EFFECTS OF DMIRO ISOFORMS ON MITOCHONDRIAL TRANSPORT, HEALTH, AND MORPHOLOGY

By

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Summary

Microtubule (MT)-based transport of mitochondria into dendrites and axons is vital for sustaining neuronal excitation and synaptic function. The mitochondrial GTPase Miro is critical for mitochondrial transport by linking MT motor proteins to mitochondria. In flies and mammals, Miro proteins contain a variable domain whose position but not sequence is highly conserved. In *Drosophila*, the shortest (Miro-S) and longest (Miro-L) isoform differ by only 21 amino acids while the medium isoform (Miro-M) is 8 amino acids shorter than Miro-L. To test the functional significance of these isoforms, we expressed each individually in *miro* null or wild type *Drosophila* neurons and found differential effects on mitochondrial transport, structure and health. Expression of Miro-L and –S fail to rescue the larval lethality of *miro* null mutants while Miro-M does. OE of Miro-L and –S but not –M is also lethal. The effects on viability correlate with effects on mitochondria. Miro-L and Miro-S primarily cause morphological changes of axonal mitochondria, and are less efficient in facilitating aspects of mitochondrial transport than Miro-M. In conclusion, our data suggest different roles and/or activities for each of the 3 Miro protein isoforms.

Introduction

Mitochondria are multi-functional organelles: besides producing most of the energy used by eukaryotic cells, they play major roles in multiple cellular signaling pathways, calcium signaling and apoptosis (reviewed by Wang, 2009). The general structure of mitochondria is that of two lipid membranes, with the inner membrane undergoing 3-dimensional folding within the mitochondrion to create structures called cristae, in which the components of the electron transport chain conduct oxidative phosphorylation (Palade 1953). Cytoplasmic glycolysis converts glucose obtained from food sources to ATP and pyruvate. Mitochondria then take up pyruvate and convert it into Acetyl Coenzyme A and eventually NADH and FADH2, to fuel oxidative phosphorylation (OXPHOS) generating ATP.

OXPHOS involves five multimeric complexes forming the electron transport chain generating a proton gradient across the inner mitochondrial membrane that results in an very negative membrane potential. This membrane potential is usually used as an indicator of mitochondrial health (Vayssier-Taussat, 2002). The main purpose of the proton gradient is to fuel ATP synthase to phosphorylate adenosine diphosphate (ADP) generating ATP. Mitochondrial OXPHOS generates on average 32 molecules of ATP per single glucose molecule while glycolysis produces only 2 ATP molecules. Considering the drastically higher ATP output of oxidative phosphorylation, the production of ATP by mitochondria is critical for the cellular function of cell with high-energy metabolism like liver cells and neurons (reviewed by Koopman, 2013).

Mitochondria are inherited maternally, with paternal mitochondria being quickly ubiquitinated upon egg fertilization, causing their impending degradation and subsequent absence from the fertilized egg (Sutovsky 1999). When a cell needs to increase the number of

mitochondria, for example, before cellular division or in situations of high energy need, mitochondria proliferate (Luciakova, 1992, Pette, 1992) by fission. Mitochondria must first enlarge (grow) by adding membrane which requires lipid transport from the endoplasmic reticulum (Toulmay 2011, Osman 2011), the main site of lipid synthesis in the cell. In addition, mitochondria must replicate their genome and obtain additional proteins (Schmidt 2010). Mitochondria grow in size until they are large enough to divide (Chan 2006).

Besides mitochondrial proliferation, fission together fusion is required in a complex cycle to regulate mitochondrial morphology, health, and function (Youle 2012). Balancing fusion and fission drives the specification of different mitochondrial morphologies from small grape-like mitochondria to large interconnected tubular networks that fit the demands of different cell types, locations, or functional requirements (reviewed by Okamoto, 2005). Mitochondrial fusion fuses both the inner and outer mitochondrial membrane and leads to a mixing of the outer membranes, the inner membranes, and the matrix contents.

Perhaps the most important consequence of mitochondrial fusion is content mixing between mitochondria. In particular, mixing of matrix components appears to be a primary role of mitochondrial fusion that is critical to preserve mitochondrial function (Song, 2009) Fusion quickly followed by fission also appears to be a critical mechanism of controlling the functional quality of mitochondria. Measurement of the mitochondrial membrane potential during single fusion and fission events showed that fission yields uneven daughter mitochondria where the depolarized daughter is less likely to become involved in a subsequent fusion and is more likely to be targeted by autophagy. These observations suggest that the integration of mitochondrial fusion, fission and autophagy forms a quality maintenance mechanism. Accordingly, fusion and fission allow the sequestration of damaged mitochondrial components into daughter

mitochondria that are segregated from the healthy mitochondrial pool and subsequently eliminated by autophagy (Twig, 2008).

Fusion of outer and inner mitochondrial membrane in higher eukaryotes is facilitated by Mitofusin 1 and 2 (Mfn1 and Mfn2) and Optic atrophy 1 (OPA1), respectively (Song 2009). The Drosophila ortholog of mitofusin 1 and 2 is MARF [Hwa (2002) Differential expression of the Drosophila mitofusin genes fuzzy onions and dmfn. Mechanisms of Development 116 213–216], which expresses both mitofusins as differential mRNA splicing products (pers. communication). In addition, the fly genome contains a second more distant homolog of human Mitofusins, Fuzzy onions (Fzo), which was the first mitochondrial fusion gene to be identified. In contrast to MARF expression, FZO expression is tightly restricted to the male germ line including spermatocytes and early spermatids, and exclusively required for the formation of two giant mitochondria that comprise the mitochondrial derivative (termed the Nebenkern) (Tokuyasu, 1973). Mutations in the Fzo gene induce sterility in males as their spermatids are unable to effectively move (Hales 1997).

Mitochondrial fission of the outer membrane is facilitated by the soluble protein dynamin-related protein 1 (Drp1) (Smirnova, 2000, Chan 2012). Mammalian DRP1 is recruited to mitochondria by the outer membrane protein mitochondrial fission 1 (Fis1). Only little is known about division of the mitochondrial inner membrane. and no specific fission proteins have been identified yet (Westermann, 2010).

In conclusion, fusion and fission are critical to adapt mitochondrial morphology to specific demands of cells and maintain a healthy population of mitochondria. When either fusion or fission is disrupted, mitochondrial functions deteriorate due to an impaired membrane potential. Vice versa, an impaired mitochondrial membrane potential prevents fusion but not

fission, and triggers a pathway that leads to the initiation of mitochondrial autophagy (mitophagy) (Narendra 2008, Zamzami 1995). A mitochondrial membrane increase in potential, or hyperpolarization, has been shown to have the opposite effect, promoting cell survival (Ward 2007).

Mitophagy appears to be regulated, at least in part, by the proteins PTEN-induced putative Kinase 1 (PINK1) and Parkin, two main proteins found to be rendered dysfunctional by genetic mutations in early-onset autosomal recessive Parkinson's Disease (Geisler, 2010). If a mitochondrion's membrane potential becomes depolarized, it is either due to a dysfunctional electron transfer chain or due to a natural uncoupling by uncoupling proteins to, for example, produce heat without generating ATP (Rousset, 2004). Starvation or depolarization of mitochondria typically induces a predominant expression of full length Pink1 (F64) on the mitochondrial surface (Becker, 2012). Pink then phosphorylates Mfn2 which recruits Parkin to mitochondria, stimulating Parkin-mediated ubiquitination of mitochondrial surface proteins such as mitofusins and a voltage-dependent anion-selective channel (VDAC) that behaves as a general diffusion pore for small hydrophilic molecules (Chen, 2013; Geisler, 2010; Poole, 2010). Ubiquitinated VDAC is critical for the prompt clearing of damaged mitochondria by autophagy (Vives-Vauza, 2009), thereby preventing cell death (Abu-Hamad, 2009). Misregulation of autophagy is a major culprit in neurodegeneration, specifically in diseases such as Alzheimer's, Huntington, and Parkinson's (Kegel, 2000; Nixon, 2005; Yang, 2007).

