Motor Neuron-specific Disruption of Proteasomes, but Not Autophagy, Replicates Amyotrophic Lateral Sclerosis*

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Background: It is not clear how protein degradation systems are involved in ALS pathogenesis.

Results: Transgenic mice with motor neuron-specific knock-out of proteasomes, but not of autophagy showed ALS phenotypes.

Conclusion: Dysfunction of proteasome may primarily contribute to the pathogenesis of ALS than that of autophagy.

Significance: Modulation of proteasome function is a promising approach toward treatment of ALS.

Evidence suggests that protein misfolding is crucially involved in the pathogenesis of amyotrophic lateral sclerosis (ALS). However, controversy still exists regarding the involvement of proteasomes or autophagy in ALS due to previous conflicting results. Here, we show that impairment of the ubiquitin-proteasome system, but not the autophagy-lysosome system in motor neurons replicates ALS in mice. Conditional knock-out mice of the proteasome subunit Rpt3 in a motor neuron-specific manner (Rpt3-CKO) showed locomotor dysfunction accompanied by progressive motor neuron loss and gliosis. Moreover, diverse ALS-linked proteins, including TAR DNA-binding protein 43 kDa (TDP-43), fused in sarcoma (FUS), ubiquilin 2, and optineurin were mislocalized or accumulated in motor neurons, together with other typical ALS hallmarks such as basophilic inclusion bodies. On the other hand, motor neuron-specific knock-out of Atg7, a crucial component for the induction of autophagy (Atg7−CKO), only resulted in cytosolic accumulation of ubiquitin and p62, and no TDP-43 or FUS pathologies or motor dysfunction was observed. These results strongly suggest that proteasomes, but not autophagy, fundamentally govern the development of ALS in which TDP-43 and FUS proteinopathy may play a crucial role. Enhancement of proteasome activity may be a promising strategy for the treatment of ALS.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by progressive loss of upper and motor neurons. Although the exact pathogenesis of ALS remains elusive, prior studies of causal genes for familial ALS, including superoxide dismutase 1 (SOD1) (1), als2 (2), TAR DNA-binding protein 43 kDa (TDP-43) (3, 4), fused in sarcoma (FUS) (5, 6), and optineurin (7), have identified diverse pathogenic cascades and key molecules both in vitro and in vivo (8). In particular, accumulation of ubiquitinated inclusions containing these gene products is a common feature in most familial ALS models and is also a pathologic hallmark of sporadic ALS.

The abbreviations used are: ALS, amyotrophic lateral sclerosis; 3-MA (3-methyladenine), Atg (autophagy-related protein), CAG (chicken β-actin promoter with cytomegalovirus enhancer), ChAT (choline acetyltransferase), CKO (conditional knockout), Cre (cycylation recombination enzyme), FRT (flippase recognition target), LC3-II (light chain 3-II), loxP (locus of crossing over P1), MAC-2 (macrophage antigen-2), Nbr1 (neighbour of BRCA1), Neo (neomycin resistance gene), Rpn (regulatory particle non-ATPase subunit), Rpt (regulatory particle triple-ATPase subunit), SOD1 (superoxide dismutase 1), SQSTM1 (sequestosome 1), UCH-L1 (ubiquitin carboxy-terminal hydrolase L1), UPS (ubiquitin-proteasome system), VACHT (vesicular acetylcholine transporter).

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This article contains supplemental Table S1 and Fig. S1.

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Disruption of Proteasome Replicates ALS

Protein quality control is a vital system for regulating cellular homeostasis and is mediated by two major pathways: the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system. The impairment of either is implicated in the neurodegeneration seen in ALS, Parkinson disease, Alzheimer disease, and polyglutamine disease (11, 12) by allowing toxic proteins to accumulate in neurons or glial cells (13, 14). For instance, and polyglutamine disease (11, 12) by allowing toxic proteins to accumulate in neurons or glial cells (13, 14). For instance, genetic mutations in Parkin (the ubiquitin ligase) and UCH-L1 (the enzyme for de-ubiquitination) are reported to cause Parkinson disease (15–17). Moreover, ablation of the Parkin gene in transgenic mice expressing its substrate, the Pael receptor, results in progressive nigral degeneration, similar to Parkinson disease (18). Furthermore, dopaminergic neuron-specific gene knock-out of Rpt2, a 26S proteasome subunit in mice, resulted in dopaminergic neurodegeneration accompanied by Pale body-like inclusion composed of alpha-synuclein (19). A role for autophagy has also been shown in Parkinson disease, Alzheimer disease, and Huntington disease (20, 21). Indeed, suppression of autophagy in neuronal cells induces neurodegeneration with robust ubiquitinated inclusions (22, 23).

