also reported to associate loosely with the 19S RP of 26S proteasomes.[9]

The second group contains auxiliary factors that regulate proteasome functions via direct binding. For example, Ecm29 is an approximately 200-kDa protein that can bind to both the RP and the 20S proteasome in yeast. Purified 26S proteasomes from *Δecm29* cells tend to dissociate into RPs and 20S proteasomes. Together with the findings of electron micrographs of Ecm29–20S proteasome complexes, these results suggest that Ecm29 stabilizes the 26S proteasomes by tethering the 20S proteasome to the RP.[126),127] The mechanism underlying this function, however, is unclear. As listed in Table 2, there are many other factors, such as p28/gankyrin, Rpn14, p27 and S5b that interact with proteasomes. Some of them are suggested to be responsible for the regulation of 26S proteasomes or the assembly of the lid and base complexes, the process is largely ambiguous to date, but the details of their functions are unknown and require further studies.

Perspectives

The UPS is essential for cells to proliferate, and consequently proteasome levels are tightly regulated. For example, the balance between 20S and 26S proteasomes fluctuates to respond to environmental conditions; e.g., while the 26S proteasome levels increase during growth and developmental stages the 26S proteasome attenuates with aging process in *Drosophila*.[128] In addition, proteasomes are predominantly distributed in the nuclei of rapidly proliferating mammalian cells and growing yeast, indicating that this localization may contribute to cell proliferation. Why the proteasome is predominantly located in this cellular compartment remains to be determined, although typical nuclear localization signals (NLSs) are found on several of the 20S proteasomal α subunits, but not the β subunits.[129] No clear NLSs have been identified in the 19S RP subunits, except Rpn2, but it is plausible that the lid and the base are transported into the nucleus independently (unpublished results); the mechanisms underlying this translocation are a complete mystery at present. In addition, the issue of nuclear export (i.e., nucleocytoplasmic transport) of proteasomes is totally open to investigation. Indeed, nuclear export signals (NESs) of 20S and 26S proteasomes remain undefined.

To date, various lines of evidence have supported the importance of proteasomes outside of their proteolytic functions, such as transcription, DNA repair, and chromatin modeling.[9] For example, the 19S RP may contribute to transcriptional control in cells, independent of the functions of the 20S proteasome.[130),131] The non-proteolytic activities of the proteasome are important for coactivator recruitment; i.e., the ATPase activity of PA700 drives a stable association of a transactivator with the SAGA histone acetyltransferase complex.[131] PA700 also acts nonproteolytically in nuclear excision repair (NER).[132],[133] Chromatin remodeling is another nonproteolytic role of PA700, with implications for both transcription and DNA repair.[131] In addition, a proteasome-derived ATPase activity mediates relocalization of the substrates of endoplasmic reticulum-associated degradation (ERAD), a function that is primarily attributed to the AAA-ATPase p97/Cdc48.[134] ERAD eliminates aberrant proteins from the ER by localizing them to the cytoplasm where they are tagged by ubiquitin and degraded by the proteasome.

As described before, PI31 and PR39 are naturally occurring proteasome inhibitors, but their physiological functions are unclear. On the other hand, membrane-permeable synthetic inhibitors have been devised; e.g., various substrate-related peptidyl aldehydes have been designed as potent inhibitors of proteasomes, such as MG-132 (*N*-carbobenzoxy-Leu-Leu-leucinal) and PSI (*N*-carbobenzoxy-L-gamma-t-butyl-L-glutamyl-L-alanyl-L-leucinal), and the non-aldehyde peptidyl inhibitor Z-L_3VS (carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone), which are often used in *in vitro* and *in vivo* experiments.[135],[136] However, caution must be exercised in their use for inferring proteasome functions, because they inhibit not only proteasomes but also cysteine proteases such as calpains and lysosomal cathepsins.[135] In contrast to these compounds, microbial metabolites, lactacystin and epoxomicin, were found to be selective proteasome inhibitors that do not affect other proteases examined so far.[137],[138] Of particular interest is bortezomib (also known as velcade or PS-341). Bortezomib as first-in-class proteasome inhibitor has proven to be highly effective in some hematological malignancies, and in fact it has been granted approval by the FDA for relapsed multiple myeloma and non-Hodgkin lymphoma (NHL) and has been used clinically in over 85 countries worldwide so far.[139] Moreover, preclinical studies demonstrate that proteasome inhibition potentiates the activity of other cancer therapeutics, and particularly, the combination of proteasome inhibition with novel targeted therapies is an emerging field in oncology.[140] Furthermore, Salinosporamide A (also called NPI-0052),[141] recently identified from the marine bacterium *Salinispora tropica*, is a potent inhibitor of 20S proteasome and exhibits therapeutic potential against a wide variety of tumors. In addition, many other proteasome inhibitors are being assessed clinically for therapeutic use.[142] Thus, proteasome inhibitors provide a powerful new tool as fashionable drugs against cancer and other diseases including inflammations.

