In vivo imaging axonal transport of mitochondria in mammals
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Supplementary Figures and Text:

**Supplementary Figure 1** Neuronal mitochondria are specifically labeled and appear normal.

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Supplementary Methods

*Note: Supplementary Movies 1–4 are available on the Nature Methods website.*
Neuronal mitochondria are specifically labeled and appear normal.

(a) Mitochondria-specific dyes (here MitoFlour Red, red) show colocalization (white arrowheads) with mitochondrial CFP (cyan) at a neuromuscular junction, but also stain non-neuronal mitochondria (e.g. in muscle). In the myelinated axon that enters the synapse (left) staining with mitochondrial dye is weak due to insufficient penetration. (b) Cross section of two axons ( thy1-mitoCFP-S x thy1-YFP-19) stained for pyruvate dehydrogenase (PDH, red), a mitochondrial matrix protein. The lower axon expresses mitochondrial CFP (cyan), while the upper one does not (both contain cytoplasmic YFP, yellow). Almost perfect co-localization can be seen between the mitochondrial fluorescent protein and the antibody in the lower axon (98.6%, 145 / 147 of transgene-labeled structures also label with the antibody). All mitochondria identified with the antibody also exhibited CFP fluorescence in transgene-expressing neurons (145 mitochondria in 50 mitoCFP-expressing axons). Transgene-labeled and unlabeled axons have similar mitochondrial densities (2.9 ± 0.2 mitochondria per cross section of mitoCFP-expressing axons versus 3.1 ± 0.1 in axons that did not express the transgene). Schwann cell mitochondria are labeled with the antibody (gray arrowhead), but did not contain mitochondrial CFP. (c - e) Neuromuscular junctions appear normal in heterozygous MitoMouse muscles. Neuromuscular junction area (c) and postsynaptic receptor density (d) are normal. (e) Confocal reconstructions show similar axonal and synaptic morphology in MitoMouse and control muscles ( thy1-mitoCFP x thy1-YFP-19, inset shows detail). (f, g) No evidence of atrophy is apparent in phalloidin-stained MitoMouse muscles. (h) Synaptic mitochondria are ultrastructurally normal in MitoMouse neuromuscular junctions ( thy1-mitoCFP-C mice, in which mitochondria are labeled in all motor axons). (i) Quantification confirms absence of mitochondrial swelling and accumulation in thy1-mitoCFP mice. (j) Living nerve-muscle explant of a thy1-mitoCFP subset mouse showing one mitoCFP-positive and one negative synapse counterstained with MitoTracker Red. (k, l) Detail from the mitoCFP-positive (k) and negative (l) synapses illustrating comparable staining patterns with MitoTracker Red (arrowheads, synaptic mitochondria; asterisks, subsynaptic myelin mitochondria; octothorps, sarcoplasmic mitochondria). (m) Quantitative analysis of MitoTracker Red staining confirms unaltered mitochondrial potential in mitoCFP-positive synapses (n = 25 synapses per group; similar results were obtained with Rhodamine123). mito- control mice, mito+: thy1-mitoCFP mouse; mito: dia.: mitochondrial diameter; mito/ axon: mitochondria per axon cross-section; cYFP: cytoplasmic YFP; ΔΨm: mitochondrial potential as measured by potential-sensitive dyes.
Expression patterns in MitoMouse lines.

(a) Coronal sections from 4 different MitoMouse brains. Each line shows expression in cortical neurons (insets). In some lines barrels in somatosensory cortex are revealed by the mitochondrial labeling (arrowheads in leftmost panel). (b - d) Expression patterns in the hippocampus (b), spinal cord (c) and cerebellum (d) in the same lines as in panel (a). Insets in (d) show mitochondrial accumulations in mossy fiber rosettes in cerebellar foliae. (e-h) All Purkinje cells are labeled in one line (e) while only a subset of Purkinje cells is labeled in another line (h). (f - g) High power images from these two lines show dense dendritic labeling in Purkinje cells. (i) Retinal expression pattern in four lines that includes retinal ganglion cells, bipolar cells, amacrine cells (arrowhead), and photoreceptors. (j) In the neuromuscular system many lines label the majority of motor axons while others only label subsets. YFP: yellow; CFP: cyan; est: cortico-spinal tract; dc: dorsal column; dg: dentate gyrus; dh: dorsal horn; gl: granule layer; inl: inner nuclear layer; ipl: inner plexiform layer; lh: lateral horn; ml: molecular layer; NMJs: neuromuscular junctions, onl: outer nuclear layer; opl: outer plexiform layer; PC: Purkinje cells; RG: retinal ganglion cells; vh: ventral horn;
Time-lapse imaging of mitochondria at branch points.

