Three-dimensional analysis of somatic mitochondrial dynamics in fission-deficient injured motor neurons using FIB/SEM

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Abbreviated title: 3D morphology of mitochondrial dynamics

Key words: mitochondria, FIB/SEM, mitophagy, dynamin-related protein 1, motor neuron, nerve injury,

RRID: AB_1500538, AB_591819, AB_1542690, SCR_014305

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Grant information: This study was supported by a Grant-in-Aid for KAKENHI on Innovative Areas (grant number 23111007) and KAKENHI (grant number

25430036, 16H05117) from Japan Society for the Promotion of Science (JSPS), Hibino Kikin (Nagoya University), and a Grant for Basic Science Research Projects from The Sumitomo Foundation.

Abstract

Mitochondria undergo morphological changes through fusion and fission for their quality control, which are vital for neuronal function. In this study, we examined three-dimensional morphologies of mitochondria in motor neurons under normal, nerve injured, and nerve injured plus fission-impaired conditions using the focused ion beam / scanning electron microscopy (FIB/SEM), because the FIB/SEM technology is a powerful tool to demonstrate both 3D images of whole organelle and the intra-organellar structure simultaneously. Crossing of *dynamin-related protein 1 (Drp1)* gene-floxed mice with neuronal injury-specific Cre driver mice, *Atf3*:BAC Tg mice, allowed for Drp1 ablation specifically in injured neurons. FIB/SEM analysis demonstrated that somatic mitochondrial morphologies in motor neurons were not altered before or after nerve injury. However, the fission impairment resulted in prominent somatic mitochondrial enlargement, which initially induced complex morphologies with round regions and long tubular processes, subsequently causing a decrease in the number of processes and further enlargement of the round regions, which eventually resulted in big spheroidal mitochondria without processes. The abnormal mitochondria exhibited several degradative morphologies: local or total cristae

collapse, vacuolization and mitophagy. These suggest that mitochondrial fission is crucial for maintaining mitochondrial integrity in injured motor neurons, and multiple forms of mitochondria degradation may accelerate neuronal degradation.

Introduction

Mitochondrial morphology varies among cell species, with certain cells having their own characteristically shaped mitochondria. Mitochondria can change their morphology by fission and fusion in response to changes of their intracellular and extracellular environments (Chan, 2012). These mitochondrial dynamics are crucial not only for cellular functions, but also for quality control within the mitochondria (Karbowski & Youle, 2003; Okamoto & Shaw, 2005). As the axons and dendrites of neurons are long and have large volumes, the efficient delivery of mitochondria to the tips of these processes is crucial for supplying ATP and for the local buffering of Ca^{2+} (Hollenback & Saxton, 2005; Mac Askill & Kittler, 2010; Sheng & Cai, 2012). In fact, abnormal mitochondrial dynamics cause several neurodegenerative phenotypes in animal models (Knott AB, 2008; Berman et al., 2009; Bilsland et al., 2010; Chan, 2012).

Multiple studies have revealed that neuronal damage induces mitochondrial fragmentation, especially in axons (Cho et al., 2009; Wang et al., 2009; Kiryu-Seo et al., 2010; Song et al., 2013). Recently, we found that the size of mitochondria in axons is reduced in a nerve injury model compared with normal conditions, with faster transportation enabling the effective delivery of

mitochondria to the damaged or regenerating axonal tip (Kiryu-Seo et al., 2016). In the same study, we successfully ablated the fission regulatory protein, dynamin-related protein 1 (Drp1), specifically in injured motor neurons using injury-inducible Cre driver mice, *Atf3*:BAC Tg mice, because the *Atf3* promoter is specifically activated in response to nerve injury (Nakagomi et al., 2003; Kiryu-Seo et al., 2011; Kiryu-Seo et al., 2016). The nerve injury-induced *Drp1*-deficient mice (Drp1 CKO), which showed accelerated neuronal degeneration, had elongated tubular mitochondria in axons and massively enlarged mitochondria in the soma (Kiryu-Seo 2016). However, we believed that the mitochondrial appearance in the soma required further study as the complexity of somatic mitochondria is beyond the capacity of observations using light microscopy.

To analyze more precisely the morphologies of somatic mitochondria in motor neurons before and after nerve injury, we performed three-dimensional (3D) electron microscopy using focused ion beam scanning electron microscopy (FIB/SEM) in this study (Knott G., 2008; Ohta et al., 2012; Ohno et al., 2015). FIB/SEM technology allows us to observe the very complicated 3D structure of mitochondria in motor neurons under normal and *Drp1*-deficient conditions.

