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Mitochondria in hippocampal presynaptic and postsynaptic compartments differ in size as well as intensity

David W. Freeman¹, Ronald S. Petralia², Ya-Xian Wang², Mark P. Mattson³, and Pamela J. Yao^{3,*}

¹Laboratory of Epidemiology and Population Science, NIA/NIH, Baltimore, Maryland 21224

²Advanced Imaging Core, NIDCD/NIH, Bethesda, Maryland 20892

³Laboratory of Neurosciences, NIA/NIH, Baltimore, Maryland 21224

Abstract

Experimental observations have hinted that, in different compartments of a neuron, mitochondria can be different in their structure, behavior and activity. However, mitochondria have never been systematically compared at the subcellular level in neurons. Using electron microscopy, we analyzed several thousands of mitochondria in the synapses of rat hippocampal neurons *in vitro* and *in vivo*. We focused on examining the intensity and size of mitochondria as these structural features have been correlated to the activity of mitochondria. We compared mitochondria in the presynaptic compartment to those in the postsynaptic compartment. We found that, at least in the synapses of hippocampal neurons, presynaptic mitochondria are smaller in diameter and overall higher in intensity (darker) than postsynaptic mitochondria. Our finding highlights the need for developing technologies that would measure the activity of individual mitochondria at single-mitochondria resolution in real time.

Introduction

Since the first electron micrographs of mitochondria were published in 1952 (Palade, 1952), electron microscopy has provided detailed, nanometer-scale information about the structure and organization of mitochondria (Palade 1953; Ernster and Schatz, 1981). While electron microscopy does not directly measure the activity of mitochondria, monitoring individual mitochondria up-close under various experimental conditions enables functional deductions from their morphological phenotypes. For instance, electron microscopic studies of rat liver cells have shown that visible changes in the electron opacity as well as the width of mitochondria are tightly linked to their metabolic state (Hackenbrock, 1966, 1968a,b). Similarly, a study of mouse embryonic fibroblasts found that mitochondria become darker under some experimental situations such as starvation (Gomes et al., 2011). And, even more recently, a study of hippocampal neurons showed that the activation of Sonic hedgehog signaling pathway increases mitochondrial activity accompanied by a concomitant increase in mitochondrial intensity as revealed by electron microscopy (Yao et al., 2017). This

*Corresponding author Pamela J. Yao, Ph.D., Laboratory of Neurosciences, NIA/NIH Biomedical Research Center, 251 Bayview Boulevard, Baltimore, Maryland 21224, Phone: 410-558-8586, Pamela.Yao@nih.gov.

finding adds to the growing recognition that the ultrastructural appearances of mitochondria are relevant to their function.

In this study, we continue this progress. We use electron microscopy to analyze the mitochondria in synapses of rat hippocampal neurons both *in vitro* and *in vivo*. We compare the mitochondria in the presynaptic compartments to those in the postsynaptic compartments of the neurons. Our analysis focuses on examining the intensity and size of mitochondria, since early studies have implicated the relevance of these ultrastructural features to the activity of mitochondria and metabolic status of cells (Colonnier and Guillery, 1964; Royer and Kinnamon, 1988; Sasaki and Iwata, 1996).

Objective

We set out to analyze two structural parameters of mitochondria, intensity and size, using electron microscopy. We compare the mitochondria in the presynaptic compartments to those in the postsynaptic compartments of the hippocampal neurons *in vitro* and *in vivo*.

Results & Discussions

We first examined cultured hippocampal neurons. We took micrographs of the neurons (from three different cultures) that had clearly visible mitochondria in their synaptic regions. Axons with presynaptic terminals in the synaptic regions were identified on the basis of the presence of synaptic vesicle clusters and a dark presynaptic active zone. Dendrites with postsynaptic structures were identified based on the presence of a postsynaptic density and enrichment of microtubules (for examples see Fig. 1A, 1B).