Neurons have an extremely high energy demand, with the brain consuming about 20% of the oxygen in the body, when only representing 2% of total body mass (Mink 1981). Furthermore, in neurons, mitochondria must travel from the cell body down the axon to the axon terminals. This distance can be 100,000 times the diameter of the cell body itself (Brown, 2000).

At synaptic terminals the metabolic demands are high, ATP being required in high levels for proper synaptic transmission. It is estimated that 28% of the oxygen used by a rat hippocampal neuron is used at the presynaptic terminal (Hall 2012).

Mitochondria in neurons are especially required at the synapse for proper vesicle exocytosis, recycling, loading, and the formation of synaptic membrane potentials (reviewed by Sheng 2012). If mitochondria do not reach the presynaptic terminal, there is not enough energy to sufficiently sustain activity there, and synaptic potentiation is inhibited (Tong 2007). Hence, proper axonal and dendritic transport of mitochondria is critical for neuronal function. Defects in mitochondrial transport in neurons have been associated with Alzheimer's Disease, Amyotrophic Lateral Sclerosis, Huntington's Disease, and Parkinson's Disease (Sheng 2012)

Axonal mitochondrial transport relies on microtubules. Microtubules are a component of the cellular cytoskeleton that gets its name from its tubular shape, comprised of rings of alternating α and β tubulin. It extends and grows in one direction, the "plus" (+) end and shrinks from the "minus" (-) end (Downing 1998). In axons, microtubules are oriented such that their + end is facing towards the growth cone or axon terminal, and the - end towards the cell body (Baas 1988, Stone 2008)

The main motor proteins responsible for microtubule-based transport of mitochondria in axons are kinesin and dynein. Kinesin facilitates + end directed motions that promote anterograde transport in axons, and dynein facilitates - end directed that promote retrograde transport in axons (Hollenbeck 2005). Kinesin utilizes the hydrolysis of ATP to facilitate its transport of mitochondria and other cargo. There are two "head" regions of the Kinesin complex that bind to microtubules and alter their conformation and affinity to microtubules in such a way to simulate "walking" down the microtubules (Rice 1999). It is suspected that dynein works in a

similar ATP hydrolysis-driven, hand-over-hand fashion, although less is known about its exact mechanism (reviewed in Gennerich 2009).

Even though mitochondria are transported in one net direction, they display a bi-directional motility that is facilitated by MT plus end-directed kinesin and minus end-directed dynein motors. Effective net transport is likely achieved by a “directional program” that favors one motor activity over the other (Morris and Hollenbeck, 1993). The nature of this mechanism is poorly understood but may require coordination of motor activities or engagement with MTs ensuring that when one type of motor is active, the other is not (Gross, 2003; Welte, 2004; Hollenbeck and Saxton, 2005).

Previous work suggested that the mitochondrial GTPase Miro (also called RhoT in human or Gem1p in yeast) is critical for the execution of the directional program of mitochondrial transport in axons (Guo 2005; Russo, 2009 #3). In flies and mammals, kinesin heavy chain (KHC, Kif5 in mammals) associates with mitochondria via the adaptor protein Milton (also called OIP98/106, and TRAK1/2), which in turn binds to Miro in the outer mitochondrial membrane (Stowers 2002; Glater, 2006; Fransson et al., 2003, 2006; Brickley et al., 2005), although human Milton can also be targeted to mitochondria independent of Miro (Koutsopoulos, 2010). In the absence of Drosophila Milton or Miro, neuronal mitochondria remain in the soma and are nearly absent from axons and dendrites (Glater et al., 2006; Guo et al., 2005; Stowers et al., 2002). Loss of Drosophila Miro (dMiro) reduces the efficacy of both antero- and retrograde mitochondrial transport in axons by selectively impairing the time mitochondria allocate to either kinesin- or dynein-mediated motions, depending on the direction of net transport. Consequently, mutant mitochondria exhibit mostly back-and-forth) motions that fail to gain any significant net distance. Accordingly, we suggested that dMiro ensures effective

mitochondrial transport by selectively promoting the kinesin activity during anterograde and dynein activity during retrograde transport (Russo, 2009).

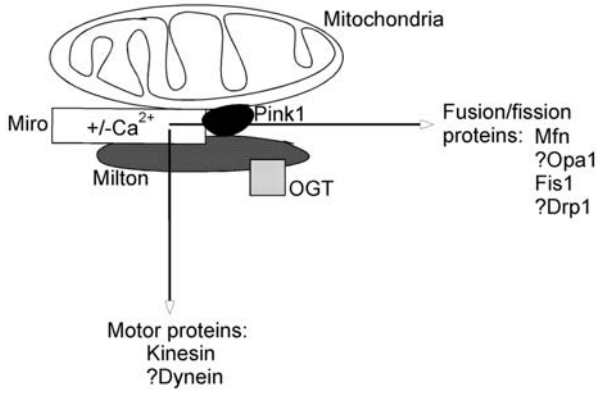

Figure 1. Miro sits in the outer mitochondrial membrane and interacts with Pink1, Milton, and proteins involved in mitochondrial fusion and fission processes such as Mfn and Fis1. The Miro Milton complex associates with motor proteins Kinesin and possible Dynein to facilitate microtubule transport (Liu 2009)

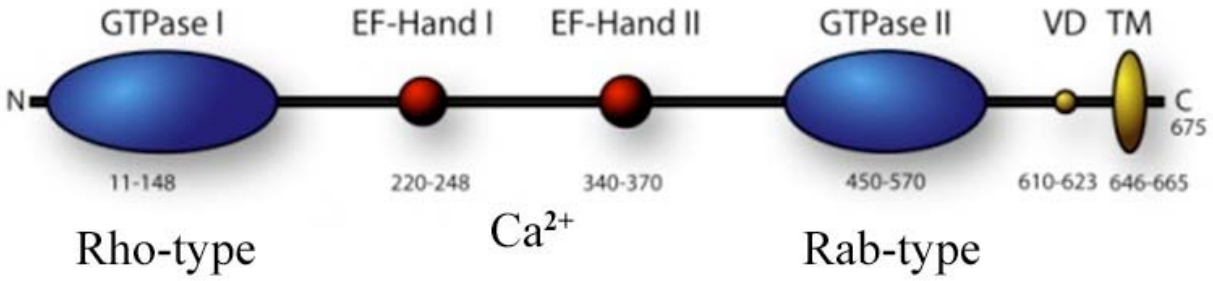

Figure 2. The Miro protein has two GTPase domains, one "Rho-like" and one "Rab-like," two calcium-binding EF-hand domains, a transmembrane domain that sits in the outer mitochondrial membrane, and a "variable domain" (VD). The variable domain is the location of the only alternative splice site on the Miro transcript.