Concomitantly, intra-mitochondrial ultrastructure such as condensation and collapse of cristae, vacuolization, and mitophagy, are able to detect by means of FIB/SEM. In this study, we revealed marked alterations of ultrastructural morphologies of mitochondria, both inside and a whole structure, under nerve injury and *Drp1*-deficient conditions by 3D reconstruction using FIB/SEM technology.

Material and Methods

Animals

All animal protocols were performed in accordance with the *University Animal Care Guidelines for the Care and Use of Laboratory Animals*, and were approved by the Nagoya University Institutional Animal Care and Use Committee.

Atf3:BAC Tg mice and *Drp1* conditional knockout (Drp1 CKO) mice were generated as reported by Kiryu-Seo *et al.* (2016). Briefly, *Atf3*:BAC Tg was created by using bacterial artificial chromosome (BAC) technology, in which mitochondria are labelled with GFP and *cre* recombinase is expressed in response to nerve injury. Drp1 CKO mice, in which *Drp1* was ablated in an injury-responsive manner, were generated by crossing *Atf3*:BAC Tg mice with *Drp1*$^{flox/flox}$ mice, which were provided by Ishihara and colleagues (Ishihara et al., 2009). *Atf3*:BAC Tg mice and *Drp1*$^{flox/flox}$ mice were crossed with C57BL/6Ncr mice for at least seven generations.

Surgical procedures

Animals (10–22 weeks old, of either sex: *Atf3*:BAC Tg mice n=4, Drp1

CKO mice n=9 for electron microscopy, *Atf3*:BAC Tg mice n=10, Drp1 CKO mice n=10 for immunohistochemistry) were anesthetized with pentobarbital (45 mg/kg). For sciatic nerve injury, a small incision was made on the skin and the right sciatic nerve at the level of mid-thigh was exposed. After cutting the nerve with a pair of scissors, the incision was closed with nylon sutures.

Specimen preparation

Immunohistochemistry

Mice were perfused transcardially with Zamboni solution containing 2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, and their spinal cords were removed. To obtain cryosections, the spinal cords were immersed in sucrose solutions, embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, Calif., USA), and frozen. Frozen sections were obtained with a thickness of 14–16 μm using a cryostat and mounted on coated glass slides. The sections were washed in 0.01 M phosphate-buffered saline (PBS) and blocked in 1% bovine serum albumin (BSA), 0.3% Triton X-100 in PBS for 20 min. Subsequently, they were incubated with primary antibody: mouse monoclonal anti-cytochrome c (1:1000; Cat# 456100, Invitrogen, Eugene,

Oregon, USA), rabbit polyclonal anti-GFP antibody (1:1000; Cat# 598, MBL International, Japan) and guinea-pig polyclonal anit-p62 antibody (1:1000; Cat#03-GP62-C, American Research Product, Belmont, MA, USA) overnight at 4°C. Next, the sections were incubated with secondary antibodies conjugated with Alexa 488 or Alexa 594 (goat anti-mouse IgG, anti-rabbit IgG, anti-guinea-pig IgG; 1:500; Invitrogen, Eugene, Oregon, USA). The images were acquired on a confocal laser-scanning microscope (Olympus FV10i, Tokyo, Japan).

Antibody characterization

The antibodies used in this study are listed (Table 1). The GFP antibody was used to identify GFP-labelled mitochondria after sciatic nerve injury. The specificity was confirmed in Kiryu-Seo et al (2016). The cytochrome c antibody was used as mitochondrial marker. Cytochrome c is the mitochondrial intermembrane-space protein and approximately 13 kDa (Lauritzen et al., 2011). The p62 antibody was used as marker of autophagy (Itakura & Mizushima, 2011). The immunogen of antibody is C-terminal domain (20 amino acids) of human p62 protein, coupled with KLH.

Conventional TEM

The mice were perfused using Karnovsky fixative solution containing 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer. The trimmed specimens were placed in the same fixative for 3–4 h at 4 °C. The specimens were then rinsed in the same buffer, post-fixed in 1% osmium tetroxide in the same buffer for 2 h at 4 °C, rinsed with distilled water, block-stained overnight in a saturated solution of uranyl acetate, dehydrated in an ethyl alcohol series, and embedded in epoxy resin (Epon812).

Following examination of semi-thin sections stained with toluidine blue to select suitable areas, ultrathin sections were cut using an ultramicrotome (UC7k; Leica Microsystems, Wetzlar, Germany). The sections were then double-stained with uranyl acetate and lead citrate, and processed for observation with a TEM (JEM-1400 EX, Plus; JEOL, Tokyo, Japan).

