GENETIC ANALYSIS OF MIRO AND MITOFUSIN PROTEIN INTERACTIONS

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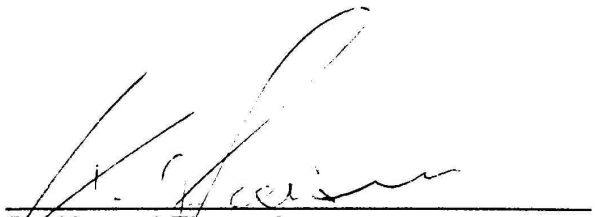

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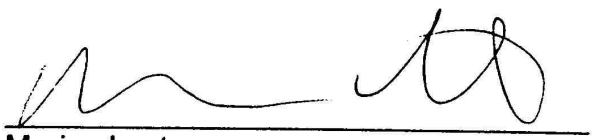

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Abstract

Mitochondria are crucial in the providing the energetic needs to cellular function, apoptosis, and damage. Mitofusin in particular is important because it helps with both mitochondrial fusion as well as interactions with mitochondrial ER. Fusion and fission are two opposing processes work together to help maintain the size, shape, and quantity of mitochondria as well as their physiological function. Some of the major molecules mediating mitochondrial fusion and fission in mammals have been discovered, but the underlying molecular mechanisms have yet to be solved. Here, we show that Marf OE has an affect on the size, quantity, and localization of mitochondria within the axons, NMJs, and ventral ganglia. We also show that Miro OE associated with Marf OE only slightly modulates this change and that Miro's EF-hand domains do not play a role in this change in structure. This is important to help better understand Miro and Mitofusin interactions.

Introduction

Drosophila melanogaster, more commonly referred to as the fruit fly, has been a useful model organism for nearly 100 years. There have been arrays of genetic and molecular tools to assist in analysis of gene interactions and functions that are unique to the fly. Drosophila is also useful because there are many conserved pathways with humans since it is a multi-cellular organism.[2] Due to these advantages; Drosophila is the ideal model in order to better observe how mitochondrial transport takes place.

Mitochondrial have shown to play a crucial role for supplying the energetic requirements for cellular function, apoptosis, and damage. Majority of ATP that

gets broken down and carried throughout the cell is produced here and without this ATP, the cell would undergo apoptosis.[12-14] Mitochondria also interact with a number of different organelles such as the endoplasmic reticulum (ER). It is still unclear how it interacts specifically in order to allow for it to function as it does as well as whether mitochondrial dynamics affect the physiology of the organism.[5,13] By studying transportation of mitochondria, we are able to gain further insight into different diseases that are caused by mutations in this transport, allowing us to push closer to possible drug therapies for future generations.

In the study conducted by Dietrich et al., mitochondria in hypothalamic neurons that produce the orexigenic peptide, agouti-related peptide (Agrp), were shown to demonstrate distinctions in regards to the size, shape, and number on the metabolic state of the animal. These changes were found to be due to the control of mitofusins 1 and 2. These are nuclear encoded proteins that are involved in mitochondrial fusion in response to feeding and overfeeding. Mitofusin 2 has also been shown to play a role in anchoring mitochondria to the ER[10]. Mitochondrial fusion in Agrp neurons is a critical response to a high fat diet to mediate weight gain by increased fat mass.[5,13] This study directly correlates to the research that I performed because by gaining more insight into the interactions of mitofusin and demonstrating that it interacts with the ER, we can therefore develop further research questions about how Marf interacts with miro (mitochondrial rho-like) and what effects it has within the mitochondria.

