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Supplemental Information

Wld^S Prevents Axon Degeneration through Increased Mitochondrial Flux and Enhanced Mitochondrial Ca^{2+} Buffering

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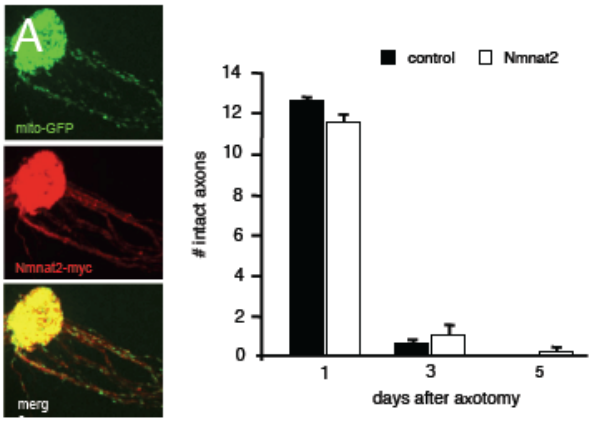
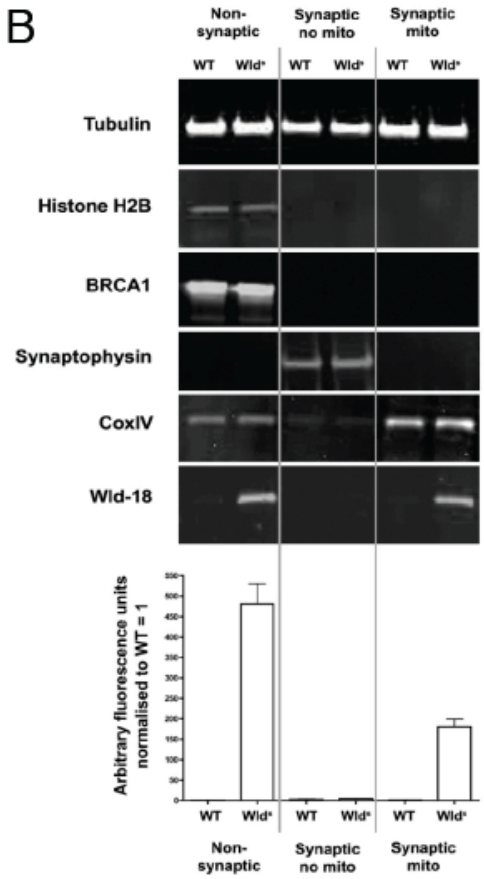
Supplemental Inventory


A
mito-GFP
Nmnat2-myc
merg
control
Nmnat2
intact axons
14
12
10
8
6
4
2
0
1
3
5
days after axotomy


B
Non-synaptic
Synaptic no mito
Synaptic mito
WT
Wld^S
Tubulin
Histone H2B
BRCA1
Synaptophysin
CoxIV
Wld-18
Arbitrary fluorescence units normalised to WT = 1
Non-synaptic
Synaptic no mito
Synaptic mito

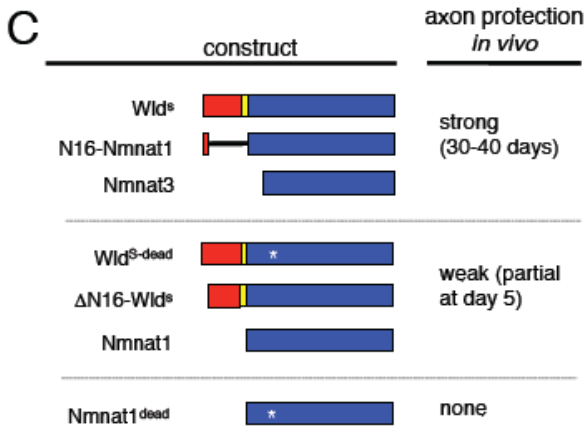

C
construct
axon protection
in vivo
Wld^S
N16-Nmnat1
Nmnat3
strong
(30-40 days)
Wld$^{S\text{-}dead}$
ΔN16-Wld^S
Nmnat1
weak (partial
at day 5)
Nmnat1^{dead}
none

