tivity by using *Drosophila melanogaster* and found age-related attenuation of the 26S proteasome activity and abundance that was associated with impaired assembly of the 26S proteasome with the 19S regulatory particle (RP) and the 20S proteasome. In a genetic gain-of-function screen, we identified *Rpn11*, which encodes one of the lid subunits in the 19S RP, as a suppressor of the age-dependent progression of a polyglutamine-induced neurodegenerative phenotype. The overexpression of *Rpn11* prevented the age-related reduction of the 26S proteasome activity, which suppressed the age-dependent accumulation of ubiquitinated proteins and extended the life span. On the other hand, the loss of function of *Rpn11* enhanced the age-related reduction of 26S proteasome activity, leading to a shorter life span, a premature age-dependent accumulation of ubiquitinated proteins, and an early onset of a neurodegenerative phenotype. Our results demonstrate for the first time that the age-related reduction of the 26S proteasome activity is a key factor in the induction of certain age-related biological changes and in the increased risk for the onset or progression of neurodegenerative diseases. Our findings imply that improving the amount and/or activity of the 26S proteasome by overexpressing a lid subunit, such as *Rpn11*, could provide an extension to the mean life span and prevent the age-dependent onset or progression of neurodegeneration.

MATERIALS AND METHODS

Fly stocks and generation of transgenic flies. Flies were raised on standard *Drosophila* medium at 25°C, and transgenic strains were generated as described previously (22). The w^{1118} strain was used as the wild-type strain. The *UAS-LacZ*, *tub-GAL80^{ts}*, *GMR-GAL4*, and *da-GAL4* (Bloomington *Drosophila* Stock Center), *UAS-MJDtr-Q78* (2), *GMR-huntingtin 120Q* (16), and *UAS-LacZIR* (20) fly strains were used in this study. The gene search (GS) system alleles were gifts from T. Aigaki. Using the temporal and regional gene expression targeting (TARGET) system, we raised flies at the restrictive temperature (18°C) before eclosion to suppress the activity of GAL4. One day after eclosion, the flies were moved to the permissive temperature (29°C) (28). For the longevity assay, more than 200 males of each genotype were collected and cultured in vials containing 20 males each. The flies were maintained at 29°C from day 2 and transferred to vials with fresh food every 3 to 4 days, and deaths were scored every day. Statistical significance was defined as a P value of <0.0001 by log-rank test.

Screening methods. To identify the suppressor for neurodegeneration-induced cell death, we adapted the P element-based GS system (33). The GS system vector contains two copies of the upstream activating sequence (UAS) enhancer adjacent to a core promoter: there is one copy near the terminal inverted repeats at each end of the vector, and each is oriented to direct transcription outward. This system combined with the "local hops" technique (34) enabled us to perform an efficient gain-of-function screen using alleles containing the GS vector at various locations. For the first screen, we performed a dominant-modifier screen for heat-shocked *GAL4* (*hs-GAL4*)-mediated *Reaper* (a proapoptotic gene in *Drosophila*)-induced lethality to identify a strong suppressor of cell death. We identified 5 suppressor alleles from 1,600 alleles. Expecting that this collection of suppressor alleles would include suppressors of neurodegeneration, in the second screen, we performed a dominant-modifier screen for *GMR-GAL4*-mediated polyglutamine-induced neurodegenerative cell death. One allele, named *DANC* (*d*efender *a*gainst *n*eural *c*ell death), was identified as such a suppressor.

Immunoblotting. Whole flies or fly heads were prepared, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted as described previously (22). All samples were separated by 10% SDS-PAGE, and the blots were probed with antihemagglutinin (anti-HA) Y11 (1:500; Santa Cruz Biotechnology), mouse antiubiquitin (1:500; Stressgen Biotechnologies), anti-β-tubulin (1:500; Chemicon), anti-α2 (1:1,000) (11), or anti-Rpn11 (1:300) primary antibody and with anti-mouse immunoglobulin G-horseradish peroxidase (1:1,000; Promega) or anti-rabbit horseradish peroxidase-linked immunoglobulin G (1:1,000; Cell Signaling) secondary antibody. For the glycerol density gradient analyses, 200 μl of each fraction was precipitated with acetone and subjected to SDS-PAGE. The relative amount of each protein was determined by densitometric analysis using Image Gauge software (Fujifilm) (22). Quantitative data were obtained as the ratio of the indicated protein signal to that of the loading control for each immunoblot and were plotted as a ratio graph.

Histology and immunohistochemistry. The heads of adult flies from each genotype were fixed and embedded in Epon resin for the preparation of semithin horizontal sections (1 μm). The sections were stained with toluidine blue and examined under a light microscope as described previously (18). Immunohistochemistry treatment of eye discs from third-instar larvae was carried out as described previously (22). The following antibodies were used: rabbit anti-HA (1:500; Santa Cruz Biotechnology) and rat anti-ELAV 7E8A10 (1:20; Hybridoma Bank). Alexa Fluor 488- and Cy3-conjugated secondary antibodies were obtained from Molecular Probes and Jackson Immunoresearch and used at 1:100. All fluorescently labeled samples were examined with an LSM5 model confocal microscope (Carl Zeiss).

Proteasome activity. Whole flies or heads alone were homogenized in buffer B (25 mM Tris-HCl [pH 7.5], 2 mM ATP, 5 mM $MgCl_2$, and 1 mM dithiothreitol). Proteasome peptidase activity in the lysates was measured with a synthetic peptide substrate, succinyl-Leu-Leu-Val-Tyr-7-amino-4-methyl-coumarin (Suc-LLVY-AMC) (Sigma).

Glycerol density gradient analysis. The whole bodies of 70 flies of the appropriate genotypes were homogenized in buffer B. The lysates were clarified by centrifugation at 20,000 × *g*, and the lysates (1 mg of protein) were subjected to 8 to 32% (vol/vol) linear glycerol density gradient centrifugation (22 h, 83,000 × *g*). The gradient was separated into 32 fractions (11), and proteasome peptidase activity in 10 μl of each fraction was measured with Suc-LLVY-AMC activity.

Plasmid construction. The expressed sequence tag clone RE07468 was purchased from Invitrogen as full-length *Rpn11* cDNA. The sequence of the clone was confirmed, and an *Rpn11* fragment was amplified with the following PCR primers: 5′-CGG GGT ACC ACC ATG GAT CGT CTG CTA CGT CTT GGA-3′ and 5′-GCT CTA GAT CAC TTA AAG ACT ATG GTG TCC A-3′. The amplicon was then inserted into the KpnI-XbaI sites of the *pUAST* vector to generate *pUAST-Rpn11*. For the RNA interference experiment, a fragment containing the first 500 bp of the *Rpn11* open reading frame was inserted into *pUAST-R57* (a gift from R. Ueda), as previously described (22).

Anti-Rpn11 antibody. The N-terminally His_6-tagged recombinant Rpn11 (His-RPN11) protein was produced in the *Escherichia coli* strain BL21(DE3) pLysS (Novagen) using *pRSET-His-Rpn11* by incubating the bacteria for 20 h at 20°C. The recombinant His-Rpn11 protein expressed in E. coli BL21(DE3) was purified on a Ni^+ column and used to immunize rabbits (Hokudo Co.).

RT-PCR analysis. For reverse transcription-PCR (RT-PCR) analysis of the genes surrounding the *DANC* allele or of other GS system fly lines, flies expressing *DANC* or other GS system genes with *hs-GAL4* were treated or not with heat shock (twice at 37°C for 30 min, with a 30-min interval between treatments), and their total RNA was prepared 3 h later by using Trizol (Invitrogen). The RNA was reverse transcribed and subjected to PCR analysis (26 cycles) using the following primers: *Rpn11* (5′-ACT TAA AGA CTA TGG TGT CCA-3′ and 5′-TGG ATC GTC TGC TAC GTC TT-3′); *Rpn9* (5′-CGG GGT ACC ACC ATG TCC AAT CCT CAG CC-3′ and 5′-GCT CTA GAC TAA TTG GTG AGG ATT TCG GC-3′); *Rpn5* (5′-CGG GGT ACC ACC ATG GAC ACC TAT TTG TT-3′ and 5′-GCT CTA GAT TAA TCC TCG ACA GCA CAC AT-3′); *Rpn2* (5′-ATG AGT CTT ACG TCC GCC GCG-3′ and 5′-GAT GCA ACT TTT CAA CCT CGT T-3′); *α6* (5′-ATG TTT CGC AAC CAG TAC GAT AG-3′ and 5′-CTA TGG ACG CTG CTC GGT TGC AA-3′); and *GAPDH* (5′-CCA CTG CCG AGG AGG TCA ACT A-3′ and 5′-GCT CAG GGT GAT TGC GTA TGC A-3′).

RESULTS

Proteasome activity decreases and ubiquitinated proteins accumulate with age. To gain insight into the cause of the age-related proteasomal dysfunction, we assessed the peptidase activities of the proteasome with age in *Drosophila*. The peptidase activities of the proteasome in lysates of wild-type fly heads were measured by Suc-LLVY-AMC-hydrolyzing activity (chymotrypsin-like activity), which cleaves peptide bonds after hydrophobic amino acids. Lysates of wild-type fly heads showed gradually decreased proteasome activity with age (Fig. 1A). The proteasome activity remarkably decreased in flies that were 20 to 30 days posteclosion, the time when age-related symptoms, such as memory impairment, are first

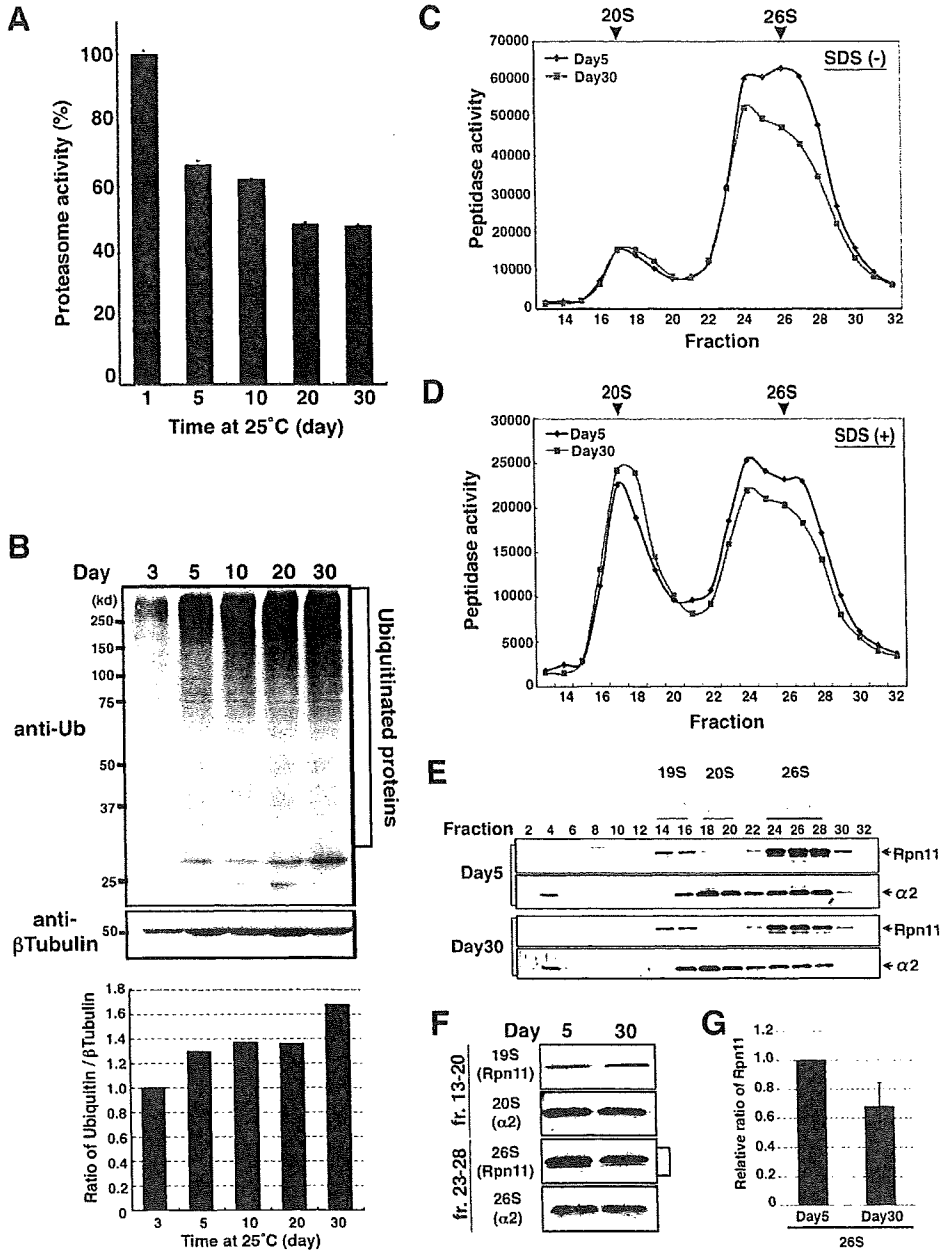

FIG. 1. The 26S proteasome activity decreases and ubiquitinated proteins accumulate with age. (A) Proteasome activity in wild-type flies decreased with age. The proteasome activity in wild-type fly heads was measured by hydrolysis of Suc-LLVY-AMC (chymotrypsin-like activity) on the indicated day posteclosion. (B) Accumulation of ubiquitinated proteins with age in wild-type flies. Wild-type fly head extracts were immunoblotted with antiubiquitin (anti-Ub) or anti-β-tubulin antibodies on the indicated day posteclosion. (C) The 26S proteasome activity in aged flies was significantly lower than that in young flies. Extracts of day 5 or day 30 flies were fractionated by glycerol density gradient centrifugation, and the Suc-LLVY-AMC hydrolysis activities were measured. The experiments were repeated three times. (D) Suc-LLVY-AMC hydrolysis activities of the same lysates shown in panel C were measured with the addition of 0.01% SDS [SDS(+)], a potent artificial activator of the 20S proteasome. The peptidase activity of the 20S proteasome did not significantly change between day 5 and day 30. (E) Immunodetection of the Rpn11 or the α2 subunit revealed a decrease in the level of this subunit with age in the 26S-containing fractions. Immunoblot analysis shows the even fractions probed with antibodies against Rpn11 and α2. (F) Fractions (fr.) 13 to 20 (19S or 20S included) or fractions 23 to 28 (26S included) shown in panel E were pooled and immunoblotted with antibodies against Rpn11 or α2. (G) The protein level of the 26S proteasome is lower in the aged fly than in the young fly. The amount of Rpn11 protein (the amount of the band indicated on panel F by a line) was determined by densitometric analysis. The graph shows the relative ratio of the amounts of Rpn11 in fractions 23 to 28 (26S included) at day 30 to that at day 5 from three individual experiments.

