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Original Article

The role of mitochondrial quality control in lumbar spinal cords of SOD1-G93A transgenic mice

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Abstract: Objective: Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that selectively affects the upper and lower motor neurons (MNs). Mitochondrial dysfunction plays an important role in ALS and it is a converging point of multiple pathological pathways in ALS. The mitochondrial quality control including mitochondrial fission and fusion as well as mitophagy is the major mechanism of repair or removal of damaged mitochondria. In the present study, we made efforts to explore possible factors for mitochondrial dysfunction through studying the mitochondrial quality control in ALS mice. Materials and methods: Transgenic hSOD1-G93A mice and their non-transgenic counterparts were divided into four groups: 60-day-old group, onset group, end stage group and control. Then each group was further equally divided into 4 subgroups with 3 mice in each subgroup. These 4 subgroups were used for protein analysis, immunohistochemical analysis, electronic microscopy study, and mitochondrial protein analysis, respectively. Results: The expression of Fis1 and p-Drp1ser616 that control mitochondrial fission revealed a gradual enhancement as the development of ALS. Conversely, the expression of OPA1 and Mfn1 that control mitochondrial fusion exhibited a gradual down-regulation in MNs of lumbar spinal cords in ALS mice. In addition, the abnormal clustering of OPA1-deficient mitochondria and the accumulation of mitochondria-containing autophagosomes in MNs of ALS mice were also observed. Intervention with rapamycin as an autophagy enhancer could obviously improve the excessive and imbalanced fission of mitochondria. Conclusion: These results indicated that impaired mitochondrial fission, fusion and mitophagy were the causes of ALS.

Keywords: Amyotrophic lateral sclerosis, mitochondrial dysfunction, fission, mitophagy

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressively degenerative disease due to the selective loss of MNs in spinal cord, brainstem, and cortex, thus resulting in paralysis and death within 3-5 years after its diagnosis [1]. Approximately 20% of familial ALS cases are correlated with the mutation of the gene encoding copper-zinc superoxide dismutase (SOD1) [2]. In order to explore the pathogenesis of ALS, transgenic mice expressing human SOD1-G93A were generated. MNs in lumbar spinal cords of these model mice revealed a gradual degeneration and the model mice had an obvious replication of human ALS phenotypes [3]. ALS involves multiple pathological mechanisms including glutamate excitotoxicity, oxidative stress, mitochondrial dysfunction and protein aggregation [1, 4]. However, no consensus regarding the causes of MN degeneration has been achieved nowadays. Mitochondrial dysfunction has basically been confirmed in ALS [5, 6]. Mitochondrial dysfunction is not only a converging point of multiple pathological pathways in ALS, but also an initial factor of ALS [7]. However, the causes for mitochondrial dysfunction are still unclear. In physiological conditions, in order to curb the accumulation of damaged mitochondria, the cell has a set of quality control mechanisms such as repairing damaged mitochondria, removing aged mitochondria, promoting mitochondrial renewal, and maintaining mitochondrial morphology and functions [8, 9]. The imbalance of mitochondrial quality control will lead to ultra-structural

changes of mitochondria including cristae remodeling characterized by fragmentation, occasional cristae dilation or vesiculation, and the disappearance of cristae membrane [10], which are extremely similar to the changes of mitochondria in ALS. Mitochondrial quality control mainly includes mitochondrial fission, fusion and mitophagy [11]. Normally, the factors of mitochondrial quality control interact with each other, which are very crucial to the function of mitochondria [12].

Mitochondrial fission and fusion are two opposite processes, which are initially discovered in yeast. Fusion is controlled by mitofusins 1 and 2 (Mfn1 and Mfn2) located in the outer membrane and optic atrophy 1 (OPA1) located in the inner membrane or inter-membrane space of mitochondria [9]. Besides fusion controlling, OPA1 can also stabilize mitochondrial cristae, increase the stability of cytochrome C in the gap of mitochondrial membrane, and protect cells from apoptosis by inhibiting the release of cytochrome C [13]. Fusion serves as a functional complementation, in which mitochondria have no severe loss of functions and can borrow some necessary components such as matrix proteins and DNA to repair the damage by fusing with "healthy" mitochondria [14]. It is the first line of defense prior to autophagy for damaged mitochondria. Fission is controlled by Drp1 that exists constitutively in the cytosolic pool and is recruited to the mitochondrial membrane after phosphorylation. The mitochondrial fission 1 (Fis1) protein located in the outer membrane acts as the downstream of Drp1 recruitment. Phosphorylation at Ser616 is potential to mitochondrial fission [15, 16]. Fission can generate uneven daughter units: one exhibits increased membrane potential and a high probability of subsequent fusion, while the other reveals the decreased membrane potential and a high probability of degeneration by mitophagy. Mitochondrial fission and fusion regulate mitochondrial morphology, distribution and function. Mutation of the genes for controlling fission and fusion can lead to mitochondrial dysfunction and neurodegenerative diseases [17-20]. In Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and other neurodegenerative diseases, mitochondrial dysfunction has already been found, and the imbalanced fission and fusion have also been confirmed [21]. However, whether mitochondrial fission and fusion are normal processes in ALS and their impact on the development of ALS are still not clear.

In mammals, autophagy can remove abnormal proteins and damaged organelles such as mitochondria and endoplasmic reticulum, which is important to maintain physiological function of cells [22]. Severely damaged mitochondria need to be removed from the cycling pool of organelles to maintain the overall mitochondrial health of the cells. This process is called mitophagy [23]. Removing damaged and aged mitochondria in time can effectively prevent the release of pro-apoptotic proteins and the production of ROS, which are key factors determining the health status of cells. Microtubule-associated protein 1 light chain 3 isoform II (LC3-II) is tightly bound to both the internal and external surfaces of the autophagosomal membranes and serves as an autophagic marker protein [24]. The p62 is closely related to maturation of autophagosomes, and can serves as a bridge between LC3-II and ubiquitin-conjugated cargos. In the process of mitophagy, p62 binds to LC3-II and ubiquitin-modified mitochondria for mitochondrial transport to autophagy vacuoles (AVs) [22]. Mitophagy is crucial to the renewal of mitochondria, and impaired mitophagy can lead to a variety of degenerative diseases. In HD, the recognition capability of AVs to mitochondria is reduced, thus leading to the abnormal accumulation of mitochondria, neuronal degeneration and necrosis [25]. In PD, it is also found to be closely correlated with to the mutation of Parkin that controls mitophagy [21]. In addition, the aggregation of LC3-II and p62 in MNs of ALS has been observed, suggesting that autophagy flux may be impaired in ALS [26, 27]. In ALS, the autophagic pathway was impaired. However, is mitophagy an exception?

Mitochondrial fission, fusion and mitophagy also have cross-interaction. Mitochondrial fission can trigger mitophagy, and mitophagy can regulate mitochondrial fission [8, 28, 29]. Overexpression of Fis1 can significantly increase mitophagy [30]. The reduction of OPA1 indicates that mitochondria have already been surrounded by AVs or will be surrounded by AVs [8]. On the other hand, the knockdown or inhibition of these genes can result in the inhibition of mitophagy and ROS generation. But the gener-

ation of ROS almost disappears [11, 31]. All above findings indicate that mitochondria fission is closely correlated with mitophagy. Rapamycin can induce autophagy by inhibiting mammalian target of rapamycin (mTOR) so that it is often used as autophagy enhancer [32]. In the present study, we have explored the possible mechanisms for mitochondrial dysfunction in terms of mitochondrial fission, fusion and mitophagy in the process of ALS.

By studying mitochondrial fission, fusion and mitophagy in ALS, we have found that mitochondrial quality control was really impaired, and then the impaired mitophagy flux and the consequent imbalance of mitochondrial quality control led to a series of pathological changes, which are all reflected in the incidence and process of ALS. These findings may be the fundamental for understanding the mechanisms of this disease and also help to identify the therapeutic targets in ALS.

