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Mitofusin 2 Regulated Transport of Mitochondria is Necessary for Axonal Integrity

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by

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ABSTRACT OF THE DISSERTATION

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Professor Robert Baloh, Chairperson

The ubiquitous finding of axonal degeneration in a number of the most prevalent neuropathologies marks the importance of understanding axonal biology and the axonal self-destruct mechanism. Though our understanding of axonal degeneration remains largely incomplete, several down-steam steps of the molecular cascade have been elucidated. While this insight has emerged from models of axon degeneration following physical injury or toxic insult, a more comprehensive understanding of the upstream events may be gained from studying primary axonopathies with defined genetic causes.

This dissertation aims to elucidate a molecular mechanism underlying the loss of axons in Charcot-Marie-Tooth Disease type 2A, which is caused by mutations in the mitofusin 2 (*MFN2*) gene. Utilizing an in vitro culture system, we find that CMT2A associated MFN2 mutants disrupt the transport of axonal mitochondria in DRG neurons. Though MFN2 has a previously defined role in facilitating mitochondrial fusion, we propose a direct role for MFN2 in mediating transport based on its interaction with key components of the mitochondrial transport apparatus and perturbation of transport in MFN2 null DRG neurons. MFN2 does not provide a direct link between mitochondria and microtubule based motors, but is poised to mediate transport by a still undefined mechanism. The ability of MFN2 to mediate transport is separate from its ability to mediate fusion as MFN2 disease mutants, that have been shown to retain their ability to fuse mitochondria, cannot rescue the transport deficit in MFN2 null neurons. Additionally, loss of mitochondrial fusion by knockdown of opa-1 is not sufficient to disrupt mitochondrial transport

despite reduced mitochondrial function. These findings may explain why mutations in or haplo-insufficiency of opa-1 leads to Dominant Optic Atrophy (DOA) but not degeneration of long peripheral axons, highlighting the potential importance of mitochondrial transport for axon integrity.

To further test our hypothesis that mitochondrial transport is critical for the integrity of axons, we expressed MFN2 mutants in cultured DRG neurons and looked for signs of degenerating axons. High levels of Ca^{2+} or reactive oxygen species (ROS) delineated a population of degenerating axons that we not observed in opa-1 knock down cultures. Mitochondria in MFN2 mutant expressing cells showed little change in membrane potential compared to a significant change in the mitochondrial membrane potential of opa-1 silenced cells; however, both groups of cultured neurons upregulated glycolysis and were sensitive to treatment with 2DG. We hypothesize that these changes in the MFN2 mutant expressing cells could be explained by lack of mitochondrial flux across segments of axon which must resort to use of glycolysis. The absence of mitochondria could cause segments of axon to become vulnerable to local perturbations in energy or Ca^{2+} levels and explain the axonal degeneration observed in culture. In this way, disrupted redistribution of mitochondria in CMT2A patients would put the longest axons at the highest risk due to the probability of incurring at least one insult along its length for which mitochondria could not compensate.

Finally we attempted to study an animal model of CMT2A to see if our in vitro findings we recapitulated in vivo. To this end we obtained a mouse line in which the R94Q mutation had been knocked in to the endogenous allele. To accurately mirror conditions in CMT2A patients we chose to analyze heterozygous mice. Though homozygous mice die by the third postnatal week, heterozygous mice are phenotypically normal showing no signs of axon loss or muscle denervation. Differential expression of the MFN2 homologue MFN1, absolute length of axons or absolute time to disease may account for the discrepancy between the mouse model and human patients. Hopefully this work will help elucidate the molecular mechanisms underlying CMT2A and contribute toward a more general understanding of why axons degenerate.

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Introduction and Background Research

Axonal degeneration in neurodegenerative disease

Recognizing that a single neuron contains a unique and compartmentalized biology in both its axon and soma has been a fundamental advance in our understanding of neuronal cell physiology. Over the last century, experimentation has revealed that axons and somas employ unique schemes of cytoskeletal organization and transport (Setou et al., 2004), appropriate different ion channels and membrane receptors (Lasiecka et al., 2008), and even instigate separate programs of self-destruction (Raff et al., 2002). The marked differences between the axon and soma necessitates that both be considered in a comprehensive description of neuronal physiology.

Evidence that axons utilize a non-apoptotic program of self-destruction combined with a growing appreciation for the prominence of axon loss in several neurodegenerative paradigms has prompted researchers to reevaluate the prevailing "apoptosis-centered" approach to understanding neurodegeneration. Many studies suggest that axonal degeneration precedes cell death in ALS (Fischer and Glass, 2007), Parkinson's disease (Cheng et al., 2010), Alzheimer's disease (Vickers et al., 2000) and Multiple Sclerosis (MS) (Dziedzic et al., 2010)and is the most important factor contributing to morbidity in MS and demyelinating peripheral neuropathies. Understanding the molecular mechanisms underlying axonal degeneration will be pivotal to both our understanding of neurodegeneration, and our ability to prevent it.

Axonal degeneration in the peripheral nervous system

Axonal degeneration occurs in the peripheral nervous system in response to number of toxic, metabolic and infectious insults as well as occurring in a number of hereditary neuropathies. Patients most commonly experience a progressive loss of sensation and/or motor function beginning in the feet and later progressing up the legs. The spreading of symptoms gives the

impression that axons are progressively degenerating distally toward their cell bodies, earning the phenomenon the name “dying back” (Cavanagh, 1964) (Blakemore and Cavanagh, 1969). Dying back neuropathies occur in a length dependent manner where the longest axons show a predilection for degeneration before their shorter counter parts. This explains why symptoms first occur in the long axons innervating the feet and later progress to affect the shorter axons innervating the hands. Causes of such dying back neuropathies include diabetes (Sima et al., 1983), excessive alcohol consumption (Low et al., 1975), acrylamide poisoning (Schaumburg et al., 1974), the chemotherapeutic agent vincristine (Silva et al., 2006) (Guiheneuc et al., 1980), AIDS (Smith et al., 1990), and a number of inherited gene mutations. Because longitudinal observation of axonal degeneration is difficult to achieve in vivo, it is still unclear if axonal degeneration always starts at the neuromuscular junction and retracts backwards, or if degeneration can be initiated at other areas along the length of an axon. An alternate explanation of the “dying back” phenomenon is that the longest axons are also more frequently targeted by disease processes and are therefore statistically more likely to degenerate first. If axons were to degenerate roughly in the order of their length, it would give the appearance of “dying back”.

At least in culture, axons do have the ability to progressively degenerate in a manner that resembles “dying back”. When a distal segment of an axon is locally deprived of nerve growth factor (NGF), the axon degenerates back toward a proximal segment still exposed to NGF (Campenot, 1982). Though this demonstrates the ability of cultured neurons to degenerate at the distal axon while keeping the proximal intact, whether or not this in vitro behavior recapitulates some aspects of what happens in a dying back neuropathy is still not clear.

Regardless of the mechanistic basis of the dying back pattern of axonal degeneration in the PNS, one fact remains constant; the longest axons are the most sensitive to degeneration. Because the longest axons in the body are in the periphery, global dying back neuropathies should emerge first in the PNS. There are, however long descending axons in the motor tracts of the spinal cord and these axons are also involved in certain length dependent neuropathies.

Axonal degeneration in the central nervous system

Though length dependent neuropathies are among the clearest examples of primary axon diseases, other neurodegenerative processes may also originate in axons. In fact, axons of the central nervous system are primary players in the development MS (Dziedzic et al., 2010) and possibly Alzheimer's disease (Vickers et al., 2000)and Parkinson's disease (Cheng et al., 2010).

Though primarily a disease of CNS myelin, MS also has a prominent neurodegenerative component. Approximately 85% of MS patients first experience a relapsing remitting (RRMS) syndrome in which discrete foci of inflammatory demyelination lead to reversible neurological deficits followed by a secondary progressive phase of the disease (SPMS) which results in an irreversible decline in neurological function. Though RRMS is predominately characterized by acute episodes of demyleination, axon loss has been demonstrated in active brain lesions (Ferguson et al., 1997; Trapp et al., 1998). During this phase, the plastic nature of the brain is able to compensate for the modest loss of axons. However, loss of axons beyond this capacity marks the transition to SPMS where the continued loss of axons underlies a gradual neurological decline (Trapp and Nave, 2008).

In addition to MS, axonal degeneration is also seen in Alzheimer's, Parkinson's and Huntington's disease. In Alzheimer's disease, dystrophic axons can be seen in contact with Aβ plaques, and it has even been suggested that the plaques may compress axons and disrupt axonal transport (Vickers et al., 2009). Degenerating axons associated with α-synuclein containing Lewy-bodies and huntingtin protein aggregates have been seen in Parkinson's and Huntington's disease respectively (Braak et al., 2001)(Li et al., 2001). Importantly, evidence suggests that axonal degeneration occurs at an early stage in these diseases and may play a driving role in their pathophysiology.

The pmn/pmn mouse is a model of motor neuron disease illustrating the importance of axonal degeneration in disease progression

Up to this point the association of axonal degeneration with a broad spectrum of neurodegenerative disease has been demonstrated by numerous studies, however the most

definitive proof that neurodegenerative processes can originate in axons has come from an animal model of progressive motor neuron (pmn) disease and an advance in the understanding of Wallerian degeneration.

The pmn mouse is a widely used model of peripheral motor neuron disease. Mice homozygous for the mutant allele develop progressive hindlimb paralysis that later involves the forelimbs and eventually the descending cortical motor neurons. By six weeks of age homozygotes die concomitant with severe loss of ventral horn motor neurons. Interestingly however, the disease first manifests as a dying-back axonopathy that begins with denervation at motor end plates (Schmalbruch et al., 1991).

To assess the differential roles of cell death and axon loss on disease progression and morbidity, pmn mice were crossed with transgenic mice overexpressing the human bcl-2 gene to inhibit apoptotic loss of motor neurons (Sagot et al., 1995). As expected, expression of bcl-2 prevented the loss of ventral horn motor neuron cell bodies. However, it had no affect on axonal degeneration, disease progression or survival. This suggested that axonal degeneration and not the loss of neuronal cell bodies was more important to the neurodegenerative phenotype.

Axonal degeneration as an actively regulated process

The realization that axons contained a unique program of self-destruction came with the discovery of a spontaneously occurring mutant dubbed the Wallerian degeneration slow (Wld^s) mouse in which distal nerve segments continue to survive and propagate action potentials up to three weeks following nerve transection (Lunn et al., 1989). The genetic basis of this defect was mapped to the distal end of chromosome 4 where a tandem triplication of an 85kb region created an aberrant fusion protein containing part of the ubiquitin ligase E4b and the entire nicotinamide adenine mononucleotide transferase 1 (NMNAT1) (Lyon et al., 1993) (Coleman et al., 1998) (Conforti et al., 2000). Further studies went on to prove that NMNAT's enzymatic activity was responsible for the slowed axonal degeneration (Araki et al., 2004), though the precise molecular mechanisms underlying the phenomenon remain unclear.

The Wld^{s} mice were also used to obtain final proof that axonal degeneration was a more important contributing factor to the death of motor neurons than apoptosis in the pmn/pmn mouse. An experiment crossing the pmn/pmn mice with the Wld^{s} mice demonstrated that double mutants showed delayed axonal degeneration, attenuated symptoms, increased life span and rescue of motor neuron number and size (Ferri et al., 2003).

Targeting axon degeneration to treat neurodegenerative disease

The pmn/pmn and Wld^{s} mice have provided us with three important insights concerning axonal degeneration: 1) Degeneration of axons can drive a neurodegenerative process leading to secondary loss of cell bodies; 2) Preventing axon loss can be an effective means of ameliorating neurodegeneration; 3) Axon degeneration is an active process that may serve as a common target in different paradigms of neurodegeneration.

Despite our increased appreciation for the role axonal degeneration in disease, we have yet to delineate the cascade of molecular events that underlie its propagation. One useful line of investigation to define the molecular pathways critical for axon maintenance and degeneration is to study an inherited axonopathy that has an established genetic cause, as this approach immediately provides candidate molecules to investigate experimentally. The line of investigation described in this dissertation takes this approach. Starting with the prior knowledge that mutations in the mitofusin 2 (MFN2) gene cause the inherited peripheral neuropathy Charcot-Marie-Tooth disease type 2A, we explore the function of MFN2 and it role in supporting axonal integrity.

Charcot-Marie-Tooth disease type 2A is an inherited axonopathy

Charcot-Marie-Tooth disease type 2A (CMT2A) is an inherited peripheral neuropathy characterized by a progressive dying back degeneration of the longest axons in the body. The dominantly inherited disease usually presents in the first or second decade of life, first manifesting symptoms in the feet and later spreading to the hands and other proximal body regions.

The most commonly identified mutations associated in with CMT2 are in the MFN2 gene (Zuchner et al., 2004) (Lawson et al., 2005) (Verhoeven et al., 2006). MFN2 is an outer mitochondrial membrane protein that has been shown to have a role in both mitochondrial fusion and transport (Chen et al., 2003) (Baloh et al., 2007). Sural nerve biopsies from CMT2A patients with identified mutations in MFN2 have demonstrated the presence of clustered mitochondrial with abnormal cristae structure in axons (Verhoeven et al., 2006). Though these data suggest that CMT2A results from an abnormality in mitochondrial function, the nature of this abnormality remains unclear. Most importantly, the reason why mutations in this ubiquitously expressed protein manifest as selective degeneration of long peripheral axons has not yet been demonstrated.

CMT2A animal models

Currently there are two transgenic animal models of CMT2A that have been reported. The first model reported utilized the Hb9 promoter, which expresses specifically in motor neurons at very high levels prenatally and declines to low levels at around three weeks of age, to overexpress the T105M MFN2 disease mutant (Detmer et al., 2008). These Hb9 transgenic animals developed a severe hindlimb gait defect, motor axon degeneration, motor neuron death and severe atrophy of the tibialis anterior muscle. Cultured motor neurons from the animals also demonstrated an abnormality in the distribution of axonal mitochondria, which were tightly clustered along the length of the axon. All of these features recapitulate to some extent the findings in human patients with CMT2A. However, the high levels of mutant protein expression early in development and the complete absence of expression following birth, suggests that the animals may suffer from a developmental syndrome rather than a neurodegenerative disease.

Importantly, the animals described in the study had to be bred to homozygosity to obtain a noticeable phenotype. This is an important point, since in human patients the gene dosage of normal to abnormal MFN2 is 1:1, however, the mice do not show an overt phenotypic consequence without significant overexpression of the mutant allele. In fact, a second transgenic

line created by the authors only expressed ~70% as much mutant protein as the first line and did not show any abnormalities even when bred to homozygosity.

An additional complication comes from the use of the HB9 promoter, which specifically expresses in motor neurons. The strong overexpression of a toxic MFN2 mutant may have caused degeneration of the motor neurons simply because it was only expressed in motor neurons. A toxic affect of the mutant in other cells types would have been missed in this animal model altogether. This is important to consider when interpreting its ability to explain the human disease where the mutant MFN2 allele is expressed throughout the body yet selectively affects motor and sensory axons.

A second CMT2A model was recently reported in which the neuron specific enolase promoter was used to drive expression of the R94Q mutant MFN2 allele (Cartoni et al.). These mice showed locomotor deficits on rotor rod tests and walked with abnormal posture where their body was kept low to the ground. These mice also showed a gene dosage affect as only the homozygous animals showed early signs of locomotor abnormalities. Despite their behavioral phenotype, neither axon degeneration nor signs of muscle atrophy were observed in these animals. Only a population shift toward smaller diameter axons with an apparent increase in the number of mitochondria in smaller diameter axons was seen in mutant transgenics. The difference between this line and the previously described ones may have been due to differences in gene dosage but unfortunately neither of these models offers an accurate recapitulation of the human disease.

An animal model of CMT2A will be extremely import both for studying mechanisms underlying axonal degeneration and testing therapeutic interventions aimed at preventing axonal degeneration. Though the two transgenic models thus far described offer some insight, an MFN2 knock-in animal (as described in Chapter 4) more closely models the genetic situation in humans, and would theoretically be the best model to understand the cell type specificity observed in CMT2A.

Mitochondrial disease: the role of mitochondrial dynamics and MFN2

Mitochondrial disorders are multisystem diseases that result from mutations in mitochondrial DNA (mtDNA) or nuclear genes that encode for mitochondrial proteins. Classical mitochondrial disorders are maternally inherited and affect proteins involved in mitochondrial respiration (Chan, 2006) (Dimauro and Davidzon, 2005). These diseases usually affect the brain and muscle presumably because energy demand is at a premium. MERRF (*m*yoclonic *e*pilepsy associated with *r*agged-*r*ed *f*ibers) and MELAS (*m*itochondrial *e*ncephalomyopathy, *l*actic *a*cidosis, and *s*troke-like episodes) syndromes are prime examples where defective mitochondrial respiration leads to neurological and myopathic symptoms. Interestingly, though both disorders stem from defective mitochondrial respiration, they are clinically distinct diseases. Patients with MERRF syndrome present with myoclonic seizures, ataxia, and muscle weakness while MELAS syndrome is predominated by stroke-like brain infarctions. This suggests that while the nervous system and musculature are sensitive to mitochondrial dysfunction in general, there is still an issue of selectivity depending on the exact cause of disease.

In addition to mutations in mtDNA that directly affect mitochondrial respiration, mutations in nuclear encoded mitochondrial genes can also cause disease. As discussed previously, CMT2A results from mutations in the MFN2 gene and selectively affects axons of peripheral motor and sensory axons. Though MFN2 does not directly participate in mitochondrial respiration, MFN2 regulates mitochondrial fusion and potentially plays a role in mitochondrial transport (Chen et al., 2003) (Eura et al., 2003) (Baloh et al., 2007). Haploinsufficiency or mutations in OPA-1, another regulator of mitochondrial fusion, also affects the nervous system resulting in dominant optic atrophy (DOA) (Delettre et al., 2000) (Alexander et al., 2000). Lastly, mutations in GDAP-1, a protein implicated in mitochondrial fission, cause CMT4A (Baxter et al., 2002) (Cuesta et al., 2002) (Nelis et al., 2002), a recessive axonal and demyleinating neuropathy.

The common theme emerging from these three diseases is the disruption of mitochondrial fusion and fission. To better understand how disruption of these processes cause disease, mitochondrial fusion and fission will be discussed below more in depth.

Molecular mechanisms of mitochondrial fusion and fission

In addition to undergoing frequent movement, mitochondria are constantly changing their shape. The bulk of mitochondrial mass in any given cell is divided into individual organelles that can fuse with each other or divide into smaller units. A balance of fusion and fission mechanisms controls the size and number of individual mitochondria. Fusion and fission regulate several important aspects of mitochondrial function: they control mitochondrial morphology, allow exchange of membrane and matrix constituents, and facilitate the induction of apoptosis by controlling release of proteins from the inter-mitochondrial membrane space (Detmer and Chan, 2007a). In mammals, the fusion machinery consists of two outer mitochondrial membrane (OMM) anchored GTPases, mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) and a single inner mitochondrial membrane (IMM) anchored GTPase, OPA1. Mfn1 and Mfn2 are thought to tether adjacent OMMs by forming homo or heterodimers and facilitate membrane fusion in a GTP dependent manner (Figure 1) (Koshiba et al., 2004)(Hales and Fuller, 1997) (Hermann et al., 1998; Santel and Fuller, 2001; Rojo et al., 2002; Eura et al., 2003; Santel et al., 2003). Following fusion of the OMMs, OPA1 then fuses the IMMs (Cipolat et al., 2004)(Song et al., 2009). Fission on the other hand is achieved by recruiting dynamin-related protein 1 (Drp1) from the cytoplasm to the OMM where it localizes in a punctate fashion with Fis1, an integral OMM protein that facilitates recruitment of Drp1 (Lee et al., 2004) (Smirnova et al., 2001). Fission is then thought to occur as molecules of Drp1 assemble into a ring and constrict the mitochondrial tubule into separate organelles.

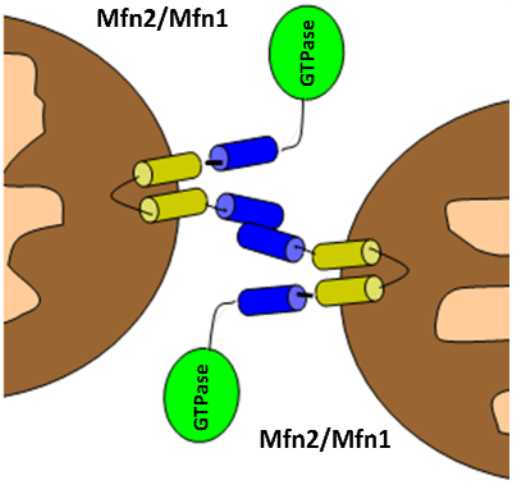

Figure 1. Representation of mitofusins tethering mitochondria before mediating fusion of the OMM.

Mitofusin 1 and 2 mediate docking and fusion of the outer mitochondrial membrane

Mfn1 and Mfn2 belong to the family of large dynamin-like GTPases. Defining structures of classical dynamins include a large GTPase domain and a self contained GTPase activating domain (GED) (Praefcke and McMahon, 2004). While the mitofusins retain the large GTPase domain, it is not clear from their structure or function that any part of the proteins acts as a GED domain. Instead, mitofusins have two coiled-coil domains separated by two transmembrane domains. Their topology is such that both the carboxy and amino terminal are oriented toward the cytoplasm with the two transmembrane domains situated in the OMM (Rojo et al., 2002). This topology is common to mitofusins and atlastins, which are also members of the large dynamin-like GTPase family and play a role in ER membrane fusion and tubulation (Figure 2) (Hu et al., 2009).

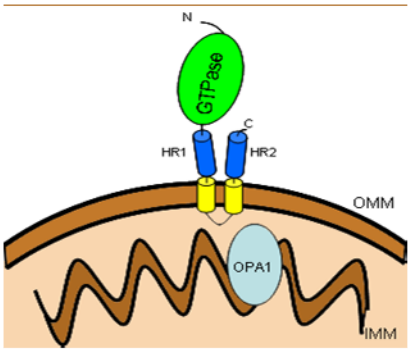

Figure 2. Common topology of Mfn2, Mfn1 and Atlastins with both C- and N-terminal oriented toward the cytoplasm.

The coiled-coil domains on Mfn1 and Mfn2 allow the molecules to form homo and hetero dimmers in cis or trans across two juxtaposed mitochondria (Koshiba et al., 2004). When two mitofusins interact in trans their second coiled-coil domains from and antiparallel coiled-coil structure and tether the two juxtaposed mitochondria together. This docking step is followed by fusion of the OMM membranes, the exact steps of which are still unclear, that is dependent on the GTPase activity of the mitofusins (Ishihara et al., 2004) (Hales and Fuller, 1997) (Hermann et al., 1998).

Both Mfn1 and Mfn2 are important to mitochondrial fusion. Mouse embryonic fibroblasts (MEFs) derived from Mfn1 or Mfn2 null animals, both show small fragmented mitochondria that are deficient in their ability to fuse (Chen et al., 2003). Notably the mitochondria also show abnormal patterns of movement but do not show a global collapse in mitochondrial membrane potential or oxidative respiration Chen, 2003 #372}(Chen et al., 2005). Mfn1 and Mfn2 null animals die before birth due to placental defects Chen, 2003 #372}. If Mfn2 is conditionally knocked out in all other tissues except the placenta, mfn2 null mice survive until 2 weeks after

birth. In contrast, if is conditionally knocked out in all other tissues except placenta, Mfn1 null mice survive into adulthood (Chen et al., 2007). It has thus become evident that Mfn1 and Mfn2 have some redundant but not completely overlapping functions. In support of this idea, one study has reported that the complete fusion of the IMM and OMM is dependent on the function of mfn1 and opa1 but not mfn2 (Cipolat et al., 2004). Mfn1 also displays and eight fold higher GTPase activity than mfn2 and more efficiently tethers mitochondria at a much higher efficiency than Mfn2 (Ishihara et al., 2004). Additionally, Mfn2 has been found on the ER membrane where it helps establish ER –mitochondrial contacts whereas mfn1 is only found on the OMM of mitochondria (de Brito and Scorrano, 2008).

Mitochondrial fusion and fission regulate mtDNA stability

mtDNA resides within the matrix of a mitochondrion as a structure called a nucleoid. A single mitochondrion can contain several nucleoids and hence several complete copies of the mitochondrial genome (Garrido et al., 2003). If one nucleoid contains an inherited or acquired mutation, the other copies can dilute out the effect of the mutation. Fusion and fission of mitochondria allows for the exchange of nucleoids and can help ameliorate the effects of a mutation (Yoneda et al., 1994)(Nakada et al., 2001). This was elegantly demonstrated in mice conditionally lacking both mitofusin 1 and 2 in muscle (Chen et al., 2010). Double knockout mice (mfn1 -/-; mfn2 -/-) had over a 10-fold reduction in the number of mtDNA copies present in their muscle, a 5-fold increase in mtDNA point mutations and a 14-fold increase in the number of mtDNA deletions by 7 weeks of age. Additionally, a synergistic lethality was demonstrated between the loss of mfn1 and a mutation in the mtDNA polymerase (making it more error prone). This experiment demonstrated that a decrease in mitochondrial fusion made mice less able to cope with mtDNA mutations.

The importance of mitochondrial fusion could then reasonably support the hypothesis that mutations in MFN2 could lead to a decrease in mitochondrial fusion that would then make patients more susceptible to spontaneously arising mutations in mtDNA. However, closer examination of MFN2 disease mutants and their affects on mitochondrial fusion along with a

possible role of MFN2 in mitochondrial transport has made the significance of mitochondrial fusion in CMT2A questionable, as discussed below.