Miro has two GTPase domains and two calcium-binding EF-Hand domains that regulate its function (Fransson 2003). The first GTPase domain appears critical for mitochondrial transport but it remains unclear how the domain facilitates transport GTPase dependent recruitment of Grif-1. (MacAskill 2009). The EF-hand domains of Miro are responsible for proper localization of mitochondria at locations throughout the cell experiencing high neuronal

activity. Ca2+ entering through NMDA glutamate receptors arrests mitochondrial transport due to Miro's EF-Hand domains (Wang 2009). Calcium binds to Miro's EF-Hand, causing kinesin to bind Miro with its motor domain and thereby dissociate from microtubule.

It has been assumed that retrograde transport of mitochondria may be critical for mitochondrial health. To support this, the membrane potential of moving mitochondria in axons was analyzed, and it was found that 90% of mitochondria with high membrane potential move anterogradely, and 80% with low membrane potential move retrogradely (Miller 2004). However, another study found no correlation between membrane potential and direction of axonal transport (Verburg and Hollenbeck, 2008). To look for a causal relationship, the effects on transport of conditions that alter mitochondrial physiology were tested. A reduction of inner-membrane potential with the electron transport inhibitor antimycin A temporarily increased retrograde transport (Baqri et al., 2009). However, an innermembrane uncoupler, CCCP, reduced transport in both directions (Baqri et al., 2009; Hollenbeck et al., 1985). Thus, the relationship between mitochondrial 'age' or physiology and transport behavior remains unresolved.

In addition to the well known GTPase and EF-hand domains, Miro also has a variable domain that is conserved between humans, mice and Drosophila. Here, Miro mRNA is alternatively spliced into three different splice variants. These are termed long, medium, and short based off of their amino acid length after splicing occurs. This is the only location on the Miro transcript that experiences alternative splicing.

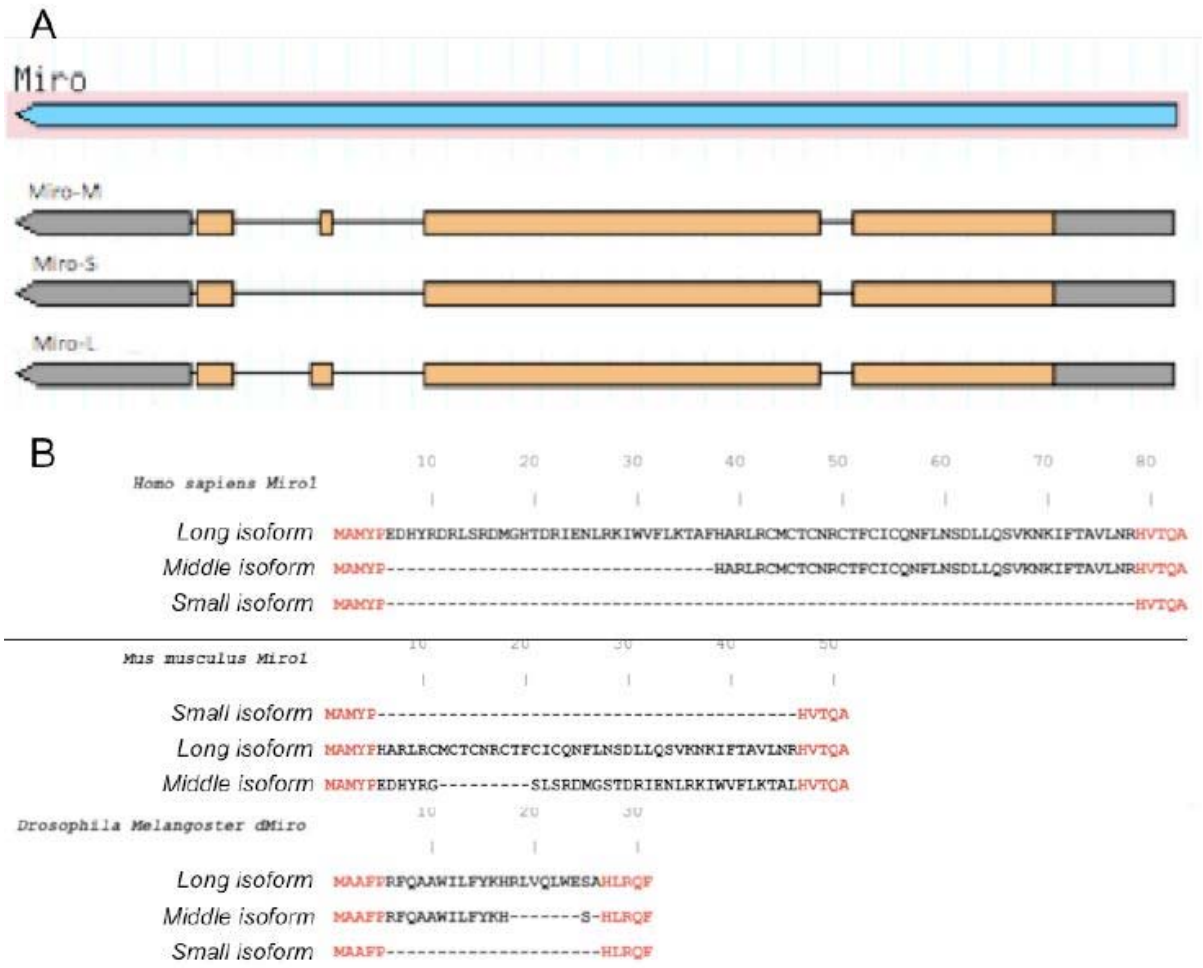

Figure 3. The Miro transcript has only one site of alternative splicing, termed the "variable domain." Human, Mouse, and Drosophila Miro all experience alternative splicing at this same location. In mice and humans, this site is longer than in flies, but the result is still 3 transcripts of different lengths. *Drosophila Miro-S* is 21 amino acids shorter than *dMiro-L*, and 8 amino acids shorter than *dMiro M*.

The difference between the long and short forms however is very minimal with regard to the length of the entire protein. In *Drosophila melanogaster*, there is only a difference of 21 amino acids between the long and short variants. All three RNA versions are present in brains of Drosophila (Guo 2005), although it must be noted that this expression could come from glial cells as well as from neurons. The existence of three isoforms of different lengths that are so well conserved suggests that it may serve some functional purpose within the cell.

There are many possibilities for a role of these different Miro isoforms. One idea could be that the differing lengths of the protein so close to the transmembrane domain could allow the Miro protein to be situated within different microdomains on the mitochondrial membrane surface. This could potentially change the function of each version and allow them to have different roles. Another potential idea is that the different parts of the variable domain that are present or missing may, in three dimensional space once the Miro protein is folded, interact with GTPase or EF-hand domains and regulate Miro function with regard to transport in that way, perhaps by regulating how long the Miro/Milton/Kinsin complex stays attached to the microtubules. The possibility exists that variable domain could be involved in Miro's relationship with the Pink1/Parkin pathway and could mediate autophagy or changes in mitochondrial morphology.

Methods

Immunostainings

Drosophila melanogaster wandering 3rd instar larva were dissected in a fillet preparation in a solution of HL-3 (Stewart 1994). The fillet includes an incision down the dorsal side of the larvae and the removal of intestines and other organs to reveal the brain and nervous system below. After dissection, the preparation was fixed with 4% paraformaldehyde solution (diluted in PBS of pH 7.3) at room temperature for 15 minutes, and washed 3 times in PBS supplemented with 0.5% Triton (PBST) for 10 minutes. Then a blocking agent "SuperBlock" (Thermo Scientific PROD # 37516) was applied for 30 minutes. The preparation was then incubated with the primary antibodies AF488-conjugated anti-GFP in a 1:2000 dilution in PBST, and Cy3-conjugated anti-HRP (1:500 dilution) overnight in 4˚C in the dark. After washing (3x for 10 minutes in PBST, 2x for 10 minutes in PBS) the preparation was post-fixed in 4% PFA for 10 minutes at room temperature. After 2 additional washes for 10 minutes in PBS, the preparation was then transferred to glass slides and embedded in VectaShield mounting medium for fluorescence (Vector Laboratories H-1000) and imaged on Olympus FV300 confocal microscope with UPlanAPO 100x Oil Iris Lens.

