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Dysfunction of mitochondrial dynamics in the brains of scrapie-infected mice

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ABSTRACT

Mitochondrial dysfunction is a common and prominent feature of many neurodegenerative diseases, including prion diseases; it is induced by oxidative stress in scrapie-infected animal models. In previous studies, we found swelling and dysfunction of mitochondria in the brains of scrapie-infected mice compared to brains of controls, but the mechanisms underlying mitochondrial dysfunction remain unclear. To examine whether the dysregulation of mitochondrial proteins is related to the mitochondrial dysfunction associated with prion disease, we investigated the expression patterns of mitochondrial fusion and fission proteins in the brains of ME7 prion-infected mice. Immunoblot analysis revealed that Mfn1 was up-regulated in both whole brain and specific brain regions, including the cerebral cortex and hippocampus, of ME7-infected mice compared to controls. Additionally, expression levels of Fis1 and Mfn2 were elevated in the hippocampus and the striatum, respectively, of the ME7-infected brain. In contrast, Dlp1 expression was significantly reduced in the hippocampus in the ME7-infected brain, particularly in the cytosolic fraction. Finally, we observed abnormal mitochondrial enlargement and histopathological change in the hippocampus of the ME7-infected brain. These observations suggest that the mitochondrial dysfunction, which is presumably caused by the dysregulation of mitochondrial fusion and fission proteins, may contribute to the neuropathological changes associated with prion disease.

1. Introduction

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative disorders that affect both humans and animals [1,2]. Scrapie is a prototypical prion disease that affects sheep and goats. Clinically, scrapie is characterized by a long latent period, progressive ataxia, tremor, wasting and ultimately death [3]. Many scrapie strains have been isolated from sheep and goats and used to examine not only the range of various pathogenesis pathways but also the neuropathological mechanisms induced by prion disease [4]. Typical features of the disease include the formation of spongiform vacuoles and astrocytosis, the formation of amyloid plaques in some cases and neuronal loss in the brain [5,6]. A key event in prion disease is the conformational misfolding of the endogenously expressed cellular prion protein (PrP^{C}) into the scrapie form of the pathogenic prion protein (PrP^{Sc}) [5].

A number of recent studies have demonstrated that mitochondria are dynamic organelles that continually undergo fission and fusion with one another [7–9]. Mitochondria can change in number and morphology within a cell during development, throughout the cell cycle and when challenged with various cytotoxic stimuli [9]. In mammals, the key molecules involved in mitochondrial fission are dynamin-like protein 1 (Dlp1, also referred to as Drp1) and fission 1 (Fis1). The process opposing fission, i.e., mitochondrial fusion, is controlled in mammalian cells by mitofusin 1 (Mfn1), mitofusin 2 (Mfn2) and optic atrophy 1 (Opa1) [7]. The sizes, shapes and interconnectivities of mitochondria are determined by their fusion and fission [9]. It has been suggested that the mitochondrial defects associated with Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) may result, at least in part, from a disruption of the fusion and fission mechanisms of mitochondria [8,9]. A recent study reported that the expression of Dlp1 is decreased and that mitochondria are

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abnormally elongated in the fibroblasts of AD patients and in neuronal cell lines overexpressing amyloid precursor protein (APP) [10]. The study suggests that APP causes an imbalance between mitochondrial fusion and fission that results in an abnormal distribution of mitochondria, which in turn contributes to mitochondrial and neuronal dysfunction. Several observations suggest a link between mitochondrial dysfunction and PD. Pink1 and parkin, which are PD-related genes, promote mitochondrial fission and inhibit mitochondrial fusion in *Drosophila* [11]. Additionally, HD research has focused on mitochondrial dysfunction. A mouse model of HD exhibits early defects in respiration and ATP production [12]. Moreover, mutant huntingtin seems to disrupt mitochondrial Ca^{2+} buffering [13] and to cause mitochondrial ultrastructural changes in the lymphoblasts of HD patients [14]. Previous studies reported that dysfunction and enlargement of the mitochondria occur by oxidative stress in animal models of prion disease [15,16]. However, the underlying mechanism responsible for this mitochondrial dysfunction remains unclear.

