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Mitofusin activation enhances mitochondrial motility and promotes neuroregeneration in CMT2A

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Human brains represent only 2% of body mass, but their high relative metabolic activity accounts for ~20% of total body adenosine triphosphate (ATP) consumption. ATP generated by neuronal mitochondria fuels nerve signaling and homeostatic repair. In the peripheral nervous system, which has greater capacity for regeneration after physical, toxic or genetic injury than the central nervous system, ATP also powers actin polymerization/depolymerization for growth cone formation and axon extension. Mitochondrial ATP generation is therefore a central component of neuronal functioning in the central and peripheral nervous systems.

The contextual roles of neuronal mitochondria are largely determined by two factors, the fitness of individual organelles and their localization within host neurons. The role of mitochondrial fitness is widely acknowledged: healthy mitochondria perform their ATP-producing function without compromising host neurons, whereas damaged mitochondria in which the respiratory chain is partially uncoupled from ATP production elaborate cytotoxic reactive oxygen species (ROS) that both damage the host neuron and can activate programmed cell death pathways. By comparison, the role of mitochondrial transport and localization has only recently become widely appreciated: almost all ~1000 mitochondrial proteins (i.e. except the 13 respiratory chain proteins encoded by the mitochondrial genome) are encoded by nuclear genes and synthesized by host cell ribosomes. Therefore, mitochondrial biogenesis takes place primarily in neuronal soma and newly formed mitochondria in the cell body are actively directed to distal areas of neuronal activity, such as synapses and growth cones. The unique architecture of neurons, especially long motor neurons innervating upper and lower extremities, requires that mitochondria traverse approximately 1 meter of axon length from neuronal soma in the spine to peripheral neuromuscular junctions. Damaged or senescent mitochondria in synapses or growth buds likewise require export for mitophagic elimination, which is necessary to prevent mitochondrial-

mediated ROS cytotoxicity at distal nerves. Thus, an interruption in mitochondrial transport might be as consequential to neuronal health as, and can contribute to, impaired mitochondrial fitness. Recently, Franco et al. (2020) demonstrated that directly enhancing mitochondrial trafficking within neuronal axons helps regenerate peripheral nerve damage in a mouse model of the rare sensorimotor neuropathy, Charcot-Marie-Tooth disease type 2A (CMT2A). These studies provide critical *in vitro* and *in vivo* support mechanistically linking mitochondrial trafficking to neuronal regeneration.

Two genetic neuropathies serve as prototypical neurodegenerative diseases having mitochondrial dysfunction: Familial amyotrophic lateral sclerosis caused by mutations of superoxide dismutase 1 (*SOD1*) is an example of loss of mitochondrial fitness (Wong et al., 1995), in this case precipitated by increased neuronal ROS levels. CMT2A caused by mutations of mitofusin 2 (*MFN2*) is an example of interrupted mitochondrial transport (Baloh et al., 2007), although the precise mechanism by which dysfunction of a mitochondrial fusion protein impairs mitochondrial trafficking remains unclear.

MFN2 and closely related MFN1 were originally described as proteins mediating mammalian outer mitochondrial membrane fusion (Dorn, 2019). MFN2 also plays important roles in mitophagy (the selective elimination of damaged mitochondria via autophagy pathways) (Chen and Dorn, 2013) and axonal mitochondrial transport (Rocha et al., 2018). The observations that mitochondrial motility is impaired in CMT2A caused by *MFN2* mutations (Baloh et al., 2007), and that CMT2A-associated mitochondrial dysmotility can be reversed by small molecule mitofusin activators (Rocha et al., 2018), suggested that pharmacological activation of endogenous normal MFN1 and MFN2 might overcome mutant MFN2 suppression of normal mitofusin functioning in autosomal dominant CMT2A. While conceptually simple, the putative mechanistic link between mitofusin dysfunction and disease progression in CMT2A has at least

4 degrees of separation: (1) MFN1/MFN2-mediated mitochondrial fusion and motility are suppressed by dominant CMT2A MFN2 mutants; (2) transport of healthy mitochondria to, and damaged mitochondria away from, neuromuscular junctions is diminished; (3) neuromuscular signaling is consequently impaired; (4) motor nerves die back from muscles; and (5) muscle mass decreases due to neurogenic atrophy. This pathophysiological complexity confounded expectations of whether mitofusin activation could reverse disease *in vivo*.