Figure S1. Nmant2 Lacks Protective Function; WldS Localizes to Synaptic Mitochondria; Schematic of neuroprotective Molecules Used in This Study, Related to Figures 1 and 2

(A) Mouse Nmnat2::Myc localizes throughout the axonal compartment in *Drosophila* axons. *22a-Gal4* was used to drive *UAS-Nmnat2::Myc* and *UAS-mCD8::mCherry*. *22a-Gal4* was used to drive *UAS-Nmnat2* in a background where axons were labeled with membrane-tethered GFP (*UAS-mCD8::GFP*). Axons were cut and the number of remaining axons were scored at the indicated time points. n≥20 antennal lobes for each. *** $p<0.001$. For all figures error bars represent ±SEM.

(B) Representative quantitative fluorescent western blots showing protein expression levels in non-synaptic preparations (all tissue excluding synaptosome fraction), synaptic (synaptosome) preparations without mitochondria, and isolated synaptic mitochondria preparations from wild-type and Wld^S mice. Tubulin is shown as a loading control; Histone H2B and BRCA1 are shown as nuclear markers to confirm that no nuclear proteins were detectable in the synaptic and synaptic-mitochondrial fractions; Synaptophysin is shown as a synaptic marker; CoxIV is shown as a mitochondrial marker; Wld^S protein levels were detected using Wld-18 antibodies. Wld^S protein was present, as expected, in the non-synaptic fraction containing nuclei. Wld^S protein was also present in the synaptic mitochondrial fraction but was undetectable in the synaptosome fraction lacking mitochondria. Quantification of Western blots (obtained from quantitative fluorescent western blots; N=3 mice per genotype).

(C) Axonal protective phenotypes for *UAS*-regulated version of each of these molecules by driving in adult ORN axons are based on *Avery et al.* (2009).