The role of mitochondrial fusion in CMT2A

The role of mitochondrial fusion in the pathogenesis of CMT2A came into question with a closer investigation of the ability of CMT2A associated MFN2 mutants to mediate mitochondrial fusion. Out of the eight disease mutants tested, four (V69F, L76P, R274Q and W740S) were able to mediate mitochondrial fusion in mfn1 -/-; mfn2 -/- double knockout cells (Detmer and Chan, 2007b). While the other mutants tested (R94Q, R94W, T105M and P251A) could not mediate mitochondrial fusion alone, all mutants tested could be complemented by mfn1 and thus mediate fusion under normal circumstances. Though it is not certain how the fusion enabling mutants could lead to disease, it was suggested that mfn1 maybe be absent or expressed at low levels in motor and sensory neurons where mitochondrial fusion would then be disrupted in CMT2A patients.

Our lab began its study by expressing six different MFN2 mutants (three of which retained their ability to mediate mitochondrial fusion) in cultured rat DRG neurons and observed both their affect on mitochondrial morphology and movement (Baloh et al., 2007). The striking finding was that all seven mutants disrupted mitochondrial movement while high-level expression of wild type MFN2 had no effect. This was the first consistent attribute that all tested MFN2 mutants demonstrated. Also remarkable was the lack of effect on levels of ATP or oxidative phosphorylation (OXPHOS) when the mutants were expressed in 293T cells. This suggested that the effect of the mutants was centered on disrupting mitochondrial transport rather than function. Unfortunately this study did not address whether MFN2 was playing a direct role in mitochondrial transport, nor provide an explanation how it might be disrupting transport.

A new focus on mitochondrial transport

Functional mitochondria are essential to cells, which rely on the organelles to produce ATP, buffer cytosolic Ca^{2+}, and orchestrate apoptosis. Crucial to the integrity of mitochondria is their

ability to undergo fusion and fission with one another to assure the integrity of their DNA. Though the association between mutations in MFN2 and CMT2A suggest a connection between mitochondrial fusion and disease, the data from our first investigation suggests that disruption of mitochondrial transport may actually be the central phenomenon explaining the degeneration of axons.

Mitochondrial transport in axons

Mitochondria have evolved to mediate a multiplicity of functions including oxidative phosphorylation, Ca^{2+} sequestration, and initiation of apoptosis. Integral to their successful functioning is their proper distribution to subcellular domains where they are required. In peripheral neurons, mitochondria are recruited to areas of high-energy demand such as active growth cones (Morris and Hollenbeck, 1993), the distal region of the initial segment (Li et al., 2004), Nodes of Ranvier (Berthold et al., 1993), and presynaptic boutons (Misgeld et al., 2007) where they may replenish ATP levels or buffer calcium. Presumably, interfering with the proper distribution of axonal mitochondria would hamper their ability to meet the needs of the intracellular environment and compromise axon viability. Indeed, several neurodegenerative disorders have been associated with abnormal mitochondrial transport (Chang and Reynolds, 2006) marking the importance of understanding this process.

The transport and distribution of mitochondria are regulated processes that bring mitochondria from the soma, where the majority of mitochondrial biogenesis is thought to occur (Amiri and Hollenbeck, 2008), across great spans of axon, up to a meter in peripheral sensory and motor neurons. Unlike other transported organelles, mitochondria move in a salutatory and bidirectional manner constantly pausing or reversing their direction of travel (Hollenbeck and Saxton, 2005). This dynamic motion presumably results from mitochondria responding to intracellular or extracellular cues. In neurons, both increased levels of ADP and cytosolic Ca^{2+} act as intracellular signals to modulate mitochondrial mobility, while NGF can affect mitochondrial trafficking from signaling outside of the cell (Mironov, 2007) (Rintoul et al., 2003) (Chada and Hollenbeck, 2003) (Chada and Hollenbeck, 2004). Though a diversity of signals may

simultaneously modulate mitochondrial transport, their affects most likely converge on common targets, the motor proteins and their adaptors that associate with mitochondria.

Motor proteins mediating transport

Axonal mitochondria move along both microtubule and microfilament tracks; fast mitochondrial movements are microtubule based while slow transport is dependent on microfilaments (Morris and Hollenbeck, 1995). Mitochondria may also remain stationary while docked to cytoskeletal elements to maintain their proper distribution (Hollenbeck and Saxton, 2005)(Kang et al., 2008). Movement along microtubule tracks is achieved by associating with kinesin or dynein motors. The stereotypic arrangement of microtubules in axons, with the plus ends orientated toward the growth cone and negative ends toward the soma, dictates that all kinesin based movement is anterograde and all dynein based movement is retrograde. Out of the six kinesin families that mediate axonal organelle transport only two have been associated with mitochondria in mammals: kinesin-1 and kinesin-3. The kinesin-1 family member Kif1B associates with both mitochondria and other transported organelles (Hollenbeck and Saxton, 2005) while the kinesin-3 family member Kif5C specifically associated with mitochondria in neurons (Nangaku et al., 1994). In mammals there are only three dynein heavy chains that are expressed in the cytoplasm (Vaisberg et al., 1996) which are thought to regulate retrograde movement of mitochondria. Interestingly, dynein mediated retrograde axonal transport seems to also depend on the presence of functional kinesin-1. Perfusing squid axoplasm with kinesin-1 antibodies produces a marked decrease in organelle transport in both anterograde and retrograde directions (Brady et al., 1990) while in Drosophila, mutations in kinesin-1 result in decreased retrograde transport in axons (Pilling et al., 2006). These observations point to an interdependent relationship between anterograde and retrograde transport mechanisms though no specific molecular complexes of kinesin and dynein heavy chains have been identified (Hollenbeck and Saxton, 2005). This phenomenon may however, be a due to the disrupted function of adaptor proteins that link cytoskeletal motors to the mitochondrial membrane and presumably regulate their function.

Mitochondrial motor protein adaptors

In mammals, there are four known adaptor proteins that link mitochondria to kinesin proteins: GRIF1, OIP106, syntabulin and kinesin binding protein (KBP) (Frederick and Shaw, 2007). GRIF1 and OIP106 are both coiled-coil domain proteins that are known homologues of the *Drosophila* Milton protein (Brickley et al., 2005). When the single *Drosophila* Milton homologue is knocked out in photoreceptors, aberrant synaptic transmission and a striking absence of mitochondria in synaptic terminals and axons is observed (Stowers et al., 2002). These findings led the further characterization of Milton as a mitochondrial associated protein that bound the *Drosophila* kinesin heavy chain (KHC). It was then assumed that Milton was necessary for anterograde transport of mitochondria out of neuronal bodies but the nature of its mitochondrial localization was still a mystery, as Milton's protein structure does not suggest it is an integral outer mitochondrial membrane (OMM) protein. Later work demonstrated that Milton interacts with the integral OMM protein dMiro and that this interaction could affect the localization of Milton to mitochondria (Figure 3) (Glater et al., 2006). To date, dMiro is the only known "receptor" for a mitochondrially localized motor protein adaptor, though other unidentified receptors are postulated to exist for syntabulin and KBP (Frederick and Shaw, 2007). Because some of the adaptor proteins such GRIF1 and OIP106 have been shown to also link other organelles to motor proteins, their specific roles in mitochondrial transport is likely regulated by dMiro which is only localized to mitochondria

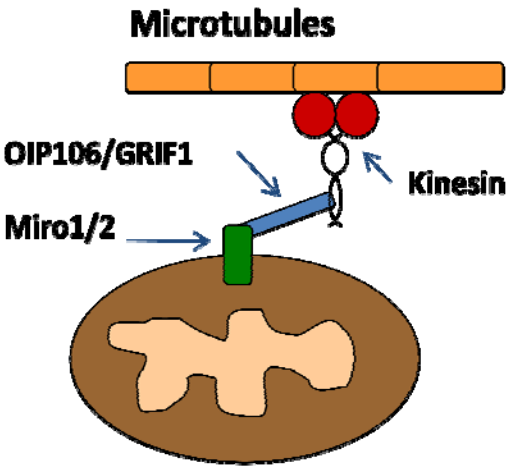

Figure 3. Schematic representation of the mitochondrial transport apparatus illustrating the indirect connection of mitochondria to kinesin.

The mitochondrial Miro GTPase proteins

The Miro gene is highly conserved with isoforms present from yeast up to humans. While single cell organisms and Drosophila only contain a single Miro gene, two separate but highly homologues isoforms of Miro exist in mammals. These two genes both encode integral outer mitochondrial membrane (OMM) proteins that are prominently expressed in the mammalian nervous system. Both Miro isofroms contain classical domains associated with signaling processes: a Rho GTPase related domain, a Rab-like GTPase domain and two calcium binding EF hand domains (Fransson et al., 2003; Fransson et al., 2006). Both Miro isoforms are anchored to the OMM by a C-terminal transmembrane domain proceeded by a short tail in the inter-mitochondrial membrane space (Figure 3). The juxtaposition of these evolutionarily conserved motifs portends a unique regulatory function for these mitochondrial proteins.

Miro proteins and axonal transport of mitochondria

In developing *Drosophila* larvae, absence of the dMiro allele results in abnormal locomotion and death during early larval stages. Other alterations in these animals include conspicuous loss of mitochondria in axons, dendrites, presynaptic terminals and NMJs along with an abnormal organization of mitochondria in neuronal cell bodies and muscle (Guo et al., 2005). Presynaptic boutons were also structurally abnormal displaying a reduced volume and appearing in aggregated “cauliflower-like” bundles. While microtubules normally extend into presynaptic boutons as easily identifiable loops, Miro mutant boutons lacked the same number of loops and often the loops failed to extend into the last bouton of an axonal branch. Despite the lack of mitochondria at NMJ’s in the knockout flies, trafficking of neurotransmitter vesicles and baseline neurotransmission appeared unperturbed. However in response to prolonged bouts of stimulation, Ca^{2+} buffering and neurotransmitter release were greatly hampered indicating that the presence of mitochondria was necessary to access reserve stores of neurotransmitter vesicles. Interestingly, reintroduction of Miro protein solely into the nervous system but not muscle rescued the knockout animals stressing the critical importance of Miro function in neurons (Guo et al., 2005).

In contrast to the loss of mitochondria in distal axons of the Miro knockout fly, overexpression of Miro in vivo led to in an increased accumulation of mitochondria in distal synaptic buttons suggesting that the protein promoted anterograde transport of mitochondria in axons (Guo et al., 2005). The connection between Miro and anterograde transport was later solidified with the identification of its binding partner Milton (Glater et al., 2006). In mammals, Miro1 and Miro2 both associate with Grif1 and OIP106, the two known Milton homologues (Fransson et al., 2006).

Disruption of mitochondrial transport is conceptually similar to directly decreasing mitochondrial function. Perhaps another measure to consider would be "effective" mitochondrial function, which would encompass both the intrinsic ability of mitochondria to generate ATP and buffer Ca^{2+} as well as their ability to effectively complete these tasks in the cell. Disrupting mitochondrial transport and preventing proper localization of mitochondria to areas of high energy and Ca^{2+} flux would therefore decrease effective mitochondrial function. Other insults that negatively affect mitochondria, such as hypoxia or mitochondrial toxins, are known to incite axonal degeneration. We hypothesize that disruption of mitochondrial transport by MFN2 disease mutants will affect axons in a similar manner to directly inhibiting mitochondrial function and lead to the degeneration of axons.

Mechanisms of axonal degeneration

Anoxia/ischemia models of axonal degeneration

The nervous system accounts for a large percentage of the body's total energy consumption. The conduction of action potentials along nerves and white matter tracks is costly, requiring energy to pump ions across the plasma membrane and establish the resting membrane potential. Despite the high demands of action potential propagation, neurons have low intrinsic energy reservoirs and require a continuous supply of oxygen and glucose. Accordingly, within minutes of onset, anoxia or hypoxia in optic nerve causes conduction block and depolarization of the resting membrane potential (Stys et al., 1990)(Malek et al., 2005). Peripheral nerves on the other hand are more resistant to anoxia or hypoxia and can remain excitable for up to 60 minutes before

succumbing to conduction failure (Kugelberg, 1944)(Waxman et al., 1995). An elegant demonstration of this phenomenon was performed with explants of dorsal spinal roots and slices of the dorsal column which contain the peripheral and central axon processes of dorsal root ganglion cells respectively. Though both the peripheral and central processes extended from the same population of cells, the peripheral axons were able to tolerate the complete replacement of O_2 with N_2 in their bath solution while the central processes were unable to conduct action potentials or maintain their resting membrane potential within minutes of hypoxia. Because the axons originate from the same cell, the difference in hypoxia tolerance is likely attributable to differences in glia or extracellular matrix surrounding the processes rather than an intrinsic difference in the metabolisms of the axons (Utzschneider et al., 1991).

In either case, disruption of energy production in axons of the CNS or PNS leads to an accumulation of axoplasmic Na^+ and Ca^{2+} and an efflux of K^+, which instigates the depolarization of the resting membrane potential and subsequent conduction block. The proposed series of events leading to the ionic dysregulation involve 1) failure of the Na^+-K^+ pump from lack of ATP 2) Na^+ accumulation through persistent Na^+ channels and K^+ efflux 3) reversal of the Na^+- Ca^{2+} driven by high axoplasmic Na^+ and consequent accumulation of intracellular Ca^{2+} (Trapp and Nave, 2008). Though influx of extracellular Ca^{2+} plays an important role in accumulation of intra-axonal Ca^{2+}, intracellular stores also play a role. In response to prolonged anoxia, Ca^{2+} can be released from ryanodine and IP_3 sensitive channel on the axoplasmic reticulum and from mitochondria through the mitochondrial Na^+- Ca^{2+} exchanger (Nikolaeva et al., 2005) (Ouardouz et al., 2003).

An abrupt increase in cytoplasmic Ca^{2+} plays a definitive role in the induction of axon degeneration. In response to increased Ca^{2+} levels, activated phosphatases dephosphorylate neurofilaments, destabilizing the intermediate filament network (Strack et al., 1997). Calcium responsive calpains also act to degrade neurofilaments as well as spectrin and other structural elements of the axon (Stys and Jiang, 2002). In addition to changes in neurofilment, fast axonal transport, which is mediated by the microtubule network, also becomes compromised. Accumulation of APP and other cargo of the fast axonal transport system are routinely used to

identify dystrophic axons in a variety of diseases (Ferguson et al., 1997; Sheetz et al., 1998; Terwel et al., 2002). But perhaps one of the most significant consequences of increase axonal Ca^{2+} is the activation of nitric oxide synthase (NOS) and the production of NO. In addition to modifying ion channels, transporters and glycolytic enzymes, NO directly inhibits mitochondrial respiration (Pacher et al., 2007). The energy deficit augments the ionic imbalance by depleting Na^{+}-K^{+} function and propagating further influx of Na^{+}, reversal of the Na^{+}-Ca^{2+} exchanger and influx of Ca^{2+}. In this way, a severe ionic imbalance in the axon will create a positive feedback loop by inhibiting any mitochondrial energy production instigating the described chain of events.

Though anoxia/hypoxia induced axonal degeneration has been extensively studied in the optic nerve, work in peripheral nerves suggests that a similar chain of events occurs. In fact the described scheme can be applied to axonal degeneration in general. Whatever the inciting insult, a severe ionic imbalance or energy deficit will propagate itself through a vicious cycle.

Demyelination, energy imbalance and axonal degeneration

The initial symptoms of MS are precipitated by inflammatory demyelination in CNS with matter. Even in these early stages, axon loss is visible. As the disease progresses, axonal degeneration becomes the predominate cause of symptoms and permanent disability. Studies into the mechanisms of axon loss in MS have elucidated a fascinating connection to axonal mitochondria suggesting that models of axonal degeneration constructed from studies of anoxia/hypoxia may also apply to MS (Trapp and Nave, 2008).

Demyleination of axons results in the rearrangement of axolemmal domains, redistributing voltage gated Na^{+} channels and Na^{+}/K^{+} pumps to denuded axon segments where energy and Ca^{2+} flux consequently increase (Arroyo et al., 2002; Devaux and Scherer, 2005). Axons stripped of myelin are essentially in the same predicament as unmyelinated nerve fibers, which deal with high-energy demands by supplying axons with greater numbers of mitochondria. Interestingly, axons in different models of CNS demyelination employ the same technique and recruit additional mitochondria to demyelinated segments (Andrews et al., 2006; Hogan et al., 2009; Witte et al., 2009). As levels of ADP and Ca^{2+} both influence mitochondrial transport

(Mironov, 2007; MacAskill et al., 2009; Wang and Schwarz, 2009), both energy demands and ionic imbalances can act as recruiting signals. Though it has not been directly tested, the failure of mitochondria to redistribute in response to these demands should compromise the axon.

Another recognized insult in the early phases of MS is the production of NO from invading immune cells (Smith and Lassmann, 2002). Increased levels of NO can directly inhibit mitochondrial respiration and compromise local supplies of ATP (Brown et al., 1995; Pacher et al., 2007). Application of NO to myelinated axons was demonstrated to induce conduction block and structural damage with high frequency electrical stimulation (Redford et al., 1997; Smith et al., 2001). These outcomes are the same seen in anoxia/hypoxia experiments and suggest that NO induces a state of "virtual hypoxia" in the axon. Exposure to NO also results in the same ionic imbalances observed in anoxia/hypoxia and its affects can be ameliorated with pharmacological blockade of Na^+ channels (Kapoor et al., 2003).

Exposure of axons to NO also occurs in Guillan-Barre syndrome (GBS), where inflammatory demyelination of peripheral axons induce a similar cascade of events to that seen in MS. Experimental exposure of peripheral nerves to NO results in conduction block, intra-axonal accumulation of Na^+ and Ca^{2+}, and inhibition of mitochondrial respiration (Kapoor et al., 2003). In addition to causing reversible conduction block, a brief exposure of sciatic nerve to NO also produces some degree of Wallerian degeneration distal to the site of administration. The same effects are seen with a brief administration of DNP, an uncoupler of the IMM (Alvarez et al., 2008). The striking conclusion is that a transient deficit in mitochondrial metabolism is sufficient to trigger axonal degeneration.

In summary, mitochondrial metabolism is essential to the integrity of axons. The ATP provided by mitochondria is necessary for the function of the Na^+-K^+ pump and the regulation of intra-axonal ion levels. Failure of mitochondrial metabolism leads to a viscous cycle of events that perpetuates an increase in axonal Ca^{2+} levels and a decrease in levels of ATP. Importantly mitochondrial metabolism can fail in various ways: mitochondrial respiration can be directly affected by a toxin or signaling intermediate like NO, or mitochondria could fail to accumulate in sufficient numbers to areas of high metabolic demand. The fact that even a brief inhibition of

mitochondrial function can trigger axonal degeneration stresses the importance of actively distributing mitochondria to areas of demand. Taken together with our initial observations in MFN2 mutant expressing neurons, a logical path from disrupted mitochondrial transport to axonal degeneration may now help to explain the loss of axons in CMT2A.

Thesis organization

This thesis describes evidence for a direct role of Mfn2 in the regulation of mitochondrial transport and the role of mitochondrial transport in supporting axon integrity. At the beginning of the study we already had data suggesting that CMT2A associated Mfn2 mutants disrupted mitochondrial transport, but we did not know if Mfn2 was directly involved in transport nor whether the disrupted transport could explain the axonal degeneration seen in CMT2A.

Chapter one describes data suggesting that Mfn2 and Mfn1 are directly involved in mediating mitochondrial transport. We show that transport of axonal mitochondria is disrupted in Mfn2 null mice and that Mfn2 and Mfn1 interact with the Miro/Milton/kinesin complex. We also provide evidence that disrupted mitochondrial fusion is not sufficient to disrupt mitochondrial transport indicating that the mitofusins play a role in mediating transport distinct from their function in mitochondrial fusion.

Chapter 2 describes evidence that CMT2A associated Mfn2 disease mutants cause axonal degeneration by disrupting mitochondrial transport and that inhibiting mitochondrial fusion alone is not sufficient to incite axonal degeneration. We also show that abnormal transport prevents mitochondria from uniformly distributing across axons, leaving segments devoid of mitochondria. We propose that the abnormal distribution of mitochondria coupled with their inability to redistribute to areas in need of ATP or Ca^{2+} buffering leads to the axon degeneration observed in CMT2A.

Chapter 3 describes the characterization of R94Q heterozygous knock-in mice. We believed that these mice would most accurately reflect the genetic condition in CMT2A patients and provide more insight than previously reported models. We found that the heterozygous

animals were unremarkable up to 6 months in age and did not display an emergent phenotype when challenged metabolically or when challenged with genetically induced demyelination.

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Chapter 2

Mitofusin 2 is necessary for transport of axonal mitochondria and interacts with the miro/milton complex

Introduction

As principal mediators of ATP production and calcium buffering, mitochondria actively distribute to areas of high energy demand and calcium flux (Berthold et al., 1993; Li et al., 2004; Misgeld et al., 2007). Based on this principle, disruption of axonal transport has long been proposed as a potential mechanism in human neurodegenerative diseases (Duncan and Goldstein, 2006; De Vos et al., 2008).

Mitochondrial transport is regulated by a series of molecular adaptors which mediate the attachment of mitochondria to molecular motors (Hollenbeck and Saxton, 2005; Kang et al., 2008; Li et al., 2009). In Drosophila, connection of mitochondria to kinesin motors involves the outer mitochondrial membrane (OMM) protein dMiro, which indirectly attaches to kinesin heavy chain via the adaptor protein Milton (Guo et al., 2005; Glater et al., 2006). In mammals, two isoforms of Miro (Miro1 and Miro2) and Milton (OIP106 and GRIF1) have been identified and are proposed to act in a similar manner (Fransson et al., 2006).

In addition to constant movement, mitochondria also undergo frequent fission and fusion events (Chan, 2006). The mitochondrial fusion apparatus consists primarily of Mitofusin 1 and Mitofusin 2 (Mfn1 and Mfn2) located in the OMM, and Opa1 located on the inner mitochondrial membrane (IMM) (Rojo et al., 2002; Chen et al., 2003; Eura et al., 2003; Wong et al., 2003; Cipolat et al., 2004). Both the mitofusins and Opa1 are dynamin family GTPases, and are believed to tether opposing membranes and facilitate their fusion. Though the precise reason for mitochondrial fusion remains unclear, interruption of mitochondrial fusion (via loss of Opa1 or

Mfn1/2) leads to loss of mitochondrial potential and oxidative phosphorylation indicating it is critical for maintaining mitochondrial function (Olichon et al., 2003; Chen et al., 2005).

Dominantly inherited point mutations in MFN2 are the most commonly identified cause of axonal Charcot-Marie-Tooth disease (designated CMT2A), however it is still not clear how mutations in this ubiquitously expressed protein lead to relatively selective degeneration of long peripheral axons (Zuchner et al., 2004; Lawson et al., 2005; Verhoeven et al., 2006). Mutations in OPA1 disrupt mitochondrial fusion and lead to degeneration of optic nerve axons, referred to as dominant optic atrophy (Votruba et al., 2003; Newman, 2005). In some cases, CMT2A mutant forms of MFN2 are unable to mediate mitochondrial fusion in fibroblasts, whereas others are completely competent to promote fusion (Detmer and Chan, 2007). Interestingly, exogenous expression of disease mutant forms of Mfn2 in cultured sensory neurons leads to a profound abnormality mitochondrial transport (Baloh et al., 2007), and mice overexpressing disease mutant MFN2 in motor neurons show markedly abnormal axonal mitochondrial distribution (Detmer et al., 2008). These data support that Mfn2 influences mitochondrial transport, however the molecular basis of this effect has not been defined, and it has only been observed in the context of Mfn2 overexpression.

Here we show that loss of Mfn2 profoundly and selectively disrupts axonal mitochondrial transport, and that Mfn2 directly interacts with OMM proteins and adaptors involved in mitochondrial transport. Furthermore we show that disrupting mitochondrial fusion via OPA1 knockdown does not itself disrupt axonal mitochondrial transport. These data indicate that Mfn2 plays an integral role in the regulation of mitochondrial transport, and have important implications for understanding the pathophysiology of CMT2A and dominant optic atrophy.

Materials and Methods

Plasmids and silencing RNAs*:* Human wild type MFN2 and MFN2 disease mutant constructs were previously described (Baloh et al., 2007). For siRNA constructs targeting sequences were generated using the Dharmicon siDesign Center and cloned into the FSP-i construct as previously described (Araki et al., 2004). Silencing was confirmed via either western blotting or quantitative PCR. Myc-tagged Miro1 and Miro2 constructs were a gift from Dr. Pontus Aspenström, and were subcloned into a lentiviral expression vector. For Mfn1, GRIF1 and OIP106 constructs cDNAs were obtained from Openbiosystems (Huntsville, Alabama), and amino terminal tags (His-Mfn1; Flag-GRIF1 and Flag-OIP106) were inserted via PCR mutagenesis and sequenced fully.

Production of Mfn2 knockout mice*:* A targeting construct which inserted the full length human MFN2 cDNA with the H361Y mutation and an HA tag, as well as a PGK-neo selection cassette into the first coding exon of mouse Mfn2 was electroporated into mouse ES cells. Clones were screened for homologous recombination via southern blotting, a properly targeted clone was injected into blastocysts to generate chimeras, and germline transmission confirmed. Due to either the PGK promoter or the removal of other endogenous mouse sequences, we found that the chimeric mRNA transcript was barely detectable, and no HA tagged protein was expressed from the targeted allele. Living homozygous $Mfn2^{H361Y/H361Y}$ mice were not observed from $Mfn2^{H361Y/+}$ x $Mfn2^{H361Y/+}$ breedings. However, timed pregnancies of $Mfn2^{H361Y/+}$ x $Mfn2^{H361Y/+}$ mice showed E12.5 embryos at 25%, indicating that they die between this time and birth, slightly later than a previously characterized Mfn2 null line (Chen et al., 2003). Immunoblotting of nervous tissue from $Mfn2^{H361Y/H361Y}$ embryos showed that no Mfn2 protein was being generated, indicating these mice represent a complete knockout of Mfn2 protein, and hence we refer to them as $Mfn2^{-/-}$ in this manuscript.

DRG cultures. Dorsal root ganglion cultures were performed from embryonic rats or mice on gestational day 15.5 or 12.5 respectively. Dorsal root ganglia were dissected out, dissociated using trypsin/EDTA and plated directly onto 24-well plates coated with laminin and poly-D-lysine.