Finally, it should be emphasized that studies of the proteasome continue to provide significant insights in the physiologic roles of these complexes. Many questions, however, remain to be uncovered.

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Profile

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Chapter 4
Protein Misfolding and Axonal Protection in Neurodegenerative Diseases

Haruhisa Inoue, Takayuki Kondo, Ling Lin, Sha Mi, Ole Isacson and Ryosuke Takahashi

Abstract Genetically engineered mouse model studies show that neuronal dysfunction caused by protein aggregation/misfolding are reversible, indicating that injured neurons are alive even under disease states. Protein misfolding/aggregation in axons and distal dominant axonal degeneration are observed in a subgroup of degenerative diseases and in certain experimental conditions. Moreover, therapeutic approaches towards axonal protection are effective in neurodegenerative disease mouse models; (a) axonal regeneration, (b) anti-Wallerian degeneration, (c) autophagy enhancement, and (d) stabilization of microtubules. These studies demonstrate that axonal protection/functional repair of axons can be general therapeutic interventions for neurodegenerative diseases.

4.1 Neuronal Dysfunction in Neurodegeneration is a Reversible Process

It had been believed that neurodegeneration is not reversible. However, recent studies of transgenic mouse models, which express abnormal proteins associated with Alzheimer's disease, diffuse Lewy body disease, Parkinson's disease (PD), Huntington's disease (HD) and tauopathies such as frontotemporal dementia develop distinct disease-related neurological impairments, elegantly show that some neurological deficits of neurodegenerative cascades can be prevented or reversed by removing abnormal proteins, without obvious alteration of the number of neuronal cell bodies [1]. Thus, neurological impairments that are associated with neurodegenerative conditions might be caused by neuronal dysfunction to some extent rather than neuronal loss. These studies also demonstrate that symptoms arise from neuronal dysfunction which precedes neuronal death [1]. In HD mice model, as in most of the other triplet repeat diseases, the mutant huntingtin proteins form misfolded nuclear aggregates,

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which are highly insoluble. The double mutant huntingtin transgenic mice, in which the bidirectional transgene expression is activated by the removal of doxycycline at birth, express high levels of both mutant huntingtin and lacZ in the striatum, cortex, and hippocampus [2, 3]. Most of striatal neurons are stained with an anti-huntingtin antibody, showing diffuse nuclear aggregates. By 8 weeks of age, striatal morphological alterations in the mutant huntingtin transgenic mice include a reduced size, reactive gliosis, and a decrease in D1 receptors (a feature seen in HD patients). All of the mice at this age also show a behavioral abnormality common to mouse models of HD: when suspended by their tails, they clasp their limbs. This behavioral phenotype was aggravated over time. Neuropathological examination demonstrated the colocalization of various molecular chaperones, ubiquitin, and proteasome subunits with the aggregated proteins. Surprisingly, abolishing the expression of mutant huntingtin by Cre-loxP system in mutant huntingtin transgenic mice with neurodegenerative phenotype results in either a halt of the disease progression or a full recovery from the disease phenotype including pathological changes [2, 3]. This observation indicates that irreversible changes that commit the neurons to persistent dysfunction or death do not necessarily take place in the neurodegenerative process. These observations suggest that therapeutic approaches aiming at elimination of misfolded proteins might be effective in treating neuronal dysfunction. Furthermore, the recovery from motor disturbances indicates that plastic changes can occur when the toxic insult ceases [3]. Recent studies of mutant huntingtin transgenic mice show that the neuronal dysfunction may be caused by misfolded mutant huntingtin protein, at synaptosomal proteasome and mitochondria, which seem to trigger vicious cycles of aberrant neuronal activity [4].

