Loss of Miro1-directed mitochondrial movement results in a novel murine model for neuron disease.

Tammy T Nguyen  
*Department of Biochemistry, University of Utah School of Medicine*

Sang S Oh  
*Department of Neurology, University of Michigan, Ann Arbor*

David Weaver  
*MitoCare Center, Department of Pathology, Anatomy, and Cell Biology, Thomas Jefferson University*

Agnieszka Lewandowska  
*Department of Biochemistry, University of Utah School of Medicine*

Dane Maxfield  
*Department of Biology, University of Utah, Salt Lake City*

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Loss of Miro1-directed mitochondrial movement results in a novel murine model for neuron disease


Departments of Biochemistry, Pathology, Neurology, University of Utah School of Medicine, Salt Lake City, UT 84112; Department of Neurobiology, University of Michigan, Ann Arbor, MI 48109; Mitocare Center, Department of Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, Philadelphia, PA 19107; Departments of Biology and Pharmacology and Toxicology, University of Utah, Salt Lake City, UT 84112; and Department of Biology, Faculty of Sciences, Kyushu University, Fukuoka 812-8581, Japan

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Defective mitochondrial distribution in neurons is proposed to cause ATP depletion and calcium-buffering deficiencies that compromise cell function. However, it is unclear whether aberrant mitochondrial motility and distribution alone are sufficient to cause neurological disease. Calcium-binding mitochondrial Rho (Miro) GTPases attach mitochondria to motor proteins for anterograde and retrograde transport in neurons. Using two new KO mouse models, we demonstrate that Miro1 is essential for development of cranial motor nuclei required for respiratory control and maintenance of upper motor neurons required for ambulation. Neuron-specific loss of Miro1 causes depletion of mitochondria from corticospinal tract axons and progressive neurological deficits mirroring upper motor neuron disease. Although Miro1-deficient neurons exhibit defects in retrograde axonal mitochondrial transport, mitochondrial respiratory function continues. Moreover, Miro1 is not essential for calcium-mediated inhibition of mitochondrial movement or mitochondrial calcium buffering. Our findings indicate that defects in mitochondrial motility and distribution are sufficient to cause neurological disease.

Ca2+-dependent motility | Miro GTPase | mitochondrial respiration

Motor neuron diseases (MNDs), including ALS and spastic paraplegia (SP), are characterized by the progressive, length-dependent degeneration of motor neurons, leading to muscle atrophy, paralysis, and, in some cases, premature death. There are both inherited and sporadic forms of MNDs, which can affect upper motor neurons, lower motor neurons, or both. Although the molecular and cellular causes of most MNDs are unknown, many are associated with defects in axonal transport of cellular components required for neuron function and maintenance (1–6).

A subset of MNDs is associated with impaired mitochondrial respiration and mitochondrial distribution. This observation has led to the hypothesis that neurodegeneration results from defects in mitochondrial motility and distribution, which, in turn, cause subcellular ATP depletion and interfere with mitochondrial calcium ([Ca2+]m) buffering at sites of high synaptic activity (reviewed in ref. 7). It is not known, however, whether mitochondrial motility defects are a primary cause or a secondary consequence of MND progression. In addition, it has been difficult to isolate the primary effect of mitochondrial motility defects in MNDs because most mutations that impair mitochondrial motility in neurons also affect transport of other organelles and vesicles (1, 8–11).

In mammals, the movement of neuronal mitochondria between the cell body and the synapse is controlled by adaptors called trafficking kinesin proteins (Trak1 and Trak2) and molecular motors (kinesin heavy chain and dynein), which transport the organelle in the anterograde or retrograde direction along axon microtubule tracks (7, 12–24). Mitochondrial Rho (Miro) GTPase proteins are critical for transport because they are the only known surface receptors that attach mitochondria to these adaptors and motors (12–15, 18, 25, 26). Miro proteins are tail-anchored in the outer mitochondrial membrane with two GTPase domains and two predicted calcium-binding embryonic fibroblast (EF) hand motifs facing the cytoplasm (12, 13, 25, 27, 28). A recent Miro structure revealed two additional EF hands that were not predicted from the primary sequence (29).

This report probes the physiological roles of mammalian mitochondrial Rho1 (Miro1), a calcium-binding, membrane-anchored GTPase necessary for mitochondrial motility on microtubules. Using two new mouse models and primary cells, the study demonstrates a specific role for Miro1 in upper motor neuron development and retrograde transport of axonal mitochondria. Unexpectedly, Miro1 is not essential for calcium-regulated mitochondrial movement, mitochondrial-mediated calcium buffering, or maintenance of mitochondrial respiratory activity. Nevertheless, a neuron-specific Miro1 KO mouse model displays physical hallmarks of neurological disease in the brainstem and spinal cord and develops rapidly progressing upper motor neuron disease symptoms culminating in death after approximately 4 wk. These studies demonstrate that defects in mitochondrial motility and distribution alone are sufficient to cause neurological disease.