Materials and methods

Mice

In order to conduct the experiment, we chose the transgenic hSOD1-G93A mice and their non-transgenic counterparts, among which the former onces were the hybrids of the female B6SJLF1 and male hemizygous carriers [B6SJL-Tg (SOD1-G93A) 1Gur/J] from the Jackson Laboratories (002726), identified by PCR-based genotyping of tail/blood DNA. Appropriate food, water and light were provided to keep them alive. They were maintained under standard accredited housing conditions with enough light and appropriate temperature and humidity; supplied with nutritional food after strict disinfection and clean water. The procedure was monitored by trained staffs to ensure the best care. According to the pathological stages, ALS mice (male) were divided into 3 groups: 60-day-old group, onset group (90-110 days of age, when the earliest abnormal gait was shown), end stage group (130-150 days, when they couldn't stand up by themselves in 30 s after being placed on either their back or side) and one control group of wild-type mice (120 days, non-transgenic mice) (n = 12, each group). Then each group was further equally divided into 4 subgroups with 3 mice in each subgroup. These 4 subgroups were used for protein analysis, immunohistochemical analysis, electronic microscopy (EM) study, and mitochondrial protein analysis, respectively. Mice were deeply anesthetized and received CO_2 inhalation before cervical dislocation. Then whole spinal cord was removed. All procedures are approved by the Ministry of Science and Technology of the People's Republic of China, and are in accordance with the guidelines of NIH for the Care and Use of Laboratory Animals.

Rapamycin intervention

Rapamycin (Sigma, R0395) was dissolved in dimethyl sulfoxide (DMSO) (25 mg/ml) and further diluted with 1:200 (v/v) NS before intraperitoneal (ip) injection [27]. ALS transgenic mice (female) born on the same day were randomly divided into 2 groups: Rapamycin intervention at the dose of 2 mg/kg body weight/day in ALS mice (ALS-Rapa, n = 3) and vehicle intervention (NS or DMSO) in ALS mice (ALS-vehicle, n = 3) was conducted. The injection started on the 90th day and terminated on the 97th day and the injection was conducted once a day. Decapitate the mice; mitochondria of the lumbar spinal cords were isolated using Percoll density gradient centrifugation for further protein analysis.

Western blot

Whole tissue from the lumbar spinal cords was prepared utilizing a total protein extraction kit (Applygen). The protein concentration was determined by using Bradford method. The proteins were sequentially separated on 10% or 12% SDS-polyacrylamide gels, transferred to PVDF membranes and blocked with 5% milk in phosphate-buffered saline (PBS), The membranes were incubated by using corresponding antibodies such as LC3-II (1:1000; Sigma, L7543), OPA1 (1:800; Abcam, ab42364), Fis1 (1:500; Abcam, ab71498), Mfn1 (1:200; Santa Cruz, sc-50330), p-Drp1 (1:600; Cell signaling, 3455), p62 (1:500; Sigma, P0067), SOD-1 (1:1000; Santa Cruz, sc-8673), actin (1:500; Santa Cruz, sc-81178), COX-1 (1:500; Mitosciences, MS105) and VDAC (1:2000; Abcam, ab14734), respectively. After an overnight incubation, the membranes were washed three times with TPBS, and then incubated with appropriate secondary antibodies (anti-rabbit IgG, 1:5000; Rockland, 23641 or anti-mice IgG, 1:5000; Rockland, 21056). The film was scanned with an Odyssey Infrared Imaging

System (LI-COR, Lincoln) and protein level was analyzed by Odyssey Infrared Imaging System Version 2.1.12.

Confocal microscopy

The lumbar spinal cords were fixed in 30% sucrose-4% paraformaldehyde solution before slicing with a Leica CM1850 freezing microtome. The frozen sections were washed three times with PBS, permeabilized with 0.3% Triton X-100 in PBS for approximately 20 min at room temperature, blocked with 10% equine serum in PBS for 30 min and then incubated with primary antibodies such as LC3-II (1:800; Sigma, L7543), OPA1 (1:500; Abcam, ab42364), Fis1 (1:500; Abcam, ab71498), Mfn1 (1:200; Santa Cruz, sc-50330), VDAC (1:2000; Abcam, ab-14734), SMI32 (1:2000; Convance, SMI-312R) and GFAP (1:500; Santa Cruz, sc-51908) diluted in 50% 0.3% Triton X-100-TBS at 4°C overnight with shaking, respectively. On the next day, the sections were washed three times with PBS, and then incubated with secondary antibodies (FITC conjugated goat anti-rabbit and Cy5 conjugated horse anti-mouse antibodies) in TBS in dark for 1 h at room temperature. The slides were analyzed by fluorescent confocal microscopy (Olympus FV1000).

Immunohistochemistry

The lumbar spinal cords fixed with 4% paraformaldehyde were cut into 20 μm sections by a Leica VT 1000S vibratome. The sections were washed three times with PBS, and then soaked in methanol with 3% H_2O_2 for 15 min. Then the sections were washed again and perforated with 0.3% Triton X-100 for 30 min. After blocked with 10% equine serum in PBS for 45 min, the sections were incubated with primary antibodies such as LC3-II (1:800; Sigma, L7543), OPA1 (1:500; Abcam, ab42364), Fis1 (1:500; Abcam, ab71498) and Mfn1 (1:200; Santa Cruz, sc-50330) diluted in 10% equine serum in PBS at 4°C overnight. Then further incubation with biotin-conjugated secondary antibodies in PBS for 2 h at room temperature, and final incubation with HRP-conjugated streptavidin for 1 h at room temperature were performed. The sections were treated with DAB colorimetric reagents and then analyzed by light microscopy (Nikon 50i).

Electron microscopy

The tissue was fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate and stored at 4°C until embedding and tomography. Conventional and tomographic electron microscopies were carried out.

Mitochondrial preparation

Mitochondria of the lumbar spinal cord were isolated from four groups of mice as described above using Percoll density gradient centrifugation with slight modification [33]. Rapidly removed the spinal cord and abandon the meninges. The tissue was cut into small pieces and homogenized in a dounce homogenizer. Samples were centrifuged at 30,700 g at 4°C for 5 min. The upper part was reserved as cytoplasm without mitochondria for further study and the lower precipitation was mixed with digitonin (Sigma, D141) solution, and then layers them on discontinuous gradients consisting of 19% Percoll, layered over 40% Percoll. Centrifugation was conducted at 30,700 g and 4°C for 10 min, and the band was enriched mitochondrial fraction. The mitochondrial fraction was diluted in isolation buffer and centrifuged for 10 min at 16,7000 g. The resuspended solution in isolation buffer was centrifuged at 6900 g for 10 min and the supernatant was discarded. Finally, the pellet was gently resuspended in 100 μL of RIPA. The concentration of mitochondria and cytoplasm was assessed by Bradford method for further protein analysis. Purity of mitochondrial fractions was evaluated via Western blot to explore cytochrome oxidase subunit I, VDAC and actin as mitochondrial and cytosolic markers, respectively. Cytochrome oxidase or VDAC was clearly expressed in mitochondrial fractions, while actin was undetectable, indicating the presence of highly purified mitochondria.

Statistical analysis

The data were analyzed using one-way ANOVA and the significant difference was considered at $P < 0.05$. All data were expressed as Mean ± SD.