FIB/SEM

The mice were perfused using half Karnovsky solution (2% paraformaldehyde, 2% glutaraldehyde in 0.05 M phosphate buffer). After selecting samples, the specimens were further immersed in the same fixative for 2 h at 4 °C and were then rinsed in the same buffer, further fixed with 1.5% potassium ferrocyanide and 2% osmium tetroxide for 1 h at 4 °C. After rinsing with distilled water, the specimens were treated with 1% thiocarbohydrazide, rinsed with distilled water, immersed in a 2% osmium tetroxide solution 1 h at

room temperature, and washed again with distilled water. For en bloc staining, the specimens were immersed in a solution of 4% uranyl acetate solution overnight and washed with distilled water. The specimens were further stained with Walton's lead aspartate solution (Deerinck et al., 2010). They were dehydrated in an ethyl alcohol series and ice-chilled acetone, and embedded in epoxy resin (Epon812) and polymerized.

FIB/SEM observation

The resin blocks were placed on a metal stub. The surfaces of the embedded specimen were exposed with a diamond knife and trimmed. The surfaces with the target motor neurons were confirmed by semi-thin section with toluidine blue staining. Especially, the injured motor neurons were detected with adhesion of microglial, which were known to adhere to soma of motor neuron in response to nerve injury at the early stage (Raivich, 2005; Raivich & Makwana, 2007; Gamo et al., 2008; Kiryu-Seo & Kiyama, 2011). Their surfaces were coated with a protective layer of carbon, which prevented charging artifacts. The metal stub with the specimens was set on the stage of FIB/SEM (Scios: FEI, Hillsboro, OR, USA). After carbon deposit on the milling area, serial images of the block face were acquired by repeated cycles of sample surface milling and imaging using the Auto Slice & View G3 operating software (FEI). Serial images of the block face were acquired by repeated cycles of sample surface milling using a focused gallium ion beam (accelerating voltage: 30 kV, current: 0.30 nA, milling step: 20 nm) and by image acquisition using SEM as compositional

contrast image from back scattered electrons (accelerating voltage: 2.0 kV, current: 0.10 nA, dwell time: 3 μs, resolution: 3072 × 2048, Horizontal Field Width (HFW) : 17.3 μm, x pixel resolution: 5.6nm/pix, y pixel resolution: 7.1nm/pix).

3D-structure reconstruction and volume analysis

Serial section images were reconstructed into 3D images and were analyzed using 3D visualization software *Amira Ver.5.0–6.0.1* (FEI, RRID: SCR_014305). The mitochondria and isolation membrane were manually traced, and the volume of representative data was measured using Amira. For the analysis in Fig. 4C, the volumes of total mitochondria in a unit cubic volume (1 μm^3) were measured using the same software.

Results

Morphology of somatic mitochondria in motor neurons using light microscopy and TEM

To identify the morphology of somatic mitochondria under light microscope, we stained spinal motor neurons using a cytochrome c (Cyt c) antibody (Fig. 1). Before the experiment, we pre-examined and confirmed that somatic mitochondrial morphologies of non-injured motor neurons between the wild-type C57BL/6 and the *Atf3*:BAC Tg mice were comparable. In *Atf3*:BAC Tg, no significant differences in Cyt c staining pattern between injured and non-injured motor neurons were identified (Fig. 1A and B), although resolution of mitochondrial structure was insufficient for detailed analysis using immunostaining and confocal microscopy alone, due to the inherent resolution limits. In Drp1 CKO mice, non-injured motor neurons showed a similar staining pattern of mitochondria with that in *Atf3*:BAC Tg (Fig. 1C) mice, whereas the injured motor neurons, without Drp1 expression, showed large round mitochondria (Fig. 1D).

Under TEM observation, similar characteristics were observed as those obtained with light microscopy. The somatic mitochondria had identical

morphologies in non-injured motor neurons of both *Atf3*:BAC Tg and Drp1 CKO mice. They also appeared similar between non-injured and injured motor neurons of *Atf3*:BAC Tg mice (Fig. 2A–C), suggesting that nerve injury might not affect the morphologies of somatic mitochondria in motor neurons. Conversely, very large and round mitochondria with rich cristae were observed in *Drp1*-deficient injured motor neurons (Fig. 2D).

Three-dimensional reconstruction of somatic mitochondria using FIB/SEM

FIB/SEM was used to acquire 3D-reconstructed images of somatic mitochondria. This analysis provided us with more precise 3D images of individual mitochondria, mitochondrial density in a unit area ($1\mu m^3$), and cristae structure within the mitochondria. Using both *Atf3*:BAC Tg and Drp1 CKO mice, somatic mitochondria in spinal motor neurons were reconstructed. In *Atf3*:BAC Tg mice that expressed Drp1 as normal, the morphologies of mitochondria appeared similar among neurons from non-injured (control), the injured 1 week (1wk), and the injured 2 weeks (2wk), although a tendency of enlargement of mitochondria was seen at 2wk after injury (Fig. 3 A–C). Many mitochondria had tubular structures and varied in length. The morphology of mitochondria in