Significance

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*This Direct Submission article had a prearranged editor.

1To whom correspondence should be addressed. Email: shaw@biochem.utah.edu.

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mitochondrial motility in axons. Loss of Miro1-directed retrograde mitochondrial transport is sufficient to cause MND phenotypes in mice without abrogating mitochondrial respiratory function. Furthermore, Miro1 is not essential for calcium-mediated inhibition of mitochondrial movement or $[\text{Ca}^{2+}]_{\text{m}}$ buffering. These findings have an impact on current models for Miro1 function and introduce a specific and rapidly progressing mouse model for MND.

**Results**

**Loss of Miro1 Results in Neural Respiratory Control Defects.** We constructed a conditional KO allele for Miro1 [Miro1loxP], hereafter referred to as Miro1 floxed (Miro1$^{f}$), in which exon 2 (encoding part of the essential GTPase I domain) is flanked with lox target sequences (loxP sites) (Fig. S1 A–C). Germline Miro1 inactivation was achieved by crossing mice harboring the Miro1$^{f}$ allele with a mouse line expressing Cre recombinase from the hypoxanthine-guanine phosphoribosyltransferase (HPRT) promoter (30) (Fig. S1 C–E). Mating Miro1$^{f}$ mice resulted in Miro1$^{-}$ (Miro1 KO) animals that were cyanotic and died shortly after birth [postnatal day (P) 0] (Fig. 1A). Miro1 KO mice were present in the population at the expected Mendelian frequencies (Fig. 1B). Fetal cardiac ultrasound at embryonic gestation day (E) 18.5 confirmed that Miro1 KO animals were alive and had normal cardiac function before birth. The early postnatal death of Miro1 KO animals

![Fig. 1. Miro1 deletion causes postnatal lethality and loss of specific neurons required for respiration. (A) Newborn WT and KO P0 mice after birth. Birth occurs at E19.5. The homozygous Miro1 KO is cyanotic. (B) Loss of Miro1 leads to postnatal lethality. (C–H) P0 Miro1 KO mice fail to aerate lung tissue. H&E staining shows normal lung tissue in all genotypes before birth at E18.5 (C–E), aerated lung tissue in WT and HET genotypes at P0 (F and G), but unexpanded lung tissue in the KO genotype at P0 (H). (Scale bar: 300 μm.) (I–N) Loss of Miro1 disrupts cranial nuclei formation in the hindbrain. (I–K) Nissl (green) and DAPI (blue) staining of the NA in the hindbrain of E18.5 animals. White dashed boxes mark the green NA region, which is present in the WT and HET genotypes but absent in the KO genotype. (L–N) Nissl (green) and DAPI (blue) staining of the facial nucleus (FN). White dashed boxes mark the FN region, which has defined margins in the WT and HET genotypes but not in the KO genotype. (Scale bar: 100 μm.) (O–T) Loss of Miro1 reduces the number of cervical motor neurons. Representative images of anti-choline acetyltransferase (ChAT) (red, motor neurons) and DAPI (blue, nuclei) labeling of cervical motor neurons in E18.5 WT (O–Q) and KO (R–T) animals. (Scale bar: 50 μm.) (U) Quantification of cervical motor neurons in WT (n = 4) and KO (n = 7) animals. Data are represented as mean ± SD. (V–Z, and A) Loss of Miro1 reduces phrenic nerve branching. Antibungarotoxin (red, neuromuscular junctions with diaphragm) and anti-neurofilament (green, phrenic nerve) labeling of floating preparations in E18.5 WT (V–X) and KO (Y–A) animals. (Scale bar: 100 μm.) (B’) Quantification of phrenic nerve branching in WT (n = 7) and KO (n = 6) animals. Data are represented as mean ± SD. (Also see Fig. S1.)

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suggests that some Miro1 functions are not shared with Miro2. Although the Miro2 gene is present in Miro1 KO mice, its expression is not up-regulated in brain tissue or Miro1 KO mouse embryo fibroblasts (MEFs) (Fig. S1 F–H).

Postmortem autopsy revealed that Miro1 KO mice had expanded lungs, suggesting that these animals die at birth because they fail to breathe. Miro1 could be required for lung tissue development or for neural inputs that control respiration. However, H&E staining of Miro1 KO lung tissue revealed no morphological abnormalities beyond a lack of lung alveoli expansion (Fig. 1 C–H). Thus, the postnatal respiratory failure observed in Miro1 KO mice is not caused by gross abnormalities in lung development.