In ALS, the presence of ubiquitinated inclusions such as skein-like and round hyaline inclusions strongly suggests dysfunction of the UPS. Indeed, we have shown that continuous expression of mutant SOD1 results in decreased proteasome activity, and that primary cultured embryonic motor neurons are vulnerable to proteasome inhibition by lactacystin (24). On the contrary, other reports have documented that proteasome activity is unchanged (25) or increased (26, 27), and it has been reported that long-term pharmacological inhibition of proteasomes does not cause motor neuron death in a slice culture study (28). Besides proteasomes, autophagy is implicated in the pathogenesis of ALS as well. Mutant SOD1 and TDP-43 are reportedly degraded by the autophagy-lysosome system, as well as by the proteasome (29, 30). Therapeutic benefit has been reported by enhancing autophagy in ALS models with lithium treatment (31) and overexpressed TDP-43 models with rapamycin treatment (32). Conversely, there is a report showing that dietary restriction, but not rapamycin (an autophagy inducer), ameliorates the symptoms of mutant SOD1 transgenic mice (33). Therefore, the predominant involvement of autophagy in ALS pathogenesis is a matter of debate. This is in part due to the complex effects of pharmacological approaches other than autophagy suppression. To clarify the exact roles of protein quality control systems, the use of mouse genetic engineering approaches, especially those targeting motor neurons, is needed.

In the present study, we investigated the effect of disrupting proteasomes or autophagy only in motor neurons. To this end, we generated conditional knock-out (CKO) mice for Rpt3, a subunit of 19S particle that formed 26S proteasome, to disrupt the 26S proteasome, or Atg7, a crucial factor for the induction of autophagy, using the Cre-loxP system. Rpt3 is a subunit of 26S proteasome of which mutations are reported in Parkinson disease patients (34), and knock-out of mice Rpt3 gene results in early-embryonic lethality (35). We show here that motor neuron-specific disruption of Rpt3, but not Atg7, induced the ALS phenotype both behaviorally and pathologically in mice. Although both types of mice displayed accumulation of ubiquitin or ubiquitinated proteins, TDP-43 and FUS pathologies were observed only in Rpt3-CKO mice. Our work has suggested the predominant role for the UPS in ALS pathogenesis, which may be mediated by TDP-43 and/or FUS proteinopathy.

Experimental Procedures

Transgenic Mice Generation

Floxed Rpt3 Mice—Using homologous recombination, a cassette containing a loxP site with a neomycin resistance gene (Neo) flanked by flippase recognition target (FRT) sites (36) and a loxP site were inserted into introns 7 and 10, respectively, of the Rpt3 gene (GenBank™ ID 23996) in the mouse C57BL/6N ES cell line RENKA (37) by electroporation. After ES cell selection, 5–10 positive transgenic ES cells were injected per 8-cell-stage embryo mice. After overnight culture, the ES cell-containing embryos that developed to the blastocyst stage were transplanted into the uterus of pseudo-pregnant ICR females. Germline-transmitted chimeric males were crossed with C57BL/6N females for the floxed Rpt3 mice to generate heterozygotes. After removing the neomycin resistance gene with the FLP-FRT system by crossing with FLP66 mice that express flippase (36), Rpt3+/floxed mice were crossed to establish homozygous floxed Rpt3 mice (Rpt3floxed/floxed) at apparent Mendelian frequencies. Rpt3floxed/floxed mice were phenotypically normal. Rpt3floxed/floxed mice were used after eight generations for this experiment. To selectively knock out Rpt3 activity in motor neurons, Rpt3delta/floxed mice were crossed with VACH-T-Cre.Fast mice (38) with the Rpt3+/floxed allele to obtain the mutant mice. All studies were performed in accordance with the Guideline for Animal Studies of the Kyoto University and the National Institutes of Health. The committee of animal handling of Kyoto University and Juntendo University also approved the experimental procedures used.

