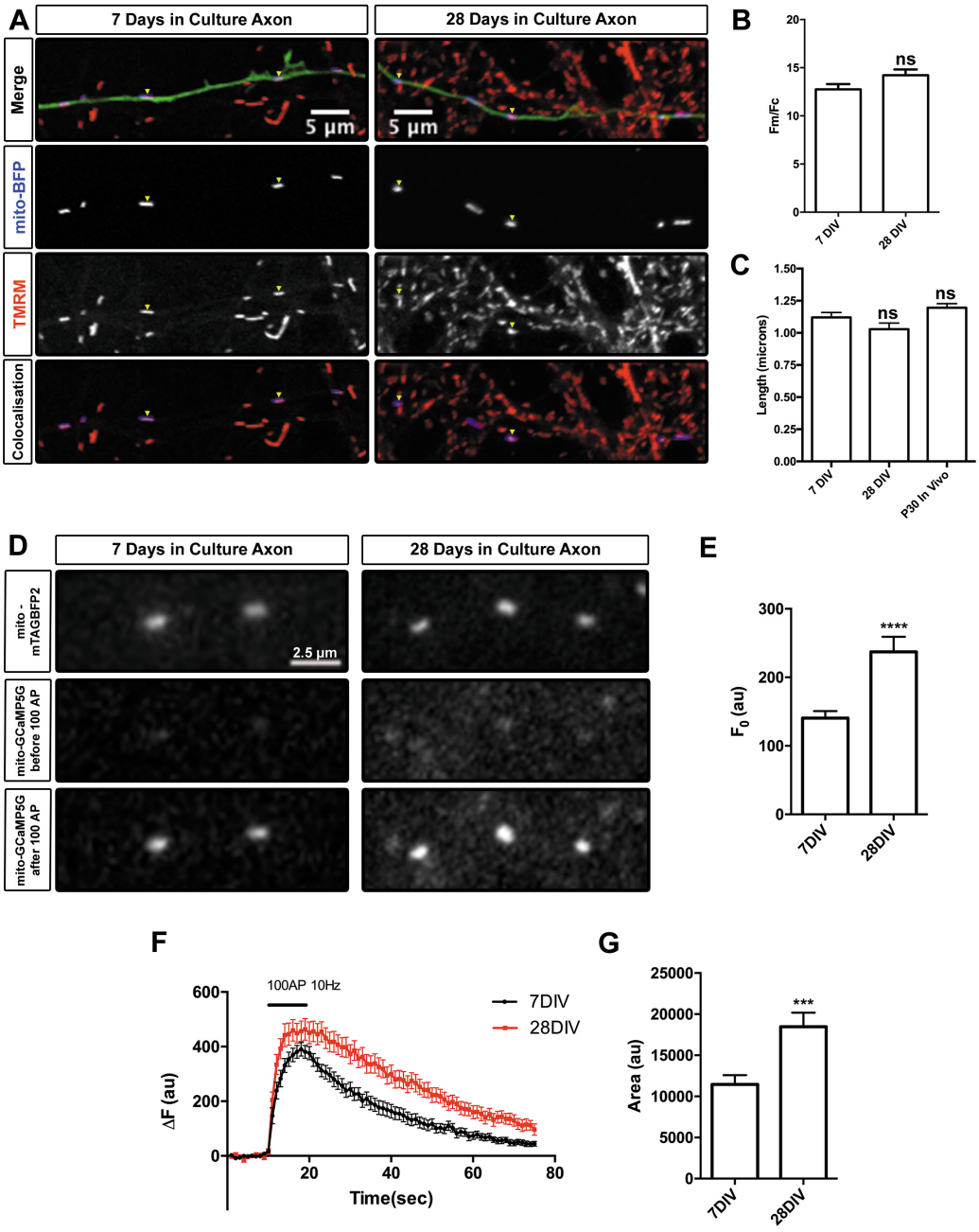
A
7 Days in Culture Axon
28 Days in Culture Axon
Merge
mito-BFP
TMRM
Colocalisation
5 µm
5 µm
B
Fm/Fc
ns
7 DIV
28 DIV
C
Length (microns)
ns
ns
7 DIV
28 DIV
P30 In Vivo
D
7 Days in Culture Axon
28 Days in Culture Axon
mito - mTAGBFP2
mito-GCaMP5G before 100 AP
mito-GCaMP5G after 100 AP
2.5 µm
E
F0 (au)
****
7DIV
28DIV
F
100AP 10Hz
7DIV
28DIV
ΔF (au)
Time(sec)
G
Area (au)
***
7DIV
28DIV