In testing the idea of activating endogenous normal mitofusins in CMT2A Franco et al. (2020) developed three major objectives. The first was to assess the relevance of mitofusin activation in human CMT2A by replicating in human CMT2A patient neurons previous observations that mitofusin activation can reverse mitochondrial dysmotility in mouse CMT2A nerve axons (Franco et al., 2016; Rocha et al., 2018; Dang et al., 2020). Because iPSC-derived neurons lose characteristic CMT2A mitochondrial phenotypes (e.g. fragmentation from loss of fusion and stasis from loss of motility), Franco et al. (2020) used microRNA-mediated neuronal conversion to directly reprogram CMT2A patient dermal fibroblasts having four different MFN2 mutations into HB9/MNX1-positive motor neurons. Reprogramming efficiency was > 90% neurons (β-III tubulin positive) and > 85% motor neurons (HB9 positive) in both normal and CMT2A cells. Compared to reprogrammed motor neurons from multiple normal subjects, all four CMT2A motor neuron lines exhibited hallmark mitochondrial fragmentation (loss of fusion), depolarization (impaired respiratory function), and dysmotility (measured as the proportion and velocity of motile mitochondria). Notwithstanding different genetic mechanisms for MFN2 dysfunction (two mutations within the MFN2 GTPase domain and two within the MFN2 coiled-coiled hydrophobic core), all four reprogrammed CMT2A motor neuron lines responded to addition of a small molecule mitofusin activator by normalizing mitochondrial morphology, polarization status and motility. Thus, human CMT2A neurons, like CMT2A mouse neurons, respond to mitofusin activation by reversing prototypical mitochondrial disease phenotypes. Moreover, in this small sample the benefit on mitochondria appeared to be agnostic to causal MFN2 mutation.

The second goal of Franco et al. (2020) was to establish efficacy in an *in vivo* mouse CMT2A model similar to the human

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condition. Because there were no published CMT2A mouse models that reproduce the time course of disease progression, pattern of neuromuscular dysfunction, neuroelectrophysiological features, and histological/ultrastructural characteristics of human CMT2A in the absence of confounding developmental defects (Dorn, 2020), they generated a new model by combining motor neuron specific *Mnx1*-Cre and flox-stop human MFN2-T105M transgenes (Franco et al., 2020). The resulting mice express mutant human (h) MFN2 T105M in mouse motor neurons, but the Cre that evokes mutant MFN2 expression is extinguished after the first week of life, and is therefore not a continuing variable. Paralleling the human pattern of childhood onset with disease progression into young adulthood, CMT2A hMFN2 T105M mice were normal up to 10 weeks of age and then exhibited a progressive decline in neuromuscular function (measured as RotaRod latency) from 20 to 30 weeks, after which the disease stabilized. Human CMT2A is clinically distinguished from more common CMT1 by neuroelectrophysiologic testing showing decreased amplitude of compound muscle action potentials (reflecting loss of neurons) without decreased nerve conduction velocity (because in CMT2A there is no loss of myelin); this was also recapitulated in MFN2 T105M mice. Finally, the characteristic histological and ultrastructural features of CMT2A were reproduced in hMFN2 T105M mice. Remarkably, daily intramuscular injection of the mitofusin activator trans-MiM111 to mice having the full-fledged CMT2A phenotype reversed all of these abnormalities within 8 weeks (Franco et al., 2020).