Conditional VACH-T-Rpt3 KO Mice—Rpt3delta/+; CAG-Cre+ mice were generated by crossing Rpt3floxed/floxed mice with CAG-Cre mice (39). After removing CAG-Cre by crossing with WT mice, Rpt3delta/+ mice were mated with Rpt3floxed/floxed mice to generate Rpt3delta/floxed mice. Rpt3delta/floxed mice were crossed with Rpt3+/floxed; VACH-T-Cre.Fast+ mice to produce Rpt3delta/floxed; VACH-T-Cre.Fast+ as mutant mice and Rpt3delta/-; VACH-T-Cre.Fast+ as controls. Female mutant and control mice were used (n = 8). There were no differences in the results between male and female mutant mice. All genotyping was performed with PCR using DNA from tail biopsies.

Conditional VACH-T-Atg7 KO Mice—Floxed Atg7 mice are described elsewhere (40). Atg7floxed/floxed mice were crossed with Atg7+/floxed; VACH-T-Cre.Fast+ mice to produce Atg7floxed/floxed; VACH-T-Cre.Fast+ (Atg7-CKO) as the mutant mice and Atg7+/-floxed; VACH-T-Cre.Fast+ as controls (Atg+control).

PCR Genotyping—Tails were lysed with 0.5% SDS and 100 mg/ml protease K. DNA was extracted from lysates with phenol/chloroform 1:1 (v/v), precipitated with isopropanol, and dissolved in 10 mM Tris-HCl, pH 7.5/1 mM EDTA. Genotyping
for the Rpt3 flox allele was carried out with primers Rpt3 fl-for and Rpt3 fl-rev. Genotyping for the Rpt3 delta allele was carried out with primer Rpt3 del-rev (the forward primer used was the Rpt3 fl-for primer). PCR conditions were 94 °C for 5 min, and then 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 74 °C for 1 min on a thermal cycler. The presence of a 330-bp band is indicative of the wild-type allele, whereas the presence of a 390-bp band is specific for the Rpt3 flox allele in Rpt3 flox allele detection PCR. The presence of a 360-bp band is specific for the Rpt3 delta allele instead of a 1800-bp wild-type allele (the 1800-bp band is not detected with this PCR method time) in Rpt3 delta allele detection PCR. Genotyping for the Cre allele, which was included in CAG-Cre and VACHT-Cre. Fast mice, was carried out with primers Cre-for and Cre-rev. PCR conditions were 95 °C for 3 min, and then 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s followed by an extension of 72 °C for 2 min on a thermal cycler. The presence of a 303-bp band is indicative of the Cre transgene. Genotyping for the Flp allele was carried out with primers Flp-for and Flp-rev. PCR conditions were 94 °C for 5 min, and then 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min on a thermal cycler. The presence of a 480-bp band is indicative of the Flp transgene. Products of the reaction were analyzed by electrophoresis in a 2% agarose gel. Genotyping for the Atg7 flox allele was carried out with primers Hind-Fw and 96–121c. PCR conditions were 94 °C for 5 min, and then 30 cycles of 98 °C for 20 s, 68 °C for 3 min, and 72 °C for 10 min on a thermal cycler. The presence of a 4.4-Kbp band is indicative of the Atg7 flox allele, whereas the presence of a 2.2-Kbp band is specific for the Atg7 delta allele in Atg7 flox allele detection PCR. Products of the reaction were analyzed by electrophoresis in a 1% agarose gel. Histology For the analysis of Rpt3-CKO mice, three mutant mice of 6, 12, and 40 weeks of age and three controls of the same ages were used; three mutant mice 2 years of age and three controls of the same age were used for Atg7-CKO mice. Mice were deeply anesthetized with pentobarbital (25 mg/kg, intraperitoneal) and fixed by cardiac perfusion with 4% paraformaldehyde (PA) buffered with 0.1 M phosphate buffer (pH 7.2) (PB) containing 4% sucrose for light microscopic immunohistochemistry and with 2% PA and 2% glutaraldehyde buffered with PB for electron microscopy. The spinal cords were removed from the mice and further fixed in the same fixatives for 2 h. The spinal cords were embedded in paraffin wax and cut into sections of 6-μm thickness according to standard protocols. For general morphological examination, we used hematoxylin (Harris) and eosin (H&E), Kluver-Barrera’s staining, and Nissl staining. For immunohistochemistry, first, endogenous peroxidase activity was quenched by incubation with 0.2% hydrogen peroxide in 0.1 M PB, pH 7.3 containing 0.2% Triton X-100 for 5 min at 37 °C. After antigen retrieval by heat/microwaving (5 min in 10 mM sodium citrate buffer, pH 6.0; except for ubiquitin), the sections were incubated with primary antibody (listed in supplemental Table S1) overnight at 4 °C. Bound antibodies were detected with the appropriate Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) with