

Mitochondrial dysfunction and pharmacodynamics of mitofusin activation in murine Charcot-Marie-Tooth disease type 2A

Antonietta Franco¹, Xiawei Dang^{1,2}, Lihong Zhang^{1,3}, Perry B Molinoff⁴, Gerald W Dorn II¹

¹Department of Internal Medicine, Washington University School of Medicine, St. Louis MO USA.

²Department of Cardiology, The First Affiliated Hospital of Xi'an Jiao Tong University, Xi'an, Shaanxi, China.

³Mitochondria in Motion Inc., St. Louis MO USA.

⁴Department of Pharmacology, University of Pennsylvania, Philadelphia PA USA.

RUNNING TITLE – Mitofusin dysfunction in CMT2A

Correspondence to:

Gerald W. Dorn II, MD

Philip and Sima K. Needleman Professor

Washington University Center for Pharmacogenomics

660 S Euclid Ave.

Campus Box 8220 St. Louis, MO 63110

Phone: 314 362-4892. Fax 314 362-8844.

Email: gdorn@wustl.edu

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Nonstandard abbreviations:

Ad – adenovirus

β -Gal – β -galactosidase

CMAP – Compound muscle action potential

CMT – Charcot-Marie-Tooth

CPR1-B – N-[(1*s*,4*s*)-4-Hydroxycyclohexyl] (1*R*,2*R*)-2-(3-phenylpropyl)
cyclopropanecarboxamide

DRG – dorsal root ganglion

FRET – Forster resonance energy transfer

HP- β -CD – 2-hydroxypropyl- β -cyclodextrin

MEFs – murine embryonic fibroblasts

MFN – human mitofusin

Mfn – mouse mitofusin
MOI – multiplicity of infection
MP-1 – mini-peptide 1 (a peptide mitofusin activator)
Neuro-EP – Neuro-electrophysiology (aka nerve conduction testing)
TMRE – tetramethylrhodamine ethyl ester
trans-MiM111 – N-(*trans*-4-Hydroxycyclohexyl)-6-phenylhexanamide
WGA – wheat germ agglutinin
WT – wild-type

Recommended section assignment: **Drug discovery and translational medicine**

ABSTRACT

Mitofusin (MFN) 1 and MFN2 are dynamin GTPase family mitochondrial proteins that mediate mitochondrial fusion that requires MFN conformational shifts, formation of macromolecular complexes on and between mitochondria, and GTP hydrolysis. Damaging MFN2 mutations cause an untreatable, largely pediatric progressive peripheral neuropathy, Charcot-Marie-Tooth (CMT) disease type 2A. We used small molecule allosteric mitofusin activators that promote MFN conformations favoring fusion to interrogate the effects of MFN2 conformation and GTPase activity on MFN2-mediated mitochondrial fusion and motility *in vitro*. We translated these findings *in vivo* by defining dose-dependent pharmacodynamic and disease-modifying effects of mitofusin activators in murine CMT2A. MFN2 catalytic GTPase activity and MFN2 conformational switching are essential for mitochondrial fusion, but the two processes are separate and dissociable. We report the first concentration-response relationships for mitofusin activators to stimulate mitochondrial transport through CMT2A neuronal axons, which is similar to their stimulation of mitochondrial fusion. In CMT2A mice, intermittent (daily short acting) and sustained (twice daily long acting) mitofusin activation were equally effective in reversing neuromuscular degeneration. Moreover, acute dose-dependent pharmacodynamic effects of mitofusin activators on mitochondrial transport through CMT2A neuronal axons anticipated those for long-term reversal of neurodegenerative phenotypes. A cross-over study showed that CMT2A neuronal deficits recurred after mitofusin activators are discontinued, and revealed that CMT2A can be ameliorated by mitofusin activation even in old (>74 week) mice. These data add to our understanding of mitochondrial dysfunction induced by a CMT2A MFN2 GTPase mutation and provide additional information supporting the approach of pharmacological mitofusin activation in CMT2A.

SIGNIFICANCE –

We interrogated the roles of MFN2 catalytic activity and allosteric activation on impaired mitochondrial fusion and neuronal transport as they impact an untreatable peripheral neuropathy caused by MFN2 mutations, Charcot-Marie-Tooth disease type 2A. Our results mechanistically link mitochondrial fusion and motility to the relaxed MFN2 protein conformation, and correction of mitochondrial abnormalities to *in vivo* reversal of neurodegeneration in murine CMT2A.

INTRODUCTION

Charcot-Marie-Tooth (CMT) disease type 2A is a largely pediatric peripheral neuropathy caused in most instances by mutations of the nuclear gene encoding a mitochondrial fusion protein, mitofusin (MFN) 2 (Zuchner et al., 2004; Verhoeven et al., 2006). Although other genetic causes have been reported (Zhao et al., 2001), the majority of CMT2A cases are produced by loss-of-function *MFN2* mutations with autosomal dominant transmission or that arise spontaneously; rarely, autosomal recessive inheritance of CMT2A caused by *MFN2* mutations has been reported (Tazir et al., 2013; Stuppia et al., 2015). While the >100 implicated *MFN2* mutations confer some heterogeneity in disease onset and progression (Verhoeven et al., 2006; Feely et al., 2011; Bombelli et al., 2014; Pipis et al., 2020), neuromuscular degeneration typically manifests in the “toddler” period of age 3-5, progresses during childhood and adolescence, and then stabilizes in young adults, producing life-long disability. Because there has been no way of correcting mutational MFN2 dysfunction, this disease remains untreatable and is managed with supportive measures.

The consequences of mitofusin dysfunction in CMT2A (Palau et al., 2009; Wolf et al., 2019) have traditionally been interrogated through overexpression of wild-type or mutant human MFN cDNAs or genetic ablation/suppression of endogenous mouse *Mfn1* or *Mfn2*. The recent development of small molecule mitofusin activators made it possible to investigate mitofusin functioning without experimentally perturbing *Mfn1* or *Mfn2* levels. To date, three chemical classes of mitofusin activators have been described, triazolureas (Rocha et al., 2018; Zacharioudakis et al., 2022), phenylhexanamides (Dang, 2020; Dang et al., 2021), and derivatives of the natural compound piperine. All of these small molecule mitofusin activators chemically mimic amino acid side chains that determine mitofusin protein conformation. Thus, when mitofusin activators bind to mitofusins they compete with endogenous peptide-peptide interactions that normally maintain a folded MFN structure unfavorable for mitochondrial fusion (Franco et al., 2016). As a consequence, mitofusins acquire a relaxed protein conformation that facilitates mitochondrial fusion and motility. This pharmaceutical mechanism of activation mimics phosphorylation-induced MFN conformational changes during natural homeostatic regulation (Li et al., 2022). Mitofusin activators exhibit a high degree of selectivity (>3 orders of magnitude) for mitofusins compared to other drug targets (Rocha et al., 2018; Dang et al., 2020; Dang et al., 2021), suggesting that they can be useful additions to the research armamentarium.

The prototype triazolurea mitofusin activators, collectively called Chimeras (Rocha et al., 2018), underwent rapid first-pass hepatic elimination and were therefore not useful *in vivo* (Dang et al., 2020). However, the equipotent phenylhexanamide, *trans*-MiM111, possessed *in vivo* pharmacokinetic properties suitable for studying MFN2 dysfunction in mice (Dang et al., 2020). An initial feasibility study of daily intra-muscular *trans*-MiM111 in murine CMT2A reversed functional, histological and neuroelectrophysiological signs of neuromuscular degeneration (Franco et al., 2020). This study employed a “burst activation” approach in which mitofusin activator plasma levels were therapeutic during only ~8 hours of every 24, and the presumed disease-relevant pharmacodynamic effect (increased mitochondrial motility in CMT2A mouse sciatic nerve axons) was completely lost between daily doses (Franco et al., 2020).

The above cited proof-of-concept study for mitofusin activation in murine CMT2A raises questions whose answers may determine whether pre-clinical development is possible or warranted. First, although transient mitofusin activation was beneficial, might sustained mitofusin activation be superior? Second, is the duration of benefit from mitofusin activation in CMT2A temporary or does it extend beyond the treatment period? And finally, how does mitofusin activator dose relate to reversal of mitochondrial dysmotility in peripheral nerves and improved *in vivo* neuromuscular function? Here, we take advantage of a recently described longer-acting phenylhexanamide mitofusin activator, N-[(1*s*,4*s*)-4-Hydroxycyclohexyl] (1*R*,2*R*)-2-(3-phenylpropyl) cyclopropanecarboxamide (CPR1-B) (Dang et al., 2021), to further interrogate mechanisms of mutant MFN2 dysfunction in CMT2A and better define the relationships between mitofusin activator pharmacodynamics and disease response.

MATERIALS AND METHODS

Mouse lines

All experimental procedures were approved by the Washington University in St. Louis, School of Medicine Animal Studies Committee; IACUC protocol number 19-0910 Exp:12/16/2022. Rosa-STOP-mMFN Thr105Met (T105M) mice (C57BL/6 Gt(ROSA)26 Sortm1 (CAG-MFN2*T105M)Dple/J) from The Jackson Laboratory (Bar Harbor, Maine, USA; Stock No: 025322) were crossed to HB9-Cre mice (B6.129S1-Mnx1tm4(cre)Tmj/J) from The Jackson Laboratory (Stock No: 006600) to generate motor neuron-targeted MFN2 T105M mice as described (Franco et al., 2020). For *in vivo* longitudinal studies, mice were randomized to treatment groups by LZ and experiments performed by AF who was blind to treatment status. Male and female mice were randomized to treatment without regard to sex. Because there were no differences between CMT2A male and female mice prior to or after completing 8 weeks of mitofusin activator treatment (**Supplemental Figure 1**), males and females were combined for analyses of *in vivo* phenotypes.

Chemicals and reagents:

Viral vectors: Viral constructs used human adenovirus Type5 (dE1/E3). Cells were transduced at a multiplicity of infection (MOI) of 50 using: adenovirus β -galactosidase (Vector Biolabs Cat#: 1080), adenovirus Mito-Ds-Red2 (Signagen Cat#: 12259), or adenovirus Cre-recombinase (Vector Biolabs Cat#: 1794); Ad-MFN2 WT and MFN2 T105M (Franco et al., 2020) were synthesized at Vector Biolabs.