In the present study, we investigated the mitochondrial fusion and fission proteins that may be involved in the mitochondrial dysfunction observed in prion disease. We show that mitochondrial fusion and fission proteins are differentially modulated in the terminal stage of an experimental mouse model of prion disease and that this modulation may contribute to the morphological damage and reduction in number of mitochondria in infected neuronal cells.

2. Materials and methods

2.1. Antibodies

The following monoclonal and polyclonal antibodies were used: mouse monoclonal anti-PrP (3F10) [17], mouse monoclonal anti-COX IV (Abcam), goat polyclonal anti-enolase (Santa Cruz), mouse monoclonal to β-actin (Sigma–Aldrich), mouse monoclonal anti-Opa1 and mouse monoclonal anti-Dlp1 (BD Transduction Laboratories), mouse monoclonal anti-Mfn2, chicken polyclonal anti-Mfn1 (Novus Biologicals) and rabbit polyclonal anti-Fis1 (Santa Cruz).

2.2. Animals and scrapie strains

Six-week-old C57BL/6 mice were obtained from the Central Laboratory Animal (Republic of Korea) and divided into two groups: one group was infected with the ME7 scrapie strain, and the other included age-matched controls. The ME7 scrapie strain was kindly provided by Dr. Alan Dickinson (Neuropathogenesis Unit, Edinburgh, UK). This scrapie strain was maintained by serial intracerebral passages of brain homogenate from a terminally affected mouse. The mice were intracerebrally inoculated with 30 μl of 1% (w/v) brain homogenate in 0.01 M phosphate-buffered saline (PBS, pH 7.4) from either a normal brain or an ME7-infected C57BL/6 mouse brain at the terminal stage of the disease. When the clinical signs of prion disease were evident in the terminal stage (160 days postinoculation, dpi), the mice were sacrificed.

2.3. Western blot analysis

Whole brains or hippocampal regions were homogenized gently in 10-fold greater volumes (w/v) of 50 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 0.25% Na-deoxycholate, 1% NP-40 and protease inhibitor cocktails (Roche). The protein concentration was determined using the BCA assay (Thermo Scientific). The homogenates were treated with Proteinase K (PK) at a concentration of 50 μg/ml. Equal amounts of protein (10–50 μg in all assays) were separated by SDS–PAGE using 10%, 12% or 15% acrylamide gels and then transferred to nitrocellulose membranes (Thermo Scientific). After blocking with 5% skim milk for 1 h, the membranes were incubated with the individual antibodies overnight at 4 °C and then incubated with horseradish peroxidase-conjugated secondary antibody in Tris-buffered saline with 0.05% Tween-20 (TBST) containing 5% skim milk for 1 h at room temperature. The blots were visualized with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The expression levels of each protein were quantified using ImageJ software (NIH).

2.4. Transmission electron microscopy (TEM)

The animals were perfused with 0.1 M PBS (pH 7.4) containing 4% paraformaldehyde and 2.5% glutaraldehyde under deep anesthesia with 16.5% urethane. The brains were removed and fixed in the 0.1 M PBS fixative that was used for perfusion. The bilateral hippocampal regions were trimmed into small pieces immediately after their surgical removal and kept in the fixative for 2 h at 4 °C. Post-fixation was performed in 0.1 M PBS with 1% osmium tetroxide followed by dehydration through a graded ethanol series and embedding in Epon 812. Ultra-thin sections (75 nm) prepared by using an ultramicrotome (RMC MTXL) were stained with uranyl acetate and lead citrate and were subsequently observed with a transmission electron microscope (JEM-1011, JEOL). The numbers of total and damaged mitochondria were counted in the hippocampal neurons in the control and ME7-infected brains and then calculated based on fifteen arbitrarily selected hippocampal neurons. The sizes of the mitochondria were measured under high magnification (×50,000) using TEM (iTEM, Olympus Soft Imaging Solutions, GmbH), and the values were calculated as the means ± SDs of the lengths and widths of thirty arbitrarily selected mitochondria from each group. Statistical analyses of the numbers and sizes of the mitochondria were performed using Jandel SigmaStat software (V 3.5).

2.5. Statistical analyses

The compared values were calculated as the mean ± SD of three brains from each group, and statistical significance was determined using Student's *t*-tests.