detectable (the normal *Drosophila* life span is 60 to 70 days) (40). This finding indicated that the reduction of proteasome activity was age dependent, as expected.

Because the proteasome maintains cellular homeostasis, we examined whether the age-related decline of proteasome activity caused the accumulation of unfolded proteins, represented by polyubiquitinated proteins. Extracts were made from wild-type fly heads or whole bodies and immunoblotted with an antiubiquitin antibody. Polyubiquitinated proteins, which showed a high-molecular-weight smear, gradually increased with the age of the flies, in both head (Fig. 1B) and whole-body extracts (see Fig. 5A). These results indicate that the reduction of proteasome activity and the accumulation of ubiquitinated proteins are observed with age.

The age-related reduction of proteasome activity results from the attenuation of the 26S proteasome. Polyubiquitinated proteins are recognized and degraded by the 26S proteasome. The 26S proteasome is a huge protein complex composed of one proteolytically active 20S proteasome and two 19S RPs, each attached to one end of the 20S proteasome (37). To compare the biochemical nature of proteasomes in young with that in aged flies, we performed an 8 to 32% glycerol density gradient centrifugation analysis and analyzed each fraction for chymotrypsin-like activity, using a peptide substrate (Suc-LLVY-AMC). The peptidase activity, particularly in the 26S proteasome-containing fractions (Fig. 1C, fractions 24 to 28) of the aged flies, was significantly lower than that of the young flies (Fig. 1C). On the other hand, the peptidase activity of the 20S proteasome, which was assayed in the presence of 0.01% SDS, a potent artificial activator of the latent 20S proteasome, did not significantly change with age (Fig. 1D).

Next, we tested the protein levels of each proteasome. Each fraction was subjected to immunoblotting with an anti-Rpn11 antibody to detect the 19S RP and the 26S proteasome or with an antibody against the α2 subunit to detect the 20S and 26S proteasomes. The immunodetection of Rpn11 and α2 revealed that the protein level of the 26S proteasome was lower in the aged fly than that in the young fly; however, the protein levels of the 20S proteasome and the 19S RP remained essentially stable with age (Fig. 1E, F, and G). These data indicate that the age-related reduction of proteasome activity results from the attenuation of the activity and amount of the assembled 26S proteasome.

Identification of *Rpn11*, a component of the 19S RP, as a suppressor of progressive neurodegeneration. Previously, we showed that the inhibition of proteasome function in flies expressing expanded polyglutamine enhances both the accumulation of ubiquitin conjugates and polyglutamine-induced neural degeneration (18). To examine whether the age-related attenuation of the 26S proteasome causes the age-dependent progression of neurodegenerative diseases, we focused on a progressive phenotype of neurodegenerative diseases. Machado-Joseph disease (MJD), also known as spinocerebellar ataxia type 3, is the most common dominantly inherited progressive ataxia caused by polyglutamine expansion. Expression of a truncated form of the human MJD protein with an expanded polyglutamine domain (MJDtr-Q78) causes progressive neural degeneration in *Drosophila* (39). The expression of *MJDtr-Q78* driven by the eye-specific promoter *GMR* caused only a slight disruption of the regular, external lattice of the eye in young flies (Fig. 2A); however, the eye morphology of these flies showed progressive degeneration over time, with significant loss of pigmentation by day 15 (Fig. 2B).

To identify the suppressor of neurodegeneration-induced cell death, we adapted the P element-based GS system (33). This system combined with the "local hops" technique (34) enabled us to perform an efficient gain-of-function screen using alleles containing the GS system vector, a P element-based vector with UAS enhancers, at various locations. In the course of performing a gain-of-function screen for suppressors of neural cell death, using fly alleles with the GS vector, we identified 1 of 1,600 alleles, which we called the *DANC* allele, that suppressed the progression of the *MJDtr-Q78*-induced degenerative phenotype (Fig. 2C and D, and see Materials and Methods). We used the inverse PCR method to determine the insertion site for the GS vector in the *DANC* strain; this vector can induce transcription bidirectionally from its insertion site (Fig. 2I). The PCR amplified a predicted gene, *Rpn11*, the expression level of which was elevated in a GAL4-dependent manner (Fig. 2J). Rpn11 is one lid component of the multiple subunits that make up the 19S RP. The 19S RP can be divided into two subcomplexes, known as the "base" and "lid" subcomplexes (see Fig. S2N in the supplemental material). Among the lid subunits, Rpn11 functions as a metalloprotease that cleaves the isopeptide bonds between a ubiquitinated substrate and the most proximal ubiquitin of the polyubiquitin chain (35, 41). We also examined another GS fly line, encoded by *GS13423*, in which the GS vector, inserted in the 5′ untranslated region of *Rpn11*, could induce unidirectional transcription (Fig. 2I) and drive *Rpn11* expression in a GAL4-dependent manner (Fig. 2K). *GS13423* also suppressed the progression of the *MJDtr-Q78*-induced rough eye phenotype (Fig. 2E and F). To confirm directly that *Rpn11* was responsible for the suppression of the *MJDtr-Q78*-induced phenotype in the *DANC* fly line, we generated the *UAS-Rpn11* transgenic fly lines and overexpressed *Rpn11* in the fly eye with *MJDtr-Q78*. As with the *DANC* or *GS13423* allele, the overexpression of *Rpn11* suppressed the *MJDtr-Q78*-induced progressive loss of pigmentation (Fig. 2G and H) without affecting *GAL4* expression by the overexpression of *Rpn11* or with age (see Fig. S1 in the supplemental material).

We next examined whether overexpression of *Rpn11* could suppress another model of expanded polyglutamine disease, the human Huntingtin (*Htt*) gene (16). The photoreceptor neurons in the wild-type fly eye are arranged in a series of repeating trapezoids visible as seven rhabdomeres within each ommatidium (Fig. 2L and M). When Htt peptides were expressed in the eye, although almost seven rhabdomeres were observed within each ommatidium at day 2 (Fig. 2N and R), the loss of rhabdomeres in each ommatidium was observed at day 15 (Fig. 2O and R). However, when *Rpn11* was coexpressed with *Htt*, the age-related loss of rhabdomeres was significantly reduced (Fig. 2P, Q, and R). These data indicate that the overexpression of *Rpn11* suppresses the age-related progression of neural degeneration induced by polyglutamine.

Overexpression of *Rpn11* ameliorates the toxicity of expanded polyglutamine. A number of studies have focused on the strong linkage between the pathogenesis of neurodegenerative diseases and protein aggregation (32). We next ad-

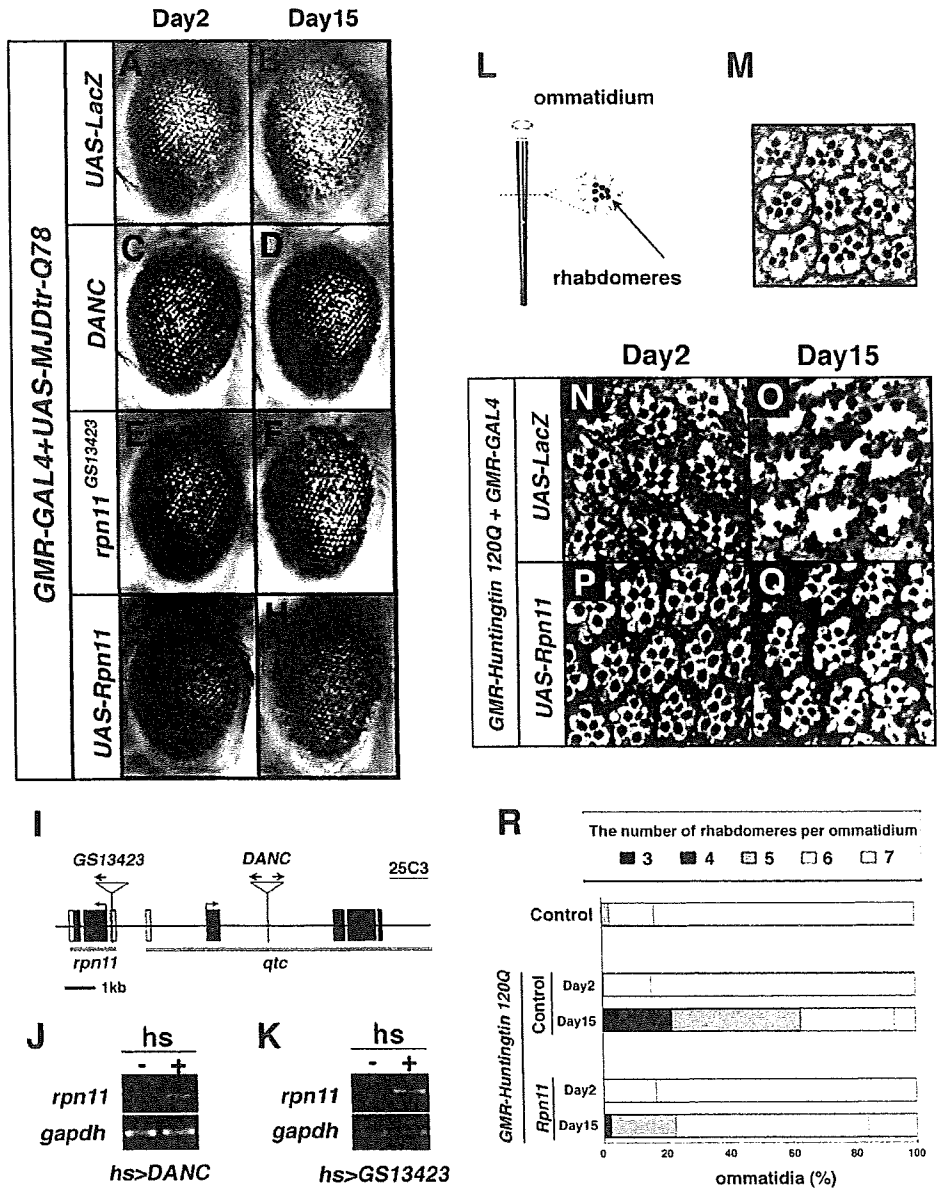

FIG. 2. Identification of *Rpn11* as a suppressor of age-related polyglutamine-induced progressive neurodegeneration. (A to H) Light photomicrographs of fly eyes expressing an expanded polyglutamine protein product of *MJDtr-Q78*, along with the indicated transgene. (A and B) Flies of genotype *w*; *GMR-GAL4/UAS-LacZ*; *UAS-MJDtr-Q78*/+ are shown at day 2 (A) and day 15 (B) posteclosion. These flies show eye degeneration, with a progressive loss of external pigment. (C to H) The progressive pigment loss phenotype was suppressed in flies expressing both *MJDtr-Q78* and *DANC* (C and D), *GS13423* (E and F), or *Rpn11* (G and H). The fly genotypes shown are *w*; *GMR-GAL4/GS13423*; *UAS-MJDtr-Q78*/+ (E and F) and *w*; *GMR-GAL4/UAS-Rpn11*; *UAS-MJDtr-Q78*/+ (G and H). (I) The genomic structures around the *DANC* and *GS13423* alleles are shown. (J and K) The *DANC* and *hs-GAL4* flies (J) and the *GS13423* and *hs-GAL4* flies (K) were heat shocked (hs+) or not (hs−), and the expression levels of the surrounding genes, as well as that of GAPDH, were analyzed by RT-PCR. Note that *rpn11* was upregulated in a GAL4-dependent manner. (L) Schematic of a single *Drosophila* ommatidium showing the regular trapezoidal arrangement of seven visible rhabdomeres within the photoreceptor neuron. (M) Representative semithin sections are shown of compound eyes from wild-type flies 2 days after eclosion. Normal ommatidia contain seven visible rhabdomeres at a given plane of the section. A representative single ommatidium is circled. (N to Q) Semithin sections of compound eyes from flies expressing *GMR-Huntingtin 120Q*, with *LacZ* as a control (N and O), or with *Rpn11* (P and Q). When *Rpn11* was coexpressed with *Htt*, the age-related loss of rhabdomeres was significantly improved (N to Q). The following genotypes are shown: *w*; *UAS-LacZ/GMR-GAL4*; *GMR-Huntingtin120Q*/+ (N and O) and *w*; *UAS-Rpn11/GMR-GAL4*; *GMR-Huntingtin120Q*/+ (P and Q). (R) Quantification of the number of rhabdomeres per ommatidium. More than 100 ommatidia per eye section were counted, and at least four eyes were sectioned for each fly line. The mean number of rhabdomeres per ommatidium ± SD for the control flies was 6.7 ± 0.5; that for the *Htt* plus control *LacZ* flies on day 15 was 5.2 ± 0.9; and that for the *Htt* plus *Rpn11* flies on day 15 was 5.9 ± 0.6. Differences between the *Htt* plus control *LacZ* flies on day 15 and the *Htt* plus *Rpn11* flies on day 15 are significant: $P < 0.0001$ (Student's *t* test).

