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A novel method to study the local mitochondrial fusion in myelinated axons *in vivo*

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ABSTRACT

Mitochondrial remodeling (replication, fission/fusion) is a dynamically regulated process with diverse functions in neurons. A myelinated axon is an extension from the cell soma of a fully differentiated neuron. Mitochondria, once synthesized in the cell body, enter the axon displaying robust trafficking and accumulation at nodes of Ranvier to match metabolic needs. This long-distance deployment of mitochondria to axons raises the issue of whether myelinated axons can function independently of the cell body to execute mitochondrial remodeling to match local demands. Mitochondrial fusion has been suggested to occur in axons in simple neuronal cultures *in vitro*. However, whether such events occur *in vivo* in an intact nervous system remains unanswered. Here we describe a novel technique which allows monitoring of mitochondrial fusion in intact sciatic nerve of frog (*Xenopus laevis*). Mitochondrial population was labeled by injecting two different MitoTracker dyes (Red and Green), spatially apart along sciatic nerves surgically and then allow to "meet" *in vivo*. At 24 h post-surgery, the sciatic nerves were taken out for mitochondrial imaging at the half-way point. During the post-injection periods, the anterograde-directed Green mitochondria meet with the retrograde-directed Red mitochondria. If fusion occurs, the merged of Green and Red fluorophores in the same mitochondrion will produce a Yellow color in merged images. The labeled mitochondria were observed with a Nikon A1 confocal microscope. Our new mitochondrial imaging method opens an avenue to separately assess the role of local axonal mitochondrial fusion, independent of the cell body of nerve fibers.

1. Introduction

Mitochondria are mobile organelles classically known to be the key energy producers within cells. However, over the last 10 years mitochondrial research has identified an important new function referred to as mitochondrial remodeling (Detmer and Chan, 2007; Baloh, 2008; Benard and Karbowski, 2009). Mitochondrial remodeling is a process collectively referring to the fusion and fission activities exhibited by these organelles. Advance in the field of mitochondrial remodeling has necessitated a conceptual transformation of the function of mitochondria from mere ATP production to the regulation of cell survival and rescue (Chen et al., 2007; Jahani-Asl et al., 2007, 2010; Tondera et al., 2009). In the area of cell rescue, a recent paper has suggested that mitochondrial fusion preserves mitochondrial integrity by stabilizing damaging mutation of mitochondrial DNA (Chen et al., 2010). Do myelinated axons have the ability to execute local, autonomous mitochondrial remodeling? Most of the studies on mitochondrial remodeling are performed on simple cell culture systems where it occurs under close range somal control (Detmer and Chan, 2007; Suen et al., 2008). While it is clear that protein synthesis and biogenesis occur at or near the soma (Kaplan et al., 2009), it is unclear whether biogenesis or remodeling of mitochondria can occur locally in myelinated axons *in vivo*, which can be 6 feet away from the soma in large mammals. Indeed, mitochondrial replication has been suggested to occur mainly in the perinuclear region of PC-12 cells (Davis and Clayton, 1996). Further, even if the cell soma provides axons with mitochondria each fully armored with remodeling machinery comprising fusion (MNF1/2, OPA1) and fission (drp1) proteins, activation of this remodeling machinery still might involve additional signaling molecules and co-factor proteins (Benard and Karbowski, 2009) synthesized only in the neuronal soma. Does the soma direct axonal mitochondrial remodeling by synthesizing and sending necessary signaling proteins long-distance, or can axons autonomously trigger local mitochondrial remodeling?

The concept of an autonomous axon functioning independently from a distant cell soma is rapidly emerging. Pioneering works on primary cultures have suggested that mitochondria-specific

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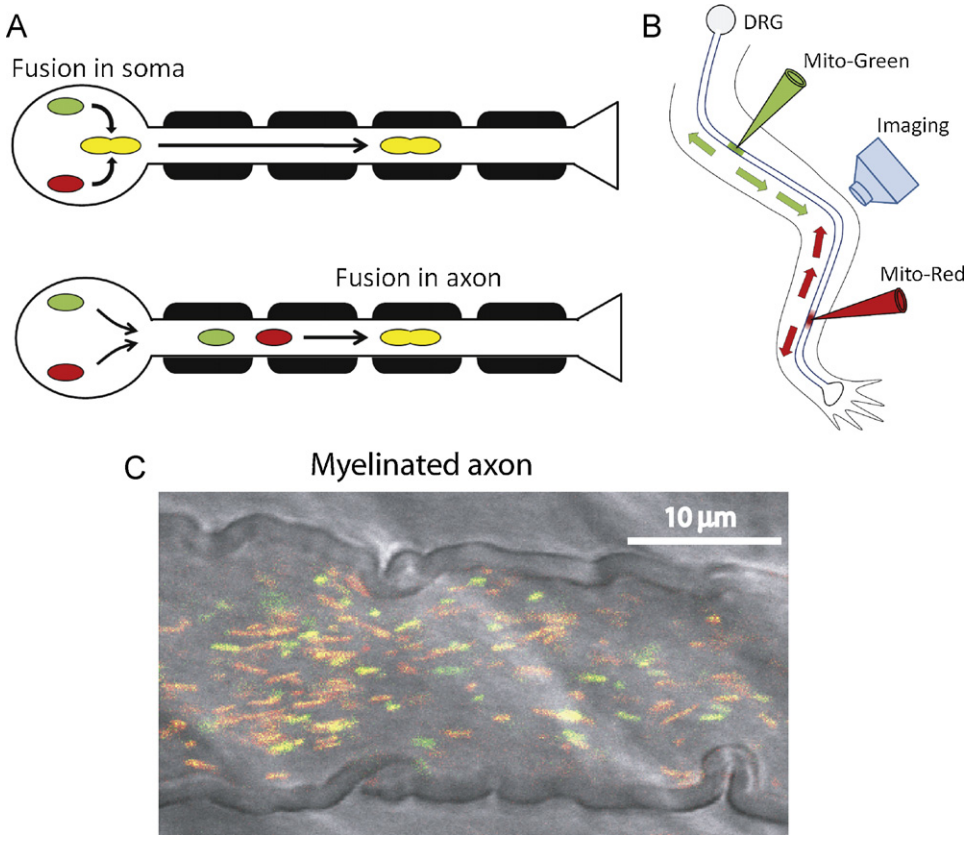

