Journal of Neuroscience Methods 262 (2016) 56–65

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Basic neuroscience

Quantification of mitochondrial morphology in neurites of dopaminergic neurons using multiple parameters

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HIGHLIGHTS

- Mitochondrial morphology of dopaminergic neurites can be quantified by at least 4 independent variables.
- A gestalt method of analysis measuring multiple parameters is required to adequately quantify mitochondrial morphology.
- Mutations with known effects on mitochondrial morphology change the relationship between morphological parameters of mitochondria.
- The method successfully differentiates control mitochondria from those damaged by Parkinson's disease-like treatment and genetic manipulations affecting mitochondrial fusion/fission.

ARTICLE INFO

Article history:
Received 24 August 2015
Received in revised form
12 December 2015
Accepted 2 January 2016
Available online 14 January 2016

Keywords:
Drosophila
Mitochondria
drp1
opa1
Parkinson

ABSTRACT

Background: Studies of mitochondrial morphology vary in techniques. Most use one morphological parameter while others describe mitochondria qualitatively. Because mitochondria are so dynamic, a single parameter does not capture the true state of the network and may lead to erroneous conclusions. Thus, a gestalt method of analysis is warranted.
New method: This work describes a method combining immunofluorescence assays with computerized image analysis to measure the mitochondrial morphology within neuritic projections of a specific population of neurons. Six parameters of mitochondrial morphology were examined utilizing ImageJ to analyze colocalized signals.
Results: Using primary neuronal cultures from *Drosophila*, we tested mitochondrial morphology in neurites of dopaminergic (DA) neurons. We validate our model using mutants with known defects in mitochondrial morphology. Furthermore, we show a difference in mitochondrial morphology between cells treated as control or with a neurotoxin inducing PD (Parkinson's Disease in humans)-like pathology. We also show interactions between morphological parameters and experimental treatment.
Comparison with existing methods: Our method is a significant improvement of previously described methods. Six morphometric parameters are quantified, providing a gestalt analysis of mitochondrial morphology. Also it can target specific populations of mitochondria using immunofluorescence assay and image analysis.
Conclusions: We found that our method adequately detects differences in mitochondrial morphology between treatment groups. We conclude that some parameters may be unique to a mutation or a disease state, and the relationship between parameters is altered by experimental treatment. We suggest at least four variables should be considered when using mitochondrial structure as an experimental endpoint.

1. Introduction

Mitochondria are a dynamic collective of organelles that, in addition to supplying to the cell with energy, keenly respond to cellular stress. They have important functional relationships with apoptosis, metabolism, and other diseases (Rugarli and Langer, 2012). However, there is seemingly no agreement on how to best describe or report the morphology of these organelles. Some

Abbreviations: DA, dopaminergic; MPP^+, 1-methyl-4-phenylpyridinium; PD, Parkinson's disease; TH, tyrosine hydroxylase.

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http://dx.doi.org/10.1016/j.jneumeth.2016.01.008
0165-0270/

studies use individual mitochondria (Wang et al., 2011), while others examine all of the mitochondria in a cell (Koopman et al., 2006; Dagda et al., 2009; Leonard et al., 2015). Also important is which cellular compartment is best to measure mitochondria. In neurons, the mitochondria in the soma are likely not as critical as mitochondria in the dendrites and/or axons for synaptic transmission (Cheng et al., 2010; Court and Coleman, 2012). Thus, measuring one parameter from individual mitochondria is not capturing the totality of the state of the network in terms of morphology.

The range of possible morphologies for mitochondria is wide and dependent on cell-type, particularly for neurons and especially for the dopaminergic (DA) neurons implicated in Parkinson's disease (PD). Mitochondria in these highly-specialized neurons are smaller and have a lower total mass compared to mitochondria in other neurons (Liang et al., 2007; Perier and Vila, 2012), and are selectively vulnerable to oxidative stress (Surmeier and Schumacker, 2013; Wiemerslage et al., 2013). In general, damaged mitochondria are fragmented, round, and swollen (Chang and Reynolds, 2006; Berman et al., 2008; Perier and Vila, 2012), and such changes in mitochondria are implicated in Parkinson's disease (PD) (Dawson et al., 2010; Nakamura et al., 2011; Saxena and Caroni, 2011; Botella et al., 2011). A difficulty in studying neurodegenerative disorders such as PD is that many of the neurons implicated in the disease are already dead by the time symptoms appear (Jellenger, 2012; Smith et al., 2012). However, mitochondria could serve as an early biomarker to screen for neuroprotective therapies by trying to reverse changes in mitochondrial morphology caused by neurodegenerative processes.

In this work, we utilize our previous cell culture model of PD-like degeneration (Wiemerslage et al., 2013) and adapt an ImageJ macro written by Dagda et al. (2009) to quantify the mitochondrial morphology specifically in DA neurons. First, we quantify different parameters of morphology using mutants (*e.g.* drp1, opa1) with known morphological defects in their mitochondria. Then we compare mitochondrial morphology between control and PD-like treatment. Lastly, we examine the specific parameters measured to determine how many parameters are needed for a comprehensive assessment of mitochondrial morphology.