4.2 Neuronal Dysfunction Is Not Treatable by Anti-Cell Death Therapy

Although an important role of apoptosis is implicated in neurodegenerative diseases, data from both humans and animal models indicate that neurodegeneration is often a long-lasting process that finish with cell death only after a prolonged period of disease state.

In PD model mice study, although peptide inhibitors of caspases block 1-methyl-4-phenylpyridinium (MPP+)-induced dopaminergic neuronal death, dopaminergic neuronal terminals are not rescued [5]. Similarly, adenovirus-mediated transgene expression of X-linked inhibitor of apoptosis protein (XIAP) blocks death of dopaminergic neurons in a N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mouse model, but does not prevent the decrease of dopaminergic terminal markers in the striatum [5]. Moreover the resistance of the dopaminergic neurons in the pro-apoptotic Bax protein knockout mice against MPTP toxicity is accompanied by a significant, although less prominent, sparing of striatal dopamine contents [6]. In the superoxide dismutase 1 (*SOD1*) transgenic mouse models of amyotrophic lateral sclerosis (ALS) study, we have also shown that overexpression of XIAP in spinal motor neurons rescues cell bodies of motor neuron without inhibition of neuronal dysfunction [7]. Similarly, removal of *Bax* gene resulted in complete rescue of cell bodies of motor neurons in ALS model mice, but denervation and axonal degeneration still occurred [8, 9]. Moreover with Bcl-2 transgenic mice crossed with *pmn* mice to block cellular apoptosis, motor neurons were completely rescued, but motor axons degenerate to the same extent as in *pmn* mice with normal levels of Bcl-2, and there is no change in muscle strength or life span [9, 10]. Consistent with these findings, although an important role for synaptic caspase activation and apoptosis has been proposed, axonal degeneration after withdrawal of trophic support occurs without activation of caspases in contrast to cell death of the cell body [9, 11]. These studies support the idea that axonal degeneration/dysfunction may proceed independently from the molecular events regulating cell death, and that apoptosis plays a critical role in neuronal cell body death, and neuronal dysfunction is not treatable by anti-cell death therapy. Therefore, anti-dysfunction therapies which target axonal degeneration/dysfunction are promising for treatment of neurodegenerative diseases.

4.3 Morphological Aspects of Neuronal Dysfunction Caused by Protein Aggregation/Misfolding in Human Neurodegenerative Disorder

It is hard to morphologically evaluate the neuronal dysfunction caused by protein aggregation/misfolding in the central nervous system, because neurons possess intricate three-dimensional structure, and are embedded deep in the brain which prevents accurate observation of cell shape. In contrast, morphological evaluation of the peripheral nervous system shows that degeneration of the cardiac sympathetic nerve occurs in PD and diffuse Lewy body disease, both of which are caused by accumulation of misfolded α-synuclein, and that degeneration of their distal axons precedes loss of their neuronal cell bodies in the paravertebral sympathetic ganglia [12]. This interesting observation suggests that distal dominant axonal degeneration precedes cell death not only in peripheral sympathetic, but central dopaminergic neurons of PD. Moreover, it is implicated that the centripetal degeneration may represent the common pathological process underlying various neurodegenerative disorders.