Results

Abnormal mitochondrial morphology in the lumbar spinal cords of SOD1-G93A transgenic mice

It has been reported that mitochondrial morphology and function were abnormal both *in vitro* and *in vivo* models of ALS [5, 6]. As expect-

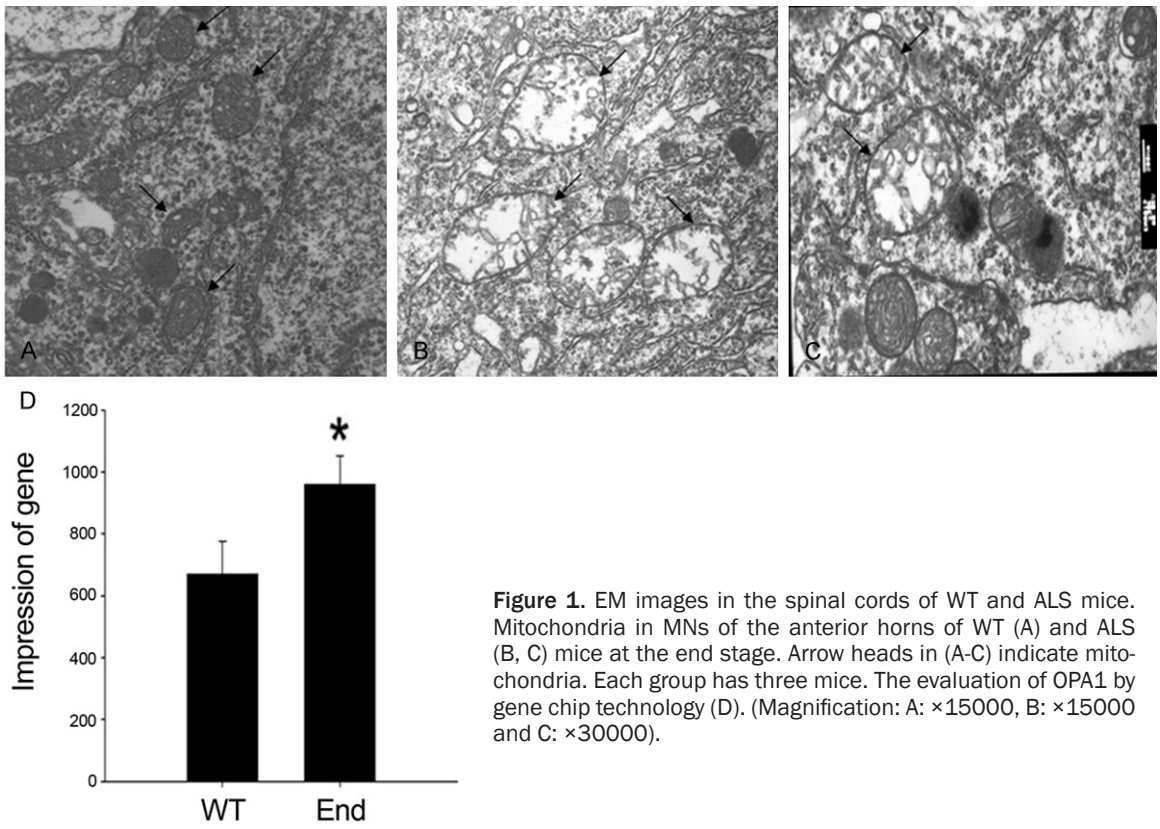

Figure 1. EM images in the spinal cords of WT and ALS mice. Mitochondria in MNs of the anterior horns of WT (A) and ALS (B, C) mice at the end stage. Arrow heads in (A-C) indicate mitochondria. Each group has three mice. The evaluation of OPA1 by gene chip technology (D). (Magnification: A: ×15000, B: ×15000 and C: ×30000).

ed, we also monitored the same change of mitochondria in the lumbar spinal cords of SOD1-G93A transgenic mice.

In order to explore mitochondrial histopathology, we conducted ultra-structural analysis with EM. For the 60 d group of ALS mice, morphological abnormalities of mitochondria in MNs of the lumbar spinal cords were not obvious. While for the onset and final stages of ALS mice, some typical changes of mitochondria such as vacuolization and swelling were observed, and the cristae disappeared (**Figure 1A-C**).

Based on the abnormal morphology of mitochondria observed in ALS mice, several components of the fusion and fission machinery that regulate mitochondrial morphology, distribution and function, were examined in ALS mice at different stages.

Accumulation of imbalanced mitochondrial fission and fusion and abnormal mitochondria in MNs of SOD1-G93A transgenic mice

In order to investigate whether mitochondrial fission and fusion were altered in the lumbar spinal cords, we measured several dynamic proteins including OPA1, Mfn1, p-Drp1 and Fis1 by western blot and immunostaining in the lumbar spinal cords of ALS mice at different stages and imbalanced mitochondrial fission and fusion were observed:

1) There was a remarkable descent of protein levels of OPA1 and Mfn1 in MNs of the lumbar spinal cords of ALS mice at onset and final stages of the disease, but for the 60 d group, the protein level of Mfn1 had no change, and the protein level of OPA1 revealed an increase (**Figure 2A**, **2B**, **2E** and **2F**). Using gene chip technology (previous works in our laboratory), we also found that the expression of OPA1 was down-regulated gradually with the advance of ALS (**Figure 1D**). Immunofluorescence (IF) for OPA1 and Mfn1 in SMI32-labeled MNs and immunohistochemistry (IHC) for OPA1 in the lumbar spinal cords of ALS mice revealed lower immunoreactivity of OPA1 and Mfn1 in MNs at onset and final stages of the disease (**Figure 3A-C**, **3E**, **3I** and **3J**). These findings were consistent with the results of Western blot for exploring OPA1 and Mfn1. Reduced protein levels of OPA1 and Mfn1 in MNs suggested that