non-injured motor neurons from Drp1 CKO mice was similar to those seen in *Atf3*:BAC Tg mice (Fig. 3D). However at 1wk after nerve injury under *Drp1* deficiency, it appeared that spherical mitochondria had one or a few long tubular processes, and in some cases spherical mitochondria appeared to be connected by tubular mitochondria (Fig. 3E). At 2wk after injury, the spherical part of mitochondrion became much bigger and round in shape, and the processes and the connecting tubular part were disappeared, presumably by merging the tubular processes (Fig. 3F). When the volume of each somatic mitochondrion was measured, the histogram of the volume demonstrated no significant changes among the control, the injured 1wk, and the injured 2wk in *Atf3:*BAC Tg mice. Most of them had volume less than 3.0×10^8 nm^3, although a minor group of larger mitochondria was found at 2wk after nerve injury (Fig. 4A). In contrast, the volume frequency in nerve-injured *Drp1*-deficient motor neurons showed dramatic increases in volume (Fig. 4B). The majority of mitochondrial volumes in injured motor neurons of *Drp1*-deficient mice were greater than 1.0×10^9 nm^3 at 2wk after nerve injury, which were over 10-fold more than their volumes in non-injured motor neurons. Less than 10% of cytoplasmic volume was occupied by mitochondria in both injured and intact neurons of *Atf3:*BAC Tg mice,

whereas, in *Drp1*-deficient mice, 15% was occupied at 1wk and 50% at 2wk after injury (Fig. 4C). Since our light and electron microscopic observations at 2wk focused on motor neurons which did not show apparent changes in cell size, the occupancy of mitochondria per unit cytoplasmic volume became noticeably larger in *Drp1*-deficient mice, suggesting that the volume of many mitochondria increased and the total volume occupied by mitochondria reached almost half of the cytoplasmic volume.

In Figure 5 the detailed morphology of an individual mitochondrion is shown. In the non-injured motor neuron of *Atf3*:BAC Tg mouse, somatic mitochondria had various shapes: multipolar (Fig. 5 A1), short tubular (Fig. 5 A2), long tubular (Fig. 5 A3), long tubular with branching (Fig. 5 A4), and small round (Fig. 5 A5). All of these mitochondria had diameter less than 500 nm. These characteristics are comparable among those in injured 1wk and 2wk neurons. Conversely, the mitochondria in *Drp1*-ablated injured motor neurons demonstrated abnormal shapes (Fig. 5 B, C). At 1wk after nerve injury, most of the *Drp1*-deficient mitochondria had one or a few large spherical bodies (up to 3 μm in diameter) and thin (30 nm to 200 nm in diameter) long processes, which

partly connected the round bodies (Fig. 5 B1, B2, B3). The thin tubular process also contained normal membrane structure and cristae. Intriguingly, the total length of the processes of single mitochondrion was occasionally longer than 20 μm (Fig. 5 B4). At 2wk after injury, most of the *Drp1*-deficient mitochondria became even larger (up to 5 μm in diameter), appearing as entire spheroidal shapes without processes (Fig. 5 C1-C4). Although the sizes of the spheroidal mitochondria varied from 5 μm to 500 nm, most mitochondria were more than 2 μm in diameter.

Degradative morphologies of mitochondria in motor neurons of Drp1-deficient mice

In comparison to mitochondria seen in non-injured neurons of *Drp1*-deficient mice (Fig.6A, B), most mitochondria became large spheroidal shapes after nerve injury and, concomitantly, intra-mitochondrial structures showed a variety of changes (Fig. 6C-G). Most of the enlarged mitochondria in injured motor neurons of *Drp1*-deficient mice initially showed densely packed cristae as shown in Fig. 2D and Fig. 6C. However, as time passed after injury, some mitochondria began to change their cristae structures. A typical change

seen in the spheroidal mitochondria was total or local sparseness of cristae (Fig. 6D-F); some cristae-rich mitochondria had several vacuoles (Fig. 6D), and some had lower density of cristae or local degradation of cristae (Fig. 6E and F). In addition, the entire collapse of the cristae structure of mitochondria without isolation membranes (Fig. 6G) and mitophagy-like structures with multi-stacked isolation membranes (Fig. 6H) were identified. The mitochondria, which have several vacuoles (Fig. 6D) or local degradation of cristae (Fig.6F), were further examined by 3D analysis to reveal intra-mitochondrial localization of those structures. More detailed 3D analyses of mitochondria clearly demonstrated localization of sparse cristae structures in single mitochondrion (Fig. 7). Intriguingly the local cristae degeneration occurred in sphere region rather than in tubular region (Fig. 7A); the vacuoles were not evenly localized, but localized in relatively central region of mitochondria (Fig. 7B).