Cultures were maintained in Neurobasal medium with B27 supplement (Invitrogen, Carlsbad, CA), 50 ng/ml NGF, 5 mM glutamine, and antibiotics. Addition of 5-fluorouracil for the first 4 days in culture was used as an antimitotic treatment.

Lentivirus production and infection. Lentiviruses were produced as described previously (Baloh et al., 2007). Briefly, HEK 293T cells were plated onto six-well plates and transfected using Mirus reagent (Mirus, Madison, WI) with a packaging vector (Δ8.91), envelope vector (vesicular stomatitis virus-glycoprotein), and transfer vector encoding the gene to be expressed. Media was changed once at 12hr and collected at 24 and 48hrs, pooled and applied directly to DRG cultures resulting in >95% infection efficiency.

Transfection of DRG cultures. To label mitochondria in a small subset of neurons within a culture, Liptofectamine 2000 (Invitrogen, Carlsbad, CA) was used according to the manufacturer's specifications to introduce a mitochondrially targeted DsRed. Briefly, 0.4ug of plasmid DNA was diluted in 25uL of OPTI-MEM and combined with 0.5uL of transfection reagent diluted in 25uL. The mixture was incubated for 20 minutes and added to cells. Media was changed 6hrs later to remove the transfection reagent.

Measurement of mitochondrial transport. For imaging of rat DRG neurons expressing Mfn2 wild type or disease constructs or silencing RNA constructs, cultures were infected at 4 days in vitro (DIV4) and subsequently transfected with mitochondrial marker on DIV7. Imaging mitochondrial movements was possible on DIV10-13. For mouse DRG neurons from Mfn2-/- mice, infections were performed on DIV4 and infected again with mitochondrial marker on DIV7. Imaging was then performed on DIV 10-13. All imaging was performed in a climate controlled chamber (In Vivo Scientific) at 37°C and 5% CO2 and images acquired with a Cool Snap HQ^2 CCD camera (Photometrics, Ottobrunn, Germany) mounted on a Nikon Eclipse Ti-U microscope. Images were aquired at 40x magnification every 1 second for 5 minutes. Kymographs were then generated and analyzed using MetaMorph Software (Molecular Devices, Sunnyvale, CA). Mitochondria were classified as either moving or stationary based on whether they achieved a displacement >2μM. Constant velocity movement segments identified on kymographs were measured for mitochondria

that were classified as moving. Measurements were pooled and the amount of time that mitochondria spent moving at a given velocities were binned and represented on a histogram. A velocity cut off of 0.1 μM/second was established and all movements below this velocity were treated as time spent paused. Pauses in movement segments were measured between consecutive anterograde or retrograde movements.

Coimmunoprecipitation. HEK293T cells were transfected using Mirus with constructs containing His-Mfn1, EGFP-Mfn2, myc-Miro1, myc-Miro2, flag-GRIF1, flag-OIP106 or Bluescript plasmid as a vector control. Cells were lysed in 1% Triton X-100, 5 mM EDTA, 300 mM NaCl, and 50 mM Tris-HCl, pH 7.5 (Glater et al., 2006) with complete protease inhibitor cocktail (Roche, Basel, Switzerland). Cells were lysed at 4°C for 30min, clarified by spinning at 10,000*g* and protein concentration determined using the BCA protein assay (Thermo Scientific, Rockford, IL). Equal amounts of protein from each lysate were raised to a final volume of 500μL, precleared with protein A sepharose beads (Invitrogen, Carlsbad, CA), incubated with 1μL anti-myc (Cell Signaling, Danvers, MA) or anti-flag (Sigma, St. Louis, MO) antibody for 1hr at RT and then incubated over night at 4°C with protein A sepharose beads. Beads were washed three times with lysis buffer and boiled in laemmli buffer before separation by SDS-PAGE.

Results

CMT2A associated MFN2 mutants disrupt both anterograde and retrograde mitochondrial transport, but do not alter transport of other organelles

Previous studies indicate that CMT2A associated MFN2 mutants produce a marked decrease in overall mitochondrial mobility in axons of cultured sensory neurons (Baloh et al., 2007), and alter mitochondrial distribution in motor axons of transgenic mice (Detmer et al., 2008). To clearly define the abnormality in mitochondrial transport in CMT2A expressing dorsal root ganglion (DRG) neurons, we introduced wild-type (wtMFN2) or mutant MFN2 constructs using lentivirus (>99% infection), followed by transfection with a mitochondrial targeted RFP which labels only a small number of neurons, allowing precise analysis of anterograde and retrograde movements in single axons. In wtMFN2 expressing cells, kymograph analysis of mitochondrial movements consistently depicted fast persistent movements in both anterograde and retrograde directions, accompanied by slower moving and stationary mitochondria. By contrast CMT2A disease mutant MFN2 (R94Q) expressing neurons showed a striking absence of the fast persistent movements, with the amount of time spent paused between anterograde and retrograde movements significantly greater in mutant expressing neurons than in wtMFN2 expressing controls (Fig.2-1A,B). Additionally, mitochondria from mutant expressing neurons moved at slower velocities in the anterograde and retrograde directions (Fig.2-1C). These findings indicate that mitochondria in MFN2 mutant expressing cells were unable to either initiate or sustain fast processive movements, supporting a disruption of microtubule based mitochondrial transport.

To determine whether this was a direct effect on mitochondrial mobility or a global disruption of all organellar transport, we performed sequential timelapse imaging of mitochondria and either endosomes or peroxisomes in single axons of DRG neurons expressing wtMFN2 or MFN2 disease mutants (R94Q and H361Y) (Fig. 2-1D-J). Axons expressing wild-type or mutant MFN2 were co-infected with a mito-RFP mitochondrial marker (Fig 2-1D) and a RhoB-GFP endosomal marker (Fig. 2-1E), and timelapse imaging with kymograph analysis was performed on endosomes and mitochondria. Despite the marked abnormality in mitochondrial transport

observed in mutant Mfn2 expressing axons (Fig. 2-1D,H), endosomes imaged in the same axons showed normal movement (Fig. 2-1E,I). Similarly, imaging of peroxisomes (visualized with a GFP-SKL fusion protein) showed normal movement in both wild-type and mutant MFN2 expressing axons (Fig. 2-1F,G,J). These observations indicate that Mfn2 disease mutants specifically alter mitochondrial transport, and do not globally disrupt organellar transport via alterations in microtubules or intra-axonal signaling pathways.

Mfn2 is necessary for the proper transport of axonal mitochondria

The specific disruption of mitochondrial transport by MFN2 disease mutants suggests that MFN2 may directly regulate microtubule-based mitochondrial transport. To determine whether MFN2 is necessary for mitochondrial transport, we cultured DRG neurons from Mfn2 knockout embryos. Lentivirus infection of DRG neurons with mito-RFP showed a marked delay in the migration of mitochondria out into axons of $Mfn2^{-/-}$ neurons compared to DRG neurons from wild type littermates (Fig. 2-2A,B). As expected, mitochondria from $Mfn2^{-/-}$ axons were smaller than controls, consistent with a mitochondrial fusion defect (Fig. 2-2D). Live imaging and kymograph analysis revealed abnormal mitochondrial movement patterns in $Mfn2^{-/-}$ neurons that mirrored the movement abnormalities seen in MFN2 disease mutant expressing neurons (Fig. 2-2A,B kymographs). This effect was again specific to mitochondrial transport as peroxisomal transport was normal in these neurons. Reintroduction of wild type Mfn2 by lentiviral infection into $Mfn2^{-/-}$ cultures fully rescued both the delay in migration and the abnormal mitochondrial movement patterns (Fig. 2-2C and kymograph). Similar to Mfn2 disease mutant expressing neurons, mitochondria in $Mfn2^{-/-}$ neurons spent a greater amount of time paused between anterograde and retrograde movements (Fig. 2-2E,F), and more time at slower velocities in both anterograde and retrograde directions than in controls (Fig. 2-2G).

Interestingly, overexpression of Mfn1 also restored mitochondrial pause time and velocity distributions to normal levels (Fig. 2-2E,F). Mfn2 and Mfn1 are highly homologous and are known

to have overlapping functions in mediating mitochondrial fusion (Chen et al., 2003). Analysis of Mfn1 and Mfn2 expression in embryonic fibroblasts and DRG neurons showed that both Mfn1 and Mfn2 are expressed in DRG neurons, though Mfn2 is higher in neurons than in fibroblasts. Therefore although both are able to mediate mitochondrial transport, the severe transport abnormalities in $Mfn2^{-/-}$ DRG neurons suggests that Mfn1 levels in these cells are not sufficient to compensate for the loss of Mfn2.

These findings indicate that Mfn2 is necessary for proper mitochondrial transport, and indicates that CMT2A associated Mfn2 mutants disrupt this function in a dominant negative fashion. The increased pause time of mitochondria observed in $Mfn2^{-/-}$axons suggests that Mfn2 may either directly promote mitochondrial attachment to microtubules, or maintain the processivity of the microtubule motors kinesin and dynein.

Mfn2 and Mfn1 interact with components of the mitochondrial transport apparatus.

The findings that loss of Mfn2 or expression of CMT2A disease mutants leads to a selective disruption of fast processive mitochondrial movements suggest that mitofusins could play a direct role in the regulation of microtubule based mitochondrial transport. We therefore investigated whether MFN2 could interact with molecules that link mitochondria to microtubule-based transport motors. Miro1 and Miro2 are OMM Rho-like GTPases that link mitochondria to kinesin, and are essential for proper mitochondrial transport in Drosophila (Fransson et al., 2006; Glater et al., 2006; Macaskill et al., 2009b; Russo et al., 2009). Co-immunoprecipitation experiments demonstrated that Mfn2 and Mfn1 are capable of interacting with either Miro1 or Miro2 when exogenously expressed in HEK293T cells, with an apparently stronger interaction observed between Mfn2:Miro2 (Fig. 2-3A,B). We next examined OIP106 and GRIF, homologues of the Drosophila Milton protein that interacts with both Miro proteins and kinesin (Fransson et al., 2006). We found that Mfn2 and Mfn1 also coimmuniprecipitated with OIP106 and GRIF1 (Fig. 2-3C-F). Finally we investigated whether the mitofusins directly interact with kinesin. Because

Kif5C, a conventional kinesin-1 superfamily member, is highly expressed in neurons we used exogenously expressed Kif5C for our coimmunoprecipitation experiments but found that neither Mfn2 nor Mfn1 directly interacted with Kif5C (Fig. 2-3G,H). Similarly no interaction between Mfn2 and kinesin light chain was observed (not shown). These experiments demonstrate that Mfn2 and Mfn1 interact with components of the mitochondrial molecular transport apparatus, providing an explanation for how loss of Mfn2 disrupts mitochondrial transport. Of note, the CMT2A associated MFN2 mutants (R94Q and H361Y) maintained the ability to interact with Miro1/2 and OIP106/GRIF1, and both Miro1 and Miro2 coimmunoprecipitated with OIP106 and GRIF1 in the presence of MFN2-R94Q or MFN2-H361Y (not shown). Therefore the disruption of mitochondrial transport observed with disease mutant Mfn2 expression is likely through altered regulation of the kinesin transport apparatus (processivity, attachment to microtubules) rather than simple disruption of Mfn:Miro:OIP106 complex formation.

Knockdown of Miro2 produces a mitochondrial transport abnormality similar to loss of Mfn2

Drosophila Miro is required for both anterograde and retrograde transport of axonal mitochondria (Guo et al., 2005; Russo et al., 2009). Although the mammalian Miro proteins interact with Milton homologues (Fransson et al., 2006), the effect of loss of Miro proteins on axonal mitochondrial transport in mammalian cells has not been examined. We used lentiviral delivered siRNAs to knock down Miro1 or Miro2 in sensory neurons and analyzed the effect on mitochondrial transport. Knockdown of Miro1 altered mitochondrial distribution in the soma, but did not affect axonal mitochondrial transport. In contrast, knockdown of Miro2 markedly disrupted axonal mitochondrial motility (Fig. 2-4). The pattern of mitochondrial movements on kymograph analysis was similar to that seen in $Mfn2^{-/-}$ neurons, with loss of fast sustained movements (Fig. 2-4A,B). Unlike loss of Mfn2, which produced concurrent mitochondrial fusion and transport defects with smaller fragmented mitochondria, loss of Miro2 did not alter the length of axonal mitochondria (Fig. 2-4C). Mitochondria in Miro2 depleted neurons spent more time paused between

anterograde and retrograde movements, and the velocity distributions were skewed toward slower velocities (Fig. 2-4D,E). Of note, overexpression of Miro2 in $Mfn2^{-/-}$ cultures was not able to rescue transport deficits (data not shown). Taken together, the fact that Mfn2 interacts directly with the Miro proteins, and that loss of Mfn2 or Miro2 disrupt axonal mitochondrial transport in a nearly identical fashion, suggests that an intact Mfn2:Miro2 complex must be present for proper axonal mitochondrial transport.

Disruption of mitochondrial fusion by knockdown of Opa1 does not affect the axonal transport of mitochondria

Some of the CMT2A disease mutant MFN2 alleles are unable to mediate mitochondrial fusion (Detmer and Chan, 2007). Given that loss of mitochondrial fusion leads to dissipation of $\Delta\psi$ and diminished oxidative phosphorylation (Lodi et al., 2004; Chen et al., 2005), it is possible that the observed alterations in mitochondrial transport could be secondary to loss of mitochondrial fusion. To investigate whether diminishing mitochondrial fusion directly or indirectly disrupts axonal mitochondrial transport, we used lentiviral delivered siRNA to knockdown Opa1 in cultured DRG neurons. Knockdown of Opa1 severely disrupts mitochondrial fusion in non-neuronal cells, leading to a decrease in mitochondrial size similar to loss of Mfn1/Mfn2 (Cipolat et al., 2004; Chen et al., 2005). Measurement of mitochondrial lengths in confirmed that the mitochondria were significantly shorter in Opa1 knockdown DRG axons (Fig. 2-5C), consistent with the expected decrease in mitochondrial fusion (Cipolat et al., 2004; Chen et al., 2005). However, timelapse imaging and kymograph analysis revealed that patterns of mitochondrial movement in Opa1 knockdown neurons were indistinguishable from controls (Fig. 2-5A,B). Mitochondria in neurons depleted of Opa1 spent equal amounts of time paused between movements in anterograde and retrograde directions, and moved at similar velocities compared to controls (Fig. 2-5D,E). These data indicate that disrupting mitochondrial fusion alone via Opa1 knockdown does not itself affect mitochondrial transport, and further supports that Mfn2, via its interactions with other proteins at the OMM, plays a direct role in regulating mitochondrial transport.

CMT2A associated MFN2 mutants cannot compensate for the mitochondrial transport abnormality in Mfn2$^{-/-}$ neurons

As described above, some CMT2A-associated MFN2 mutants are unable to mediate mitochondrial fusion (R94Q), whereas others are fusion competent (L76P, W740S) (Detmer and Chan, 2007). However, exogenous expression of all of these mutants produced a mitochondrial transport deficit in DRG neurons (Baloh et al., 2007), suggesting that the ability of CMT2A mutants to mediate fusion and transport may be separable. To determine whether the role of Mfn2 in mitochondrial fusion could be dissociated from its role in transport, we expressed the L76P and W740S mutants (fusion competent), and the R94Q mutant (fusion incompetent), in cultured Mfn2$^{-/-}$ neurons and assessed their ability to rescue the transport defect. In contrast to wtMFN2, none of the CMT2A associated mutants were able to completely normalize the time mitochondria spent paused between movements, or to fully restore the faster movement segments seen on velocity distributions (Fig. 2-6A-C), with the fusion incompetent R94Q allele being least capable of rescuing the defect in transport in Mfn2$^{-/-}$ neurons. Therefore, CMT2A MFN2 mutants were not able to normalize axonal mitochondrial transport in DRG axons, whether they are fusion competent (L76P, W740S) or incompetent (R94Q). This finding indicates that disruption of axonal mitochondrial transport correlates better with pathogenicity of CMT2A associated MFN2 mutants than the loss of the ability to mediate mitochondrial fusion.

Discussion

These experiments provide evidence for a direct role for Mfn2 in mediating mitochondrial transport, distinct from its role in mitochondrial fusion. Both loss of Mfn2, and expression of CMT2A-associated MFN2 disease mutants, specifically alter mitochondrial movement patterns causing mitochondria (but not other organelles) to move at slower velocities and pause for greater lengths of time, consistent with an inability to attach/move via the microtubule-based kinesin and dynein transport systems. Together with the finding that Mitofusins interact with Miro proteins and OIP106/GRIF1, these data indicate that Mfn2 is a key component of the linker/adaptor complex between mitochondria and kinesin/microtubules.

Mfn2 as a multifunctional regulator of mitochondrial dynamics and function

Outer mitochondrial membrane proteins are well positioned to play a key role in coordinating and regulating diverse aspects of mitochondrial function. Perhaps it should not be a surprise then that Mitofusins have been reported to perform numerous functions, including the regulation of mitochondrial fusion (Chan, 2006), ER-mitochondrial tethering (de Brito and Scorrano, 2008), apoptotic cell death and outer membrane permeability (Suen et al., 2008), oxidative phosphorylation and gradient coupling (Pich et al., 2005), and microtubule-based mitochondrial transport.

Our data indicates that regulation of mitochondrial transport by Mfn2 is achieved by interacting with the Miro:Milton complex. The increased pause time and slower movement velocities in both anterograde and retrograde directions due to loss of Mfn2 closely resemble the mitochondrial movement abnormalities seen in Miro2 depleted DRG neurons, and in dMiro knockout flies (Russo et al., 2009). Other reports found that overexpression of Miro proteins can either increase (Guo et al., 2005) or decrease (Russo et al., 2009) axonal mitochondrial transport. Importantly, we observed that overexpression of Miro2 could not rescue the transport deficit in $Mfn2^{-/-}$ neurons, indicating that a functional Mfn:Miro complex is required to cooperatively mediate mitochondrial transport.

While Miro proteins have only been shown to interact with anterogradely moving kinesin motors, loss of either Mfn2 or Miro2 led to disruption of both anterograde and retrograde mitochondrial transport, similar to loss of dMiro in flies (Russo et al., 2009). The Mfn:Miro complex may also bind to dynein and direct alternating engagements of either motor with microtubule tracks. However this does not have to be the case, as anterograde and retrograde organellar transport are coordinately regulated, and selective disruption of either kinesins or dynein/dynactin typically diminishes bidirectional transport, though the exact reason remains unclear (Brady et al., 1990; Waterman-Storer et al., 1997; Martin et al., 1999; Deacon et al., 2003). The increased time mitochondria spend paused in axons from neurons lacking Mfn2 or Miro2 may also indicate a disruption of motor processivity. Taken together, this suggests that the Miro2:Mfn2 complex plays a role in regulating the processivity of kinesin motors, and in orchestrating the switch between engaging anterogradely moving kinesins and retrogradely moving dyneins (Russo et al., 2009).

Given the interaction between Miros and Mitofusins, it is perhaps not surprising that several studies have suggested that Miros may also influence mitochondrial morphology and fusion. Examples include the expression of constitutively active Miro1 producing long thread-like mitochondria in COS-7 cells, hippocampal neurons, and H9c2 cells (Fransson et al., 2006; Saotome et al., 2008; MacAskill et al., 2009a), and the overexpression of dMiro in Drosophila increasing the mean length of axonal mitochondria (Russo et al., 2009). These studies highlight the notion that membrane transport and fusion are intimately related processes that must be coordinately regulated to achieve proper organellar trafficking, and it is likely that molecular adaptor complexes at the outer mitochondrial membrane will serve dual roles in transport and fusion.

Alterations in mitochondrial dynamics in human disease

Mitochondrial dysfunction is involved in numerous neurodegenerative and metabolic diseases, however whether mitochondrial dysfunction plays a primary role in disease pathogenesis, or is simply a consequence of other aspects of cellular dysfunction (disruption of protein homeostasis,

environmental toxins, etc), is unclear. In this context, studying the molecular mechanisms by which mutations in mitochondrial proteins (such as MFN2 or OPA1) cause human disease is helpful, as altered mitochondrial function likely plays a primary role in disease. One of the fundamental mysteries of inherited neurodegenerative diseases is the selective vulnerability of particular populations of neurons to mutations in universally expressed proteins. CMT2A and dominant optic atrophy are associated with mutations in MFN2 and OPA1 respectively, both ubiquitously expressed proteins essential for mitochondrial fusion (Alexander et al., 2000; Delettre et al., 2000; Zuchner et al., 2004). However, instead of leading to identical human diseases, mutations in MFN2 cause a severe peripheral neuropathy affecting the longest axons in the body, whereas mutations in OPA1 (typically via haploinsufficiency) result in degeneration of optic nerve axons. Our findings suggest that the fundamental difference in the pathogenesis of these diseases lies in the fact that loss of OPA1 disrupts mitochondrial fusion without altering mitochondrial transport. Interestingly, early changes in mitochondrial dynamics are seen in neurons expressing mutant forms of neurofilament which cause CMT2E, suggesting that alterations in mitochondrial transport and distribution may be a final common pathway leading to axonal degeneration in multiple types of CMT (Baloh, 2008; Tradewell et al., 2009). A viable working hypothesis is that the long peripheral nerves affected in CMT are more vulnerable to disruption of mitochondrial transport and distribution than other cell types. Defining evidence for or against this hypothesis from patient samples or animal models remains to be demonstrated, and is a focus of ongoing research.

Although our studies indicate Mfn2 is clearly required for mitochondrial transport, the precise molecular mechanism by which Mfn2 disease mutants disrupt this process remains unclear, as they maintain the ability to interact with Miro1/2 and with OIP106/GRIF1. These data indicate the CMT2A disease mutants do not simply disrupt formation of the molecular adaptor complex linking mitochondria to kinesins, but instead disrupt the proper function of this complex, and hence further studies will be needed to understand exactly how Mfn2 disease mutants alter the properties of the adaptor complex on a molecular level.

The role of Mfn1 and Mfn2 in controlling mitochondrial dynamics

Though Mfn2 and Mfn1 are highly homologous proteins with overlapping functions, several lines of evidence have shed light on properties which are divergent between them. Mfn1 more effectively tethers mitochondrial membranes and alone is sufficient to mediate fusion with Opa1 (Cipolat et al., 2004; Ishihara et al., 2004). Though both Mfn1 and Mfn2 null mice show early embryonic lethality due to a placental defect (Chen et al., 2003), loss of Mfn1 outside of the placenta (using a Cre-lox system) led to no detectable abnormalities, whereas similar non-placental loss of Mfn2 caused severe multisystem defects and early postnatal death (Chen et al., 2007). Additionally, recent evidence suggested that Mfn2 but not Mfn1 is involved in ER-mitochondrial tethering (de Brito and Scorrano, 2008).

In our study, loss of Mfn2 alone is sufficient to produce a severe mitochondrial transport deficit in DRG neurons, which could be restored by expression of either Mfn2 or Mfn1, consistent with the fact that both can interact with the Miro:Milton complex. Indeed, we found that exogenous expression of Mfn1 was able to compensate for loss of Mfn2 in DRG neurons, raising the possibility that identifying drugs which would augment Mfn1 expression may represent a valid therapeutic strategy for CMT2A.

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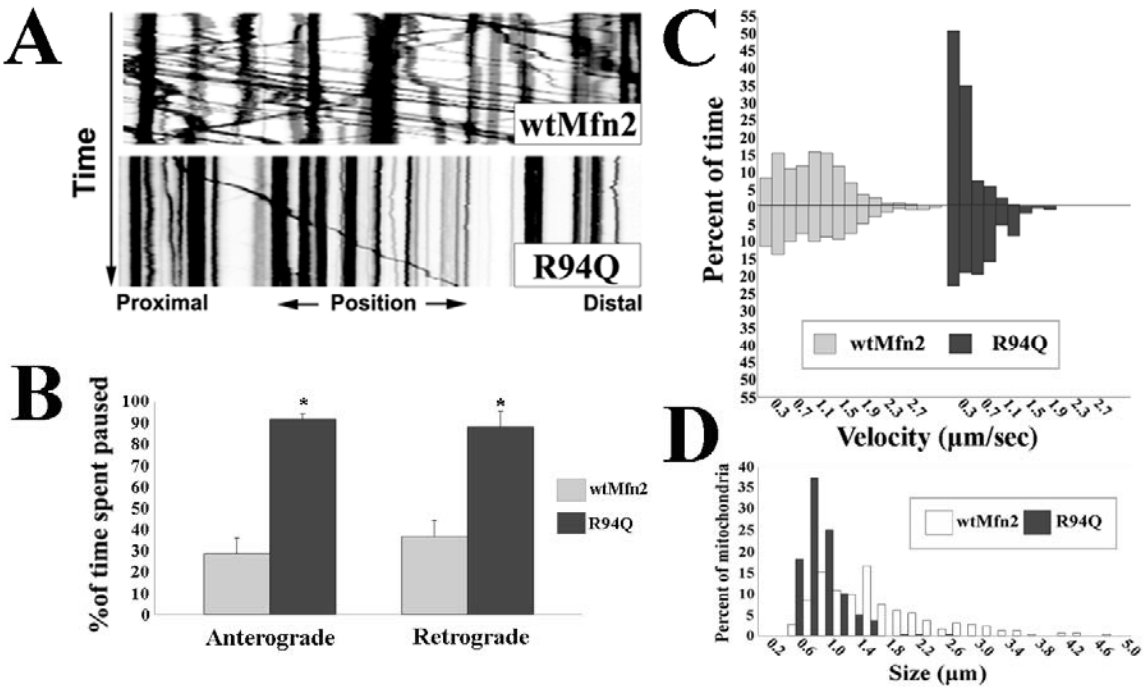

Figure 2-1. **CMT2A-associated MFN2 mutants alter the transport of axonal mitochondria.** Mitochondria in cultured DRG neurons expressing wtMFN2 or R94Q were labeled with mito-RFP and imaged by time-lapse microscopy. *A* , Kymograph analysis of mitochondrial movements in R94Q-expressing cells reveal diminished numbers of moving mitochondria. *B* , Mitochondria from R94Q-expressing neurons spent more time paused between anterograde and retrograde movements than did mitochondria from controls. (*$p < 0.005$, *t* test; *n* = number of axons from which image stacks were created). Each condition contained a total of at least 500 observed mitochondria. *C* , Velocity distributions representing the amount of time that mitochondria from wtMFN2- or R94Q-expressing neurons spent moving at indicated velocities. Anterograde velocities are above the *x*-axis, and retrograde velocities are below the *x*-axis. There was a shift in both the anterograde and retrograde velocity distributions of mitochondria from R94Q-expressing neurons toward slower velocities. The differences were statistically significant as determined by rank-sum test analysis ($p < 0.001$). *D* , Size–frequency distributions of axonal mitochondria from wtMFN2-, R94Q-, and H361Y-expressing cells show that CMT2A disease mutants decrease mitochondrial lengths.