The final goal of Franco et al. (2020) was to elucidate mechanisms for observed CMT2A phenotype reversal after mitofusin activation. As currently understood, the pathophysiology of forearm and foreleg neuromuscular degeneration caused by loss-of-function MFN2 mutations in CMT2A consists of the following: (1) genetic mitochondrial dysfunction and dysmotility leads to a dearth of healthy mitochondria at neuromuscular junctions; (2) this compromises neuromuscular signaling and evokes distal neuron die-back; and 3. the end result is neurogenic muscular atrophy (**Figure 1**). If this is correct, then restoration of function in mitofusin activator-treated CMT2A mice implies that the neurons which had previously died back were regenerated, thus reestablishing neuromuscular integrity. Alternately, restoration of mitochondrial fusion alone might have engendered
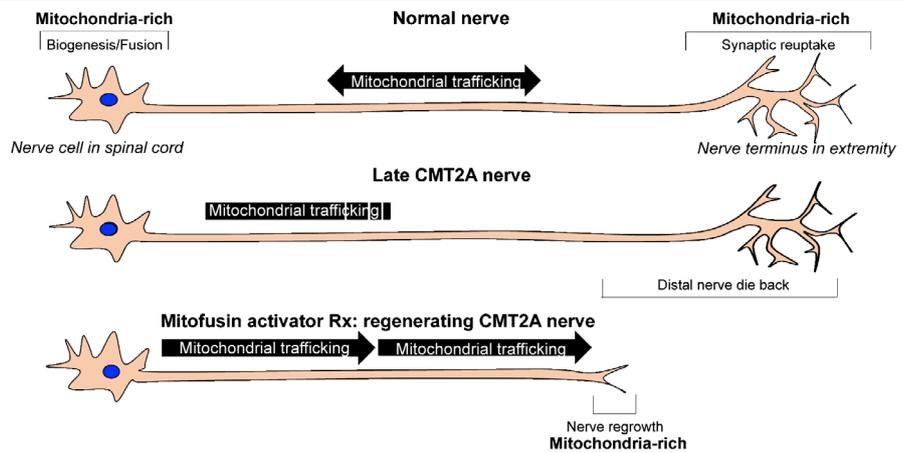

Figure 1 │ **Schematic depiction of relationship between mitochondrial transport, neuron die-back, and neuron regeneration in CMT2A.**
Normal nerves (top) have bidirectional mitochondrial transport that delivers healthy organelles to and removes damaged organelles from, nerve termini. In CMT2A nerves (middle) interruption of distal mitochondrial transport impairs nerve function and repair, prompting dying back of the termini. Restoration of mitochondrial transport by activated mitofusins (bottom) promotes nerve regeneration that reverses *in vivo* neuromuscular dysfunction. CMT2A: Charcot-Marie-Tooth disease type 2A.

neuroprotective effects. However, in support of a connection between mitochondrial trafficking and neuronal regeneration, enhancing mitochondrial motility by genetically ablating the neuronal anchoring protein syntaphilin was previously shown to promote neuronal regrowth after physical injury (Zhou et al., 2016).

Franco et al. (2020) performed a series of *in vivo* and *in vitro* experiments to more fully understand the connection between mitofusin activation, mitochondrial transport/localization, and neuronal regeneration. Reasoning that impaired mitochondrial trafficking to neuromuscular junctions and neuronal die-back in CMT2A would result in a decreased mitochondrial occupancy of junctional synapses and a reduced total number of junctional synapses, respectively, they used immunohistology to quantify junctional synapses (anti-acetylcholine receptor) and their resident mitochondria (anti-cytochrome oxidase IV) in mouse foreleg muscles. Consistent with a link between mitochondrial trafficking and neuronal regrowth, tibialis muscles of vehicle-treated CMT2A mice had fewer than half the normal number of junctional synapses per muscle myocyte, and only ~half the normal density of mitochondria within those synapses; mitofusin activation reversed both of those abnormal metrics (Franco et al., 2020).