Mitofusin is a mitochondrial membrane protein that is found in mammals and facilitates mitochondrial fusion and helps with operation and maintenance of

the mitochondrial network. As shown in figure 1, this is a complex network that is constantly undergoing fusion and fission, which needs to remain balanced in order to permit membrane interactions as well as exchange between DNA and mitochondria. Mitochondrial fusion is essential for embryonic development and is also known to interact with miro in mammals, which affects transport of mitochondria.[5-6] Fusion is also important for mitochondrial transport and localization in neuronal processes.[21] Conditional MFN2 knockout mice show degeneration in the Purkinje cells of the cerebellum, as well as improperly localized mitochondria in the dendrites. MFN2 also associates with the MIRO-Milton complex, which links the mitochondria to the kinesin motor.[1]

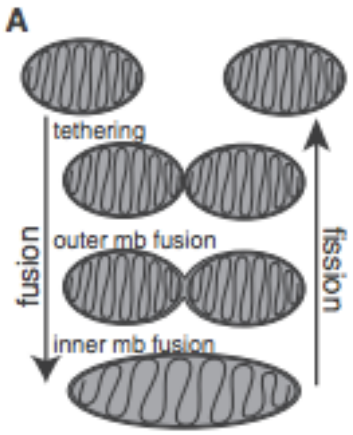


Figure 1. Shows the multistep process of fusion and fission that interact with mitochondria dynamics.[5]

The mitochondrial Rho-GTPase (Miro) protein family has the potential to connect and interact with mitochondrial dynamics via signaling pathways. There are two different GTPase domains as well as two Ca^{2+} binding EF hand domains that are present in orthologs of human Miro. Perinuclear aggregates of mitochondria are seen with overexpression of constitutively active human Miro in COS7 cells as well as an increase in the number of events of apoptosis.[17] Yeast

cells that are lacking Miro (Gem1p) show a breakdown of the tubular mitochondrial network, but this is not caused with faults with fission or fusion.[6,17] Both GTPase domains as well as EF-hand motifs are required for Gem1p function.[17] In cultured cells, upon a decrease in mitochondrial membrane potential, PINK1 kinase recruits Parkin, a ubiquitin E3 ligase, which ubiquitinates several mitochondrial targets, including Mitofusin-1 (MFN1) and Miro, to facilitate the degradation of mitochondria via mitophagy.[1,14] ER forms close contacts with mitochondria, essential for calcium regulation in cellular microcompartments. Miro is a mitochondrial receptor for kinesin via Milton that facilitates the transport of mitochondria on microtubules in a Ca^{2+}-regulated manner. Upon synaptic activity in neurons, influx of glutamate and Ca^{2+} halts mitochondrial transport via Miro to position them at sites of synaptic activity that require Ca^{2+} uptake and ATP.[6,17]

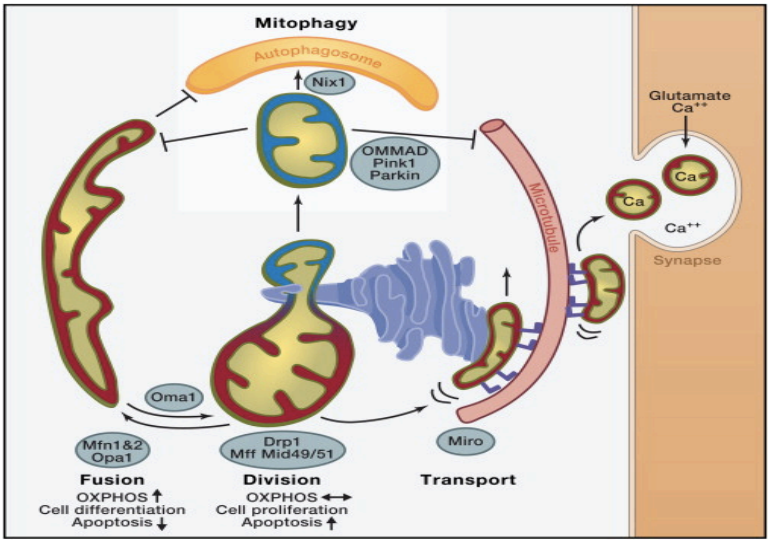


Figure 2. Shows the role of mitochondrial interactions. The mitochondria with high membrane potential are shown in red. These mitochondria also have a high level of oxidative phosphorylation (OXPHOS). Low membrane potential mitochondria are shown in blue, which allow for Mitofusin 1 or 2 (MFN1, MFN2) to be able to mediate mitochondrial outer-membrane fusion in a tissue-specific manner.[2]