Live Imaging

Live imaging of mitochondria in larval nerves

Drosophila melanogaster wandering 3rd instar larva containing the transgene mitoGFP (allowing live fluorescence of mitochondria) were dissected as described in HL-6 solution (Macleod 2002) supplemented with 0.6 mM Ca and 7 mM L-glutamic acid. Imaging was performed on Olympus FV300 confocal microscope, with an Olympus LUMPlanFl 60x water

lens. With brain on left side of the lens for directional tracking purposes, 200 seconds of consecutive images 1 second apart of a 1024 x 280 image were taken with Ar488 laser, at 5% intensity of a nerve just proximal from the Ventral Nerve Cord (VNC) of the larva brain.

Rate of mitochondrial transport (Flux)

Drosophila melanogaster wandering 3rd instar larva were dissected in a filet preparation in prepared solution of HL-6 (Stewart 1994) with 0.6mM Ca and 7mM L-glutamic acid. Imaging was performed on Olympus FV300 confocal microscope, with LUMPlanFl 60x water lens With brain on left of lens for directional tracking purposes, the fluorescence of the mitochondria in a clip of a 1024 x 800 box was "photobleached" in the nerve proximal to the VNC with Ar 488 and HeNe 543 lasers at maximum intensity for 60 seconds. Then 200 consecutive images were taken 1 second apart.

Analysis of mitochondrial motility in motor axons

In Photoshop, two images "in-frame" with one another that were 120 seconds apart were selected. The earlier image was stripped of all colors except green, the later image stripped of all colors except magenta, and the two set together in an overlay (see Figure 7). Mitochondria that remained stationary during the 120 seconds had both green present from the first image, and magenta present from the second image, and so appeared white. White mitochondria were scored as stationary. Green mitochondria were originally at that location at time 0 and were elsewhere at time 120, and therefore were scored as motile mitochondria. Magenta mitochondria are uncounted as they may represent mitochondria that have entered the frame.

Analysis of Mitochondrial Density in axons

Mitochondrial density was quantified by marking mitochondria with Image J "Cell Counter," separately counting motile (green) mitochondria and stationary (white) mitochondria from overlays of data described above.

In Image J, this series of images was viewed as a movie, scrubbed through and the number of mitochondria that enter the bleached space from each direction scored. Retrograde movement was towards the cell body (to the right) and anterograde towards the nerve terminal (to the left).

Measurements of Mitochondrial Length

Mitochondrial length was determined from images taken to analyze mitochondrial flux. In Image J, the "oval" marking tool was used to select a region of interest (ROI) surrounding individual mitochondria . The Feret's diameter was collected for each mitochondrion's profile. This was done for mitochondria travelling both anterogradely and retrogradely, and the results were analyzed separately.

Analysis of mitochondrial membrane potentials using TMRM

Drosophila melanogaster wandering 3rd instar larva were dissected in filet preparation in prepared solution of HL-6 (cite) with 0.1 mM Ca and 7 mM L-glutamic acid (reduced calcium for reduced larval movement when imaging).

Larva were incubated in 20 nM tetramethylrhodamine mesthyl ester (TMRM) in HL-6 in the dark for 25 minutes, rinsed briefly and imaged with 5 nM TMRM in HL-6 using a confocal microscope. Neuromuscular junction was located using 0.5% Ar 488 to prevent photobleaching.

Once found, it was imaged with 2% Ar 488 and 3% HeNe 543 lasers. Gamma was set to 1.0, intensity of green channel low was 1, high was 4095. The intensity of the red channel low was 200, high was 4095. PMT for both channels was 650v. An NMJ stack of 0.8 μm size slices was collected. In Image J, whole stacks were analyzed together and mitochondria encircled with freehand drawing tool. Each mitochondrion, or cluster of mitochondria, was saved as a single Region of Interest, the channels split into green and red, and the mean intensity of each object/region recorded. The GFP in the green channel was intended for use as a baseline to compare the intensity of TMRM to. GFP levels come from transgene mitoGFP that sits in the mitochondrial matrix. TMRM is a fluorescent dye that is a reliable measurement of membrane potential, as it is attracted to the negative charges in the mitochondrial matrix, inside of the inner mitochondrial membrane. Higher intensity of TMRM indicates higher membrane potential.

Results

Percent Motility

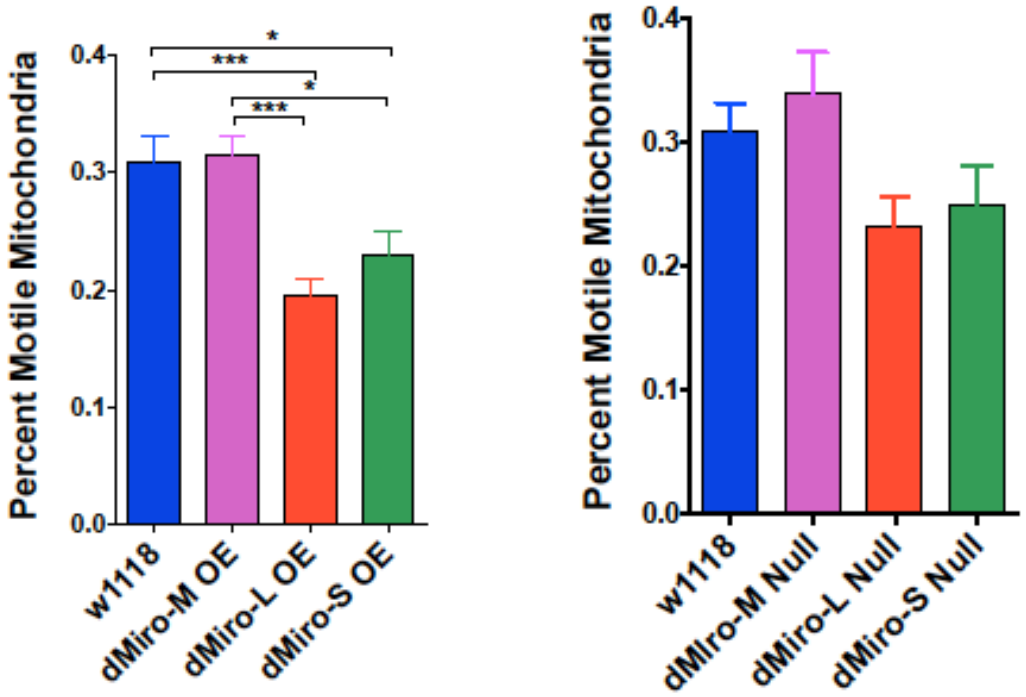

Figure 4. Percent of motile mitochondria in proximal nerve axons of *Drosophila melanogaster* larvae. Larvae were from fly lines that had *dMiro-M*, *dMiro-L* or *dMiro-S* inserted via p-element into genomes from both wildtype and *miro* null backgrounds. Statistical analysis was performed with ANOVA and Bonferonni post test, statistical differences indicated by asterisks (* - $p<0.5$, *** - $p<0.001$).