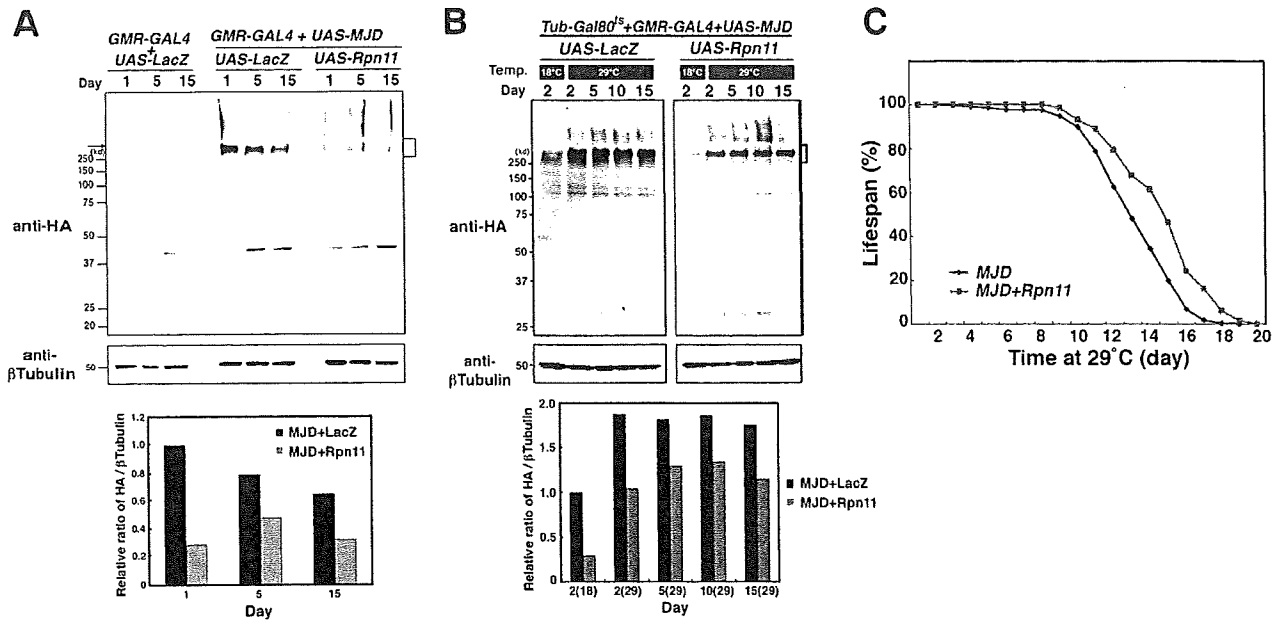

FIG. 3. Overexpression of *Rpn11* ameliorates the toxicity caused by expanded polyglutamine. (A) The coexpression of *Rpn11* with *MJDtr-Q78* (MJD+Rpn11) significantly decreased the polyglutamine-induced aggregation. The expanded polyglutamine protein in flies ran as an SDS-insoluble complex in the stacking gel and at the top of the separating gel and was detected by immunoblot analysis with an anti-HA antibody, with which *MJDtr-Q78* was tagged. Fly heads from each line of the indicated genotype were subjected to immunoblotting with an anti-HA antibody and an anti-β-tubulin antibody as a loading control. Fly genotypes were *w*; *GMR-GAL4/UAS-LacZ*, *w*; *GMR-GAL4/UAS-LacZ*; *UAS-MJDtr-Q78/+* and *w*; *GMR-GAL4/UAS-Rpn11*; *UAS-MJDtr-Q78/+*. (B) The overexpression of *Rpn11* after eclosion suppressed the aggregation induced by the eye-specific expression of the polyglutamine protein. Fly genotypes were *w*; *tub-GAL80^{ts}/UAS-LacZ*; *GMR-GAL4/UAS-MJDtr-Q78/+* and *w*; *tub-GAL80^{ts}/UAS-Rpn11*; *GMR-GAL4,UAS-MJDtr-Q78/+*. Graphs show the ratio of HA to β-tubulin (A and B). The relative amounts of each protein were determined by densitometric analysis. For the anti-HA antibody, the band indicated on the figure by a line was analyzed. (C) The coexpression of *Rpn11* and *MJDtr-Q78* in adult flies partially suppressed the short life span of *MJDtr-Q78* flies (the mean life span ± SD for *MJD* flies was 12.3 ± 0.1 days, $n = 640$; that for *MJD* plus *Rpn11* flies was 13.8 ± 0.16 days, $n = 260$; log-rank test, $P < 0.0001$). Fly genotypes were *w*; *tub-GAL80^{ts}/+*; *da-GAL4/UAS-MJDtr-Q78* and *w*; *tub-GAL80^{ts}/UAS-Rpn11*; *da-GAL4/UAS-MJDtr-Q78*.

dressed whether the suppression of polyglutamine-induced neural degeneration by *Rpn11* was accompanied by a change in polyglutamine protein aggregate formation. To do this, we performed an immunoblot analysis of HA-tagged MJDtr-Q78. The expanded polyglutamine protein ran as an SDS-insoluble complex at the top of the separating gel and in the stacking gel; however, the amount of SDS-insoluble complex was significantly decreased in flies expressing *MJDtr-Q78* with *Rpn11* (Fig. 3A).

We next determined whether *Rpn11* expression is required during development or only during adulthood to suppress the polyglutamine protein aggregation. We adapted the TARGET system to control gene expression temporally (28). In this system, the GAL4-UAS system is conditionally regulated by a temperature-sensitive allele of *GAL80*. At a restrictive temperature (18°C), the activity of GAL4 is repressed, whereas this repression is relieved by a temperature shift to the permissive temperature (29°C). When *MJDtr-Q78* was expressed after eclosion, using the TARGET system, the SDS-insoluble complex was clearly detected from day 2 to day 15. However, when *Rpn11* was coexpressed after eclosion, the SDS-insoluble complex was significantly reduced (Fig. 3B).

To address whether *Rpn11* suppressed neurodegeneration by modulating the toxicity of the aggregated polyglutamine proteins, we assessed the life span of *MJDtr-Q78* flies overexpressing *Rpn11*. The coexpression of *Rpn11* with *MJDtr-Q78* during adulthood in the whole bodies of adult flies partially suppressed the *MJDtr-Q78*-induced shortening of the life span (the mean life span ± standard deviation [SD] for flies expressing *MJDtr-Q78* alone was 12.3 ± 0.1 days, $n = 640$; that for flies expressing *MJDtr-Q78* plus *Rpn11* was 13.8 ± 0.16 days, $n = 260$; log-rank test, $P < 0.0001$) (Fig. 3C). Thus, the overexpression of *Rpn11*, even occurring only in the adult flies, reduced the polyglutamine-induced toxicity by suppressing the accumulation of expanded polyglutamine proteins.

Other lid subunits, but not subunits of the base or the 20S proteasome, may suppress the polyglutamine-induced phenotype. Next, we assessed whether the suppressive effect of *Rpn11* on the progression of the polyglutamine-induced neurodegenerative phenotype was unique among proteasome components. For this experiment, we used GS fly lines that expressed different components of the 26S proteasome in a GAL4-dependent manner (see Fig. S2K to M in the supplemental material). We found that the overexpression of several lid subunits (see Fig. S2N in the supplemental material), *rpn9* (see Fig. S2C and D in the supplemental material) and *rpn5* (see Fig. S2E and F in the supplemental material), as well as *rpn11*, suppressed the progression of the polyglutamine-induced neurodegenerative phenotype. However, neither the *rpn2* base subunit (see Fig. S2G and H in the supplemental material) nor the 20S α6

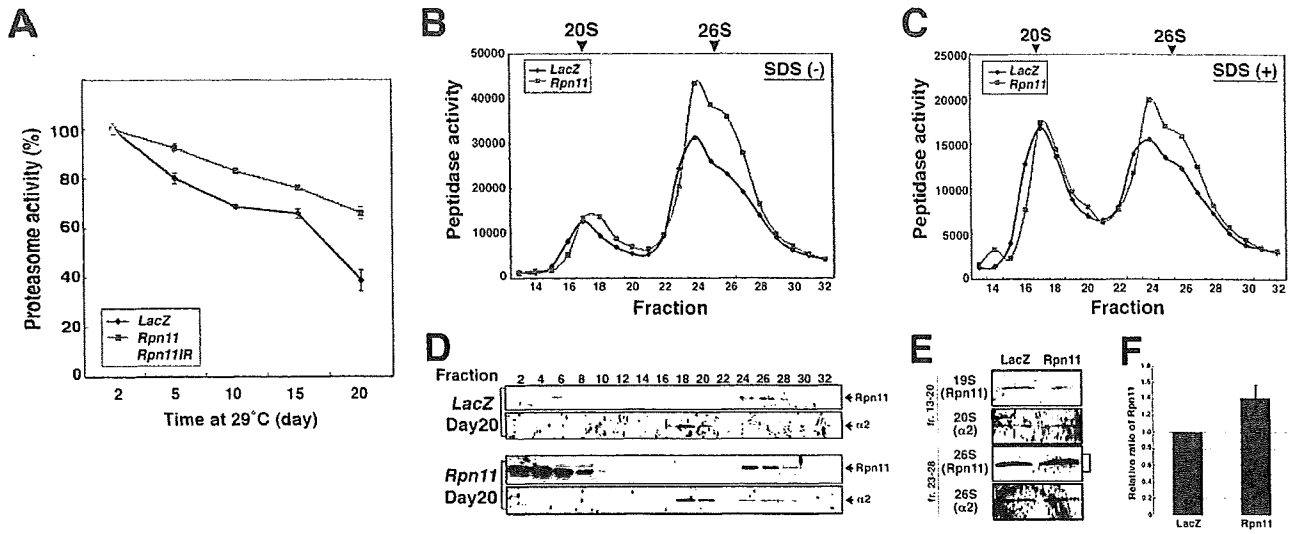

FIG. 4. Overexpression of *Rpn11* suppresses the age-related reduction of the 26S proteasome activity. (A) Proteasome activity in whole *Drosophila* with overexpression or knockdown of *rpn11* after eclosion. Fly genotypes were *w*; *tub-GAL80^{ts}/UAS-LacZ*; *da-GAL4/+*, *w*; *tub-GAL80^{ts}/UAS-Rpn11*; *da-GAL4/+*, and *w*; *tub-GAL80^{ts}/+*; *da-GAL4/UAS-Rpn11IR*. (B) The peptidase activity in the 26S proteasome fraction of 20-day-old flies expressing *Rpn11* was significantly higher than that of control flies. Extracts of flies overexpressing *LacZ* or *Rpn11* at day 20 were fractionated, and the Suc-LLVY-AMC hydrolysis activities were measured. The experiments were repeated three times. (C) The peptidase activity of the 20S proteasome in flies expressing *LacZ* was not different from that of flies expressing *Rpn11*. Suc-LLVY-AMC hydrolysis activities of the lysates used for panel B were measured with the addition of 0.01% SDS [SDS(+)]. (D) The amount of Rpn11 in the 26S proteasome fraction was significantly greater in flies overexpressing *Rpn11* at day 20 than that in control flies. Immunoblot analysis for the even fractions shown in panel B was performed with anti-Rpn11 or anti-α2 antibody. (E) Fractions (fr.) 13 to 20 or fractions 23 to 28 in panel D were pooled and immunoblotted with anti-Rpn11 or anti-α2. (F) The amount of Rpn11 in the 26S fractions was significantly greater in flies overexpressing *Rpn11*. The amount of Rpn11 protein (the amount of the band shown in panel E by a line) was determined by densitometric analysis. The graph shows the ratio of the amount of Rpn11 in fractions 23 to 28 (26S included) in flies expressing *Rpn11* at day20 to the amount of Rpn11 in fractions 23 to 28 in flies expressing *LacZ* at day 20 from three individual experiments.

subunit (see Fig. S2I and J in the supplemental material) could do so. Whereas GS system-based gene overexpression might induce expression of genes that are not closed to the GS system insertion site, these results suggest that the additional expression of at least some lid subunits suppresses the progression of expanded polyglutamine-induced degeneration, which implies that they might also have the ability to suppress the age-dependent impairment of proteasome activity.