3. Results

3.1. Imbalanced expression of mitochondrial fusion and fission proteins in infected brains

First, we identified deposits of PK-resistant PrP^{Sc} in ME7-infected brains in the end stage; the normal PrP^{C} proteins were completely degraded by the PK treatment, as shown in Fig. 1. To determine whether the mitochondrial fusion and fission proteins are affected by prion infection, their expression patterns were investigated in whole brains of the control and ME7-infected mice at the end stage of disease (160 dpi) (Fig. 2). Of the mitochondrial fusion and fission proteins (fusion proteins: Opa1, Mfn1 and Mfn2; fission proteins: Dlp1 and Fis1), Western blot analysis revealed that only Mfn1 was differentially expressed in the infected whole brains compared to the age-matched control brains (Fig. 2A and B). It has previously been reported that the hippocampal regions are more severely damaged than any other regions in brains infected with the ME7 scrapie strain [18]. Thus, to compare the expression levels of the mitochondrial fusion and fission proteins in various brain regions, we dissected both the control and ME7-infected brains into the following regions: cerebral cortex, hippocampus, cerebellum, striatum and brainstem. We found that the levels of Mfn1 and Fis1 were significantly

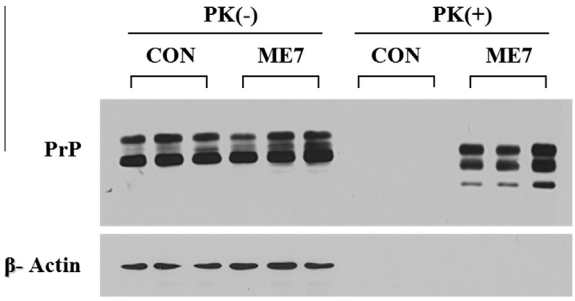

Fig. 1. Deposition of PK-resistant prion isoforms in ME7-infected mice. Whole brains of control ($n = 3$) and infected mice ($n = 3$) in the end stage (160 dpi) were homogenized and blotted with anti-PrP antibody (3F10). A portion of each sample was treated with PK (PK 50 μg/ml). β-Actin was used as a loading control.

increased in the hippocampi of the infected brains, whereas the Dlp1 levels were significantly decreased at the end stage (Fig. 2C and D). Additionally, the levels of Mfn1 and Mfn2 were increased in the cerebral cortex and striatum, respectively, of infected brains (data not shown). In the cerebellum and brain stem, none of the five mitochondrial fusion and fission proteins was significantly differentially expressed between the control and infected mice (data not shown). These results indicate that the differential regulation of mitochondrial fusion and fission proteins may be involved in the mitochondrial dysfunction of the ME7-infected brains, particularly in the hippocampus [16].

3.2. Analysis of fission proteins in the hippocampal cytosol of infected brain

To examine the localization of the mitochondrial fusion and fission proteins in the organelles and the expression levels of these proteins in the cytosol and mitochondria, mitochondrial and cytosolic fractions were isolated from the hippocampi of control and ME7-infected mice; again the hippocampus was chosen because this region is known to be severely damaged in ME7-infected brains [18]. Immunoblot analyses revealed that Dlp1 levels in the mitochondrial fractions from the hippocampi were not altered by ME7 infection (Fig. 3A and B) but were significantly decreased in the cytosolic fractions of the infected hippocampi; this finding is consistent with the decrease in Dlp1 levels in the affected hippocampi that is illustrated in Fig. 2C and D. The concentration of Opa1, which is primarily localized to the inner membrane of the mitochondria, was not altered in the mitochondria or cytosol isolated from the hippocampi of the infected brains (Fig. 3C and D).

3.3. Morphological changes in the mitochondria in the hippocampal region of infected brain

The modulation of mitochondrial dynamics due to fusion and fission protein concentration probably contributed to alterations in mitochondrial shape [9]. Electron microscopic analyses were performed to assess the relationship between mitochondrial fusion and fission proteins and mitochondrial shapes in the hippocampal