Supplemental Figure 1: Functional properties of mitochondria in immature and mature axons (Related to Figure 1 and Figure 2)

(**A**) Imaging of mitochondrial membrane potential in 7DIV and 28DIV cortical axons in culture. (Top three panels) Representative images of ex utero electroporated cortical axons labeled with cytoplasmic Venus (green), mito-mTAGBFP2 (blue, 2nd panels from the top), as well as all mitochondria with a membrane potential from all cell types via 20nM TMRM (red, 3rd panels from the top). (Bottom panels) The colocalisation threshold tool in Fiji/ImageJ was used to create overlapping pixel maps from the mito-mTAGBFP2 (blue) and TMRM (red) image panels above. Overlapping pixels are shown in purple and demonstrate that mito-mTAGBFP2 labeled mitochondria load with TMRM and therefore have a membrane potential. Yellow arrowheads mark a few mitochondria across all panels. (**B**) Quantitation of the ratio of TMRM fluorescence in mitochondria versus the cytoplasm for each labeled mitochondria in the 7 and 28DIV axons in culture. There is no significant difference between the ratio at 7 vs. 28DIV, revealing there is no difference in mitochondrial membrane potential in immature versus mature axons. N= 82 or 191 mitochondria from 6 or 8 axons for 7 or 28DIV respectively. Mann-Whitney p= 0.7630, Mean ± SEM (**C**) Mitochondrial length was measured across multiple live imaging conditions. There is no significant change in length in any of the conditions suggesting that the imaging parameters are not harming the mitochondria. N= 146, 168 or 149 mitochondria from 12, 13 or 10 axons for 7, 28DIV or P30 *in vivo* respectively. One way ANOVA p= 0.1765, Mean ± SEM (**D**) (Top panels) Axonal mitochondrial labeled with mito-mTAGBFP2 via ex utero electroporation at E15.5 and cultured to 7DIV or 28DIV. (Middle panels) Matrix calcium visualized with mito-GCaMP5G before stimulation (F_0) at 7DIV or 28DIV. (Bottom panels) Matrix calcium visualized with mito-GCaMP5G after stimulation with 100AP at 7DIV or 28DIV. (**E**) Matrix calcium is elevated in resting mitochondria from mature axons compared to resting mitochondria in immature axons. Mann-Whitney p= <0.0001, Mean ± SEM. (**F**) Time course of mitochondrial calcium uptake into the matrix upon stimulation with 100AP for 7DIV axons and 28DIV axons, Plotted as ΔF, Mean ± SEM. (**G**) Quantitation of the area under the curves in (F) showing an increase in the amount of mitochondrial Ca^{2+} import upon stimulation. N= 16 or 18 axons for 7 or 28DIV respectively. Mann-Whitney p= <0.0005, Mean ± SEM. Results E-G suggest that mitochondria in mature axons experience higher levels of calcium but are still able to respond to neuronal activity.