The mitophagy-like structures were occasionally, but significantly, observed in *Drp1*-deficient motor neurons at 2wk after injury under electron microscopic observation (Fig. 6H); the occurrence of mitophagy was also confirmed by the appearance of p62-immunopositive structures, which were

adhering to the surfaces of some large mitochondria (Fig. 8). 3D reconstructions of mitophagy images revealed that isolation membranes wrapped a large mitochondrion either completely or partially with a small unwrapped region remaining (Fig. 9A and B). In some cases, the lysosome attaching to unwrapped region of mitochondria was observed (Fig. 9B).

Discussion

The present FIB/SEM technology allows for the reconstruction of 3D images of organelles; the images of several hundreds of serial sections can be obtained by SEM and the resolution of these images are comparable to those obtained with TEM. After examining 3D morphologies of somatic mitochondria of motor neurons before and after nerve injury using FIB/SEM, we found no significant changes in morphology of somatic mitochondria, although the size of axonal mitochondria became smaller (Kiryu-Seo et al, 2016). *Drp1* deficiency in injured motor neurons, however, induced drastic changes of somatic mitochondrial morphology as well as the degeneration of mitochondria demonstrating huge spheroidal morphology with local or total cristae collapse, and mitophagy.

Axon injury does not alter morphologies of somatic mitochondria

We recently found that axonal mitochondria became smaller in size and the axonal transport became faster after nerve injury in *Atf3*:BAC Tg mice (Kiryu-Seo et al., 2016). These could be purposeful changes immediately after injury, effectively delivering mitochondria to the growing or regenerating axon

tips to provide energy and Ca^{2+} buffering locally. We therefore initially assumed the mitochondrial fission might be promoted in soma as well. However, light microscopic observation did not indicate a clear change, and we thus analyzed 3D morphology of somatic mitochondrial in *Atf3*:BAC Tg mice by FIB/SEM. Unexpectedly, the present FIB/SEM analysis did not show significant alterations of 3D images of somatic mitochondria in size, volume and morphologies in response to nerve injury. It is possible that there is a difference of morphological response between axonal and somatic mitochondria after nerve injury because the morphologies of mitochondria are primarily distinct between in axon and soma under normal condition (Hollenbeck & Saxton 2005). The change of axonal mitochondrial morphology in response to nerve injury might occur somewhere near or in the axon, such as the axon hillock, the initial segment and axon proper. Further precise and systematic 3D analysis of mitochondrial morphology throughout neuron including soma, dendrite and axon (tip) after nerve injury would be necessary, and this may reveal existence of a local regulation of subcellular mitochondrial dynamics in neurons.

Spheroidal formation of mitochondria by Drp1 deficiency

In our experimental model using Drp1 CKO mouse, we found that nerve injury triggers *Drp1* deletion, and the change of mitochondrial morphologies from tubular shapes to the entire spheroidal shapes occurs in 2wk. This would be because the fission protein Drp1 loss results in mitochondria connecting together through normal fission/fusion dynamics being restricted to fusion only. Intriguing morphologies were seen at 1 week after *Drp1* deletion. At this time point, *Drp1* deficiency appears to exert two morphological changes simultaneously: one is the formation of long and thin tubules, the other is local enlargement leading to sphere formation. Most of these mitochondria have one or a few enlarged spheres together with single or multiple long and thin processes, as seen in Fig. 5. In yeast, maintenance of mitochondrial morphology 1 (MMM1) was identified as a responsive protein involved in maintaining tubular formation of mitochondria in addition to the fission responsible dynamin-related GTPase, Dnm1 (Burgess et al., 1994, Otsuga et al., 1998; Bleazard et al., 1999; Sesaki & Jensen, 1999). *MMM1* disruption in yeast resulted in spherical mitochondria similar to those seen in this study, while fission deficiency showed a long and interconnected net structure (Hobbs et al., 2001; Okamoto & Shaw, 2005). Currently, there are no reports on proteins required for tubulation

maintenance in eukaryotic cells; however, we believe that Drp1 might be involved in the suppression of long tubulation in motor neurons. Furthermore, it would be possible that the ablation of *Drp1* triggers the collapse of mitochondrial integrity accompanied by excess oxidative damage, and leads to spherical morphology of mitochondria. In consistent with this, previous report has showed that *Drp1*-deficient mouse embryonic fibroblasts (MEFs) alter mitochondrial shape from elongation to enlargement under oxidative stress while *Drp1*-deficient Purkinje cells prevents mitochondrial enlargement with treatment of antioxidant (Kageyama et al., 2012).