The age-related reduction of proteasome activity is suppressed by the overexpression of *Rpn11*. To assess whether the ameliorative effect of *Rpn11* on the progression of expanded polyglutamine-induced degeneration was caused by preventing the age-related attenuation of the proteasome, we analyzed proteasome activity in flies expressing *Rpn11* with age. We used the TARGET system to express *Rpn11* only after eclosion, because *Rpn11* expression only in the adult was sufficient to reduce the polyglutamine-induced toxicity by suppressing the accumulation of expanded polyglutamine proteins (Fig. 3B and C) and because the additional expression of proteasome subunits during development might have affected development and reproduction. With the TARGET system, *Rpn11* expression was prevented when flies were raised at 18°C and was permitted when the flies were switched to 29°C at 1 day posteclosion (28). The overexpression of *Rpn11* after eclosion significantly prevented the age-related reduction of proteasome activity seen in the *LacZ* control, especially at day 20 (Fig. 4A).

To examine how the overexpression of *Rpn11* prevented the age-related reduction of proteasome activity, extracts of 20-day-old flies expressing *Rpn11* were fractionated by 8 to 32% glycerol density gradient centrifugation. When *Rpn11* was overexpressed, the peptidase activity in the 26S proteasome-containing fraction of 20-day-old flies was higher than that in the control flies (Fig. 4B). However, the peptidase activity of the 20S proteasome did not significantly change (Fig. 4C). The immunodetection of Rpn11 or α2 revealed that the amount of Rpn11 in the 26S proteasome fractions was significantly greater in flies overexpressing *Rpn11* (Fig. 4D, E, and F), even though the protein levels of the 19S RP and the 20S proteasome showed almost no changes (Fig. 4E). These results suggest that the ectopic expression of *Rpn11* after eclosion helped to maintain the activity and amount of the 26S proteasome with age.

Overexpression of *Rpn11* suppresses the age-related accumulation of ubiquitinated proteins and extends the life span. Next, to examine whether the maintenance of the 26S proteasome by the ectopic expression of *Rpn11* could also suppress the age-dependent accumulation of misfolded proteins, as it did with the accumulation of polyglutamine proteins (Fig. 3A and B), fly extracts were immunoblotted with the antiubiquitin antibody. Although the amount of polyubiquitinated proteins gradually increased with age in the control flies, this age-dependent accumulation was significantly suppressed when *Rpn11* was overexpressed after eclosion (Fig. 5A). If the age-dependent accumulation of polyubiquitinated proteins is associated with proteotoxicity in vivo, it is possible that the age-

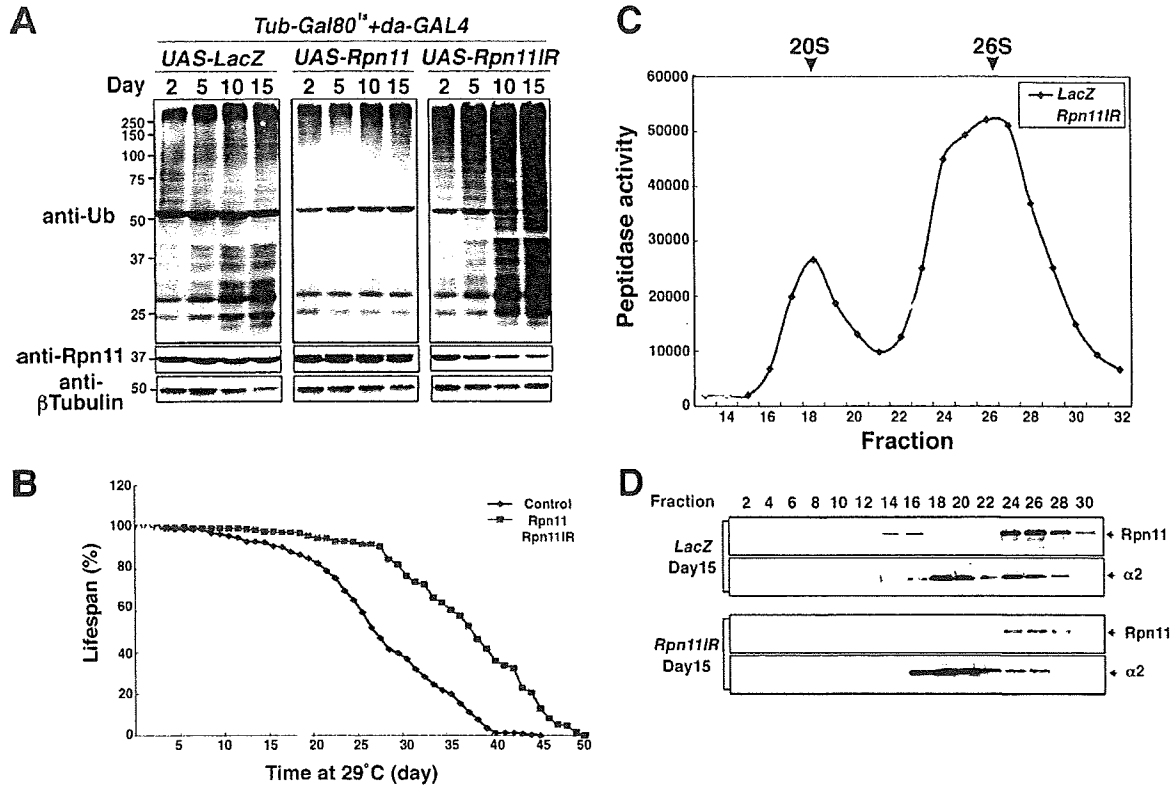

FIG. 5. *Rpn11* is required to suppress the age-dependent accumulation of ubiquitinated proteins and to extend the life span. (A) The accumulation of ubiquitinated proteins with age in the adult body was suppressed by the overexpression of *Rpn11* after eclosion and enhanced by the knockdown of *rpn11*. Whole flies from each line of the indicated genotype at the indicated day posteclosion were subjected to immunoblotting with antiubiquitin (anti-Ub), anti-Rpn11, and anti-β-tubulin antibodies. Fly genotypes were *w*; *tub-GAL80^{ts}/UAS-LacZ*; *da-GAL4/+*, *w*; *tub-GAL80^{ts}/UAS-Rpn11*; *da-GAL4/+*, and *w*; *tub-GAL80^{ts}/+*; *da-GAL4/UAS-Rpn11IR*. Numbers at left are molecular masses (in kDa). (B) The overexpression of *Rpn11* significantly extended the life span compared with that of control flies (the mean life span ± SD of control flies was 27.2 ± 0.56 days, n = 220; and for *Rpn11*-overexpressing animals was 36.8 ± 0.53 days, n = 240; log-rank test, $P < 0.0001$). On the other hand, the ubiquitous expression of *Rpn11IR* in adult flies reduced their life span (the mean life span ± SD for control flies was 27.2 ± 0.56 days, n = 220; and for the *rpn11* knockdown line was 11.1 ± 0.18, n = 320; log-rank test, $P < 0.0001$). Fly genotypes were *w*; *tub-GAL80^{ts}/UAS-GFP*; *da-GAL4/+*, *w*; *tub-GAL80^{ts}/UAS-Rpn11*; *da-GAL4/+*, and *w*; *tub-GAL80^{ts}/+*; *da-GAL4/UAS-Rpn11IR*. (C) Knockdown of *rpn11* led to a decrease in the 26S proteasome activity. Extracts of flies expressing *LacZ* or *Rpn11IR* ubiquitously for 15 days after eclosion were fractionated by 8 to 32% glycerol gradient centrifugation. Genotypes shown are *w*; *tub-GAL80^{ts}/UAS-LacZ*; *da-GAL4/+* and *w*; *tub-GAL80^{ts}/+*; *da-GAL4/UAS-Rpn11IR*. (D) Immunodetection of Rpn11 or α2 revealed that the knockdown of *rpn11* inhibited the assembly of the 26S proteasome. The levels of Rpn11 and α2 in the fractions containing the 26S proteasome (fractions 24 to 30) were significantly decreased. Immunoblot analysis was performed for each fraction, using antibodies against Rpn11 or α2.

dependent accumulation of polyubiquitinated proteins affects the life span. Therefore, we compared the life span of control flies with that of flies expressing *Rpn11* after eclosion. The overexpression of *Rpn11* during adulthood significantly extended the mean life span compared with that of control flies (mean life span ± SD for control flies was 27.2 ± 0.56 days, n = 220; life span for *Rpn11*-overexpressing animals was 36.8 ± 0.53 days, n = 240; log-rank test, $P < 0.0001$) (Fig. 5B). Thus, the overexpression of *Rpn11* suppresses the age-dependent accumulation of ubiquitinated proteins and extends the mean life span in flies. These results imply that the maintenance of the 26S proteasome with age prevents the age-related aggregation-mediated toxicity.

Knocking down of *rpn11* enhances the age-related accumulation of ubiquitinated proteins and shortens the life span, accompanied by reduced 26S proteasome. To examine whether impairment of the 26S proteasome activity enhanced the age-related aggregation-mediated toxicity, we assessed the accumulation of ubiquitinated proteins and the life span in the *rpn11* knockdown flies. We generated transgenic flies bearing an inverted repeat (IR) fragment of the *Rpn11* cDNA in the *pUAST* vector (*Rpn11IR*) that would specifically inhibit *rpn11* expression in a GAL4-dependent manner via a mechanism of RNA interference. When *rpn11* was knocked down in whole-body extracts after eclosion by an *Rpn11IR*, the age-dependent reduction of proteasome activity was enhanced (Fig. 4A), along with the attenuation of the activity and amount of the 26S proteasome (Fig. 5C and D), suggesting that the assembly of the 26S proteasome was impaired. In the *rpn11* knockdown flies, the accumulation of ubiquitinated proteins was clearly enhanced with age (Fig. 5A). In addition, the mean life span in the *rpn11* knockdown flies was severely impaired (mean life span ± SD for control flies was 27.2 ± 0.56 days, n = 220; and for the *rpn11*