Study	System	PNS or CNS	Neurons imaged	Compartment	Stage/Age	Anesthesia	% Stationary Mitochondria	Measurement of Motility
Obashi and Okabe 2013	Mouse - culture	CNS	Hippocampal	Axon	19-21 DIV	N/A	~ 95% over 180 mins	-
Current Study	Mouse - culture	CNS	Cortical L2/3	Axon	21-28 DIV	N/A	98 ± 4% over 30mins, 68 ± 25% over 12hours	-
Misgeld et al 2007	Mouse - in vivo	PNS	Sciatic nerve	Axon	?	Yes - ?	~ 87% over 5 mins	> 9 mito/min
Plucinska et al 2012	Zebrafish - in vivo	PNS	Rohon-Beard	Axon	2-3 dpf	No - ?	99% distal, 83% proximal over 10 mins	~ .8 mito/min
Sajic et al 2013	Mouse - in vivo	PNS	Saphenous nerve	Axon	8-12 weeks	Yes - terminal urethane	-	~ 1 mito/axon in niave/sham
Sorbata et al 2014	Mouse - in vivo	PNS	Spinal Cord	Axon	6-12 weeks	Yes - ketamine/xylazine	93 ± 6% over 5 - 15 mins	~ .8 mito/min
Takihara et al 2015	Mouse - in vivo	CNS	Retinal Ganglion Cells	Axon	2-25 months	Yes - ketamine/xylazine	-	~ 10 mito/100µm axon/3 mins
Current Study	Mouse - in vivo	CNS	Cortical L2/3	Axon	P30/45 awake	No	93 ± 4.5% over 10 - 20 mins	~ 1 mito/axon
Misgeld et al 2007	Mouse - explant	PNS	Sciatic nerve	Axon	?	N/A	87 ± 1% over 5 mins	9 ±.3/min
Ohno et al 2011	Mouse - slice culture	CNS	Purkinje Cell	Axon	P8-9	N/A	-	~1 mito/5min
Jackson et al 2014	Rat - slice culture	CNS	Hippocampal	Dendrites	P6-12	N/A	~60% over 15 mins	-
Faits et al 2016	Mouse - retina whole mount	CNS	Retinal Ganglion Cells	Dendrites	P9-P21	N/A	~70% at P9, ~100% at P21 over 35 mins	-

Supplemental Table 1: Comparison of mitochondrial motility in mature cultures, slice cultures and *in vivo* (Related to Figure 1 and Figure 4)

Measurements of motility and/or the percentage of stationary mitochondria are compared across multiple publications that imaged mitochondrial dynamics under many different conditions and in many different neuronal types. The table demonstrates that in most studies, control/wildtype neurons show limited mitochondrial motility in mature neurons.

Supplemental Experimental Procedures

Animals

All animals were handled according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Columbia University. Time-pregnant CD1 females were purchased from Charles Rivers. Timed-pregnant hybrid F1 females were obtained by mating inbred 129/SvJ females, and C57Bl/6J males in house [S1].

***In utero* cortical electroporation**

In utero electroporations were performed as detailed in [S1] with the exception that CD1 mice were used. Plasmids concentrations were 1μg/μl for pCAG mito-YFP and pCAG tdTomato.

Primary cortical culture and *ex utero* electroporationCortices from E15.5 CD1 mouse embryos were dissected in Hank's Buffered Salt Solution (HBSS) supplemented with Hepes (2.5 mM), $CaCl_2$ (1 mM, Sigma), $MgSO_4$ (1 mM, Sigma), $NaHCO_3$ (4mM, Sigma) and D-glucose (30 mM, Sigma), hereafter referred to as cHBSS. Cortices were dissociated in cHBSS containing papain (Worthington) and DNAse I (100 μg/ml, Sigma) for 20 min at 37°C, washed three times and manually triturated in cHBSS supplemented with DNAse. Cells were then plated at 7.5 x 10^4 cells per 35mm glass bottom dish (Mattek) coated with poly-D-lysine (1 mg/ml, Sigma) and cultured for 3-28 days in neurobasal medium supplemented with B27 (1X), FBS (2.5%), L-glutamine (2 mM). One third of the medium was changed every 5 days thereafter with non-FBS containing medium. *Ex utero* electroporation was performed as previously published in [S1].

Constructs

pCAG VGLUT1-venus, pCAG mVenus and pCAG mito-DsRED1 were previously described in [S1]. pCAG mito-YFP was created by placing the DNA encoding YFP-mito (from pYFP-Mito; Clontech) 3' to the CAG promoter using PCR. pCAG mito-mTAGBFP2 was created by placing the DNA encoding mTAGBFP2 (from the Addgene plasmid #34632) 3' to the CAG promoter and a matrix mitochondrial targeting sequence (from cytochrome C subunit VIII) using PCR. pCAG-tdTomato and pCAG-mTAGBFP were created by cloning the DNA encoding tdTomato (Clontech) or mTAGBFP (Evrogen) 3' to the CAG promoter using PCR. pCAG mEmerald-LAMP1 was created by cloning the DNA encoding LAMP1-mEmerald from mEmerald-Lysosomes-20 (from the Michael Davidson collection (Addgene plasmid #54149)) 3' to the CAG promoter by PCR. pCAG mito-mEos2 was created by cloning the DNA encoding mEos2 (from the Michael Davidson collection (Addgene plasmid #54510)) 3' to the CAG promoter and the mitochondrial targeting sequence described above via PCR. pCAG mCardinal was created by cloning the DNA encoding mCardinal (from the Michael Davidson collection (Addgene plasmid #54590) 3' to the CAG promoter using PCR. pCAG mito-GCaMP5G was created by cloning the DNA encoding GCaMP5G (from Addgene plasmid #31788) 3' to the CAG promoter and mitochondrial targeting sequence described above using PCR.