In previous studies using *Drp1* knockout mice, distinct changes in mitochondrial morphology were reported in different cells under TEM: for instance, elongated patterns occurred in MEFs, and enlargement patterns were observed in Purkinje cells (Ishihara et al., 2009; Wakabayashi et al., 2009). In this study, the enlarged mitochondria with elongated processes were observed at least 1wk after *Drp1* deletion with FIB/SEM analysis. After 1 further week (i.e. 2wk), almost all thin processes had disappeared and all mitochondria had become round spheroidal shapes, suggesting that spherical bodies might merge membranes of processes. Collectively, *Drp1* deletion initially promotes the

formation of long tubular mitochondria, perhaps by connecting nearby mitochondria, with subsequent local enlargements in the longer tubular mitochondria. Eventually, by merging the tubular processes and other spherical mitochondria, which are connected by the processes, one enlarged sphere became much larger.

As described above, these intriguing morphological changes of mitochondria in Drp1CKO injured neurons were seen at 1wk after injury. The previous reports using the same Drp1-deficient model (Kiryu-Seo et al., 2016) showed decreased mitochondria integrity, including reduced membrane-potential detected in injured axon with marker TMRM, and in injured soma with accumulation of Parkin, a maker for dysfunctional mitochondria (Narendra et al., 2008). Therefore it is likely that the mitochondrial morphological changes of Drp1CKO mice shown in this study were correlating with the mitochondrial function to some extent.

Mitochondrial degradation in Drp1-deficient motor neurons after nerve injury

Regarding the intra-mitochondrial structure, the spherical-shaped

regions of mitochondria seen at 1 week and around half of the spheroidal mitochondria at 2 weeks after *Drp1* deficiency appeared to have cristae-rich structures. Some of these had extremely cisterna-enriched structures, which is similar to the previously termed the mito-bulb structure (Ban-Ishihara et al., 2013). Ban-Ishihara et al. (2013) reported that *Drp1* deficiency induces mito-bulb formation, and that the enclosure of Cyt c into the highly stacked cristae suppressed its release from mitochondria under apoptotic stimuli. Therefore, the appearance of highly stacked cisterna in *Drp1*-deficient motor neurons may be a protective response for neurons to prevent apoptotic cell death. However, at 2 wk after *Drp1* deficiency, the proportion of the mitochondria with highly stacked cristae was decreased, and mitochondria with cristae collapse and vacuoles appeared. In these mitochondria at 2wk we observed the disruption of the inner membrane, collapse of normal cristae and even rupture of the outer membrane (Fig. 6). One of the most intriguing observations in this study was that the increase in mitochondrial total volume, which occupied almost half of the cytoplasm. The increase in number of swollen and spheroidal mitochondria at 2wk after *Drp1* deficiency might contribute to the higher rate of mitochondrial occupancy in cytoplasm.

In our previous study, *Drp1* deficiency accelerated nerve injury-induced motor neuron death (Kiryu-Seo et al., 2016). Because mitochondrial swelling could occur late in apoptosis after release of Cyt c and loss of the mitochondrial membrane potential (Sun, 2007), the accumulation of swollen and spheroidal mitochondria might lead to the acceleration of motor neuron death.

Mitophagy-like structures in injured motor neurons of Drp1-deficient mice

In addition to intra-mitochondrial degradation patterns, several mitophagy-like structures in which large mitochondria were wrapped by the isolation membrane were observed in Drp1 CKO mice 2wk after *Drp1* deficiency. This was rarely observed in normal and at 1wk after *Drp1* deficiency. This would suggest that two degradation patterns occur simultaneously in this model: non-mitophagic and mitophagic degradations. Mitophagy is a selective degradative system of mitochondria by autophagy, and the autophagosome selectively targets and engulfs membrane-depolarized mitochondria to digest by fusion with the lysosome (Bjørkøy et al., 2005; Lemasters 2005; Komatsu et al., 2007; Mizushima, 2007; Xie & Klionsky, 2007; Narendra et al., 2008; Kanki et al., 2009; Nakatogawa et al. 2009; Okamoto et al., 2009;). Concomitantly the

depolarization of membrane potential of enlarged mitochondria in *Drp1*-deficient mice has been reported (Kiryu-Seo et al., 2016), and some of the large spheroidal mitochondria were p62-immunopositive in this study, similar to previous studies (Kageyama et al., 2012; Kiryu-Seo et al., 2016). These may indicate that mitophagy and mitochondrial spheroid formation are induced simultaneously by stresses such as *Drp1* deficiency. Furthermore, mitophagy could be induced to prevent toxicities induced by mitochondrial degeneration, although many mitochondria release toxic materials into the cytoplasm, leading to the acceleration of cell death (Kim et al., 2007). This could be why nerve injury-induced motor neuron death is accelerated in *Drp1*-deficient mice, and that keeping the integrity of mitochondrial dynamics is a crucial mechanism for motor neuron survival and regeneration after nerve injury.