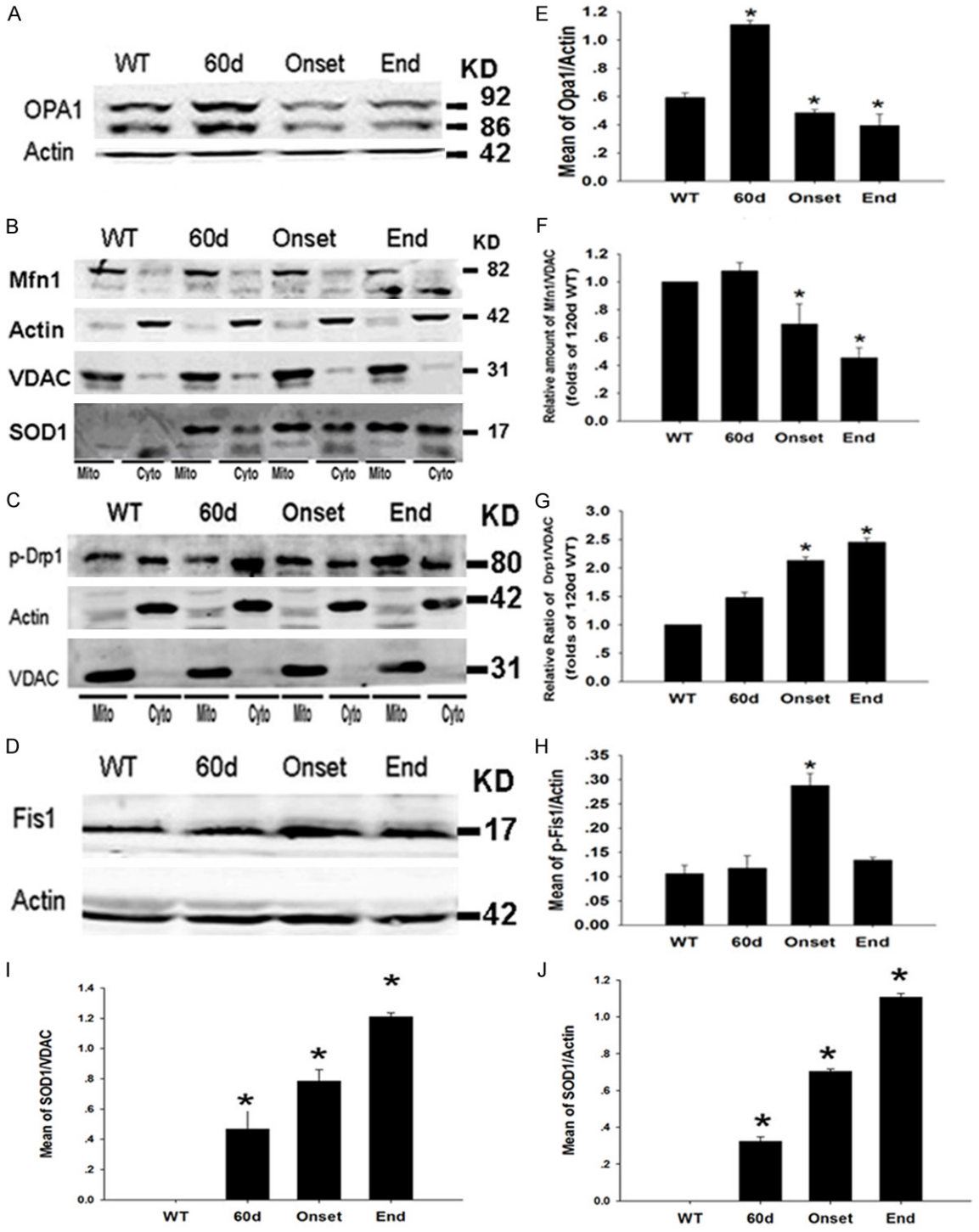

Figure 2. Analysis of fission and fusion proteins in the lumbar spinal cords of WT and ALS mice at different stages. Western blot analysis for protein levels of OPA1 (A), Mfn1 (B), p-Drp1 (C), Fis1 (D) and mutant SOD1 (B) in the spinal cords. Quantitative analysis of OPA1 relative to b-actin (E: $*P < 0.05$, total protein), Mfn1 relative to VDAC (F: $*P < 0.05$, isolated mitochondria), p-Drp1 relative to VDAC (G: $*P < 0.05$, isolated mitochondria), Fis1 relative to b-actin (H: $*P < 0.05$, total protein), mutant SOD1 relative to VDAC (I: $*P < 0.05$, isolated mitochondria) and mutant SOD1 relative to actin (J: $*P < 0.05$, isolated mitochondria) in the lumbar spinal cords. Mito = mitochondrial fraction, cyto = cytoplasm without mitochondria.

mitochondrial fusion present a gradual decreasing trend during the development of disease and the accumulation of OPA1 and Mfn1-deficient mitochondria in MNs of ALS mice.

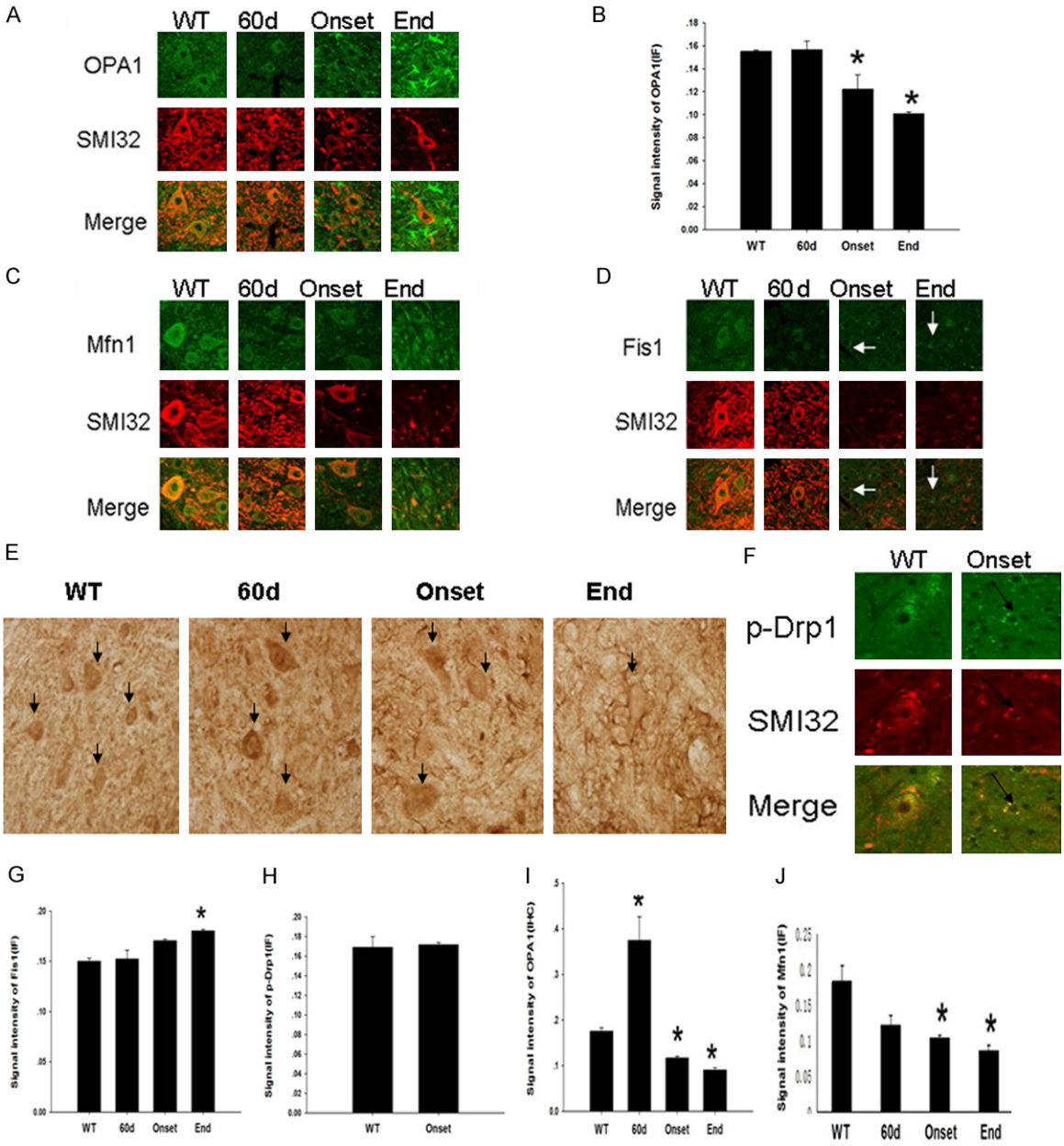

Figure 3. Immunochemical analysis of fission and fusion proteins in SMI32-labeled MNs in the spinal cord anterior horns of the WT and ALS mice. Double labeling of OPA1 and SMI32 in WT and ALS mice at different stages (A), double labeling of Mfn1 (C), Fis1 (D), p-Drp1 (F) and SMI32 in WT and ALS mice. Immunochemical analysis of OPA1 in WT and ALS mice at different stages (E). Quantitative analysis of immunoreactivity of OPA1 (B and I), Mfn1 (J), Fis1 (G) and p-Drp (H) in MNs. Arrow heads indicate Fis1 and p-Drp1 positive foci in (D and F). Arrow heads indicate OPA1 positive MNs in (E).