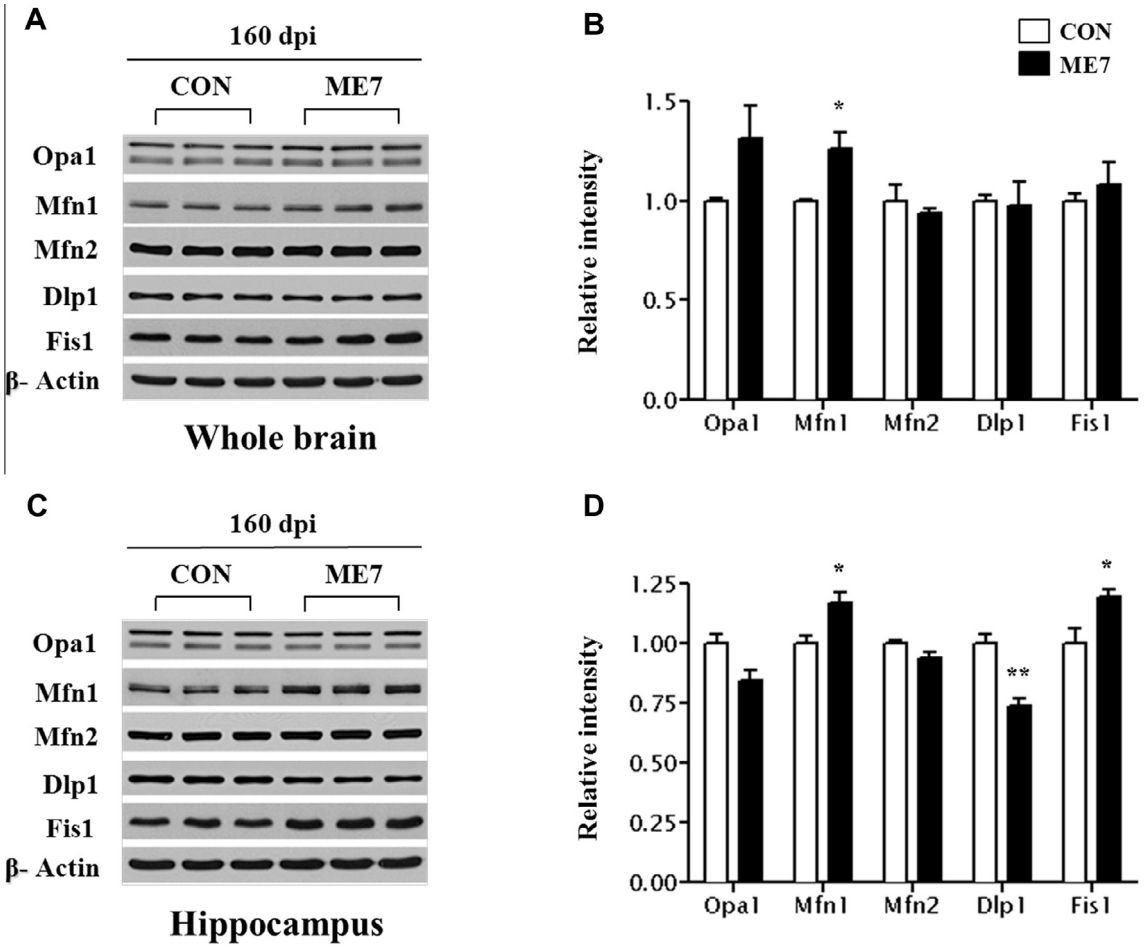

Fig. 2. Differential expression of mitochondrial fusion and fission proteins in hippocampi of ME7-infected mice. The mitochondrial dynamic proteins of the whole brains and hippocampal regions of the control ($n = 3$) and infected mice ($n = 3$) in the end stage of disease were blotted. β-Actin was used as a loading control for A and C. The intensities of the bands in panels A and C were measured and quantified (B and D). The values are expressed as the mean ± SD ($n = 3$). CON: control (white bars); ME7: ME7-infected (black bars). Statistically significant differences are indicated ($^{*}p < 0.05$ and $^{**}p < 0.01$).

