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Ultrastructural evidence for impaired mitochondrial fission in the aged rhesus monkey dorsolateral prefrontal cortex

Yury M. Morozov*,[1], Dibyadeep Datta [1], Constantinos D. Paspalas, Amy F.T. Arnsten*

Department of Neuroscience, Yale University School of Medicine, New Haven, CT, USA

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ABSTRACT

Dorsolateral prefrontal cortex mediates high-order cognitive functions that are impaired early in the aging process in monkeys and humans. Here, we report pronounced changes in mitochondrial morphology in dendrites of dorsolateral prefrontal cortex neurons from aged rhesus macaques. Electron microscopy paired with 3D reconstruction from serial sections revealed an age-related increase in mitochondria with thin segments that intermingled with enlarged ones, the 'mitochondria-on-a-string' phenotype, similar to those recently reported in patients with Alzheimer's disease. The thin mitochondrial segments were associated with endoplasmic reticulum cisterns, and the mitochondrial proteins Fis1 and Drp1, all of which initiate mitochondrial fission. These data suggest that the 'mitochondria-on-a-string' phenotype may reflect malfunction in mitochondrial dynamics, whereby fission is initiated, but the process is incomplete due to malfunction of subsequent step(s). Thus, aged rhesus monkeys may be particularly helpful in exploring the age-related changes that render higher cortical circuits so vulnerable to degeneration.

1. Introduction

The newly evolved dorsolateral prefrontal cortex (dlPFC) mediates high-order cognitive functions (Fuster, 2001) that are impaired early in the aging process in both monkeys and humans (Herndon et al., 1997; Moore et al., 2006; Morrison and Baxter, 2012). A prototypic cognitive function executed by the dlPFC involves working memory, the ability to use representational knowledge to guide behavior, thought, and emotion. Neurons in layer III of the dlPFC excite each other to maintain persistent firing across the delay period in a working memory task (Goldman-Rakic, 1995). Pyramidal cells in dlPFC deep layer III have extensive recurrent connections (Kritzer and Goldman-Rakic, 1995) on long, thin spines with N-Methyl-D-aspartate receptor-NR2B synapses (Wang et al., 2013b). These dlPFC layer III connections are weakened by increased calcium-cyclic adenosine monophosphate (cAMP)–dependent protein kinase A (PKA) signaling, which opens nearby K^+ channels to gate network connections (Arnsten, 2015; Arnsten et al., 2012). This process is regulated by the phosphodiesterase, PDE4A, which declines with advancing age (Carlyle et al., 2014), leading to increased PKA phosphorylation of tau (Jicha et al., 1999), and increased cAMP-PKA opening of K^+ channels, which reduces neuronal firing during working memory (Wang et al., 2011). There is also a marked loss of thin dendritic spines from monkey layer III dlPFC with advancing age (Du et al., 2010; Dumitriu et al., 2010; Luebke et al., 2010), the spines that mediate recurrent excitatory connections and are the focus of cAMP-calcium-K^+ channel gating. Thus, these circuits are particularly vulnerable to advancing age (Morrison and Baxter, 2012).

The persistent neuronal firing generated by dlPFC recurrent circuits during working memory is highly energy demanding. Thus, it is of interest that the dlPFC has higher mRNA expression of mitochondrial proteins compared to other cortical regions (Chandrasekaran et al., 1992). Importantly, oxidative stress indicative of mitochondrial pathology has been identified in the dlPFC as part of the progression in Alzheimer's disease (AD) (Ansari and Scheff, 2010). Ultrastructural studies of the aging rhesus monkey dlPFC may help to illuminate this process, as these analyses provide clarity rarely possible in human postmortem tissue. For example, one recent study of axon terminals in layer III of the monkey dlPFC identified mitochondrial morphological abnormalities (i.e., "donut" morphology) with increasing age (Hara et al., 2014), indicating that mitochondrial changes may play a role in age-related cognitive decline. Given the great expansion of layer III dlPFC dendrites in primate evolution (Elston, 2003; Elston et al., 2006), the current study tested the hypothesis that mitochondrial morphology in

* Corresponding authors at: Department of Neuroscience, Yale University School of Medicine, PO Box 208001, 333 Cedar Street, New Haven, CT 06520-8001, USA. Tel.: +1-203-785-4330; fax: +1-203-785-5263.
E-mail addresses: Yury.morozov@yale.edu (Y.M. Morozov), amy.arnsten@yale.edu (A.F.T. Arnsten).
[1] These authors contributed equally to this work.

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dlPFC dendrites may also be altered with aging and that changes in mitochondria might reveal laminar differences.

Mitochondria are extremely dynamic, multifunctional organelles that play important roles in maintaining cellular health. Proper cellular function demands a balance between opposing mitochondrial dynamics—fusion and fission. Fusion may allow mitochondria to compensate for defects by sharing components; while fission segregates the damaged segments of mitochondria that then undergo mitophagy in the autophagosome, preserving the integrity of the mitochondrial network (Cho et al., 2010; Friedman and Nunnari, 2014; Palmer et al., 2011; Youle and van der Bliek, 2012). An equilibrium between fusion and fission may also balance energy versus toxic reactive oxygen species (ROS) production, where mitochondrial fission may provide a respite from high ROS production (Nasrallah and Horvath, 2014). Therefore, alterations in mitochondrial dynamics may lead to cell pathology.