In ALS study, there are data supporting the hypothesis that the pathology of ALS starts with distal axonal degeneration [9]. Neuropathological studies, by quantitative morphometry, demonstrate a distal-to-proximal gradient of axonal pathology in phrenic nerves from ALS patients [9, 13]. Moreover, an autopsy case of an ALS patient, who died unexpectedly during a minor surgical procedure, revealed severe denervation and reinnervation changes demonstrated by electromyography, but there were no detectable changes in the corresponding spinal motor neuronal cell bodies [9, 14]. Threshold tracking, which measures axonal excitability, is an alternative electrophysiological technique that demonstrates early abnormalities in

ALS patients. An apparent increase in persistent Na+ current and a decrease in K+ conductance is observed in two ALS patients [9, 15, 16], and these changes are more prominent distally than proximally [9, 17]. Genetical studies of ALS also provide further evidence for the potential importance of axonal pathology in ALS [9, 18].

4.4 Protein Misfolding and Axonal Degeneration in Experimental Animal Models

Recent studies demonstrate that genetically engineered mice with misfolded protein accumulation display axonal degeneration phenotype [19].

One of the excellent examples is the knockout mouse of an essential autophagy gene, *Atg7*, whose alterations have also been observed in several neurodegenerative diseases [20, 21]. Ablation of panneuronal autophagy causes ubiquitin-p62 positive aggregation in neuronal cell body [20, 21]. Conditional knockout of *Atg7* in Purkinje cells initially causes cell-autonomous, progressive dystrophy (manifested by axonal swellings) and degeneration of the axon terminals [22]. Consistent with suppression of autophagy, no autophagosomes are observed in these dystrophic swellings [22]. Axonal dystrophy of mutant Purkinje cells proceeds with little sign of dendritic or spine atrophy, indicating that axon terminals are much more vulnerable to autophagy impairment than dendrites. This early pathological event in the axons is followed by Purkinje cell death. Furthermore, ultrastructural analyses of mutant Purkinje cells reveal an accumulation of aberrant membrane structures in the axonal dystrophic swellings, indicating that the autophagic machinery component Atg7 is required for membrane trafficking and turnover in the axons, and that impairment of axonal autophagy as a possible mechanism for axonal degeneration associated with neurodegeneration [22]. Accordingly, significant accumulation of ubiquitinated proteins is noted in Atg7-deficient brain, but their levels, especially insoluble ubiquitinated proteins, are lower than in Atg7-deficient liver, and formation of the inclusion is found in restricted groups of neurons. Several ubiquitin-positive aggregates are recognized in Atg7-deficient brain regions in the presence of mild neuronal loss [22]. Direct degradation of aggregates/misfolded protein by autophagy is contradictory to the recent hypothesis that the generation of protein aggregates represents a protective mechanism [23]. However, the primary targets of autophagy are likely to be diffuse cytosolic proteins, not inclusion bodies themselves, suggesting that inclusion body formation in autophagy-deficient cells is an event secondary to impaired general protein turnover [23]. However, it is still possible that misfolded proteins in soluble or oligomeric states could be preferentially recognized by autophagosomal membranes, which might also be mediated by ubiquitin–p62–LC3 interactions [23, 24].

A recent study also showed that axonal degeneration is relevant to autophagy caused by protein mislocalization [25]. Adaptor protein-4 (AP-4) is a member of the adaptor protein complexes, which control vesicular trafficking of membrane proteins. Although AP-4 has been suggested to contribute to basolateral sorting

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in epithelial cells, its function in neurons is unknown. A recent study showed that disruption of the gene encoding the β subunit of AP-4 resulted in increased accumulation of axonal autophagosomes, which contained alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and transmembrane AMPA receptor regulatory proteins (TARPs), in axons of hippocampal neurons and cerebellar Purkinje cells both *in vitro* and *in vivo* [25]. AP-4 indirectly associates with the AMPA receptor via TARPs, and the specific disruption of the interaction between AP-4 and TARPs causes the mislocalization of endogenous AMPA receptors in axons of wild-type neurons. These results indicate that AP-4 may regulate proper somatodendritic-specific distribution of its cargo proteins, including AMPA receptor-TARP complexes and that protein mislocalization may disturb the autophagic pathway(s) in neurons [25].