Acknowledgements

The authors wish to acknowledge the Division for Medical Research Engineering, Nagoya University Graduate School of Medicine, for the use of electron microscopy (Scios, JEM-1400Plus, EX), ultramicrotome (UC7k), and 3D analysis software (Amira), and thank K. Itakura for maintenance of the equipment. We also thank N. Tawarayama and Y. Itai for their excellent assistance with animal care, A. Asano for secretarial assistance and technical staff in the Division for Research of Laboratory Animals, Center for Research of Laboratory Animals and Medical Research Engineering.

Conflict of interests

All authors have no conflict of interests to declare.

Authors contributions

H.T. and H.K. designed the experiments and H.T., S.K.-S. and H.K. wrote the manuscript. H.T. carried out the electron microscopy (TEM, FIB/SEM) study, performed three-dimensional reconstruction, analyzed the data and prepared figures. S. K.-S. generated *Atf3*:BAC Tg and performed immunohistochemistry. H. H. assisted the three-dimensional reconstruction analysis. K. O. and N. K. contributed FIB/SEM analysis. N.I., M.N. and K. M. provided *Drp1*$^{flox/flox}$ mice.

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Figure legends

Fig 1. Immunohistochemical identification of mitochondrial morphology in injured motor neurons of *Atf3*:BAC Tg and Drp1 conditional knockout (CKO) mice

Cytochrome c (Cyt c) was immunostained to identify mitochondria in motor neurons of non-injured (control) *Atf3*:BAC Tg (A), nerve-injured *Atf3*:BAC Tg (B), non-injured Drp1 CKO (C), and nerve-injured Drp1 CKO (D) mice. Only *Drp1*-deficient motor neurons after nerve injury (D) demonstrated significantly enlarged mitochondria, whereas others were not significantly different. These images are representative data. *Scale bar* = 10 μm

Fig 2. Conventional transmission electron microscopy (TEM) images of mitochondria in injured motor neurons of *Atf3*:BAC Tg and Drp1 conditional knockout (CKO) mice

Representative conventional TEM images of motor neuron mitochondria in non-injured (control) BAC Tg (A), injured BAC Tg (B), non-injured Drp1 CKO (C), and injured Drp1 CKO (D). Mitochondria seen in (A)–(C) appear similar. These mitochondria have relatively smaller sizes and are spreading throughout the cytoplasm. The intra-mitochondrial structures are similar among A–C; however, very large round mitochondria with very intense cisternae are seen in *Drp1*-deficient injured motor neurons (D). *Scale bar* = 2 μm

Fig 3. Three-dimensional (3D) reconstructed images of mitochondria in injured motor neurons demonstrated by FIB/SEM

3D structures of individual mitochondrion in soma near nucleus are reconstructed with segmented images obtained by focused ion beam / scanning electron microscopy (FIB/SEM). Mitochondria in non-injured (Control) and injured motor neurons at 1 week (wk), and 2 wk after injury were reconstructed using *Atf3*:BAC Tg and Drp1 conditional knockout (CKO) mice. All mitochondria observed in area were reconstructed. In *Atf3*:BAC Tg mice, relatively longer and tubular mitochondria were seen in control (A) and injured motor neurons at 1 wk (B) and 2 wk (C). No significant morphological changes were observed among (A)–(C), although a slight tendency of mitochondrial thickening was seen in nerve injured *Atf3*:BAC Tg (C). In injured motor neurons of Drp1 CKO mice, although the morphology seen in the control (D) was about the same as those in (A–C), dramatic mitochondrial changes occurred at 1 wk (E) and 2 wk (F) after injury. Both enlargement and elongation of mitochondria can be seen in (E), and almost all mitochondria show spheroidal shapes in (F). These images are representative data. *Scale bar* = 5 μm

Fig 4. Mitochondrial volumes in motor neurons

(A) Frequency distribution of individual mitochondrial volumes in *Atf3*:BAC Tg mice (199 mitochondria in control motor neurons, 128 mitochondria in injured motor neurons at 1 wk, 141 mitochondria in injured motor neurons at 2 wk). The histogram pattern among control, 1 wk, and 2 wk are similar, suggesting the volume pattern of mitochondria does not alter by nerve injury. **(B)** Frequency distribution of individual mitochondrial volumes in Drp1 CKO mice (31 mitochondria in control motor neurons, 30 mitochondria in injured motor neurons

at 1 wk, 31 mitochondria in injured motor neurons at 2 wk). The volume of an individual mitochondrion shifted to much higher volumes after Drp1 deficiency. **(C)** Sum of individual mitochondrion volume observed in the unit area ($1\mu m^3$) was calculated and the ratio of mitochondrial occupancy in the cytoplasm was obtained. A marked increase of mitochondrial total volume was seen in Drp1 CKO mice.