Antibodies and stains: Mouse monoclonal anti-mitofusin 2 was from AbCAM (Cat#: ab56889; 1:1000 dilution), rabbit polyclonal anti-cytochrome c oxidase subunit 4 (COX-IV) was from AbCAM (Cat#: ab16056; 1:1000 dilution), mouse monoclonal anti-GAPDH was from AbCAM (Cat#: ab8245; 1:3000 dilution), α -bungarotoxin Alexa-Fluor 594 was from ThermoFisher (Cat#: B12423; 0.5 μ g/ml), Alexa-Fluor 488 goat anti-rabbit was from ThermoFisher (Cat#: A11008; 1:400 dilution), fluorescein-conjugated wheat germ agglutinin (WGA) was from Invitrogen (Cat#: W834; 1:50 dilution), MitoTracker Orange was from Thermo Fisher (Cat#: M7510), and tetramethylrhodamine ethyl ester (TMRE) was from Thermo Fisher (Cat#: T669), and Hoechst nuclear stain was from Thermo Fisher (Cat#: H3570).

Chemical compounds: *Trans*-MiM111 and CPR1-B (Dang et al., 2020; Dang et al., 2021) were initially obtained from Mitochondria in Motion, Inc. under terms of an MTA, and later re-synthesized at WuXi Apptech. Chimera C (Rocha et al., 2018; Dang et al., 2020) was re-synthesized at Paraza Pharma. Each small molecule mitofusin activator was maintained as a 10 mM stock in DMSO. For *in vivo* administration the final vehicle was 10% DMSO/ 90% (70% water/30% 2-hydroxypropyl- β -cyclodextrin (HP- β -CD; Sigma Cat#: 332607). Mitofusin activating peptide MP1 (Franco et al., 2016) was synthesized at ThermoFisher.

Cultured cells

Mfn2 null and *Mfn1/Mfn2* double null murine embryonic fibroblasts (MEFs) were purchased from American Type Culture Collection (ATCC Manassas, Virginia, USA, CRL-2993 and CRL-2994 respectively) and cultured under standard conditions as described (Dang et al., 2020).

Dorsal root ganglion (DRG) neurons were isolated from ~ 8 week old MFN2 T105M flox-stop transgenic mice and cultured as described (Franco et al., 2020). After 5 days in culture, DRGs were transduced with adenovirus-Cre to induce MFN2 T105M expression, and with adenovirus-mitoRFP to label neuronal mitochondria. Mitochondrial motility was assessed 48 hours thereafter by time-lapse video confocal imaging (Franco et al., 2020) after overnight treatment with *trans*-MiM111 or CPR1-B (100 nM) or vehicle (DMSO).

Live cell mitochondrial imaging in *Mfn2* null MEFs

Concentration-dependent fusogenic effects of mitofusin activators were assayed in MitoTracker Orange-stained live cells independently by two investigators in different laboratories using separate techniques and equipment.

Mitochondrial aspect ratio was measured by AF using confocal microscopy of individual MEFs exactly as described (Rocha et al., 2018). Briefly, images were acquired at room temperature on a Nikon Ti Confocal microscope using either a 60X1.3 NA oil-immersion objective or 10X0.3 NA dry objective. Cells were maintained in Krebs-Henseleit buffer (138 NaCl, 3.7 nM KCL, 1.2 n M KH₂PO₄, 15 nM Glucose, 20 nM HEPES pH: 7.2-7.5, and 1mM CaCl₂) during imaging. MitoTracker Orange was excited with the 549 nm laser and emission monitored at 590 nm. Mitochondrial aspect ratio was quantified using Image J software.

The % of Mfn2 null MEFs with filamentous mitochondria (Detmer and Chan, 2007) was measured by LZ using a Keyence BZ-X800 Fluorescence Microscope with automatic image acquisition (excitation 549 nm, emission 590 nm; eight 40x images per well) of 12-well plates. The proportion of cells with filamentous mitochondria in each image, corrected for vehicle, was indexed to the same value for 1 μ M *trans*-MiM111.

Interpolated D-R curves from both methods were generated using Prism software (version 9) and the data reported as EC₅₀ (nM) and E_{max} (% *trans*-MiM111) with 95% CI.

Mitochondrial motility studies

Mitochondria of cultured DRG neurons mitochondria were labeled with Adeno-mitoDsRed2 for 48h (excitation: 561 nm; emission: 585 nm). Mitochondria of sciatic nerves mitochondria were labeled *ex vivo* with TMRE (200nM). Mitochondrial movement was tracked in cultured neurons or explanted sciatic nerves using time-lapse confocal imaging (0.2 frames/sec) at 37°C on a Nikon A1Rsi Confocal Microscope with the 40x oil objective. Kymographs and quantitative data were generated using an Image-J velocity measurement tool plug-in as described (Rocha et al., 2018).

GTPase assay

Mfn1/Mfn2 double-null MEFs were transduced with adenoviral vectors encoding wild-type MFN2, MFN2 T105M, or β -galactosidase (β -Gal) at a multiplicity of infection (MOI) of 50. 72 hours later mitochondria were isolated (Frezza et al., 2007) and total mitochondrial GTPase activity assayed using 100 μ g of mitochondria protein in triplicate for each condition and components of the GTPase-Glo assay kit from Promega (#V7681; Madison, WI, USA) following the manufacturer's protocol. In some studies, mitofusin activators Chimera or *trans*-MiM 111 (1 μ M) or their DMSO vehicle were included in the assay. Luminescence was quantified on a Promega GloMax Luminometer. MFN2-specific GTPase activity is GTPase activity in MFN2 expressing cells minus that for β -Gal expressing cells.

Mitofusin conformation Forster Resonance Energy Transfer (FRET) assay

Wild-type and T105M MFN2 were engineered with Cerulean fluorescent protein fused to the amino terminus and Venus fluorescent protein fused to the carboxyl terminus. Mfn1/Mfn2 double-null MEFs were transduced for 48 hours with adenovirus expressing the FRET-MFN2 proteins (50 MOI) for 48 hours, the cells harvested, and mitochondria isolated (Frezza et al., 2007). The FRET assay is described in detail in (Dang et al., 2020). Briefly, 65 µg of mitochondrial protein in 100 µl 10mM Tris-MOPS (pH 7.4), 10mM EGTA/Tris, 200mM sucrose was added to each well of a polystyrene 96 well assay plate (Costar CAT: 3916). Mitofusin agonist peptide MP-1 (5 µM), a small molecule mitofusin activator (1 µM), or their respective vehicles (water or DMSO) were added for 4 hours at room temperature with gentle shaking. Data were acquired on a Tecan Safire II multi-mode plate reader as follows: FRET – Excitation 433 nm, Emission 528 nm; Cerulean – Excitation 433 nm, Emission – 475 nm. FRET signals were normalized to their respective cerulean signals.

Western Blotting

Protein electrophoresis and immunoblotting used standard techniques. Briefly, whole cell lysates were size-separated on 10% polyacrylamide mini-gels at 30 milliamps for ~1 hour at room temperature before transferring to nylon membranes. After blocking for 1 hour with 5% milk, primary antibodies against Mfn2 (1:500, Abcam ab56889) or GAPDH (1:3000, Abcam ab8245) were added for 1 hour. Immunoreactive proteins were visualized using a horseradish peroxidase (HRP) linked anti-mouse IgG from Cell Signaling Technology (1:3000, cs7076) and imaged on a LI-COR Odyssey detection system.

Evaluation of mouse CMT2A phenotype

HB9-Cre/MFN2 T105M flox-stop mice were aged to 50 weeks to develop the complete CMT2A phenotype (Franco et al., 2020). Rotarod and neurophysiological tests of sciatic nerve/tibialis muscle function were performed at baseline and 4 week intervals thereafter as described (Franco et al., 2020). For studies comparing *trans*-MiM111 to CPR1-B and the cross-over substudy, baseline evaluation of Rotarod latency and CMAP amplitude was followed by randomization of 23 mice to receive, by oral gavage, *trans*-MiM111 (50 mg/kg once daily), CPR1-B (60 mg/kg twice daily) or daily vehicle (10% DMSO/90% [30% HP-β-CD] in water). The *trans*-MiM111/CPR1-B comparison study was terminated after 8 weeks, at which time 5 of

the 10 *trans*-MiM111 treated mice, the 5 CPR1-B treated mice, and 3 of the 8 vehicle-treated mice were euthanized by anesthesia overdose and tissue specimens obtained for histological studies.

The remaining 9 mice (4 *trans*-MiM111 treated and 5 vehicle treated) continued in a crossover study: Treatments were discontinued for a 16 week washout period and the treatment groups crossed over for 8 additional weeks. One mouse (subsequently determined to be in the *trans*-MiM111 treatment group) was euthanized for rectal prolapse unrelated to the study; these data were not included in the analysis.

Histological studies

Tibialis nerves and gastrocnemius muscles were isolated and prepared for frozen sections. For histology and immunohistology, nerves were fixed in 4% paraformaldehyde for 2 hours, transferred to 30% sucrose in PBS overnight (4 degrees C), embedded in optimal cutting temperature medium (OCT, Tissue-TEK Cat: 4583), and stored at -80 degrees C until frozen sectioned. Gastrocnemius muscles were fixed in Acetone for 10 minutes (room temperature), washed 3 times with PBS, treated for 15 minutes in 10 % Triton-X in PBS, and washed twice more with PBS. 10% goat serum was added for 15 minutes followed by Alexa Fluor 594 conjugated α -bungarotoxin overnight to stain acetyl choline receptors. Mitochondria were stained with anti-COX IV. Skeletal myocyte sarcolemma was visualized using fluorescein-conjugated wheat germ agglutinin. Mitochondrial area within neuromuscular synapses was calculated as the number of green/red pixels using Image J.

Data presentation and statistical analyses

Data are reported as mean \pm SEM, mean \pm SD, or mean with 95% confidence intervals as specified. Two-group comparisons used Student's t-test; multi-group comparisons used one-way ANOVA except for time course-by-treatment comparisons that used two-way ANOVA. Post-hoc ANOVA analyses used Tukey's test to obtain individual statistical comparisons. $P < 0.05$ was considered significant.