It is still unknown how Miro and Marf interact specifically, although we know that they do interact somehow, at least within Drosophila. By looking at the double mutants of Marf and Miro, we can gain more insight into how Miro could affect the functions of Marf, or vice versa. There have been several mutants of miro studied that show that the mitochondria are long and stringy as well as unable to compartmentalize normally. This brings up the questions of could miro then be affecting mitochondrial fusion and fission by affecting Marf function? Does Marf affect mitochondrial transport and is this because it affects miro? This therefore leads to the objective of my research, which is aimed at answering questions: Does Marf OE change mitochondrial localization, size, or morphology? If this is found to be true, then does Miro OE modulate this change? Again, if this found to be the case, then does this require Miro's EF hand (Ca-sensing) domains, and if so which one?

Materials and Methods

Fly Stocks

Flies were raised on standard medium (http://flystocks.bio.indiana.edu/bloom-food.htm) with dry yeast at 25°C. The strain *w^{1118}, P[w+; UAS::mito-GFP]* (abr. mitoGFP) expressing GFP tagged by an N-terminal mitochondrial localization signal was obtained from W. Saxton (Indiana University, Bloomington, IN). To express mitoGFP and dMiro, we used the enhancer trap strain *w^{1118}; P[w+, OK6::Gal4]*, which drives expression in motor neurons and some interneurons of the ventral ganglia, salivary glands, wing discs, and a subset of tracheal

branches.[6,17] The following genotypes were examined: control: w-; OK6-Gal4/+;UAS-mitoGFP/+. Marf OE: w-;OK6-Gal4/+;UAS-Marf-HA/UAS-mitoGFP. Miro OE with Marf OE: w-;UAS-miro-2D/OK6-Gal4;UAS-Marf-HA/UAS-mitoGFP. EF-KO OE with Marf OE: UAS-miro-EFQ5/w-;OK6-Gal4/+;UAS-Marf-HA/UAS-mitoGFP. E234K OE with Marf OE: w-;UAS-miro-E234K/OK6-Gal4/+;UAS-Marf-HA/UAS-mitoGFP. E354K OE with Marf OE: w-;UAS-miro-E354K/OK6-Gal4/+;UAS-Marf-HA/UAS-mitoGFP. Crosses were set up in vials and parents were moved after 7 days to ensure the right generation was being imaged.

Generation of myc-tagged dMiro transgenes

dMiro cDNA (RE) was sequentially PCR-amplified to replace the original ATG start codon with a consensus Kozak sequence followed by a N-terminal myc tag (primer1: 5′-ATCTCTGAAGAAGATCTGGGACAGTACACGGCGTCG-3′; primer 2: 5′-ATGGAGCAGAAACTCATCTCTGAAGAAGATCTG-3′; primer 3: 5′-AATTAATGCGGCCGCACCATGGAGCAGAAACTCATC-3′). The PCR fragment was then sub-cloned into a pCR2.1-TOPO vector. After confirming the DNA sequence by DNA sequencing, the myc-tagged cDNA was subcloned into a pUAST P element vector and transgenic strains containing P were generated.[17,20]

Immunostainings

Dissected third instar larvae were fixed with 4% paraformaldehyde for 30 minutes at RT, washed 3 times with PBS (PBS, containing 0.2% Triton X-100) for 5

minutes, blocked with PBS containing 0.2% Triton X-100, 0.2% BSA for 1 hour, incubated in a 1/1000 dilution of rabbit anti-GFP AlexaFluor488-conjugate (Molecular probes) and goat anti-HRP Cy3-conjugate (Jackson Laboratories, West Grove, Pa) overnight at 4°C. These were kept shielded from the light after this and for the remainder of the immunostaining. Then it was washed with PBS with 0.2% Triton X-100 for 20 minutes 3 times and washed for 2 minutes in PBS before observing it under the microscope.

