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Evidence for the Drp1-dependent Mitochondrial Fission in the Axon of the Rat Cerebral Cortex Neurons

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흰쥐 대뇌 피질 신경세포의 축삭에서 Drp1 의존적 미토콘드리아의 분열

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ABSTRACT

Neurons utilize a large quantity of energy for their survival and function, and thereby require active mitochondrial function. Mitochondrial morphology shows dynamic changes, depending on the cellular condition, and mitochondrial dynamics are required for neuronal development and function. In this study, we found that the length of mitochondria in the distal axon is significantly shorter than that of mitochondria in dendrites or proximal axons of cerebral cortical neurons, and the reason for this difference is the local fission within the axon. We also found that suppression of Drp1, a key regulator of mitochondrial fission, resulted in significant elongation of mitochondria in axons. Collectively, these results suggest that local mitochondrial fission within the axon contributes to region-dependent mitochondrial length differences in the axons of cortical neurons.

Keywords : Axons, Cortical neurons, Drp1, Fission, Mitochondria

INTRODUCTION

Mitochondrion is an essential organelle for the survival of cells, because it synthesizes ATP, buffers intracellular calcium, and isolates key apoptogenic factors from cytosolic activation (Mattson et al., 2008; Tait & Green, 2010). The term "*mitochondria*" is derived from the Greek words "*mitos*" (thread) and

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"*khondrion*" (grain), indicating the heterogeneity of mitochondrial morphology (Bereiter-Hahn & Voth, 1994; Collins et al., 2002; Chan, 2006). In fact, mitochondrial morphology is dynamic, changing by continuous fission and fusion within cells. Changes in the mitochondrial morphology depend on cellular context, such as metabolic rate, cell division, and cellular stresses (Yu et al., 2006; Taguchi et al., 2007; Tondera et al., 2009; Gomes et al., 2011). The assessment of mitochondrial morphology is thus considered an important reference for understanding cellular conditions. Considering the large amount of energy that neurons necessarily consume for their survival and function, mitochondria are especially important to these cells. Accumulating reports suggest a significant contribution of mitochondrial dynamics to neuronal development and function. Abnormal mitochondrial morphology is frequently observed in many central nervous system (CNS) diseases, such as Alzheimer disease, Parkinson disease, Charcot-Marie-Tooth neuropathy, and optic atrophy (Knott et al., 2008). Impairments of mitochondrial morphology in these neurological diseases are, at least in part, due to failure of the normal regulation of mitochondrial dynamics. For instance, genetic mutations in *OPA1* are found in hereditary optic atrophy, and this gene functions is known to be related to mitochondrial fusion (Delettre et al., 2000; Davies et al., 2007). Furthermore, a genetic knockout of *Drp1*, which is responsible for mitochondrial fission, results in abnormal neuronal apoptosis and impairment of axonal growth; this observation supports the importance of mitochondrial dynamics in neuronal development and function (Ishihara et al., 2009; Wakabayashi et al., 2009). Neurons are highly polarized, and the axons and dendrites are functionally and morphologically distinct. Most CNS neurons have a single thin elongated axon with numerous arborized branches, but they have many short and thick dendrites with spines. On the other hand, peripheral sensory neurons show bipolar morphology, with both peripherally and centrally projecting axons. Axons and dendrites show different cytoskeletal arrangements and selective transport of cargo proteins, rendering them functionally specialized. Despite the importance of mitochondrial dynamics in neurons, mitochondrial dynamics in polarized neurons has not yet been extensively addressed. For instance, mitochondrial biogenesis and dynamics have been studied in dendrites of CNS neurons (Li et al., 2004) and axons of peripheral nervous system (PNS) neurons (Li et al., 2004), but they have not been compared. To this end, we examined the difference in mitochondrial length between dendrites and axons, and mitochondrial dynamics in CNS axons.

MATERIALS AND METHODS

1. DNA constructs

pEGFP-C1 and pDsRed1-mito were purchased from Clontech (Japan). For generation of GFP-DRP1^{K38A}, we amplified DNA fragment of DRP1^{K38A} from pcDNA-DRP1^{K38A} (Smirnova et al., 1998) by using polymerase chain reaction. Forward and reverse primer were 5′-atc tcg agc tAT GGA GGC GCT AAT TCC TGT-3′ and 5′-agg aat tcT CAC CAA AGA TG AGT CTC CC-3′, respectively. DNA fragment of DRP1^{K38A} was then inserted between XhoI and EcoRI sites of pEGFP-C1.