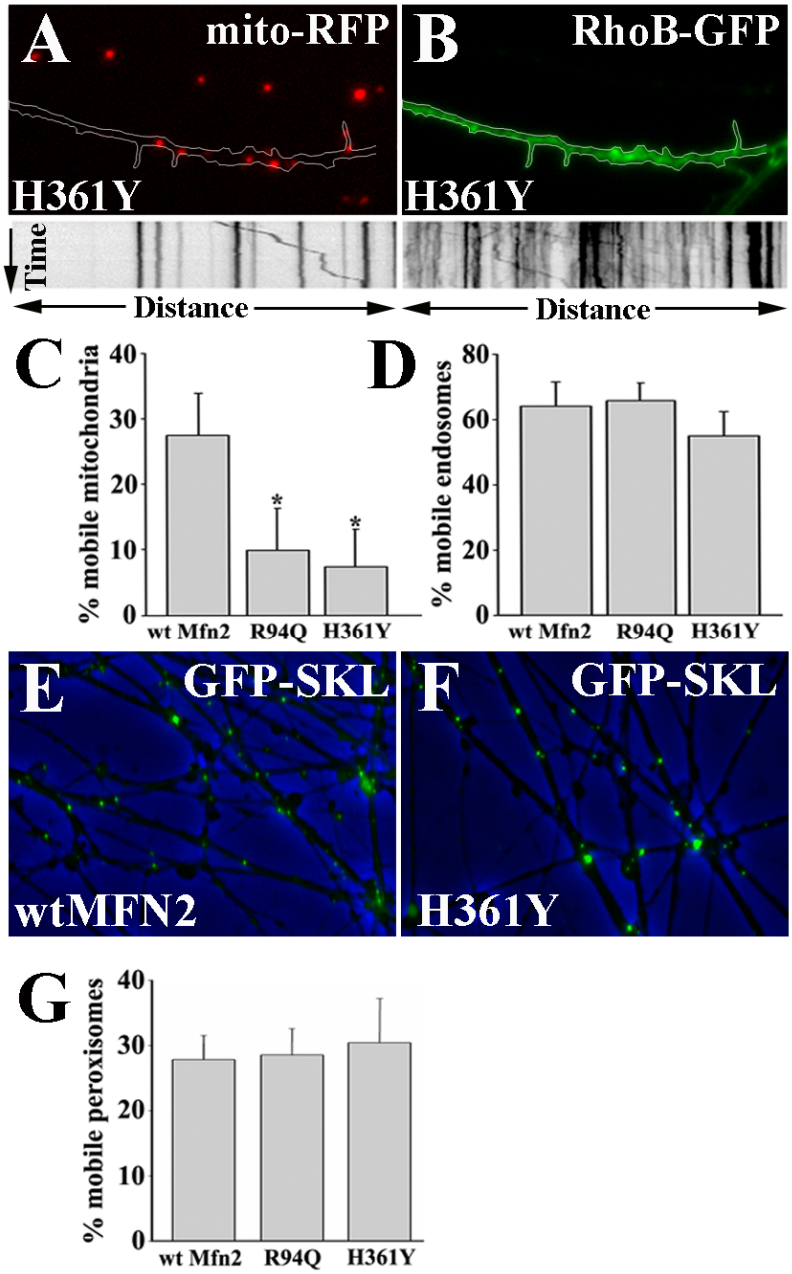

Figure 2-2. **CMT2A-associated MFN2 mutants specifically disrupt mitochondrial transport.** *A* , *B* , DRG neurons expressing the H361Y or R94Q MFN2 disease mutants were coinfected with mito-RFP (*A*) and RhoB-GFP (*B*) mitochondrial and endosomal markers, respectively. Mutant-expressing neurons revealed diminished mitochondrial mobility (*A* , kymograph) in the same axons that showed normal endosomal transport (*B* , kymograph). *C* , *D* , The percentage of mobile mitochondria is significantly decreased in R94Q- and H361Y- compared with wtMFN2-expressing neurons (*$p < 0.001$), whereas the percentage of mobile endosomes in these axons was normal. *E* , *F* , Overlay images of phase contrast and EGFP-SKL (which labels peroxisomes) in wtMFN2 (*E*)- and H361Y (*F*)-expressing DRG axons. *G* , Similar to endosomes, the percentage of mobile peroxisomes was unaltered by the expression of MFN2 mutants.

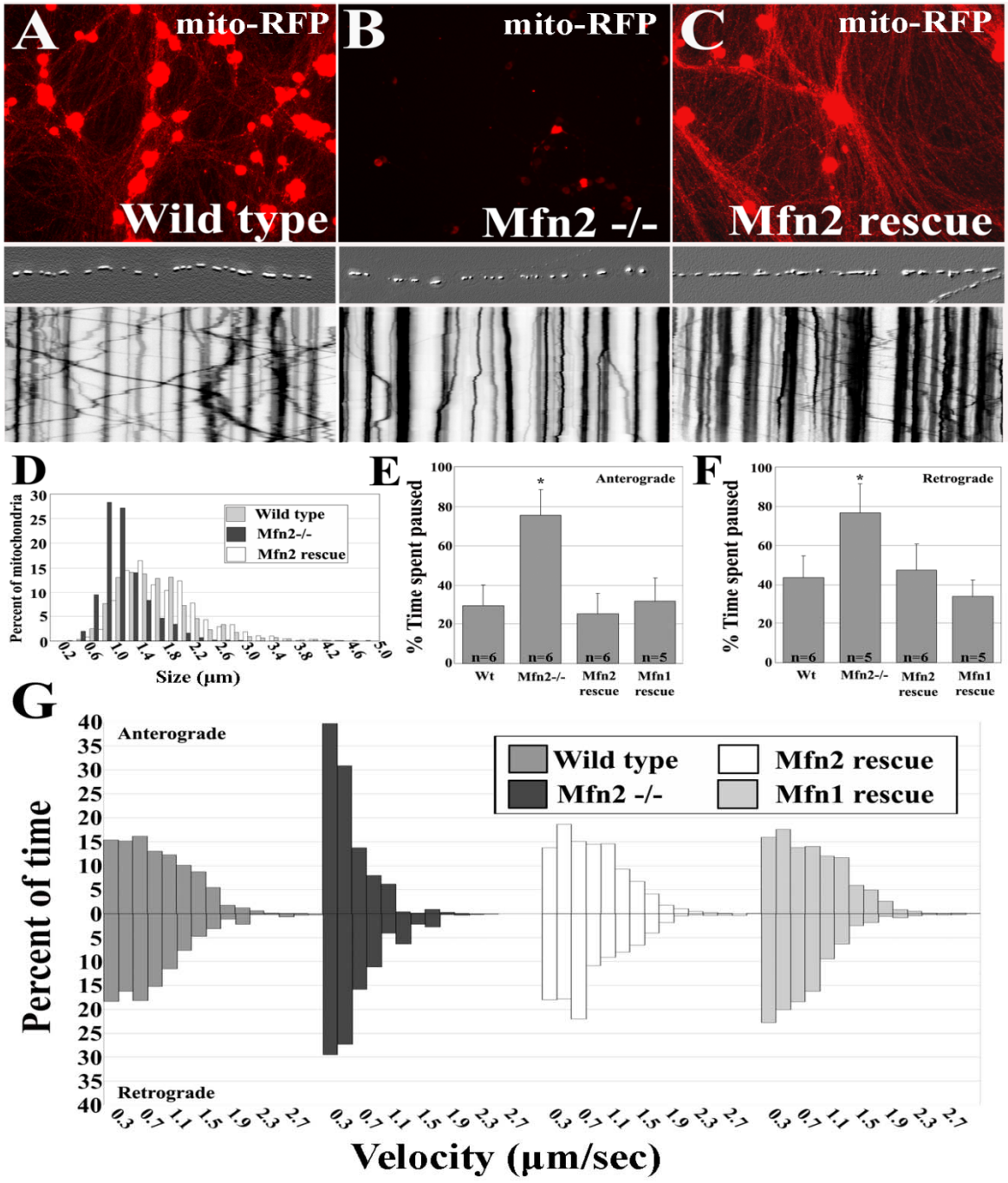

Figure 2-3. **Mfn2 is required for normal axonal transport of mitochondria.** To assess the effects of loss of Mfn2 on mitochondrial mobility, we analyzed mitochondrial transport in DRG neurons cultured from Mfn2 knock-out animals. *A* – *C* , DRG cultures from wild-type (*A*) or $Mfn2^{-/-}$ (*B*) mice infected with mito-RFP and imaged 4 d after infection at 10× magnification coupled with corresponding kymographs from single axons. Corresponding axons are presented

above each kymograph as an embossed image for clarity. *B* , Diminished numbers of mitochondria are observed in Mfn2$^{-/-}$ axons due to a delay in the overall outward migration of mitochondria that is corrected by reintroduction of wild-type human MFN2 (*C* , Mfn2 rescue). *D* , Size–frequency histogram of mitochondria shows a decrease in the lengths of mitochondria observed in Mfn2$^{-/-}$ axons that is restored with reintroduction of MFN2 (Mfn2 rescue). *E* – *G* , Kymograph analysis of mitochondrial movements in individual MFN2$^{-/-}$ axons showed a profound abnormality in transport, resembling the deficit observed with expression of MFN2 disease mutants. The moving mitochondria from Mfn2$^{-/-}$ axons spent more time paused between movements (*E* , *F*) (*$p < 0.001$, *t* test; *n* is number of axons from which kymographs were generated), moved at slower velocities in the anterograde and retrograde directions (*G*), and were completely rescued by expression of either Mfn2 or Mfn1.

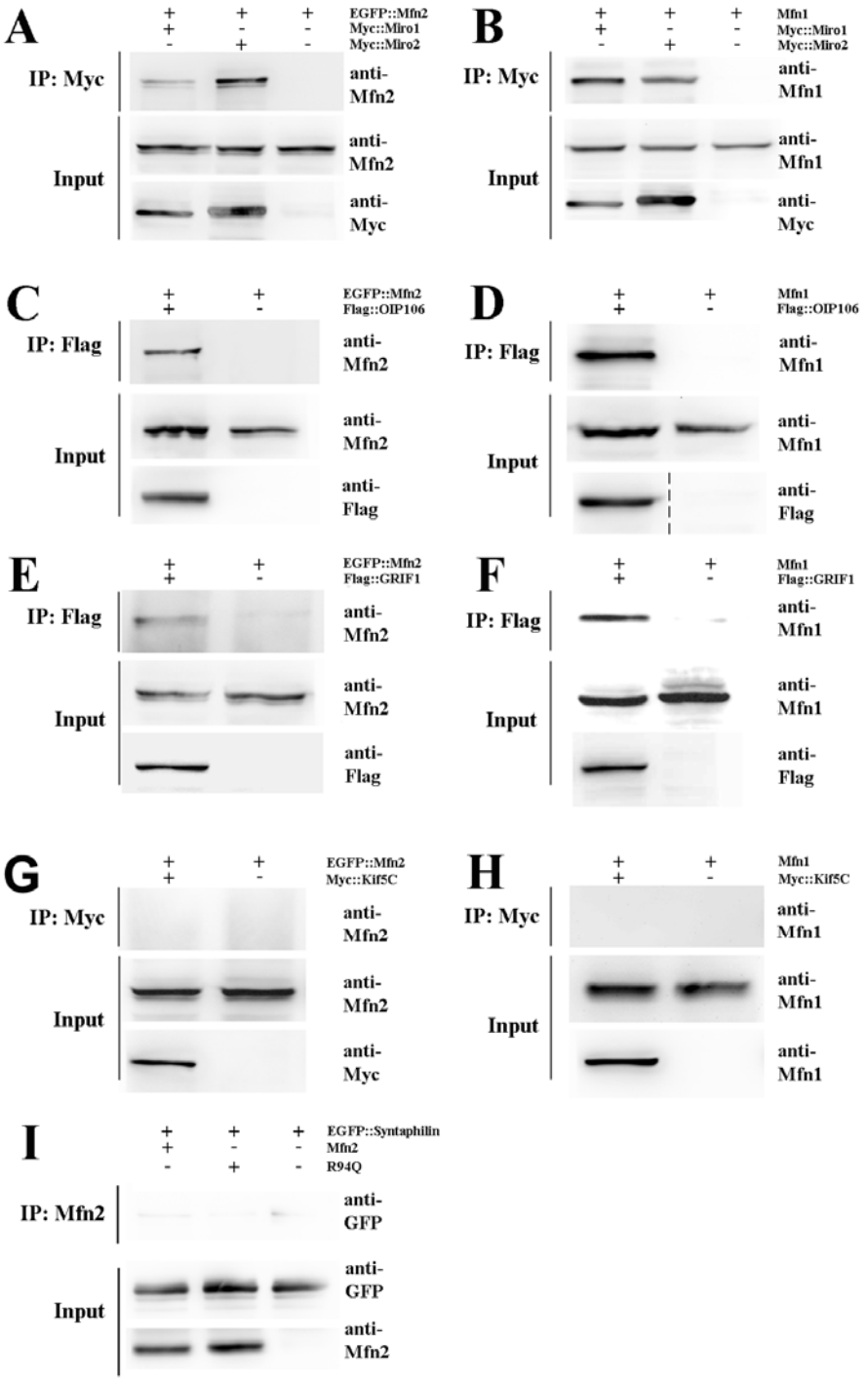

Figure 2-4. **Mfn2 and Mfn1 interact with Miro and Milton proteins, key components of microtubule-based mitochondrial transport.** HEK 293T cells were transiently transfected with the indicated epitope-tagged constructs, followed by immunoprecipitation (IP) and immunoblotting. *A* , *B* , Both Mfn2 and Mfn1 were able to interact with Miro1 and Miro2 by

coimmunoprecipitation. The Mfn2:Miro2 interaction was consistently more robust than the Mfn2:Miro1 interaction, suggesting there may be selectivity for the formation of this complex. *C – F* , Mfn2 and Mfn1 interact with the Milton homologues OIP106 and GRIF1, proteins known to function as linkers between mitochondria and kinesins motors. *G* , *H* , Neither Mfn2 nor Mfn1 coimmunoprecipitated with Kif5C, indicating that they do not directly link mitochondria to kinesin. *I* , Additionally, Mfn2 was unable to interact with syntaphilin, an outer mitochondrial membrane protein that anchors mitochondria to microtubules.

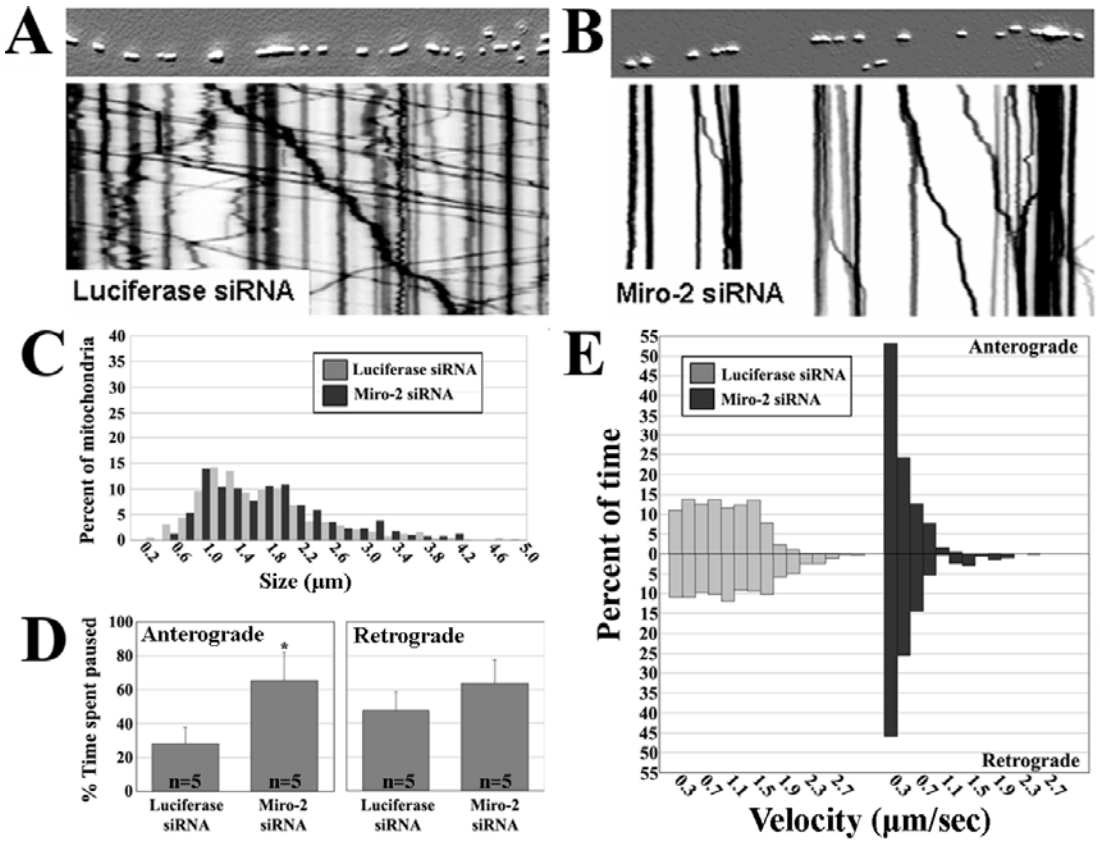

Figure 2-5. **Depletion of Miro2 produces a mitochondrial transport abnormality similar to that observed with loss of Mfn2 in DRG neurons.** *A* , *B* , Kymograph analysis reveals that siRNA-mediated knockdown of Miro2 dramatically altered patterns of mitochondrial transport. *C* , Size–frequency histogram of axonal mitochondria demonstrated that depletion of Miro2 altered mitochondrial transport without changing mitochondrial morphology. *D* , Similar to Mfn2$^{-/-}$ cultures, mitochondria spent a greater percentage of time paused between anterograde movements in Miro2 knockdown cultures (*$p < 0.001$, *t* test; *n* is the number of axons from which image stacks were created). Pauses between retrograde movements trended toward longer pause times but did not reach statistical significance. *E* , Mitochondria velocity distributions were also skewed toward slower movements in Miro2 knockdown cultures, similar to effects seen with loss of Mfn2.

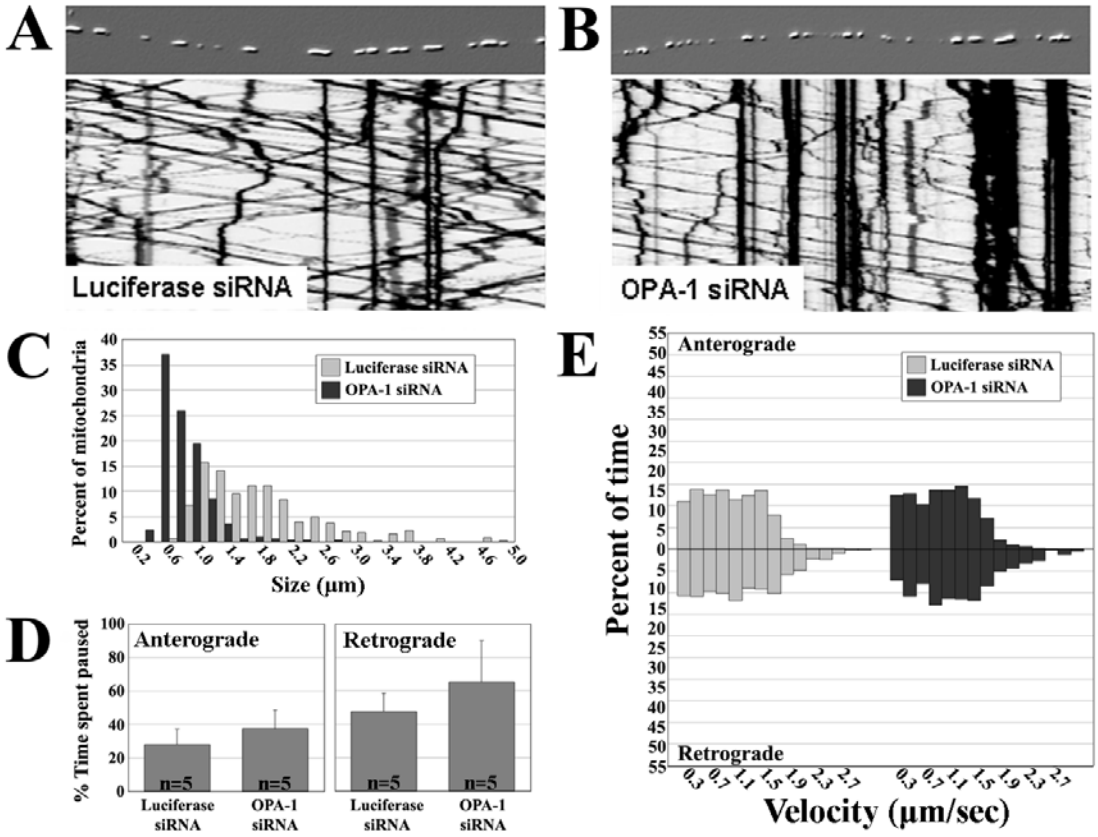

Figure 2-6. **Disruption of mitochondrial fusion by knockdown of Opa1 does not alter mitochondrial axonal transport.** To assess whether disrupting mitochondrial fusion is itself sufficient to alter mitochondrial transport, we used siRNA to knock down Opa1 in DRG neurons. *A*, *B* , Embossed images of representative axons and the corresponding kymographs showing Opa1 knockdown (>90%) did not alter mitochondrial movement patterns. *C* , Mitochondrial size–frequency histogram showed that loss of Opa1 significantly decreased mitochondrial length similar to that seen in Mfn2$^{-/-}$ cultures, reflective of decreased mitochondrial fusion. *D* , *E* , Pause times between anterograde or retrograde movements (*D*) and velocity of movements (*E*) were not altered by Opa1 knockdown (*n* is the number of axons from which kymographs were generated), unlike loss of Mfn2, indicating that loss of mitochondrial fusion alone does not alter mitochondrial transport.

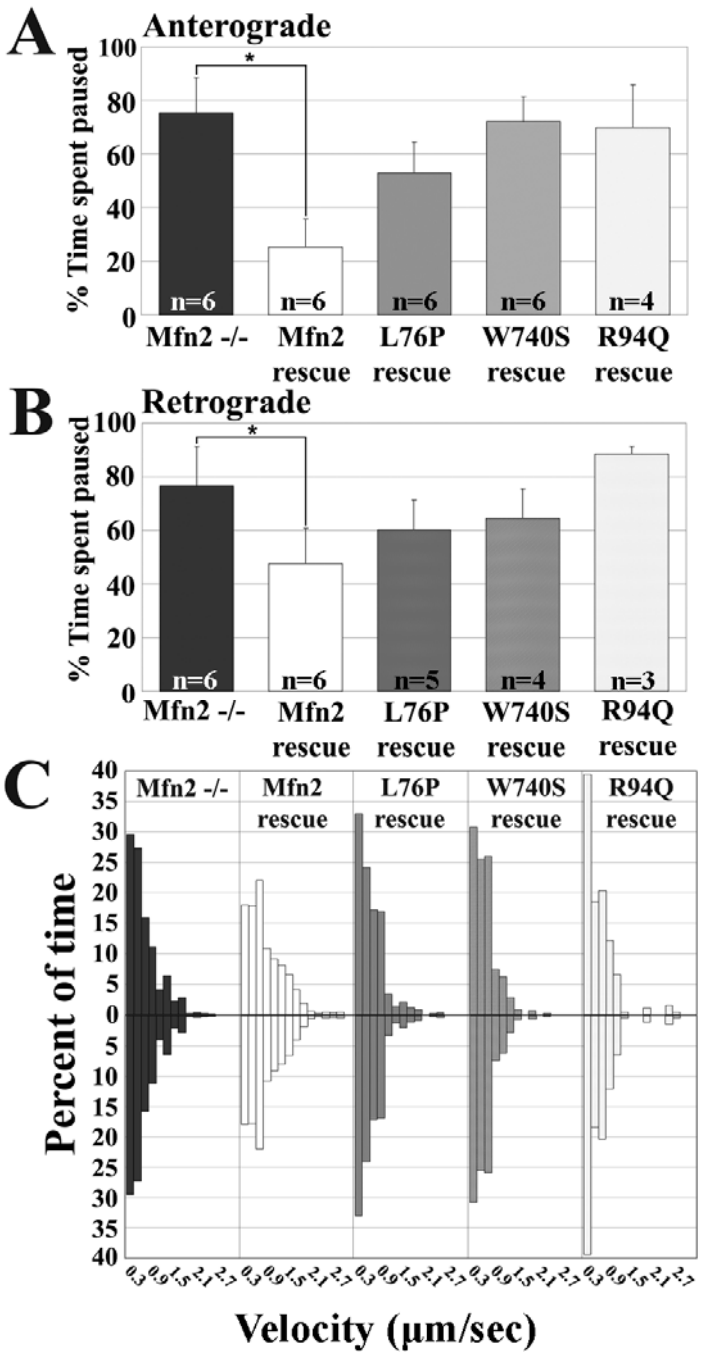

Figure 2-7. **Fusion-competent, CMT2A-associated MFN2 mutants cannot compensate for the mitochondrial transport abnormality in Mfn2$^{-/-}$ neurons.** To compare the ability of fusion-competent (L76P, W740S) and fusion-incompetent (R94Q) MFN2 mutants to mediate mitochondrial transport, we reintroduced these mutants into Mfn2$^{-/-}$ neurons and assessed their ability to rescue the mitochondrial transport abnormality. As compared with wtMFN2, neither the fusion-competent (L76P, W740S) nor fusion-incompetent (R94Q) mutants were able to fully restore mitochondrial pause time (*A* , *B*) (*n* is number of axons from which kymographs were generated) or mitochondrial velocities (*C*).