Dual color time-lapse imaging of cultured neurons

Imaging was performed between 3-28DIV in cHBSS with a 60x (1.4NA) oil objective on a Nikon Ti-e inverted microscope equipped with a Lumencor Spectra-x light engine and Andor iXon Ultra 897 EM-CCD camera. A custom quad-band excitation/mirror/emission cube (based off Chroma, 89400) followed by clean up filters (Chroma, ET525/50, ET600/50, ET435/26) were used for excitation and emission. Live imaging was done in a Tokai Hit chamber system with the following settings: top heater – 37°C, Bottom heater - 40°C, Bath heater - 37°C, Objective heater - 37°C, CO_2 – 125 mL/min of 5% CO_2, 21.4% O_2, 73.6% N_2. Videos were acquired at 0.1 fps for 30 minutes with mitochondria and presynaptic sites while videos were acquired at 2 fps for 3 minutes with lysosomes and mitochondria. Dual channel kymographs were created in NIS Elements by drawing a line along the imaged axon shaft. These kymographs were then analyzed for mitochondrial, lysosomal and VGLUT1 dynamics using NIS Elements. The Colocalisation Threshold program in FUJI/ImageJ was used to create overlapping pixel maps.

Mito-mEos2 photo-conversion and imaging

Imaging was performed on 28-30DIV distal axons of cortical neurons in cHBSS with a 40x (.95NA) air objective at 1.5x zoom on a Nikon Ti-e inverted microscope equipped with a Nikon A1 confocal. Live imaging was done in a Tokai Hit chamber with the same settings as above. Videos were acquired once every 15 minutes for 12 hours following photo-conversion of a small section of the distal axon (30 to 50 micrometers). All imaging acquisition was performed with NIS Elements and photo-conversion was done with the A1 Stimulation module. Settings for stimulation were determined experimentally based on the expression level of mEos2. Settings were 4% laser power (405nm, Coherent, 100mW) and 24 seconds stimulation of the selected region of interest. The stimulation cycle was repeated one more time on some

bright samples to photo-convert as much green mEos2 as possible (maximum of 48 seconds 405nM exposure). Mitochondrial morphology was not affected by this stimulation protocol. For confocal microscopy imaging sessions, we had 15 μW of power through the objective (40X, 0.95NA), the objective was properly filled for diffraction limited imaging, and the area of the focal spot was on the order of ~0.31 μm^2. Under these illumination conditions, using published absorption cross sections for YFP, a given fluorophore residing at the focus of the light would be excited every ~2x10^-7 seconds meaning under continuous illumination over 98% of the time the fluorophore would not be excited. Semi-automated tracking was performed on photo-converted mitochondria using the NIS Elements tracking module.

TMRM and mito-aGCaMP5G imaging

TMRM (Life Technologies) 10 mM stock was diluted in cHBSS to 10μM. Neuron cultures were then loaded with 20nM TMRM in cHBSS for 20 minutes at 37C. After 20 minutes, the medium was replaced with cHBSS containing 5nM TMRM for the imaging session. Live imaging was performed as above with the 60x objective on the confocal microscope. The Colocalisation Threshold program in Fiji/ImageJ was used to create overlapping pixel maps. For mitochondrial Ca^{2+} imaging, we used modified normal tyrode solution which contained (in mM): 145 NaCl, 2.5 KCl, 10 HEPES pH7.4, 2 $CaCl_2$,1 $MgCl_2$,10 glucose. For blocking spontaneous activity, APV (50μM, Tocris) and CNQX (20μM, Tocris) was added. Action potentials were triggered by 1ms current injections with a concentric bipolar electrode (FHC) placed 20μm away from labeled axons. We applied 100APs at 10Hz with 20V using the stimulator (Model 2100, A-M systems) and imaged with 1s interval during 90sec. Images were analyzed in Fiji (Image J) using a Time Series Analyzer (v3.0) plugin. Mito-GCaMP5G signal and nearby backgrounds were selected by circular ROIs and intensities were measured by the plug-in. After intensities were corrected for background subtraction, ΔF values were calculated from (F-F0). F0 values were defined by averaging 10 frames before stimulation.