2. Neuron culture and gene transfection

Rat cerebral cortex neurons were cultured as previously described (Eun et al., 2010). Briefly, embryonic (E16) cortex were separated and dissociated into single cells with trypsin. Cells (each 10^5 cells/cm^2) were then seeded onto poly-D-lysine coated class coverslip, and maintained with neurobasal media containing B27 supplement. We transfected plasmids into rat cerebral cortex neurons by using Calphos mammalian transfection kit (Japan, Clontech) as manufacturer's manual. On 10 days in vitro (DIV), GFP and DsRed-mito were transfected into rat cerebral cortex neurons. For suppression of endogenous DRP1 activity, we transfected GFP-DRP1^{K38A} with GFP and DsRed-mito on DIV2. Mitochondrial morphology was examined under a confocal microscope (Germany, Carl Zeiss, LSM510), and the mitochondrial length was measured with Image J program (USA, NIH). Mitochondrial length was measured from only separated mitochondria.

3. Live imaging

Cells on coverslip were placed on the stage-mount live imaging chamber (Korea, Live Cell Instrument) where CO_2 (5%) and temperature (37˚C) were controlled. Images were captured every 5 seconds for 10 minutes under inverted fluorescence microscope (Germany, Carl Zeiss, Axiovert 2000) with imaging software program MetaMorph (USA, Molecular Devices).

4. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 10 minutes, and briefly washed with PBS. Cells were then incubated with blocking solution (3% BSA and 0.1% Triton-X100 in PBS) for 30 minutes, and primary antibody was added. Antibodies used

in this study are as follows: anti-Drp1 (USA, Santacruz Biotechnology) and MAP2 (USA, Millipore). After 2 hours, coverslips were washed with PBS twice, and secondary antibody mix was incubated for 30 minutes. After washes, coverslips were mounted and the labeling was monitored under a fluorescence microscope.

RESULTS

1. Regional differences in mitochondrial length in the neurites of cerebral cortical neurons

The morphology of mitochondria in cerebral cortical neurons was visualized by transfection of a plasmid expressing a mitochondria-targeted DsRed fluorescent protein (DsRed-mito). For the assessment of whole-cell morphology, GFP plasmid was co-transfected (Fig. 1A). Based on the morphology, we identified dendrites (thick and short processes with dendritic spines) and axons (thin processes with rare arborization), and measured the length of mitochondria within dendrites, and both proximal (100～200 μm from the cell body) and distal (＜200 μm from the axonal tip/growth cone) axonal segments (Fig. 1B-1E). Quantification of the mitochondrial length revealed that the mean length of mitochondria within distal axons was significantly shorter than in dendrites and proximal axons. However mitochondria in dendrites and proximal axons were similar in length. These results indicate that mitochondria within axons undergo fission during axonal transport.

2. Live imaging of mitochondrial fission within the axon

To provide direct evidence for the fission of mitochondria within the axon, we performed live imaging. Axons were identified by their morphology, and the mitochondrial changes were monitored for 30 seconds, in 5-second intervals. During this period, we observed 4 fission events and 1 fusion event within the 30-μm monitoring window (Fig. 2).