RESULTS

Equal responsiveness of mitochondrial fusion and transport to mitofusin activation

The appellative function of mitofusins is initiation and promotion of mitochondrial fusion (Santel and Fuller, 2001; Dorn, 2019). Accordingly, the adverse effects of mitofusin mutants and the benefits of mitofusin activators have been described almost exclusively in terms of mitochondrial fusion, most commonly as the observed change in organelle length or aspect ratio (length/width) (Detmer and Chan, 2007; Dang et al., 2022). However, accumulating evidence underscores a critical role for mitochondrial dysmotility in some neurodegenerative diseases, including CMT2A (Baloh et al., 2007; De Vos et al., 2008; Sheng and Cai, 2012; Dorn, 2020a; Schiavon et al., 2021). Indeed, mitochondrial dysmotility in neuronal axons may be a biomarker for neurodegeneration in this disease, and improved mitochondrial motility is postulated to predict therapeutic response (Rocha et al., 2018; Franco et al., 2020; Dang et al., 2022). Because mitofusin activator dose-response relations for mitochondrial motility have not been defined, we compared concentration-dependent effects of two pharmaceutically acceptable mitofusin activators, *trans*-MiM111 and CPR1-B (**Figure 1**), on mitochondrial fusion and motility.

Increased mitochondrial fusion manifests as mitochondrial elongation in mouse embryonic fibroblasts (MEFs) lacking *Mfn2*, in which mitochondria are atypically short or “fragmented” at baseline (Chen et al., 2003). Mitochondrial elongation can be assayed either as the increase in proportion of cells having predominantly filamentous mitochondria (Detmer and Chan, 2007) or as the increase in mitochondrial aspect ratio (organelle length/width) within individual cells (Dang et al., 2022). Here, fusogenic activities of MiM111 and CPR1 were similar when measured by either method (**Figures 2A, 2B; Table 1**). As reported (Dang et al., 2020; Dang et al., 2021), both MiM111 and CPR1 exhibited stereoisomer-specific fusogenic activity (**Figure 2A**).

The same compounds were evaluated for their effects on mitochondrial motility in dorsal root ganglion (DRG) neurons derived from mice expressing the human CMT2A MFN2 T105M mutation (Franco et al., 2020). This mutant is representative of the majority of CMT2A mutations located within the MFN2 GTPase domain (Feely et al., 2011); mitochondrial motility is severely depressed in neuronal processes of these DRG neurons (Franco et al., 2020). As shown in **Figure 2C**, concentration-dependent effects of *trans*-MiM111 and CPR1-B to increase mitochondrial motility in MFN2 T105M neuronal processes paralleled their induction of

mitochondrial elongation in fibroblasts (**Figure 2C**, compare to Figures 2A and 2B). EC_{50} and E_{max} values are in **Table 1**. Similar mitofusin activator concentration-response relationships for mitochondrial fusion and transport strengthen the proposition that these processes are co-regulated by mitofusins (Baloh et al., 2007; Schiavon et al., 2021).

Dissociation of MFN2 GTPase activity and allosteric activation

Mitofusins are dynamin-family GTPases that undergo conformational changes to physically link mitochondria and promote fusion of their outer membranes (Santel and Fuller, 2001; Detmer and Chan, 2007; Knott et al., 2008; Franco et al., 2016; Dorn, 2020b; Dorn, 2022). However, it is unclear how MFN GTPase activity relates to MFN protein conformation. This question has both scientific and clinical relevance as a majority of CMT2A-linked MFN2 mutations occur within the MFN2 GTPase domain (Feely et al., 2011) and are postulated to impair catalytic GTPase activity.

We considered that a combined genetic and pharmacological approach could help inform a more complete mechanistic understanding of how mitofusin-mediated mitochondrial fusion is impacted by mitofusin GTPase activity and protein conformation. Wild-type MFN2 or CMT2A mutant MFN2 T105M were expressed in murine embryonic fibroblasts (MEFs) lacking both *Mfn1* and *Mfn2*, thus avoiding confounding effects of endogenous mitofusins; adenovirus carrying β -Gal was the negative control, reflecting responses in the absence of any mitofusin target (**Figure 3A**). Compared to β -Gal expressing mitofusin null cells, WT MFN2 evoked mitochondrial elongation by increasing organelle fusion (**Figure 3B**) (Detmer and Chan, 2007; Dang et al., 2022). By contrast, mitochondria of cells expressing MFN2 T105M remained severely fragmented, indicating that this CMT2A mutant has little or no intrinsic fusogenic activity (**Figure 3B**). Representative triazolurea (Chimera C) and phenylhexanamide (*trans*-MiM111) mitofusin activators did not induce mitochondrial elongation in β -Gal expressing mitofusin null cells that lack their protein targets (i.e. absence of both *Mfn1* and *Mfn2*), nor did they correct absence of intrinsic fusogenicity in cells expressing CMT2A mutant MFN2 T105M (**Figure 3B**).

Absence of mitochondrial fusogenicity stimulated by MFN2 T105M recapitulates dysfunction reported for the MFN2 K109A mutant (4 amino acids distal in the GTPase domain) engineered to be GTPase defective (Detmer and Chan, 2007). Yet, a previous report described

increased (not decreased as expected) GTP hydrolysis with T105M substitution into a fragmentary MFN2 protein lacking amino acids 1-21 and 401-705 (Li et al., 2019). We posited that deletion of almost half of the MFN2 protein might have produced an artificial result, for example by removing MFN2 from its physiological membrane insertion site and perturbing its orientation and/or oligomerization. Moreover, normal mitochondrial mechanisms that regulate GTPase activity were not present in the published study design. Here, we compared GTP hydrolysis by MFN2 WT and T105M expressed as intact proteins in mitofusin null cells, permitting evaluation of the complete proteins in their natural state. When MFN2 GTPase activity is assayed *in situ*, adenovirally-expressed WT MFN2 accounts for approximately half of total mitochondrial GTPase activity (**Figure 3C**). By comparison, MFN2 T105M contributed no GTPase activity (**Figure 3C**). We further observed that mitofusin activators did not affect mitochondrial GTPase activity under any of the experimental conditions (**Figure 3C**). Thus, the CMT2A MFN2 GTPase domain mutation T105M abrogates enzymatic GTPase activity, which can account for its inability to promote mitochondrial fusion that requires GTP hydrolysis (Cohen and Tareste, 2018).

As introduced above, mitofusins are believed to undergo one or more conformational transitions that enable trans-organelle tethering and membrane fusion. Multiple models have been proposed envisioning different MFN structures, topologies, and multimeric complexes (reviewed in (Cohen and Tareste, 2018; Dorn, 2020b; Dorn, 2022)). Although the precise structures remain uncertain, MFN2 conformational shifts have been reliably and reproducibly measured using Forster resonance energy transfer (FRET) of recombinantly expressed MFN2 proteins engineered with amino- and carboxyl-terminal chromophores (Franco et al., 2016; Dang et al., 2020). In these assays a mitofusin activating peptide, MP1 (Franco et al., 2016), induces a relaxed conformation indicated by a lower FRET signal (increased distance between amino and carboxyl termini). Here, we assayed conformational switching induced by small molecule mitofusin activators in comparison with MP1. As previously reported (Dang et al., 2020; Dang et al., 2021), *trans*-MiM111 and CPR1-B promoted a relaxed WT MFN2 conformation (**Figure 3D, left**). Thus, conformational switching induced by mitofusin activator stereoisomers paralleled their stimulation of mitochondrial fusion and motility.

To our knowledge, the impact of a CMT2A MFN2 mutation on conformational switching has not been described using intact proteins *in situ*. We observed that MFN2 T105M shifted to

the relaxed/active conformation in response to MP1, *trans*-MiM111 and CPR1-B in a manner identical to WT MFN2 (**Figure 3D, right**). Thus, MFN2 T105M is not impaired in its ability to change conformation. Since MFN2 T105M is GTPase defective (**Figure 3C**), this finding dissociates mitofusin conformational shifting from GTP hydrolysis. The ability of mitofusin activators to act allosterically and promote the relaxed/active MFN2 conformation in GTPase-deficient MFN2 T105M mutant further dissociates these two processes.

Non-superiority of sustained vs burst mitofusin activation for reversing murine CMT2A

The above results indicate that *trans*-MiM111 and CPR1-B have similar effects on mitochondrial fusion and motility *in vitro*, and that they promote the relaxed/active MFN2 conformation without affecting catalytic GTPase activity. However, these two mitofusin activators have different *in vivo* pharmacokinetic and pharmacodynamic properties: *trans*-MiM111 is short acting and induces transient “burst” mitofusin activation when administered once daily, whereas CPR1-B has emerged as a longer acting compound that can provide sustained mitofusin activation (Dang et al., 2020; Dang et al., 2021). We took advantage of these features to test the hypothesis that sustained mitofusin activation would provide a more rapid or complete CMT2A response than burst activation. Because both mitofusin activators reportedly have acceptable oral bioavailability (50-70%) (Dang et al., 2020; Dang et al., 2021), we also took the opportunity to determine the efficacy of oral mitofusin activator administration in this CMT2A model.

Neuromuscular degeneration in MFN2 T105M these mice manifests functionally as an impaired ability to remain on a rotating cylinder (Rotarod latency) and electrophysiologically as decreased hind-limb compound muscle action potentials (CMAP) measured from sciatic nerves and tibialis muscles. Juvenile MFN2 T105M mice appear normal, but by 50 weeks of age Rotarod latency and CMAP amplitude decreases to approximately 50% of normal controls (Franco et al., 2020). Here, 50 week old MFN2 T105M mice recapitulated these features and were randomized to vehicle treatment, burst mitofusin activation with *trans*-MiM111 (50 mg/kg given orally once daily), or sustained mitofusin activation with CPR1-B (60 mg/kg given orally twice daily) (**Figure 4A**). Sustained and burst mitofusin activation improved RotaRod latency and increased CMAP amplitude to the same extent after both 4 and 8 weeks of treatment (**Figures 4B, 4C**). Likewise, both mitofusin activator treatment protocols restored neuronal

innervation of lower limb muscles measured as the density of neuromuscular junctions and mitochondrial residency within neuromuscular synapses, which are characteristically reduced in this CMT2A model (Franco et al., 2020) (**Figures 4D, E**). Thus, *in vivo* disease mitigation in MFN2 T105M CMT2A mice was similar after transient (*trans*-MIM111) vs sustained (CPR1-B) MFN activation.