We examined the neural respiratory control pathway in Miro1 KO mice. This pathway consists of two networks that control involuntary respiration (31). The first is the central neural respiratory network located in the brainstem, which includes cranial motor neurons of the nucleus ambiguous (NA). Although Nissl staining labeled the NA in brainstems of E15.5 and E18.5 Miro1WT (WT) and Miro1Het (HET) animals, this structure was absent in Miro1KO (KO) mice (Fig. 1 I–K). We also observed marked disorganization of neurons that innervate facial muscles in Miro1 KO mice (the facial nucleus; Fig. 1 L–N). By contrast, organization of cranial nerve neurons X and XII, which innervate the viscera and tongue, respectively, was normal in Miro1 KO mice (Fig. S1 J). Thus, loss of Miro1 function during early development causes remarkably specific defects in brainstem cranial motor neurons.

The second network required for involuntary respiratory control is mediated by phrenic nerve innervation of the dia-

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Fig. 2. Miro1 NKO mice develop upper MND phenotypes. Images of open (A) and clasped (B) hind-limb reflex in P45 control and Miro1 NKO (Miro1F/–; Eno2Cre/+ ) mice, respectively. (Scale bar: 10 mm.) Images of P45 control (C) and Miro1 NKO (D) animals. NKO mice exhibit kyphosis and have stiff tails and hind limbs. (E) Average weights of control (black) and Miro1 NKO (gray) mice at the indicated postnatal ages (n = 10). Data are represented as mean ± SD. (F) Average composite phenotype score for control (black) and Miro1 NKO (gray) mice at the indicated postnatal ages (n = 10). Data are represented as mean ± SD. (G) Kaplan–Meier survival curve for control (Miro1WT and Miro1Het; red and gray lines, respectively), Miro1 NKO (black line), and Miro1 MNKO (Miro1F/–; Mnx1Cre/+ ; dashed blue line) mice (n = 10). Representative images of H&E-stained brainstem (H and I) and anterior horn lumbar spinal cord (J and K) of P30 littermate control (n = 6) and Miro1 NKO (n = 5) mice, respectively. Arrows mark eosinophilic Bunina-like bodies in Miro1 NKO mice that are not present in control mice. (Scale bar: 50 μm.) Representative images (magnification: 1,000×) of lumbar spinal cord cross-sections from P30 Miro1 control (L) and Miro1 NKO (M) mice. Red arrowheads mark mitochondrial profiles in axons. The red arrow marks an axon lacking mitochondrial profiles in Miro1 NKO. (Scale bar: 2 μm.) (N) Percentage of axons with mitochondrial profiles in control and Miro1 NKO lumbar spinal cord sections (n = 2,564 axons from four control animals and 4,602 axons from four Miro1 NKO animals). Data are represented as mean ± SD. (Also see Fig. S2 and Movies S1–S3.)
phragm muscle. The phrenic nerve originates from motor neurons located in the cervical spinal cord at levels C3–C5 (C, cervical spine). Anti-choline acetyltransferase (ChAT) labeling and quantification revealed that Miro1 KO mice had significantly fewer motor neurons in this region compared with WT littermate controls (Fig. 1 O–U). Neuroadfiment labeling experiments also showed that the phrenic nerve of Miro1 KO mice had fewer axonal branches than controls (Fig. 1 V–P). These combined data indicate that death of Miro1 KO mice is caused by specific defects in motor neurons required for breathing after birth.

**Miro1 Neuron-Specific KO Mice Develop Upper MND Phenotypes and Pathology.** The postnatal lethality of Miro1 KO mice prevented studies of Miro1 function in postmitotic neurons later in development. To circumvent this problem, we crossed the conditional Miro1 Cre mouse with a line expressing the Cre recombinase from the enolase 2 (Eno2) promoter (32) to yield a Miro1 neuron-specific KO (NKO; Miro1F/Cre–/Eno2Cre+−) mouse. Eno2-Cre mice express the recombinase in the cerebral cortex, hippocampus, and spinal cord (Fig. S2 A–F). Miro1 KO animals were alive at birth and indistinguishable from control littermates. By P14, however, these mice displayed hind-limb claspng, an early sign of neuronal deficit (Fig. 2 A and B). As they matured, Miro1 KO mice failed to gain weight and developed spinal curvature (kyphosis), a stiff tail, hind-limb spasticity, and severe movement defects (Fig. 2 C–F and Movies S1–S3). These phenotypes resemble symptoms observed in human upper MND, became more severe with age, and resulted in premature death by approximately P35 (Fig. 2G).

Further analyses identified a histological marker of neurodegenerative disease in Miro1 NKO mice. The cerebral cortex and hippocampal regions of NKO mice were similar to those of littermate controls (Fig. S2 G–J). However, the brainstem and lumbar spinal cord regions of the Miro1 NKO mice contained aggregate bodies that were absent in control animals (Fig. 2 H–K). These structures resemble Bunina bodies, which are eosinophilic inclusions commonly observed in degenerating neurons of patients with ALS (33–35). By contrast, the Bunina-like structures in Miro1 KO tissues appeared to be extracellular and may correspond to remnants of dead neurons. The combination of motor defects and histological markers for MND indicates that loss of Miro1 causes MND in mice.