Mitochondrial GFP Imaging

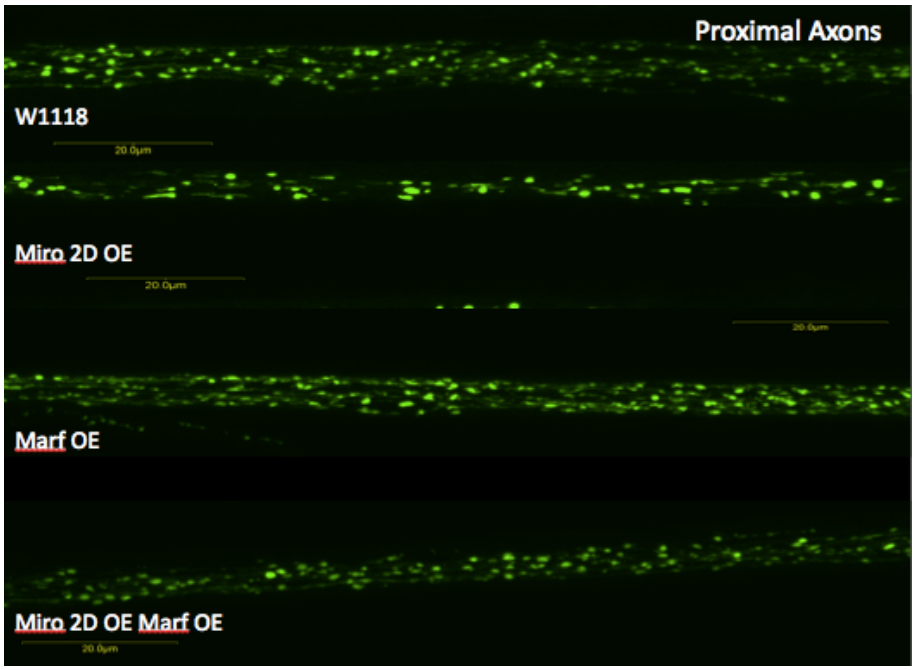
The mitochondria in motor neurons of dissected third larvae were immunostained as described previously. The dissected preparation was then submersed in HL-3.2 solution (containing 7 mM L-glutamate and 0.1 mM $CaCl^2$) in order to be viewed with an upright Olympus microscope BX50WI equipped with a confocal laser scanner (FluoView300) and a 60X water immersion objective (LUMPLANFL N.A. 0.9). The dissected larvae were orientated under the microscope so that the ventral ganglion (VG) appeared on the right of the acquired image and the following segmental nerves were aligned horizontally across the image. The 488 nm excitation line of the multi-argon laser (Mellet Griot, 150mW) was attenuated to 4% of its maximum power, with all other lasers at 0%. Fluorescence emission was monitored through a low-pass optical filter with a cut-off at 510 nM (BA510IF, Olympus). The pinhole was fully opened to allow maximum “focal depth”. Image size of each region of interest (ROI) was recorded for m67s3, the ventral nerve cord, and the proximal axons, which were imaged at 2x zoom. The

settings of the Argon laser were set to 4% and the HeNe543 laser was 1%. The PMT was also set to 600 for both channels.

Results

MITOCHONDRIAL GFP IMAGING

Mitochondrial GFP imaging was performed after being immunostained in order to visualize the size, location, and quantity of neuronal mitochondria with w^{1118}, Marf OE, Miro 2D OE, Miro OE with Marf OE, EF-KO OE with Marf OE, E234K OE with Marf OE, and E354K OE with Marf OE. These different genotypes were imaged within the NMJs, ventral ganglia, and proximal axons. The following images show what was observed.