The above result supported the thesis that mitofusin activation promotes neuron regeneration in CMT2A, but the *in vivo* data were entirely correlative. Moreover, the observed relationship between mitochondrial localization within junctional synapses and enhanced mitochondrial

motility is inferential. Therefore, Franco et al. (2020) isolated dorsal root ganglion (DRG) neurons from mice carrying the hMFN2 T105M flox-stop allele, activated the mutant MFN2 transgene *in vitro* using adenoviral Cre, and measured DRG outgrowth in the presence or absence of the mitofusin activator. (Note, *in vitro* activation of hMFN2 T105M with adeno-Cre was necessary for this experiment because DRGs contain sensory neurons and the MNX1/HB9 promoter used *in vivo* drives predominantly motor neuron expression.) The CMT2A DRG platform permitted live cell studies of mitochondrial aspect ratio (an indirect measure of fusion), mitochondrial motility and residency within axonal termini (linking axonal transport to distal neuron localization), and axon growth/branching (a metric of axon regeneration after isolation and plating), which was not possible in fixed tissue. Mitofusin activation strikingly improved each of the measured endpoints, normalizing mitochondrial aspect ratio, motility and distal axon residency, and markedly accelerating axonal outgrowth. Importantly, the time courses of these responses, in which motility was maximized only 2 hours after mitofusin activation and axonal outgrowth was markedly accelerated within 24 hours, but mitochondrial fusion was corrected only after 48 hours, supported a causal relationship between mitochondrial transport and neuronal regrowth (Franco et al., 2020).

Finally, because *de novo* DRG axon outgrowth might be mechanistically distinct from axon regrowth after injury in CMT2A, Franco et al. (2020) isolated neonatal cortical neurons from hMFN2 T105M flox-stop mice and cultured the neurons in wells connected

to empty wells by linear microchannels. Cortical neuron outgrowth resulted in long, non-branching axons that grew through the microchannels into the empty wells, wherein they underwent terminal branching. This *in vitro* arrangement mimics *in vivo* neuron architecture of soma, linear axon body and distal branched axon. Because mouse CMT2A neurons grow very poorly (as in the above DRG studies), adeno-Cre was used to induce hMFN2 T105M expression after the cultured cortical neurons had grown through the microchannels and developed complex arborizations (i.e., 10 days *in vitro*). In this way, mature CMT2A neurons were created that subsequently underwent distal aspiration axotomy, amputating the distal branched axons while leaving the proximal linear axons intact. Amputated neurons were maintained in culture in the presence or absence of mitofusin activator and early mitochondrial readouts of fusion (aspect ratio) and motility (% motile and velocity) were related to axon regrowth after 3 days. Mitochondrial aspect ratio was not affected by axotomy, whereas mitochondrial motility in CMT2A neurons (already diminished by half compared to normal neurons treated identically) was further reduced after axotomy, but restored by mitofusin activation. Importantly, axon regrowth in CMT2A neurons was doubled by mitofusin activation.

These studies are the first to mechanistically link mitofusin activation, enhanced mitochondrial transport to distal regions of the neuron, and accelerated neuronal regeneration. The novel *in vitro* and *in vivo* CMT2A disease models employed in these studies integrated loss of MFN2 function with restoration of function to demonstrate a central role for mitofusins in mitochondrial motility. The underlying principle is that accelerated regeneration of damaged nerves can be achieved by facilitating delivery of healthy mitochondria to, and damaged mitochondria away from, terminal axon growth areas. Indeed, this paradigm may apply to other genetic neurodegenerative diseases, including amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, wherein abnormalities of mitochondrial structure, transport and/or function contribute to observed neuropathology (Knott et al., 2008). In these conditions we envision a vicious pathophysiologic cycle wherein primary genetic mutations encoding diverse proteins that directly or indirectly damage mitochondria evoke oxidative stress and mitotoxicity, resulting in additional mitochondrial damage and a feedback loop that culminates in metabolic insufficiency from mitochondrial drop-out and neuronal death from mitochondrial pathway apoptosis. According to this scenario, interrupting the cycle at any point could prove therapeutic. Thus, interventions like mitofusin activation that promote general mitochondrial resistance to an array of noxious insults may have broad therapeutic utility.

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