In order to analyze whether there is any difference in ratio of stationary versus motile mitochondria in axons, mito-GFP tagged mitochondria were live imaged in larvae motor axons of Drosophila. Images that were 120 seconds apart were pseudo-colored in green and magenta, respectively. Merging the two pseudo-colored images revealed stationary mitochondria in white while motile mitochondria appear green or magenta (Fig 5).

Expression of Miro-L, -M or -S in *miro* null mutants caused no significant effects on the percent of motile mitochondria, even though expression of Miro-L and –S in a Miro null background showed a trend reducing the percentage of motile mitochondria (Fig 4). This

suggests that each protein isoform alone does not shift the balance between motile and stationary mitochondria. However, since there is a trend towards a significant difference, it is suspected that significance may be reached if more data were collected.

Overexpression (OE) of Miro-L or -S in otherwise wild type flies caused a significant decrease in the percent of motile mitochondria compared to both control and Miro-M OE (Fig. 4). This suggests that overexpression of Miro-L or -S in combination with endogenous Miro expression can alter the “transport state” by either prolonging a stationary state of mitochondria or prematurely terminating a motile state. Alternatively, the morphological changes induced by Miro-L and –S create circumstances unfavorable for transport.

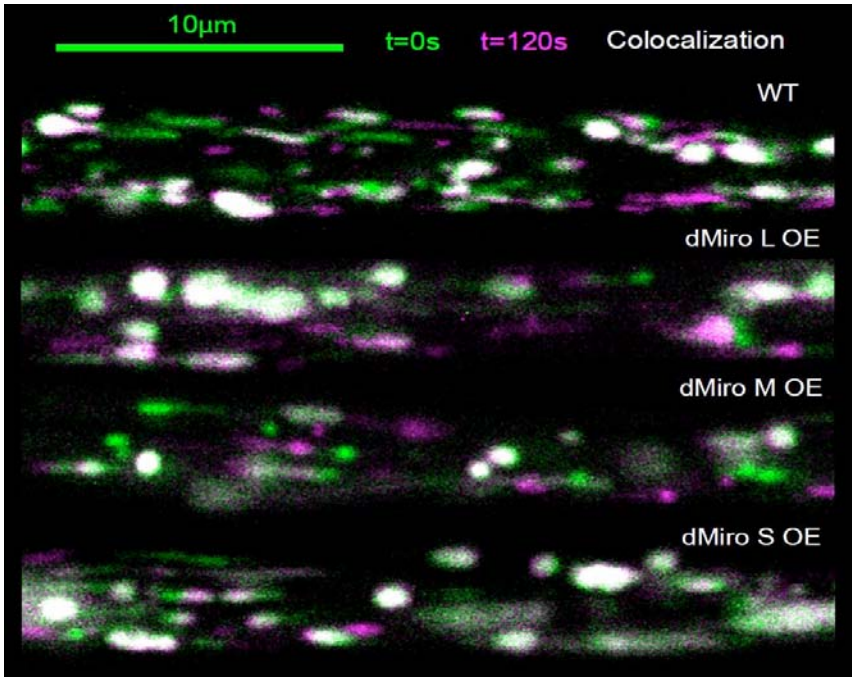

Figure 5. In proximal motor axons of *Drosophila melanogaster* larvae, two images taken 120 seconds apart with an Ar488 laser were set together in an overlay. The first was image colored green and the second colored magenta. When the two images were overlaid together, green and magenta became white. White mitochondria were scored as stationary, green as motile. Density was quantified based on number of stationary, motile, and total mitochondria within the total nerve area shown.

Density:

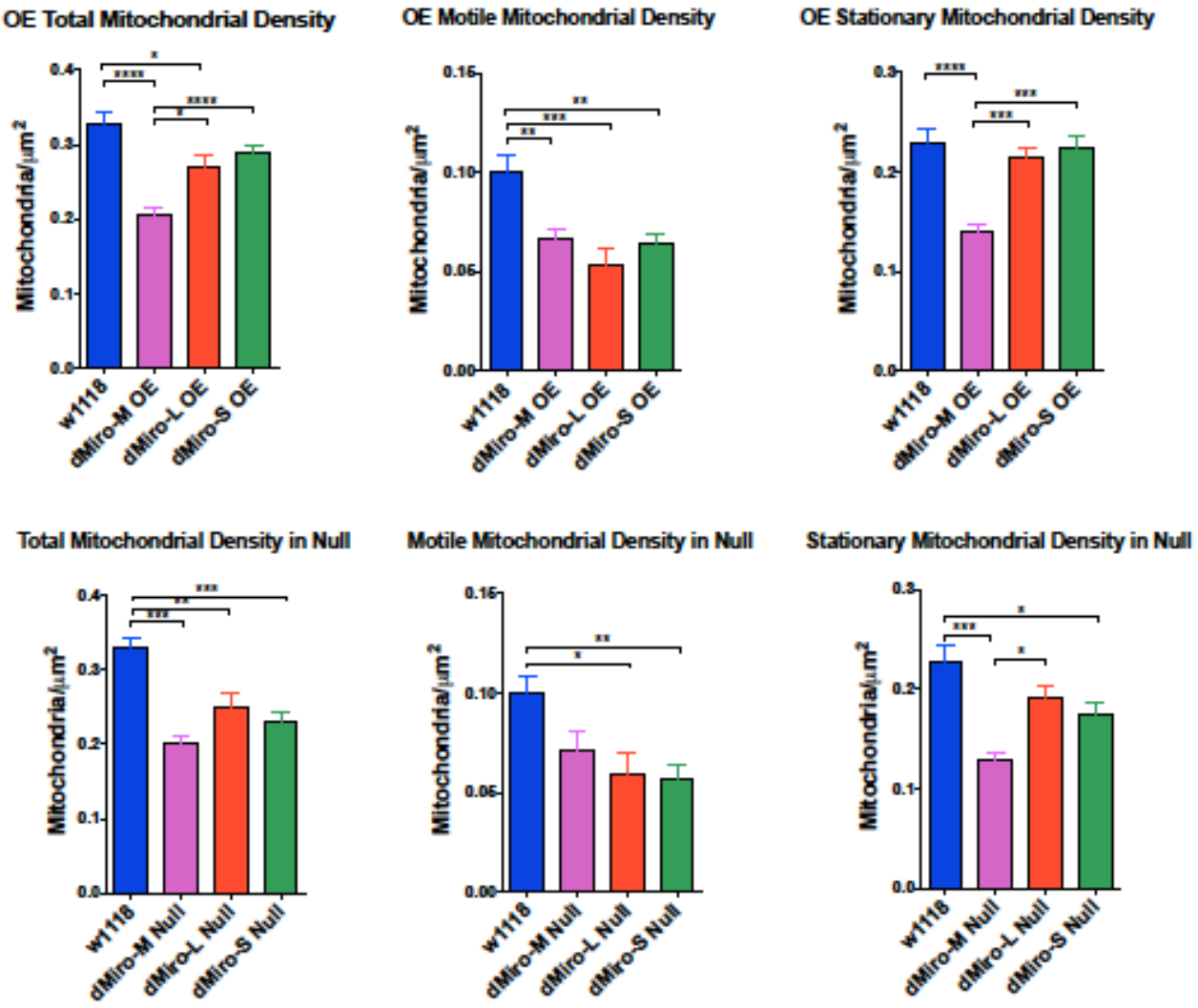

Figure 6: Mitochondrial density taken from proximal motor axons of *Drosophila melanogaster* larvae. Motile mitochondria were scored from overlays as green mitochondria. Stationary mitochondria were scored from overlays as white. Total density was derived from the combination of these two values. Larvae were from fly lines that had *dMiro-M*, *dMiro-L* or *dMiro-S* inserted via p-element into genomes from both wildtype and *miro* null backgrounds. Statistical analysis was performed with ANOVA and Bonferonni post test, statistical differences indicated by asterisks (* - $p<0.5$).