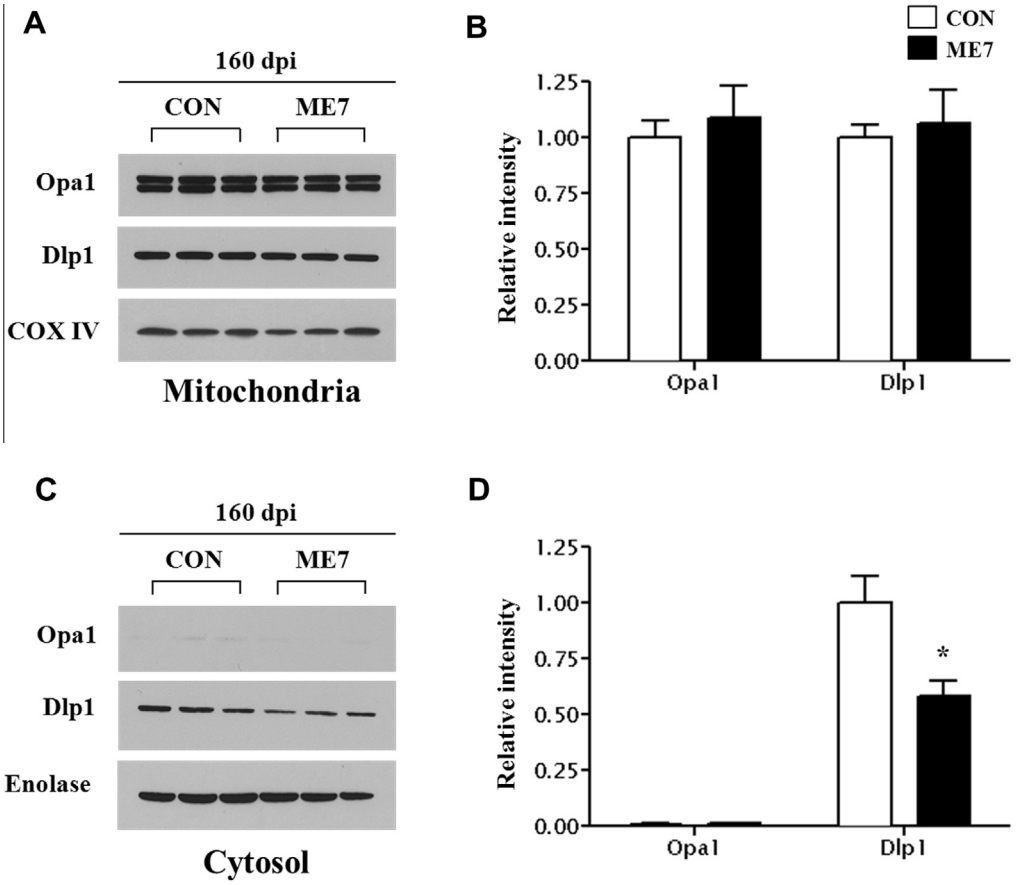

Fig. 3. Subcellular localization of mitochondrial fusion and fission proteins in hippocampi of ME7-infected mice. Opa1 (fusion) and Dlp1 (fission) from the mitochondria (A) and cytosolic (C) fractions of the hippocampi of the control ($n = 3$) and ME7-infected mice ($n = 3$) at the end stage of the disease were blotted (160 dpi). β-Actin was used as a loading control. The intensities of the bands in panels A and C were measured and quantified (B and D). The values are expressed as the mean ± SD ($n = 3$). CON: control (white bars); ME7: ME7-infected (black bars). Note that both of the Opa1 bands are primarily localized to the mitochondria of hippocampi in both the control and infected specimens, whereas substantial amounts of Dlp1 were localized to the cytosol. Statistically significant differences are indicated ($^*p < 0.05$).

region. As shown in Fig. 4, the mitochondria in the normal hippocampal neurons were present in a variety of shapes, including rod, round and elliptical shapes. The cristae were normal in appearance. In contrast, enlarged mitochondria with damaged cristae were clearly observed in many of the hippocampal neurons in infected brains (Fig. 4A). The numbers of total neuronal mitochondria were significantly decreased in the hippocampi of the infected mice compared to those of controls (Fig. 4B). Additionally, the lengths and widths of the mitochondria in the hippocampal neurons of infected brains were significantly increased compared to those of the controls, which is consistent with the observation of enlarged hippocampal mitochondria visualized in Fig. 4B. The morphological changes seen in mitochondria may be involved in the mitochondrial dysfunction in the hippocampi of ME7-infected mice.