Restoration of mitochondrial motility in CMT2A mice anticipates reversal of neuromuscular dysfunction.

Phenotypic reversal after mitofusin activation in CMT2A mice is thought to accrue from enhanced mitochondrial fusion and improved transport of axonal mitochondria to neuromuscular synapses (Dorn, 2021). The results reported in Figure 2 above revealed similar concentration-dependent mitofusin activator effects on mitochondrial fusion and neuronal transport *in vitro*. However, it is not known how these *in vitro* findings translate to *in vivo* reversal of CMT2A-associated neuromuscular degeneration. To provide clarity on this issue we assayed dose-dependent effects of mitofusin activation on mitochondrial motility in CMT2A sciatic nerve axons (a disease-relevant pharmacodynamic endpoint) and compared the acute pharmacodynamic response to chronic effects on Rotarod and CMAP phenotype testing. Because there were no differences between *trans*-MiM111 and CPR1-B *in vitro* or *in vivo* (*vide supra*), these experiments were performed only with *trans*-MiM111.

First, we defined the dose-dependent *in vivo* effects of *trans*-MiM111 on mitochondrial transport in CMT2A mice. The mitofusin activator was administered by oral gavage to naïve (i.e. no previous mitofusin activator treatment) MFN2 T105M CMT2A mice. Six hours thereafter the mice were sacrificed and their sciatic nerves placed in short term organ culture for time-lapse confocal imaging of axonal mitochondria (Rocha et al., 2018). Approximately ~25% of axonal mitochondria are motile in a normal mouse sciatic nerve, and this value is reduced to <5% in CMT2A mice (Franco et al., 2020). The 50 mg/kg dose of *trans*-MiM111 used in the disease reversal study (see Figure 4) increased mitochondrial motility to the normal range (**Figure 5A**). The minimum dose tested, 10 mg/kg, significantly improved mitochondrial motility to approximately half the normal value (**Figure 5A**), whereas the highest dose (300 mg/kg) increased mitochondrial motility to approximately 1.5 times normal (**Figure 5A**).

Next, we determined the impact of *trans*-MiM111 daily dose on the CMT2A mouse neurodegenerative phenotype. As in Figure 4, CMT2A mice were aged to 50 weeks to develop the full-blown disease and then treated with oral *trans*-MiM111 at 10, 30 or 50 mg/kg/day. (The highest *trans*-MiM111 dose for chronic studies was limited to 50 mg/kg/day by compound solubility). Rotarod latency and CMAP amplitude were measured after 4, 8 and 12 weeks of treatment. Both metrics of neuromuscular degeneration were improved at all drug doses, but the treatment duration required for improvement was inversely related to drug dose (**Figures 5B and 5C**). Thus, the MFN2 T105M CMT2A mouse model exhibits analogous dose-dependent effects of mitofusin activation on the acute pharmacodynamic endpoint of mitochondrial motility and on long-term phenotype reversal.

CMT2A recurs after withdrawing mitofusin activators

Gradual dying-back of peripheral neurons due to impaired delivery of healthy mitochondria to distal neurons is a postulated mechanism for neuropathy progression in CMT2A (Dorn, 2021). Accordingly, we envisioned that correcting mitochondrial dysfunction through mitofusin activation involves enhanced delivery of healthy mitochondria to neuronal growth buds and synapses, thus contributing to neuronal repair and regeneration. Because mitofusin activation does not correct intrinsic dysfunction of MFN2 T105M (*vide supra*), fusion/fission dynamics are seemingly corrected through activation of endogenous non-mutant Mfn1 and Mfn2. For this reason, we considered that mitofusin activation might have to be maintained for long-term disease amelioration and tested this notion in a randomized, blinded cross-over study of oral *trans*-MiM111 in CMT2A MFN2 T105M mice (**Figure 6A**). As in previous CMT2A mouse cohorts treated with *trans*-MiM111 [(Franco et al., 2020); *vide supra*], both functional and electrophysiological metrics of neuromuscular integrity improved within weeks of mitofusin activator treatment (**Figures 6B, 6C**). However, when the mitofusin activator was discontinued, neuromuscular dysfunction recurred over an 8-12 week period (**Figures 6B, 6C**). Remarkably, therapeutically naïve 74 week old CMT2A mice in the cross-over study design responded to mitofusin activation with improved exercise capacity and neuroelectrophysiological integrity comparable to the 50 week old mice (**Figures 6B, 6C**). Thus, removal of the mitofusin activator is associated with disease recurrence, but even older CMT2A mice responded to mitofusin activation with marked improvement in the disease phenotype.

DISCUSSION

Mitochondrial fusion mediated by mitofusin GTPases is essential to organelle fitness and function (Chen and Chan, 2005). Interrupting mitochondrial fusion by combined genetic ablation of Mfn1 and Mfn2 in mouse nervous system, skeletal muscle or heart adversely impacts homeostasis of those tissues (Chen et al., 2007; Chen et al., 2010; Chen et al., 2011; Kasahara et al., 2013). By contrast, interrupting mitochondrial motility through germ-line genetic ablation of the Miro1 mitochondrial transport adaptor protein preferentially affects the neurological system (Nguyen et al., 2014; López-Doménech et al., 2016). These and other findings support a paradigm where mitochondrial fusion is broadly important to cellular health, but mitochondrial motility and transport have particular importance to long neurons (Sheng and Cai, 2012). Consistent with this idea, the progressive peripheral neuropathy CMT2A caused in most instances by mutations of MFN2 has been variously attributed to defective mitochondrial fusion, motility or both (Frank, 2006; Herzig and Martinou, 2008; Chen and Chan, 2009; Dorn, 2020a). Here, we used recently developed research platforms and new reagents to interrogate the consequences of mutational MFN2 dysfunction on mitochondrial fusion and motility, and to better understand how mitofusin activation can reverse CMT2A-associated mitochondrial pathology and neuromuscular degeneration. We found that: 1. MFN2 GTPase activity and conformational shifting are independent and mechanistically unrelated; 2. mitochondrial fusion and motility are enhanced by small molecule mitofusin activators over a similar range of drug concentrations, and improved mitochondrial motility in CMT2A neuronal axons predicts improved neuromuscular phenotype; and 3. *in vivo* mitofusin activation must be maintained over weeks, but need not be sustained throughout the day, to reverse and protect against neurodegeneration in murine CMT2A. The preclinical results are summarized in Figure 7.

MFN2 and MFN1 physically connect mitochondria across cytosolic space and mediate the critical initial steps of reparative mitochondrial fusion (Chen and Chan, 2005). Uniquely, MFN2 can act as a mitochondrial receptor for Parkin in PINK1-Parkin mediated mitophagy (Chen and Dorn, 2013; Li et al., 2022). Finally, MFN2 (and possibly MFN1) can positively affect mitochondrial motility through incompletely understood mechanisms (Misko et al., 2010). Multifunctionality of MFN2 complicates interpretation of the many published studies that relied upon gene ablation or mRNA suppression to modulate MFN2 function, which we eschewed.

MFN2 tasking is thought to be determined by its binding to different protein partners (Dorn, 2020b; Dorn and Dang, 2022). Thus, MFN-MFN interactions mediate mitochondrial fusion, MFN-Parkin pairing mediates mitophagy, and MFN-Miro pairing likely regulates mitochondrial transport. MFN2 protein partnering is, in turn, determined by the phosphorylation status of 3 MFN2 PINK1 kinase sites, T111, S378 and S442. Phosphorylation of T111 and S442 promote Parkin binding and mitophagy (Chen and Dorn, 2013; Li et al., 2022), whereas S378 phosphorylation does not affect mitophagy, but instead acts as an “on-off switch” for mitochondrial fusion (Li et al., 2022). MFN2 S378 is located in the so-called “zipper domain” (MFN2 367-384) that regulates protein conformation via internal peptide-peptide interactions (Franco et al., 2016; Rocha et al., 2018). S378 de-phosphorylation increases the probability of MFN2 “un-zipping” into a more relaxed protein conformation that favors mitochondrial fusion. The mitofusin activating peptide MP1 (Franco et al., 2016) and the peptidomimetic small molecule mitofusin activators used herein (Rocha et al., 2018; Dang et al., 2020; Dang et al., 2021) act by disrupting the intra-molecular S378-regulated peptide-peptide interaction, thereby inducing a change in conformation that is more conducive to both mitochondrial fusion and motility.

The role of GTP hydrolysis as a determinant of mitofusin macromolecular structure has been studied using partial proteins in solution (Misko et al., 2012; Cao et al., 2017; Yan et al., 2018; Li et al., 2019), but whether GTP hydrolysis controls mitofusin conformation *in situ* remained unclear. Our data show that GTPase activity, which is abrogated by the MFN2 T105 mutation, is dispensable for conformational shifting. Furthermore, allosteric mitofusin activators induced a relaxed MFN conformation without affecting GTP hydrolysis. Thus, GTP hydrolysis is necessary for mitochondrial fusion, but does not regulate and is not impacted by allosteric mitofusin activation.

Of the many putative functions for MFN2, its modulation of mitochondrial transport is probably the least understood. Multiple groups have observed impaired mitochondrial motility in CMT2A neurons (Baloh et al., 2007; Misko et al., 2012; Franco et al., 2020; Mou et al., 2021). Baloh suggested that MFN2 modulates transport by interacting with Miro proteins (Misko et al., 2010). The current observations that mitofusin activator concentration-response relationships for mitochondrial fusion and transport are virtually identical suggest that a relaxed mitofusin conformation is conducive to both mitochondrial fusion and transport. Likewise, parallel dose-

response relationships for *trans*-MiM111 to enhance mitochondrial motility in therapeutically naïve CMT2A mouse sciatic nerve neurons, and to reverse neurodegeneration in CMT2A mice, supports any therapeutic approach that can restore mitochondrial motility in this condition (Dorn, 2021). It is notable that a low *trans*-MIM111 dose (10 mg/kg/day), which only partially corrected mitochondrial dysmotility in CMT2A sciatic nerves, was capable of improving neuromuscular degeneration in the mice, although over a longer time period than higher doses. One question raised by these results is whether mitofusin activation might also help correct mitochondrial dysmotility in the other neurodegenerative conditions, such as amyotrophic lateral sclerosis (Ligon et al., 2005; De Vos et al., 2007).