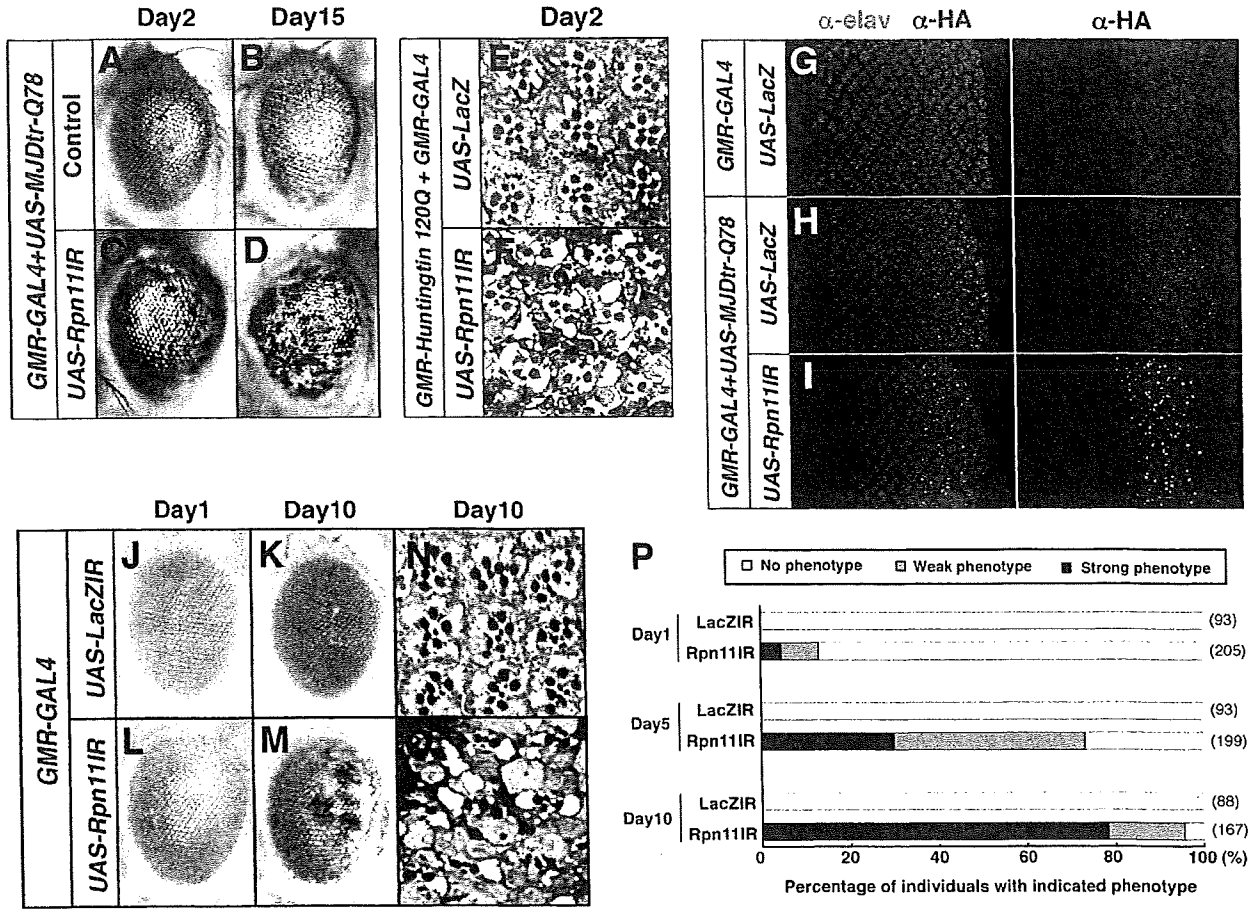

FIG. 6. Knocking down *rpn11* enhances the toxicity of expanded polyglutamine and causes the age-related onset of a neurodegenerative phenotype. (A to F) Knocking down *rpn11* clearly enhances the phenotype of polyglutamine-induced neurodegeneration. Light microscopy (A to D) and semithin-section (E and F) images of the compound eyes are shown. The following genotypes are shown: *w*; *GMR-GAL4/UAS-LacZ*; *UAS-MJDtr-Q78*/+ (A, B) and *w*; *GMR-GAL4*/+; *UAS-MJDtr-Q78/UAS-Rpn11IR* (C and D), *w*; *UAS-LacZ/GMR-GAL4*; *GMR-Huntingtin120Q*/+ (E), and *w*; *GMR-GAL4*/+; *GMR-Huntingtin120Q/UAS-Rpn11IR* (F). (G to I) Polyglutamine aggregation was significantly enhanced when *rpn11* was knocked down. Eye imaginal discs of wandering third-instar larvae were immunostained with anti-ELAV (green) and anti-HA (magenta), which *MJDtr-Q78* was tagged with. ELAV is expressed in all photoreceptor neurons. The following genotypes are shown: *w*; *GMR-GAL4/UAS-LacZ* (G), *w*; *GMR-GAL4/UAS-LacZ*; *UAS-MJDtr-Q78*/+ (H), and *w*; *GMR-GAL4*/+; *UAS-MJDtr-Q78/UAS-Rpn11IR* (I). (J to O) Knocking down *rpn11* caused the severe neural degeneration. Light microscopy (J to M) and semithin-section (N and O) images of fly eyes expressing *Rpn11IR* (L, M, and O) or control protein (J, K, and N) are shown. The following genotypes are shown: *w*; *GMR-GAL4/UAS-LacZIR* at day 1 (J) and day 10 (K and N), respectively, and *w*; *GMR-GAL4/UAS-Rpn11IR* at day 1 (L) and day 10 (M and O), respectively. (P) The *Rpn11IR*-induced neural degeneration is progressive. Quantification of the appearance of black dots on the surface of the eye with age is shown. A weak or strong phenotype is categorized as the appearance of one black dot or the appearance of more than two black dots, respectively. Numbers of individuals counted are shown at the right. Progressive phenotypes are seen in flies in which *rpn11* was knocked down.

knockdown fly line, life span was 11.1 ± 0.18 days, $n = 320$; log-rank test, $P < 0.0001$) (Fig. 5B).

Knocking down of *rpn11* enhances the toxicity of expanded polyglutamine and causes the age-related onset of a neurodegenerative phenotype. Next, we examined whether the impairment of the 26S proteasome activity enhances the toxicity of expanded polyglutamine. When *rpn11* was knocked down in the *MJDtr-Q78*-expressing fly eye, the *MJDtr-Q78*-induced progressive loss of pigmentation was clearly enhanced (Fig. 6A to D). In addition, when *Rpn11IR* was coexpressed with *Htt*, the loss of rhabdomeres was severe even at day 2 (Fig. 6E and F).

To determine whether knocking down of *rpn11* enhanced polyglutamine aggregation, we examined polyglutamine aggregation formation in developing eye discs of larvae expressing polyglutamine. Polyglutamine aggregation was observed in eye discs of larvae expressing expanded polyglutamine protein, which was detected with antibody to the HA tag (Fig. 6H). When *rpn11* was knocked down, polyglutamine aggregation was significantly enhanced (Fig. 6I).

Next, we examined whether the impairment of the 26S proteasome activity leads to a neurodegenerative phenotype. While the eye-specific knockdown of *rpn11* resulted in an almost normal phenotype on day 1 after eclosion (Fig. 6L and P), by day 10 after eclosion, black dots progressively appeared on the surface of the eye with age (Fig. 6M and P). In addition,

retinal sections showed that knocking down *rpn11* caused the severe loss of rhabdomeres (Fig. 6N and O). These data indicate that impairment of the 26S proteasome leads to retinal degeneration in otherwise wild-type individuals and to early onset of polyglutamine-induced defects.

DISCUSSION

Although aggregation-mediated toxicity is involved in both normal aging and neurodegenerative disease, the reason that aggregation-mediated toxicity emerges late in life has not been elucidated. Our results demonstrate for the first time that an age-related reduction of the 26S proteasome activity affects longevity and could underlie the induction of certain effects of aging and the age-dependent increased risk of the onset and/or progression of neurodegeneration in vivo.

In this study, we demonstrated that the age-related reduction of proteasome activity results from the attenuation of both the activity and amount of the 26S proteasome, which is associated with impaired assembly of the 26S proteasome from the 19S RP and the 20S proteasome in vivo. Age-related proteasome dysfunction has been reported in studies on humans, other mammals, and flies (3, 19). Thus, the age-related reduction of proteasome activity is evolutionarily conserved. Importantly, our data indicated that the reduction of the 26S proteasome activity with age might result from a defect in its assembly. This indication is supported by our data that the lysates from the young flies showed two peaks of peptidase activity (Fig. 1C, fractions 24 and 26) among the 26S proteasome fractions, which represented the 26S proteasome with one or two RP caps, but aged flies showed only one peak (Fig. 1C, fraction 24), which indicates that the 26S proteasome with one cap was the predominant form (Fig. 1C).

The free 20S proteasome is almost inactive and cannot degrade multiubiquitinated proteins because the pores leading into the catalytic chamber are closed. The opening of these gates is triggered by the 19S RP attached to one end of the 20S proteasome (6). The assembly of the 26S proteasome is known to be ATP dependent, and a recent report indicates that ATP binding is sufficient to promote the assembly of the 26S proteasome from the 19S RP and the 20S proteasome (25). That report's data indicate that no additional assembly factors are required in vitro (25). Therefore, we at first suspected that an age-associated decline in ATP levels caused the disassembly of the 26S proteasome. However, no decline in ATP levels could be detected, even in flies that were 30 days posteclosion (data not shown), despite the obvious decline of proteasome activity (Fig. 1A). Thus, specific regulators besides ATP may affect the assembly of the 26S proteasome in vivo.

Previous reports have identified extraproteasomal proteins that promote the assembly of the 26S proteasome. For example, Hsp90, an ATP-regulated chaperone, promotes the assembly and maintains the stability of the 26S proteasome in *Saccharomyces cerevisiae* (14). Ecm29 has also been identified as a stoichiometric binding partner for the yeast 26S proteasome and is proposed to enhance its stability by binding to both the 20S proteasome and the 19S RP (24). It is possible that these specific regulators of the assembly or stability of the 26S proteasome are affected by age and cause the age-related decline of the 26S proteasome activity.

Our results demonstrate that an age-related reduction of the 26S proteasome activity could be key to the age-related accumulation of misfolded or unfolded proteins and the duration of the life span. This idea that the homeostasis of general protein folding is important during aging is supported by recent findings for the role of chaperones in promoting longevity in *C. elegans* and other organisms. For example, overexpression of either the HSP70 or HSP16 chaperone, which resists protein misfolding, increases the life span (38, 42), whereas reduced chaperone expression by *heat shock factor-1* (*hsf-1*) knockdown shortens the life span (13). Although the direct effects of the proteasome function on life span have been less well explored, a recent report suggests that AIRAP (arsenic-inducible proteasomal 19S regulatory particle-associated protein), which associates tightly with the 19S proteasome, is involved in regulating the life span. The report showed that *C. elegans* lacking *aip-1*, a homologue of mammalian *AIRAP*, exhibit a shortened life span (43). Taken together with our findings, these results support our idea that maintenance of the 26S proteasome activity with age should promote longevity, implying that the age-related decline of the 26S proteasome activity is an important element in determining longevity.

Although genetic variants, behaviors, and environmental factors have been associated with increased risk of disease (27), our genetic evidence indicates that the age-related decline of the 26S proteasome activity could be a key risk factor for the progression or late onset of neurodegenerative diseases, because the overexpression of *Rpn11* suppressed the progression of polyglutamine-induced neurodegeneration with the maintenance of the 26S proteasome activity during aging. It is possible that Rpn11 functions as a metalloprotease and suppresses the polyglutamine-induced neurodegeneration by upregulation of the deubiquitinating activity. However, because it is reported that Rpn11 could not work by itself (35, 41), it is unlikely that Rpn11 functions as a metalloprotease. In fact, we measured the deubiquitinating activity in flies overexpressing *Rpn11*, but it did not change significantly compared with that of the control flies (data not shown).

Although further study is required to determine the precise details of how *Rpn11* suppresses the age-related impairment of proteasome activity, our data indicate that this mechanism could involve the promotion of 26S proteasome assembly. We revealed that GS system-based overexpression of some lid subunits, such as *Rpn11*, *-9*, and *-5*, suppressed the progression of polyglutamine-induced neurodegenerative phenotypes (see Fig. S2 in the supplemental material). Some reports indicate the existence of a core interaction cluster composed of the lid components Rpn11, -9, -5, and -8 (7, 15). It is possible that the expression of any given lid subunit could promote assembly of the lid complex, which could conceivably drive the assembly of the 26S proteasome by increasing the pool of 19S RPs. Although it is conceivable that the overexpression of *Rpn11* could lead to the upregulation of other proteasome components, our quantitative PCR data showed that overexpression of *Rpn11* did not affect the expression of other proteasome components or of the autophagy-related gene *atg5* (see Fig. S3 in the sup-

plemental material). These lines of evidence suggest that Rpn11 could promote the assembly of the 26S proteasome from free subunits without the upregulation of other lid subunits.

We have examined age-related attenuation of the 26S proteasome as one component in a process of protein degradation. Because the network of protein homeostasis is a complex process, it is possible that chaperone and autophagy could also gradually decrease and play an important role in the late onset or progression of neurodegenerative diseases (29, 30). Given the importance of maintaining the 26S proteasome activity, the mechanism for the late onset or progression of neurodegenerative diseases may be revealed by future studies of specific regulators of the 26S proteasome, for example, the 19S lid subunits such as Rpn11, some unidentified lid assembler, or a specific assembler for the 26S proteasome. This knowledge might be translated into the development of valuable therapeutic tools for the treatment of progressive age-related neurodegenerative diseases, including polyglutamine-induced disease.