tetrahydrochloride as the chromogen and observed under an Olympus BX-51 microscope (Olympus Corporation, Tokyo, Japan). For double immunofluorescence staining, primary antibodies were visualized with Alexa Fluor 488-labeled goat anti-rabbit IgG (1:200, Molecular Probes, Eugene, OR) and Cy5-labeled goat anti-mouse IgG (1:100, Chemicon International, Temecula, CA). The slides were mounted with Vectashield with DAPI (Vector Laboratories) and observed under a Keyence BZ-9000 microscope and camera (Keyence Corporation, Osaka, Japan). We assessed the staining specificity by replacing the primary antibodies with an appropriate amount of non-immune rabbit serum or phosphate-buffered saline solution containing 3% bovine serum albumin. No deposits of reaction products were seen in these sections. For Atg7-CKO mice, the fixed samples were processed for paraffin embedding, cut into 5-μm sections with a microtome (RM2245; Leica, Germany), and immunostained according to the method described previously (41) with primary antibodies listed in supplemental Table S1. For Nbr1 and Atg7 staining, deparaffinized sections were autoclaved for 20 min in 10 mM Na-citrate buffer (pH 6.1) before incubation with rabbit anti-Nbr1 antibody. Electron Microscopy The fixed samples were cut into slices less than 1 mm, postfixed with 2% OsO4 buffered with 0.1 M PB (pH 7.2), block-stained in 1% aqueous solution of uranyl acetate, dehydrated in a graded series of alcohols, and embedded in Epon 812 (TAAB). After confirming that semi-thin sections from the Epon-embedded blocks of spinal cords contained the ventral horn, silver-colored sections were cut with an ultramicrotome (Ultracut UC6; Leica Microsystems). The ultrathin sections were then stained with uranyl acetate and lead citrate and observed with an electron microscope (HT7700; Hitachi). Statistical Analysis Two-group data analysis was performed using the two-tailed unpaired Student’s t test with unequal variance. For data analysis of more than two groups, the significance of differences among test values was determined with the two-tailed multiple t test with Bonferroni correction following ANOVA (6 comparisons in 4 groups or 15 comparisons in 6 groups). Statistical significance is shown in each figure as appropriate. RESULTS Generation of Spinal Motor Neuron-specific Rpt3 Conditional Knock-out Mice—A floxed Rpt3 construct was generated in which exons 7–10, encoding the majority of the conserved ATPase domain (42), were flanked by loxP sites and replaced with a neomycin resistance gene (Neo) that was adjoined by
flippase recognition target (FRT) sites (36). The floxed Rpt3 gene construct was inserted into the Rpt3 gene in the mouse C57BL/6N ES cell line RENKA (37) by electroporation (Fig. 1A). To selectively ablate Rpt3 activity in spinal motor neurons, we crossed the floxed Rpt3 mice with VACHT-Cre.Fast mice, which express Cre in a spinal motor neuron-specific manner (38). Cre expression in VACHT-Cre.Fast mice is developmentally regulated, and ~50% of spinal motor neurons express Cre, the level of which reaches a maximum by 5 weeks of age (38). We generated Rpt3^+/−; VACHT-Cre+ as a control mouse line, and Rpt3^delta/flox; VACHT-Cre+ (Rpt3-CKO) as the mutant mouse line (Fig. 1B). The number of Rpt3/choline acetyltransferase (ChAT) double-immunopositive spinal motor neurons in the mutant mice was reduced to approximately half of that in control mice at 6 weeks of age (Fig. 1C). Moreover, Rpt3-negative/ChAT-positive cells, comprising about 50% of the total spinal motor neurons, were observed exclusively in Rpt3-CKO mice, indicating that Rpt3 expression was deficient in about half of the spinal motor neurons in a Cre-dependent manner (Fig. 1C). Immunohistochemistry using serial spinal cord sections revealed that Rpt3 was effectively depleted in the ChAT-positive motor neurons (Fig. 1D, arrows). In agreement with this finding, ubiquitin immunoreactivity was increased in the cytoplasm of these cells, validating the severe impairment of the UPS (Fig. 1D). Moreover, we investigated the 26S proteasome subunits in Rpt3-CKO mice at 6 weeks of age, including Rpt6, a 19S base proteasome subunit connected directly to Rpt3 (43), Rpn2, a subunit required for 19S particle formation, and α6, a 20S proteasome subunit. We confirmed the reduction of immunoreactivity for Rtp6 and Rpn2 in the nuclei and cytosol only in Rpt3-CKO mice, strongly suggesting that the 19S particle was disassembled in Rpt3-deficient motor neurons (Fig. 1E).