Given the known role of Miro1 in mitochondrial movement, we postulated that defects in Miro1 NKO mice might be accompanied by changes in axonal mitochondrial distribution. We tested this hypothesis by quantifying negative-stained mitochondrial profiles in lumbar spinal cord axons of Miro1 NKO mice at P25–P30, ages when movement phenotypes are severe. Only 49.5% of lumbar spinal cord axons in Miro1 NKO mice contained mitochondrial profiles, compared with 69.6% in littermate controls (Fig. 2 L–N). There were no obvious changes in axon myelination of Miro1 NKO mice relative to controls. Thus, the movement phenotypes observed in Miro1 NKO mice are most likely due to defects in mitochondrial distribution that lead to motor neuron dysfunction.

**Miro1 Is Required for Retrograde Axonal Mitochondrial Movement.** The behaviors and histological markers observed in Miro1 NKO mice are consistent with a role for Miro1 in maintenance of upper motor neurons. In support of this interpretation, Mnx1-Cre driven KO of Miro1 in motor neurons of the developing CNS (excluding the brain) caused no obvious phenotypes [Fig. 2G; Miro1 MNKO (Miro1F–/Mnx1Cre+−) mouse] (36, 37). Thus, defects observed in Miro1 NKO mice are likely caused by loss of Miro1 function in upper motor neurons that originate in the cerebral cortex and travel down the spinal cord. To explore the cellular basis of this defect, we examined the distribution of mitochondria and their molecular motors in primary cortical cultures from Miro1F/+, Miro1F/−, and Miro1−/− animals. In colocalization studies, anti-Tom20-labeled mitochondria were present in MAP-2-labeled dendritic processes of Miro1 WT, HET, and KO primary cortical neurons (Fig. S3 A–I). Moreover, colocalization of axonal mitochondria with a kinesin (Fig. S3 J–L) or dynemin (Fig. S3 M–O) motor was similar in cultures from all three genotypes.

To determine whether Miro1 loss altered axonal mitochondrial movement, we performed confocal time-lapse imaging on primary Miro1 WT and KO cortical neurons transfected with GFP outer membrane protein 25 (OMP25) (mito-GFP) (Fig. 3 A and B). Kymographs generated from time-lapse images revealed the behavior of individual mitochondria within an axon (50–150 μm in length) (38). Stationary mitochondria appear as vertical lines, and moving mitochondria appear as diagonal lines, with the slope representing the velocity (Fig. 3 C and D). Approximately 35% of mitochondria were motile in Miro1 KO cortical neurons, similar to the percentage observed in Miro1 WT (Fig. 3E) and reported in the literature for WT neurons (20, 39, 40). However, the motile mitochondria in Miro1 KO axons spent less time in motion compared with WT (Fig. 3F). These data raised the possibility that processesivity of mitochondrial movement is impaired in Miro1 KO cortical neurons. Indeed, we observed that mitochondria in Miro1 KO axons exhibited a fourfold reduction in overall retrograde velocity compared with WT (Fig. 3G). By contrast, no significant difference in Miro1 KO anterograde velocity was observed relative to WT (Fig. 3G). Additional analysis revealed that the instantaneous velocities of axonal mitochondria in primary cortical neurons were similar to those reported previously and no different between Miro1 KO and WT (41) (Fig. S3P). Finally, we observed a twofold reduction in retrograde run length in the Miro1 KO cortical neurons, although anterograde run lengths were unaffected (Fig. 3H). These data indicate that loss of Miro1 in cortical neurons preferentially affects retrograde axonal mitochondrial movement.

**Miro1 Loss Causes Mitochondrial Distribution Defects Without Concomitant Changes in Respiration.** It is hypothesized that damaged axonal and/or synaptic mitochondria need to be shuttled back to the cell body for general repair or turnover by mitophagy. In addition, a previous study reported that pharmacological disruption of mitochondrial respiration increased retrograde mitochondrial transport in axons (42). According to this model, a defect in Miro1-mediated retrograde movement is predicted to interfere with mitochondrial quality control and cause the accumulation of respiratory compromised mitochondria. However, histochemical enzyme assays of cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) revealed no COX-negative, SDH-positive neurons in the cerebral cortex or hippocampus of Miro1 WT and KO embryos (Fig. 4 A–D and Fig. S4). In addition, negative staining and transmission EM detected mitochondria with normal cristae ultrastructure in axons of Miro1 WT and KO animals, suggesting that mitochondrial respiration was intact (Fig. 4 E and F). Thus, mitochondrial respiration likely continues in these affected tissues when Miro1 is disrupted. We also examined mitochondrial membrane potential in WT and KO primary cortical neurons by double labeling with potential-independent (MitoTracker Green; Molecular Probes) and potential-dependent [tetramethylrhodamine methyl ester (TMRM); Biotium] dyes. There were no differences in the pattern of mitochondrial staining (Fig. 4 G–I), and for both genotypes, the majority of mitochondria were labeled with TMRM. Variability in the intensity of TMRM staining within and between different WT and KO axons was observed, however, both cultures displayed the same range of TMRM/MitoTracker Green fluorescence intensity ratios (Fig. S4E). The fact that TMRM labeled mitochondria of KO axons indicates that these organelles continue to respire.