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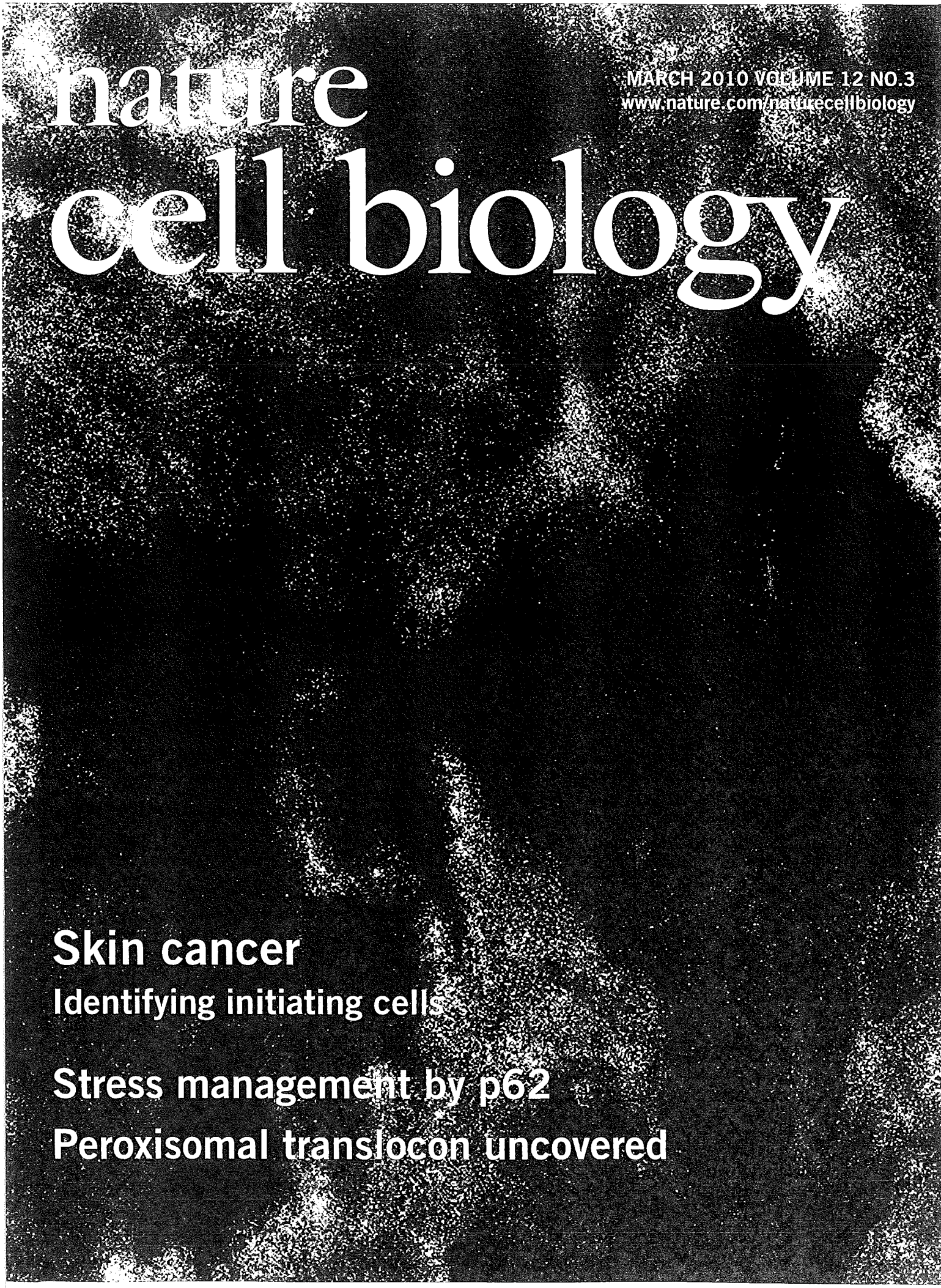

Skin cancer

Identifying initiating cells

Stress management by p62

Peroxisomal translocon uncovered

neuron injuries, and neuropathogenic pathways cause rapid formation and accumulation of autophagosomes in neurons. As a result, the intracytoplasmic area occupied by autophagic activity is sharply increased and the capacity of autophagic degradation could be maximally expanded. During the process of autophagy induction, neurons may undergo a significant change in autophagy regulation, involving a deregulation process that allows neurons to transition from basal level (neuron-specific process) to the activated state (well-conserved and known as induced autophagy) involving large-scale biosynthesis of autophagosomes. Here we summarize some examples of induced autophagy in neurons.

3.2.1. Hypoxic-ischemia

A combined hypoxic-ischemia (H/I) procedure in rodents has been widely used as a model for studying human ischemia or stroke. H/I causes extensive CNS neuron damage or injury and consequent neuronal death. Two recent studies of mouse models, one in neonatal brains [8] and the other in adult brains [65], showed that H/I induced robust formation of autophagosomes within a very short period of time (hours), concomitant with increased production of LC3-II levels. This induced autophagy occurred in different types of neurons in the hippocampus and striatum, suggesting that it is not cell type-specific. The rapid appearance of autophagic hallmarks in these two *in vivo* models demonstrated that H/I is a powerful stimulus for autophagy induction in CNS neurons. At the cellular level, H/I produces pro-inflammatory cytokines and simultaneous activation of both pro-survival (e.g., upregulation of Hsp70, phosphorylation of ERK and AKT) and pro-apoptotic signaling pathways (e.g., release of cytochrome *c* and AIF from mitochondria, cleavage of caspase-9 and -8) [65]. Therefore, at present, it is unknown which signals of autophagy induction are triggered by the H/I procedure. Interestingly, Koike et al. further demonstrates that mice deficient in essential autophagy gene, *Atg7*, show nearly complete protection from H/I-induced neuron death, arguing strongly for the involvement of autophagy in a pro-death pathway in this specific setting [8]. Although the significant role of autophagy in triggering cell death pathways has yet to be clarified, this study suggests that H/I-induced autophagy, rather than an epiphenomenon of neuronal death, is an active process that controls cell fate. More importantly, inhibition of autophagy represents an attractive strategy for drug design in alleviating neuronal damage associated with H/I injury.

3.2.2. Excitotoxic stimuli

Many excitotoxic stimuli provoke neurodegeneration through poorly defined cell mechanisms. The Clarke group has previously used NMDA (glutamate mimetic)-treated organotypic hippocampal slices to investigate this mechanism [66]. They found that, within 2 h following the drug administration, numerous autophagosomes appeared in CA1 and CA3 pyramidal neurons, concomitant with signs of neurodegeneration. Moreover, this induction of autophagosome formation was inhibited by treatment of a JNK-signal blocking peptide, which also prevented neuronal death [66]. In another excitotoxicity-related model with focal injection of kainic acid, hippocampal extracts contain a significant increase in LC3-II levels as early as 4 h following the injection [67]. Consistent with autophagic activity elicited in hippocampus, local administration of kainic acid caused a rapid increase in autophagosomes and autolysosome formation in the cytoplasm of striatal cells. In addition, it also augmented the ratio of LC3-II/LC3-I, LAMP2, cathepsin B, release of cytochrome *c*, and activation of caspase-3, suggesting stimulation of both apoptosis and autophagy [68]. Pre-treatment of autophagy inhibitor 3-methyladenine (3-MA) reversed the change in LC3-II levels, autophagosome formation, and loss of striatal cells [68]. In *Lurcher* mice, a genetic mouse model of excitotoxicity associated with Purkinje cell-specific degeneration, constitutive activation of glutamate δ2 receptor results in autophagy induction, preceding Purkinje cell degeneration. Moreover, the mutant glutamate δ2 receptor-mediated cell toxicity in transfected cells was blocked with autophagy inhibitor 3-MA [69].

Current knowledge indicates that excitotoxicity occurs when excitatory glutamate receptors, such as those for NMDA or kainic acid, are overexcited by high dosage of these ligands. The activated receptors allow the receptor channels to open, resulting in Ca^{2+} influx and consequent activation of a number of enzymes, including phospholipases, endonucleases, and proteases, such as calpain. Recent studies show that Ca^{2+} triggers strong stimulation of autophagic activity [70]. Therefore, it is possible that excitotoxic-related signals induce elevated levels of autophagy by a common Ca^{2+}-mediated pathway. Furthermore, the above studies suggest that manipulation of autophagy, by blocking its activation, may be beneficial in the treatment of excitotoxic-associated neuronal death.

3.2.3. Methamphetamine and MPP^+

Administration of methamphetamine (METH) in animals generally produces selective degeneration of dopamine (DA) neuron terminals without significant cell body loss [71]. This model is currently used to study specific aspects of neuron terminal dystrophy and degeneration in PD. To investigate the mechanism underlying the METH-mediated toxicity, the Sulzer group found that METH treatment promoted the rapid formation of autophagosomes, particularly in neuronal varicosities and, ultimately, within cell bodies of DA neurons from midbrain neuronal cultures [72]. A recent study in PC12 cells showed a similar response to METH administration, and provided additional evidence that suppression of autophagy, by blocking PI-3K class III activity, precipitates neuronal death. This study implicates a beneficial role of METH-induced autophagy in neural protection, perhaps through repairing or remodeling injured neurons [73].

A related PD cell model is the treatment of the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+), which produces mitochondria-targeted injury and contributes to parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine in mammals. Administration of MPP^+ in primary DA neuronal culture elicited an increased profile of intracellular autophagosomes, which was suppressed by extracellular signal-regulated protein kinase (ERK) kinase, a MEK inhibitor [74]. However, it is unclear why 3-MA, an inhibitor for autophagy, did not impede autophagosome formation induced by MPP^+ in this setting. Interestingly, RNAi knock-down of autophagy gene LC3 or block of MEK with a specific inhibitor in MPP^+-treated human neuroblastoma SH-SY5Y cells reduced formation of autophagosomes and cell death. One plausible explanation for MPP^+-induced autophagy is through injured mitochondria, which elicited signals involving MEK for increased autophagic activity. Although the result shows that reduced autophagy is correlated with delay of cell death, whether or not autophagy acts as an executor of cell death is unclear [74].

3.2.4. Proteasome inhibition: a compensatory induction

Emerging evidence implicates inhibition of the ubiquitin–proteasome system (UPS) in neurodegenerative diseases, such as AD and PD. Autophagy-lysosomal degradation and UPS are the two major proteolytic pathways, but they differ in various aspects including their substrates, capacity, and molecular and cellular machinery. Previous study of the effects of chronic low-level proteasome inhibition on neural homeostasis indicated that inhibition of UPS causes increased autophagic activity in clonal SH-SY5Y cells [75]. The view that increased autophagic activity is a compensatory response of impaired UPS system is supported by a few other studies. Pandey et al. used the fly genetic system to provide *in vivo* evidence that significantly strengthened and expanded this view. They showed that histone deacetylase 6 (HDAC6), a microtubule-associated deacetylase that interacts with polyubiquitinated proteins, plays a critical role in this compensated induction of autophagy for impaired UPS function. Moreover, they found that forced expression of HDAC6 is able

to rescue degeneration associated with UPS dysfunction in an autophagy-dependent fashion [76]. This study raises an important question regarding the cross-talk between the two main cellular degradation routes – autophagy and UPS – and whether or not HDAC6 is positioned at the intersection of these two pathways. Interestingly, a more recent report shows that genetic deletion of 26S proteasomes in mouse brain causes neurodegeneration and Lewy-like inclusions in midbrain DA neurons, which is accompanied by increased expression of autophagy genes and formation of numerous vacuoles resembling autophagosomes [77]. Although the mechanism of interaction between the two pathways is unclear, the compensatory up-regulation of autophagy for degradation of protein aggregates, which normally exceed the capacity of UPS degradation, should be explored as drug target for the treatment of neurodegenerative diseases.

3.2.5. Lysosomal enzyme/lipid storage or suppression: autophagy "vicious circle"

A number of studies exploring autophagy in lysosome-related neurological diseases provide a new understanding of the pathogenic mechanisms of "old" diseases. A subset of these diseases, also known as lysosomal storage disease, is caused by deficiencies of lysosomal hydrolases, resulting in accumulation of undigested materials in the lysosomal or related compartment. These disorders include Niemann–Pick type C (NPC) [78,79], mucolipidosis type IV with mutations in Mucolipin 1 (MCOLN1), multiple sulfatase deficiency and mucopolysaccharidosis type IIIA [80]. A common cellular pathologic feature in these diseases and their animal models is the manifestation of aberrant autophagic activity as evidenced by the accumulation of autophagosomes and increased levels of LC3-II, accompanied by neurodegeneration. Direct deletion of the genes encoding lysosomal enzymes such as cathepsin D (CD−/−) or combination of cathepsin B and L (CB−/− and CL−/−) in mouse brain, resulted in a similar phenotype [81]. The current view is that lipid trafficking molecules or lysosomal enzymes are required for the delivery, fusion or clearance of autophagosomes; deficiencies in these molecules causes disruption of this process, resulting in build-up of autophagosomes/autolysosomes unable to complete the digestion. This view supports a hypothesis that impaired autophagic activity is part of the disease mechanism for lysosomal storage diseases due to the inability of neurons to clear autophagosomes. However, these studies also provide evidence that otherwise amends this hypothesis. Pacheco et al. showed that, in addition to impaired autophagy flux, there is also induction of autophagy in NPC by signaling through a complex of the class III phosphoinositide 3-kinase/beclin-1 [79]. A significant number of nascent autophagosomes (immature or before fusion with lysosomes) are found in CD−/− or CB−/− and CL−/− brains, suggesting that they are newly synthesized autophagosomes [81]. In a study of fibroblasts obtained from patients carrying mucolipidosis type IV mutation, Vergarajauregui et al. suggest that the autophagosome accumulation is due to increased *de novo* autophagosome formation and delayed fusion of autophagosomes with late endosomes/lysosomes [80]. Therefore, the available evidence indicates that there is induced synthesis of autophagosomes in addition to the compromised autophagosome fusion or clearance in lysosomal storage diseases.