(a1, a2) Mitochondrial transport at an axonal trifurcation in a *MitoMouse* (thy1-mitoCFP-S x thy1-YFP-I6). (a1) Anatomical overview of the branch point as revealed by cytoplasmic YFP-labeling. (a2) Time-lapse series (frame rate at 1 Hz) showing two mitochondria coursing through the branch point. Top panel shows a mitochondrion (highlighted red) that enters the leftmost branch #1. Below, another mitochondrion (highlighted green) enters the middle branch #2. As mitochondrial flow is divided at branch points, the number of moving mitochondria drops towards more distal locations. Typically axons in the triangularis sterni muscle supply 50 synapses (50 ± 3.1, n = 9 axons). As expected, the number of moving mitochondria at synaptic entry points is about 30 times lower than the number moving in the proximal unbranched part of an intercostal axon (total number at synaptic entry points: 0.18 ± 0.03 min⁻¹, n = 19 versus proximal axons: 9.6 ± 0.6 per min, n = 38). Given the number of mitochondria per synapse (343 ± 45, see Figure 1) and the number of mitochondria that enter synapses (0.12 ± 0.02 per min; n = 15 synapses), the half-life of mitochondria in synaptic terminals appears to be around 2 days. (b) Image of an integrated time-lapse series of 1700 frames (45 min at 1 Hz) revealing the organelle tracks that enter the three branches. Image has been edge-enhanced to improve contrast (Supplementary Movie 3).
**Supplementary Figure 4**

**Repetitive in vivo imaging of synaptic mitochondria.**

(a, b) A neuromuscular junction in the sternomastoid muscle of a double transgenic *thy1-mitoCFP-S x thy1-YFP-H* mouse was imaged on two consecutive days. (c, d) Another example of a neuromuscular junction that was first imaged in vivo using wide-field microscopy (c) and on the next day processed for confocal reconstruction (d). AChR: bungarotoxin staining of acetylcholine receptors; cYFP: cytoplasmic YFP.
SUPPLEMENTARY METHODS

Transgenic mice. To achieve selective labeling of neuronal mitochondria, we used two well-established neuronal promoters: the thyl-promoter (kindly provided by Dr. Joshua Sanes, Harvard University)\(^1\) to drive the expression of CFP in thyl-mitoCFP mice, and the neuron-specific enolase (nse)-promoter (kindly provided by Dr. Gregory Cox, The Jackson Laboratory)\(^2,3\) to express YFP in nse-mitoYFP mice. N-terminal in-frame fusions were generated between CFP or YFP and the mitochondrial targeting sequence from subunit VIII of human\(^4\) or mouse cytochrome c oxidase, subunit VIII, gene (cox8) respectively. This portion of cox8 is sufficient for mitochondrial localization of fusion proteins in vivo and in vitro\(^5,7\).

Transgenic MitoMouse lines were generated by standard pronuclear injection. Twenty-five (thyl) and 10 (nse) independent potential founders were identified. Thirteen transgenic lines were established and further characterized (thyl-mitoCFP-B, -C, -D, -E, -F, -H, -K, -M, -O, -P, -S; nse-mitoYFP-X, -Y). Heterozygotes of all lines bred normally and showed no neurological abnormalities. When we bred lines to homozygosity, we observed that homozygote (but not heterozygote) mice of two of the high expressing lines (thyl-mitoCFP-K and -O) showed a coarse tremor after 3 months of age. Homozygotes from other MitoMouse lines showed no such abnormalities. No conclusions presented in this study are based on the use of lines that show neurological abnormalities as homozygotes. For the characterization of mitochondrial morphology and distribution we used double-transgenic mice generated by breeding thyl-mitoCFP mice with thyl-YFP mice, which express cytoplasmic YFP in all (line thyl-YFP-16) or a subset (line thyl-YFP-H) of cortical and motor neurons. One line, which
we found to be particularly suitable for studies of adult motor neurons \( \textit{thy1-mitoCFP-S} \), was used for most of our analysis of mitochondrial transport.

**Tissue preparation, staining, confocal and electron microscopy.** \textit{MitoMouse} tissue was fixed either by transcardial perfusion or submersion (4% paraformaldehyde in phosphate-buffered saline) and sectioned using a cryostat or vibratome. Sections were stained using standard immunohistochemistry protocols with directly fluorophore-conjugated primary antibodies directed against pyruvate dehydrogenase (clone 9H9; Molecular Probes). Fluorescently-labeled bungarotoxin (BTX; to reveal acetylcholine receptors at synapses), phalloidin (to outline muscle fibers), and mitochondria-specific dyes (MitoFluor Red 589, MitoTracker Red CMXRos, Rhodamine 123) were purchased from Molecular Probes and used according to the manufacturer’s protocols. Confocal stacks were obtained using an FV1000 confocal microscopy system (Olympus; objectives: x10/0.3; x20/0.8 oil; x60/1.42 oil) and processed using Metamorph (Universal Imaging) and Photoshop (Adobe) software.

For electron microscopy we used standard methods on glutaraldehyde/paraformaldehyde fixed tissue from \textit{thy1-mitoCFP-C} animals and cytoplasmic \textit{thy1-CFP} controls\(^1\).