Mitofusin activators were only recently described and, to our knowledge, are the only small molecules that directly enhance mitochondrial fusion and transport. Multiple mitofusin activator chemical backbones have been described (Rocha et al., 2018; Dang et al., 2021; Zhang et al., 2022), each of which chemically mimic MP1, a MFN2-derived peptide that competitively inhibits peptide-peptide interactions which determine MFN1 and MFN2 folded versus unfolded tertiary structure (Franco et al., 2016; Rocha et al., 2018). Competing peptide MP1 and small molecules that mimic MP1 critical peptide side chains (Rocha et al., 2018) promote MFN extended or unfolded conformations, thereby evoking mitochondrial fusion and motility. This mechanism of action has been independently validated for the commercially available compound “MASM7” (Zacharioudakis et al., 2022), which was renamed from, but is chemically identical to, previously described Chimera parent compound “B01” (Rocha et al., 2018). Importantly, each of the mitofusin activators employed herein exhibits identical fusogenic activity for MFN1 and MFN2 (i.e. in *Mfn2* null and *Mfn1* null cells, respectively), and lacks fusogenic activity in cells lacking mitofusins (i.e. *Mfn1/Mfn2* double null cells) (Figure 3B; Ad- β Gal transduced) (Dang et al., 2020; Franco et al., 2020; Dang et al., 2021).

Our study comparing oral administration of a shorter- vs a longer-acting mitofusin activator demonstrated equivalent phenotype reversal, supporting the sufficiency of intermittent or “burst” mitofusin activation in this pathophysiology. If one accepts the idea that delivery of healthy mitochondria to, and removal of damaged mitochondrial from, neuromuscular junctions is central to CMT2A reversal, then non-superiority of continuous vs burst mitofusin activation can be explained. Mitochondria at neuronal termini generate ATP that fuels neuromuscular signaling and neuronal repair (Sheng and Cai, 2012). Accordingly, impaired transport of

mitochondria to neuromuscular junctions contributes to neuronal degeneration because healthy mitochondria can't replace damaged mitochondria where they are needed, and damaged mitochondria accumulate with cytotoxic consequences. But, the “mitochondrial shuttle system” does not have to run continuously. If mitochondrial senescence takes weeks, but intermittent replacement can be achieved daily, then neuronal function and repair should be maintained with intermittent or burst activation. Our data showing that mitochondrial transport can be pushed to supraphysiological levels by very high mitofusin activator doses raise the question of whether “more” is necessarily “better” in this context. Because mitochondria being actively transported are not doing their proper work generating ATP to maintain local neuronal metabolism, we think that the therapeutic goal should be a normal level of mitochondrial motility.

Our study has limitations. First, we focused on a single CMT2A mutation, MFN2 T105M. We selected this mutant for detailed study in part because it is an exemplar for many other MFN2 amino terminal GTPase domain mutations that are the most prevalent cause of CMT2A. Additionally, complementary *in vitro* and *in vivo* experimental platforms are available for this particular CMT2A mutation. It is comforting that similar mitochondrial dysfunction has been characterized, and responsiveness to mitofusin activation demonstrated, in patient fibroblasts and reprogrammed motor neurons carrying this and several other CMT2A mutations (Franco et al., 2020; Dang et al., 2022). Nevertheless, our *in vitro* mechanistic results indicating that MFN2 GTPase activity has no effect on MFN2 conformational switching and the observed *in vivo* parallels between dose-dependent mitofusin activator pharmacodynamic effects on mitochondrial motility and neurodegenerative phenotype reversal will need to be tested for CMT2A MFN2 mutations that are not GTPase-defective, especially within the MFN2 hydrophobic core and carboxyl terminus. A second limitation is our exclusive use of murine cells and mouse models to relate *in vitro* mitochondrial pathology in live cells with *in vivo* neuromuscular phenotype. This is necessary until mitofusin activators are approved for human use. However, published results studying the same MFN2 T105M mutation in human CMT2A patient dermal fibroblasts (Dang et al., 2022) and reprogrammed neurons (Franco et al., 2020) suggest that the types of findings we report here are translatable across species.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: GWD, PBM

Conducted experiments: AF, XD, LZ

Contributed new reagents or analytic tools: GWD

Performed data analysis: AF, XD, LZ, GWD

Wrote or contributed to the writing of the manuscript: GWD

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FOOTNOTES

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Reprint requests to:

Gerald W. Dorn II, MD

Philip and Sima K. Needleman Professor

Washington University Center for Pharmacogenomics

660 S Euclid Ave.

Campus Box 8220 St. Louis, MO 63110

Phone: 314 362-4892. Fax 314 362-8844.

Email: gdorn@wustl.edu

FIGURE LEGENDS

Figure 1. *Mitofusin activators used in these studies.* (top) Chimera C is the prototype for first generation triazolurea class of mitofusn activators that are not suitable for *in vivo* studies. (middle) *trans*-MiM111 and (lower) CPR1-B are second generation phenylhexanamide mitofusin activators that have stereoisomer-specific activity and can be used *in vivo*.

Figure 2. *Mitofusin concentration-response relationships for mitochondrial fusion and motility.*

A. Fusogenicity measured as the proportion of Mfn2 null MEFs having a preponderance of elongated filamentous mitochondria. Comparisons to each compound's less active stereoisomer are shown. **B.** Fusogenicity measured as mitochondrial aspect ratio (organelle length/width) in Mfn2 null MEFs. **C.** Mitochondrial motility measured as % motile mitochondria in neuronal processes of MFN2 T105M expressing murine DRGs. Means±SEM of 3 experiments with 8 (A), ~15 (B), or 4-5 (C) replicates. Representative images are shown to the right; mitochondria are stained red. Scale bars are 10 μ m. In Figures 2 and 4 *trans*-MiM111 is black and CPR1-B is red.

Figure 3. *Physiochemical abnormalities of CMT2A mutant MFN2 T105M.* **A.** Immunoblot showing adenoviral-mediated expression of wild-type (WT) and mutant T105M MFN2 in Mfn1/Mfn2 double knockout MEFs. GAPDH is loading control. **B.** Fusogenicity measured as the increase in mitochondrial aspect ratio after adenoviral transduction and treatment with Chimera C (Chim C) or *trans*-MiM111 (MiM111). Adeno β -Gal is negative control for MFN2 expression. * = $p < 0.05$ vs same treatment for Ad β -Gal; # = $p < 0.05$ vs same treatment for Ad MFN2 WT (2-way ANOVA). **C.** GTPase activities after adenoviral transduction and treatment with Chimera C (Chim C) or *trans*-MiM111 (MiM111). * = $p < 0.05$ vs same treatment for Ad β -Gal; # = $p < 0.05$ vs same treatment for Ad MFN2 WT (2-way ANOVA). **D.** Conformational relaxation of WT (left) and T105M (right) MFN2 assessed by FRET. MP-1 is positive control mitofusin agonist peptide. * = $p < 0.05$ vs Veh for same MFN2. **B-D.** Each marker represents a separate experiment performed with ~20 (B) or 3 (C, D) replicates.

Figure 4. *Sustained and burst mitofusin activation reverse neuromuscular degeneration in murine CMT2A.* **A.** Schematic depiction of experimental design. Once daily MiM111 confers transient mitofusin activation (Black); twice daily CPR1-B confers sustained mitofusin activation

(red). **B, C.** Improvement in neuromuscular function of 50 week old MFN2 T105M CMT2A mice after mitofusin activation. **B** is Rotarod latency; **C** is hindlimb neuroelectrophysiological study compound muscle action potential (CMAP) amplitude. Results for each mouse are reported as % change in baseline. * = $p < 0.05$ vs baseline; # = $p < 0.05$ vs vehicle at same time. **D.** Mitochondria (green, labeled with anti-COX IV) residency within acetylcholine receptors at neuromuscular synapses (AChR; red, labeled with Alexa Fluor conjugated α -bungarotoxin). Group quantitative data are to the right. **E.** Tibialis muscle myocyte cross sectional area. **D, E:** * = $p < 0.05$ vs vehicle (ANOVA); each marker represents a different mouse.

Figure 5. *Dose-dependent effects of mitofusin activation on mitochondrial motility and neuromuscular integrity in CMT2A mice.* **A.** Mitochondrial motility in CMT2A mouse sciatic nerve axons. (*left*) Representative kymographs showing motile mitochondria in neuronal axons 6 hours after indicated oral dosing of MiM111; mitochondria appear black. Raw data on top; bottom panels are re-tracings emphasizing motile mitochondria (blue is antegrade and red is retrograde transport). (*right*) Quantitative group mean mitochondrial motility data. Each marker represents a different neuronal axon from 2-3 mice per condition. * = $p < 0.05$ vs WT, # = $p < 0.05$ vs vehicle by ANOVA. **B and C.** Effects of orally administered MiM111 given once daily on CMT2A mouse Rotarod latency (**B**) and CMAP amplitude (**C**). * = $p < 0.05$ vs baseline, # = $p < 0.05$ vs vehicle at same time point, by 2-way ANOVA.

Figure 6. *CMT2A recurs after mitofusin activation is withdrawn in MFN2 T105M mice.* **A.** Schematic depiction of experimental design for this substudy, which was a continuation of the experiment depicted in Figure 4. Red X indicates where one mouse was withdrawn due to an unrelated medical issue. **B and C.** Rotarod latency (**B**) and CMAP amplitude (**C**) results. Bold lines indicate MiM111 treatment group within grey background treatment periods. * = $p < 0.05$ vs corresponding vehicle group (t-test).

Figure 7. *Schematic depiction of current study pre-clinical findings.* CMT2A mutant MFN2 T105M is GTPase defective and therefore exhibits impaired fusion despite normal conformational shifting (unfolded monomeric configuration shown). Reduced motility of mitochondria (green ovals) through MFN2 T105 expressing neurons, together with loss of

reparative mitochondrial fusion, diminishes delivery of healthy mitochondria to neuronal termini. The consequence is neuromuscular degeneration in CMT2A mice. Burst and sustained mitofusin activation of endogenous normal MFN1 and MFN2 enables MFN-MFN oligomerization (putative toroid structure shown) and restores mitochondrial motility and respiratory fitness, thereby reversing limb denervation and normalizing neuromuscular function.