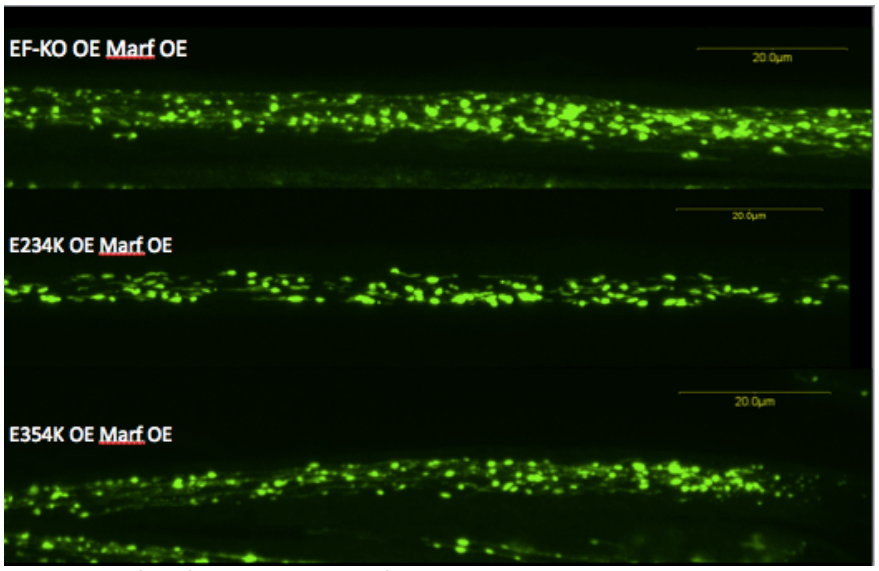
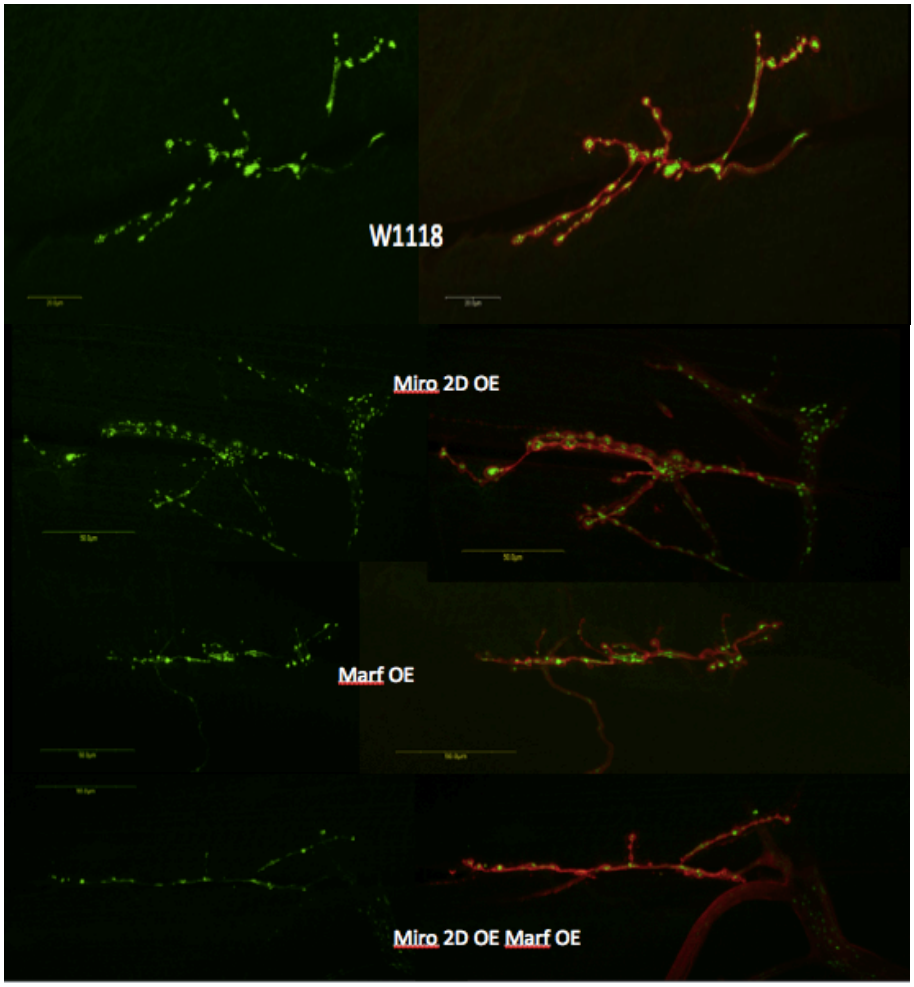


Figure 3. Confocal imaging of the proximal axons showing the change in size and shape with each of the different genotypes listed. Scale bar is 20um.