Chapter 3

Active redistribution of the mitochondrial network supports axon integrity

Introduction

Recognizing that axons employ a non-apoptotic program of self-destruction has been a fundamental advance in our understanding of neurodegeneration. The regulated nature of this process suggests that relevant molecular players could be targeted for therapeutic intervention. The need for such targets is highlighted by the prominence of axon loss in several neurodegenerative paradigms. Many studies suggest that axonal degeneration precedes cell death in ALS (Fischer and Glass, 2007), Parkinson's disease (Cheng et al.), Alzhiemer's disease (Vickers et al., 2000) and Multiple Sclerosis (MS) (Dziedzic et al.) and may be the most important factor contributing to morbidity in MS and a number of demyelinating neuropathies. Understanding the molecular mechanisms underlying axonal degeneration will be pivotal to both our understanding of neurodegeneration and our ability to prevent it.

Because of their high-energy requirements and low intrinsic energy reservoirs, neurons require continual delivery of glucose and oxygen. Within minutes of onset, anoxia in optic nerve causes conduction block and degeneration of axons (Stys et al., 1990) (Malek et al., 2005). A similar scenario of "virtual hypoxia" instigates axonal degeneration in MS where increases in nitric oxide (NO) levels inhibit mitochondrial respiration (Trapp and Nave, 2008). The integrity of an entire axon is dependent on all of its local energy demands being met; energy failure in one area can compromise the entire segment distal to the site. In peripheral nerve explants, a local, transient application of DNP (a mitochondrial membrane decoupler) inhibits mitochondrial respiration at the site of exposure and triggers Wallerian degeneration of the distal axon (Alvarez

et al., 2008). Axons are thus dependent on mitochondrial production of ATP and even a brief failure to meet local energy demands can lead to the degeneration of the entire distal axon segment.

To meet local energy demands in axons, mitochondria are actively transported to areas of high-energy demand including the distal portion of the initial segment (Li et al., 2004), Nodes of Ranvier (Berthold et al., 1993), and presynaptic boutons (Misgeld et al., 2007). These areas also experience high Ca^{2+} flux, which mitochondria help regulate by taking up cytosolic Ca^{2+} through the mitochondrial Ca^{2+} uniporter (Murchison and Griffith, 2007). Both ADP and Ca^{2+} have been shown to regulate movement of mitochondria, explaining their localization (Mironov, 2007) (Macaskill et al., 2009) (Wang and Schwarz, 2009). Though the dependence of axons on the continual redistribution of mitochondria is logically implicit, it has not been experimentally demonstrated.

In a previous study, we gave evidence for a direct role of mitofusin 2 (MFN2) in the transport of axonal mitochondria that is distinct from its ability to mediate fusion of the outer mitochondrial membrane (Misko et al., 2010). Charcot-Marie-Tooth disease type 2A (CMT2A), a primary peripheral axonopathy, is most commonly associated with mutations in the MFN2 gene (Zuchner et al., 2004) (Lawson et al., 2005) (Verhoeven et al., 2006). Because MFN2 has a well established role in mitochondrial fusion, disruption of this process was first assumed to underlie the axon degeneration in CMT2A. However, later investigation demonstrated that some of the MFN2 disease mutants retained their ability to mediate mitochondrial fusion (Detmer and Chan, 2007). We have found that MFN2 plays a direct role in mediating mitochondrial transport distinct from its role in fusion and that MFN2 disease mutants disrupt mitochondrial transport in a dominant fashion (Baloh et al., 2007; Misko et al., 2010).

In this study we asked whether disruption of mitochondrial transport could explain the loss of axons in CMT2A. We find that expression of MFN2 disease mutants trigger axonal degeneration in cultured DRG neurons. Because specific disruption of mitochondrial fusion by opa1 knock-down is not sufficient to cause degeneration, the affect of the MFN2 mutants is better

explained by their ability to disrupt mitochondrial transport. Furthermore, we show that abnormal transport impedes the uniform distribution of mitochondria across axons. This creates axon segements devoid of mitochondria that are more reliant on glycolysis and in a state of “virtual hypoixia” making them more prone to metabolic failure.

Materials and Methods

Plasmids and silencing RNAs. Human wild-type MFN2, MFN2 disease mutants, mouse Mfn1 and opa1 siRNA constructs were previously described (Misko et al., 2010).

Dorsal root ganglion cultures. Dorsal root ganglion (DRG) cultures were obtained from embryonic rats gestational day 15.5. Dorsal root ganglia were dissected out, dissociate using trypsin/EDTA, and plated directly onto 24-well plates coated with laminin and poly-D-lysine. Cultures were maintained in Neurobasal medium with B27 supplement (Invitrogen), 50 ng/ml NGF, 5 mM glutamine, and antibiotics. Addition of 5-fluorouracil for the first 3 d in culture was used as an antimitotic treatment.

Lentivirus production and infection. Lentiviruses were produced as described previously (Baloh et al., 2007). Briefly, human embryonic kidney (HEK) 293T cells were plated onto six-well plates and transfected using Mirus reagent with a packaging vector (8.91), envelope vector (vesicular stomatitis virus-glycoprotein), and transfer vector encoding the gene to be expressed. Media was changed once at 12 h and collected at 72hrs, and applied directly to DRG cultures resulting in 95% infection efficiency.

Calcium and ROS imaging. Cell permeant Fluo-4 AM (Invitrogen) was incubated at a final concentration of 1μM with cultured neurons for 30min at 37°C, washed with media and incubated again for 30min before imaging. 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCFDA) was loaded at a final concentration of 1μM for 10min at 37°C in Neural Basal media without B27 supplement, washed out and incubated again for 20min before imaging. All microscopy including time-lapse imaging was performed with in a climate-controlled chamber (In

Vivo Scientific) at 37°C and 5% CO2 and images were acquired with a Cool Snap HQ 2 CCD camera (Photometrics) mounted on a Nikon Eclipse Ti-U microscope.

Measuring mitochondrial distribution and trasnport. Trasnfection of DRG neuron cultures with mito-RFP and time-lapse imaging of mitochondrial movement were performed as previously described (Misko et al., 2010). To assess mitochondrial distribution, we adapted the protocol from (Miller and Sheetz, 2004) with minor changes. Time-lapse image stacks of axonal mitochondria within ~100μm axon segments were used to create kymographs using MetaMorph software (Molecular Devices) for accurate identification of individual mitochondria. At least 10 different axons from separate neurons were imaged and divided into 10μm bins. The number of mitochondria per bin was counted and bins were pooled for each condition. Distributions were described by comparing the average number of mitochondria (μ) per bin to the variance (var) such that μ>var is considered uniform, μ=var random and μ<var clustered. To determine significance levels we used a χ^2 analysis of the goodness of fit of the Poisson distribution. For each condition the frequency distribution of bins with a certain number of mitochondria (*i*=0,1,…5) was determined (f_{Ai}) and compared with the frequency distribution predicted by the Poisson distribution (f_{Ei}). The predicted frequency distribution was predicted as follows:

$$P(X) = (e^{-\mu})\,\mu^X / X! \qquad (1)$$

where P(X) is the probability of there being X mitochondria in a bin an μ being the average number of mitochondria per bin as empirically determined for the condition being compared.

$$f_{Ei} = P(X)(n) \qquad (2)$$

where n is the total number of bins counted. The χ^2 statistic is then calculated by comparing the observed and predicted frequency distributions.

$$\chi^2 = \Sigma_{(i=0 \text{ to } i=5)} (f_{Ai}-f_{Ei})^2/f_{Ei} \qquad (3)$$

where *i* is the number of mitochondria per bin and degrees of freedom is d_f=5-2=3.

Mitochondrial flux represents the average number of mitochondria passing through the center of a bin in 1min.

Estimating $\Delta\psi$. To measure mitochondrial membrane potentials we adopted the protocol developed by (Verburg and Hollenbeck, 2008) 2008. Briefly, DRG cultures were incubated with 20nM tetramethylrhodamine methyl ester (TMRM) for 20min at 37°C, which was then exchanged for media containing 5nM TMRM in which images were acquired. A volume corrected mitochondrial to cytoplasmic fluorescence ratio (F_m/F_c) was calculated for at least 500 mitochondria per condition by measuring the fluorescence of mitochondria and the fluorescence of the immediate surrounding axoplasm and applying the formula $F_m/F_c = (Fl_{mitochondria} – (2/3)\ Fl_{cytoplasm})/(1/3)\ Fl_{cytoplasm}$.

Measuring axonal mitochondrial content. For imaging with Mitotracker green, cultured neurons were incubated with 75nM Mitotracker Green (Invitrogen) for 30min at 37°C, washed and imaged. Labeling with TMRM is described above. To measure mitochondrial content, images of mitochondrial fluorescence were filtered with an inverted mexican hat kernel and thresholded and the total number of pixels representing mitochondria were counted. Phase images of corresponding areas were background subtracted and thresholded and corresponding pixels representing axons were used to normalize the mitochondrial content.

2-deoxy-glucose treatments. Media was changed on cultures 12hrs prior to 2DG administration to minimize fluctuations in metabolism from week to week. 2DG was added to cultures at a final concentration of 15mM in H_2O and imaged at 48hrs.

Results

CMT2A associated MFN2 mutants cause axonal degeneration in cultured DRG neurons.

In addition to promoting mitochondrial fusion, MFN2 directly mediates mitochondrial transport, a function that CMT2A associated MFN2 disease mutants disrupt in a dominant fashion. To determine whether mitochondrial transport is critical for axonal integrity, we introduced wild type (wtMFN2) or disease mutant MFN2 (R94Q and H361Y) constructs using lentivirus into cultured DRG neurons. As previously reported, axonal mitochondria imaged by time-lapse microscopy demonstrated abnormal movement patterns in cultures expressing the R94Q (Fig.1) or H361Y (not shown) MFN2 disease mutants 1 week post infection (Fig.1 A and B). The R94Q and H361Y disease mutants are not able to mediate fusion. Accordingly, mitochondrial lengths in these cultures were also decreased indicating inhibition of mitochondrial fusion (Fig.1 C).

Concomitant with these changes, we could not detect signs axonal degeneration by light phase microscopy. To employ a more sensitive approach, we labeled cultures with Fluo-4 or DCFDA dyes which detect Ca^{+2} and reactive oxygen species (ROS) levels respectively. In R94Q but not wtMFN2 expressing cultures a fraction of axons that appeared beaded or dilated could be visualized with either dye (D-O). Observation of cell bodies over three days revealed that 96.4+/-3% and 97.2 +/-3% percent of cell bodies remained viable in wtMFN2 and R94Q expressing cultures respectively. Additionally, virtually no cell death was detected in either condition with propidium iodide staining, though a 24 hours treatment with ionomycin resulted in death of all cells verifying the staining technique (Fig.1 P). The lack of cell body death confirms that the observed degeneration was originating in axons and not a secondary consequence of cell death. Taken together, these data suggest that MFN2 disease mutants cause axons to degenerate concomitant with disruption of mitochondrial transport and fusion.

MFN2 disease mutants cause axons to actively degenerate in cultured DRG neurons.

To ensure that the elevated levels of intra-axonal Ca^{2+} and ROS were labeling actively degenerating axons and were not simply marking a transient event in otherwise healthy axons,

were performed time lapse microscopy on R94Q expressing cells 1 week after infection. Imaging with Fluo-4 dye over a 12hr time period captured numerous degenerative events (Fig. 3 A-C). Three patterns of degeneration could be distinguished: 1) An axon displays a uniform rise in intracellular Ca^{2+}, beads and then fully degenerates (seen as disappearance of axon after loss of membrane integrity and inability to retain dye). 2) A dilated axon segments swells to a spheroid like shape and "pops". 3) An axon breaks apart from a single point and the resulting ends retract away from the break point before disappearing. From the onset of increased intracellular Ca^{2+} most axons fully degenerate within ~3hrs.

Disruption of mitochondrial fusion is not sufficient to cause axonal degeneration in cultured DRG neurons.

siRNA mediated knock-down of opa1 in DRG neurons has been shown to disrupt mitochondrial fusion while leaving mitochondrial transport intact (Misko et al., 2010). We next asked whether disruption of mitochondrial fusion was sufficient explain the degeneration of axon caused by MFN2 disease mutants. To this end, we used a previously reported siRNA against opa1 (siopa1) to selectively disrupt mitochondrial fusion and looked for signs of degeneration at 1 week post infection (the same period of time at which MFN2 mutant expressing cell show signs of degeneration). Cultures were transfected with mito-RFP and imaged by time-lapse microscopy to demonstrate normal patterns of mitochondrial transport (Fig. 3 A-B; kymographs). While movements appeared normal, mitochondria were markedly smaller indicating that fusion had been disrupted (Fig. 3 C). At this same time point imaging with Fluo-4 and DCFDA dye showed a complete lack of axon degeneration (D-I). These results suggest that disrupting mitochondrial fusion alone is not sufficient to cause axon degeneration and that perturbation of mitochondrial transport rather than fusion causes degeneration of axons in MFN2 disease mutant expressing neurons.

Axonal mitochondria are abnormally distributed in MFN2 disease mutant expressing DRG neurons.

In axons, mitochondria distribute to areas of high energy and Ca^{2+} flux where they supply ATP and buffer ions critical to the survival of the axon. In cultured DRG neurons, mitochondria distribute uniformly along the axon (Miller and Sheetz, 2004). We asked whether abnormal transport of mitochondria compromised the ability of mitochondria to maintain a uniform distribution, which could contribute to decreased axon stability in MFN2 disease mutant expressing cultures. Mitochondria in DRG neurons infected with wtMFN2, R94Q or siopa1 constructs were labeled with transfect mito-RFP and imaged by time-lapse microscopy (Fig. 4 A-D). Kymographs were used to assist in the identification of all stationary mitochondria, which are represented as black dots underneath kymographs. Notably, while the mitochondrial distribution in wtMFN2 or siopa1 expressing cells appeared uniform (Fig 4. A and D), occasional axon segments in R94Q expressing cells were devoid of mitochondria (Fig. 4 C; arrow head) while other segments appeared normal (Fig. 4 B). To quantitatively describe distribution patterns, axons were divided into 10μm bins and mitochondria were counted in at least 10 bins per axon across 10 different axons. A mitochondrial population was considered uniformly distributed if the average number of mitochondria per bin (μ) was greater than the variance (var), random if μ=var and clustered if μ<var. By this measure, mitochondria were uniformly distributed in wtMFN2 and siopa1 expressing cells but randomly distributed in R94Q expressing cells (Fig.4 E). Comparing observed distributions to a Poisson distribution with the same mean by using a chi-square goodness of fit test confirmed that mitochondrial distributions in wtMFN2 ($p=0.000525$) and siopa1 ($p=0.001446$) axons differed significantly from the Poisson distribution while the population in R94Q expressing neurons did not ($p=.0288$) (Fig.4 E). The random distribution of mitochondria could arise from abnormal mitochondrial movement patterns in mutant expressing neurons. To highlight this point, we calculated mitochondrial flux across segments of axon between stationary mitochondria. On average, mitochondrial flux decreased 6 fold in R94Q expressing cells compared to wtMFN2 and siopa1 neurons (Fig. 4F). The lack of movement could

hamper the ability of mitochondria to maintain a uniform distribution across the axon leaving mitochondrially depleted areas vulnerable to fluctuations in ATP or Ca^{2+}.

To obtain a more complete picture of the mitochondrial network's state, we also estimated the mitochondrial content of axons. Images of axons labeled with MT Green (labels mitochondria independent of $\Delta\psi$) were obtained at 40x and used to estimate the total mitochondrial mass normalized to axonal area. Total mass was decreased ~20% and 40% in R94Q and siopa1 expressing DRG neurons respectively compared to control (Fig. 5 A). A similar decrease of ~15% was seen in R94Q expressing cultures using TMRM dye (labels mitochodnria proportional to $\Delta\psi$), but a slightly larger ~60% decrease was noted in siopa1 expressing cells (Fig. 5 B). This difference could be explained by the presence of mitochondria that have lost their membrane potential in opa1 knock-down cells, which is consistent with previous reports (Chen et al., 2005). Finally, because virally transduced mito-RFP gets incorporated into somatic mitochondria, estimation of mitochondrial mass with this marker approximates the rate of mitochondrial transport out of cell bodies and into axons. We were surprised to notice an ~50% decrease in mitochondrial content in siopa1 cells compared to control which was higher than the 40% decrease seen with MT Green (Fig. 5 C). Rather than reflecting a deficit in transport, which we have not observed by time-lapse microscopy, the decrease could represent the smaller size of mitochondria in opa1 knock-down cells. The more severe ~70% decrease observed in R94Q expressing cells however (Fig. 5 C), correlates well with the observed transport deficit.

While mitochondrial mass is decreased in R94Q expressing neurons, it cannot alone explain the degeneration of axons since opa1 knock-down cultures also experience a more severe deficit in mitochondrial content but show no signs of degeneration. Instead we hypothesize that disrupted mitochondrial transport leads to the uneven distribution of mitochondria in axons of MFN2 mutant expressing cells. This lowers the ability of the axons to compensate for random fluctuations in energy and Ca^{2+} levels which then triggers the stochastic loss of axons we observed in these cultures.

R94Q and siopa1 expressing neurons are reliant on glycolytic production of ATP.

Mitochondrial production of ATP and buffering of Ca^{2+} are dependent on maintenance of $\Delta\psi$. Employing a previously described technique we used TMRM dye to calculate the F_m/F_c value of axonal mitochondria as an estimate of $\Delta\psi$ (see Materials and Methods)(Verburg and Hollenbeck, 2008). One week after infection with viral constructs, the mitochondria in R94Q expressing neurons maintained an F_m/F_c distribution identical to that of mitochondria in wtMFN2 controls (Fig. 6 A and B). Mitochondria in siopa1 infected neurons however, showed a shift in the population distribution toward lower values of F_m/F_c. (Fig. 6 C) consistent with reported effects of opa1 knock-down in fibroblasts. These data suggest that mitochondria in opa1 knock-down cells may not perform oxidative respiration or buffer Ca^{2+} as efficiently as mitochondria in control or R94Q expressing cells.

We also noticed that the media in R94Q or siopa1 expressing cultures began to turn yellow one week after infection. To determine whether the change in media pH was due to increased out put of lactic acid, we measured media lactate levels. Media from both R94Q and siopa1 expressing cultures contained more lactate compared to controls (Fig. 6 D). These data suggested that the neurons in R94Q and siopa1 expressing cultures were increasing their utilization of glycolysis. To test their reliance on glycolytic production of ATP, cultures were treated with 15mM 2-deoxy-glucose (2DG) to inhibit glycolysis. Within 48hrs signs of axonal degeneration became apparent in R94Q and siopa1 expressing neurons but not the wtMFN2 control. Notably, cell bodies did not appear to be undergoing apoptosis or necrosis suggesting that axons were more sensitive to the inhibition of glycolysis. From the $\Delta\psi$ measurements we had expected that decreased mitochondrial function in opa1 knock-down cells would foster a reliance on glycolysis, however, it was less expected in R94Q expressing cells where mitochondrial $\Delta\psi$ appears unaltered. One possibility is that the reliance on glycolysis of R94Q and siopa1 expressing cells may be due to the observed decrease in axonal mitochondrial mass. Alternatively, lack of proper mitochondrial distribution and movement in R94Q expressing neurons may leave some axon segments dependent on glycolysis.

Tetrodotoxin treatment prevents axonal degeneration in MFN2 disease mutant expressing cells.

The cascade of molecular events perpetuating the degeneration of hypoxic axons in has been well studied. Energy deficits in an axon lead to failure of the Na^+-K^+ pump, which allows Na^+ to accumulate in the cytoplasm. This forces the Na^+-Ca^{2+} exchanger to reverse directions and triggers an influx of Ca^{2+} that elicits the final stages of axon degeneration (Stirling and Stys). In experimental hypoxia paradigms, addition of TTX to rat optic nerve obstructs influx of extracellular Na^+ and prevents downstream degeneration (Wolf et al., 2001). We reasoned that the abnormal distribution of mitochondria in MFN2 mutant expressing cells would leave axonal segments devoid of mitochondria in a state of energy crisis and that treatment of these cells with TTX could prevent their degeneration. To this end we treated R94Q or H361Y expressing DRG cultures with 1 or 0.25 μM TTX on DIV4 through DIV11 when cultures were imaged with DCFDA dye. Treatment with TTX markedly decreased the number of DCFDA positive axons in mutant expressing cultures (Fig. 7 A and C). To quantify the effect, fluorescent images were converted into binary format (Fig. 7 B and D) and fluorescent pixels were counted. Treatment with either 1 or 0.25 μM TTX shows a significant protective effect in both R94Q and H361Y expressing cells (Fig. 7 E).

Expression of Mfn1 ameliorates degenerative effects of the R94Q disease mutant on cultured DRG neurons.

A previous study revealed that expression of exogenous Mfn1 could restore the axonal transport of mitochondria in Mfn2 null DRG neurons (Misko et al., 2010). Though TTX could never be used as a therapeutic agent against axon degeneration, we wondered whether expression of exogenous Mfn1 could prevent degeneration of axons in cultures of R94Q expressing neurons. Following the initial infection of cells with virus containing an R94Q construct on DIV3, cells were infected again on DIV4 with lentivirus containing an Mfn1 construct. When cultures were imaged with Fluo-4 dye on DIV12, signs of axonal degeneration were completely absent (Fig. 8 A-D).

Introduction of Mfn1 also increased the number of mitochondria transported into axons and the mitochondrial flux of mitochondria along the axon (Fig. 8 E and F). These data support that manipulating levels of Mfn1 in affected neurons of CMT2A patients may offer a viable therapeutic target for drug development.

Discussion

These experiments demonstrate that CMT2A associated MFN2 disease mutants trigger the selective degeneration of axons due to the disruption of mitochondrial transport rather than fusion. Abnormal transport slows the incorporation of somatic mitochondria into axons and prevents mitochondria from distributing uniformly across axons. Segments of axon left devoid of mitochondria must rely on the more inefficient process of glycolytic ATP production and thus have reduced energy reserves. This state of local "virtual hypoxia" in axons is supported by the ability of TTX to prevent the degeneration of axons in MFN2 disease mutant expressing cultures. Finally, the partial rescue of mitochondrial transport by exogenous expression of Mfn1 prevents MFN2 disease mutant induced axon degeneration and maybe a viable target for drug development in the treatment of CMT2A.

Mitochondrial function in the maintenance of axon integrity

The conduction of action potentials along nerves and white matter tracks is costly, requiring energy to pump ions across the plasma membrane and establish the resting membrane potential. Despite the high demands of action potential propagation, neurons have low intrinsic energy reservoirs and require a continuous supply of oxygen and glucose.

Disruption of energy production in axons initiates a series of events leading to axonal degeneration: 1) failure of the Na^+-K^+ pump from lack of ATP 2) Na^+ accumulation through persistent Na^+ channels and K^+ efflux 3) reversal of the Na^+- Ca^{2+} driven by high axoplasmic Na^+ and consequent accumulation of intracellular Ca^{2+}. High intra-axonal Ca^{2+} marks a late stage in axon degeneration leading to the activation of calpains, and degradation of the neurofilament network (Trapp and Nave, 2008).

Remarkably, even a transient local deficit in mitochondrial metabolism can be sufficient to trigger axonal degeneration. Experimental exposure of peripheral nerves to NO, which inhibits mitochondrial respiration, initiates Wallerian degeneration that spreads distal to the administration site, even when the NO is removed within 3 hours of exposure. The same effects are seen with a

brief administration of DNP, an uncoupler of the IMM (Alvarez et al., 2008). Similar local deficits are likely to arise when disruption of mitochondrial transport leave segments of axon devoid of mitochondria, creating a state of local "virtual hypoxia". Our data suggest that this mechanism may be the inciting cause of axon degeneration in CMT2A.

Though our study focuses on CMT2A, the importance of mitochondrial mobility is also relevant to other neurodegenerative diseases. In demyleinating disorders such as multiple sclerosis (MS), mitochondria redistribute to denuded axon segments, presumably to meet the increased energy demands of action potential propagation (Andrews et al., 2006; Hogan et al., 2009; Witte et al., 2009). In damaged axons in brains of humans with multiple sclerosis and of mice with cuprizone-induced demyelination, evelvated levels of cytosolic HDAC1 were shown to directly inhibit mitochondrial transport (Kim et al.). The increased need for mitochondria at denuded axon segments and pathological mechanisms that inhibit mitochondrial transport are directly opposed. Developing drugs to increase the efficacy of mitochondrial transport may tip the balance in favor of increasing transport and improve the survival of demyelinated axons by increasing their mitochondrial reserves.

Disrupted mitochondrial transport has also been implicated in Alzhiemer's disease where Aβ42 has been shown to disrupt mitochondrial distribution in a Drosophila model of Alzhiemer's disease (Iijima-Ando et al., 2009). Though axonal transport is globally affected in Alzhiemer's, disrupted mitochondrial transport may be an early event, instigating axon degeneration and the development of axon spheroids.

We are only beginning to understand the downstream consequences of disrupted mitochondrial transport. As our understanding develops, new molecular players and signaling pathways may emerge with wide spread implications for a number of neurodegenerative disease.