From these electron micrographs, we first measured the “darkness” or intensity of individual mitochondria. Comparing the mitochondria in the presynaptic terminals with those in the postsynaptic dendrites, the intensity measurements showed neuron-to-neuron or synapse-to-synapse variations. In some synapses, the mitochondria were noticeably darker in the presynaptic terminals than in the postsynaptic dendrites (Fig. 1A, 1B), whereas in other neurons, presynaptic mitochondria appeared similar to postsynaptic mitochondria (*data not shown*). However, the values averaged over ~600 randomly selected mitochondria exhibited a clear trend: the intensity of the presynaptic mitochondria was statistically significantly greater than the intensity of the postsynaptic mitochondria (Fig. 1C, presynaptic 168.3 ± 2.3 vs postsynaptic 161.0 ± 1.8 , $p < 0.0001$, Mann-Whitney *U* test).

The size of the mitochondria was also different between the two synaptic compartments. The average size, as determined by area, of the presynaptic mitochondria was roughly half the size of the postsynaptic mitochondria (Fig. 1D, presynaptic $0.077 \mu\text{m}^2 \pm 0.010$ vs postsynaptic $0.146 \mu\text{m}^2 \pm 0.012$, $p < 0.0001$, Mann-Whitney *U* test). As an additional assessment of the physical size of the mitochondria, we measured the smallest diameter of the mitochondria. We found a much higher proportion of presynaptic mitochondria that had a small minimum Feret diameter, the smallest distance between two points along the edges of the mitochondria, compared to postsynaptic mitochondria (Fig. 1E). Consistently, the difference between the average minimum Feret diameter of the presynaptic and postsynaptic

mitochondria was statistically highly significant (Fig. 1F, presynaptic 0.188 ± 0.009 vs postsynaptic 0.239 ± 0.009 , $p < 0.0001$, Mann-Whitney *U* test).

We next examined the neurons in the hippocampus. We took micrographs from the stratum lucidum of the CA3 region of the hippocampus (from three rats). We chose mossy terminal synapses because they typically have abundant presynaptic mitochondria. The postsynaptic processes were the proximal portions of the apical dendrites of the pyramidal cell neurons, including the basal connection of the dendrites to the soma.

In these hippocampal synapses, examples of dark presynaptic mitochondria were readily visible (Fig. 1G, 1H; and suppFig1 for additional examples), although some variations existed. Despite the variations, the intensity values averaged from the measurement of ~2,500 mitochondria (from three rats) showed that the difference between presynaptic and postsynaptic compartments was statistically significant: the presynaptic mitochondria were significantly darker than the postsynaptic mitochondria (Fig. 1I, presynaptic 169.4 ± 0.7 vs postsynaptic 165.0 ± 0.9 , $p < 0.0001$, Mann-Whitney *U* test). Notably, assessments of the mitochondrial size showed that average area (Fig. 1J, presynaptic 0.038 ± 0.001 vs postsynaptic 0.087 ± 0.005 , $p < 0.0001$, Mann-Whitney *U* test) and minimum Feret diameter (Fig. 1K, 1L, presynaptic $0.167 \mu\text{m} \pm 0.002$ vs postsynaptic $0.210 \mu\text{m} \pm 0.004$, $p < 0.0001$, Mann-Whitney *U* test) were smaller in presynaptic mitochondria, indicating that presynaptic mitochondria are thinner than postsynaptic mitochondria.

Early ultrastructural studies have noted darker mitochondria in some axonal terminals of a few neuronal types, including neurons in lateral geniculate nucleus (Colonnier and Gullery, 1964; Montero and Scott, 1981) and the spinal cord (Sasaki and Iwata, 1996). While the hippocampal neurons and the mitochondria within have been examined under electron microscopy (Nafstad and Blackstad, 1966), the physical properties of the mitochondria, such as intensity, were not specifically investigated. In a study of electron microscopic characterization of cultured hippocampal neurons, some mitochondria were visibly darker and thinner in axons than in dendrites (see Figure 5 in Bartlett and Banker, 1984). But this difference was incidental to the main focus of the study; it was neither quantitatively analyzed nor explicitly discussed.