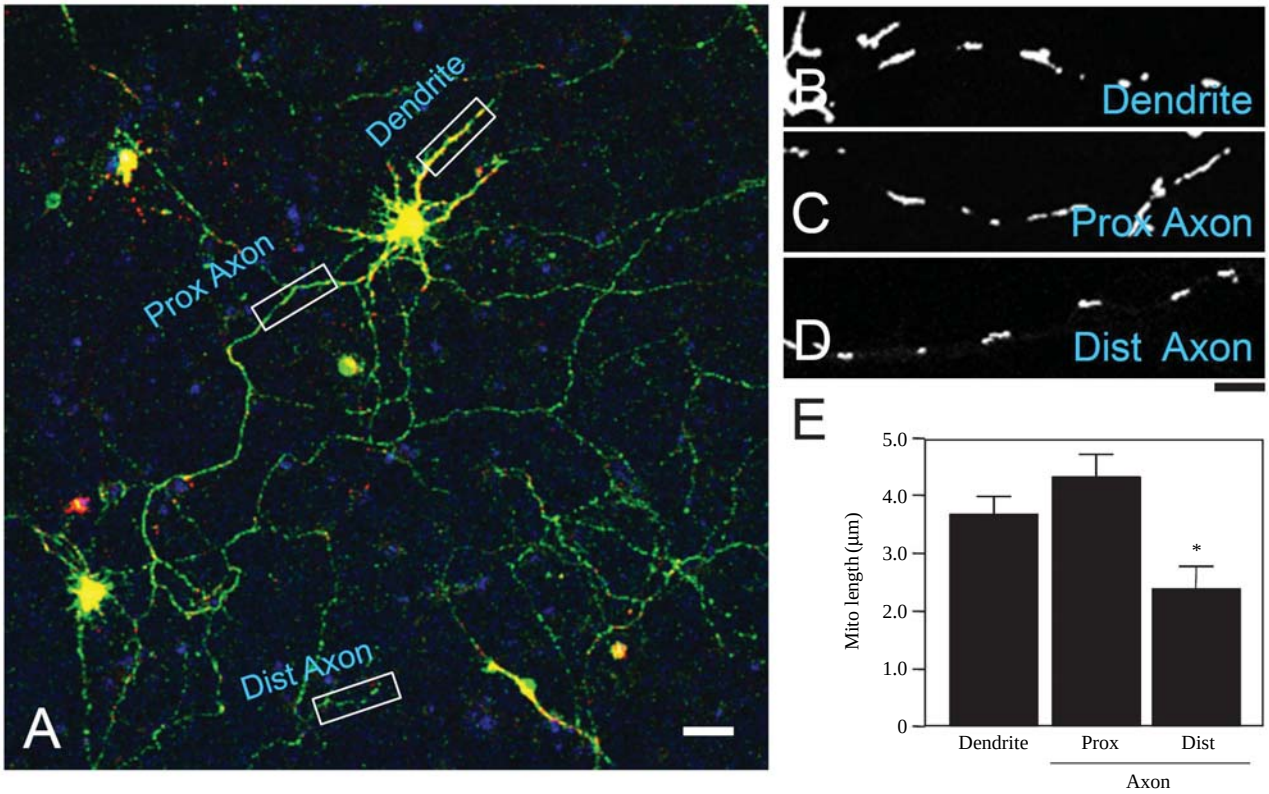

Fig. 1. Differences in the mitochondrial length in different regions of a neuron. A: Cerebral cortex neurons were transfected with GFP and DsRed-mito plasmids. Typical neuron with double-transfection is shown. Dendrites, proximal axon, and distal axon regions are marked by boxes (Scale bar=10 μm). B-D: Large magnification views of DsRed-labeled mitochondria in dendrite (B), proximal (C) and distal (D) axon (Scale bar=5 μm). E: Quantification of mitochondrial lengths in three neuronal subregions. At least 50 mitochondria from ＞10 neurons were measured. *P＜0.05 in Students' t-test comparison.