**Behavioral Abnormalities in Rpt3-CKO Mice**—Behavioral analysis of Rpt3-CKO mice revealed that motor neuron-specific proteasome inhibition caused a progressive motor deterioration. Rpt3-CKO mice displayed significantly decreased rotarod performance after 26 weeks of age (Fig. 2A). This performance (16–36 weeks of age) and remained stable after 36 weeks of age. Body weight (Fig. 2B) and grip strength (Fig. 2C) were significantly reduced in Rpt3-CKO mice compared with controls. Onset of the ALS phenotype was observed at 10 weeks...
of age when the mice displayed disturbed and tremulous hindlimb movement with tail suspension. Other measures showed obvious differences later than 20 weeks (Fig. 2D). At the advanced stage, mice presented with severely deformed spines, indicating weakness of the paraspinal muscles (Fig. 2D). Of note, no survival difference was observed between Rpt3-CKO and control mice until at least 48 weeks of age. There was no gender difference in our mice.

**Replication of Pathological Features of ALS in Rpt3-CKO Mice**—Immunohistochemical analysis using anti-ChAT antibody showed progressive loss of spinal motor neurons in Rpt3-CKO mice from 6 weeks of age (Fig. 3A). Abnormal motor neurons with eosinophilic cytoplasm were found in the mutant spinal cords at 12 weeks of age (Fig. 3B, arrows). Notably, chromatolytic neurons and basophilic inclusions were present at 12 weeks of age (Fig. 3C, arrows). These cytopathological findings resemble those observed in the spinal motor neurons of sporadic ALS patients (Fig. 3C, c and d).

Next, we investigated the presence of pathological hallmarks of sporadic ALS, including TDP-43 (3, 4), FUS (5, 6), optineurin (7), and ubiquilin 2 (44). As shown in Fig. 4, immunohistochemistry demonstrated aberrant staining patterns of these proteins, including mislocalization and inclusions of TDP-43 (Fig. 4A, a–c) and FUS (Fig. 4A, e–g), accumulation of optineurin (Fig. 4A, i–k), and ubiquilin 2 inclusions (Fig. 4A, m–o). As shown in supplemental Fig. S1, TDP-43 displayed various cytoplasmic distributions with occasional nuclear inclusions (supplemental Fig. S1D, arrows). Mislocalized TDP-43 or FUS showed diffuse distribution in the cytosol at 6 weeks of age (asymptomatic stage; Fig. 4A, b and f), whereas aggregate formation of TDP-43,
FUS, or ubiquilin 2 was observed after 12 weeks of age when the mice were paralyzed (Fig. 4A, c, g, or o). Double immunofluorescence analysis demonstrated colocalization of TDP-43 with FUS (Fig. 4B, a–c) and ubiquilin 2 (Fig. 4B, d–f) within cytoplasmic inclusions. On the other hand, p62/SQSTM1, autophagy-specific substrate, was not detected in motor neurons and their surroundings in Rpt3-CKO mice at 12 weeks of age (data not shown). Quantification by counting cells with stronger staining of mislocalized than nuclear TDP-43 or vice versa revealed that the ratio of motor neurons with mislocalized to nuclear TDP-43 was maximal at 12 weeks of age (Fig. 4C). Interestingly, the ratio decreased at 40 weeks when motor neuron loss was most prominent (Fig. 4C), indicating that cytoplasmic TDP-43-positive motor neurons had disappeared.