Because it is not possible to measure mitochondrial respiratory capacity directly using mixed primary neuronal cultures, we generated MEFs from Miro1 WT, HET, and KO mice. In

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**Miro1** and HET MEFs, mitochondria labeled with MitoTracker had the characteristic tubular morphology and were distributed throughout the cytoplasm and in filopodia (Fig. 5A, B, and D and Fig. S5 A–C). By contrast, mitochondria in the Miro1 KO MEFs were clustered around the nucleus (perinuclear) and absent from filopodia (Fig. 5C and D and Fig. S5 D–F). Fluorescence recovery after photobleaching and time-lapse imaging indicated that some mitochondria in KO MEFs were motile over short distances despite the distribution defect (Fig. S5 G–P). Further analyses revealed no gross changes in the organization of actin, intermediate filaments, or microtubules (Fig. S5 Q–Y). The morphology and distribution of the endoplasmic reticulum (ER), peroxisomes, and lysosomes were also unaffected in these cells (Fig. S5 Z–H). The mitochondrial distribution defect in Miro1 KO MEFs was rescued by overexpression of Miro1 (three spliced variants) or Miro2 (Fig. 5E). The ability of both Miro1 and Miro2 to rescue mitochondrial distribution defects in Miro1 KO MEFs indicates that the two genes have some overlapping functions. In the case of Miro2, this rescue requires overexpression, because Miro2 transcript and protein are present but not up-regulated in Miro1 KO MEFs, which display a mitochondrial distribution defect (Fig. S1 F–H).

Despite the mitochondrial distribution defect in Miro1 KO MEFs, we observed no change in inner mitochondrial membrane potential. There were no differences in the pattern of mitochondrial staining when Miro1 WT,Het, and KO MEFs were dual-labeled with potential-independent (mito-GFP) and potential-dependent (MitoTracker Red; Invitrogen) markers (Fig. 5G–R). Moreover, direct measurements of oxygen consumption rates (an indicator of mitochondrial respiratory capacity) revealed no differences between Miro1 WT, HET, and KO MEFs (Fig. 5F). Thus, although Miro1 is required for proper mitochondrial distribution in MEFs, this defect does not detectably alter mitochondrial membrane potential or respiratory function. Our combined cellular analyses suggest that MND-like phenotypes in Miro1 NKO mice are a direct result of defects in mitochondrial motility and distribution.

**Miro1 Is Not Essential for [Ca^{2+}]_{mito} Uptake or Calcium Inhibition of Mitochondrial Motility.** A previous overexpression study suggested that the Ca^{2+}-binding activity of Miro1 EF-hand motifs is not required for mitochondrial-mediated calcium buffering (26). However, it is not known whether changes in mitochondrial distribution caused by Miro1 loss affect the efficiency of calcium uptake by the organelle. To test the physiological importance of Miro1 for [Ca^{2+}]_{mito} uptake, cytosolic calcium ([Ca^{2+}]) and [Ca^{2+}]_{mito} concentrations were measured simultaneously in intact Miro1 WT and KO MEFs transfected with mitochondrial-targeted inverse pericam (a [Ca^{2+}]_{mito} sensor) and loaded with fura2/acetoxymethyl ester (a ratiometric cytoplasmic calcium indicator dye) (26). Temporal changes in [Ca^{2+}]_{mito} and [Ca^{2+}]_{cyt} concentrations were measured as fluctuations in fluorescence intensity of fura2 and inverse pericam in response to increasing intracellular calcium. To stimulate a global increase in [Ca^{2+}], the ER calcium store was first depleted by treatment with thapsigargin (an inhibitor of the ER Ca^{2+} ATPase) in Ca^{2+}-free buffer, whereupon readdition of CaCl2 evokes entry of exogenously added calcium via plasma membrane store-operated channels. Using this approach, we observed no difference in the rate or extent of [Ca^{2+}]_{mito} rise in Miro1 WT and KO MEFs (Fig. 6A and B). Thus, Miro1 does not directly affect mitochondrial Ca^{2+} uptake. More importantly, the defective distribution and perinuclear clustering of mitochondria in Miro1 KO MEFs does not appear to interfere with mitochondrial buffering of [Ca^{2+}].