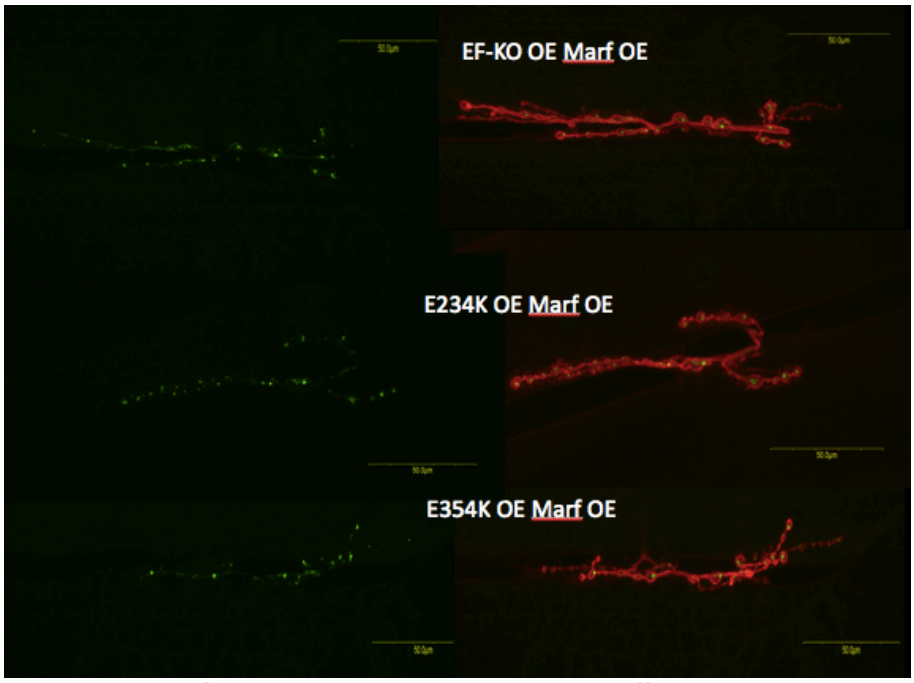
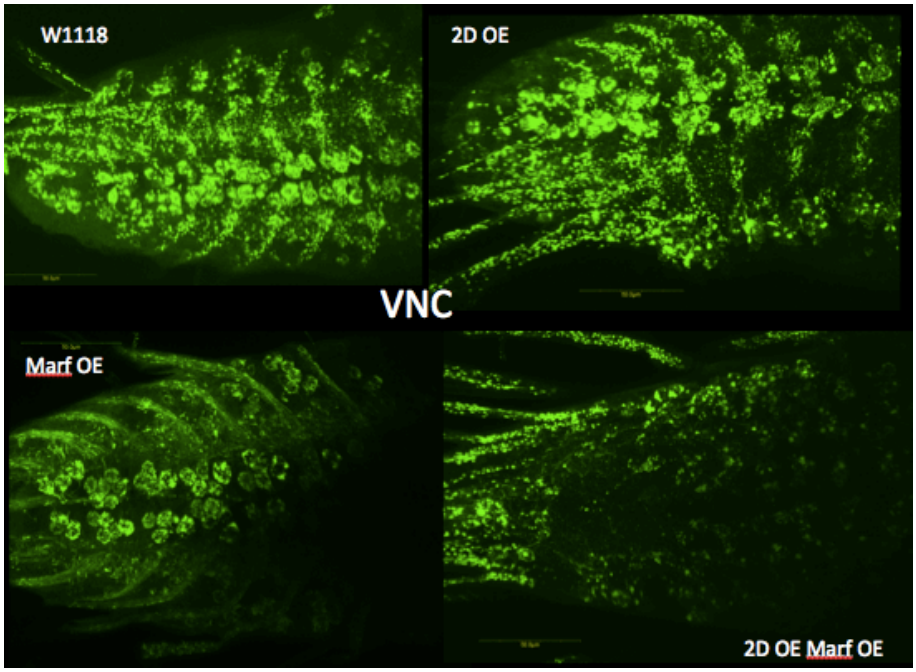


Figure 4. Mito-GFP (green) alone showing the differences in quantity, size, and localization of the presynatic mitochondria and overlap of Mito-GFP and anti-HRP (red) channels in genotypes as shown. Scale bar is 50um.