The selective degeneration of axons in CMT2A

A striking feature of CMT2A is the selective degeneration of neurons with long axonal despite the ubiquitous expression of MFN2 in all cells. It is reasonable to consider that long axons may be

more susceptible to deficits in mitochondrial transport based on their length for different reasons. First, abnormal transport could significantly delay or even prevent the migration of newly synthesized mitochondria in the cell body from reaching the most distal axon segments. Though we observed this phenomenon in our culture system, diminished mitochondrial populations are not evident in sural nerve from CMT2A patients (Verhoeven et al., 2006; Vallat et al., 2008). There is also evidence that mitochondrial biogenesis may take place in axons negating the dependency on transport of mitochondria from the soma (Amiri and Hollenbeck, 2008; Vincent et al., 2010). Alternatively, we suggest that a subtle deficit in regulation of mobility may perturb proper mitochondrial distribution and compromise axon integrity in CMT2A patients. This mechanism could also explain the length dependence of the axon degeneration as the longer length of the axon provides a greater area over which random fluctuations in ATP and Ca^{2+} thus giving rise to more events for which immobile mitochondria could not compensate. Degeneration triggered in a single location would soon lead to degeneration of the remaining distal axon.

It is also possible that apart from their sensitivity to disrupted mitochondrial transport neurons may express lower levels of Mfn1 relative to other cell types. We have previously reported that levels of Mfn1 mRNA are similar in mouse embryonic fibroblasts and DRG neurons and Mfn1 is present at the protein level in DRG cultures (Misko et al., 2010). Notably, in DRG neurons levels of Mfn1 protein were slightly lower and appeared as two distinct bands on western blot analysis, one of the expected and one of lower molecular weight. Though it is hard to know if the lower levels of Mfn1 are enough to explain the lack of compensatory function in neurons, future efforts toward understanding transcriptional and translation regulation of Mfn1 could be useful in developing

Targeting Mfn1 in the treatment of CMT2A

We currently lack a clear understanding of how Mfn1 is regulated at a transcriptional or post-transcriptional level. One possible approach to increasing Mfn1 in neurons would be to identify small molecule that stimulate expression of the gene. Efforts are now being made to map the Mfn1 promoter region and develop an in vitro assay that will allow high through put screening of

compounds that could increase Mfn1 expression. Selectively targeting of the Mfn1 locus will be extremely important however, as increasing the expression of the mutant Mfn2 alleles in a CMT2A patient would be counterproductive. Thus potential drugs will have to both increase the expression of Mfn1 while not affecting levels of Mfn2

Though the implications are unclear, Mfn1 may also be post transcriptionally or post translationally processed as suggested by western blot data in DRG neurons. Either type of processing may remove functional full length Mfn1 protein and could therefore be a therapeutic target. While Morpholino oligomers could be used to favorably affect splicing, enzymes that may cleave Mfn1 have not yet been identified.

Whichever the case, a deeper understanding of the regulation and function of Mfn1 could hold the key to treating patients with CMT2A.

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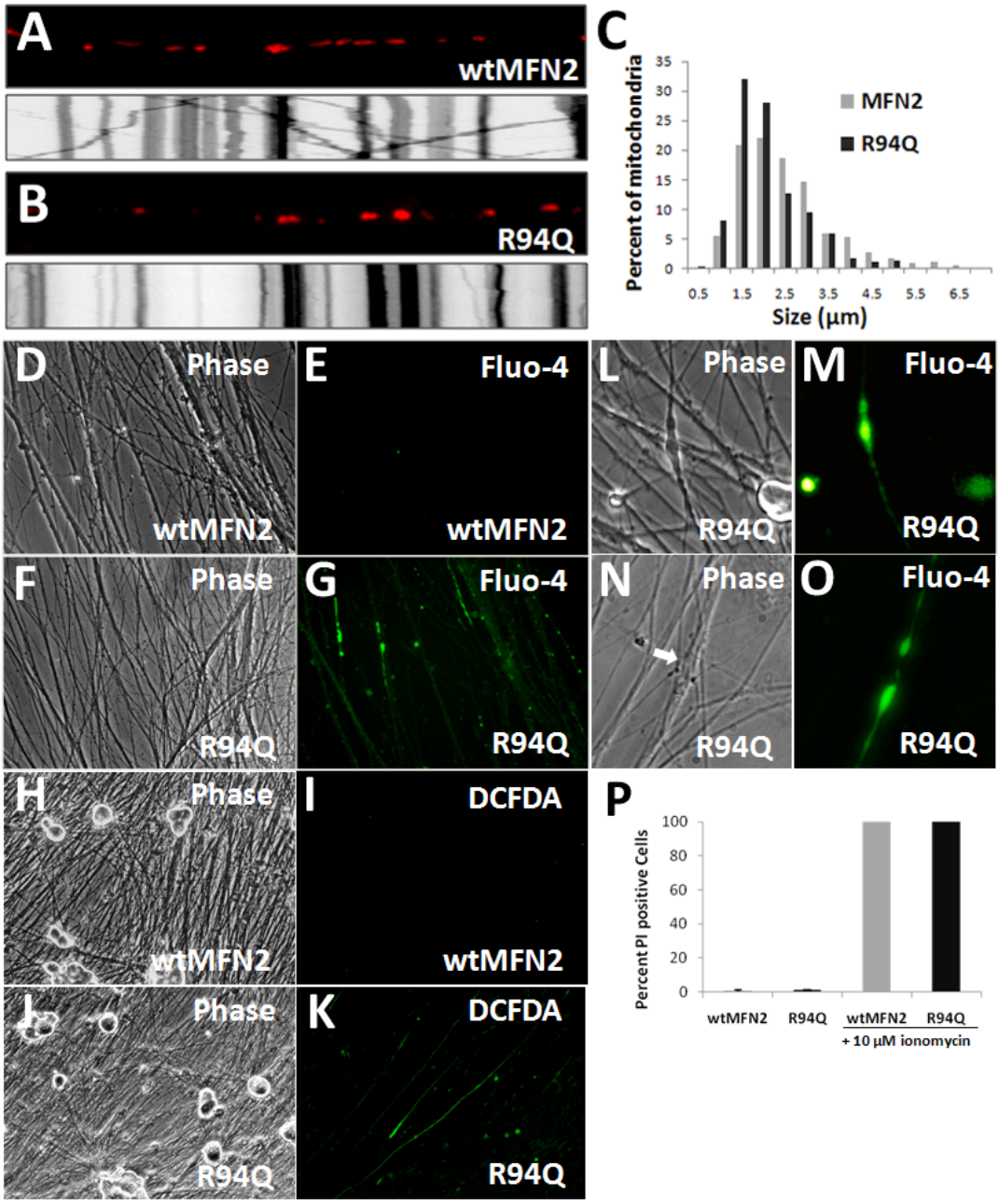

Figure 3-1: **CMT2A associated MFN2 mutants cause axonal degeneration in cultured DRG neurons.** (A-B) Mitochondria in cultured DRG neurons expressing virally transduced wtMFN2 or R94Q constructs were labeled with mito-RFP and imaged by time-lapse microscopy. Consistent with our previous reports, axonal mitochondria in R94Q expressing neurons show abnormal

movement patterns (A-B; kymographs) 1 week after infection. (C) Size frequency histogram demonstrating shorter mitochondrial lengths indicative of disrupted fusion in R94Q expressing neurons. (D-O) Imaging with Fluo-4 and DCFDA dyes revealed a fraction of axons in R94Q expressing cultures with elevated levels of intra-axonal Ca^{2+} and ROS respectively. Axons labeled by either dye develop a dilated or beaded appearance (L-M) indicative of degeneration. Lack of cell death observed with propidium iodide staining indicates that axons are the primary site of degeneration in R94Q expressing neurons (P).

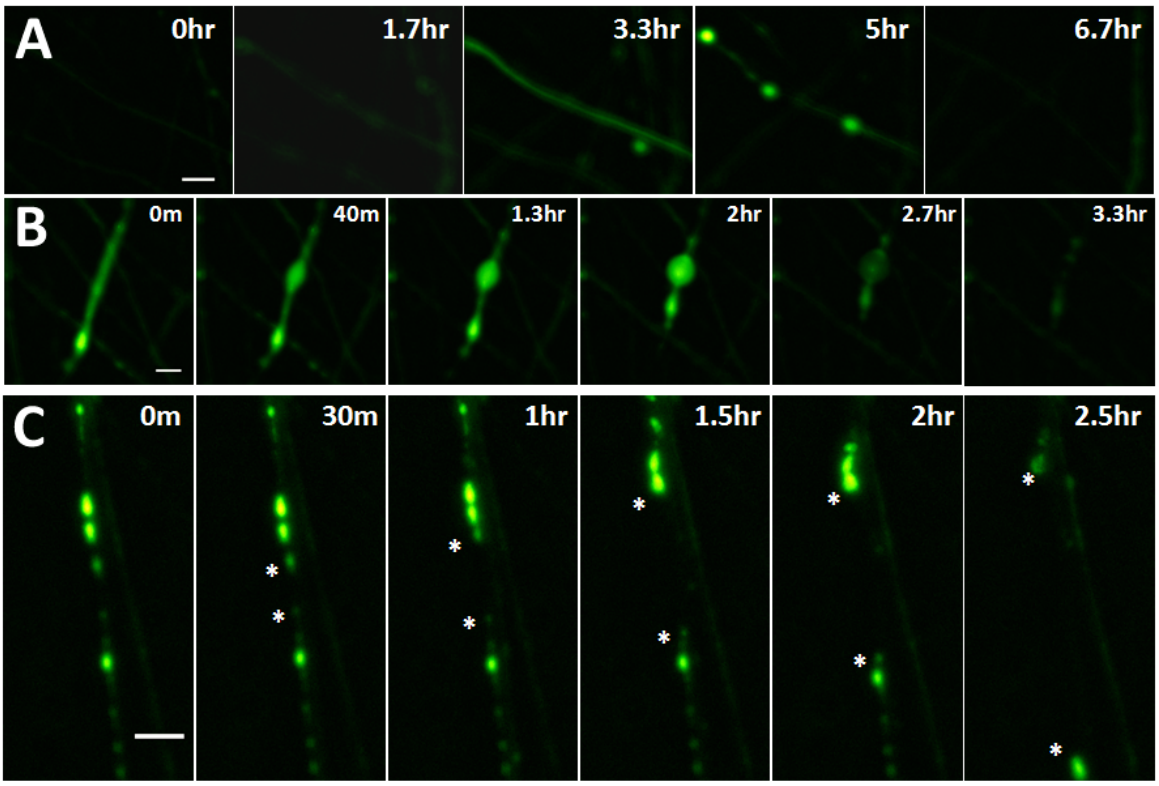

Figure 3-2: **Axons of R94Q expressing DRG neurons are actively degenerating.** R94Q expressing neurons were incubated with Fluo-4 dye and axons demonstrating elevated Ca^{2+} levels were visualized by time-lapse microscopy (A-C; 40X magnification). Three main patterns of degeneration were noted. First, several axons that were not initially visible with Fluo-4 dye demonstrated a rapid elevation in intra-axonal Ca^{2+} levels followed by beading of the membrane and finally disappearing, presumably from the inability to retain dye following loss of membrane integrity (A). Second, segmental axonal dilations were noted which swelled to a spheroid like shape before completely degenerating (B). Finally, a number of already beaded axons lost structural integrity at a single point resulting in retraction of axon ends from the break point (C; asterisks label ends of break point).

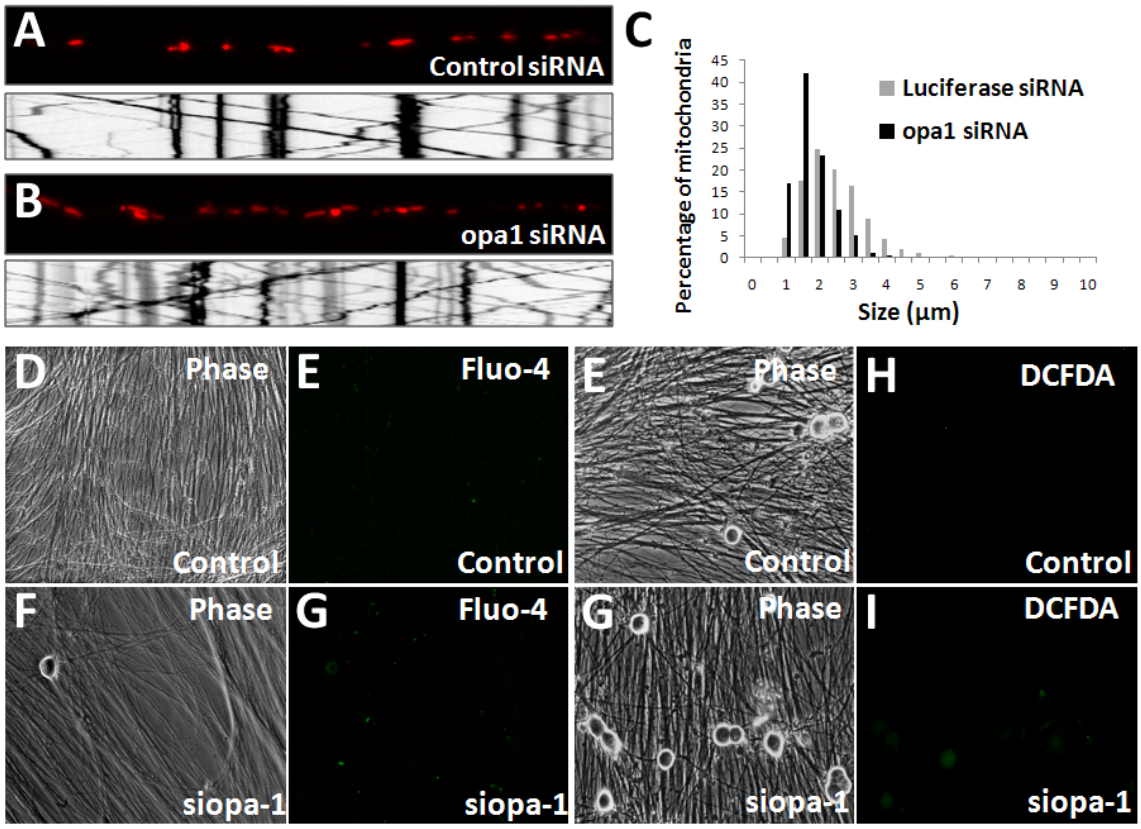

Figure 3-3: **Disruption of mitochondrial fusion is not sufficient to cause axonal degeneration.** Mitochondria in cultured DRG neurons expressing virally transduced control siRNA or opa1 targeted siRNA were labeled with mito-RFP and imaged by time-lapse microscopy. As reported previously, knock-down of opa1 leaves mitochondrial transport intact (A and B; kymographs) but results in smaller fragmented mitochondria (C) due to disrupted fusion. Imaging with Fluo-4 (D-G) or DCFDA (E-I) dyes revealed that signs of axonal degeneration were absent in opa1 knock-down cultures.

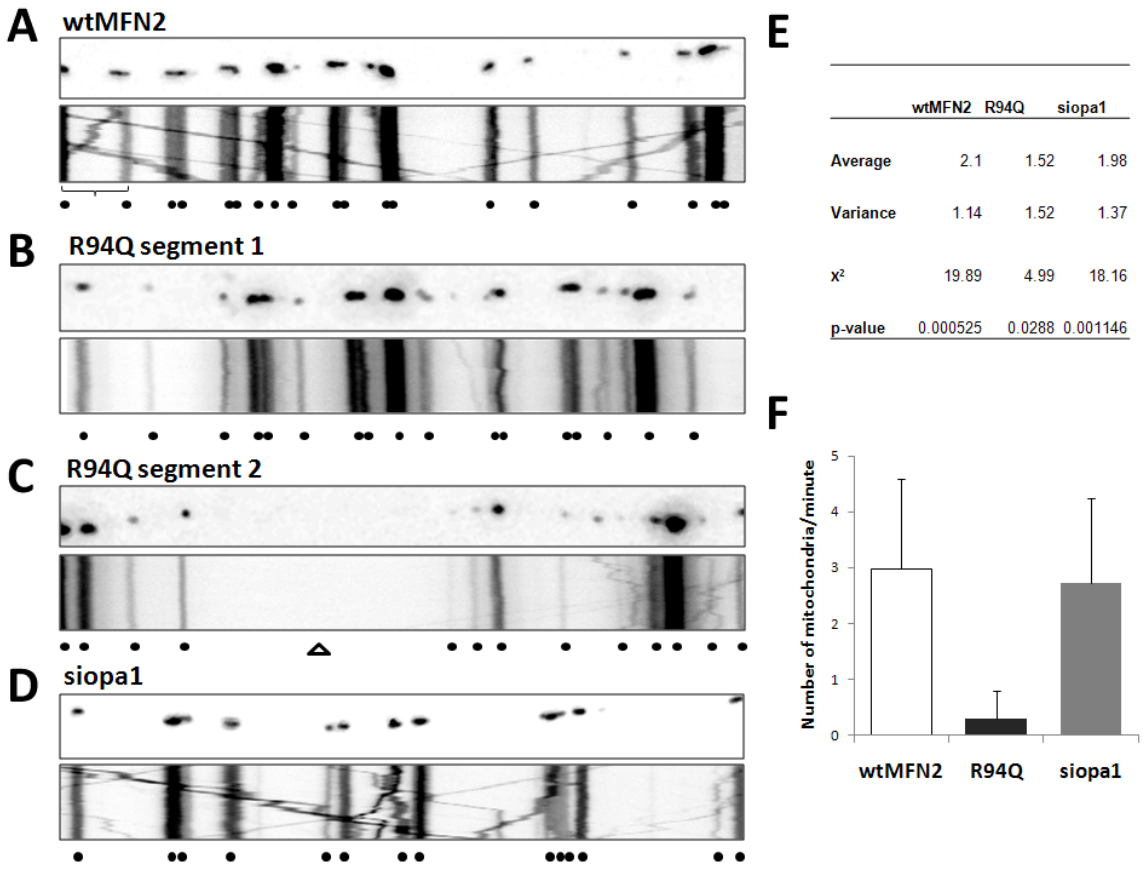

Figure 3-4: **Axonal mitochondria are abnormally distributed in R94Q expressing neurons.** Mitochondria in cultured DRG neurons expressing virally induced wtMFN2, R94Q or siopa1 constructs were labeled with mito-RFP and imaged by time-lapse microscopy 1 week after infection. (A-D) Kymographs were used to assist in the proper identification of fluorescently labeled mitochondria. Individual mitochondria are represented as black dots below kymographs. While mitochondria were evenly distributed along axons of wtMFN2 and siopa1 expressing neurons, R94Q expressing cultures contained both axons having evenly distributed mitochondria (R94Q segment 1) or axons displaying abnormal segments that were devoid of mitochondria (R94Q segment 2; arrow head). (E) Axons from 10 neurons were divided into 10μm bins and the number of mitochondria was counted per bin. Distributions were described by comparing the average number of mitochondria (μ) per bin to the variance (var) such that μ>var is considered uniform, μ=var random and μ<var clustered. While wtMFN2 and siopa1 expressing cell showed uniform distributions of mitochondria, mitochondria in R94Q expressing cells were distributed

randomly. The measured distributions were also compared to a Poisson distribution with a chi-square goodness of fit test. Only distributions from wtMFN2 and siopa1 expressing cells were significantly different, confirming the random distribution of mitochondria in R94Q expressing cells. (F) Within each bin, the number of mitochondria that moved past the center mark in 1 minute was used to estimate mitochondrial flux.

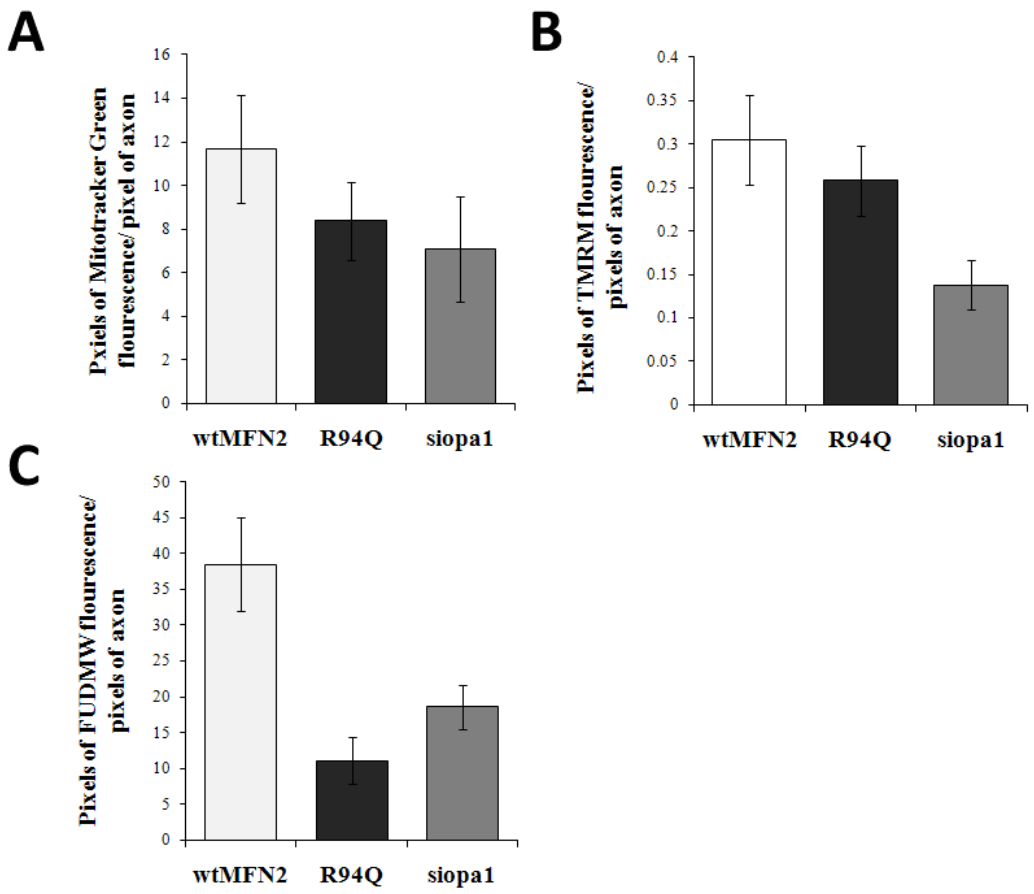

Figure 3-5: **Quantification of axonal mitochondrial content and transport into axons.** Mitochondria in axons of cultured DRG neurons were labeled with Mitotracker Green (A), TMRM (B) or Lenti-viral transduced mito-RFP (C), 1 week after cultures were infected with wtMN2, R94Q or siopa1 constructs. (A) Total mitochondrial content estimated with Mitotracker Green and normalized to axon area (labels mitochondria independent of $\Delta\psi$) showed decreases in the mitochondrial content of R94Q and siopa1 expressing cells. Changes could reflect decreased numbers, size or movement of mitochondria in axons. (B) Relative content of polarized mitochondria in axons using $\Delta\psi$ sensitive TMRM dye showed the most severe decrease in siopa1 expressing cultures. (C) Because transduced mito-RFP is incorporated into mitochondria in the soma, measurement of axonal mitochondrial content using mito-RFP estimates the rate at which mitochondria are moving into axons. Taken together with measurements using Mitotracker Green, the severe decrease in mitochondrial content of R94Q expressing cells reflect the decreased movement of mitochondria into axons.

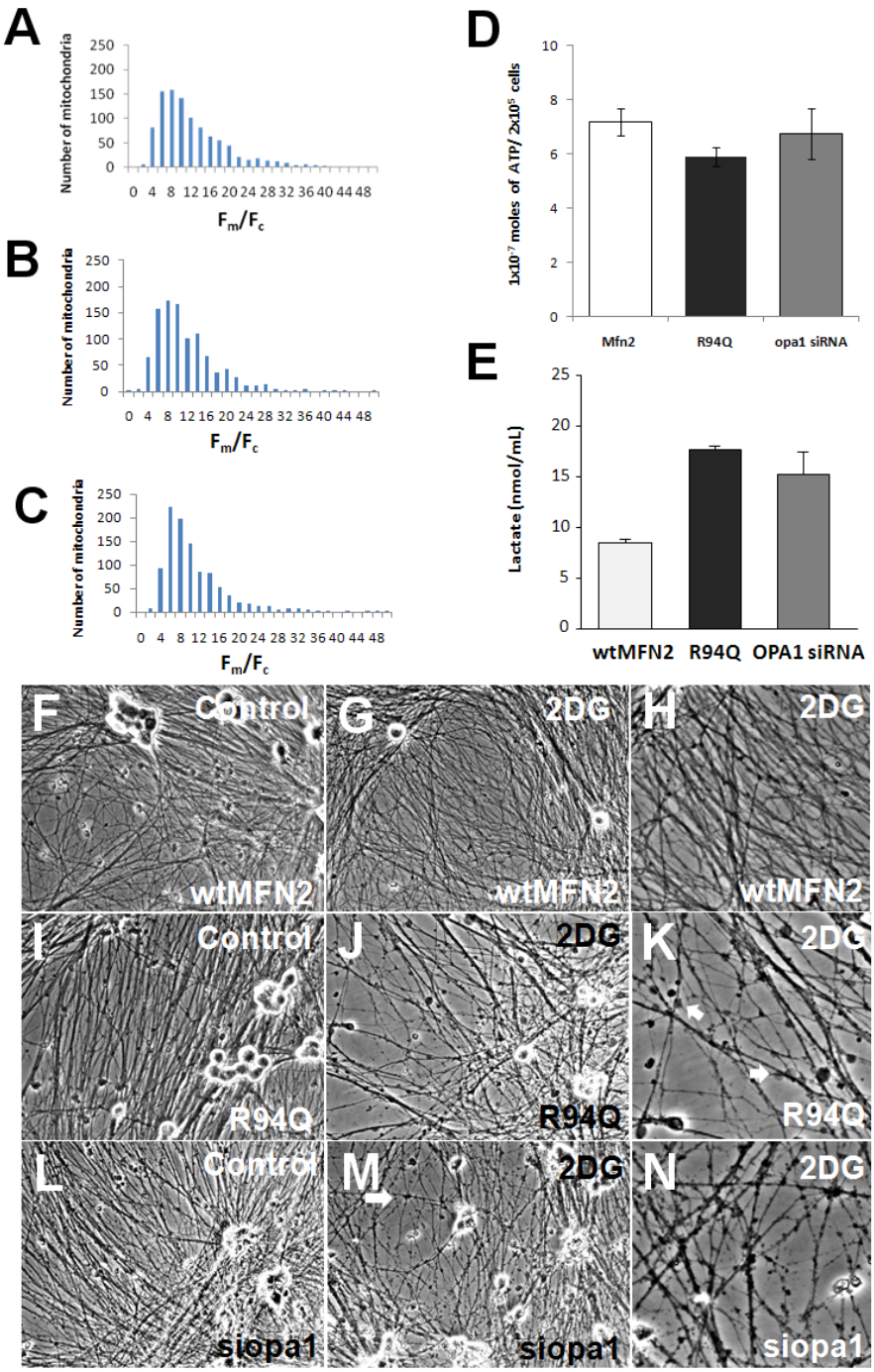

Figure 3-6: **R94Q and siopa1 expressing DRG neurons are reliant on glycolytic production of ATP.** (A-C) TMRM dye was used to calculate F_m/F_c (an estimate of $\Delta\psi$ that corrects for cytoplasmic background) values of mitochondria in wtMFN2 (A), R94Q (B) and siopa1 (C) expressing cells. Only the population of mitochondria in opa1 knock-down cells show a shift to lower values of $\Delta\psi$. (D) Increased levels of lactate in the media of R94Q and siopa1 expressing

cells indicate increased use of glycolysis. (E-M) Treating cells with 15mM 2DG for 48hrs produces signs of axonal degeneration in R94Q and siopa1 expressing cells, supporting their shift to a more glycolytic profile.