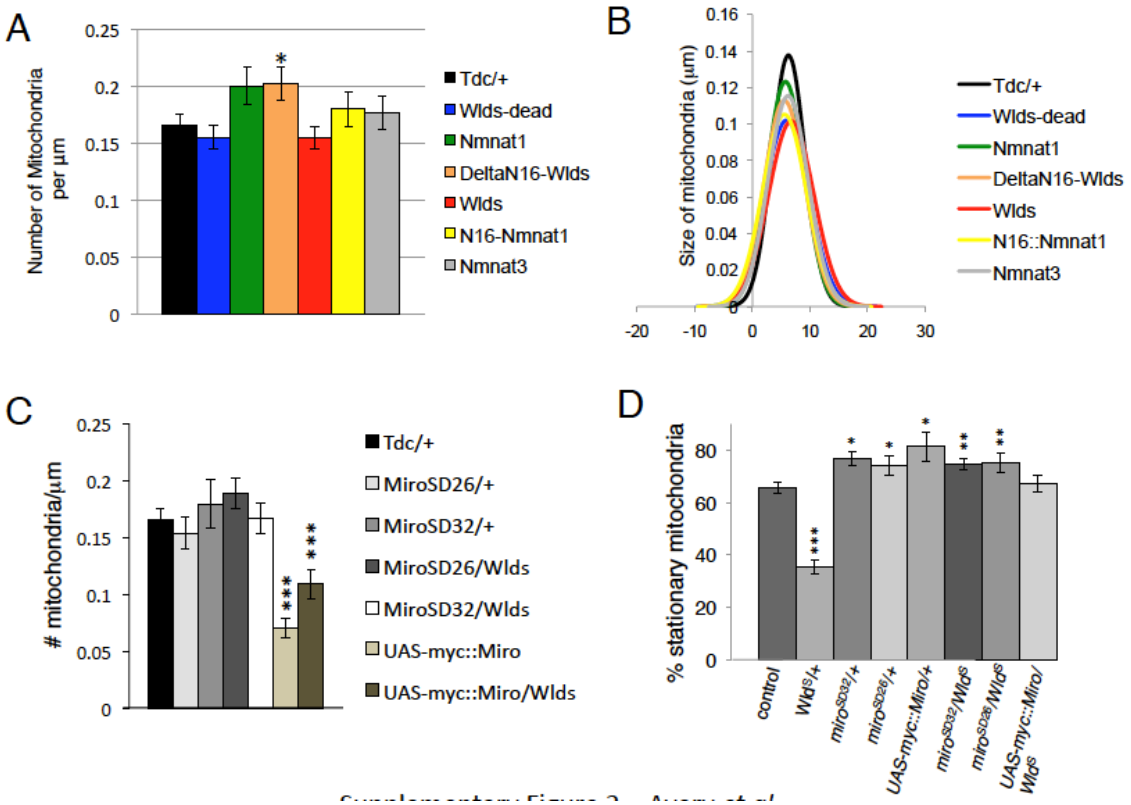


Figure S2. Wld^S Does Not Affect the Number or Size of Mitochondria in Live Animals; Suppression of Mitochondrial Movement by *miro* Mutants, Related to Figure 2

(A) Wld^S and Wld^S-derived molecules were expressed in *tdc2-Gal4*$^+$ neurons (along with mCD8::mCherry and mito::GFP, as described Figure 1C) and the total number of mitochondria per µm along axons was determined in live open filet preparations of larvae. *tdc2-Gal4/+* was used as a control. *p<0.05.

(B) Average distribution of length of mitochondria (measured along axon shaft, in microns) for genotypes listed in (A).

(C) Axons and mitochondria were labeled with mCD8::mCherry and mito::GFP, respectively, and mitochondrial numbers were calculated in the indicated genetic backgrounds. ***, p<0.001.

(D) Mitochondrial flux was assayed in axons expressing Wld^S in the presence or absence of *miro* mutations, or a *UAS-myc::Miro* construct that acts as a dominant negative. n≥10 live samples for each genotype. *, p<0.05; **, p<0.01; ***, p<0.001.