Expression *of Miro-L*, *-M*, or *-S* in a *miro* null background significantly decreased the total density of mitochondria in motor axons (Fig. 6). *Miro-M* significantly decreased the density of stationary but not motile mitochondria. *Miro-S* significantly decreased the density of both

stationary and motile mitochondria while Miro-L reduced the density of motile but not stationary mitochondria (Fig. 6). This suggests that Miro-L and Miro-S alone are deficient in transport initiation or maintenance.

Interestingly, the presence of endogenous Miro significantly altered the effects of expressing individual Miro isoforms. Overexpression of Miro-M significantly decreased motile, stationary, and therefore total mitochondrial density, and in fact was the only genotype with changes in stationary density.. The overexpression of all three isoforms decreased motile mitochondrial density. This suggests that too much of any single isoform impairs mitochondrial transport dynamics.

Flux:

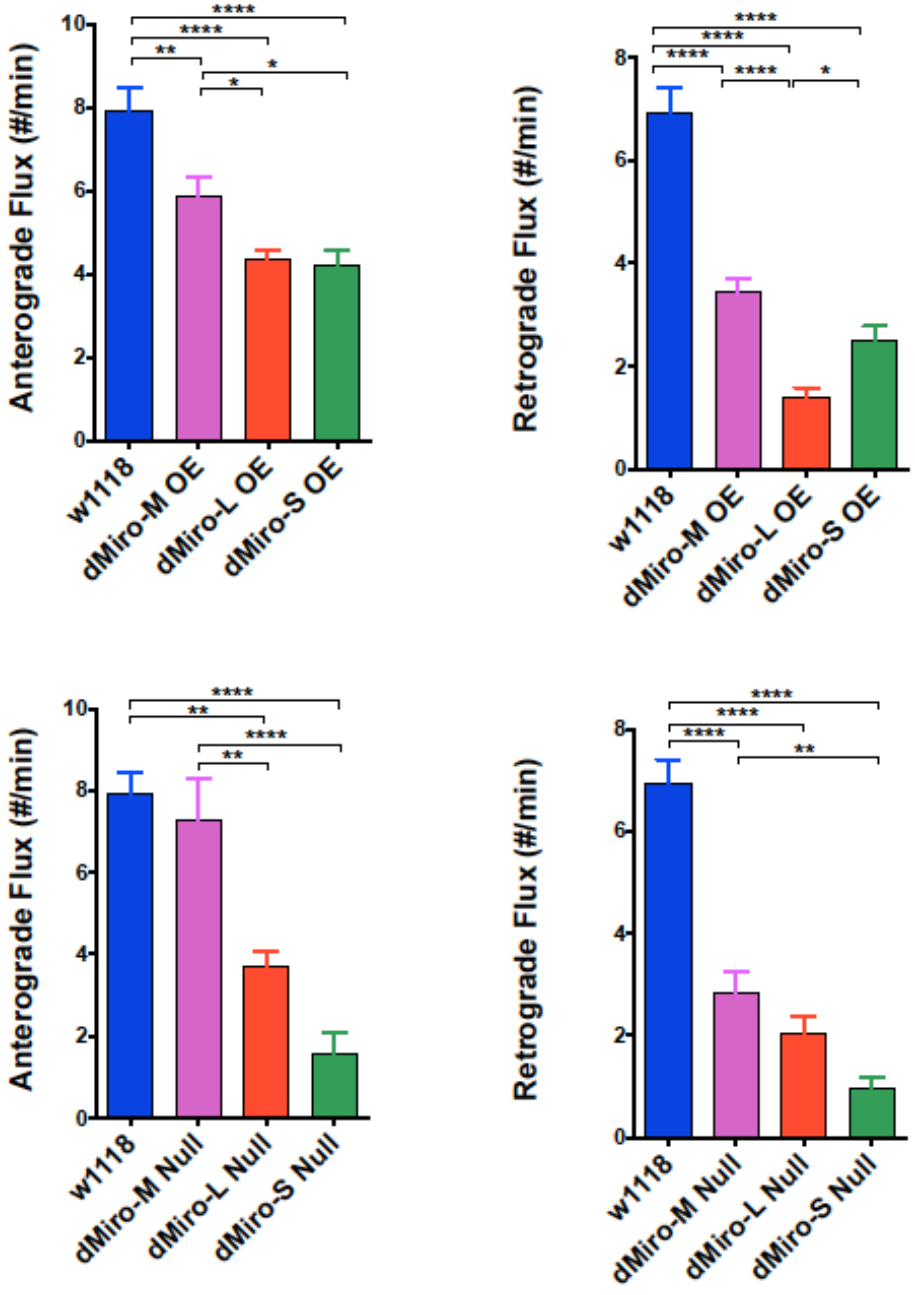

Figure 7. Flux represents the number of mitochondria moving in each direction past a specific reference points during two minutes in proximal motor axons of *Drosophila melanogaster* larvae. Anterograde flux is towards the axon terminal, retrograde flux towards the cell body. Larvae were from fly lines that had *dMiro-M*, *dMiro-L* or *dMiro-S* inserted via p-element into genomes from both wildtype and *miro* null backgrounds. Statistical analysis was performed with ANOVA and Bonferonni post test, statistical differences indicated by asterisks (* = $p<0.5$).

To measure the rate of mitochondrial transport (flux), proximal motor axons were live imaged and the numbers of mitochondria moving either antero- or retrogradely during a 2 minute interval were analyzed. Miro-S and -L but not Miro-M expression in a *miro* null background caused a significant decreased in both antero- and retrograde flux (Fig. 7). This indicates that Miro-M alone is sufficient to facilitate a normal rate of anterograde transport. In contrast, Miro-S and –L are apparently not sufficient to either trigger a normal rate of transport out of the cell body or facilitate normal transport kinetics in the axon.

Unexpectedly, Miro-M expression in nulls was not able to restore a normal rate of retrograde transport even though the rate of anterograde transport was normal (Fig. 7). This indicates that Miro-M mutant mitochondria in the periphery of the axon either become dysfunctional and are unable to undergo transport, have an impaired signaling pathway necessary to trigger retrograde transport or are unable to undergo efficient dynein motions. Importantly, this indicates that Miro-M alone is insufficient to mediate a critical aspect of retrograde transport. In contrast, the reduced rate of retrograde transport in Miro-L and –S expressing axons is proportional to the reduced rate of anterograde transport, and therefore likely a secondary consequence of a primary defect of either initiating or maintaining anterograde transport.

Overexpression of each Miro isoform caused a significant decrease in both retrograde and anterograde mitochondrial flux. This is especially interesting because expression of Miro-M alone did not impair anterograde movement, but did when overexpressed. This suggests that the overexpression of *Miro-M* in the presence of the other isoforms may be less effective at initiating or maintaining transport. There is also an especially significant retrograde flux impairment induced by overexpression of *Miro-L* (Fig. 7). This may be caused by a specific impairment in

the ability of Miro-L to initiate retrograde transport, or a defect in mitochondria that developed in, or en route back from, the axon terminal, and prevented their transport.