Glial Activation in the Spinal Cord of Rpt3-CKO Mice—We next performed immunohistochemistry for glial cells in the spinal cord of Rpt3-CKO mice. In particular, we focused on astrocytes, because recent evidence shows that they play crucial roles not only in disease progression (45), but also in disease initiation (46). Glial fibrillary acidic protein (GFAP)-positive astrocytes were obviously detectable in the anterior horns of the spinal cords as early as 6 weeks (asymptomatic age; Fig. 5, A, a, b, g, and Ca). The robust astrogliosis persisted in the mutant spinal cords until 40 weeks of age (Fig. 5, A, a–f, i–l, and Ca). On the other hand, many activated microglia, which express galectin-3/MAC-2 (47), were found near motor neurons at 12 weeks, but fewer were found at 40 weeks of age (Fig. 5, B and Cb). In control mice, marginally reactive astrocytes and MAC-2-positive activated microglia were observed until 40 weeks of age, and their distribution was not concentrated in anterior horns (Fig. 5, A–C).

Lack of Overt ALS Phenotype in Transgenic Mice with Defective Autophagy in Motor Neurons—We also investigated a role for autophagy in motor neurons in ALS pathogenesis, using a similar genetic engineering approach. We generated Atg7<sup>flox/flox</sup>; VACHT-Cre.Fast+ (Atg7-CKO) mice by crossing floxed Atg7 mice (22) with VACHT-Cre.Fast mice (Fig. 6A). Surprisingly, Atg7-CKO mice developed normally and showed neither an apparent defect in motor performance nor motor neuron loss at least at 2 years of age (Fig. 6B, a and b). Moreover, Atg7 negative motor neurons containing inclusion bodies were detected at this old age, demonstrating that autophagy-deficient motor neurons survive for life (Fig. 6B, c and d). As shown in Fig. 6C, Atg7-CKO mice at 2 years of age showed marked staining of ubiquitin and ubiquilin 2 (Fig. 6C, f and p), which were also detected in Rpt3-CKO mice (Fig. 1, Df, Fig. 4, N and O). As expected, p62/SQSTM1 and Nbr1, autophagy-specific substrates (48, 49), also accumulated (Fig. 6C, g and h). Electron microscopy revealed that motor neurons in Atg7-CKO mice had inclusions (asterisks) in which amorphous structures that similar to previous reported (22) accumulated, and large inclusions were found at 2 years of age (Fig. 6D). On the other hand, Atg7-CKO mice showed no cytoplasmic mislocalization or aggregate formation of TDP-43, FUS, or optineurin (Fig. 6C, m–p).

DISCUSSION

In the present study, we provide compelling evidence that a motor neuron-specific defect in proteasomes, but not
autophagy, replicates ALS in mice. Our Rpt3-CKO mice express Cre in ~50% of spinal motor neurons (38). This population of motor neurons with defective proteasomes was found to produce an ALS-like phenotype. Immunohistochemistry validated the efficient disruption of 26S proteasomes in motor neurons, in which ubiquitin, Rpt6, Rpn2, and the 6E subunit of the 20S proteasome accumulated together with several hallmarks of ALS, including TDP-43, FUS, and ubiquilin 2. Our data also suggest that protein quality control by proteasomes predominates in governing motor neuron survival compared with that by autophagy. Surprisingly, inhibition of autophagy in motor neurons was not as toxic as proteasome inhibition despite the robust increase in ubiquitinated substances.

Of note, TDP-43 proteinopathy was evident only in Rpt3-CKO mice, but not in Atg7-CKO mice. This result conflicts with the previous findings in a cell culture study in which TDP-43 was shown to be degraded through both autophagy and proteasomes (30, 50). Indeed, inhibition of either prolonged the half-life of TDP-43 in the pulse-chase study. However, immunofluorescence analysis demonstrated that overt cytosolic aggregates of TDP-43 are induced only in the presence of lactacystin, a specific proteasome inhibitor, but not 3-MA alone, an autophagy inhibitor (30). Therefore, the proteasome may be a dominant site for TDP-43 degradation, especially misfolded TDP-43, whereas autophagy may partially compensate to eliminate TDP-43. The population of motor neurons with mislocalized TDP-43 gradually increased until 12 weeks of age. Of note, TDP-43 proteinopathy and Rpt3 down-regulation were not concurrent in our mice. Rpt3 reduction became obvious as early as 5 weeks of age, 7 weeks before the overt accumulation of TDP-43, consistent with the idea that TDP-43 proteinopathy is caused by proteasome inhibition (Fig. 7).