4.5 Therapeutic Approaches to Treat Neuronal Dysfunction by Axonal Protection

4.5.1 Axonal Regeneration

From previous studies showing that neuronal dysfunction, which may morphologically reflect axonal degeneration by misfolded proteins, precedes neuronal cell death, we hypothesized that axonal regeneration may protect axons from degeneration and have therapeutic effects against neuronal dysfunction in neurodegeneration [26]. We have tested this hypothesis using anti-LINGO-1 antagonists in experimental PD models induced by either oxidative (6-hydroxydopamine) or mitochondrial (MPTP) toxicity [25]. LINGO-1 is the nervous system-specific leucine-rich repeat Ig-containing protein, and associated with the Nogo-66 receptor (NgR) complex and is endowed with a canonical EGF receptor (EGFR)-like tyrosine phosphorylation site, playing a critical role as an inhibitor of axonal regeneration (Fig. 4.1) [27, 28]. LINGO-1 antagonists, which block signal transduction of LINGO-1 complex (Fig. 4.2) [28], include decoy protein LINGO-1-Fc, Lenti-virus-dominant negative LINGO-1, and anti-LINGO-1 blocking antibody. We examined the role of LINGO-1 in cell damage responses of dopaminergic neurons. In LINGO-1 knockout mice, dopaminergic neuronal survival is increased and behavioral abnormalities are reduced compared with wild-type ones. This neuroprotection is accompanied by increased Akt phosphorylation [26]. Similar *in vivo* neuroprotective effects on midbrain dopaminergic neurons are obtained in wild-type mice by blocking LINGO-1 activity using LINGO-1-Fc protein which inhibit LINGO-1 function. Neuroprotection and enhanced neurite growth are also demonstrated for midbrain dopaminergic neurons *in vitro* [26]. LINGO-1 antagonists improve dopaminergic neuronal survival in response to MPTP in part by mechanisms that involve activation of the EGFR/Akt signaling pathway through a direct inhibition of the binding LINGO-1 to EGFR (Fig. 4.3) [26]. LINGO-1 is also upregulated in compromised, probably dysfunctional, neurons in spinal cord injury [29] or kainic acid injection

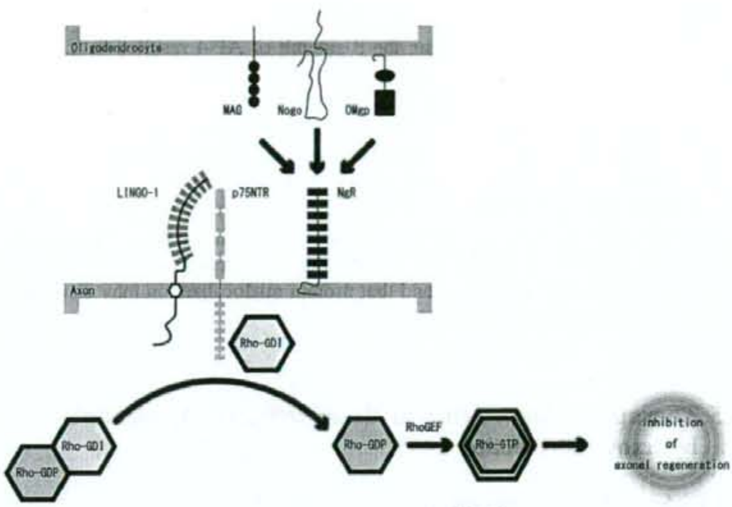

Fig. 4.1 Molecular signaling of Nogo-66 receptor (NgR) complex. The potential role of LINGO-1 is revealed as a component of NgR complex, which is comprised of NgR and p75 neurotrophin receptor (p75NTR) or an orphan TNF receptor Taj/Troy [27, 28]. Activated p75NTR binds the RhoA-GTP dissociation inhibitor (Rho-GDI), thus enabling RhoA activation via the exchange of GDP for GTP, and inhibits axonal regeneration upon binding to inhibitory molecules such as myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp), and Nogo-66 (Nogo) expressed in oligodendrocytes[40, 41]