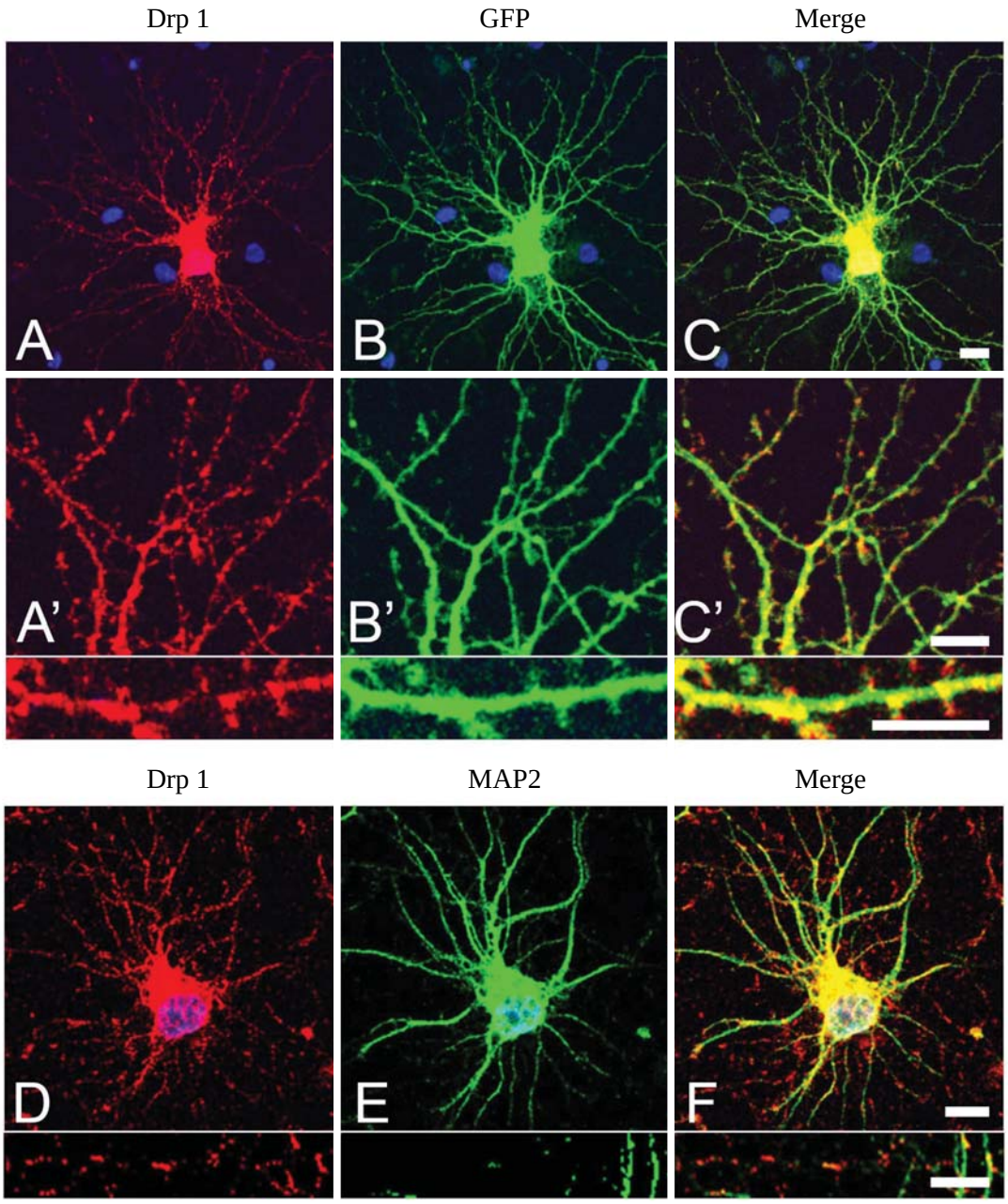

Fig. 2. Immunostaining of Drp1 in neurons (Scale bars=10 μm). A-C: GFP-transfected cortical neurons were labeled with anti-Drp1 antibody. Drp1 is distributed in the neuronal cell bodies and most neurites. A'-C' show large magnification images of dendrites. Note that Drp1 is localized in the dendritic shafts and some large spines. D-F: Drp1 and MAP2 were double labeled, and similar results were obtained. Some MAP2-negative axons also exhibited punctate form labeling or Drp1 (bottom).

3. Distribution of Drp1 in neurites

Mitochondrial fission requires the large GTPase protein Drp1. Therefore, for the mitochondrial fission within the axon, Drp1 protein should be present. To evaluate this, we next investigated the localization of Drp1 protein in cortical neurons (Fig. 3). Drp1 protein was enriched in neuronal cell bodies and neurites. When we examined the dendrites (which were readily identifiable by the dendritic spines in GFP-transfected neurons), Drp1 was expressed in the dendritic shafts and spines (Fig. 3A-3C); this finding is consistent with previous observations (Li et al., 2008). Double staining of MAP2 (dendrite marker) and Drp1 yielded similar results (Fig. 3D-3F). In addition to dendrites, axons (that are negative for MAP2) exhibit a punctate pattern