4. Discussion

In this study, we demonstrated for the first time that the expression patterns of mitochondrial fusion and fission proteins were altered in an experimental mouse model of prion disease; i.e., ME7 scrapie-infected mice. Of the mitochondrial fusion and fission proteins examined, the levels of Mfn1 were found to be significantly increased in whole brains of the ME7-infected mice. Within the different dissected regions of the ME7-infected brains, we found differences in the expression levels of various mitochondrial fusion and fission proteins compared to controls. Particularly notable were the alterations in the expression of Mfn1 in the cerebral cortex; Mfn1, Dlp1 and Fis1 in the hippocampus; and Mfn2 in the striatum.

A recent study suggested that most neurodegenerative diseases may be associated with the dysregulation of mitochondrial fusion and fission proteins [19]. Mitochondrial fusion and fission are regulated by large dynamin-related GTPases. Mitochondrial fusion, which is regulated by three large GTPases (Mfn1, Mfn2 and Opa1), involves the coordinated fusion of both the outer and inner mitochondrial membranes [20]. The overexpression of mitofusins causes the normally punctate mitochondria to become elongated, whereas the repression of mitofusins causes the fragmentation of the mitochondrial network in tissue culture cells [21]. Interestingly, we found increased level of Mfn1, which is a transmembrane protein that localizes to the outer membranes of mitochondria, in the homogenates of whole brain, hippocampus and cerebral cortex of the infected brain. In addition the expression level of Mfn2 was increased in the striatum of the infected brain (Data not shown). Although electron microscopy analyses did not reveal the detailed membrane structures of the mitochondria in this study, initial outer membrane fusion may have occurred in the brains of the infected mice due to increases in the Mfn1 and Mfn2 proteins. Moreover, although there may be distinct pathways or mechanisms for Mfn1 and Mfn2, a functional interplay between the two proteins may be involved in the control of mitochondrial fusion [22].

Different strains of mouse-adapted scrapie have been reported to preferentially target different specific brain regions; as noted previously, the ME7 strain is known to be particularly associated