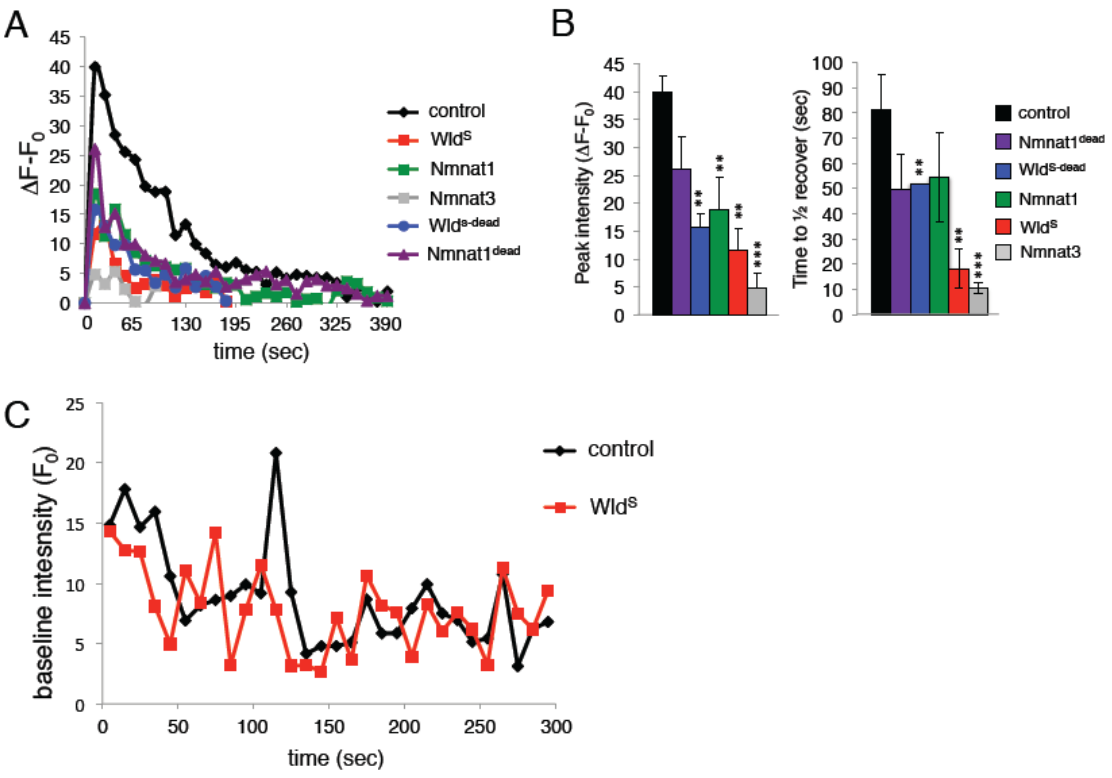


Figure S3. *In vivo* Laser Axotomy Induces a Dramatic Rise in Axonal Ca^{2+} that Is Suppressed by Wld^S: Responses in the Proximal Axon Compartment, Related to Figure 2

(A) Axonal Ca^{2+} was monitored as in Figure 2, but in proximal segments of severed axons. Representative traces showing Ca^{2+} responses in axon fragments distal to the injury site over time in control, Wld^S, Nmnat1, Nmnat3, $Wld^{S\text{-}dead}$, and $Nmnat^{dead}$ expressing axons.

(B) Quantification of peak Ca^{2+} intensities and time to ½ recovery from average peak intensity for each genotype listed. n≥5 live samples for each genotype and time point. **, $p<0.01$; ***, $p<0.001$.

(C) Baseline axonal Ca^{2+} levels were measured by GCaMP3 fluorescence over a 3 minute window in intact axons. n=5 axons per genotype, averages are shown.