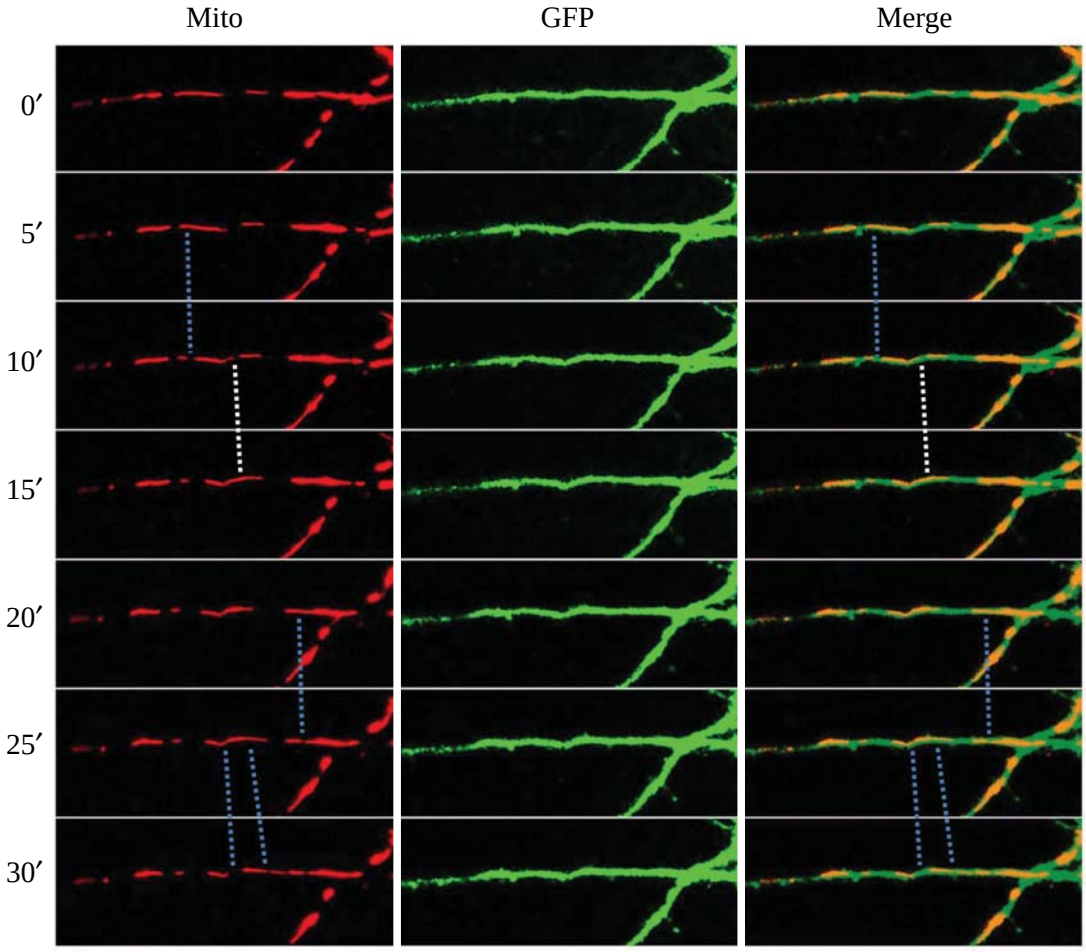

Fig. 3. Live imaging of mitochondrial fission within the axon (Scale bar=10 μm). Thin and long GFP-expressing process (middle panels) indicates that it is an axon. Images were taken every 5 second. Fission was marked by blue dotted lines, and fusion was marked by white dotted line.

of Drp1 immunoreactivity. These results suggest that Drp1 is ubiquitously distributed in cell bodies and neuronal processes.

4. Elongation of axonal mitochondria by suppression of Drp1 activity

Because Drp1 is expressed in axons, and mitochondrial fission occurs within the axon, Drp1 may be mechanistically responsible for the mitochondrial fission within the axon. To address this, we tested whether suppression of Drp1 modifies the length of axonal mitochondria. It is known that mutation in the GTPase domain of Drp1 (Drp1^{K38A}) efficiently suppresses endogenous Drp1 activity in a dominant-negative fashion (Smirnova et al., 2001). We therefore overexpressed this Drp1^{K38A} mutant in cultured neurons to suppress endogenous Drp1 activity (Fig. 4). After Drp1^{K38A} expression, we found a marked increase in the length of mitochondria in all neurites-including both proximal and distal portions of axons-suggesting that Drp1 mediates mitochondrial fission occurring within the axon.