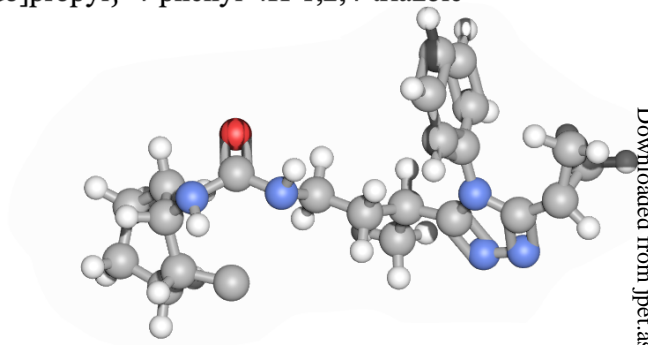
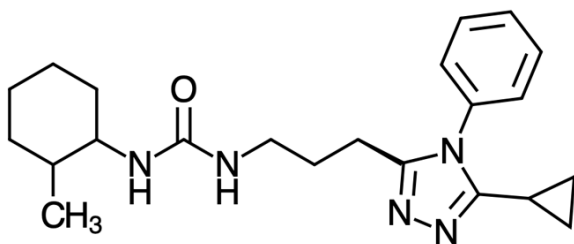
TABLE

Compound	Fusogenicity				Motility	
	% filamentous mito		Mito aspect ratio		% motile mito	
	EC ₅₀ (nM)	E _{max} (%)	EC ₅₀ (nM)	E _{max} (%)	EC ₅₀ (nM)	E _{max} (%)
<i>cis</i> -MiM111	NA	38 (32-45)	ND	ND	ND	ND
<i>trans</i> -MiM111	3.6 (2.1-6.0)	93 (82-104)	3.2 (2.1-4.7)	89 (82-96)	3.3 (2.0-5.3)	96 (86-105)
CPR1-A	NA	14 (8.2-19.7)	ND	ND	ND	ND
CPR1-B	3.3 (2.0-5.1)	87 (79-95)	4.9 (3.4-6.9)	96 (89-104)	11.2 (5.8-21)	99 (82-115)

Table 1. Calculated data from Figure 2 showing comparative activities of small molecule mitofusin activators for mitochondrial fusion vs mitochondrial motility. Fusogenicity was measured in Mfn2 null MEFs. Motility was measured in MFN2 T105M expressing mouse DRGs. Data are means (95% CI). NA = not applicable; ND is not determined. All concentration-response relations were indexed to independent *trans*-MiM111 studies.

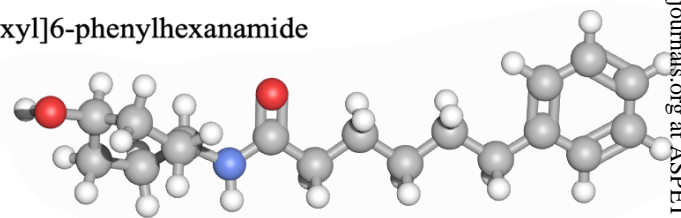
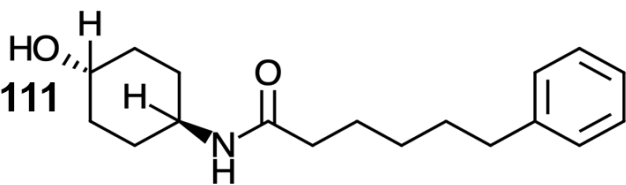
5-Cyclopropyl-3-{1-methyl-3-[3-(2-methylcyclohexyl)ureido]propyl}-4-phenyl-4H-1,2,4-triazole

Chimera C



N-[(1*s*,4*s*)-4-Hydroxycyclohexyl]6-phenylhexanamide

***trans*-MiM111**



N-[(1*s*,4*s*)-4-Hydroxycyclohexyl](1*R*,2*R*)-2-(3-phenylpropyl)cyclopropanecarboxamide

CPR1-B

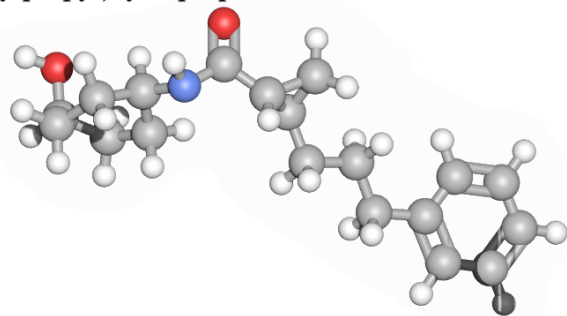
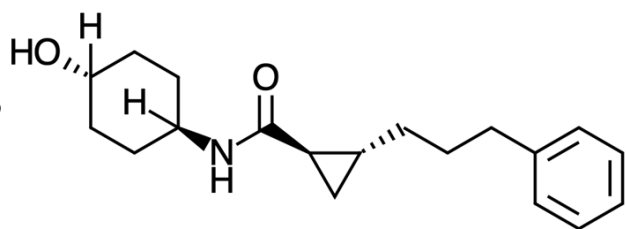
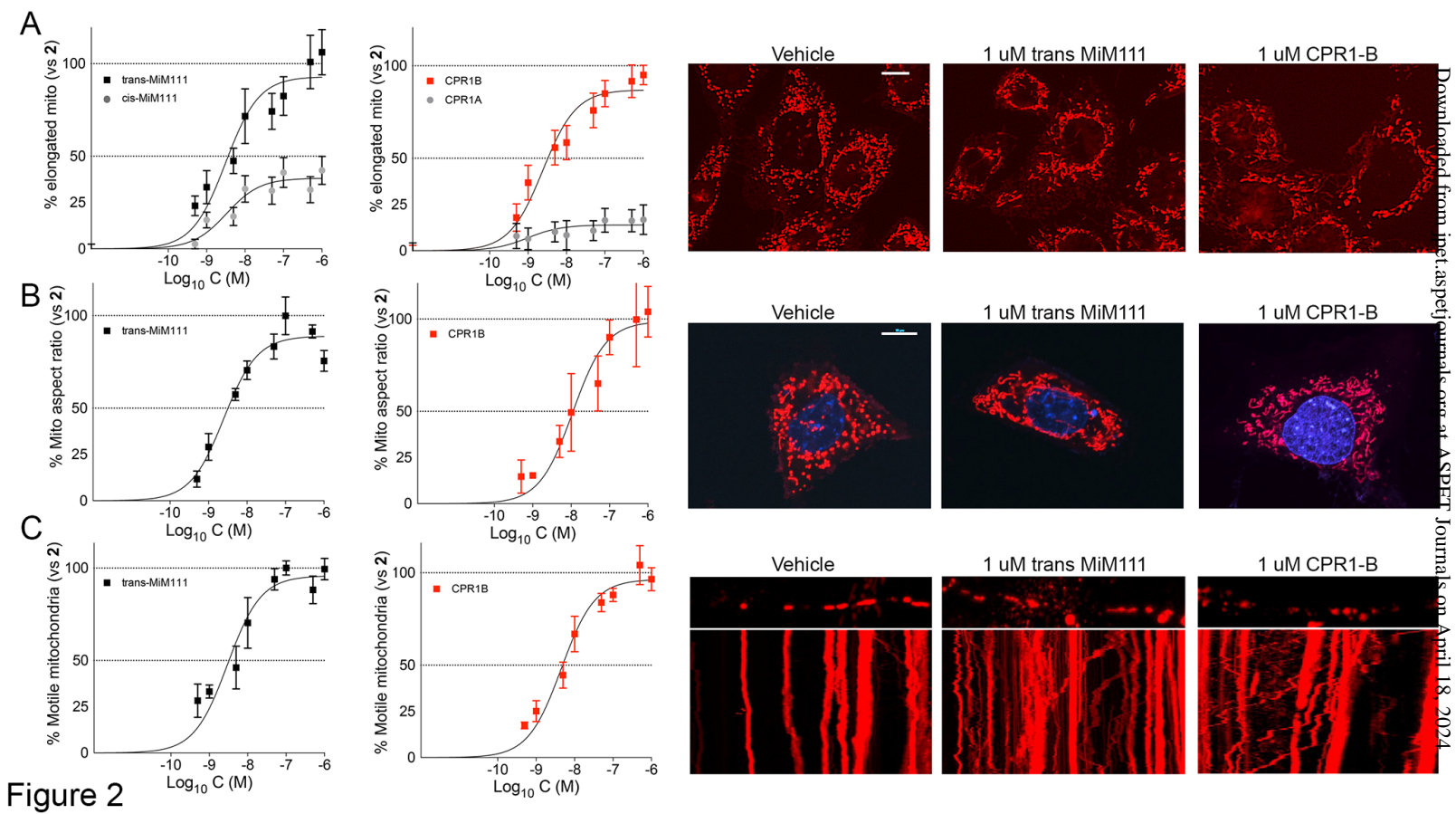