Mitochondrial fission is initiated by interactions with the calcium-containing smooth endoplasmic reticulum (SER), where SER cisterns accumulate in the vicinity of the mitochondria and determine the sites of constriction and subsequent division (Friedman and Nunnari, 2014; Friedman et al., 2011). Fission is initiated by the dynamin-like GTPase, dynamin-related protein 1 [Drp1; also known as DLP1; (Smirnova et al., 2001)], which translocates from the cytosol into the outer mitochondrial membrane (OMM), where it interacts with its primary receptor—mitochondrial fission protein 1 (Fis1). Drp1 oligomers assemble into rings/spirals around the OMM, leading to the final membrane constriction and scission (Ingerman et al., 2005; James et al., 2003; Yoon et al., 2003). The efficacy of Drp1 in fission is determined by its GTPase activity, which is inhibited by PKA signaling (Cereghetti et al., 2008, 2010). As PKA activity is increased in the aged dlPFC (Carlyle et al., 2014), mitochondrial dynamics might be disrupted in these vulnerable circuits.

Here, we used electron microscopy (EM) paired with immunocytochemistry and 3-dimensional (3D) reconstruction from serial sections to examine mitochondrial morphology and molecular expression patterns in the aging rhesus macaque dlPFC. We detected age-related morphogenetic abnormalities resulting in accumulation of mitochondria with intermingled thin ("pinched") and enlarged segments, indicative of mitochondrial dysfunction. This phenotype has just been detected in AD brains, where it has been termed "mitochondria-on-a-string" (MOAS) (Zhang et al., 2016). Thus, the discovery of this phenotype in the aged rhesus monkey may help reveal the etiology of mitochondrial deficits in human AD.

2. Materials and methods

2.1. Animals

All animal protocols were approved by the Yale University Institutional Animal Care and Use Committee (IACUC) and comply with the NIH guidelines for animal care and use. The study utilized brains from young adult (female 7 year-old [yo], and 11 yo), and aged (female 26 yo, 27 yo, 31 yo; and male 33 yo) rhesus macaques from the Yale colony.

2.2. Tissue processing for immunolabeling

Animals were deeply anaesthetized with pentobarbital (0.03 mL/10g of body weight) and perfused transcardially by the fixative containing 4% paraformaldehyde (wt/vol), 0.2% picric acid, and 0.05% glutaraldehyde in 0.1 M phosphate buffer (Carlyle et al., 2014). Coronal 60-μm-thick sections of the dlPFC were cut with a vibratome, cryoprotected with 30% sucrose and stored at −80 °C. All sections of the dlPFC went through freeze-thaw cycles in liquid nitrogen to augment penetration of immunoreagents and were processed free-floating for immunocytochemistry and EM as described below.

After defrosting and extensive washing, the sections were blocked in 5% bovine serum albumin, and incubated with mouse anti-DLP1 monoclonal (Drp1; clone 8; dilution 1:100; BD Biosciences, San Jose, CA, USA), or rabbit anti-TTC11 polyclonal antisera (Fis1; 1:100; Novus Biologicals USA, Littleton, CO, USA) overnight at room temperature. The sections were then immersed in respective solutions of biotinylated goat anti-mouse or anti-rabbit IgGs (1:300) and developed by Elite ABC kit (all from Vector Laboratories, Burlingame, CA, USA) with Ni-intensified 3,3′-diaminobenzidine-4HCl as a chromogen. Some of the sections incubated with anti-DLP1 serum were labeled with made-in-goat anti-mouse secondary serum conjugated with 1-nm gold particles (Aurion, Wageningen, The Netherlands; 1: 80). Silver-intensification of gold was performed with R-Gent SE-LM kit (Aurion) according to the manufacturer's instructions. Specificity of the methods was tested by omitting the primary antibody from the staining procedure. No staining was observed in negative control sections. The sections were postfixed with 1% OsO_4, (or 0.5% for gold/silver labeling) dehydrated and embedded in Durcupan (ACM; Fluka, Buchs, Switzerland) on microscope slides and coverslipped.

2.3. EM and 3D reconstruction

For EM investigations, cortical layers were identified at low magnification using a light microscope, then selected areas from distinct layers were dissected and re-embedded into Durcupan blocks. Serial 70-nm-thick ultrathin sections were obtained, contrasted and evaluated as previously described (Morozov et al., 2006, 2016). All images were acquired using a JEM1010 (JEOL, Tokyo, Japan) transmission electron microscope at 80 kV and Multiscan 792 digital camera (Gatan, Pleasanton, CA, USA). For EM tomography of mitochondria, 250-nm-thick sections from Durcupan-embedded dlPFC layer III slices from 31 and 33 yo animals were prepared as above and submitted to Department of Cell Biology (Yale University, New Haven, CT, USA).

For 3D reconstruction of mitochondria, 30–35 serial images were made with 15,000× magnification. Neuropil fragments were chosen for the 3D reconstruction in a random manner (but, avoiding as possible, cell bodies, blood vessels and groups of the myelinated axons) by an investigator blinded to the animal's age and to laminar locations. The micrographs were aligned using the computer program Reconstruct (Fiala, 2005), publicly available at http://www.bu.edu/neural/Reconstruct.html. We concentrated on mitochondria within dendritic shafts that were identified in serial micrographs by straight shape (in contrast to curved axons and astroglial processes). Mitochondrial profiles were traced, and the 3D images of reconstructed mitochondria were obtained and analyzed using Reconstruct software.

2.4. Data analysis

Neuropil segments (n = 4–5/animal) from cortical layers II–VI of each animal were used for 3D reconstruction and quantitative analyses of mitochondria. The neuropil segments for each cortical layer was identified and dissected based on cytoarchitectural features of dlPFC. All identifiable dendritic mitochondria were used for quantitative EM with the exception of truncated mitochondria, which were defined as mitochondria with detectable segments shorter than 1 micron. Identity of the analyzed neurons (e.g., pyramidal cell vs. interneuron) and laminar location of the cell body