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In vivo time-lapse imaging of mitochondria in healthy and diseased peripheral myelin sheath☆

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ABSTRACT

The myelin sheath that covers a large amount of neurons is critical for their homeostasis, and myelinating glia mitochondria have recently been shown to be essential for neuron survival. However morphological and physiological properties of these organelles remain elusive. Here we report a method to analyze mitochondrial dynamics and morphology in myelinating Schwann cells of living mice using viral transduction and time-lapse multiphoton microscopy. We describe the distribution, shape, size and dynamics of mitochondria in live cells. We also report mitochondrial alterations in Opa1^{delTTAG} mutant mice cells at presymptomatic stages, suggesting that mitochondrial defects in myelin contribute to OPA1 related neuropathy and represent a biomarker for the disease.

1. Introduction

Mitochondrial functions are essential for the maintenance of the nervous system. Indeed many neurodegenerative diseases such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis and peripheral nerve diseases involve dysfunctional mitochondria (de Moura et al., 2010; Cartoni and Martinou, 2009; Schroder, 1993). While the final outcome of these diseases is neuronal degeneration, previous data suggest a critical role for glial mitochondria in this process.

Indeed recent studies indicate that functional mitochondria are required in the myelin sheath to preserve axonal function and integrity (Viader et al., 2013). The myelin sheath covers a large amount of neurons both in the central (CNS) and the peripheral nervous system (PNS). Oligodendrocytes and Schwann cells in the CNS and PNS, respectively, wrap axons with multiple turns of compacted plasma membrane in order to electrically isolate successive axonal segments. Each myelinated segment is separated from the next one by a node of Ranvier, where the machinery that allows the saltatory conduction of action potentials is concentrated (Eshed-Eisenbach and Peles, 2013). Mitochondria are abundant in the axon and they are enriched at nodes of Ranvier (Edgar et al., 2008; Ohno et al., 2011). These axonal mitochondria are crucial to repolarise the nodal membrane and therefore to maintain the nerve conduction.

The importance of mitochondria in the function of myelinated axons is further illustrated by the Charcot–Marie–Tooth peripheral nerve diseases that are due to mutations in genes involved in mitochondrial dynamics such as *GDAP1* and *MFN2* (Cartoni and Martinou, 2009; Cassereau et al., 2011). Intriguingly *GDAP1* has been involved both in axon-related disease CMT2K (Cassereau et al., 2011) and myelin-related disease CMT4A (Kabzińska et al., 2014), suggesting that mitochondria of both glia and axons are involved. Moreover mutations in OPA1 (optic atrophy 1), a gene essential for mitochondria fusion, were recently shown to give rise to systemic neurodegeneration including a peripheral neuropathy with features of axonal degeneration and demyelination (Bonneau et al., 2014; Yu-Wai-Man et al., 2010). Together these data indicate that glial and axonal mitochondria are central to the maintenance of the intimate axon–myelin relationship and the modulation of their physiology may provide an opportunity to treat peripheral nerve diseases.

However investigating the myelinating glia–axon relationship is not straightforward as it involves complex cellular interactions that are difficult to reproduce in vitro. Furthermore, while in vivo methods

Abbreviations: mSC, myelinating Schwann cell; CMV promoter, cytomegalovirus promoter; OPA1, optic atrophy 1; AAV, adeno-associated virus; CAG promoter, cytomegalovirus beta-actin beta-globin promoter; ROI, Region of Interest; OPO, optical parametric oscillator.

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have been developed to analyze mitochondrial dynamics in myelinated axons (Romanelli et al., 2013), the complementary technique for observing mitochondria in the surrounding myelinating Schwann cells (mSC) has not yet been described.

Here we detail a method to enable the visualization of mitochondria in myelinating glia in living mice using a multiphoton microscope. By contrast to existing published approaches to study axonal mitochondria (Misgeld et al., 2007), our method does not require engineered mutant mice and can therefore be directly applied to any mouse model of neuropathy. Moreover, this method allows longitudinal imaging for hours under physiological in situ conditions, without requiring nerve excision or displacement. Using this method, we show that presymptomatic OPA-1^{delTTAG} mice, mimicking human mitochondrial disease with peripheral neuropathy, display strong mitochondrial defects in their myelinating Schwann cells. This suggests that these mitochondrial defects can be used as a biomarker of the disease at presymtomatic stages and they may be the cause of the peripheral nerve disease.

2. Materials and methods

2.1. Cloning

pMito-dsRed2 (Clontech, Ref. 632421) was digested with NheI/ NotI, blunted and cloned into pAdtrack-CMV (Quantum Biotechnologies, Inc.), pSICOR (Addgene, Ref. 11579) or pAAV-MCS (Cell Biolabs, Inc.) under the control of a CMV (pAdtrack-CMV, pSICOR) or a CAG (pAAV-MCS) promoter. Clones were validated by sequencing.