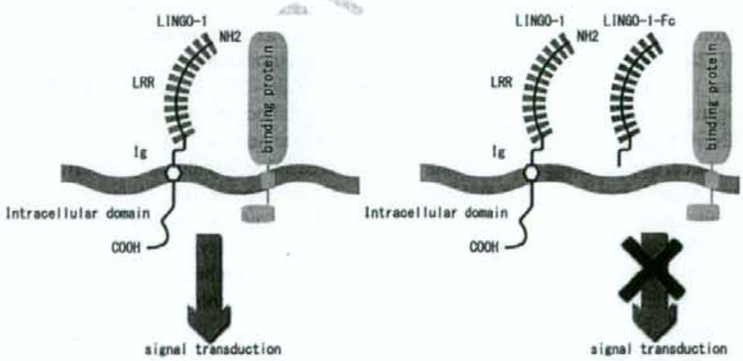

Fig. 4.2 Functional mechanisms of LINGO-1 antagonist(s). LINGO-1-Fc, one of LINGO-1 antagonists, is the soluble, truncated form of LINGO-1, and inhibits LINGO-1 modulating signaling transduction by inhibiting LINGO-1 to bind its binding protein(s) [26, 28]

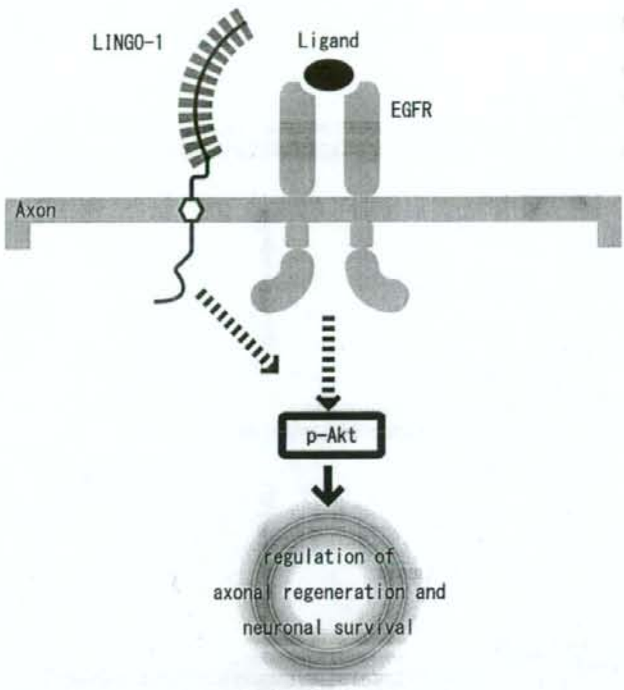

Fig. 4.3 LINGO-1 effect on EGF receptor (EGFR). LINGO-1 binds EGFR, and regulates EGFR expression level, leading to control axonal regeneration and neuronal survival via phosphorylation of Akt [26, 28]

[30]. We found that LINGO-1 expression is elevated in compromised, dysfunctional neurons including in the substantia nigra of PD patients compared with age-matched controls and in animal models of PD after neurotoxic lesions [25]. These results show that inhibitory agents of LINGO-1 activity can protect dopaminergic neurons from degeneration caused by PD. It is necessary to test whether LINGO-1 inhibition of function has protective effects on genetic PD models and/or other neurodegenerative disease models in the future.

4.5.2 Anti-Wallerian Degeneration

Axonal degeneration in "dying back" disorders seems to be different from Wallerian degeneration which is triggered by focal lesion (Fig. 4.4) [19]. However, apparent differences in the directionality of degeneration have been controversial [19].