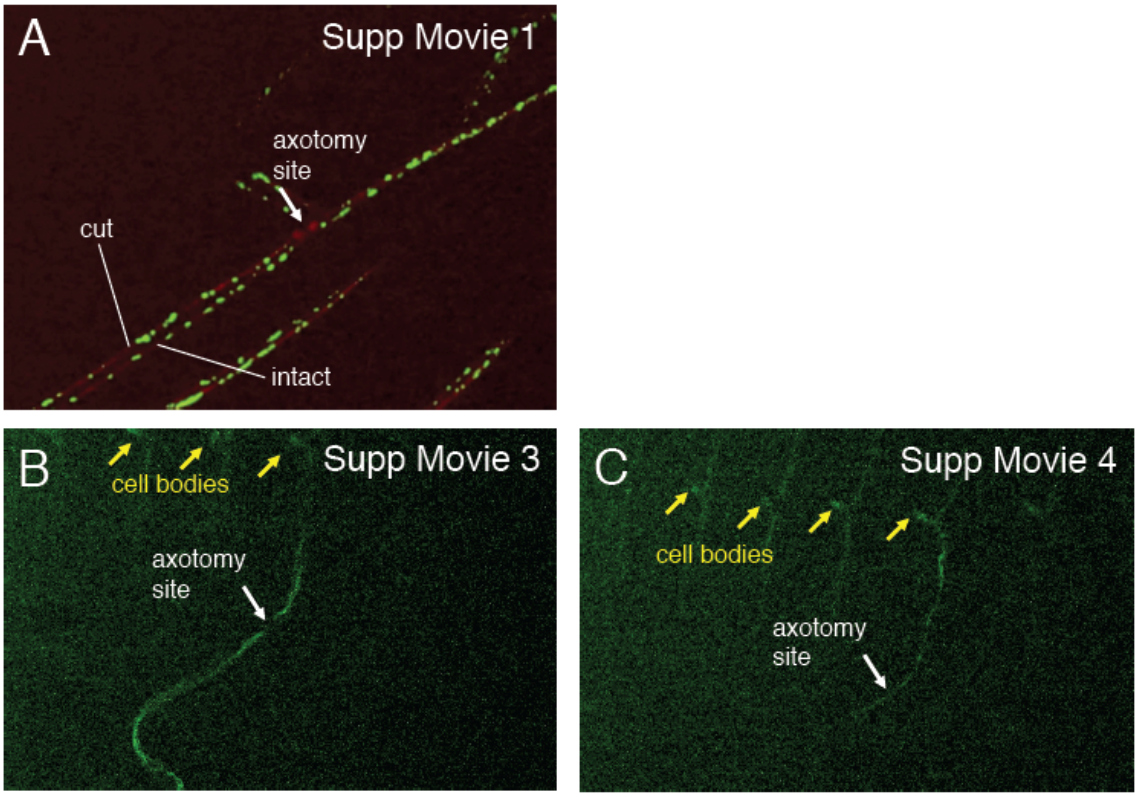


Figure S4. Visual Aid for Movie Interpretation, Related to Movies S1–S4

Stop frame images from each of the movies indicated are provided to show sites of laser axotomy in live preparations.

(A) Supplementary Movie 2 – Note that only the upper of the two axons was cut, and that mitochondrial motility in the lower axon remains robust.

(B) Supplementary Movie 4 – Laser axotomy in control animals leads to a dramatic spike in axonal Ca^{2+} in the severed axon (but not in other local axons).

(C) Supplementary Movie 5 – Laser axotomy leads to minimal axonal Ca^{2+} responses in Wld^{S}.

Supplemental Movies

All live imaging was performed in open-filet preparations of third instar *Drosophila* larvae. The *tdc2-Gal4* driver, which labels 3 axons per nerve (e.g. Figure 2A) was used to drive *UAS-mCD8::mCherry* to label axons, *UAS-mito::GFP* to label mitochondria, or *UAS-GCaMP3* to image axonal Ca^{2+}.

Supplemental Experimental Procedures

Injury Protocol

Flies were anesthetized on CO_2 pads and their 3rd antennal segments removed. *Drosophila* were kept at 25°C for 5, 10, 15, 20, or 50 days after ablation. All uninjured flies were dissected one week after eclosion unless noted as age matched.

Drosophila Stocks, Molecular Biology, and Transgenics

The following stocks were used: *OR22a-Gal4*, *pUAST-mCD8::GFP*, *pUAS-mitoGFP.AP2*, *pUAS-mitoGFP.AP3*, *pTdc2-Gal4.C2*, *pUAS-mCD8-mCherry* (*Drosophila* Bloomington Stock Center), *miro^{SD32}/TM6*, *miro^{SD26}/TM6* [1], *pUAS-Myc-Miro* (K. Zinsmaier, University of Arizona), *UAS-gCaMP3* (L. Looger, Janelia Farms Research Campus), *pUAST-Wld^{S}*, *pUAST-N16::Nmnat1*, *pUAST-Nmnat1*, *pUAST-Nmnat1^{dead}*, *pUAST-Wld$^{S\text{-}dead}$*, *pUAST-Nmnat3*, *pUAST-Nmnat2*, and *pUAST-ΔN16-Wld^{S}* [2].

pUAST-Nmnat2::myc was made by PCR amplifying amino acids 1-306 of mouse Nmnat2, cDNA clone BC098007 (Open Biosystems), with NotI sites at both ends, excluding the stop codon at 3' end. *pUAST-Nmnat3::myc* was made by PCR amplifying amino acids 1-244 of mouse Nmnat3, cDNA clone BC092086 (Open Biosystems), with NotI sites at both ends, excluding the stop codon at 3' end. Both were cloned into a pUAST-myc vector. *pUAST-mCD8::mcherry* was made by PCR amplifying 726bp of mCherry from *pKR5-mCherry*. An *EcoRI* site was added to the 5', and *XbaI* added to 3' after stop codon and then it was cloned into the EcoRI/Xba site of pUAST. Transgenic flies were made by BestGene, Inc.

Immunolabeling and Confocal Microscopy

Standard methods were used for dissection, fixation, and antibody labeling of the *Drosophila* adult brain [2]. Primary antibodies were used at the following dilutions: mouse anti-Myc (9E10, Covance Innovative Antibodies) 1:1000. Secondary antibodies (Jackson Immunoresearch) were used at 1:200. Images were then mounted in Vectashield, and viewed with a Intelligent Imaging Innovations Everest spinning disk confocal microscope.