Fig 5. Three-dimensional (3D) images showing detail of each mitochondrial morphology

(A) Representative 3D images of five normal mitochondrial morphologies seen in non-injured motor neurons of *Atf3*: BAC Tg mice: (A1) multipolar shape, (A2) short tubular, (A3) tubular, (A4) tubular with branching, and (A5) small sphere shape. **(B)** Representative 3D images of four mitochondrial morphologies observed in injured motor neurons of Drp1 CKO mice at 1 week after nerve injury: (B1, B2) a large single sphere accompanied by complicated long thin processes, (B3) multiple spheres connected by thin processes, and (B4) extremely long tubular processes without the spherical body. **(C)** Representative 3D images of mitochondria seen in *Drp1*-deficient injured motor neurons at 2 weeks after injury: (C1-C4) most of the mitochondria demonstrated spheroidal shapes of various diameters. Most mitochondria were over 4 μm in diameter, although some small mitochondria were also observed. *Scale bar = 2 μm*

Fig 6. Degradation patterns of spheroidal mitochondria in injured motor neurons of *Drp1*-deficient mice demonstrated by conventional TEM

(A,B) TEM images of mitochondria in motor neuron of non-injured (control) Drp1 CKO show small tubular or round shape and possess normal cristae structure. **(C)** More than half of the large spheroidal mitochondria in injured motor neurons of *Drp1*-deficient mice show cristae-rich morphologies at 1wk. **(D)** Several vacuoles were occasionally found in cristae-rich mitochondria at 2wk. **(E)** Some large spheroidal shape mitochondria showed degrading cristae at 2wk. **(F)** Occasionally, some large mitochondria had two distinct cristae morphologies divided by a membrane partition at 2wk. **(G)** Small numbers of large mitochondria showed entire degradation of the cristae structure, as well as both the outer and inner membrane rapture at 2wk. **(H)** Mitophagy-like structure showing a large mitochondria wrapped by multi-stacked isolation membranes at 2wk. These images are representative data. *Scale bar = 1 μm*

Fig 7. Three-dimensional (3D) reconstruction of some characteristic degradation patterns of mitochondria

(A1-A4) Series of scanning electron microscopy (SEM) images obtained by FIB/SEM showing a representative mitochondria classified in Fig. 5F. The single mitochondrion contains two components: the right-sided smaller region has relatively normal cristae, whereas the large left-sided region has very sparse and degrading crista. **(A5)** A 3D image was reconstructed from SEM images of (A1-A4). The partially degrading sphere has a normal long and thin tubular tail (A5). **(B1-B6)** Series of SEM images of a representative mitochondria, which has vacuoles similar to those mentioned in Fig. 5D. **(B7)** A 3D image was reconstructed from SEM images of (B1-B6). Most of vacuoles were localized in

the central region of the mitochondria. These images are representative data. *Scale Bar = 1 μm*

Fig 8. Localization of p62 on large spheroidal mitochondria

Mitochondria in *Drp1*-deficient injured motor neurons are labeled with anti-GFP antibody (green), because mitochondria in *Atf3*:BAC Tg mice expressed GFP after nerve injury. The huge mitochondria in the injured motor neuron of *Drp1*-deficient mice are also labeled by p62 antibody (magenta). p62-immunopositive signals are localized on some GFP-positive mitochondria (arrows). The image is a representative data. *Scale bar = 10 μm*

Fig 9. Three-dimensional (3D) analysis of the mitophagy-like structure in injured motor neurons of *Drp1*-deficient mice

(A1-A6) Series of scanning electron microscopy (SEM) images obtained from FIB/SEM demonstrating a huge mitochondria (asterisk) surrounded by an isolation membrane (arrowheads). **(A7)** A 3D image of a mitophagy-like structure was reconstructed with SEM images of (A1-A6) (mitochondria: green, isolation membrane: pink). **(A8)** A 3D image showing the engulfed mitochondria located inside an isolation membrane. **(A9)** A 3D image of a cross-section of (A7). **(B1-B6)** Other representative images of mitophagy. Multiple and dense isolation membranes (arrowheads) wrapped the mitochondria (asterisk). The lysosome (arrow) was attaching at the border of wrapped and un-wrapped region of isolation membrane. **(B7)** 3D images of the isolation membrane (pink), mitochondria (green) and lysosome (purple) reconstructed with SEM images of

(B1-B6). **(B8)** The reconstructed image of the wrapped mitochondria. **(B9)** 3D images of a cross-section of (B7). These images are representative data. *Scale Bar =1 μm*