The deficiency in lysosomal degradation is expected to affect the net outcome of all forms of autophagy, including macroautophagy, microautophagy and chaperon-mediated autophagy. In line with the concept that blocking one type of degradation (e.g., proteasome or CMA) stimulates compensatory pathways (e.g. autophagy), intracellular accumulation of non-degraded materials resulting from lysosomal enzyme deficiency is expected to feed back to the upstream signaling which triggers the biosynthesis of autophagosomes. Since autophagosome degradation through lysosomes is impaired, it creates a "dilemma" for neurons to dispose autophagosomes, amassing a large number of autophagosomes of various stages. Thus, we propose a hypothesis that explains the sequence of the events in the disease, considering lack of detectable autophagosomes in healthy neuron. The accumulation of undigested materials due to lysosomal deficiency triggers *de novo* autophagosome biosynthesis in lysosomal storage disease. Impaired lysosomal degradation will trap these autophagosomes, causing build-up of the induced autophagosomes, and more undigested materials. This can continue in circles, and as a result of this rampant autophagy "vicious circle", neurons may exhaust their energy and generate various toxic species that are harmful.

3.3. ESCRT: autophagy meets endocytosis

Previous studies from the Seglen group showed that autophagosomes can fuse with endosomes and form a hybrid product, termed amphisomes, providing early evidence of interaction between autophagy and endocytosis [82]. Although this observation suggests the convergence of autophagosomes and endosomes, perhaps under specific settings, the significance of this process has not been fully appreciated. Recently, several studies showed that mutations in CHMP2B, a subunit of the endosome sorting complex required for transport (ESCRT)-III, are linked to frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) and prevents autophagic degradation [83]. Along with these studies, genetic and ultrastructural analysis in *Drosophila melanogaster* showed that subunits of the ESCRT- I, -II and -III, as well as their regulatory ATPase Vps4 and the endosomal PtdIns(3)P 5-kinase Fab1, are all required for normal autophagy function [84,85]. These studies are important in several aspects. First, they reveal a novel mechanism involving dysfunctional autophagy in the pathogenesis of specific types of FTD and ALS. Second, since the ESCRT proteins are known to function in the sorting of transmembrane proteins into the inner vesicles of the multivesicular body (MVB) during endocytosis [86], these studies suggest that MVB or endocytic pathways are critical routes for the trafficking of autophagosomes to lysosomes, and defective ESCRT function prevents fusion or maturation of autophagosomes. Third, they suggest that neurons are particularly vulnerable to abnormal function in the autophagosome-MVB (endosomes) pathway, and this may be associated with dendritic maintenance [83].

Several outstanding questions, however, arise from these studies. The ESCRT machinery is evolutionarily conserved [86], and its function in MVB biogenesis has been shown in many species including yeast and human. Curiously, although ESCRT/MVB, as well as autophagy, are highly conserved and well-characterized in yeast, the interaction between ESCRT/MVB and autophagy has not been demonstrated. Therefore, it raises a question whether the ESCRT-MVB pathway is only important for autophagy in higher eukaryotes, including fly and mammals. Furthermore, amphisomes are not detected in yeast, and autophagosomes fuse directly with the vacuole, which is mediated by SNARE proteins [87]. A related question is whether ESCRT-MVB represents the primary, if not the only, route for delivery of autophagosomes to lysosomal degradation, and whether this route occurs only in specific cell/tissue context. Previous studies also suggest that a considerable proportion of endocytosed cargo merges with autophagic pathway prior to being degraded by lysosomes in neurons [20].

It is also conceivable that the ESCRT-MVB pathway is actually part of the yet-to-be-defined basal autophagy process in neurons. Formation of ubiquitin- or p62/SQSTM1-labeled inclusions and autophagosomes accumulation are two important features associated with cells or neurons deficient in ESCRT components. Interestingly, time-course studies show that the formation of ubiquitinated inclusions occurs prior to the appearance of autophagosomes in cortical neuronal culture expressing mSnf7-2 siRNA or CHMP-2B^{intron5}. The early formation of ubiquitinated protein inclusions in these neurons defective in ESCRT function phenocopies results from *Atg5* or *Atg7* deficient neurons [6,7], suggesting that the ESCRT-MVB pathway is likely related to the *Atg5* or *Atg7*-regulated function, which is required

for the maintenance of protein homeostasis or basal autophagy function. Since impaired ESCRT pathway does not affect biosynthesis of autophagosomes (unlike *Atg5* or *Atg7*), autophagosome biosynthesis is induced as a response to the increasing levels of ubiquitinated proteins. Thus, an alternative hypothesis is that formation of ubiquitin- or p62-labeled protein inclusions is the early response of neurons to the deficiency in basal autophagy caused by dysfunctional ESCRT, whereas the accumulation of autophagosomes is the secondary response to the increased levels of undigested protein and/or a result of impaired ESCRT function, which may be related to autophagosome fusion or maturation. The relationship of ESCRT with basal autophagy should be explored in the future.

4. Specialized neuronal autophagy in the axons

4.1. Role of neuronal autophagy in axonal homeostasis

The axon is a highly specialized neuronal compartment that performs many functions independently from the soma. Accumulating evidence has revealed that, not only is autophagy uniquely regulated in the neuron, but it may also have a distinct function and regulation within the axonal compartment, independent from the dendrites and soma. Early morphological evidence showed that, following axotomy [88] or excitotoxic injury [4], double-membrane vacuoles resembling autophagosomes started accumulating and localized in dilated axon terminals. In later studies, Hollenbeck described the sequestering of autophagic substrates in autophagosomes at the most distal region of the axon, which were retrogradely transported to the soma [89]. Mammalian autophagosomes form in various regions throughout the cytoplasm. Upon maturation, they move toward lysosomes, which are primarily located in the juxtanuclear cytoplasm of the cell body [90,91]. More recently, autophagosome formation in response to an excitotoxic insult was monitored in *Lurcher* mice expressing GFP-LC3. The constitutive activation of the GluRδ2 receptor resulted in an excitotoxic injury that triggered a rapid and robust accumulation of GFP-LC3-labeled autophagosomes in axonal dystrophic swellings [54]. The link between increased autophagy activity and axonal dystrophy would suggest local biosynthesis of autophagosomes at the distal axon terminals.

To elucidate the physiological role of neuronal autophagy, mutant mice containing a neural cell type-specific deletion of *Atg7* were generated. Establishment of these mutant mice allowed the study of cell-autonomous events in cerebellar Purkinje cells deficient in autophagy. Characterization of the mutant Purkinje cells revealed the accumulation of aberrant organelles and membrane structures in dystrophic axon terminals. This result suggests a specific role for neuronal autophagy in the maintenance of membrane homeostasis at the axon terminal. We hypothesize that this highly specialized neuronal autophagy is required for keeping the balance of the membrane network, which normally involves cycling of membranous structures or vesicles, at the axon terminals to support synaptic activity. This particular function of autophagy is no surprise considering that the typical autophagic process involves dynamic membrane rearrangement and turnover. Future study should investigate in detail how this "self-eating" process participates in axonal membrane turnover and what membrane substrates axonal autophagy removes under physiological conditions. The answers to these questions are expected to advance our current understanding of autophagy in the neuron, as well as the disease processes that are associated with dysfunctional autophagy in the neuron and the axon.

4.2. MAP1B–LC3 interaction regulates autophagosome formation and possibly axonal transport

LC3 is best known as an autophagosome-associated protein. Despite the abundance of LC3 in neurons, the vast majority of LC3 is in soluble form and is not associated with autophagosomes. To understand neuron-specific autophagosome formation, Wang et al. identified and analyzed the interaction between LC3 and microtubule-associated protein 1B (MAP1B), which is an abundant protein in the axons and plays an important role in regulating microtubule stability. They found that MAP1B binds to LC3 with high affinity, and overexpression of MAP1B in non-neuronal cell culture significantly reduces the number of LC3-associated autophagosomes, presumably through the MAP1B–LC3 interaction [54]. Since MAP1B (or MAP1A) is highly expressed in neurons, this result may potentially explain the scarcity of the autophagosomes in healthy neurons and the lack of GFP-LC3 puncta in GFP-LC3 transgenic brain. In contrast to MAP1B, phosphorylated MAP1B (MAP1B-P) was found to be associated with LC3-autophagosomes. It was further shown that LC3 was associated with increased levels of MAP1B-P in dystrophic axon terminals, coincident with the presence of a large number of autophagosomes [54]. Moreover, during development, MAP1B-P is highly expressed and is most concentrated in the distal axons of growing neurons [92]. The conserved role of MAP1B-P in axonal growth or repair during development or injury implicates autophagy in the remodeling of axon terminal structures for regeneration. Thus, we propose that interactions of LC3 with MAP1B or MAP1B-P provide a mechanism that regulates autophagosome formation in neurons or the axons.

The hypothesis that neuronal autophagy maintains axonal homeostasis is also in line with the idea that autophagy substrates are removed from the axons through axonal transport back to the soma where lysosomes perform degradation [89,93]. Interestingly, recent studies have revealed a role of MAP1B in retrograde axonal transport [54]. It is possible that the MAP1B-P–LC3 interaction may regulate axonal transport of "autophagic cargo". In support of this idea, microtubules and the dynein motor complex were previously shown to be required for autophagy process [90]. Dynein, the minus-end directed motor protein, mediates retrograde transport and interacts with the dynactin protein complex, which is implicated in cargo binding [94]. Inhibition of dynein impeded rapid movement of autophagosomes in mouse embryonic fibroblasts (MEF) cells and inhibited clearance of mutated alpha-synuclein or huntingtin by preventing fusion with the lysosome [90,95]. Furthermore, microtubule networks may facilitate the transport of LC3-positive autophagosomes [96], but are not required for fusion with lysosomes [54,97,98].

Although limited, recent studies involving live imaging showed that GFP-LC3 "dots" undergo retrograde transport in primary cerebellar granule cells [93]. In addition, both anterograde and retrograde transport of GFP-LC3-labeled autophagosomes were detected in neurites of differentiated PC12 and normal rat kidney cells [91,99]. Although bidirectional movement was observed, Jahreiss et al. propose an overall bias for microtubule-dependent movement toward the nucleus, which is mediated by dynein [91]. In summary, we anticipate that future study, using live-imaging, should elucidate the dynamic process of autophagy in neuron or axon.

4.3. Ulk1 and axonal autophagy

Additional evidence supporting the hypothesis that autophagy is important in axonal transport and function comes from the study of yeast Atg1 and its homologue, Unc-51 (*C. elegans uncoordinated-51*). Atg1/Unc-51 is a highly conserved serine/threonine kinase. In initial *C. elegans* screenings, *unc-51* mutants displayed disruptions in axon ultrastructure, including the presence of abnormal membranous structures in the axon and large swellings in the terminal [100]. The murine homologue, Unc51.1, is required for neurite extension in primary cerebellar granule neurons and governs vesicular membrane organization during outgrowth [101]. Thus, Unc-51 may provide a potential link between local membrane dynamics and autophagy at the axon terminal. Interestingly, the human homolog, Unc-51-like

kinase 1 (ULK1), interacts with two MAP1B-LC3 related proteins: Golgi-associated ATPase enhancer (GATE-16) and $GABA_A$ receptor associated protein (GABARAP), and have recently been shown to associate with p62/SQSTM1 [102,103]. Nerve growth factor (NGF) binding with TrkA receptors facilitates K-63 polyubiquitination of ULK1, promoting its association with the UBA domain of p62/SQSTM1 and recruiting ULK1 into the active TrkA complex. ULK1 interacts with syntenin and SynGAP (which regulate endocytosis) and results in the trafficking of NGF-bound TrkA receptors into endocytic vesicles [104]. These results provide a possible mechanism for the cross-talk between two important membrane trafficking pathways – autophagy and endocytosis – and their role in axonal physiology. One possibility is that, by fusion with autophagosomes, some types of membrane compartments, including endosomes, are removed from the axons and delivered to lysosomes for degradation. This process may be essential to maintain the homeostasis of the axonal membrane network [58].

4.4. Axonal autophagy and axonopathy

Axonal dystrophy, a hallmark of axonopathy, can be triggered by neuronal injuries, excitotoxicity, and various neurodegenerative conditions. Despite the prevalence of this pathology, the molecular mechanisms underlying axonopathy, as well as the connection between axonopathy and neurodegeneration, remain poorly understood. Dysfunctional autophagy has recently been implicated in axonal dystrophy. Severe axonal swellings were observed when autophagy was suppressed in the mouse brain [6,7]. Genetic ablation of *Atg7*, specifically in Purkinje cells of mutant mice, however, resulted in cell-autonomous axonal dystrophy and degeneration, implying an essential role for autophagy in membrane trafficking and turnover in axons. In contrast to *Lurcher* mice, axon terminals of *Atg7* deficient mice lacked autophagosome-like vesicles, but amassed abnormal organelles and membranous structures [58]. Moreover, mice with Purkinje-cell specific deletion of either *Atg5* or *Atg7*, exhibited axonal dystrophy much earlier than dendritic tree atrophy and cell death [57,58]. Thus, overactive or insufficient autophagy may contribute to axonopathy, which is a prominent feature of human neuropathology.