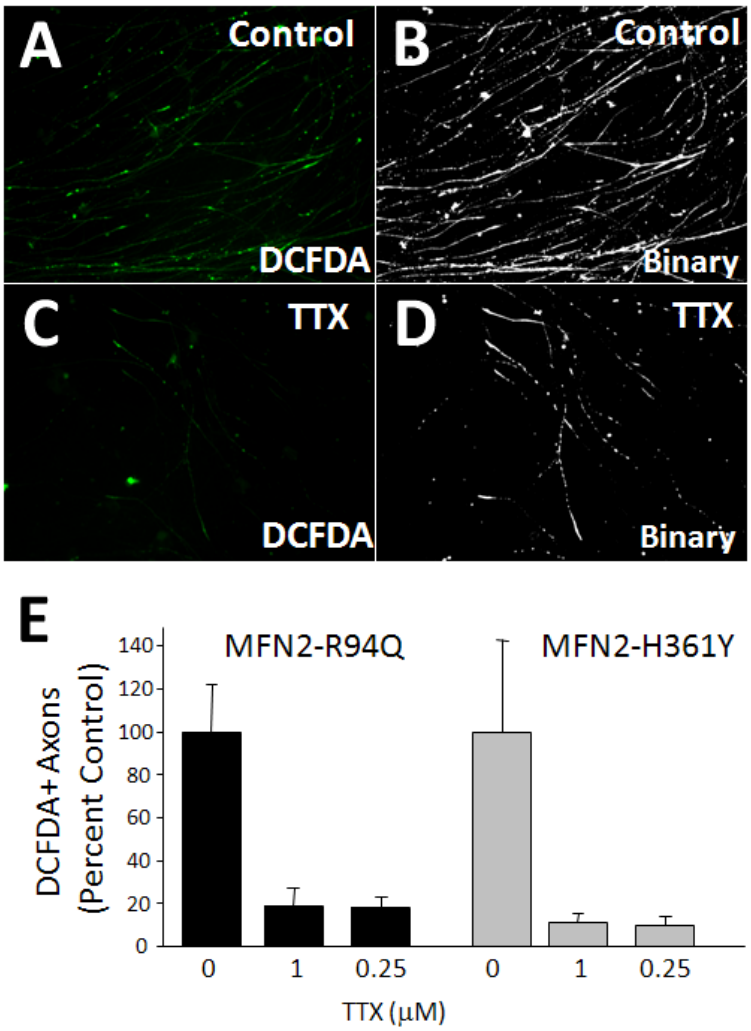

Figure 3-7: **TTX treatment prevents axonal degeneration in cultured DRG neurons expressing MFN2 disease mutants.** Based on TTX's ability to delay axonal degeneration by preventing influx of Na^+ in models of optic nerve hypoxia, we treated DRG cultures expressing the R94Q or H361Y disease mutants with TTX on DIV4 through DIV11. (A and C) DCFDA imaging showed a marked decrease in the number of axons with elevated levels of ROS. To quantify the effect, images were converted into binary format (B and D) and fluorescent pixels were counted. (E) Compared with untreated controls, DRG cultures treated with TTX show a significant decrease in the number of DCFDA positive degenerating axons one week after infection with R94Q or H361Y viral constructs.

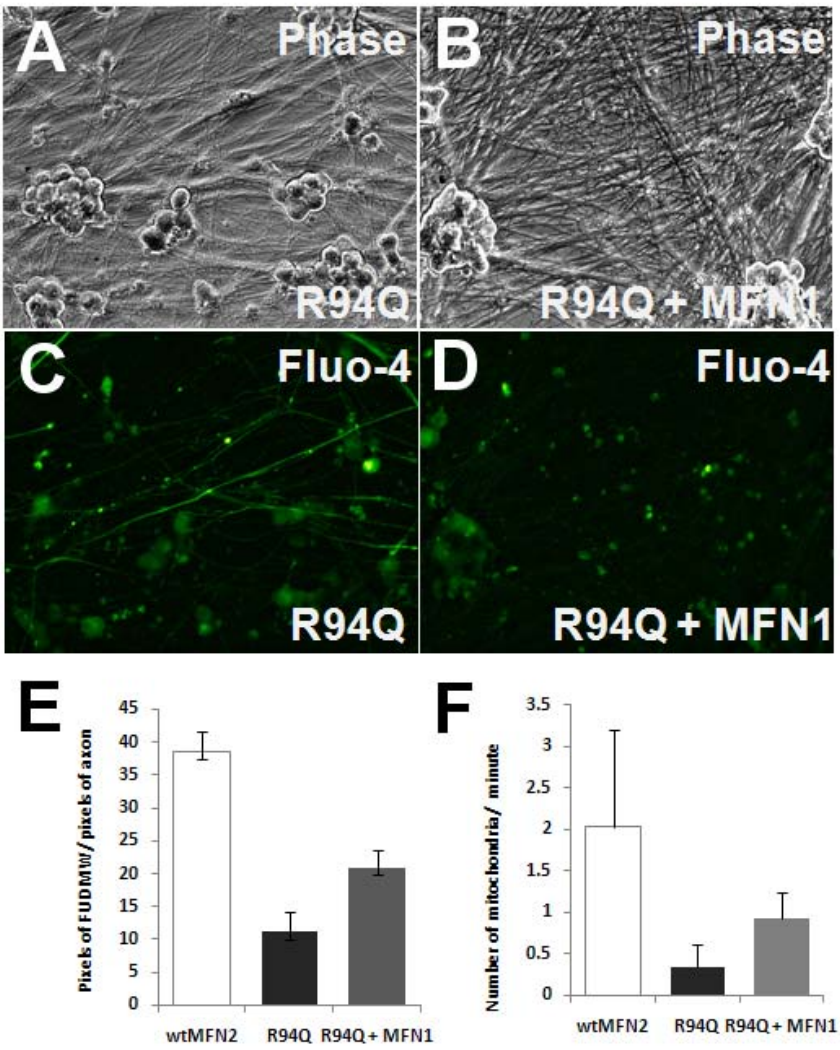

Figure 3-8: **Expression of Mfn1 ameliorates degenerative effects of the R94Q disease mutant on cultured DRG neurons.** Based on our previous findings that increased Mfn1 expression could restore mitochondrial transport in Mfn2 null DRG neurons, we reasoned that overexpression of Mfn1 could ameliorate the degenerative affects of MFN2 disease mutants. (A-D) Cultured DRG neurons were infected with R94Q construct on DIV 3 and subsequently infected with control or Mfn1 constructs on DIV4. Fluo-4 imaging performed on DIV 12 showed a striking absence of degenerating axons in cultured neurons expressing both R94Q and Mfn1. Expression of Mfn1 also increased the net transport of mitochondria out of the soma and into axons (E) as well as increasing the local flux of mitochondira (F).

Chapter 4

An R94Q knock-in mouse model of CMT2A

Introduction

Understanding the mechanisms that orchestrate axonal degeneration is key to our understanding of neurodegenerative processes and our ability to prevent them. Though the importance of axon degeneration is highlighted by its ubiquitous appearance in a number of neurodegenerative diseases (Raff et al., 2002), there are several peripheral neuropathies that are primary axon diseases (Zuchner and Vance, 2006). One of these neuropathies, Charcot-Maire-Tooth Disease type 2A (CMT2A), is most commonly linked to mutations in the *mitofusin 2* (*MFN2*) gene and may serve as a useful model to gaining a more general understanding of how axons die.

CMT2A is a hereditary peripheral neuropathy characterized by dying back degeneration of the longest axons in the body. Patients usually present within the first two decades of life and show varying degrees of motor and sensory disability. Electrophysiology studies reveal normal to slightly decreased nerve conduction velocities with significantly decreased amplitudes. As the longest axons are affected first, symptoms typically progress from the feet to the hands and affect motor more severely than sensory function. Muscle atrophy following denervation can leave patients wheel chair bound leading to a high morbidity rate associated with CMT2A.

To date, an animal model that mirrors the genetic condition in CMT2A patients has not been established, though two separate transgenic overexpressing mice have been reported. The first utilizes the motor neuron specific Hb9 promoter to drive expression of the T105M MFN2 dominant disease mutant in a transgenic mouse model (Detmer et al., 2008). Though the animals exhibit an abnormal gait, loss of motor axons and atrophy of the tibialis anterior muscle, which are consistent with the phenotype of CMT2A, there are several problems with this line. First, the animals had to be bred to homozygosity to produce a phenotype. This ultra high expression of a mutant allele is not congruent with human patients who only have one copy of the mutant allele.

Secondly, the Hb9 promotor expresses at high levels early during development and shuts off shortly after birth. Consistent with this pattern, the animals appear to have a developmental defect rather than a neurodegenerative disease. The second model reported uses the prion protein promotor to express the R94Q disease mutant in a transgenic mouse model (Cartoni et al.). Though the mutant allele expresses in all neurons, this model does not have a neurodegenerative phenotype. Though gait abnormalities are observed when the animal is bred to homozygosity, loss of axons or signs of muscle denervation are not observed.

In contrast to previous models, knocking in a dominant mutant allele using homologous recombination and breeding the mice to heterozygosity would construct a more accurate mouse model of CMT2A. To this end, we had obtained an R94Q knock-in mouse line (Detmer and Chan, 2007) and examined the phenotype of heterozygous mice. Surprisingly, the heterozygous mice show no overt phenotype though their homozygous littermates die within three weeks of age. Examination of distal nerve fibers revealed no loss of axons and no change in the profile of fiber diameters. Additional stresses to the animals, including peritoneal injection of 2-deoxy-glucose or introducing a mutant PMP22 allele to induce a demyelinating stress also did not produce a remarkable phenotype in R94Q heterozygous animals.

Materials and Methods

Nerve conduction and electromyography

Animals were anesthetized with avertin and placed in the prone position on a heated pad to maintain body temperature. Nerve conduction velocity and EMG studies were performed with a Viking Quest portable EMG machine (Nicolet). EMG studies were obtained using a 27-gauge monopolar needle electrode with a 70 × 500 μm recording surface (PRO-37SAF; Electrode Store) was inserted into the tibialis anterior (TA) or gastrocnemius/soleus muscles. A 29-gauge reference needle electrode (GRD-SAF; Electrode Store) was inserted subcutaneously in close approximation to the recording electrode and a subdermal ground electrode was placed on the back. Spontaneous electrical activity was recorded for 90 s.

Nerve histology and axon counts

Dissected tibial nerves were fixed over night at 4°C in 3% glutaraldehyde followed by secondary fixation in 1% osmium tetroxide, sequential dehydration in 50, 75, 90 and 100% ethanol and embedding in plastic resin. Two hundred to 400nm nerve sections were stained with toludine blue and used to count the total number of axons in the tibial nerve.

Imaging mitochondrial distribution in tibial nerve and neuromuscular junctions

Dissected Tibial nerves and soleus muscles from MitoK/+ mice were fixed for 1hr in 4% paraformaldehyde and washed in phosphate buffered saline. Nerves were teased, desiccated at -20°C overnight and visualized with a (SCOPE) using a 60X oil immersion objective. Soleus muscles were incubated for 30min with (%) alexa433-conjugated alpha-bungratoxin, washed and whole mounted under a coverslip and visualized in the same manner as tibial nerves.

DRG cultures and mitochondrial movement analysis

Mouse pups from R94Q heterozygous mice breedings were obtained at embryonic day 12.5. Dorsal root ganglia were dissected, trypsinized for 15 minutes at 37°C and plated as spot cultures

on a 24well plate coated with poly-D-lysine and laminin. Cells were maintained in Neural Basal media (Invitrogen) B27 supplement (Invitrogen), 50ng/mL of NGF, 5mM glutamine and antibiotics. Cells were treated with 5-flurouracil for the first 3 days to eliminate non-neuronal cells. To visualize mitochondrial movements, cultures were infected with Lenti-virus carry a mitochondrially targeted RFP on the third day in culture. Four days after infection imaging was performed in a climate-controlled chamber (In Vivo Scientific) at 37°C and 5% CO2 and images were acquired with a Cool Snap HQ 2 CCD camera (Photometrics) mounted on a Nikon Eclipse Ti-U microscope. Images were acquired at 40X magnification every 1s for 5min. Kymographs were then generated and analyzed using MetaMorph software (Molecular Devices).

Fluo-4 calcium imaging

After 1 week in culture mouse DRG neurons were incubated with 25μM Fluo-4 dye (Invitrogen) for 30min at 37°C. Media was changed to wash out excess dye followed by a 30min incubation at 37°C to allow complete esterification of intracellular Fluo-4. Relative levels of intra-axonal calcium were then observed using epiflourescent microscopy in a climate-controlled chamber (In Vivo Scientific) at 37°C and 5% CO2.

Results

Mice heterozygous for the R94Q allele do not show an overt neurodegenerative phenotype.

In the hopes of obtaining a mouse model of CMT2A, we examined knock-in mice heterozygous for the R94Q mfn2 allele (Detmer and Chan, 2007). Mice up to a year in age appear behaviorally normal, display a normal gait and no not show signs of muscle atrophy. Electrophysiology studies revealed normal conduction velocities along the sciatic nerve in heterozygous animals compared to littermate controls at 2 months (Wt: 36.00 +/-4.82 m/s; R94Q/+: 35.00 +/-3.16; Fig.1A) and 6 months (Wt: 37.00 +/-3.61 m/s; R94Q: 39.00 +/-2.00 m/s; Fig.1B) of age. This indicated that patterns of myelination are normal in the heterozygous animal as expected. Conduction amplitudes were also did not differ between mutant and control animals at 2 months (Wt: 5.37+/-1.19 m/s; R94Q: 5.36+/-1.56 m/s; Fig.1A) or 6 months (Wt: 7.63+/-0.62 m/s; R94Q: 7.14+/-2.19 m/s: Fig.1B) of age. Additionally, EMG findings were unremarkable at 2 and 6 months of age (Fig.1C and D). The normal electrophysiology findings corroborate the overtly normal phenotype of the animals.

Plastic embedded section of tibial nerve from R94Q/+ and Wt littermates showed no signs of Wallerian degeneration (Fig.2A-D) and axon counts were normal (Fig.2E and F). To assess whether mitochondria were properly distributed along axons in mutant mice, dihybrid mutants of the R94Q and MitoK allele were created. MitoK transgenic mice express a mitochondrialy targeted CFP molecule driven by the Thy1 promotor in neurons. Tibial nerves from double mutants and controls were teased and the mitochondrial content in single axons examined. Consistent with the normal phenotype of the heterozygous mice, mitochondria appeared normally distributed along axons (Fig.3A and B). Though there was a wide inter-axonal variation in mitochondrial content, the intra-axonal variation was low, which was true in both mutant and control animals. Whole mount examination of soleus muscles stained with fluorescent alpha-bungratoxin revealed that mitochondria populated synaptic boutons in both R94Q/+ and Wt animals and that all postsynaptic membranes were appropriately paired with a presynaptic bouton (Fig.3 and D). Finally we cultured DRG neurons from E12.5 R94Q/+ embryos or littermate

controls. After infecting cultures with a mitochondrially targeted RFP molecule, we observed normal patterns of mitochondrial transport in R94Q/+ axons (Fig.4A and B). Consistent with the absence of a mitochondrial transport deficit, no signs of axon degeneration were detected using Fluo-4 dye (Fig.4C-F).

Surprisingly, a single R94Q allele, which produces a severe and early onset form of CMT2A, does not seem to affect the peripheral nervous system of mice. Contrary to our predictions, mitochondrial distribution was unaltered in peripheral axons indicating that a single R94Q allele is not sufficient to disrupt mitochondrial transport in mouse neurons. We speculate that the differences between mice and humans could be attributable to the shorter absolute length of mouse axons, an absolute length of time necessary for disease manifestation that is longer than a year, or compensatory mechanisms present in the mouse such as higher expression levels of mitofusin 1.

Challenging R94Q heterozygous mice with 2-deoxy-glucose or demyelination does not bring out a degenerate phenotype.

We reasoned that R94Q/+ mice might already employ compensatory mechanisms to deal with the presence of on dysfunctional MFN2 allele and that challenging the mice might push axons beyond their compensatory capacity. Because R94Q overexpressing cultured DRG neurons are sensitive to challenge with 2DG (chapter 3), we administered two different daily regimens of intraperitoneally injected 2DG over a 7 day period and looked for signs of axonal degeneration in plastic embedded tibial nerve sections. Neither the 750mg/kg or 1000mg/kg regimens produced signs of Wallerian degeneration or axon loss (Fig.5A and B). Importantly, the doses we used represented maximally tolerable doses of 2DG as an injection of 1500mg/kg caused mice to become catatonic and seize.

Axonal mitochondria have been show to localize to areas of denuded axon in models of multiple sclerosis. We have also been able to show that axonal mitochondria localize to demyelinated axon segments in peripheral nerves of Trembler J (TrJ) mice (unpublished observation), which have a demyelinating disease caused by a point mutation in the PMP22 gene

(Valentijn et al., 1992). We reasoned that creating a demyelinating stress that necessitates mitochondria to relocate might bring out a subtle mitochondrial transport deficit in the R94Q/+ mice. Because a single TrJ allele is sufficient to create a severe demyleinating phenotype we created dihybrid mutants that were heterozygous for both the R94Q and TrJ alleles. These mice look phenotypically like TrJ/+ littermates and display intention tremors and hind limb grasping when lifted by the tail. On examination of plastic embedded sections of tibial nerve, both TrJ/+ and R94Q/+;TrJ/+ mice display signs of demyleination and axon loss (Fig.6A-D). Axon loss in these animals is consistent with previous electrophysiological evidence of axonal degeneration in the TrJ mouse (Meekins et al., 2007). The degree of fiber loss however, did not differ between TrJ/+ and R94Q/+;TrJ/+ mice. Though these mice were examined at 2 months of age, the degree of demyelination at this time point is sufficient to trigger mitochondrial relocalization. Though further observation of these mice over longer time points will be pursued, we expect there is a low likely hood of these mice developing a more severe phenotype.

Discussion

Despite the severe degenerative phenotype produced in humans from the presence of a single R94Q allele, mice heterozygous for the allele appear completely normal. One possible explanation for this difference is the shorter length of mouse axons. As discussed in Chapter 3, we hypothesize that longer axons are more susceptible to perturbations in mitochondrial mobility because of the longer distances across which metabolic or ionic imbalances may accrue. Because there can be an ~10 fold difference in the length of a human and mouse leg, axons in mice may be less likely to degenerate from subtle abnormalities in mitochondrial transport. Alternatively, there could be a dependency on mitochondrial transport down the entire length of the axon making transport over greater distances less efficacious. Another possible explanation could involve the shorter life span of mice. Rather than depending on the relative age of the mouse, accumulation of insults necessary to cause axonal degeneration could take an absolute amount of time beyond 1 year. In humans CMT2A usually appears within the first two decades of life. Therefore it could take as long as 20 years for the phenotype to show. Lastly, the repertoire of expressed genes in mouse peripheral neurons could differ from humans and confer resistance to the mutant MFN2 allele. Specifically, the level of the MFN2 homologue MFN1 may be expressed at higher levels in the mouse nervous system and compensate for the function of mutant allele.

The resilience of the R94Q/+ mice to metabolic or demyelinating stress suggests that mitochondrial transport may be completely normal in these animals. This is consistent with the absence of mitochondrial transport abnormalities or axonal degeneration in Drg neurons culture from heterozygous mice. It is important to note that the disrupted transport and axon degeneration we observed in our previous culture conditions (chapter 3) involve high levels of mutant protein expression that may out-compete or overwhelm compensatory mechanisms that preserve function in heterozygous R94Q mice.

Analyzing the R94Q homozygous mice may offer more insight as removing the one wild type mfn2 allele will create a functional null. Though the animals are likely to display pathology in

multiple tissues, it may be possible to assess the effect of disrupted mitochondrial transport by analyzing mice at early time points. Alternatively, R94Q knock-in mice could be crossed to mfn1 null mice to prevent its compensatory action.

Though generating an animal model of CMT2A has been challenging, such a model would be invaluable both in understanding the pathophysiology of CMT2A and testing drug therapies aimed at stopping axonal degeneration. For these reasons, future attempts at generating mutant lines are sure to drive us closer to obtaining a suitable model of CMT2A.

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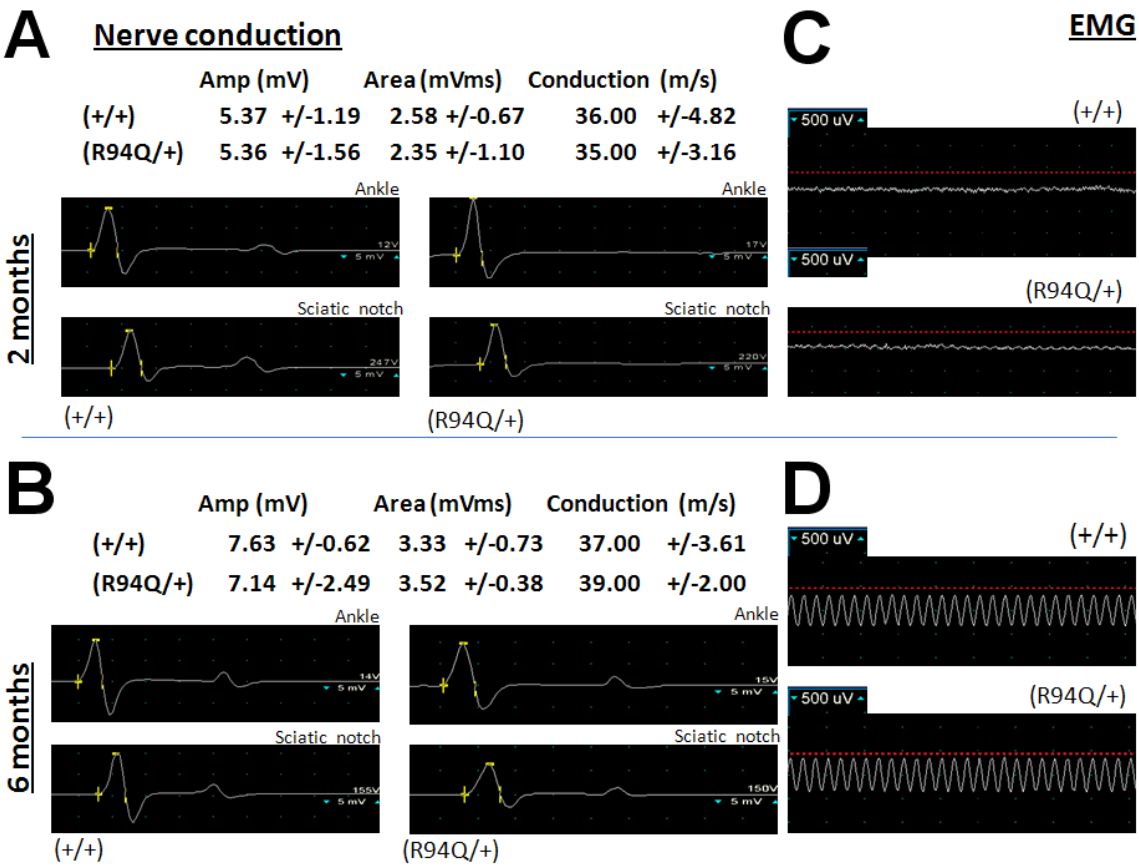

Figure 4-1**: Electrophysiology studies of R94Q heterozygous knock-in animals at 2 and 6 months of age.** (A and B) Nerve conduction studies were performed on 2 month and 6 month old R94Q/+ mice across the sciatic nerve. Nerve conduction velocity and conduction amplitude were identical between mutant and littermate control animals at both time points. (C and D) Electromyograms taken at 2 and 6 months of age are unremarkable indicating normal innervation of the gastrocnemius muscle.