Despite the clear difference in TDP-43 pathology between proteasome inhibition and autophagy disruption, the exact molecular machineries involved in proteasome-specific cascades are unclear. Both systems have been implicated in several neurodegenerative diseases, including Huntington disease, Parkinson disease, and Alzheimer disease, as well as ALS. Protein degradation via proteasomes is regulated by the substrate-specific ubiquitin-proteasome pathway, the impairment of which induces diverse toxic cascades via accumulation of misfolded or dysfunctional proteins in the cytosol or other organelles such as the endoplasmic reticulum or mitochondria (51). On the other hand, autophagy is chiefly a bulk degradation system, which serves for quality control of organelles such as mitochondria as well as various membranous or cytoplasmic proteins (52). Familial ALS-linked mutant SOD1 proteins are
Degradation of ubiquitinated proteins, regardless of the carboxyl fragments of TDP-43 (60). Previous reports documented that TDP-43 interacts with FUS to induce the expression of histone deacetylase 6 in the nucleus, and the carboxyl terminus of TDP-43 is reportedly required for this interaction (59). Therefore, the carboxyl fragments of TDP-43, subsequent to its aberrant cytoplasmic mislocalization, may promote the recruitment of FUS during the process to form inclusions. Otherwise, a detrimental environment due to proteasome inhibition may induce stress granules, possibly providing the opportunity for assembly of these stress granule proteins, regardless of the carboxyl fragments of TDP-43 (60).

The different temporal profiles between astrocytosis and microgliosis are also interesting in interpreting the pathogenesis of ALS related to proteasome dysfunction. Active astrocytes proliferated at 6 weeks of age, whereas microgliosis was most robust at 12 weeks when the loss of motor neurons was evident (Fig. 7). Although the exact role of these types of gliosis remains unclear, our finding indicates that astrocytes serve as early participants in cell death.

The role of proteasome inhibition in cell death is not established in cultured cells. For instance, selective disruption of the specific 19S proteasome subunits Rpn10, Rpn11, and Rpt5 induces the accumulation of ubiquitinated inclusions, but does not cause cell death in cultured neurons (54). On the contrary, reports have shown that inhibition of 20S particle activity leads to apoptotic cell death accompanied by the accumulation of ubiquitinated proteins (55–58). For the first time, we have provided evidence that proteasome impairment in motor neurons directly links to pathogenesis of ALS, based on the successful in vivo elimination of proteasome components in mice. It should be noted that the proteasome disruption in half of spinal motor neurons lead to motor neurons loss by 75%. This suggests the participation of non-cell autonomous machineries such as glial involvement or propagation of unidentified toxic substrates from the dead motor neurons.

Pathological findings in our mice included hallmarks of sporadic ALS, including chromatolysis, eosinophilic round hyaline inclusions, skein-like inclusions, and basophilic inclusions. Moreover, these inclusions were immunoreactive for TDP-43, FUS, optineurin, and ubiquilin 2. In addition, TDP-43 mislocalization preceded the aberrant staining of FUS in our mice. A previous report documented that TDP-43 interacts with FUS to induce the expression of histone deacetylase 6 in the nucleus, and the carboxyl terminus of TDP-43 is reportedly required for this interaction (59). Therefore, the carboxyl fragments of TDP-43, subsequent to its aberrant cytoplasmic mislocalization, may promote the recruitment of FUS during the process to form inclusions. Otherwise, a detrimental environment due to proteasome inhibition may induce stress granules, possibly providing the opportunity for assembly of these stress granule proteins, regardless of the carboxyl fragments of TDP-43 (60).

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modulators or responders to the development of ALS, not only of the progression (45, 61). Moreover, considering that proteasome impairment preceded the sequential activation of astrocytes followed by microglia, signaling molecules released from motor neurons may be crucially involved in the glial activation in ALS. The identification of such molecules may expand our understanding of ALS.

In conclusion, we have shown that dysfunction of 26S proteasomes in motor neurons is sufficient to induce cytopathological phenotypes of ALS. Thus, UPS dysfunction may primarily contribute to the pathogenesis of sporadic ALS, and preservation and/or activation of UPS may represent an effective therapeutic approach to overcome ALS.

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