2.2. Viral particles production

Lentiviral and adenoviral production have been previously described in Gonzalez et al. (2014) and He et al. (1998) respectively. Briefly, to produce high-titer adeno-associated virus (AAV), three 15 cm dishes of 70–80% confluent HEK293T cells were transfected with 71 μg of pAAV expression vector, 20 μg of pAAV capsid and 40 μg of pHelper (Cell Biolabs, Inc.). The medium was collected two days after transfection, pooled and centrifuged 15 min at 2000 rpm to spin down floating cells. In parallel, cells were scraped, collected in PBS, lysed by 4 successive freeze–thaw cycles and centrifuged 15 min at 5000 rpm to discard cell debris. The cleared supernatant and the cleared medium were pooled and filtered with a 0.22 μm syringe driven filter. The viral solution was first filtered through a cation-exchange membrane Mustang S acrodisc (Pall Corporation) to remove empty particles and then through an anion-exchange membrane Mustang Q acrodisc (Pall corporation) to retain infectious viral particles. Viral particles were then eluted in 10 mM bicine, 0.61 M NaCl, 2 mM MgCl2 and 10% glycerol buffer and concentrated using centrifugal concentrators Amicon. This protocol routinely produced a titer of 10^{11} PFU/ml. (for further details see Okada et al., 2009).

2.3. Mouse strains

Swiss mice (Janvier, France) were used for all experiments unless otherwise stated. The construction of the Opa1 knock-in mice with the recurrent OPA1 c.2708_2711delTTAG mutation was described in Sarzi et al. (2012). Experiments were carried out on 9 weeks old Opa1$^{+/mut}$ (Opa1^{delTTAG}) mice and Opa1$^{+/+}$ littermate controls. Mice were kept in the animal facility of the Institute for Neurosciences of Montpellier in clear plastic boxes and subjected to standard light cycles. All animal experiments were conducted in accordance with the French Institutional and National Regulation CEEA-LR-11032.

2.4. In vivo virus injection in the sciatic nerve

The protocol has been detailed previously (Gonzalez et al., 2014). Briefly, 6 weeks old mice were anesthetized and the incision area was shaved and cleaned using betadine solution. After incision, the *gluteus superficialis* and *biceps femoris* muscles were separated to reveal a cavity crossed by the sciatic nerve. The nerve was gently lifted out using a fine spatula and a thin glass needle filled with viral solution (8 μl) was introduced into the nerve with a micromanipulator. This solution was injected over 30 min with short pressure pulses using a Picopump (World Precision Instrument) coupled to a pulse generator. After injection, the nerve was replaced into the cavity, the muscles were readjusted as before and the wound was closed.

2.5. Drug administration

Oligomycin A (Sigma, Ref. 75351) was stored in ethanol at 8 mg/ml and then diluted in 1 ml of sterile PBS at 80 μg/ml. The aqueous solution was warmed up to 37 °C for 10 min and 2 μl were injected into the mouse sciatic nerve using a Hamilton syringe. Alternatively the aqueous solution was diluted at 800 μg/ml in 3% low melting agarose solution and the drug-containing agarose solution was used to submerge the exposed nerve.

2.6. Immunohistochemistry

The dissected nerve was washed in L15 medium, fixed in Zamboni's fixative (Stefanini et al., 1967) for 10 min at room temperature, washed in PBS, and incubated in successive glycerol baths (15, 45, 60, 66% in PBS) for 18 to 24 h each before freezing at −20 °C. The nerves were cut in small pieces in 66% glycerol and the perineurium sheet removed. Small bundles of fibers were teased in double-distilled water on Superfrost slides, dried overnight at room temperature, and the slides stored at −20 °C. For immunostaining, the teased fibers were incubated for 1 h at room temperature in blocking solution (10% goat serum, 0.2% TritonX100, and 0.01% sodium azide in PBS). Then, the samples were then incubated with integrin-β1 primary mouse antibody (1/100, Chemicon, Ref. MAB1997), ATP synthase 1 primary mouse antibody (1/100, Life Scientific Thermo Fisher, Ref. A21351), PTEN primary rabbit antibody (1/200, Cell Signaling, Ref. 9188), ECCD2 primary mouse antibody (1/100, BD biosciences, Ref. 610181), Neurofascin primary rabbit antibody (1/500, abcam, Ref. 31457) or mito-Tracker Green TM (500 nM, Life technology, M-7514) in blocking solution overnight at 4 °C. The next day, the samples were washed in PBS and incubated for 1 h at room temperature with secondary donkey antibodies coupled to Alexa488 or Alexa647 (1/1600, Molecular probes, Ref. A21202 and 940076 respectively). Finally the samples were washed in PBS and mounted in Immu-mount (Thermo Scientific). Images were acquired at room temperature using a 20× or 63× objective, a Zeiss confocal microscope LSM710, and its associated software.

2.7. Electron microscopy

WT and Opa1^{delTTAG} mouse sciatic nerves were fixed in situ in the cavity containing the nerve for 20 min with 4% PFA and 2.5% glutaraldehyde, in 0.1 M phosphate buffer (pH 7.3). Then nerves were removed and postfixed overnight in the same buffer. After washing 30 min in 0.2 M PBS phosphate buffer, the nerves were incubated with 2% osmic acid in 0.1 M phosphate buffer for 90 min at room temperature. Then, samples were washed in 0.2 M PBS phosphate buffer, dehydrated using ethanol gradient solutions and embedded in epoxy resin. For electron microscopy of sciatic nerves, ultrathin (70 nm) cross-sections were cut and stained with 1% uranylacetate solution and lead-citrate and analyzed using a HITACHI H7100 electron microscope at the 'Centre des Resources en Imagerie Cellulaire' (CRIC) (for further details see Cotter et al., 2010).