**In vivo imaging: equipment and settings.** The sciatic nerve was surgically exposed in anaesthetized and ventilated \textit{thy1-mitoCFP} mice\(^8\). To avoid movement artifacts, the hindlimb was embedded in bite registration polymer (Densply). The surgical opening was filled with warmed (33-37°C) lactated Ringer’s solution. Mitochondrial transport was documented by stream acquisition (system description see below) at a frame rate of about 4Hz for periods up to 5 min. The resulting image stacks were aligned using Autoquant software (Autoquant Imaging) and evaluated in Metamorph (Universal Imaging). For the analysis of degenerating and regenerating axons, intercostal nerves were transected \(^9\). After surgical closure of the wound, mice were injected with atipamezole to speed recovery, placed in a
heated recovery chamber and given buprenorphine for analgesia. All procedures were approved by the IACUC of Harvard University, Cambridge, MA.

**Time-lapse imaging of nerve-muscle explants: equipment and settings.** Explants of the triangularis sterni muscle were prepared from *thy1-mitoCFP* mice as previously described. Mice were killed with pentobarbital. The rib cage (with the attached triangularis sterni muscle and its innervating intercostal nerves) was isolated by paravertebral cuts, pinned on a Sylgard-coated dish (Dow Corning) using insect pins, and maintained on a heated stage (33-37°C; Warner) in Neurobasal A medium (Invitrogen) bubbled with 95% O₂/5% CO₂. Recordings were done in myelinated unbranched intercostal axons, at branch points in intramuscular fascicles or at entry points of neuromuscular junctions. Intercostal axons run very superficially in places, mostly derive from motor neurons (the triangularis sterni contains no sensory organs), and have accessible neuromuscular junctions that can be easily maintained in explants or denervated *in vivo*. Mitochondria in the explants had an appearance that was indistinguishable from the morphology seen in fixed tissues ([Figure 1](#)) and their transport was comparable to the transport seen in the sciatic nerve *in vivo*. Mitochondrial transport rates were stable in explants over 2-3 hours (data not shown), so experiments in explants were restricted to <2 hours.

Axonal transport of mitochondria was analyzed using a BX51 microscope (Olympus) equipped with x20/0.5 and x100/1.0 dipping cone water immersion objectives, an electronic filter wheel with shutter (Sutter), a z-stepper (Ludl) and a cooled CCD camera (Sensicam QE, Cooke) controlled by Metamorph Software. The following filter sets were used: CFP, Chroma 31044v2; YFP, Chroma 61008 with individual excitation filters inserted into the filter wheel. Images were acquired at rates of 0.5-1 Hz using exposure times of 250-1000 msec for 5 min. Images and movies were edited in Metamorph and Photoshop (Adobe). For enhanced clarity, some movies were high- and low-pass filtered, and their intensity was inverted. To
make low-intensity detail more visible, gamma-enhancement was used in final representations (except in Supplementary Figure 1). In high-resolution panels, pixel dimensions were set according to the Nyquist criterion.

**Quantitative and statistical analysis.** Confocal image stacks and time-lapse recordings were analyzed as follows: The size of neuromuscular junctions was determined by threshold measurements of the area of bungarotoxin staining on confocal stacks taken from the triangularis sterni muscle. The size of the muscle fibers was measured on rotated confocal projections as the circumference of individual fibers revealed by phalloidin staining. Mitochondrial potential was determined in living nerve-muscle explants as the fluorescence intensity in synaptic mitochondria over non-mitochondrial background. The following parameters of mitochondrial transport were quantified based on time-lapse recordings of individual axons: The diameter of axons was measured on a single plane focused on the center of the axon (the cross sectional area was calculated assuming cylindrical axon geometry). The number of anterogradely and retrogradely transported mitochondria was determined as the number of fluorescent mitochondria per minute crossing a vertical line in either direction. The proportion of moving mitochondria was estimated in individual axons as the relation of the number of moving versus the number of resting mitochondria in a given frame of the recording. The speed of mitochondria was measured as the distance covered by individual mitochondria during continuous movement over 10-12 consecutive frames. Results are expressed as mean ± standard error of the mean unless indicated otherwise. Statistical significance was assumed when p < 0.05 in unpaired t-tests or Mann-Whitney-U tests using Prism software (Graph Pad).

**Calculation of synaptic half-life of mitochondria.** We estimated the half-life of mitochondria in neuromuscular synapses as follows: First, we obtained series of high-resolution confocal images of neuromuscular synapses (n = 5) and their innervating motor
axons in *thy1-mitoCFP-S* mice (e.g. Figure 1 c). As individual mitochondria could not be resolved within synapses (Figure 1 c2), we obtained the overall fluorescence integral within synapses, by measuring the fluorescence in a defined synaptic area in single optical sections and then multiplying this value with the total synaptic area measured from maximum intensity projections. Then we estimated the fluorescence intensity corresponding to a unit length of mitochondria by measuring the fluorescence of single axonal mitochondria (which can be resolved, Figure 1 c1) and by dividing this value by their length. To determine synaptic half-life of mitochondria, we measured the synaptic influx of mitochondria and assumed steady-state conditions, as well as an average mitochondrial length of 1.5 µm (Figure 1 e; we choose the length of moving mitochondria, as the calculation is based on the number of mitochondria that enter the synapse).

**SUPPLEMENTARY REFERENCES**