2) Protein levels of p-Drp1 and Fis1 in the lumbar spinal cords of ALS mice at onset and final stages of the disease increased, but at the stage of 60 d, the protein levels of p-Drp1 and Fis1 had no changes (**Figure 2C**, **2D**, **2G** and **2H**). IF analysis of Fis1 and p-Drp1 in MNs in the lumbar spinal cords of ALS mice revealed that p-Drp1 and Fis1 immunofluorescence turned into a population of puncta in SMI-32-labeled MNs at the onset and final stages of ALS mice, while p-Drp1 and Fis1 immunofluorescence lightly distributed homogeneously in the cytoplasm of MNs in WT mice and 60 d ALS mice (**Figure 3D**, **3F**). Quantitative analysis

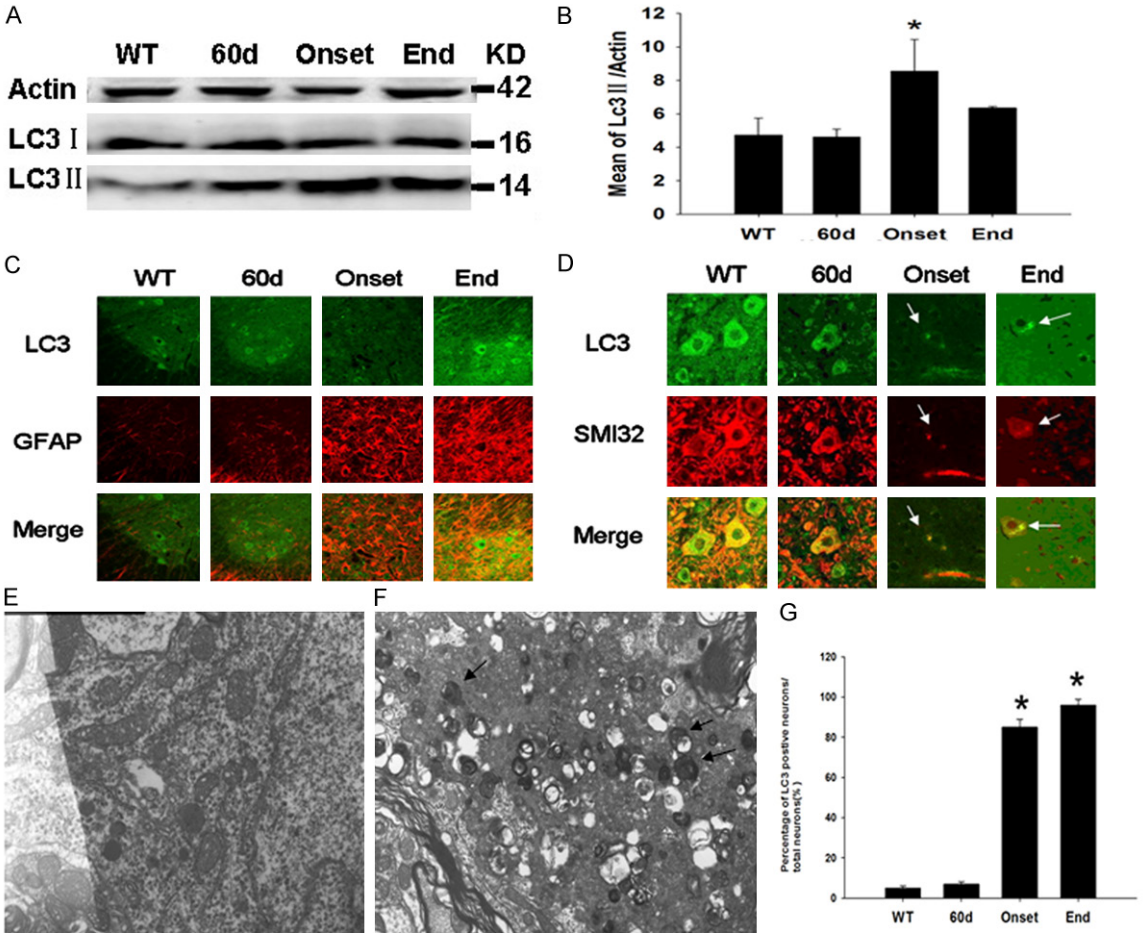

Figure 4. Analysis of LC3 and EM images of lysosomes in MNs of WT and ALS mice. Western blot analysis for protein levels of LC3-II (A) in the spinal cords. Quantitative analysis of LC3-II relative to b-actin in the spinal cords (B: $*P < 0.05$). Immunochemical analysis of LC3 in the spinal cords of WT and ALS mice, double labeling of LC3 and SMI32 (D), double labeling of LC3 and GFAP in WT and ALS mice at different stages (C). The EM images of MNs in WT (120 d, E) and ALS mice (F) at the end stage; arrow heads in F indicate accumulated lysosomes in MNs. Quantitative analysis of LC3 puncta-positive neurons in the spinal cord of ALS (G). Arrow heads in (D) indicate LC3 positive foci. (Magnification: E: ×12000, F: ×12000).

showed that the immunoreactivity of Fis1 in MNs was increased at the final stage when compared with WT mice. Although at the onset stage, there was an increasing trend, no statistically significant difference was observed (**Figure 3G**). Similarly, no statistically significant difference in immunoreactivity of p-Drp1 in MNs at the onset stage was observed either when compared with WT mice (**Figure 3H**). The total protein level of p-Drp1 had no change, but the population of p-Drp1 targeted to mitochondrial membrane revealed an increase. The above findings indicated that mitochondrial fission was excessive and the mitochondria rich in p-Drp1 and Fis1 were accumulated in MNs of ALS mice.

3) Protein level of mutant SOD1 in mitochondria revealed an increasing trend, meanwhile, mutant SOD1 located in cytosolic fraction also exhibited an increasing trend in the lumbar spinal cords of ALS mice from the stage of 60 d to final stage of the disease (**Figure 3I**, **3J**). These findings illustrated the accumulation of mutant SOD1 in mitochondria and cytosol, and the impaired degeneration of mutant SOD1.

Mitochondrial fission can trigger mitophagy, and mitophagy can regulate mitochondrial fission [8, 28, 29]. It has been reported that overexpression of Fis1 can significantly increase mitophagy [30]. The reduction of OPA1 suggests that mitochondria have already been sur-

rounded by AVs or will be surrounded by AVs [8]. Therefore, the accumulation of mitochondria lacking of OPA1/Mfn1 and rich in p-Drp1/Fis1 and the accumulation of mutant SOD1 in mitochondria and cytosol indicate that autophagy is impaired in ALS mice. Our results indicated that the excessive and imbalanced fission of mitochondria was closely related with the development of the disease. Impaired mitochondrial fission and fusion might be responsible for abnormal mitochondrial function and morphology that have been reported in ALS. In order to explore the role and function of mitochondrial quality control in ALS, autophagy and mitophagy should be considered as the key factors.

Increase of LC3-II and AVs in MNs of the lumbar spinal cords of SOD1G93A mice during the progress of ALS

LC3-II level in our transgenic mice was testified by western blot. Quantitative analysis showed that the protein level of LC3-II in the spinal cords of ALS mice was increased at the onset stage, and at the final stage when compared with WT mice, however, no statistically significant difference was observed. Similarly, no significant change between 60 d ALS mice and WT mice was observed either (**Figure 4A**, **4B**).

IF analysis showed that LC3 immunofluorescence revealed a population of puncta in SMI-32-labeled MNs at the onset and final stages, while LC3 immunofluorescence lightly distributed homogeneously in the cytoplasm of MNs in WT mice and 60 d ALS mice (**Figure 4D**). Increased population of LC3 positive neurons was detected at the onset stage, and revealed a significant difference at the end stage (**Figure 4G**). Double staining of LC3 and glial cell marker GFAP showed that GFAP positive cells did not have LC3 green foci (**Figure 4C**). Our results suggested that LC3 could increase in MNs but not in glial cell of ALS mice.

In order to explore whether there is an accumulation of AVs in MNs in SOD1 G93A transgenic mice, we conducted EM analysis to reveal a lot of AVs and lysosomal in the axon of MNs at the onset and final stage of ALS (**Figure 4E**, **4F**).

Impaired mitophagy flux in ALS, and LC3-II translocation from cytosol to mitochondria

In order to further clarify the situation of mitophagy, we conducted the exploration of mitophagy by different methods.

1) IF for VDAC, a mitochondrial marker, revealed various populations f VDAC-positive mitochondrial clusters in MNs at the onset and final stages of ALS, but not in WT mice and 60 d ALS mice (**Figure 5B**). Double staining of LC3 and VDAC showed that VDAC immunopositive clusters co-localized with LC3-positive puncta in MNs of ALS, indicating that the development AVs' recognition of mitochondria was normal. With the advance of ALS, the co-localization clusters of LC3-II and VDAC was obvious and the population of neurons containing LC3 puncta that co-localized with VDAC was also increasing as the development of ALS (**Figure 5B**, **5F**).