Live Imaging

3rd instar wandering larvae were filet-prepped in HL6 [3], and glued to the live imaging chamber with Surgi-Lock 2oc (Healthypets.com). Starting near the ventral nerve cord, 3 sections of the nerves were imaged in the larva for 5 minutes each.

Kymographs

2D movies were aligned with Image J plugin “align slice”. The Image J plugin “multiple kymograph” was then used to create the kymograph.

Laser Ablation

Wandering 3rd instar larvae were filet-prepped in HL6 (MacLeod et al., 2002) and glued to a perfusion chamber (Warner Instruments, RC-30) with Surgi-Lock 2oc (Healthypets.com). Live images were acquired for 5 minutes, after which a small section of a single nerve (identified with mCD8-mCherry) was laser ablated with a 435 wavelength laser at 70% power. Images were then collected every 5 minutes afterwards for a total of 20-60 minutes. Larvae were perfused with fresh HL6 in throughout the recordings.

Quantification of Images and Movies

For adult brain axotomy, Z stacks were compressed with ImageJ software, and intact GFP+ axons were counted [2]. For live imaging, 4D movies were compressed to 2D along the Z axis. The number of GFP^+ mitochondria were counted in the first frame of the movie, and stationary mitochondria were counted throughout the timeframe. For gCaMP3 quantification, 4D movies were compressed to 2D, average intensity from an uncut nerve was subtracted from the average intensity of a cut nerve (either distal or proximal) at each timepoint. We corrected for background by subtracting the average background intensity from the 5 minute baseline movie from each sample. Error bars represent SEM as determined by ANOVA.

Mouse Synaptosome Preparation

Mice were sacrificed and brains rapidly removed before the striatum was dissected. Synaptosomes were produced as previously described [4]. Striata were then homogenised in an ice-cold isotonic sucrose solution (0.32 M sucrose, 1 mM EDTA, 5 mM Tris-HCl, pH 7.4). Homogenate was centrifuged in a fixed-angle rotor at 900*g* for 10 min and the supernatant (S1) was collected. The pellet (P1) was resuspended in sucrose solution and centrifuged again at 900*g* for 10 min. The resulting supernatant (S1’) was combined with S1 and centrifuged in a fixed angle rotor at 20,000g for 15min. The supernatant (S2) was retained as non synaptic striatal protein and the pellet (P2) containing synaptosomes was washed in a Krebs-like buffer (118.5 mM NaCl, 4.7 mM KCl, 1.18 mM $MgCl_2$, 0.1 mM K_2HPO_4, 20 mM Hepes, 1.3 mM $CaCl_2$, 10 mM glucose, pH 7.4) then centrifuged at 14,000*g* for 10min. Mitochondria were separated from synaptosomes as previously described [5].

Quantitative Fluorescent Western Blotting

Briefly, protein was separated by SDS/Polyacrylamide gel electrophoresis on pre-cast NuPage 4-12% Bis Tris gradient gels (Invitrogen) and then transferred to PVDF membrane overnight. The membranes were then blocked using Odyssey blocking buffer (Li-COR) and incubated with primary antibodies as per manufacturers instructions (BRCA1 – Calbiochem (220KDa, Cat. No. op107); Histone H2B – Lake Placid

Biologicals (15KDa, Cat. No. AR-0139); Tubulin – AbCam (50KDa, Cat. No. AB18207); Synaptophysin – Dako (38KDa, Cat. No. A0010); CoxIV – (Mitosciences (57Kda, Cat. No. MS404); Wld18 – generated by Dr Michael Coleman (Babraham Institute). Odyssey secondary antibodies were added according to manufacturers instructions (Goat anti rabbit IRDye 680 and Goat anti mouse IRDye 800). Blots were imaged using an Odyssey Infrared Imaging System (Li-COR Biosciences). Scan resolution of the instrument ranges from 21-339μm and in this study blots were imaged at 169μm. Quantification was performed on single channels with the analysis software provided.

Supplemental References

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