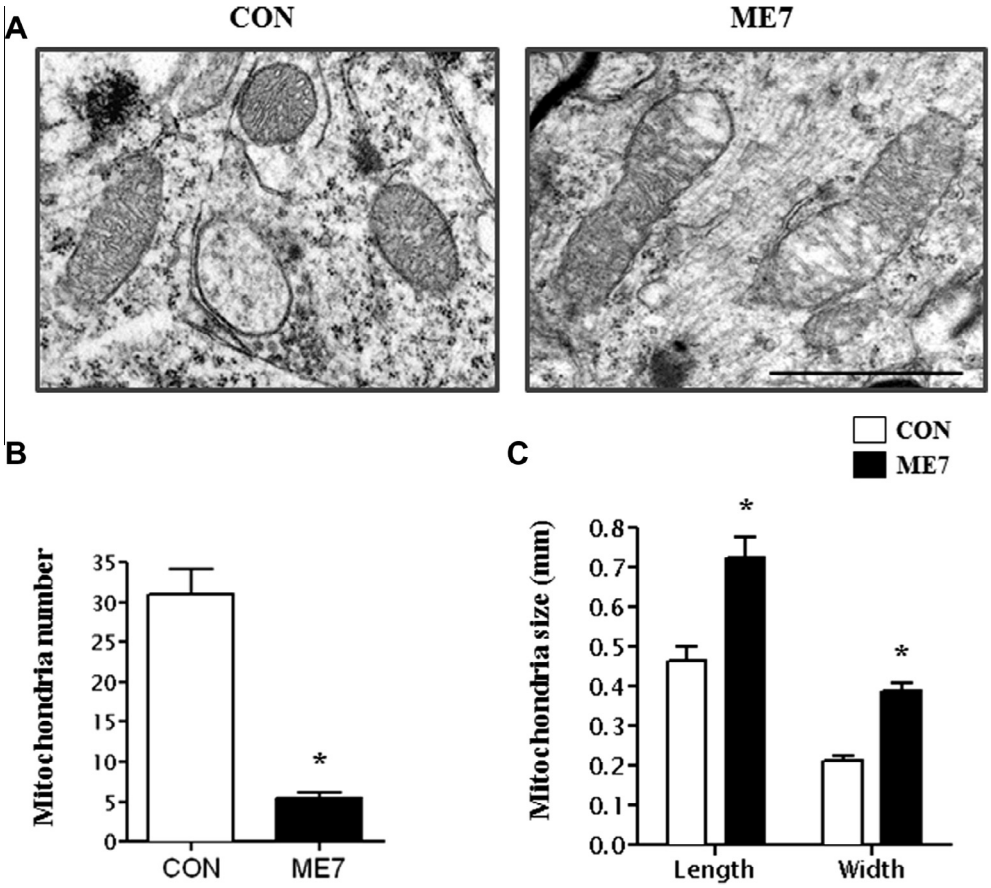

Fig. 4. Electron microscopic analysis of the mitochondria in hippocampal neurons of ME7-infected mice. (A) Control mitochondria were elliptical in shape and exhibited compact cristae, whereas the infected mitochondria were enlarged and exhibited partially swollen cristae. Scale bar, 1 μm. (B) The total number of mitochondria in the hippocampal neurons of the control and ME7-infected mice (the total number of mitochondria were counted from 15 neuronal cells from each group). (C) The lengths (white bar) and widths (black bar) of the mitochondria in the hippocampal neurons of the control and ME7-infected mice (the lengths and widths of 30 mitochondria in each group were measured). Statistically significant differences are indicated ($^{*}p < 0.01$). CON: control; ME7: ME7-infected.

with hippocampal damage [23]; therefore, we focused on the hippocampal region of the ME7-infected brains. Our study demonstrated that ME7 infection led to significant increases in the levels of Mfn1 and Fis1 in the hippocampi of the ME7-infected brains, whereas Dlp1 was significantly decreased. Dlp1 localizes in several organelles, including the endoplasmic reticulum, microtubules and peroxisomes, but primarily resides in the mitochondria and cytosol [24]. We isolated the cytosolic fraction of the hippocampal region and investigated the changes in the expression of mitochondrial fusion and fission proteins in both the purified mitochondria fraction and the cytosol. Dlp1 levels were decreased in cytosolic fractions but not in mitochondria fractions. These observations imply that imbalances in mitochondrial dynamics may contribute to the enlargement and the degeneration of mitochondria that occurs in the hippocampi of scrapie-infected mice.

Changes in the shape of the mitochondria have been observed in ME7 scrapie-infected brain even in the preclinical stage of infection [25]. The mitochondrial enlargement demonstrated during the end stage of prion disease in this study was also observed in a previous study that demonstrated structural abnormalities of the mitochondria in the neurons of the hippocampi and cerebral cortices of brains from scrapie-infected hamsters [15]. Fragmentation and clustering of mitochondria in the soma have been observed in the brains of patients with sporadic AD, and alterations in the expressions of several fusion and fission proteins have been observed in these brains [26]. Thus, we sought to determine the relationship between expression levels of the mitochondrial fusion and fission proteins and the alterations in the shapes, numbers and sizes of the mitochondria in a prion disease model. In the ME7 mouse model, we found that the total number of neuronal mitochondria was reduced significantly and that a number of enlarged and degenerated mitochondria appeared. These mitochondria had damaged cristae and matrix. The relationship between the mitochondrial damage and the induction of neurodegeneration and disease is uncertain. Clearly the fact that the number of mitochondria was reduced combined with degeneration of the remaining mitochondria would lead to a loss of neuronal function. It needs to be noted that the loss of mitochondria could affect the quantity of proteins observed; this is particularly important in instances where the level of protein decreased (e.g., Dlp1 in the cytosol). Furthermore, in those instances where there was no change in protein levels (e.g., Opa1), the reduced number of mitochondria could mask an increase.

Growing evidence suggests that the delicate balance between mitochondrial fission and fusion is critical for mitochondrial functions, including energy production, Ca^{2+} signaling, reactive oxygen species (ROS) production, apoptosis and senescence [7,27]. Dysregulation of the mitochondrial fusion and fission proteins in neurons may be a common pathway leading to the mitochondrial and neuronal dysfunctions that are critical in the pathogenesis of prion disease. It remains unclear, however, how the alterations in mitochondrial fusion and fission proteins that were observed in

this study contribute to neurodegeneration in prion diseases. Taken together, the findings of this study suggest that the disruption of the balance of mitochondrial fusion and fission proteins, which may be a common feature of prion diseases, is involved in the mitochondrial and neuronal dysfunction in the brains of ME7-infected mice.

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