We also tested the effect of Miro1 loss on calcium-mediated inhibition of mitochondrial movement. In this case, Miro1 WT and KO MEFs expressing mitochondrial matrix-targeted YFP were loaded with fura2 to monitor [Ca^{2+}]_{mito} concentration. Exogenously added Ca^{2+} was used to set [Ca^{2+}], at concentrations known to allow basal motility ([Ca^{2+}] = 100 nM) or to cause partial ([Ca^{2+}] = 400–600 nM) or maximal ([Ca^{2+}] > 1 μM) motility inhibition, as determined by calculating the differences between successive images in a time series (43) (Fig. S6A and B). To ensure rapid equilibration of the cytoplasm with added extracellular calcium [Ca^{2+}], cells were initially depleted of the cation by incubation with EGTA, thapsigargin, and ionomycin (a Ca^{2+} ionophore). As shown in Fig. 6C, dose–response plots of normalized motility inhibition as a function of calcium concent-
Discussion

Previous work established that Miro GTPases function with adaptors and motor proteins to transport mitochondria along cytoskeletal tracks (7, 22, 44). Additional investigations in cultured cells suggested that Miro function would be critical in neurons, which move mitochondria long distances to sites where ATP and calcium buffering are needed. The studies described here revealed an unexpectedly specific requirement for Miro1 in upper motor neuron development and postmitotic function. Moreover, targeted disruption of Miro1 in the cerebral cortex causes retrograde mitochondrial motility defects in cortical neurons, depletion of mitochondria from corticospinal tract axons, and rapidly progressing upper MND without concomitant changes in mitochondrial respiration.

The germ-line Miro1 KO mouse completes embryogenesis but fails to breathe and dies at birth. Our analyses indicate that loss of the brainstem NA contributes to this breathing defect. It is possible that Miro1 is required for the survival of motor neurons that form the NA. Preliminary studies of E15.5 Miro1 KO embryos showed reduced labeling with apoptotic markers in this region compared with WT. Thus, the neuronal cell bodies that normally populate the NA may simply be absent, rather than dying. Studies are underway to determine whether Miro1 is required for specification, migration, or postmigration survival of neurons that populate this structure. We also documented a decrease in the number of phrenic nerve branches that innervate the diaphragm in KO animals. It has been known for many years that mitochondria are enriched in active growth cones and at sites of neuronal branching (45). In Miro1-deficient animals, mitochondrial depletion from these sites could interfere with branch initiation or compromise maintenance of branches after they form. In support of this model, depletion of the Miro adaptor Trak1 is reported to decrease axonal branching (24). In addition, a specific kinase pathway was recently shown to support axon branching in cortical neurons by promoting immobilization of mitochondria at presynaptic sites via the docking protein syntaphilin (39, 46–48). Although the neuronal defects described here are the most likely cause of early mortality in Miro1 KO mice, changes in additional neuronal populations or tissues may also contribute to this phenotype.

Before this study, the available evidence suggested that Miro1 and Miro2 interchangeably form complexes with adaptors (Trak1 and Trak2 in mammals or Milton in Drosophila) and motors (kinesin heavy chain and dynein) to transport mitochondria in both the anterograde and retrograde directions (12–22, 24, 25). Our finding that Miro1 loss selectively affects retrograde mitochondrial transport and run length in cortical axons demonstrates that Miro1 and Miro2 contribute unequally to bidirectional mitochondrial movement in neurons. Additional evidence supports the idea that the composition of mitochondrial movement complexes modulates their activities. In hippocampal

1.2 mM calcium to calcium-depleted cells (leading to a rise in [Ca\(^{2+}\)], from ~100 nM to 300–1,000 nM) resulted in a significant decrease in mitochondrial motility in both WT and KO axons (paired t test of nonnormalized motility figures before and after calcium, P < 0.001 for both) (Fig. 6D and E). The degree of motility inhibition was not significantly different between WT and KO axons. Further addition of calcium (5 mM) and ionomycin (10 μM) resulted in total arrest of motility in both genotypes (Fig. 6E and Fig. S6C), although calcium may not be the only mechanism at play under the latter conditions. Threshold values for motility calculation were determined algorithmically and were not significantly different between WT and KO in any of the experiments. Together, these results demonstrate that the loss of Miro1 does not prevent the Ca\(^{2+}\)-dependent inhibition of mitochondrial motility and argue against an essential role for Miro1 in calcium control of mitochondrial motility.

Fig. 4. Miro1 loss does not abolish mitochondrial bioenergetic function. COX/SDH activity histochemical double labeling of representative cross-sections of E18.5 Miro1 WT (A and B, n = 3) and KO (C and D, n = 3) embryos. Brown staining reflects COX activity (A and C), and SDH activity results in blue staining (B and D) (controls treated with COX inhibitor). (Scale bars: 50 μm.) (Also see Fig. S4.) (E and F) Representative cross-sectional images of axons from the lumbar spinal cord of P30 Miro1 WT and NKO animals. Arrows mark cristae structure within mitochondrial profiles. Arrowheads mark myelin sheaths. (Scale bar: 0.5 μm.) Double labeling of primary cortical Miro1 WT (G and H) and KO (I and J) neurons with mitochondrial membrane potential-independent MitoTracker Green (MTG) and potential-dependent TMRM (red). Panels show the same field of view before (G and I) and 2 min after (H and J) treatment with 10 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (+CCCP*). (G–J, Insets, Upper Right) Magnified views of the boxed area are displayed as Insets in G–J. (Scale bar: 10 μm. Magnification: 3x.)

tration were essentially indistinguishable for Miro1 WT and KO MEFs. Miro1 WT and KO MEFs also displayed similar IC\(_{50}\) measurements of 400–500 nM [Ca\(^{2+}\)]. Moreover, the extent of maximum inhibition was unaffected by loss of Miro1. The average ratio of pixel counts at maximum inhibition as a fraction of basal motility calculated for each cell was identical (12% for both WT and KO) (Fig. S6 A and B).