DISCUSSION

This study provided evidence that mitochondrial fission in the axons of CNS neurons is mediated by Drp1. We addressed this issue with independent experimental approaches. First, we observed that mean mitochondrial length is significantly shorter in the distal axon than in the proximal axon, suggesting progressive fission of mitochondria during axonal transport. On the other hand, mitochondrial length in the axons and dendrites at similar distances from the cell body were similar, suggesting that absence of size-based differential sorting of mitochondria for axonal versus dendritic transport. Progressive shortening of mitochondrial length in the axons may be explained by differ-

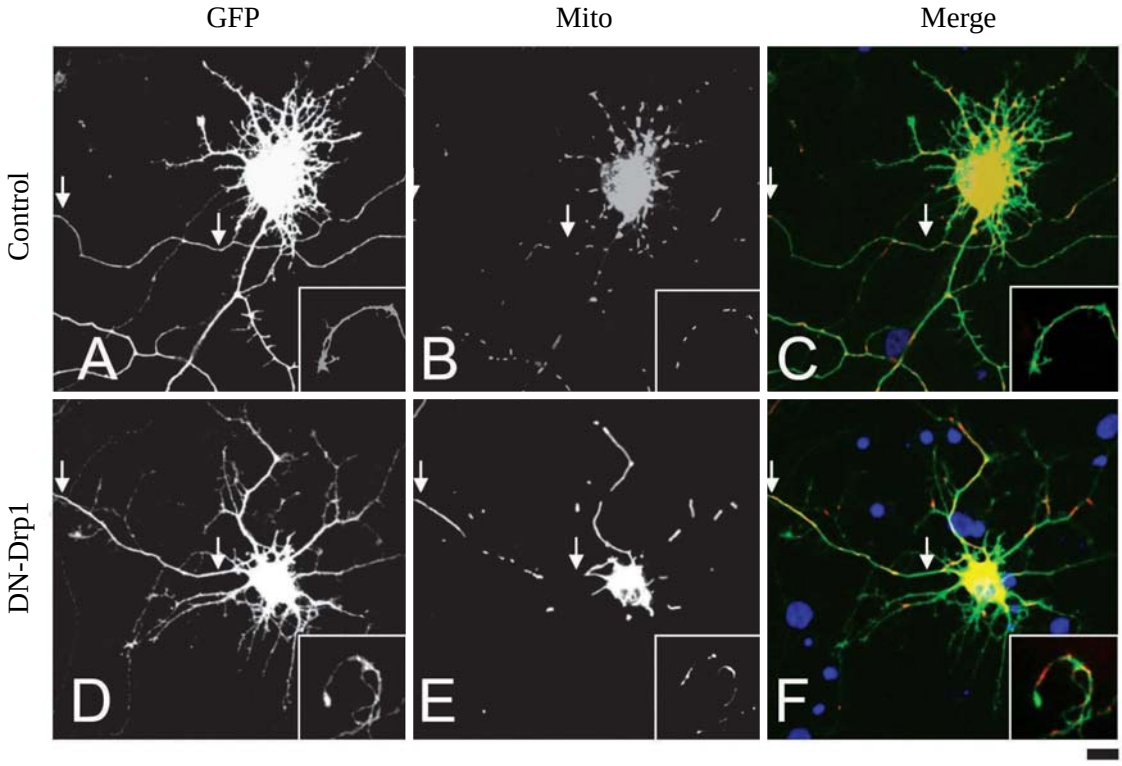

Fig. 4. Overexpression of dominant-negative Drp1 ($Drp1^{K38A}$) resulted in the mitochondrial elongation in axons (Scale bar=10 μm). Comparing to the control transfection (A-C), $Drp1^{K38A}$ transfected neurons (D-F) exhibited long mitochondria in both proximal (arrows) and distal (insets) region of the axons. GFP signal (A, D) and mitochondrial signal (B, E) are separately shown, and C, F show the merged images.

ences in the speed of mitochondrial transport, depending on the length. We directly monitored mitochondrial dynamics by live imaging, and found both mitochondrial fission and fusion events within the axon. Mitochondrial fission in the axon was previously shown in sensory ganglion neurons (Amiri & Hollenbeck, 2008), suggesting that mitochondrial fission commonly occurs in CNS and PNS axons. Although we did not quantitatively address this observation, the frequency of fission appeared to be higher than that of fusion. This may explain the overall reduction of mitochondrial length in distal axons. In addition, we showed the presence of the key fission molecule Drp1 in the axon. Within the axon, Drp1 exhibited punctate or elongated patterns of immunoreactivity, suggesting that a large subset of Drp1 is localized to mitochondria.