Accumulating evidence indicates that axonal degeneration precedes neuronal cell body death and undergoes a self-destruct mechanism that is distinct from apoptotic death in the soma [105,106]. A classic example of axonal degeneration is Wallerian degeneration, in which transected axons or neurites undergo complete fragmentation distal to the site of injury [106]. In mice carrying the spontaneous-occurring slow Wallerian degeneration (Wld^s) mutation, Wallerian degeneration is markedly delayed following transection. However, the "dying back" model, a progressive retrograde degeneration of the distal axon, may better reflect the chronic injury associated with neurodegenerative diseases [106]. NGF deprivation induced accumulation of autophagosomes in the distal tips of PC12 neurites supporting the hypothesis that autophagy induction contributes to dying back degeneration in which distal neurites are more fragile than proximal segments. Indeed, knocking down levels of *Atg7* or *Beclin1* caused a significant delay of neurite degeneration after NGF deprivation in sympathetic neurons [107]. These findings suggest that autophagy activation contributes to neurite degeneration, thus providing a valuable clue about the possible involvement of autophagy in the mechanism of axonal degeneration.

In summary, based on current evidence, we propose that basal autophagy plays an important role in the maintenance of axonal homeostasis by the removal of "autophagic cargo" from distal axons or terminals through retrograde axonal transport. Although the "autophagic cargo" can be associated with LC3, it is largely unclear whether they are in the form of typical autophagosomes [55]. Future study should investigate the nature of "autophagic cargo" in healthy neurons or axons. In contrast, pathological conditions can induce autophagy involving stimulated local synthesis of autophagosomes in axons. Degradation by the autophagic process may occur in the axons, which may require the accessibility of lysosomes or late endosomes in the axons. Or degradation may take place in the soma, which then requires retrograde transport of autophagosomes. Nonetheless, the inability of axons to either degrade or transport a large number of autophagosomes produced locally would have deleterious consequences to the axons, causing axonal dystrophy and degeneration (Fig. 2).

5. Specific function of p62/SQSTM1 in autophagy-mediated protein degradation

5.1. Role of neuronal autophagy in protein homeostasis

One of the critical findings in the study of *Atg5* or *Atg7*-deficient neurons is the recognition that autophagy is required to

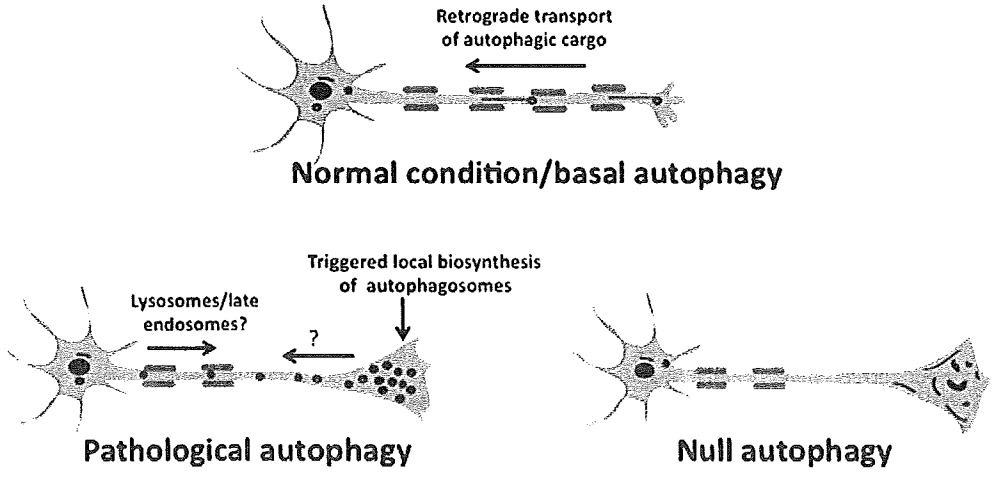

Fig. 2. Proposed model for neuronal autophagy in the axons. Under normal conditions, basal autophagy maintains axonal homeostasis by removal of "autophagic cargo" (top). The "autophagic cargo" undergoes retrograde axonal transport to the soma and fuses with lysosomes for degradation. Stress or injury can induce local biosynthesis of autophagosomes, resulting in their accumulation in axons and axon terminals. It has been suggested that, under pathological conditions, precursors of degradative vesicles or lysosomes may be anterogradely transported to the axon terminal and contribute to the degradation of autophagosomes (bottom left). Neurons deficient in autophagy amass proteins, organelles, and aberrant membrane structures at axon terminals, resulting in gross axonal swellings or dystrophy (bottom right).

suppress spontaneous protein aggregation. Loss of these autophagy genes causes accumulation of polyubiquitinated proteins. These appear as inclusion bodies, which increase in size and number with aging. However, proteasome function, which is generally known to cause abnormal ubiquitin-mediated proteolysis when impaired, is not compromised. Thus, blocking neuronal autophagy results in delayed global turnover of cytoplasmic components, accumulation of misfolded and/or unfolded proteins, followed by ubiquitination and the formation of inclusion bodies. Furthermore, these findings reveal an important role for basal autophagy in the protein clearance or protein homeostasis in neurons. While autophagy is absolutely required to degrade proteins for the maintenance of homeostasis in healthy neurons, its function becomes even more prominent in neurons expressing disease-related proteins. Recent studies show that autophagy contributes to the degradation of aggregate-prone proteins such as polyglutamine containing proteins and mutant alpha-synuclein [15]. Although it remains largely unclear how autophagy may achieve selective degradation of these disease-related proteins, expression of disease-related proteins in cells or neurons stimulates autophagy activation, and elevated autophagy is correlated with reduced levels of protein aggregates, concomitant with a decrease in neurotoxicity [61,108].

5.2. Link of p62/SQSTM1 to autophagic degradation

Emerging evidence suggests that p62/SQSTM1, originally identified as an ubiquitin-associated protein, provides a link between autophagy and selective protein degradation. Recent studies show that p62/SQSTM1 is an LC3-interacting protein [103,109]. Because p62/SQSTM1 can bind a large number of proteins through its multiple protein–protein interaction motifs, it may mediate diverse signaling pathways including cell stress, survival, and inflammation. Structural analysis reveals that p62/SQSTM1 N-terminal *P*hox and *Bem1* (PB1) domain exhibits self-oligomerization, and the C-terminal *ub*iquitin-*a*ssociated (UBA) domain can bind ubiquitinated proteins, suggesting a link between p62/SQSTM1 and disease-related protein inclusion formation. Consistent with the notion that p62/SQSTM1 is likely more related to autophagy-lysosome degradation, lysosomal inhibition, but not proteasomal inhibition, resulted in marked accumulation of p62 as well as LC3-II. Moreover, ablation of autophagy in neurons leads to a rapid and robust increase in p62 protein levels [54,109]. These observations suggest that p62 is selectively degraded by autophagy via the LC3-interaction. In addition, since autophagosome formation and long-lived protein degradation are intact in *p62*-knockout mice, p62 is considered a specific substrate of autophagy rather than a molecule involved in autophagosome formation.

Recent studies have identified the *L*C3 *r*ecognition *s*equence (LRS) in uncharacterized linker regions between the zinc finger and UBA domains in murine p62. The LRS is comprised of 11 amino acids (Ser334–Ser344), which include an acidic cluster and hydrophobic residues (DDD or DEE and WXXL or WXXV). Interestingly, this sequence is almost identical to a previously reported *L*C3-*i*nteracting *r*egion (LIR) of human p62. The crystal structure of the LC3-LRS complex, solved at 1.56 Å resolution, reveals that the acidic cluster of Asp337–Asp339 in LRS interacts with basic residues in the N terminus of LC3, and that the Trp-340 and Leu-343 residues are inserted into two hydrophobic pockets, exposed on the ubiquitin domain of LC3. LC3 has basic residues at its N-terminal α-helix surface, and these residues are involved in the interaction with the acidic cluster of LRS, whereas the other two Atg8 mammalian homologues, GATE-16 and GABARAP, have acidic residues in their respective N-terminal α-helical surfaces [110]. It is thus conceivable that p62 is a more favorable target for LC3 than GATE-16 or GABARAP.

5.3. Role of p62 in inclusion body formation

Characterization of protein inclusions in autophagy-deficient cells or neurons reveals that nearly all inclusions are positive for ubiquitin and p62 [109]. This raises an important question regarding the role of p62 in the formation of protein inclusions. To answer this, Komatsu et al. crossed p62−/− mice to mutant mice with autophagy deficiency in specific tissues. They found that loss of *p62* greatly reduces the formation of ubiquitinated protein inclusions resulting from impaired autophagy in mice. Similar studies were also performed in fruit flies and again the results indicate that the presence of p62 is necessary for the occurrence of protein inclusions in neurons [111]. To understand the molecular basis of this function of p62, it was shown that p62 proteins harboring mutations in LRS, escape efficient degradation by autophagy, leading to inclusion formation despite normal autophagy in cells. In addition, degradation of the PB1 mutant of p62, which is defective in oligomerization, is markedly attenuated, but no protein inclusions occur. These results suggest that increased levels and oligomerization of p62 are necessary for the formation of inclusion bodies, and furthermore, oligomerization of p62 via PB1 is a critical event for facilitating their degradation by autophagy.

Considerable evidence has shown that p62 is a component of protein inclusion bodies found in human disorders such as in liver injuries (e.g., alcoholic hepatitis, steatohepatitis, and α1-antitrypsin deficiency) and neurodegenerative diseases (e.g., AD, PD, and ALS). Although autophagy is beneficial in the clearance of toxic protein aggregates and prevents cellular toxicity, whether or not p62 is indeed essential for the formation of disease-related inclusions and the exact role of p62 in the disease process remains unknown at present. Intriguingly, the formation of ubiquitin-positive aggregates induced by proteasome inhibition is also suppressed in *p62*-deficient cells, suggesting that p62 is a general mediator of inclusion formation. Thus, we hypothesize that p62 normally functions as an adaptor that links certain proteins to autophagy machinery for degradation. When cellular degradation systems (proteasomes or autophagy-lysosomes) are compromised or overwhelmed, p62 levels are sharply increased (especially with impaired autophagy) and it simply behaves as "glue" that bonds many autophagic protein substrates together, manifesting as protein aggregates and eventually large protein inclusions.

5.4. Potential mechanism of neurodegeneration caused by autophagy-deficiency

Emerging evidence indicates that soluble proto-fibrils or small oligomers of disease-related proteins are the cytotoxic species, whereas large inclusion bodies derived from the sequestration of toxic protein oligomers are protective. Therefore, we can hypothesize that the presence of p62 is protective in suppressing the toxicity of proto-fibrils or protein oligomers, given the role of p62 in facilitating the formation of relatively less toxic inclusion bodies. Accordingly, loss of p62 in autophagy-deficient neurons would exacerbate the neurodegenerative process because toxic proteins would accumulate intracellularly and no inclusion bodies would form. Surprisingly, in *Atg7/p62* double knockout mice, loss of p62 was found to be associated with reduced liver dysfunction or little change in the neurodegenerative process caused by autophagy-deficiency, despite the absence of any inclusion bodies [109]. Apart from the specific function of p62 in liver, these results seem to argue against a critical role of protein inclusions in the context of cytotoxicity, especially in the absence of disease-related proteins. Alternatively, they may suggest that the proteins accumulated upon impairment of autophagy are largely neutral or at least neurons are tolerant to the build-up of those proteins. In this regard, the degradative function of autophagy in protein homeostasis control is likely more significant towards disease-related, aggregate-prone proteins than normal long-lived protein substrates of autophagy.

So what could be the primary cause that leads to neuronal death when neuronal autophagy is impaired? As discussed above, analyses of knock-out mice with Purkinje cell-specific *Atg7* deletion (*Atg7*$^{flox/flox}$; Pcp2-Cre) demonstrated that loss of Atg7 initially causes cell-autonomous, progressive dystrophy (manifested by axonal swelling) and degeneration of the axon terminals followed by cell-autonomous Purkinje cell death and mouse behavioural deficits. Furthermore, the mutant Purkinje cells developed aberrant organelles in the swelling axons, suggesting the important role of autophagy in the regulation of local axonal membrane trafficking and turnover [58]. The study also implicates impairment of axonal autophagy as a mechanism for axonopathy associated with neurodegeneration. Interestingly, such axonopathy associated with *Atg7*-deficient Purkinje cells and hypothalamic neurons is still observed in *Atg7*/*p62*-double knockout neurons, indicating that the development of axonopathy in *Atg7*-deficient neurons is *p62*-independent [109]. Therefore, we hypothesize that axonal dystrophy and degeneration provide an important mechanism for the neurodegeneration caused by autophagy-deficiency.

6. Conclusion and perspectives

The past several years have witnessed a rapid growth of autophagy research. With emerging evidence that links the aberrant autophagic activity to various neurodegenerative diseases, it is of upmost importance to understand the basic process of neuronal autophagy at molecular and cellular levels. This would aid in revealing the exact role of autophagy in the neuropathogenesis associated with different diseases. Currently, the particular challenge facing us is to establish robust and physiological cellular and animal models that allow us to dissect neuronal autophagy, and to identify specific factors that contribute to the regulation of neuronal autophagy process. We anticipate that, with the development of new experimental approaches, particularly in the area of cell biology, mouse genetics, live-imaging and proteomics, we will make significant progress in this field and provide valuable knowledge to the design of drugs targeted at autophagy in the treatment of neurodegenerative diseases.

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