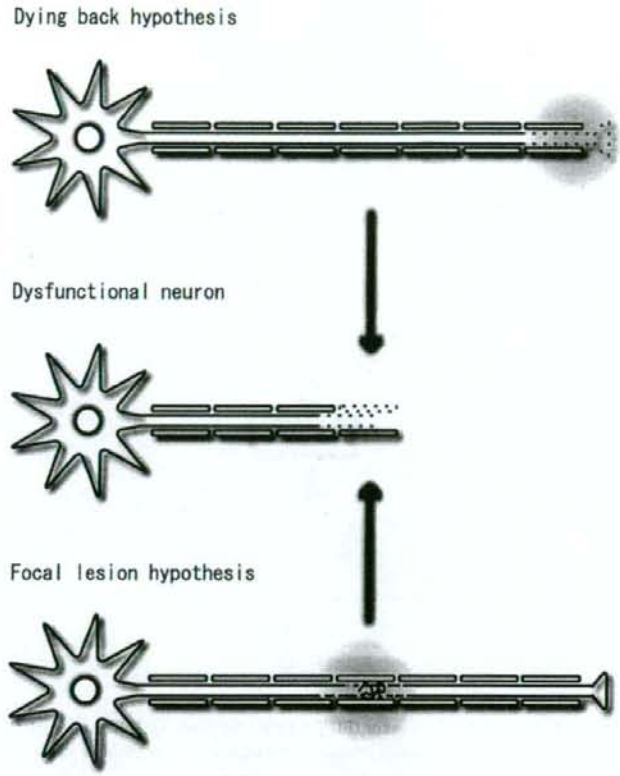

Fig. 4.4 "Dying back" and focal lesion models of axonal degeneration/dysfunction. The centripetal axonal degeneration in neurodegenerative disease may be caused either by the "dying back" process or by repetitive Wallerian degeneration from focal lesion(s) [19]

Wallerian degeneration is a simple experimental model of axonal degeneration, in which the distal stump of an injured axon degenerates rapidly after a reproducible latent phase [19]. In Wallerian degeneration slow (*WldS*) mice, Wallerian degeneration in response to axonal injury is delayed because of a mutation that results in overexpression of a chimeric protein (WldS) composed of the ubiquitin assembly protein Ufd2a and the nicotinamide adenine dinucleotide (NAD) biosynthetic enzyme Nmnat1 [31]. With the discovery of the *WldS* mouse, the hypothesis could be tested. In *WldS* mice, injury-induced Wallerian degeneration is delayed ~tenfold (for 2–3 weeks) by a dominant mutation that acts intrinsically in neurons. In cross-breeding with progressive motor neuronopathy (*pmn*) mice and myelin protein zero (P0) null mutants, a model of Charcot-Marie-Tooth disease, WldS significantly delayed axonal degeneration [19]. In the central nervous system, WldS also protects against both genetic and toxic insults. Some nigrostriatal axons, which degenerate in PD, are spared and remain functional after 6-hydroxydopamine lesions in *WldS* mice [19, 32]. Axonal spheroids, which are presumably composed by misfolded protein(s) accumulation, are reduced in number in the gracile tract of mice with gracile axonal dystrophy (*gad*), deficient in ubiquitin carboxyterminal hydrolase L1 (UCHL1) crossed with *Wld*S mice [19, 33]. Not all axonal degeneration is delayed by WldS. WldS have modest effect on the ALS model mice with protection of the terminal axon only at its early stage [34, 35]. The failure to protect axons under certain circumstances indicates the existence of multiple axonal degeneration mechanisms. WldS may have protective effect(s) in rapidly degenerative or acute disorders.

4.5.3 Autophagy Enhancement

It is reasonable to assume that autophagy could represent a therapeutic target for axonal degeneration because the deletion of essential components of autophagy causes axonal degeneration, and relevance(s) of autophagy with degeneration are observed in several neurodegenerative diseases [23]. Autophagy enhancement by the regulatory protein kinase complex Target of Rapamycin (TOR) inhibitors such as rapamycin and its analogue CCI-779 protects against neurodegeneration seen in polyglutamine disease models in Drosophila and mice [23, 36]. A screened small molecule enhancers of rapamycin improve the clearance of mutant huntingtin and α-synuclein, and protect against neurodegeneration in a fruit-fly HD model [23, 37]. These results provide us with the evidence supporting autophagy enhancement as a therapeutic strategy against the toxicity of misfolded proteins in neurodegenerative diseases.