The present study provides a thorough comparison of the differences in intensity and size of mitochondria between the presynaptic and postsynaptic compartments of hippocampal synapses. Our analyses of two different systems – *in vitro* and *in vivo* – have demonstrated a remarkably similar trend: the presynaptic mitochondria are narrower in diameter and overall darker in electron density than the postsynaptic mitochondria. Do darker mitochondria indicate their higher activity? A definitive answer to this question would require tools that can simultaneously assess both the physical properties such as electron density or intensity, and biochemical activities, such as ATP production of mitochondria, at the resolution of a single mitochondrion in real time in live neurons. Before such accurate and powerful tools are devised, though, we can infer from studies on the correlation between the ultrastructural features and function of mitochondria. Among these correlative studies is a recent report on cultured hippocampal neurons in which populations of darker mitochondria were directly proportional to an overall higher activity of mitochondria (Yao et al., 2017). If the

“darkness” signifies activity, the existence of darker mitochondria in the presynaptic terminals of the hippocampal neurons (this study) and of other neurons (Colonnier and Gullery, 1964; Montero and Scott, 1981; Royer and Kinnamon, 1988; Sasaki and Iwata, 1996; Redd et al., 2000) most likely reflects high demands in energy supply and Ca^{2+} buffering, both of which are attributed to the functions of mitochondria, and are results of presynaptic transmission.

It is worth noting that not every presynaptic terminal of the hippocampal neurons has darker mitochondria. We have observed variations at the level of synapses, neurons, or even animals. For example, of the three rats examined, the hippocampi from two of them had visibly darker presynaptic mitochondria, but the third rat did not exhibit a statistically significant difference in the intensity between the presynaptic and postsynaptic mitochondria (suppFig. 2). This variability may simply reflect the fact that mitochondria are ‘multitasking’ (Friedman and Nunnari, 2014) as well as the dynamic nature of energy demands and consumption in different compartments of a neuron under diverse conditions at any given time (Rangaraju et al., 2014).

The difference in the size of mitochondria, on the other hand, is almost invariable between the two synaptic compartments. This is particularly true in terms of the diameter of the mitochondria: the presynaptic mitochondria by and large are thinner than the postsynaptic mitochondria (Fig. 1J, K). This finding is consistent with a previous 3D reconstruction study reporting that axonal mitochondria are small and thin (Popov et al., 2005). A critical next step will be to determine whether thinner presynaptic mitochondria are merely an adaptation to the generally narrower axonal space, or whether their thinness might, or might also, indicate a population of mitochondria that are functionally more active.

Limitations

This is a correlative study, despite the large number of samples analyzed *in vitro* and *in vivo*.

Conjectures

It will be useful, and should be feasible, to systematically examine additional ultrastructural features of mitochondria, including cristae morphology and cristae junction width, in different types of neurons from different areas of the brain, as well as compare mitochondria in normal brains to those in diseased brains.

It will be also important to be able to assay the activity of individual mitochondria not only at the nanometer spatial scale but also in real time in live neurons. Such methodologies would provide unprecedented knowledge of mitochondria, their roles in subcompartments of neurons, and their contributions to physiology and pathology of neurons.

Methods

Animals

Timed pregnant rats were used as the source of embryonic brains to establish cultures of hippocampal neurons. Hippocampal tissues from postnatal day 37 rats were used for

electron microscopy. All animal procedures were approved by the NIA and the NIDCD Animal Care and Use Committees, and complied with the NIH Guide for Care and Use of Laboratory Animals.

Primary culture of rat hippocampal neurons

Cultures of hippocampal neurons were prepared from embryonic day 18 rat brains as described (Kaech and Banker 2006). Dissociated neurons were plated at a density of ~100–150 cells/mm² on polylysine (1 mg/ml)-coated glass coverslips (no. 1.5). Embryos from three rats were used to prepare the three cultures, and one coverslip from each culture was included in the electron microscopic analysis. The neurons were grown in Neurobasal medium supplemented with B27 (Invitrogen). The age of the cultures used was 19–20 days.