2) The protein levels of LC3-II and p62 were detected in the isolated mitochondria by western blot to observe the combination of these proteins. At the disease stage of 60 d, LC3-II and p62 were mainly located in cytosolic fraction, and there was no significant difference in LC3-II and p62 when compared with WT mice. However, the protein levels in the lumbar spinal cords were increased at the onset and final stage of ALS mice (**Figure 5A**, **5C** and **5D**).

3) With the elevation of LC3-II and p62 combined with mitochondria in the spinal cords, LC3-II in the cytosolic fraction revealed a decrease from the onset stage, and the reduction was more apparent at the final stage (**Figure 5A**, **5E**). Abnormal distribution of LC3-II between mitochondrial and cytosolic fractions was observed, indicating that the shift of LC3-II from cytosol to mitochondria might be somewhat related to the accumulation of mutant SOD1 in cytosol.

4) Through EM analysis, the accumulation of mitochondria surounded by AVs in ALS mice at the final stage was detected (**Figure 5G**, **5H**).

Taking protein level of p62 as the marker of autophagic flux, p62 is combined with mature autophagic vesicles and is degraded within lysosomes [34]. On the basis of these studies, the accumulation of p62 and LC3-II in mitochondria was detected. The accumulation of LC3-II in mitochondria revealed the increase of mitophagy; meanwhile, the degradation of p62 within lysosomes was reduced, suggesting that mitophagy flux might be impaired in ALS. The accumulation of mitochondria surrounded by

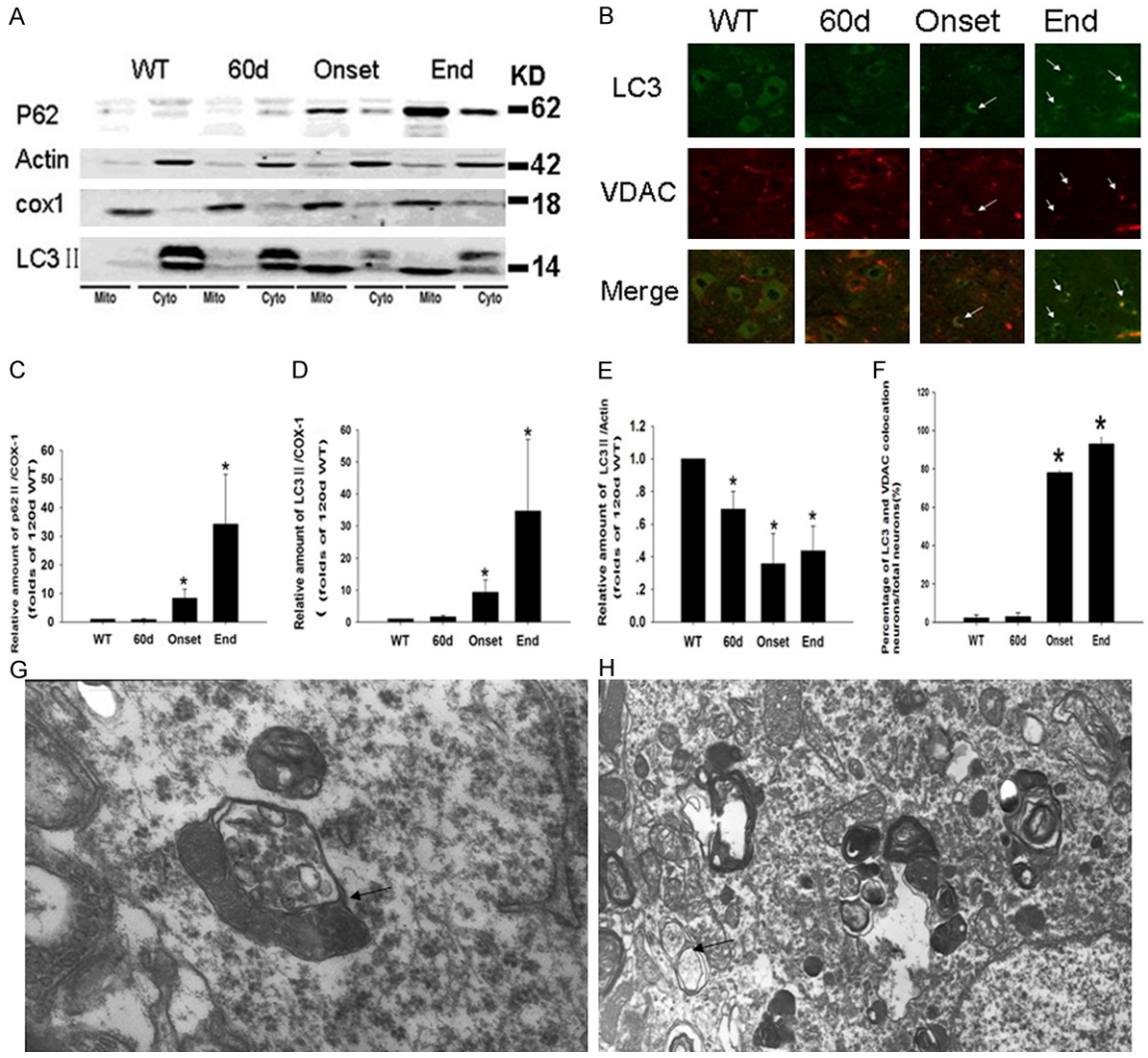

Figure 5. The EM images of mitochondria and AVs in ALS mice at the end stage and the distribution of LC3-II and p62 in mitochondrial and cytosolic fractions in the lumbar spinal cords of WT and ALS. Isolation of mitochondria, western blotting analysis for protein levels of LC3-II and p62 in mitochondria and cytosolic fractions of ALS at different stages (A). Double labeling of LC3 and VDAC in WT and ALS mice at different stages (B). Quantitative analysis for p62 relative to VDAC in mitochondria of the lumbar spinal cords (C: $*P < 0.05$); quantitative analysis of LC3-II relative to VDAC in mitochondria (D: $*P < 0.05$); quantitative analysis of LC3-II relative to b-actin in cytosolic fractions (E: $*P < 0.05$). Quantitative analysis of neurons containing LC3 puncta that co-localize with VDAC in the spinal cords of ALS mice (F). Mitochondria were degenerated by autolysosomes (G); Arrowheads in (H) indicate mitochondria surrounded by AVs. Arrowheads in (B) indicate LC3 and VDAC positive foci. Mito = mitochondrial; cyto = cytoplasm without mitochondria.

AVs was also convicted due to the abnormal distribution of autophagy flow and the accumulation of mitochondrially-targeted autophagosomes. The increased autophagosomes in ALS could contribute to the accumulation of mitochondrially-targeted autophagosomes. Mutant SOD1 was mainly located in the cytoplasm; therefore, there may be no correlation between mutant SOD1 and autophagic reaction.