Drp1 is a large GTPase protein that is essential for mitochondrial fission. For fission, Drp1 proteins are recruited to the mitochondrial outer surface, where they form oligomers. Through GTPase activity, oligomerized Drp1 proteins change their conformation to squeeze mitochondria, which provides the necessary mechanical force to complete fission (Chan, 2006). A point mutation in the GTPase domain (K38A) completely eliminates GTPase activity, resulting in a blockade of the fission process (Smirnova et al., 2001). Overexpression of $Drp1^{K38A}$ prevents endogenous Drp1 action in a dominant-negative fashion. By overexpressing the $Drp1^{K38A}$ mutant in neurons, we found that the length of mitochondria in the axon increased, suggesting that Drp1 is responsible for the axonal mitochondrial fragmentation.

The importance of mitochondrial fission in dendrites has been proposed, based on the observation that suppression of mitochondrial fission inhibited the transport of mitochondria into the dendritic spine and synaptic plasticity (Li et al., 2004). Drp1-knockout mice showed impaired mitochondrial transport (Ishihara et al., 2009), suggesting that mitochondrial fission is required for the efficient delivery of mitochondria to both axons and dendrites. The impairment of mitochondrial transport may be related to axonal growth retardation and deficits in synaptic plasticity. In this respect, local fission of mitochondria in the axon may contribute to the efficient transport of mitochondria along the axon. The functional significance of axonal mitochondrial fission will need to be further addressed in the future.

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＜국문초록＞

신경세포는 생존 및 정상적인 기능을 위하여 다량의 에너지를 소비하므로, 미토콘드리아의 기능이 매우 중요하다. 미토콘드리아는 신경세포 내에서 신경돌기를 따라 이동하기도 하고, 세포 내 여러 상황에 따라 접합과 절단을 반복하면서 그 분포와 형태가 역동적으로 변화한다. 역동적인 미토콘드리아의 형태 변화는 주로 GTPase 단백질인 Dynamin-related protein-1 (Drp1)에 의한 절단에 의해 조절되는 것으로 알려져 있다. 그러나, 중추신경계 신경세포에서의 미토콘드리아 분포 및 형태 변화 조절에 대해서는 비교적 연구가 미흡한 실정이다. 이 연구의 저자들은 미토콘드리아에 선택적으로 표적화되는 DsRed-mito 플라스미드를 일차 배양한 대뇌겉질 신경세포에 유전자 도입하여, 가지돌기 및 축삭에 분포하는 미토콘드리아의 길이와 역동성을 분석하였다. 흥미롭게도, 축삭 말단 부위에 분포하는 미토콘드리아의 길이가 세포체 근처의 축삭에 분포하는 미토콘드리아에 비하여 유의미하게 짧았다. 또한 Drp1 단백질이 가지돌기와 축삭에 다량 분포하며, 형광현미경하에서 이뤄진 실시간 촬영을 통해 축삭내에서 미토콘드리아의 절단이 활발하게 나타나는 것을 관찰하였다. 이를 통해, 축삭 말단 미토콘드리아의 길이 감소는 축삭 내 분포하는 Drp1 단백질의 활성에 의한 것으로 생각할 수 있었다. 위 가설을 검증하기 위하여, Drp1의 우성음성돌연변이 단백질을 신경세포에 유전자 도입하여 내재적 Drp1의 활성을 억제한 결과, 축삭 내 미토콘드리아 길이의 유의미한 증가가 관찰되었다. 이러한 결과들을 종합할 때, 대뇌겉질 신경세포에서 미토콘드리아의 절단은 축삭 내에서 지엽적으로도 진행되며, 이에 의하여 축삭내 위치에 따른 미토콘드리아의 길이 변화가 조절되는 것으로 생각되었다.