Electron microscopy

Electron microscopy of cultured neurons was performed according to published protocols (Petralia and Wenthold, 1999; Petralia et al., 2010; Yao et al., 2017). Neurons grown on coverslips were fixed in 2% paraformaldehyde/2% glutaraldehyde in phosphate buffer at room temperature for 30 minutes. Following washes, the neurons were fixed in 1% osmium tetroxide in cacodylate buffer for 30 minutes, and dehydrated in an ethanol series (including 10 minutes in 1% uranyl acetate in 50% ethanol), and then in propylene oxide and embedded in epon. The coverslips were removed with hydrofluoric acid, and thin sections were examined on electron microscopes (JEOL JSM-1010 and JSM-2100) without further staining. Micrographs were taken from synaptic regions of the neurons. Three different cultures were examined.

Electron microscopy of hippocampal sections were performed following published protocols (Petralia and Wenthold, 1999; Petralia et al., 2010). Three P37 male Sprague-Dawley rats were perfused with fixative and hippocampal sections were cryoprotected, frozen and then embedded in Lowicryl HM-20 in a Leica AFS freeze-substitution instrument. Thin sections were stained with uranyl acetate and lead citrate, and examined on the electron microscope. Micrographs were taken from mossy terminal synapses in the stratum lucidum of the CA3 region of the hippocampus.

Measurements of mitochondria, data analysis and statistics

This study focused on the mitochondria in the presynaptic and postsynaptic compartments of hippocampal neurons. For the hippocampus, we took ~440 micrographs of the CA3 region of the hippocampus from three rats. We chose mossy terminal synapses in the stratum lucidum of the CA3 region because these synapses typically have abundant presynaptic mitochondria. For cultured hippocampal neurons, we took ~500 micrographs of the synapses from three different cultures. The synapses from the hippocampus and cultures were identified based on the presence of clearly visible synaptic vesicle clusters, a synaptic cleft, and a postsynaptic density. We used imageJ (v1.49) to analyze several structural characteristics of mitochondria. Prior to image processing, a global scale was set based upon the scale bar associated with each micrograph. The coloration of each micrograph was inverted and mitochondria were traced using the freehand tool. The intensity (mean gray value) within the traced areas was measured and recorded. The area and minimum feret

diameter were calculated based upon the standard set scale for each image. Statistical comparisons were performed with GraphPad Prism v6.0 using the Mann-Whitney *U* Test. All results are expressed as mean $\pm$ 95% CI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Palade GE (1952) The fine structure of mitochondria. *The Anatomical Record* 114: 427–451. [PubMed: 12996882]
- Palade GE (1953) An electron microscope study of the mitochondrial structure. *J Histochem Cytochem* 1:188–210. [PubMed: 13069686]
- Ernster L, Schatz G (1981) Mitochondria: A historical review. *J Cell Biol* 91: 227s–255s. [PubMed: 7033239]
- Hackenbrock CR (1966) Ultrastructural bases for metabolically linked mechanical activity in mitochondria. *J Cell Biol* 30:269–297. [PubMed: 5968972]
- Hackenbrock CR (1968a) Ultrastructural bases for metabolically linked mechanical activity in mitochondria. II. Electron transport-linked ultrastructural transformations in mitochondria. *J Cell Biol* 37:345–369. [PubMed: 5656397]
- Hackenbrock CR (1968b) Chemical and physical fixation of isolated mitochondria in low-energy and high-energy states. *Proc Natl Acad Sci* 61:598–605. [PubMed: 4176482]
- Gomes LC, Di Benedetto G, Scorrano L. (2011) During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. *Nat Cell Biol* 13:589–598. [PubMed: 21478857]
- Yao PJ, Manor U, Petralia RS, Brose RD, Wu RT, Ott C, Wang YX, Charnoff A, Lippincott-Schwartz J, Mattson MP. (2017) Sonic hedgehog pathway activation increases mitochondrial abundance and activity in hippocampal neurons. *Mol Biol Cell*. 28:387–395. [PubMed: 27932496]
- Colonnier M, Guillery RW (1964) Synaptic organization in the lateral geniculate nucleus of the monkey. *Z Zellforsch Mikrosk Anat* 62:333–355 [PubMed: 14218147]
- Montero VM, Scott GL (1981) Synaptic terminals in the dorsal lateral geniculate nucleus from neurons of the thalamic reticular nucleus: a light and electron microscope autoradiographic study. *Neuroscience* 6:2561–2577. [PubMed: 7322350]
- Royer SM, Kinnamon JC. (1988) Ultrastructure of mouse foliate taste buds: synaptic and nonsynaptic interactions between taste cells and nerve fibers. *J Comp Neurol*. 270:11–24. [PubMed: 3372732]
- Sasaki S, Iwata M. (1996) Ultrastructural study of synapses in the anterior horn neurons of patients with amyotrophic lateral sclerosis. *Neurosci Lett* 204:53–56. [PubMed: 8929976]
- Nafstad PH, Blackstad TW. (1966) Distribution of mitochondria in pyramidal cells and boutons in hippocampal cortex. *Z Zellforsch Mikrosk Anat*. 73:234–245. [PubMed: 5982710]
- Redd EE, Pongstaporn T, Ryugo DK. (2000) The effects of congenital deafness on auditory nerve synapses and globular bushy cells in cats. *Hear Res* 147:160–174. [PubMed: 10962182]
- Bartlett WP, Banker GA. (1984) An electron microscopic study of the development of axons and dendrites by hippocampal neurons in culture. I. Cells which develop without intercellular contacts. *J Neurosci*. 4:1944–1953. [PubMed: 6470762]
- Friedman JR, Nunnari J. (2014) Mitochondrial form and function. *Nature* 505:335–343 [PubMed: 24429632]