Significant improvement of excessive and imbalanced fission of mitochondria as well as promotion of mitophagy in the presence of rapamycin

In order to further clarify the correlation between excessive mitochondrial fission and abnormal mitophagy, we explored the correlation between the distribution of autophagy flow

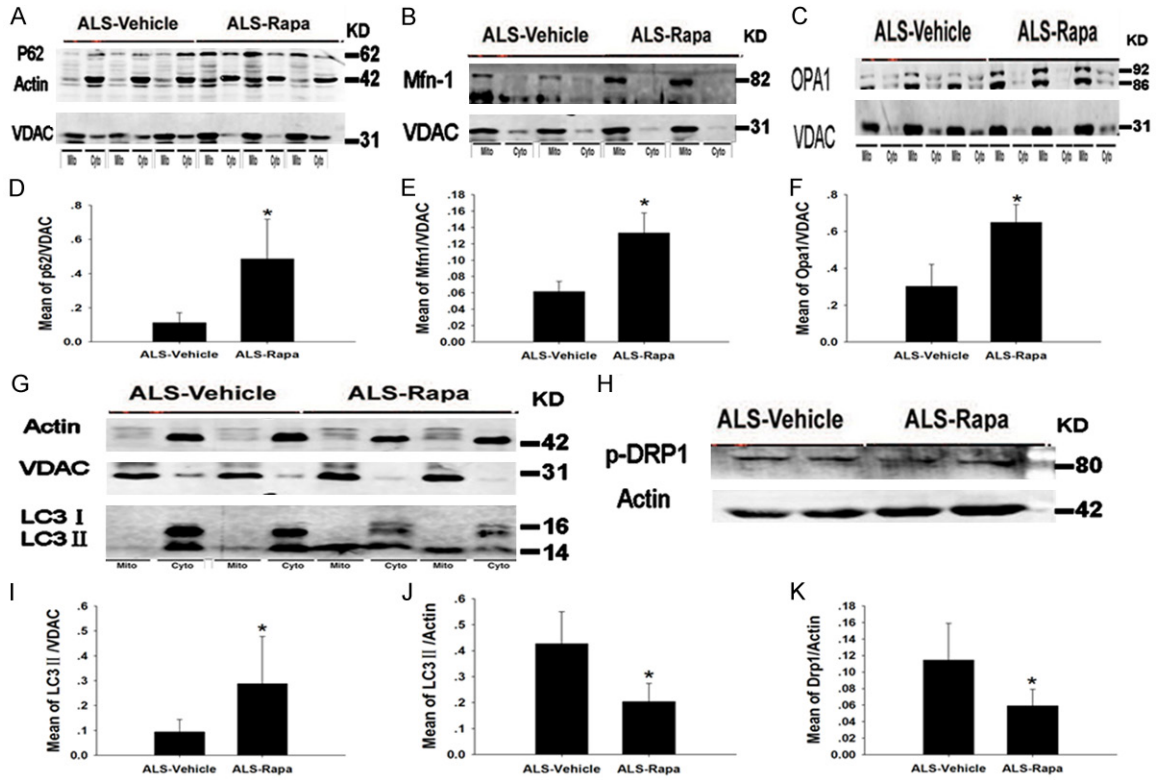

Figure 6. Analysis of fission/fusion proteins and the distribution of LC3-II and p62 in mitochondria and cytosolic fractions in the lumbar spinal cords of ALS-vehicle and ALS-Rapa mice. Western blotting analysis for protein levels of p62 (A) and LC3-II (G) in mitochondria and cytosolic fractions. Western blot analysis for protein levels of OPA1 (C), Mfn1 (B) and p-Drp1 (H) in the lumbar spinal cords. Quantitative analysis of p62 relative to VDAC in mitochondria (D: $*P < 0.05$), Mfn1 relative to VDAC (E: $*P < 0.05$), OPA1 relative to VDAC (F: $*P < 0.05$), p-Drp1 relative to b-actin (K: $*P < 0.05$), LC3-II relative to VDAC in mitochondria (I: $*P < 0.05$), LC3-II relative to b-actin (J: $*P < 0.05$). Mito = mitochondrial; cyto = cytoplasm without mitochondria.

and the aggregation of mutant SOD1. The ALS mice with short-term intervention of rapamycin revealed following findings:

1) Compared with the ALS-vehicle mice, the expression of p-Drp1 was down-regulated in ALS-Rapa mice, while the expression of OPA1 and Mfn1 was gradually enhanced. These results suggested that rapamycin could significantly improve excessive and imbalanced fission of mitochondria and promote mitochondrial fusion in ALS (**Figure 6B**, **6C**, **6E**, **6F**, **6H** and **6K**).

2) Compared with the ALS-vehicle mice, the expression of p62 and LC3-II in mitochondria was up-regulated, but the protein level of LC3-II in cytosol was decreased in ALS-Rapa mice. These results showed that more accumulation of mitochondrially-targeted autophagosomes was observed under the intervention of rapamycin, suggesting the process of impaired mitophagy in ALS mice (**Figure 6A**, **6D**, **6G**, **6I** and **6J**). Rapamycin as a classic autophagic activator could significantly improve the excessive and imbalanced fission of mitochondria, indicating that the excessive fission of mitochondria was closely correlated with impaired mitophagy flux in ALS.

Discussion

Amyotrophic lateral sclerosis is a progressively degenerative disease characterized by selective motor neuron death; however, its causes are still not clear. Increasing evidences have shown that mitochondrial dysfunction plays an important role in the pathogenesis of ALS as it is the converging point of multiple pathological pathways in ALS. Mitochondrial quality control including mitochondrial fission, fusion and mitophagy is closely related to mitochondrial morphology, distribution and function [8]. The imbalance of mitochondrial quality control can lead to ultra-structural change of mitochondria [10], which are extremely similar to mitochon-

dria in ALS. Moreover, mitochondrial quality control is also closely related to other neurodegenerative diseases such as AD and PD [35].

Mitochondrial dysfunction has been confirmed in ALS [5, 6]. In the present study, we also have gained a preliminary understanding of the occurrence of abnormal mitochondria during the development of this disease. Some studies report its attribution of the poisonous mutant SOD1, but mitochondrial morphology and function are closely related to fission and fusion. In order to explore the molecular basis of mitochondrial dysfunction in ALS, the expression of fission proteins (p-Drp1/Fis1) and fusion proteins (Mfn-1/OPA1) was evaluated through western blot and immunostaining methods. The expression of abnormal mitochondrial fission/fusion in MNs at different stages was also measured. At the disease stage of 60 days, the increased protein level of OPA1 suggested that mitochondrial fusion was more than mitochondrial fission, and that would contribute to the great benefit of the fusion to the mitochondria at the early stage of mitochondria damage. At the onset stage, mitochondrial fission exceeded mitochondrial fusion. At the end stage, the expression of fission proteins became increasingly higher, but excessive mitochondrial fission in MNs could result in a series of side-effects on MNs, thus leading to the loss of MNs [10]. Impaired mitochondrial fission, fusion might be responsible for abnormal mitochondrial function and morphology that have been reported in ALS. Compared with the onset stage, the level of Fis1 in the lumbar spinal cords was slightly reduced, but it was still higher than that in WT mice. The slight reduction of Fis1 at the end stage might contribute to the loss of MNs, because the remained neurons were rich in Fis1. Through the evaluation by gene chip technology, the decreased expression level of mitochondrial fusion genes provided the information that excessive fission of mitochondria might be natural reaction and self-regulation in the body. The excessive fission is of great benefit to trigger mitophagy, initiation of mitochondrial quality control and protection of mitochondria [8, 36]. At this situation, the quality control of intact mitochondria is used as a protective mechanism of the cells. Similarly, if one part of mitochondrial quality control is damaged (for example, impaired mitophagy), the cell will result in the exacerbation of mitochondrial fission by a negative feedback loop for making up mitophagy disorders because mitochondrial fission has a strong role in promoting mitophagy. However, at this situation, the quality control of mitochondria becomes an “accomplice” of ALS. The accumulation of dysfunctioned mitochondria and mutant SOD1 in MNs indicate that autophagy or mitophagy is impaired in ALS mice. In order to understand the position and role of mitochondrial quality control in ALS, we not only need to know the change in mitochondrial fission and fusion, but also understand the downstream of mitochondrial quality control-mitophagy.