Figure 1



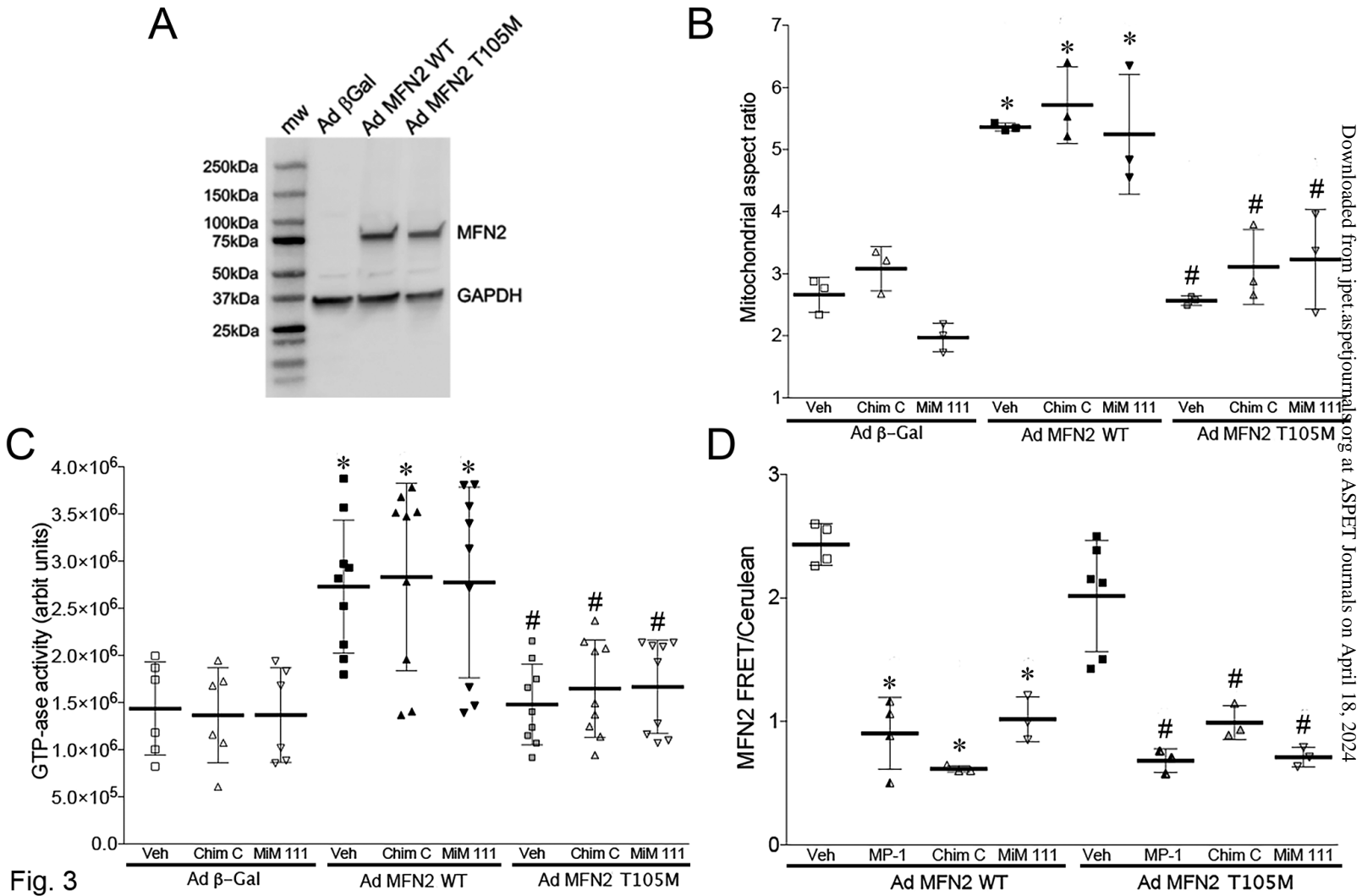


Fig. 3

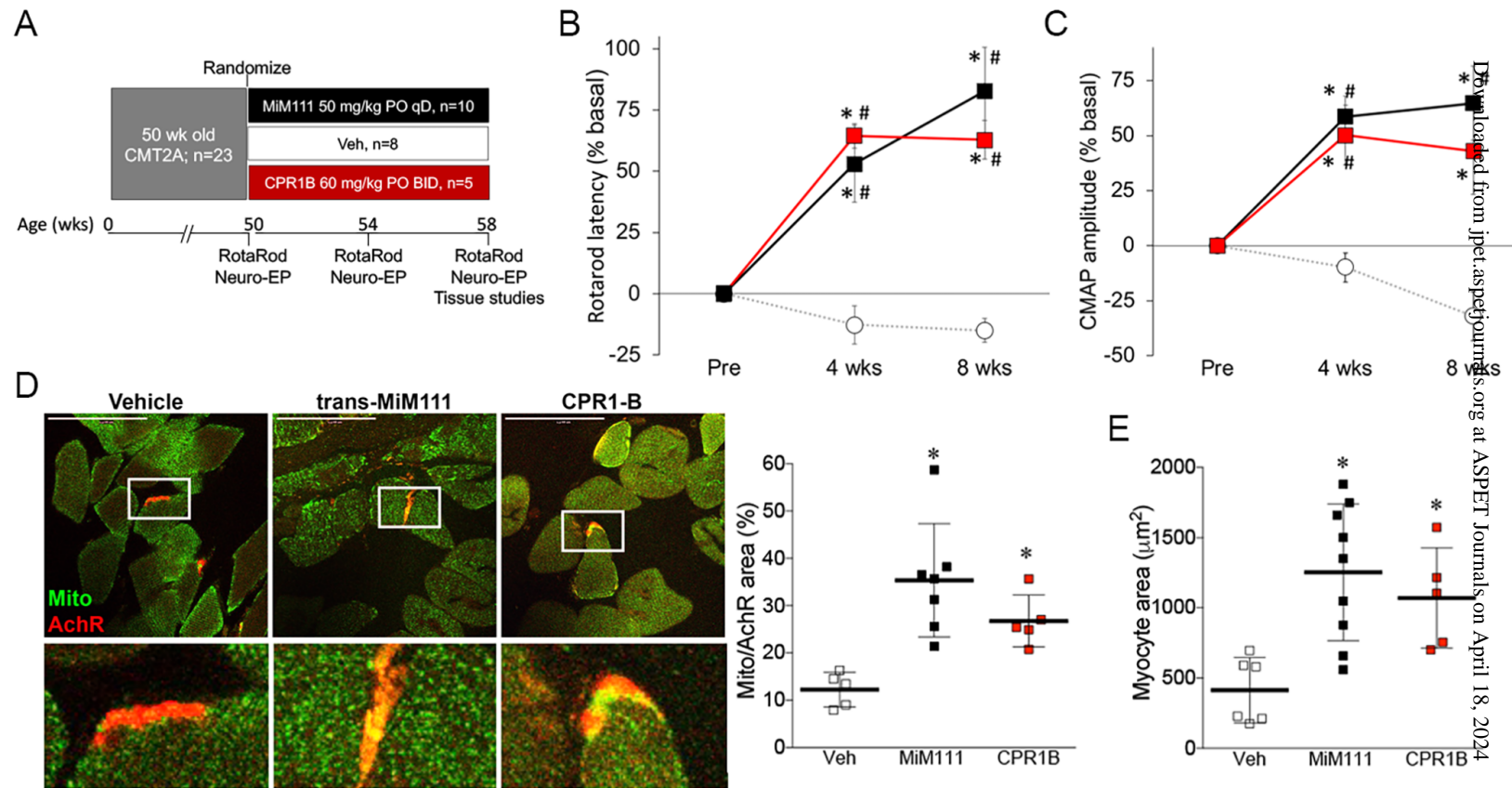


Figure 4

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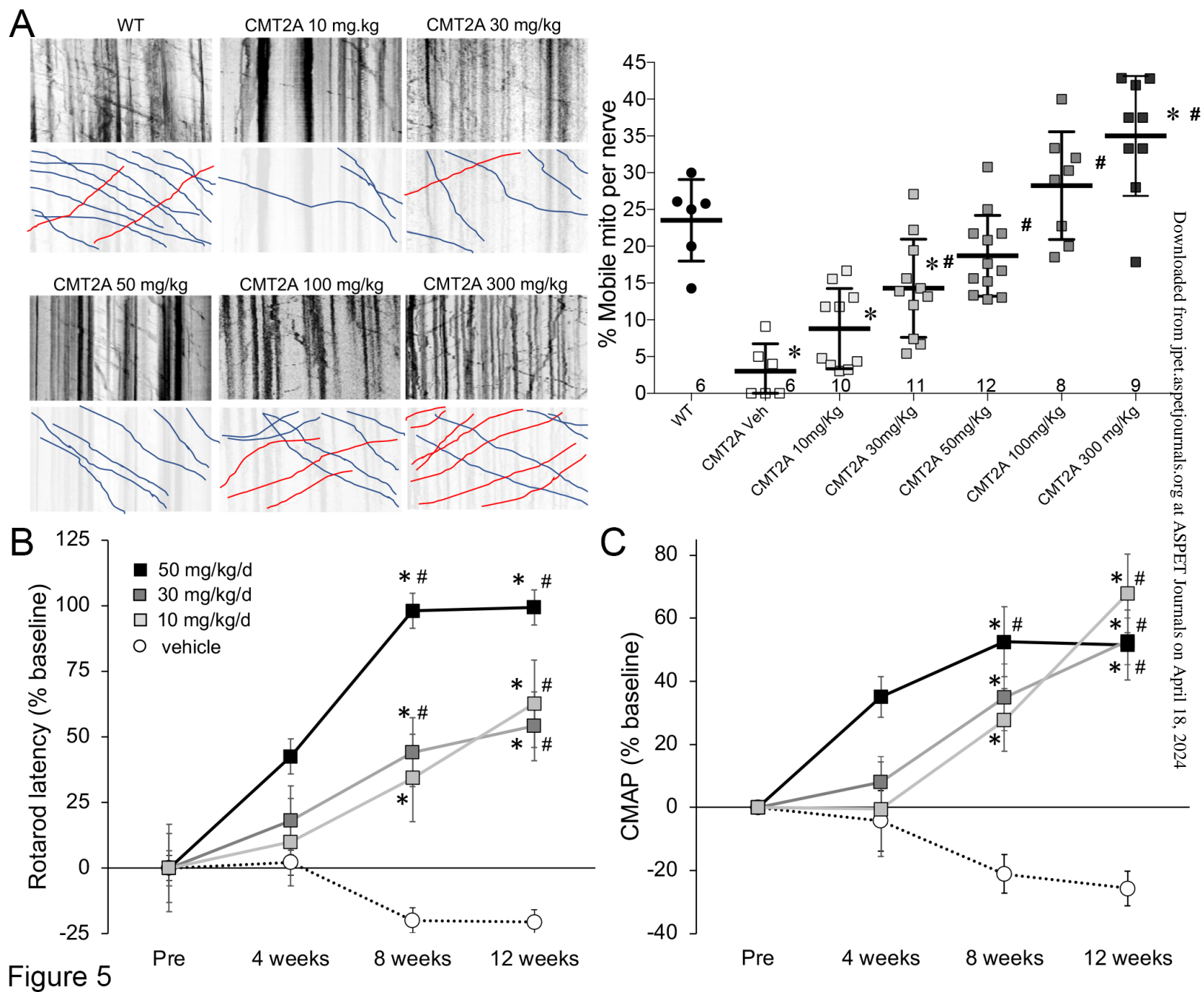