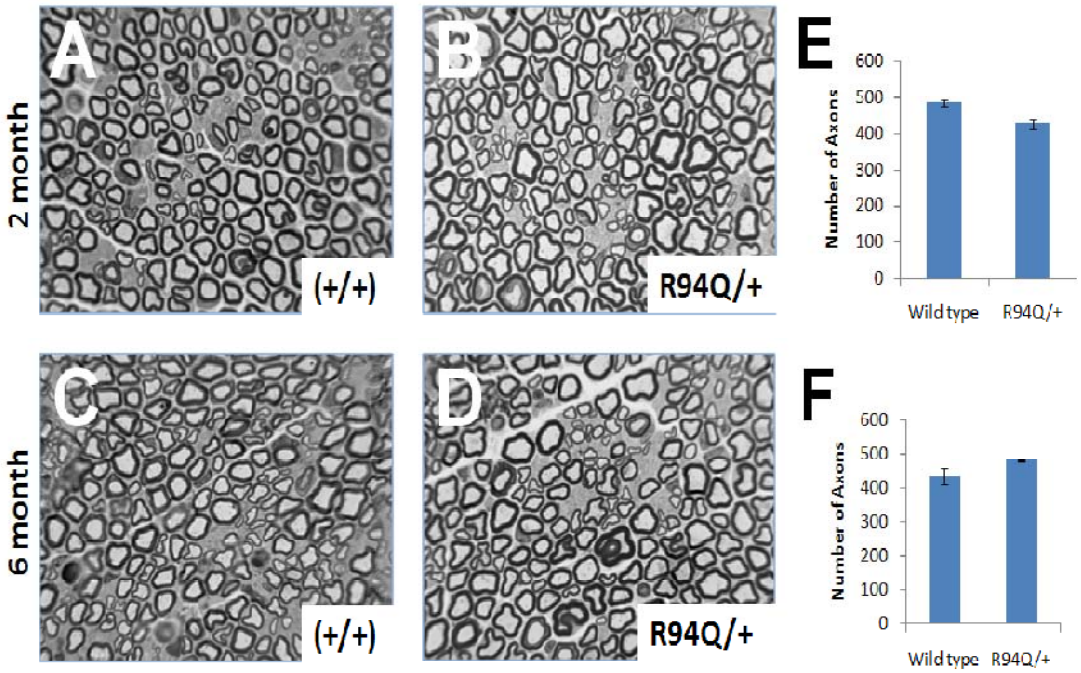

Figure 4-2**: Tibial nerve sections from R94Q heterozygous mice reveal the absence of Wallerian degeneration or axon loss.** Plastic embedded sections of tibial nerve from 2 month (A and B) and 6 month (C and D) old mice show intact nerve fibers and normal patterns of myelination. Axon counts (E and F) confirm the absence of axonal degeneration in R94Q/+ mice.

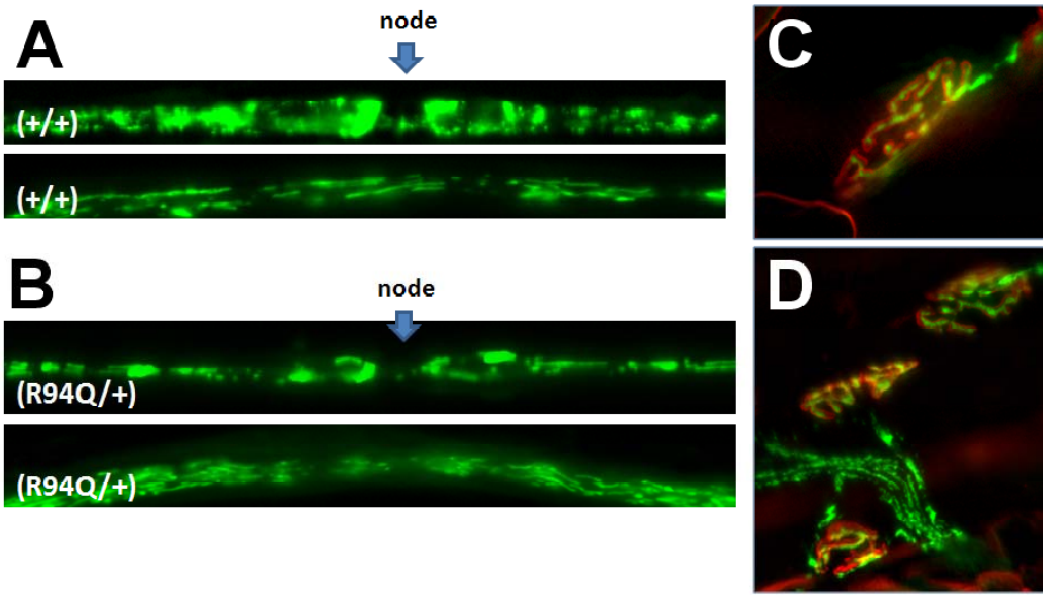

Figure 4-3**: Mitochondria show normal distribution in R94Q heterozygous tibial nerve and properly populate presynaptic terminals at neuromuscular junctions.** (A and B) Teased nerve fibers from R94Q/+;MitoK/+ mice and MitoK/+ littermate controls reveal relatively uniform distribution of mitochondria across nodes and internodal regions. Though intraxonal variation in mitochondrial content was qualitatively low, inter axonal variability was high in both R94Q/+;MiotK/+ and MitoK/+ preparations. (C and D) In whole mount preparations of soleus muscle stained with fluorescent bungratoxin, mitochondria are shown to properly populate presynaptic boutons and every presynaptic terminal was properly matched with a post-synaptic density in both mutant and control mice.

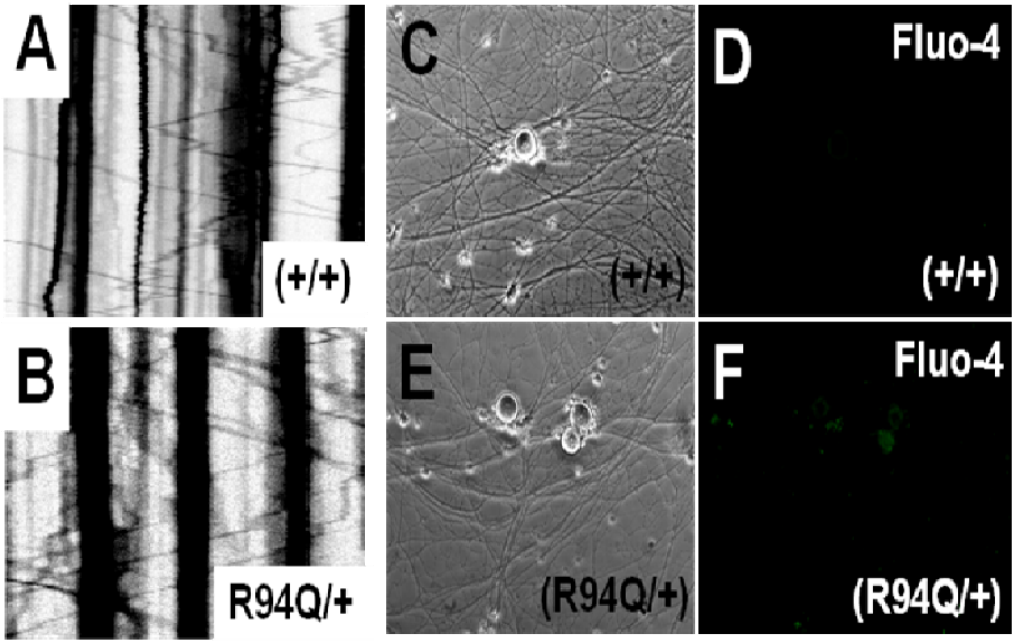

Figure 4-4**: Mitochondiral transpost and axonal integrity are uncompromised in Drg neurons cultured from R94Q heterozygous mice.** Time-lapse microscopy and kymograph analysis of mitochondiral transport in Drg neurons cultured from R94Q/+ and control mice (A and B) revealed normal patterns of transport in mutant neurons. (C-F) Fluo-4 staining of R94Q/+ and control Drg neurons show that axonal degeneration is absent in cultures of mutant neurons.

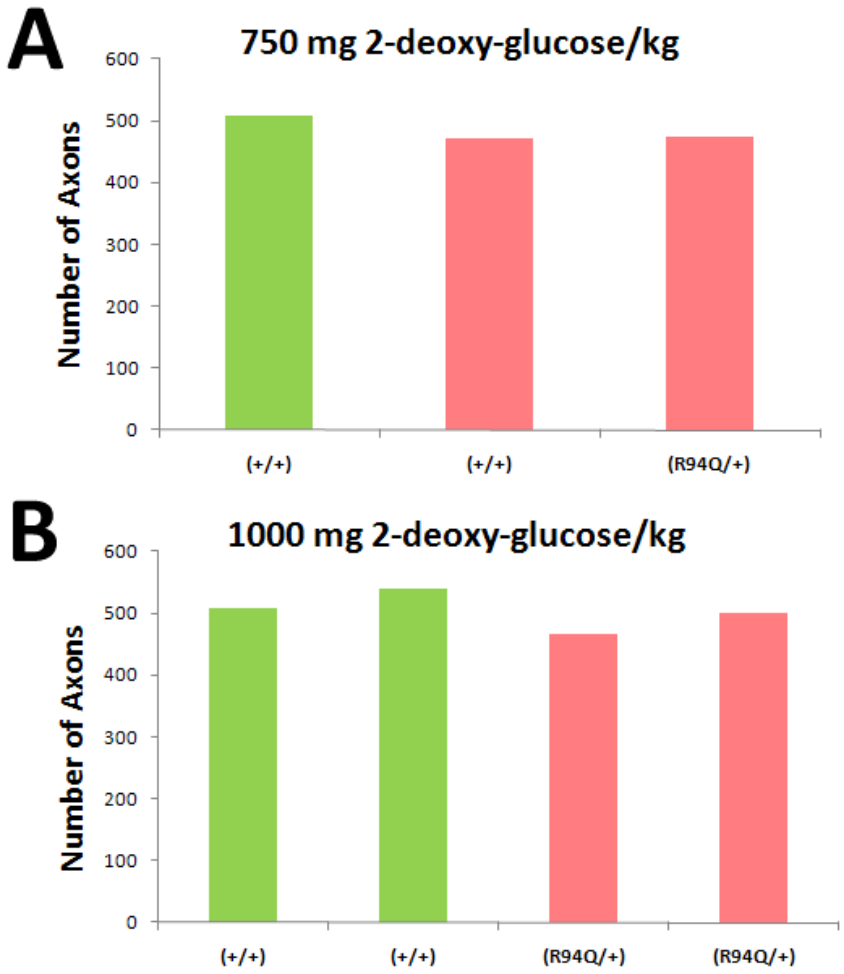

Figure 4-5**: R94Q heterozygous mice display normal tolerance to 2-deoxy-glucose.** 2DG was administered via i.p. injection at 750 mg/kg (A) or 1000 mg/kg (B) every day for 7 days. Plastic embedded sections of tibial nerve were used to estimate the number of axons remaining after the 2DG regimen. Neither regimen produced differential loss of axons in R94Q/+ mice compared to controls.

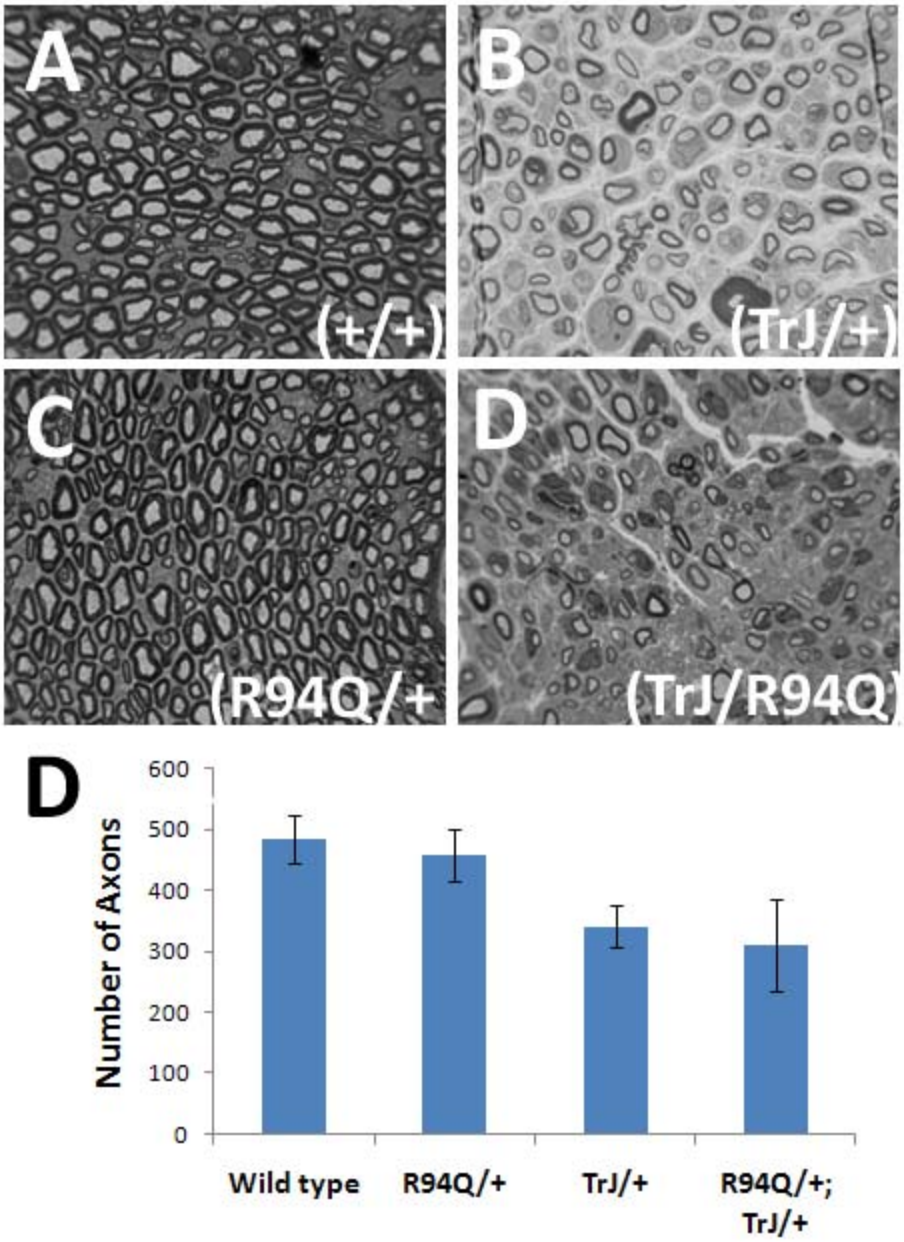

Figure 4-6**: R94Q/+; TrJ/+ dihybrid mutants do not display an increase in axon loss.** While plastic embedded sections of +/+ (A) and R94Q/+ (C) tibial nerve appeared normal, while TrJ/+ (B) and R94Q/+;TrJ/+ sections showed obvious axonal loss and signs of Wallerian degeneration. Quantification of axon loss (F) revealed that the amount of axon loss did not differ significantly between TrJ/+ and R94Q/+;TrJ/+ mice indicating that axons from R94Q/+ mice did not display an increased sensitivity to demyleination.

Chapter 5

Conclusions and Future Directions

The work described in this dissertation helps to establish a new role for mitofusins in the transport of axonal mitochondria and provides evidence that proper mitochondrial movement and distribution support axonal integrity. We also demonstrate that inhibition of mitochondrial fusion is not sufficient to disrupt transport or cause axonal degeneration in culture, arguing that MFN2's role in mitochondrial transport maybe more pertinent to the pathogenesis of CMT2A than its role in fusion. This difference also helps to clarify why mutations in MFN2 and OPA1, both known to mediate mitochondrial fusion, have distinct neurological consequences. We have also identified overlapping roles of MFN1 and 2 in mediating mitochondrial transport, which may offer hope as a target for therapeutic intervention in CMT2A. If MFN1 can be upregulated in the affected cell population, the effects of mutant MFN2 may be ameliorated.

Though this work helps begins to clarify the molecular mechanisms underlying CMT2A, there is much future work is needed to fully understand the role mitofusins play in mitochondrial transport and elucidate pathways leading to axon degeneration. In conjunction, identifying the regulatory elements controlling levels of neuronal MFN1 could offer an avenue for the first viable treatment for CMT2A. The following sections will discuss both the contextual significance of some of our findings and point out areas of future research to be pursued.

Mitofusins regulating mitochondrial transport

Mitochondrial transport and fusion are continuous processes. In order to reach a target membrane, mitochondria must be actively transported. In fact, if mitochondrial transport is disrupted, fusion will also be disrupted as a result of immobility. Control of fusion and transport must be integrated to assure that actively fusing mitochondria are not being transported away from the target membrane. The selection pressure to integrate these two processes may have

given rise to the novel regulatory role of mitofusins in transport. Since both mfn1 and 2 are endowed with this function, the molecular predecessor of the two isoforms must have acquired the role. Only one isoform of mitofusin is present in yeast, suggesting that a mitofusin predecessor was already an integral component of mitochondrial transport regulation before the appearance of metazoan animals.

Along with OPA1, DRP1 and several other proteins, mitofusins are categorized as members of the large dynamin-like GTPase family. The classic dynamin molecule mediates vesicle membrane tubulation and scission, which is directly analogous to DRP1's function in mitochondrial membrane fission. Mitofusins and OPA1 however, seem to perform the opposite function and mediate mitochondrial membrane fusion. Common to both processes, however, are membrane deformation and the generation of force to fuse or separate the membranes. These abilities seem to underlie the primary functions of all members of the large dynamin-like GTPase family. The application of membrane deformation and force generation to the regulation of transport however, is not easy to surmise. Over the course of natural history, mitofusins may have developed a unique function in the control of transport.

A mechanistic explanation of how mitofusins regulate transport is still unclear. In chapter one of this dissertation evidence is given to support the direct of mfn2 and mfn1 in mediating mitochondrial movements. Loss of mitochondrial mobility in axons of mfn2 null mice and the interaction of mfn2 and mfn1 with the miro/milton complex, argue strongly for their direct involvement. It was also shown that mitofusins do not act as a direct link between the outer mitochondrial membrane and microtubule-based motors and MFN2 disease mutants do not disrupt the miro/milton or milton/kinesin interactions. Interestingly, recent studies have demonstrated that the motor domain of Kif5 can bind to miro in the presence of elevated intra-axonal Ca^{2+}, disengaging Kif5 from microtubules and allowing mitochondria to cease moving. Because mitofusins bind to miro proteins, they could feasibly regulate this interaction. Loss of this regulatory function would explain the cessation of mitochondrial movement in mfn2 null DRG neurons. The case of the dominant negative MFN2 disease mutants is a bit trickier. If a single mitochondrion has multiple miro/milton/kinesin complexes, there is no reason why one complex

containing a mutant MFN2 protein would inhibit movement propagated by another complex on the same mitochondrion with a wild type MFN2 molecule. If multiple functional units are required per mitochondrion, however, it could explain the markedly slower mitochondrial movements we observed with overexpression of mutant proteins. This would also fit with the observation that higher levels of mutant protein expression lead to more severe transport deficits, which would reflect the increased presence of miro/milton/kinesin complexes interacting with a mutant MFN2 molecule. Alternatively, if a mitochondrion contains only one miro/milton/kinesin complex, the presence of mutant MFN2 protein would easily cause a severe disruption of transport.

Though the number of the number of miro/milton/kinesin complexes per mitochondrion is still unknown, testing the ability of MFN2 to abrogate the interaction of kinesin motor heads with miro proteins would be straight forward. It is however only one possible explanation and another molecular player that interact with mitofusins could be key in their ability to regulate transport.

Mitofusin 2 also interacts with PINK1 kinase, which localizes to mitochondria and decreases the frequency of their movements (unpublished correspondence). Our preliminary data shows that MFN2 and MFN2 disease mutants coimmunoprecipitate with PINK1. Though the mutants do not show an increased binding affinity for PINK1, their abnormal function may play an unforeseen role in balancing the affect of PINK1 activity on mitochondria.

Finally we are only beginning to understand the architecture of the mitochondrial transport apparatus. There are probably many more proteins that regulate the transport of mitochondria that may interact structurally or functionally with mitofusins. As future studies begin to elucidate the complete composition of the transport complexes we may be able to fit together the missing pieces of the puzzle.

Mitochondrial transport and axonal degeneration

Based on the work presented in this dissertation, we hypothesize that mitochondrial transport acts not only to deliver mitochondria to distal sites in the axon but also to actively redistribute mitochondria along axons. Meeting local energy and Ca^{2+} buffering demands in this way is

probably essential for the integrity of the axons in vivo. We also postulate that the susceptibility of long axons in CMT2A may be due to this phenomenon. Because a single critical insult along an axon is capable of causing degeneration of the entire axon distal to the site, longer axons would be more susceptible to defects in mitochondrial redistribution simply because the probability of one such critical insult occurring is greater with increasing axon length.

We feel that this explanation is more likely than a catastrophic deficit in the delivery of mitochondria to the distal axon based on 2 of our observations: 1) loss of mfn2 in cultured neurons results in abnormal mitochondrial movement patterns but mitochondria still populate the entire axon and 2) mitochondrial transport in r94Q heterozygous mice appears grossly normal suggesting that the transport deficit in CMT2A patients may be subtle. Though Chapter 3 of the dissertation discusses how our data supports the importance of local mitochondrial movement in supporting axon integrity, there are several topics that should still be addressed.

Mitochondiral biogenesis in neurons

The current dogma holds that mitochondrial biogenesis occurs exclusively in the soma of neurons. If this is true, given the average velocity of a moving mitochondria is ~1μm/sec, it would take a mitochondria originating in the cell body of a motor neuron approximately 11.5 days to reach the presynaptic terminals innervating muscles of the feet. The estimated half-life of mitochondrial protein in the brain is around 24 days. This would mean that a mitochondrion would have turned over roughly 24% of the proteins it started with in the soma by the time it reached the neuromuscular junctions in the feet. It's hard to know if this decrement would have any functional consequence on a mitochondrion and it may stand to reason that supplying new mitochondria solely from the cell body is a viable strategy. Furthermore, translation of genes encoded in the mitochondrial genome could continue at this distant location. Mitochondrial genes encoded in the nucleus could only be newly synthesized if mRNA were transported down the axon and translated; however, transport of mRNA down axons has been well established for numerous genes and translation of mRNA in axons is beginning to be appreciated. Accordingly, distally stationed mitochondria may be able to rejuvenate their entire repertoire of proteins.

If mitochondria can only be synthesized in the cell body and distributed to the distal axon by active transport, than disruption of mitochondrial transport by MFN2 disease mutants could severely decrease the population of viable mitochondria in distal nerves of CMT2A patients. This would opposeour view on the central importance of local mitochondrial redistribution in generating a length dependent neuropathy. The two explanations however, are not mutually exclusive. Hampered delivery of mitochondria down axons and deficits in local redistribution of mitochondria could both contribute to the disease pathophysiology.

Emerging data does exist to support that occurrence of mitochondrial biogenesis in axons. The ability of mitochondria to replicate their genome within axons coupled with the ability of axonal mitochondria to increase in number by active fission argues that mitochondrial pools in distal axons may not be reliant on transport of new mitochondria from the soma. If this holds true, it would lend further credence to our idea that local redistribution of mitochondria is sufficient to explain the length dependent degeneration of axons and that trans-axonal transport of mitochondria is not necessary to explain the phenomenom.

Mitochondria in the axons of CMT2A patients

Little is currently known concerning the affects of MFN2 disease mutants on mitochondrial function and transport in CMT2A patients. Conflicting stories have arisen over the functional status of mitochondria in patient fibroblasts. Mitochondrial respiration appears either completely normal or slightly increased (due to a decoupling effect and inefficient oxidative phosphorylation) while mitochondrial fusion is unaffected by several different mutant alleles. The status of mitochondrial function and transport in patient neurons however is completely unknown. Currently our lab is developing several lines of induced pluripotent stem cells from CMT2A patient fibroblasts. After differentiating these cells into neurons, we hope to directly address the function and mobility of mitochondria in patient neurons.

Direct observation of axonal mitochondria in CMT2A patients has come from two sources: 1) EM of sural nerve biopsies and 2) immunoflourescent visualization of mitochondria in

sensory nerve endings present in patient skin biopsies. In both cases, mitochondria appear to properly populate distal nerve segments, though EM of sural nerve sections reveals scattered clusters mitochondria with abnormal cristae against a back drop of normal appearing distribution of mitochondria. This argues against a severe transport deficit that limits trans-axonal transport but would be consistent with a subtle defect that disrupts local mitochondrial movements and distribution.

Increasing the levels of neuronal MFN1 to treat CMT2A.

Excitingly, increasing levels of MFN1 in cultured DRG neurons expressing MFN2 disease mutants, attenuates the mitochondrial transport deficit and eliminates signs of axon degeneration. While we have shown that mRNA levels of mfn1 are nearly identical in mouse embryonic fibroblasts and DRG neurons, protein levels of mfn1 are slightly lower in DRG neurons and appears as two bands on western blot, one lower in weight than expected. Mfn1 may be differentially spliced or post translationally processed specifically in neurons. It has be speculated that neurons may be selectively vulnerable to mutations in mfn2, if neuronal levels of mfn1 were too low to compensate for the dysfunctional mfn2 allele. Perhaps the lower weight molecular variant of mfn1 present in mouse DRG neurons does not retain the functions of the full length protein and neurons are left with lower levels of mfn1 than fibroblasts, which are not affect in CMT2A. Alternatively, the detectable amounts of mfn1 protein in mouse neurons may explain why the heterozygous R94Q mice described in chapter 4 do show a phenotype. Further work to determine the relative expression levels of MFN1 in human neurons would help clarify the situation.

Once the expression pattern of MFN1 in human neurons is elucidated, further efforts can be made to increase its prese456789 ce by way of small molecules. If MFN1 mRNA levels are low, mapping of the MFN1 promotor region would allow construction of a high through put assay to identify drugs that would increase expression levels. Alternatively, if diminished levels of MFN1 are caused by alternative splicing patterns, morpholino oligomers could be designed to favor the

full length MFN1 mRNA product. Either way, we to focus our future efforts on testing the viability of this treatment strategy and keep a cure for CMT2A within our sights.