The conversion of unconjugated LC3-I to LC3-II is a useful indicator of autophagy so that the process can be monitored by its change in subcellular distribution (from diffusion to puncta appearance in AVs) and the observation of molecular weight shift upon Western blot analysis [24]. Autophagy flux is monitored by protein level of p62, which is associated with mature AVs and is degraded within autolysosomes. Therefore, its level can be used as a surrogate measurement of autophagic vesicle lysosome fusion, or autophagic flux [22]. As reported, the change of autophagy, such as the increased protein level of LC3-II, the increased population of LC3 positive MNs and the accumulation of autophagosomes can further illustrate the impaired autophagy flux in ALS. In our study, the accumulation of mitochondria lacking OPA1 or rich in p-Drp1 in MNs of ALS mice has confirmed the impaired mitophagy flux. In order to monitor the recognition of AVs and conjugation to mitochondria, the co-localization of AVs and mitochondria was observed by laser confocal microscopic technology, and the protein level of LC3-II and p62 in mitochondria and cytoplasm was measured by western blot in isolated mitochondria. For the ALS mice at the 60 days stage, no obvious co-localization of LC3 and VDAC was observed. Compared with the WT mice, there were no significant change in LC3-II and p62 in mitochondria, and the protein level of LC3-II and p62 in the cytoplasm had no significant change at this stage. Our results showed that there was no accumulation of LC3-II and p62 in mitochondria, and mitophagy flux might be normal at this stage. At the onset stage of ALS mice, the increased co-localization of LC3 and VDAC was observed, meanwhile, an accumulation of p62 and LC3-II in

mitochondria was also observed, suggesting the increase in the binding of p62 and ubiquitin-modified mitochondria, but mitophagy flux was impaired and there was an accumulation of mitochondrially-targeted autophagosomes. Besides, at this stage, LC3-II in cytoplasm begins to decline, while LC3-II in mitochondria reveals an increase, which suggests the abnormal distribution of AVs. At the end stage, the level of LC3-II and p62 in mitochondria revealed the continuous increase, indicating that continuous accumulation of mitochondrially-targeted autophagosomes resulted in more serious mitophagy disorders. In contrast, LC3-II in the cytoplasm exhibits the further reduction, suggesting that the increased shift of AVs from cytoplasm to mitochondria resulted in the abnormal distribution of autophagy flow as the development of ALS. Mutant SOD1 in cytoplasm increased gradually with the process of the disease, but autophagy flow in cytoplasm revealed a decreasing trend, indicating that the decline of autophagy flow were correlated with the accumulation of mutant SOD1 in cytoplasm. Originally, it is believed that autophagy is a non-selective process with random engulf of cytosolic components and organelles [37, 38], but recent studies have revealed that the time points when the cargos were surrounded by AVs were different, indicating that the choice of "cargos" was procedural [22]. So the abnormal distribution of autophagy flow can be attributed to the priority choice of AVs to the mitochondria or the accumulation of mitochondrially-targeted autophagosomes caused by impaired mitophagy, which, in turn, may result in insufficient degradation of abnormal proteins in the cytoplasm, and lead to further accumulation of mutant SOD1 in the cytoplasm. At the same time, our results also confirmed that the elevation of protein level of LC3-II and the accumulation of AVs in MNs of ALS mice had no direct correlation with the mutant SOD1 in the cytoplasm, and the accumulation of AVs might be caused by impaired mitophagy.

Both impaired mitophagy and excessive fission of mitochondria can be observed during the whole development of ALS. As the most important components of mitochondrial quality control such as mitochondrial fission, mitochondrial fusion and mitophagy are not independent, but interactive [28, 29]. Theoretically, mitochondrial fission can promote mitophagy, thus, as a result, if mitophagy is impaired, the cell should increase mitochondrial fission by a negative feedback loop for making up the disorder of mitophagy. In order to further clarify the correlation between excessive mitochondrial fission and impaired mitophagy in ALS mice, the distribution of autophagy flow in mitochondria and cytosol after the activation of autophagy is explored in ALS mice with rapamycin intervention at the onset stage of the disease. Rapamycin initially used as an immunosuppressive agent is a classic autophagic activator and can induce autophagy by inhibiting the mammalian target of rapamycin (mTOR) [32]. This study has demonstrated that rapamycin can effectively inhibit excessive fission and promote mitochondrial fusion. Mitophagy is not only necessary for the delivery of mitochondria to lysosomes, but also contributes to mitochondrial fission and fusion [39]. Our results showed that excessive mitochondrial fission was closely related to impaired mitophagy in ALS so that we could conclude that rapamycin could significantly improve imbalanced mitochondrial fission and fusion by autophagy induction. While, the high level of p62 and LC3-II in mitochondria from the lumbar spinal cords of ALS-Rapa mice has demonstrated the more obvious accumulation of mitochondrially-targeted autophagosomes, further indicating the impaired mitophagy in ALS. Although autophagy induction can increase mitochondrially-targeted autophagy flow, the increased autophagy flow in cytosol is still not observed and there is no beneficial effect on the degradation of mutated SOD1, which suggests that the intervention of rapamycin may be no useful for ALS. Conversely, mTOR affected by autophagy activator may increase the content of mutant SOD1 in cytosol due to the shift of autophagy flow. It is also reported that long-term application of rapamycin in SOD1-G93A transgenic mice can cause a slight increase in the content of mutant SOD1 in the lumbar spinal cords [27], which is useful for explaining the abnormal protein aggregation in MNs during the treatments of ALS.

ALS is a chronic neurodegenerative disease, so it is helpful to understand the development of ALS by dynamic observation at different stages. For 60 days of ALS mice, mitochondrial quality control might still be normal. With the advance of ALS, the disorder of mitochondrial quality control is appeared, which can exert a

bad effect on mitochondrial renewal, and then result in a series of pathological changes. The effects of impaired mitochondrial quality control on the physiological function of MNs in ALS are shown as follows: 1. The accumulation of a large number of dysfunctional and OPA1-lacking mitochondria in the cell are susceptible to the release of cytochrome C and apoptosis-inducing factors, thus increasing the sensitivity of the cells to stress stimuli and leading to apoptosis. 2. Excessive and imbalanced mitochondrial fission can result in a series of side-effects on MNs. 3. Abnormal distribution of autophagy flow also can result in the reduced degradation capacity for abnormal proteins in the cytoplasm, thus correspondingly leading to the accumulation of mutant SOD1 in the cytoplasm. In the present study, we have discovered that the impression of mutant SOD1 in mitochondria is increasing as the development of ALS, and the mitochondrial dysfunction may be attributed to the "poisonous" of mutant SOD1 and impaired mitochondria quality control. Although the shift of autophagy flow can cause the relative lack of degradation capacity for abnormal proteins in the cytoplasm and result in the accumulation of mutant SOD1 in the cytoplasm, whether the shift of autophagy flow is a direct reason for the accumulation of mutant SOD1 needs to be further investigated. The relationships among the accumulation of mutant SOD1, impaired mitochondrial dynamics and the shift of autophagy flow also need to be further explored to uncover the underlying mechanisms of impaired mitophagy. Due to impaired mitochondrial fission and fusion as well as mitophagy, mitochondria quality control as a protective function for the cells becomes an "accomplice" of ALS.

A series of pathological changes caused by impaired mitophagy have been confirmed. The increasing recruitment of AVs by mitochondria indicated that the recognition process of AV to mitochondria is normal. We hypothesize that the problem may due to the fusion of AVs with lysosomes or (and) lysosomal dysfunction. Although the underlying mechanisms for impaired mitophagy are still not clear, certain measures can be taken in light of the corresponding pathological changes in ALS. In AD, the excessive mitochondrial fission is observed in ALS, and the application of mitochondrial fission inhibitor midiv-1 can significantly attenuate the progression of disease [40]. In the ALS model, the drug suppressing the degradation of OPA1 can also significantly delay the progression of disease [41]. Impaired mitophagy, excessive mitochondrial fission and the accumulation of mutant SOD1 all involve in the occurrence and progress of ALS. Therefore, the suppression of ALS progression just by inhibiting mitochondrial fission, promoting mitochondrial fusion or promoting autophagy is not enough. If it is possible, the drugs improving mitochondrial dynamics combined with the drugs promoting autophagy may have a surprising potential on ALS treatment.

Disclosure of conflict of interest

None.

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