- Rangaraju V, Calloway N, Ryan TA. (2014) Activity-driven local ATP synthesis is required for synaptic function. *Cell*. 156:825–835. [PubMed: 24529383]
- Popov V, Medvedev NI, Davies HA, Stewart MG. (2005) Mitochondria form a filamentous reticular network in hippocampal dendrites but are present as discrete bodies in axons: a three-dimensional ultrastructural study. *J Comp Neurol*. 492:50–65. [PubMed: 16175555]
- Kaech S, Banker G (2006). Culturing hippocampal neurons. *Nat Protoc* 1:2406–2415. [PubMed: 17406484]
- Petralia RS, Wenthold RJ (1999). Immunocytochemistry of NMDA receptors. *Methods Mol Biol* 128:73–92. [PubMed: 10320974]
- Petralia RS, Wang YX, Hua F, Yi Z, Zhou A, Ge L, Stephenson FA, Wenthold RJ (2010). Organization of NMDA receptors at extrasynaptic locations. *Neuroscience* 167:68–87. [PubMed: 20096331]

Figure 1.

Presynaptic mitochondria are darker and smaller than postsynaptic mitochondria.

(A) (B) Representative electron micrographs show synaptic regions of cultured hippocampal neurons. *SV*, synaptic vesicle cluster in the presynaptic terminal; *D*, postsynaptic dendrite; *m*, mitochondria. Scale bars = 0.5 μm . (C) Individual mitochondria were traced and intensity (mean gray value) within the traced areas was measured using imageJ. (D) The size in term of area of presynaptic mitochondria is smaller than that of postsynaptic mitochondria. (E)(F) Analysis of the minimum diameter of mitochondria showed that presynaptic mitochondria

have significantly smaller diameters. In (C), (D), and (F), ****p value < 0.0001, calculated with a Mann-Whitney *U* test.

(G) (H) Representative electron micrographs show mossy terminal synapses of the adult hippocampus. Notice darker and smaller mitochondria in synaptic vesicle (*SV*) – containing presynaptic terminals than in postsynaptic dendrites (*D*). Scale bars = 1 μ m (I) The average intensity of presynaptic mitochondria is greater than that of postsynaptic mitochondria. (J) The average area of presynaptic mitochondria is also greater than that of postsynaptic mitochondria. (K)(L) The minimum diameter of presynaptic mitochondria is smaller than that of postsynaptic mitochondria. In (I), (J), and (L), ****p value < 0.0001, calculated with a Mann-Whitney *U* test.