Length:

Motile mitochondrial length was measured from flux images. Surprisingly, very long, thin structures often formed a continuous network extending throughout the length of the images taken from *Miro-S* or *Miro-L* animals, and were therefore difficult to quantify. A method of quantifying the thin structures is necessary for complete capture of these morphological changes. An analysis of overall mitochondrial size would also be beneficial, as extremely large, “blown-up” mitochondria were often seen associated with the long and short isoforms as well. Mitochondria with such drastic morphological defects were not observed to move. This quantification has yet to take place due to the complexity of the measurements necessary. Morphological changes in motile mitochondria were observed to a lesser degree however, and were more readily quantifiable.

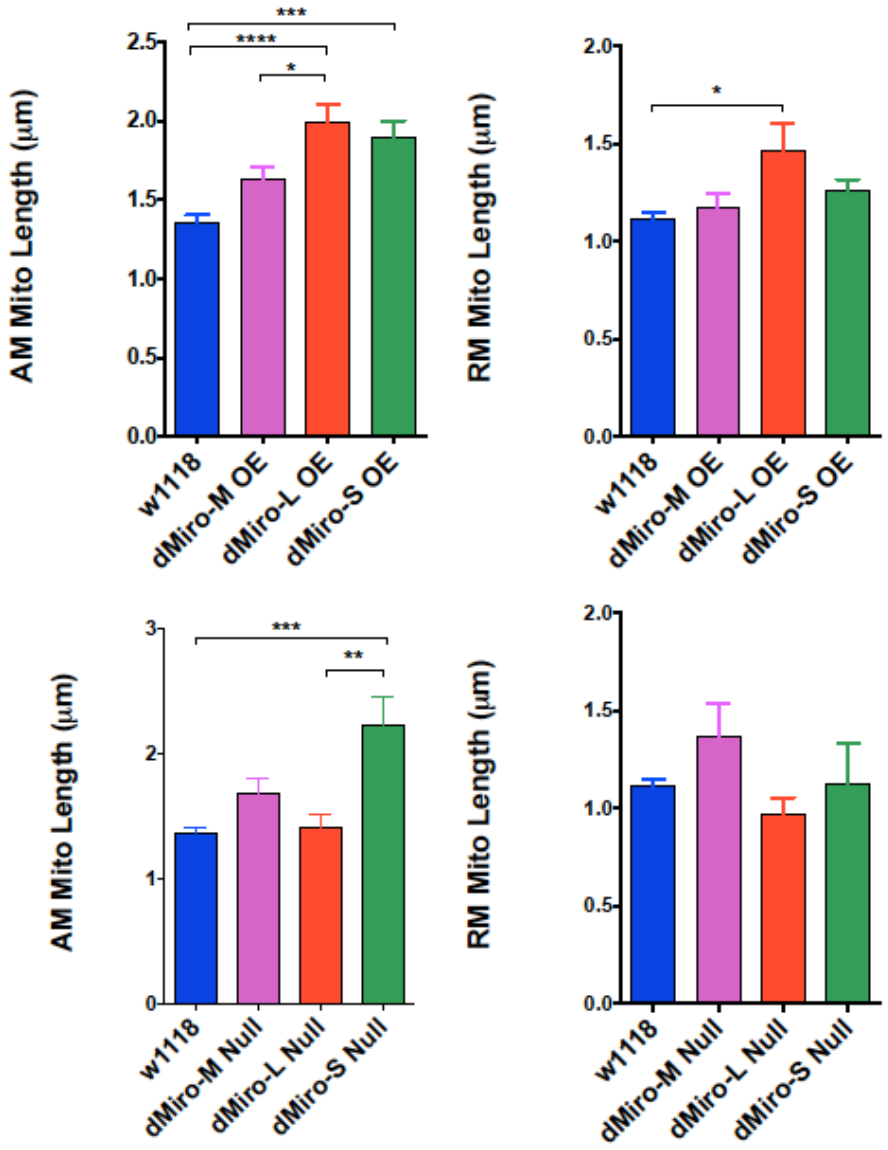

Figure 8: Motile mitochondrial length in proximal motor axons of *Drosophila melanogaster* larvae as measured with Feret's diameter. Larvae were from fly lines that had *dMiro-M*, *dMiro-L* or *dMiro-S* inserted via p-element into genomes from both wildtype and *miro* null backgrounds. Statistical analysis was performed with ANOVA and Bonferonni post test, statistical differences indicated by asterisks (* - $p<0.5$).

Overexpression of Miro-L induced a significant increase in mitochondrial length of motile mitochondria that moved both antero- and retrogradely (Fig. 8). This effect was not seen when Miro-L was expressed in a *miro* null background suggesting that the dominant effect of Miro-L

OE requires the presence of another Miro isoform. Miro-S induced a significant increase in mitochondrial length, both when overexpressed or expressed in a *miro* null background. This indicates that Miro-S has a dominant affect on mitochondrial length. Miro-M did not induce any significant changes in mitochondrial length.

Analyzing mitochondrial "health" through membrane potential using TMRM:

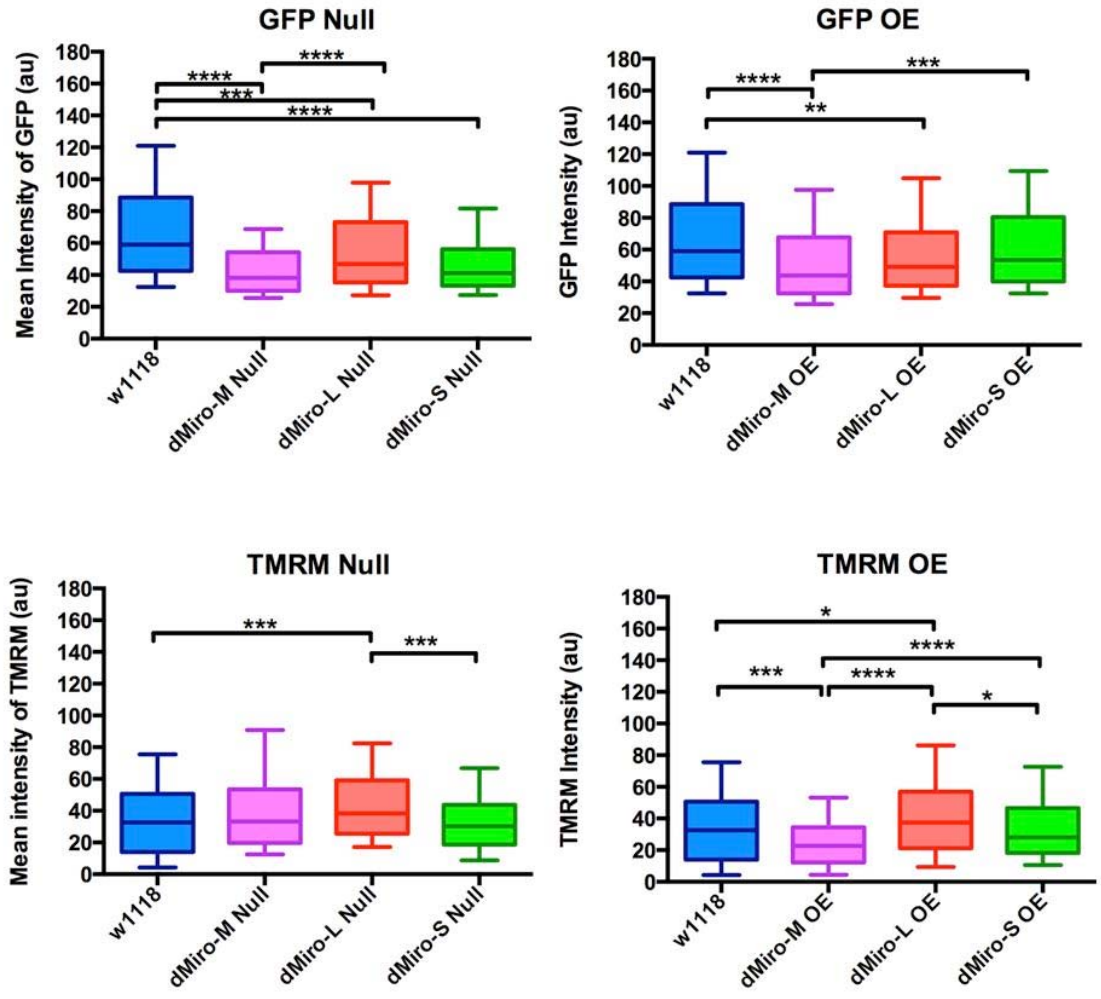

Figure 9: GFP intensity derived from transgene mitoGFP present in all genotypes. TMRM dye was attracted to cations in mitochondria and can therefore be used as an assessment of mitochondrial membrane potential. Mean intensity of individual mitochondria and mitochondrial clusters were collected for animals in each genotype. . Larvae were from fly lines that had *dMiro-M*, *dMiro-L* or *dMiro-S* inserted via p-element into genomes from both wildtype and *miro* ull backgrounds. Statistical analysis was performed with ANOVA and Tuckey post test, statistical differences indicated by asterisks (*) ($p<0.5$).

To measure potential effects of the Miro isoforms on mitochondrial health, we measured the mitochondrial membrane potential using Tetramethylrhodamine methyl ester (TMRM). TMRM is a cationic dye that is attracted to the positive charge of protons within the mitochondrial matrix, and is therefore a good indicator of membrane potential (Scaduto 1999). To adjust for variability in dye loading from preparation to preparation, we also measured the fluorescence intensity level of the mitoGFP tag, which would allows us to normalize the TMRM measurements if the mitoGFP fluorescence is similar in all genotypes. Surprisingly, we found that GFP intensity levels are statistically significantly different among all genotypes (Fig. 9). Expression of all Miro isoforms in a *miro* null background significantly decreased the levels of GFP fluorescence. A decrease in GFP intensity was also seen for Miro-L and -M OE (Fig. 9). Interestingly, in both genetic backgrounds the variants with the lowest GFP intensities were seen when Miro-M was expressed in a Miro null background. This suggests that all the isoforms, especially Miro-M, somehow decreases either mitoGFP intensity or mitoGFP expression levels in mitochondria.

When overexpressed or expressed in a *miro* null background, Miro-L induced an increase in TMRM intensity, suggesting that those mitochondria are not unhealthy, and if anything may promote cell survival (Ward 2007). Overexpression of Miro-M interestingly decreased TMRM levels suggesting that this mitochondria may be less healthy, which is surprising considering the viability of Miro-M for rescuing miro null lethality when Miro-L and Miro-S cannot do the same.

Discussion

Our primary goal was to examine the potentially different roles of the three dMiro isoforms Miro-L, -M, and -S. When Miro-L or Miro-S expression was driven in all *miro* null mutant neurons of *Drosophila* by the paneuronal driver elav, they both failed to rescue the lethality of the *miro* null mutant. However, Miro-M expression restored a normal viability. Miro null mutant larvae expressing only Miro-L or Miro-S did not eclose from their pupal cases, and often did not even pupate, dying at or before the 3rd instar larval stage.

Since Miro is primarily known as a "mitochondrial transport protein," the drastic phenotypic differences in viability among the three isoforms could have been caused by differences in their ability to facilitate transport of mitochondria. This hypothesis appears to have some support based on our data. The expression of Miro-L or Miro-S decreased the density of motile mitochondria in proximal axons and altered the ratio of motile versus stationary mitochondria in axons. Consistently, Miro-M but not Miro-L and –S expression restored a normal rate of anterograde mitochondrial transport in *miro* null mutants. This suggests that Miro-L and -S may be deficient in their ability to either properly initiate, perform, or maintain transport of mitochondria in axons. A more comprehensive analysis of mitochondrial transport will be required to differentiate between these possibilities.

It is interesting to note that Miro-M expression significantly decreased both the density of motile and stationary mitochondria in both wild type and *miro* null backgrounds. However, expression of Miro-M restored a normal rate of anterograde transport (flux), while the rate of retrograde transport was much reduced. This produces a paradox: if mitochondria are moving into the axon, but aren't coming back, why then is the density reduced instead of being increased? One obvious suggestion is that, while Miro-M may be competent in restoring near-

wild type levels of anterograde transport, it may cause survival problems that result in increased autophagy of axonal mitochondria.

Miro-S and Miro-L appear to have less of a role in transport, or at least in driving transport, than Miro-M. However, they do appear to have a role in regulating mitochondrial morphology. Both isoforms increase the length of motile mitochondria, especially of anterogradely moving mitochondria. The effect of Miro-S and -L appears dominant.. Both, electron microscopy and confocal images of mitochondria in the neurons displayed abnormal morphologies, from a slight increase in length of motile mitochondria, to the formation of mitochondria large enough to fill an entire axon, to the proliferation of networks of thin mitochondrial tendrils that fill axons. These morphological changes suggest a role of Miro in mitochondrial fusion and fission.

To examine a potential differential role of all three isoforms for mitochondrial health, we analyzed the mitochondrial membrane potential. TMRM dye will distribute itself within polarized mitochondria in a Nernstian manner. Membrane potential-driven accumulation of TMRM within the inner membrane region of healthy functioning mitochondria results in a dramatic increase in TMRM-associated fluorescence. When the mitochondrial membrane potential collapses in apoptotic or metabolically stressed cells, the TMRM is dispersed throughout the cell cytosol at a concentration that yields minimal fluorescence. When this dye was used to assess membrane potential and subsequent “health” of mitochondria, expression of Miro-L showed a significant increase in mitochondrial membrane potential. This is puzzling, because this indicates a hyperpolarization of mitochondria, which is known to promote cell survival. It was expected that these mitochondria, with such apparent morphological and hypothesized defects in fusion/fission, quality control, and autophagy pathways would have

severe defects in membrane potential. Equally surprising is the fact that Miro-M induces a decrease in TMRM intensity and therefore membrane potential. By all accounts these mitochondria appear morphologically consistent with mitochondria in wild type organisms, but the data indicates that they are somehow less healthy. It can only be concluded that this membrane potential and “health” of mitochondria cannot be correlated with organism viability associated with these Miro isoforms.

The intensity of the mitoGFP tag was measured for the purpose of normalizing TMRM levels. However, GFP levels were reduced in all genotypes, which is surprising. Hence, mitoGFP fluorescence cannot be used to normalize TMRM measurements. It seems likely that the in GFP fluorescence is caused by a defect in protein import since mRNA transcription and processing can be ruled out as culprits. The reduced mitoGFP fluorescence could also result from less mitochondrial matrix space, which could be caused by uncoordinated fusion or fission of the outer and inner membrane. Nevertheless, these data potentially suggest that role for Miro in the coupling of translation with mitochondrial protein import.

Drosophila Miro has proven to be a complex, multi-faceted protein involved in more than just mitochondrial transport, and its various protein isoforms may drive its pleiotropic nature. Miro-M appears to drive transport, especially in the anterograde direction, where Miro-L and -S appear to be less transport-competent. It is apparent that Miro-L and -S are involved in other functions that affect mitochondrial morphology, potentially fusion/fission processes or autophagy. However mitochondrial health is not impaired in these morphologically distinct mitochondria, and therefore it is unlikely that these changes result in the lethality associated with these isoforms.

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