Fig. 1. The nature of mitochondrial fusion in a fully differentiated myelinated axon *in vivo*. (A) Two models for origin of fused mitochondria in axons *in vivo*. Top – fusion in soma precedes transport to axons. Bottom – transport to axon precedes local fusion. (B) Experimental design to monitor the mitochondrial fusion in frog sciatic nerve. MTG and MTR are locally injected (0.2–0.4 μl) 4 cm apart in sciatic nerves of anaesthetized frogs to label two populations of uni-color Green and Red mitochondria in axons. This is followed by imaging of encounters of Red and Green mitochondria in single axons in between injection sites of freshly isolated nerves at 6, 24 h after injection. Fusion produces Yellow mitochondria. (C) A typical, identifiable myelinated axon with Green, Red and Yellow mitochondria labeled with MTG and MTR, 60× objective lens. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

precursor proteins can be locally synthesized in axons (Kaplan et al., 2009) and that mitochondrial fusion/fission occurs in axons (Amiri and Hollenbeck, 2008). However, the extrapolation from *in vitro* to *in vivo* is a major challenge, particularly in the case of myelinated axons. A fully differentiated axon in an intact nervous system is several orders of magnitude longer than its counterpart in culture, has undergone myelinogenesis and operates in a morphological, cellular and biochemical environment unlike any that *in vitro* conditions can mimic.

Here we demonstrate a new technique which allows monitoring the mitochondrial fusion in live sciatic nerve of frog *in vivo*. We believe that this technique will be useful in assessing the role of mitochondrial remodeling in neurological disorders involving axonal pathology.

2. Materials and methods

Female adult frogs were obtained from Nasco (Fort Atkinson, WI). Mito-Tracker dyes, Rhod-2 and Dimethyl Sulphoxide (DMSO) were purchased from Invitrogen (Carlsbad, CA). All other chemicals were of analytical grade, obtained from Sigma (Sigma–Aldrich, St. Louis, MO).

2.1. Differential dye injection in sciatic nerve

All animal experiments were carried out according to the guidelines from Institutional Animal Care and Use Committee (IACUC). Sciatic nerves from 45 female adult frogs were used. All surgical procedures and dye injections were performed at room temperature. The fusion detection technique was modified from our previous studies on mitochondrial motility (Zhang et al., 2010). Briefly, frogs were anaesthetized by submersion in water containing ethyl 3-aminobenzoate, methanesulfonate (2 g/l water) for 10–15 min. Once anaesthetized, the skin was cut and sciatic nerve was exposed near the sciatic notch (the site where the nerve exits the spinal cord). A sharp glass micropipette (2–5 μm), mounted to a holder and controlled by a fine 3-dimensional manipulator, was used to locally inject MitoTracker-Green-FM (MTG) into the sciatic nerve. At 4 cm downstream, MitoTracker-Red-CMXRos (MTR) was locally injected similarly. The injection pipette contained 0.2–0.4 μl of 0.5 mM Mito-Tracker (dissolved in DMSO), was gently injected under visual guidance through a microscope at 20× magnification (Ghabriel and Allt, 1982). The frog was allowed to recover from surgery at 6, 24 h post-surgery. The frog was then killed and the sciatic nerves were taken out for mitochondrial imaging at the half-way point (*i.e.*, 2 cm from each injection site).

2.2. Confocal microscopy

Time lapse imaging of individual nerves were performed using a Nikon A1 laser scanning confocal microscope (Nikon Instruments Inc. Melville, USA). Sciatic nerves, labeled with MitoTracker, were bathed in a frog Ringer solution containing (mM): NaCl, 100; KCl, 3; $CaCl_2$, 2.0; $MgSO_4$, 1.0; HEPES, 3; $NaHCO_3$, 20; and glucose, 10; sodium pyruvate, 2.5; malic acid, 2.5. The solutions were fully oxygenated with 95% O_2 and 5% CO_2. All mitochondrial imaging