Importantly, Miro1 was also not essential for Ca\(^{2+}\)-dependent mitochondrial motility inhibition in cortical neurons. Addition of
neurons, the Trak1 and Trak2 adaptors differentially control mitochondrial movement in axons and dendrites, and they preferentially bind either the kinesin or dynein motor proteins, respectively (24). Although not yet tested, Miro1 and Miro2 may also bind adaptors (and, indirectly, motors) with different affinities and preferentially transport mitochondria in the retrograde or anterograde direction. The effect of Miro1 disruption on retrograde movement could also be indirect. Anterograde and retrograde motors have been shown to bind mitochondria simultaneously or associate with mitochondria moving in both directions (16, 49, 50). Thus, the selective activation and inactivation of bound motors could coordinate bidirectional movement of the organelle (51). Loss of Miro1 from the mitochondrial surface might disrupt interactions between, or regulation of, Miro2-associated kinesin and dynein motors in a manner that preferentially impairs retrograde transport.

The retrograde transport defect we observe in cortical neurons may not result in the same type of axonal mitochondrial depletion we observed in lumbar spinal cord sections from Miro1 NKO mice (Fig. 2 L–N). Axons analyzed in culture are much shorter than those of the corticospinal tract in the mouse, which could explain this difference. In addition, the process of culturing cortical tissue may alter both the type and physiology of neurons relative to those in vivo. Finally, cells in culture, which are chronologically "younger" than neurons of a P30 Miro1 NKO mouse, may not be mature enough to display this phenotype.

The retrograde transport defect we observe in cortical neurons may not be recapitulated in nonpolarized cells. When Miro1 is absent from both MEFs (Fig. 4 E and F) and astrocytes (Fig. S3H), mitochondria collapse near the nuclear periphery. In MEFs, dynein moves mitochondria toward microtubule minus ends near the nucleus and kinesin moves mitochondria toward microtubule plus ends at the cell periphery. Thus, the perinuclear mitochondrial clustering in MEFs is predicted to result from defects in kinesin-mediated motility toward the cell periphery. Alternatively, loss of Miro1 in these cells may cause mitochondria to become anchored near the nucleus. In particular, physical connections between the ER membrane and the outer mitochondrial membrane are known to coordinate the functions of these organelles in many organisms (52). Fluorescently tagged Miro proteins localize at these ER-mitochondrial attachment sites in both yeast and mammalian cells (53). Interestingly, a role for the yeast Miro homolog in separating newly divided mitochondria from ER-mitochondrial attachment sites was recently reported (54). Whether this function is conserved in mammals and is responsible for the mitochondrial perinuclear clustering that occurs when Miro1 is absent is under investigation.

It is well established that elevated cytoplasmic calcium inhibits mitochondrial movement in a variety of cell types, allowing the organelle to collect at sites where energy is needed (7, 17). In addition, mitochondria accumulate and sequester the elevated cytoplasmic calcium associated with synaptic activity in neurons (55). Failure to buffer elevated calcium is implicated in neuronal cell death via excitotoxicity (2, 56). Previous work strongly suggested that Miro proteins were the \([Ca^{2+}]_m\) sensors, because \(Ca^{2+}\) binding to the EF-hand motifs in Miro appeared to halt kinesin-driven mitochondrial movement (15, 16, 26). Thus, we were surprised to find that calcium-mediated mitochondrial stopping and \([Ca^{2+}]_m\) buffering were unchanged when Miro1 was absent. It is possible that Miro2 is responsible for the bulk of the calcium sensing that occurs in vivo. There may also be a Miro-independent calcium-sensing mechanism responsible for regulating

![Diagram](image-url)
mitochondrial movement under normal conditions or when Miro1 is absent. The Miro1 NKO mice develop age-dependent, progressive neurological symptoms that most closely resemble SP (57, 58). Although mitochondrial distribution defects accompanied by mitochondrial respiratory defects are associated with a subset of MNDs, only a few of these are attributable to primary mutations that disrupt mitochondrial respiration (6, 59). In the remaining cases, it has been difficult to unravel the relative contributions of mitochondrial distribution defects or mitochondrial respiratory defects to disease progression. We now show that loss of mammalian Miro1 function can have a negative impact on mitochondrial distribution without disrupting mitochondrial respiratory function. When combined with the severe neurological phenotypes observed in the Miro1 NKO mouse, these findings demonstrate that primary defects in mitochondrial motility and distribution can cause neurological disease.