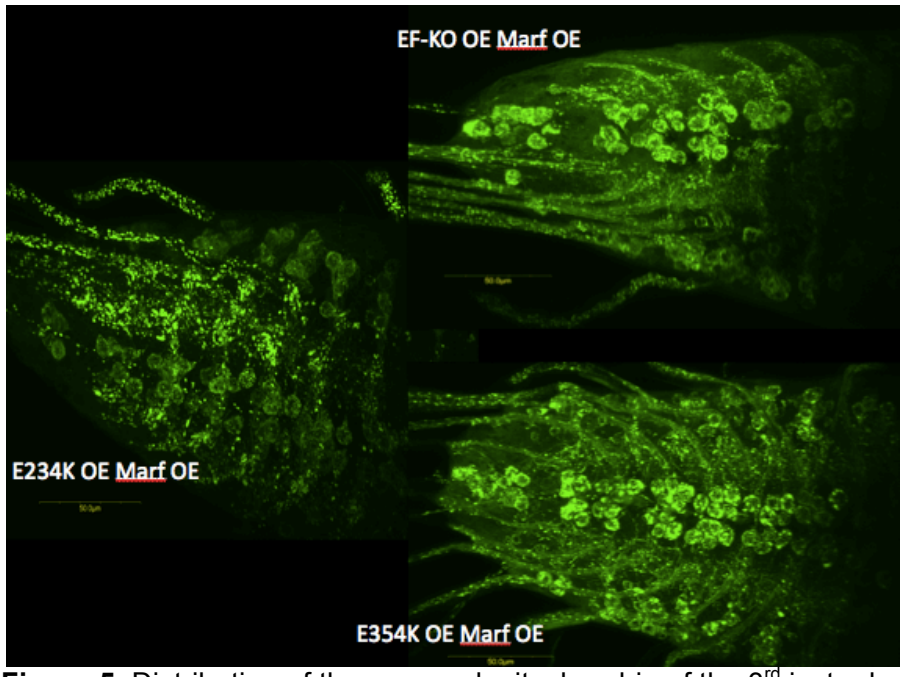


Figure 5. Distribution of the neuronal mitochondria of the 3rd instar larvae in the ventral ganglia. Showing how the neurophil as well as accumulation in cell bodies are lost in Miro 2D overexpression with Marf overexpression. Mitochondria were visualized by expression of MitoGFP (green) with Gal4 driver *elav.* Scale bar is 50um.

Discussion

Mfn1 and Mfn2 localize to the mitochondrial outer membrane and cause aberrations in mitochondrial morphology when overexpressed.[3] My research demonstrates this point because Marf OE does indeed change mitochondrial size and quantity, Miro OE is able to slightly alter this change, but Miro's EF-hand domains don't play a role in modulating this change. Mouse studies have provided similar results with clear evidence that Mfn1 and Mfn2 are crucial for mitochondrial fusion.[3,21] These findings were confirmed by research performed by Murley et al. because Miro1/2 is thought to be involved in the connection between mitochondria and motor proteins, which help provide transportation along the microtubules. If there is a mutation with Miro 1/2, there is a reduction in the movement of mitochondria. This suggests that there are likely enhanced

contacts between mitochondria and the ER.[9-12] The contact is also thought to be maintained after fission due to the ER tubules being coupled specifically within the division site of the mitochondria.[16,18]

EF-hand regions play a relatively minor role in the maintenance of mitochondrial morphology and distribution, with only EF-hand I acting to stabilize Gem1 expression levels.[12] These results coincide with the findings from this study because since Gem1 is thought to be a regulatory protein, it makes sense that the EF-hand domains wouldn't affect the expression of Marf.