Surgery

To image mitochondrial motility *in vivo* we implanted mice with a cranial window above the somatosensory cortex contralaterally to the electroporated hemisphere. First the mice were anesthetized with isoflurane and treated with buprenorphine (0.1 mg/kg, subcutaneous) to minimize post-operative discomfort. We then exposed the skull and drilled a slightly smaller than 3-mm diameter circle centered over the somatosensory cortex. We removed the bone and dura, and irrigated the tissue with sterile chilled cortex buffer (125 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM CaCl2 and 2 mM MgCl2) to stop any sporadic bleeding. A sterilized glass coverslip (3 mm diameter) was placed over the craniotomy and secured to the bone with tissue adhesive (Vetbond, 3M). Finally, a stainless steel headpost was attached to the posterior part of the skull with dental acrylic (Unifast Trad; GC America Inc. IL USA). We monitored the mice every 12 hours for three days after surgery, administering buprenorphine to minimize any signs of discomfort. For acute imaging (P10-12) we anaesthetized the mice with a cocktail of ketamine – xylazine (i.p. 80 and 5 mg/kg body weight for ketamine and xylazine respectively) then performed the surgery described above. During the imaging session the depth of the anesthesia was checked regularly by pinching the mice's hind leg and anesthetics was applied in case of any sign of muscle contraction. For awake imaging (P30-45), after the three day recovery period mice were hand habituated followed by head restraint on a freely moving treadmill for increasing amounts of time for at least three more days before the first imaging session. Mice imaged awake at P30 had a minimum of six days recovery before the first imaging session while mice imaged at P45 had at least twenty one days of recovery before the first imaging session.

***In vivo* 2-photon microscopy imaging**

We use an *in vivo* resonant galvo-based multi-photon microscopy system (Bruker) and an ultra-fast pulsed laser beam (Chameleon, Coherent; tuned to 920-nm wavelength, average 20-40mW out of the objective) controlled with an electro-optical modulator to excite mito-YFP and tdTomato through a 20X or 40X objective (Nikon). Distilled water served to connect the water immersion objective with the window. Green and red fluorescence were separated with an emission filter cube set (green, HQ525/70m-2p; red, HQ607/45m-2p; 575dcxr). Fluorescent light was detected with photomultiplier tubes (green GFP fluorescence, GaAsP PMT; red tdTomato fluorescence, multi-alkali PMT) operated with PrairieView software. Once mice were head-fixed, we used goniometers (Edmund Optics) to adjust the angle of the mouse's head up to 10 degrees to make the imaging window parallel to the objective. Timelapse image series were collected in red (tdTomato signal) and green (mito-YFP signal) channels by acquiring 30 frames at 0.1 Hz for 10 – 15 minutes. We can estimate the level of two-photon excitation following the prescriptions given in Denk et al, 1990 [S3] combined with the known parameters of our laser and

microscope, and the 2-P cross section of YFP at 920nm. Under these conditions, any given fluorophore in the focus of the beam has a less than 10% chance of being excited per laser pulse. We are still very far from saturation conditions, and direct non-linear damage is highly unlikely. Per scan the total energy delivered into the diffraction limited spot is on the order of 6nJ, and most of that is not absorbed, so local heating is also insignificant. Time-series were motion-corrected based on the static tdTomato signal as described in [S2] and the same displacements were applied to the green channel.

Statistics

All statistical analysis was performed in GraphPad Prism 6. Statistical tests used are labeled in the figure legends. Graphs were created in GraphPad Prism 6 or Microscoft Excel.

Supplemental References

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