Figure 5

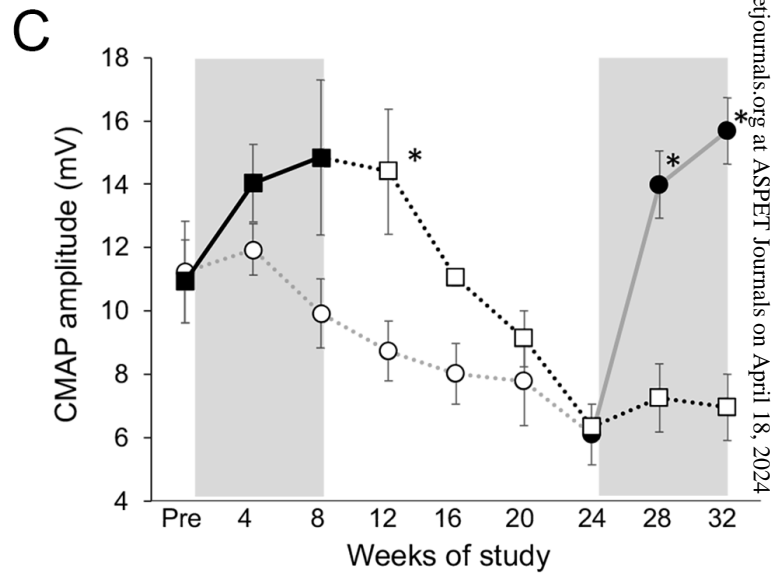
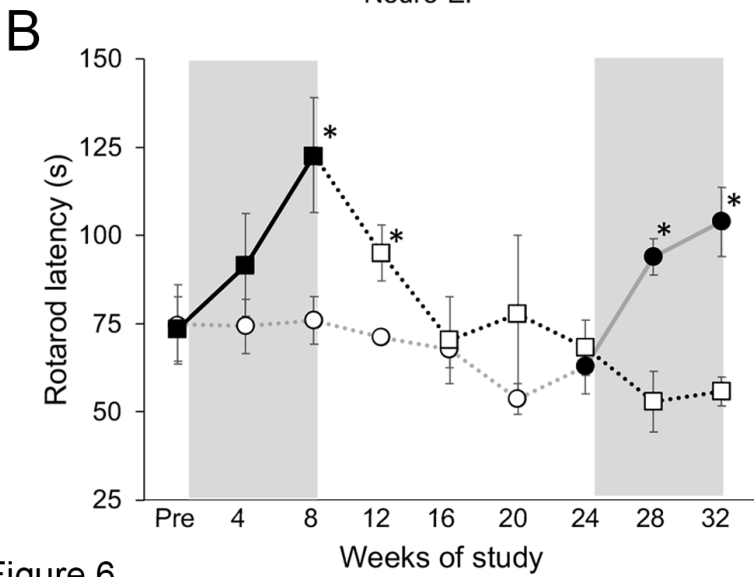
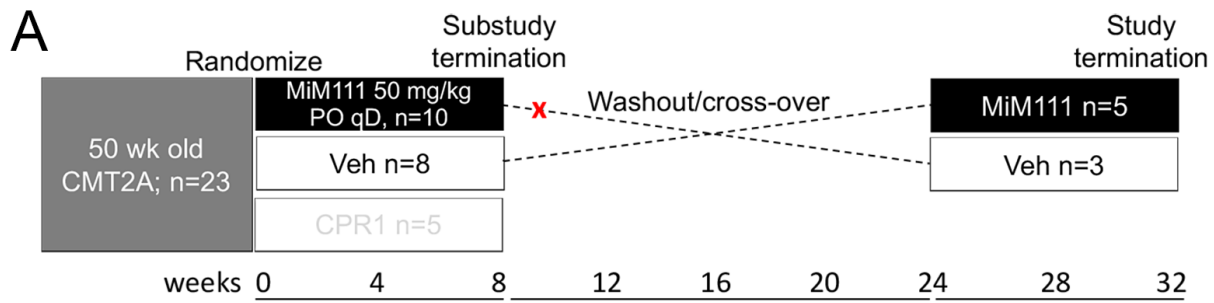


Figure 6

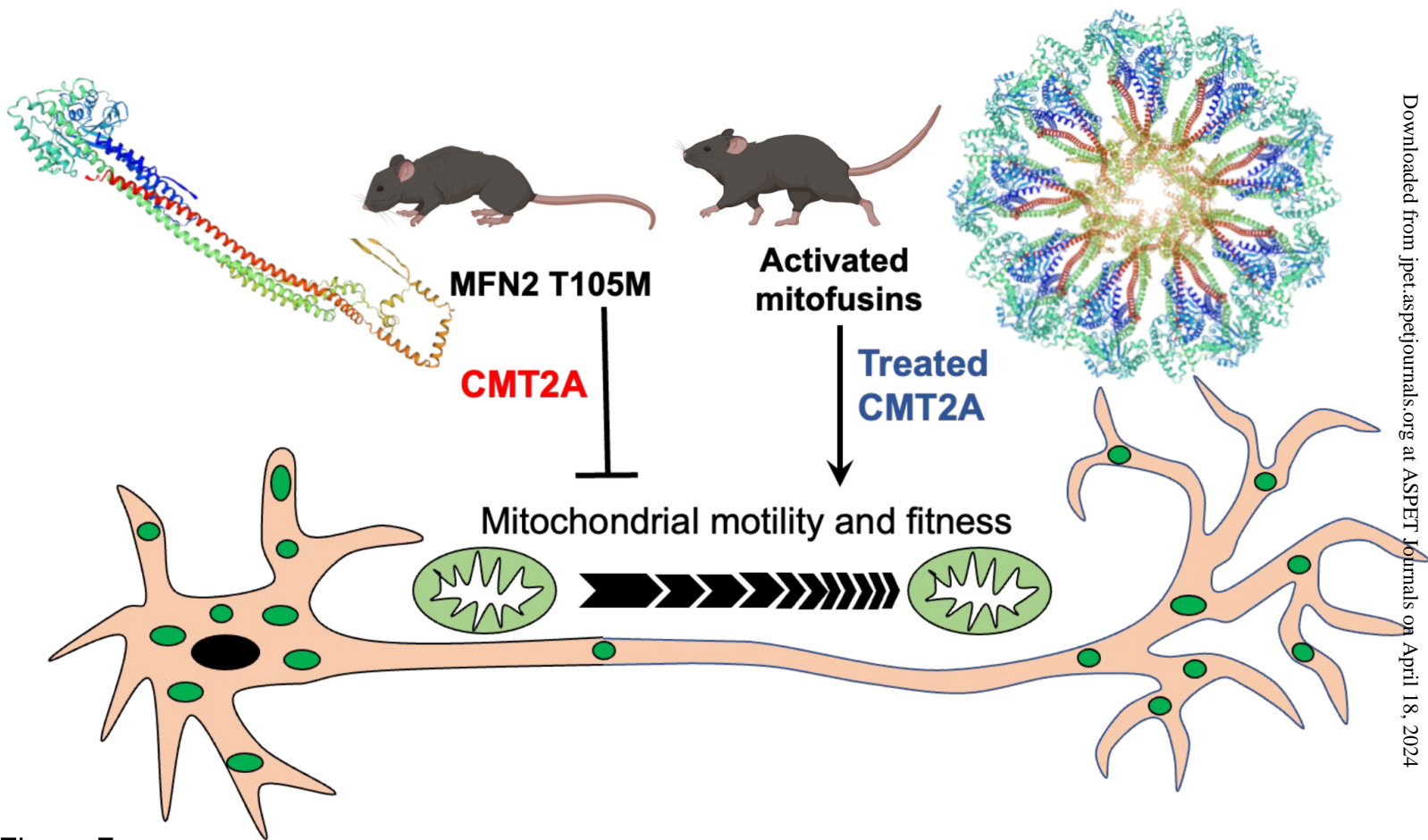


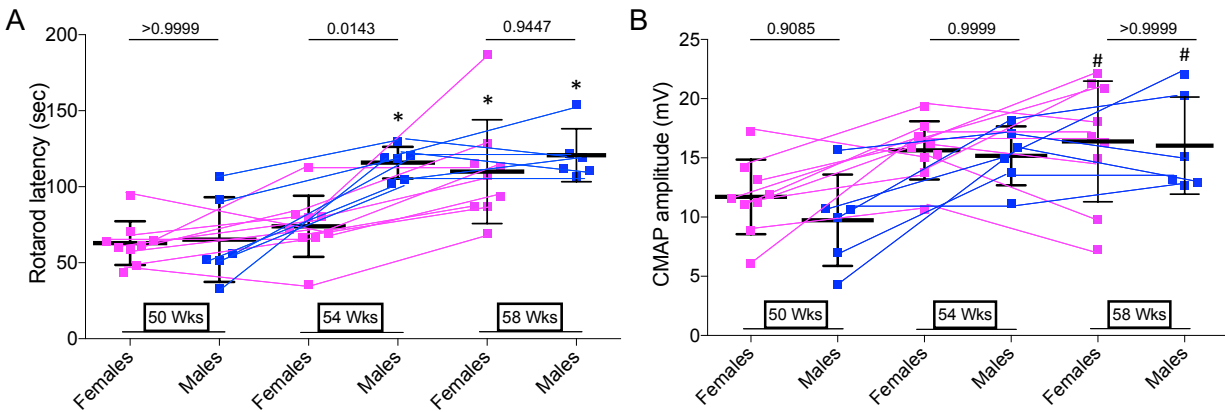
Figure 7

Mitochondrial dysfunction and pharmacodynamics of mitofusin activation in murine Charcot-Marie-Tooth disease type 2A

Antonietta Franco¹, Xiawei Dang^{1,2}, Lihong Zhang^{1,3}, Perry B Molinoff⁴, Gerald W Dorn II¹

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SUPPLEMENTAL INFORMATION



Supplemental Figure 1. Comparison of female vs male CMT2A mouse response to daily oral administration of the small molecule mitofusin activator trans-MiM111. **A.** Rotarod latency. **B.** CMAP amplitude. Each mouse is a square; pink is female and blue is male. Group mean data are shown as means ± SD. P values (2-way ANOVA) for male vs female comparisons are shown at top. * = p < 0.05 vs same sex pretreatment baseline (50 week age); # = p < 0.1 vs same sex pretreatment baseline (50 week age).