When the rate of mitochondrial fusion has been reduced, the mitochondrial then begin to fragment off into short tubular structures or small spheres due to an increase in the amount of fission with the decrease in the amount of fusion.[3] Based on this, we can concluded that mitochondrial morphology is regulated by a balance between both fusion and fission. These results support the findings from my research because as seen in figure 3, with overexpression of miro, the mitochondria get clipped into short tubules or round spheres as opposed to the WT that a large amount of a combination between long tubular structures and small spheres. We also see that with Marf OE there is an increase in extremely long tubular structures and there appears to be less of the small spheres of mitochondria (figure 3). This could suggest that there is an increase in the amount of fusion as opposed to fission. With the Miro OE with Marf OE, there seems to be a significant decrease in the amount of long tubular mitochondria and since Mitofusin's function is to create these tubular structures, in the 2D OE with Marf OE this is being repressed (figure 3). In the EF-KO mice

we are seeing similar results to those of the Marf OE with long tubules and a large quantity of mitos, which we would expect based on some of the other data collected within the lab (results not shown). We were interested in seeing if the EF-hand domains played a role because it was thought that the ER was using these domains to help get the Ca^{2+} sensors off of the ER and begin moving, but based on the results, this doesn't appear to be the case[17]. This shows that it doesn't require Miro's EF hand domains. This information is useful because very little is known about the exact interactions and functions of EF-hand domains.

In the NMJ, we are seeing similar findings as in the axons with Marf OE having a large amount of punctae of GFP fluorescence and very long thin structures (Figure 4). In the Miro OE with Marf OE, we are seeing a very small amount of punctae and very few of them being associated with the synaptic boutons as compared to the control. In the EF-KO OE with Marf OE and the E354K OE with Marf OE, we are only seeing the effects of Marf OE, which allows us to conclude that Miro's EF hand domains are not required to see this change. In the E234K OE with Marf OE we see results similar to Miro 2D, which is what we would expect because this is used as somewhat of a control within most experiments (Figure 4).

In the ventral ganglia we see Marf OE causes there to be a "fluffy" appearance of mitochondria in the neuropil as well as bright mitoGFP expression within the neuronal somata (Figure 5). With the OE of both Miro and Marf, we see that this "fluffiness" is lost in the neuropil and the mitochondria are small fragments. We also see that the mitoGFP in the cell bodies is dull, but this could

be due to the Z stack not going deep enough. Similar to the NMJs, in the EF-KO OE with Marf OE and the E354K OE with Marf OE in the ventral nerve cord, we see comparable distribution and localization with the Marf OE and in the E234K OE with Marf OE we see similar localization and distribution with Miro OE, which is again something that is still unknown as to what exactly is causing these results.[8]

As with all research, there could be some error that took place such as leaving the dissection in the HL-3.2 buffer for too long during the dissection causing some of the results to be skewed due to the animal not being healthy before it is fixed, but this is why at least 3 animals were examined per genotype to be able to compare the animals and discover the patterns.

Conclusion

The main purpose of this project was to observe the effects of overexpression with miro-mitofusin interactions. Studies were performed on different genotypes of Drosophila in order to see the effects of Marf OE associated with Miro OE, the two EF hand domains OE (E234K and E354K), and EF-KO OE. By performing immunostainings in order to observe the differences with each genotype, we were able to conclude that Marf OE causes a change in the shape and quantity of mitochondria and that Miro OE is able to slightly alter this change, but no entirely rescue the Miro WT. After concluding this, we took it a step further and discovered that Miro's EF-hand domains aren't required for this change (Figure 3-4). Furthermore, the ventral ganglia showed an increased amount "fluffiness" within the neuropil because of the long tubular mitos that are associated with

Marf OE as well as an increased amount of florescence within the neuronal somata (Figure 5).

The main challenge for this project was getting fast and accurate dissections so that you were able to fully see all of the regions of interest under the florescent microscopy. Another challenge was taking the right image splice to ensure that you aren't going too deep into another structure or too shallow so that you aren't able to fully visualize the structure. By continuing to practice and develop my skills, I think that these challenges can be improved and ultimately result in more precise images. The next step for this project could be to determine if the GTPase domains play a role in this observed change since we determined that EF-2 is required and perform statistical analysis using ImageJ to see if there is any statistical differences between the size, shape, and distribution of mitochondria within the NMJs and axons. Another interesting question that could be studied is whether Marf KO has any affect as opposed to overexpression.

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