Materials and Methods

Mouse Experiments. Generation of Miro1+/+, Miro1−/−, KO, and NKO mice is described in SI Materials and Methods. All experiments were approved by the University of Utah Institutional Animal Care and Use Committee. All controls were phenotypic WT littermates of the mutant animals (Miro1+/+, Eno2Cre+/+, Miro1+/− Eno2+/−, and Miro1−/− Eno2−/−). A blinded examiner performed behavioral analysis and physical examinations. Mice were

Fig. 6. Miro1 loss does not interfere with mitochondrial Ca\(^{2+}\) uptake or Ca\(^{2+}\)-mediated inhibition of mitochondrial motility. (A) Graphs depict the average synchronized traces ± SEM of \([\text{Ca}^{2+}]_c\), (Upper) and \([\text{Ca}^{2+}]_m\), (Lower) in the indicated MEFs during store-operated calcium entry as determined by fura2 and inverse pericam (iPcam), respectively. Included in the means are all cells that showed maximum \([\text{Ca}^{2+}]_c\), in the range of 1–3 μM (WT, n = 11; KO, n = 23). (B) \([\text{Ca}^{2+}]_m\) as a function of \([\text{Ca}^{2+}]_c\), during the rising phase of \([\text{Ca}^{2+}]_c\), is shown for each cell included in the means. (C) Dose–response relationship between \([\text{Ca}^{2+}]_c\), and motility inhibition in Miro1 WT (black; IC\(_{50}\): 498 ± 18 nM, n = 20) and KO (red; IC\(_{50}\): 492 ± 15 nM, n = 22). (D) Kymographs generated from single processes in WT or KO primary cortical neurons. Overlay of fura2 fluorescence at 340 nm (red) and 380 nm (green) excitation (Top), MitoTracker Green fluorescence (Middle, grayscale), and MitoTracker Green overlaid with calculated motility (Bottom, red). (Scale bar: 10 μm.) Iono, ionomycin. (E) Plot shows the mean ± SEM of \([\text{Ca}^{2+}]_c\) and mitochondrial motility inhibition in cortical neurons at rest, after 1.2 mM CaCl\(_2\), and after 5 mM CaCl\(_2\) plus Iono. Data are derived from those processes where \([\text{Ca}^{2+}]_c\) rose to the range of 0.3–1.0 μM after the addition of 1.2 mM CaCl\(_2\). (WT, n = 33 from three embryos; KO, n = 23 from five embryos). (Also see Fig. S6.)
MEFs and Cortical Neuronal Culture. MEFs were isolated from Miro1 KO, Het, and WT littermate E14.5 embryos and immortalized by lentiviral transduction with large t antigen (61). Primary MEF cells were cultured in DMEM (Invitrogen) supplemented with 10% (vol/vol) FBS (Sigma), l-glutamine (Invitrogen), penicillin/streptomycin (Invitrogen), and 50 mM β-mercapto-ethanol (Sigma). Lipofectamine 2000 (Invitrogen) was used to transfect either 5 μg of plasmids harboring Myc-tagged variants of Miro1 or Miro2 and 1 μg of a plasmid harboring GFP-OOMP25 (provided by the laboratory of D. Chan, California Institute of Technology, Pasadena, CA) (62). Primary mixed cultures of cortical neurons were prepared from four WT and five KO E14.5 mice, as described previously (63, 64). The cultures were plated at a low density (100,000–150,000 cells) on poly-L-lysine-coated, eight-well, glass-bottomed chamber slides (Lab-Tek II; Thermo Scientific), or 12-mm glass coverslips placed in 60-mm dishes. Cultures were maintained in a humidified incubator at 37 °C with 5% (vol/vol) CO2.

NGF-Dependent mitochondrial transport to axons and dendrites. Embryos were monitored within 45 min of laparotomy. The culture medium was replaced with fresh plating media [MEM (Sigma), 10% (vol/vol) horse serum (Invitrogen), 5,000 units of penicillin and 5,000 μg of streptomycin, 30 mM glucose, 4 mM l-glutamine, and 8 μg of Ara-C (Sigma)] every 2–3 d. Before imaging and transient transfection, cortical culture media were changed to Neurobasal A feeding media (Neurobasal A supplemented with 2% [v/v] (Invitrogen), 500 units of penicillin and 500 μg of streptomycin, 0.5 mM l-glutamine (Invitrogen), and 4% [v/v] HP3825, 0.5 μM of GFP-OOMP25 was transected with using 1 μg of GFP-OOMP25 using 2.5 μl of Lipofectamine 2000, and was imaged 1–4 d later. Additional details are provided in SI Materials and Methods.

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