2. Methods

2.1. Fly stocks

All lines were obtained from the Bloomington *Drosophila* Stock Center (BDSC) unless otherwise noted. Flies were kept at 25 °C and raised on standard cornmeal agar diet. A 'Cantonized' white eye stock w^{1118} served as wild-type. Morphometric control experiments used *drp1*/*CyO*-GFP and *opa1*/*CyO*-GFP (gifts from Dr. Leo Pallanck, University of Washington).

2.2. Drosophila *primary neuronal cultures*

Cultures were prepared as previously described in Park and Lee (2006). Briefly, mid-gastrula embryos at developmental stage 7 were harvested in a laminar-flow hood and plated onto round, glass coverslips (Bellco Glass, Inc., Vineland NJ, USA). Cultures were incubated in 4–5% CO_2 at 24–25 °C for up to 9 days *in vitro* (DIV). Culture medium (DDM1) was a mixture of high glucose Hams's F-12/Delbecco's medium (Irvine Scientific, Santa Ana, CA), L-glutamine (2.5 mM; Irvine Scientific), HEPES (20 mM), and four supplements: putrescine (100 μM), progesterone (20 ng/mL), transferrin (100 μg/mL), and insulin (50 μg/mL). At 3 DIV, all cultures had 50% of the culture medium replaced with new medium.

2.3. Pharmacological treatments

All drugs were added to cultures at 3 DIV after baseline images were acquired, except for experiments with delayed use of rescue therapy. Drug remained in the dish once treated (*i.e.* was never washed out). Cultures were handled and treated in a laminar flow hood (Forma Scientific, model 1849). Drugs dissolved in ddH2O were sterilized by filtration through a 0.2 μm cellulose acetate filter before use/storage. 1-methyl-4-phenylpyridinium (MPP^+) iodide (Sigma) was prepared as a 40 mM stock solution dissolved in ddH2O and stored in darkness at −70 °C. MPP^+ was handled according to guidelines reviewed in Przedborski et al. (2001).

2.4. Immunofluorescence assay

Cultures were fixed with 4% paraformaldehyde for 40 min on ice, and then washed 3 times, 10 min for each wash. Wash solution was 10 mM phosphate buffered saline containing 0.5% bovine serum albumin. All washes were at room temperature (≈25 °C). Blocking and permeabilization was performed using 0.1% Triton X-100 and 5% normal goat serum (Sigma) for 30 min on ice. After 1 more wash for 10 min, permeabilized cultures were incubated overnight (≈16 h) at 4 °C with a 1:1000 ratio of primary antibody (mouse anti-tyrosine hydroxylase, ImmunoStar) diluted in wash. The next day, primary antibody was removed and cultures were washed 3 times, 10 min for each wash. Cultures were next treated with a 1:2000 ratio of secondary antibody (FITC or TRITC labeled goat anti-mouse, Invitrogen) diluted in wash and placed on ice for 1 h. Secondary antibody was then removed and cultures were again washed 3 times, 10 min for each wash. Coverslips were next mounted onto glass slides upon rows of fluorogel with Tris buffer (Electron Microscopy Sciences), covered with an extra drop of fluorogel, then topped with coverglass (Electron Microscopy Sciences), and edges sealed with clear fingernail polish.

2.5. Microscopic detection of mitochondria in dopaminergic neurons

To visualize mitochondria from DA neurons, cultures were stained with both MitoTracker Orange (Invitrogen) and anti-TH antibody (ImmunoStar). At 7 DIV but before staining, cultures were treated with 50 nM MitoTracker Orange (Invitrogen) in the original culture medium for 1 h at 25 °C prior to immunostaining. Culture medium was then removed. Neuronal cultures were fixed and stained as usual (described above) with anti-TH primary antibody and FITC-labeled secondary antibody. The colocalization of signals between MitoTracker and anti-TH staining specifically identified mitochondria in DA neurons for analysis.

2.6. Image acquisition

Prepared/stained cultures were viewed under a fluorescence microscope (Olympus IX71 with 100 W Mercury lamp). Mitochondria were observed with a LUCPLFLN 40× lens (NA, 0.60). Two fluorescence filter sets were also used as following: Chroma 41017 (FRITC) and 41002 (TRITC). Images were taken using Spot CCD digital camera (2 megapixels, Diagnostic Instruments, Sterline Heights, MI). For a 12-bit image, the intensity ranges from 0 (no signal) to 4095 (maximum saturation). To determine the best threshold for our analysis of mitochondria, the raw MitoTracker signal of several images of mitochondria was compared to its "ideal" threshold level. The ideal threshold level was considered the one where a manual count of the mitochondria from the raw image gave the same results as the Analyze Particles function in ImageJ. The mean