Tsc2, also known as tuberin, is a GTPase activating protein that regulates the G protein Rheb, an activator of mTOR (mammalian Target of Rapamycin) [38]. Tuberous sclerosis is a single-gene disorder caused by heterozygous mutations in the *TSC1* or *TSC2* genes and is frequently associated with mental retardation, autism and epilepsy [38]. Even individuals with tuberous sclerosis and a normal intelligence quotient are commonly affected with specific neuropsychological problems, including long-term and working memory deficits [38]. Mice heterozygous for the deletion of the *Tsc2* gene in *Tsc2*(+/−) mice show deficits in learning and memory [38]. A recent study showed that hyperactive hippocampal signaling led to abnormal long-term potentiation in the CA1 region of the hippocampus and consequently to deficits in hippocampal-dependent learning in TSC mice [38]. Moreover, a brief treatment with the mTOR inhibitor rapamycin in adult mice rescues not only the synaptic plasticity, but also the behavioral deficits in this animal model of tuberous sclerosis, demonstrating that treatment with mTOR antagonists ameliorates cognitive dysfunction in the TSC mice model [38]. Autophagy may be included in axon and/or synaptic dysfunction in degeneration via the mTOR signaling pathway(s).

4.5.4 Stabilization of Microtubules

In tauopathy model(s), misfolded mutant tau protein causes axonal dysfunction/degeneration [39]. Microtubule-binding drugs can be therapeutically beneficial in tauopathy models by functionally substituting for the microtubule-binding protein tau, which is sequestered into inclusions of human tauopathies and transgenic mouse models [39]. Mutant tau transgenic mice treated with paclitaxel (Paxceed) showed that fast axonal transport in spinal axons is restored, and that microtubule numbers and stable tubulins are increased compared with sham treatment [39]. Moreover, Paxceed ameliorated motor impairments in tau transgenic mice [39]. Thus, microtubule-stabilizing drugs have therapeutic potential for axonal dysfunction/degeneration, in tauopathies by offsetting losses of tau function that result from the sequestration of this microtubule-stabilizing protein into filamentous misfolded inclusions [39].

4.6 Concluding Remarks

Accumulating experimental evidence suggests that protection/functional repair of axons (Fig. 4.5) can be a general therapeutic strategy for neuronal dysfunction caused by misfolded protein(s) deposition in neurodegenerative disease(s). The translation of these results into human disease therapies should be done in the near future.

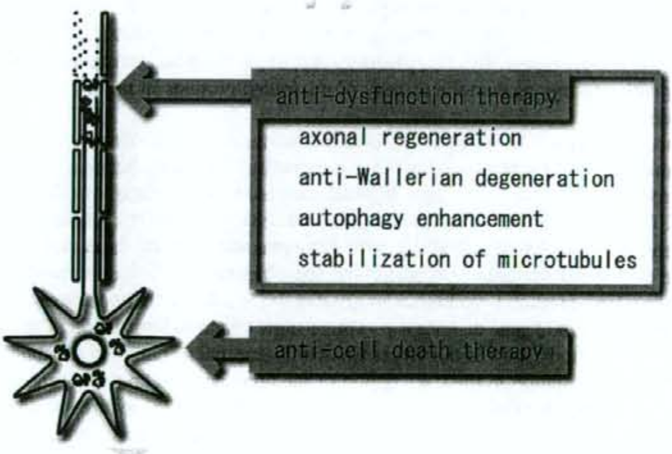

Fig. 4.5 Anti-dysfunction therapy for axonal protection and anti-cell death therapy for inhibiting neuronal loss. Neuronal dysfunction in neurodegeneration is reversible. Axonal regeneration, anti-Wallerian degeneration, autophagy enhancement, and/or stabilization of microtubules may be effective as anti-dysfunction therapies by protecting axon from neurodegenerative diseases, although anti-cell death therapies may inhibit only neuronal cell body loss