MOUSE MODELS OF CHARCOT-MARIE-TOOTH TYPE 2E FOR THERAPEUTIC DEVELOPMENT

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INTRODUCTION

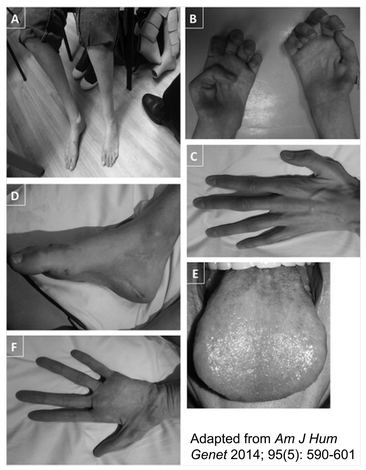
- Charcot-Marie-Tooth (CMT) is a group of inherited peripheral neuropathies with an incidence of approximately 1 in 2,500.
- CMT2 is a slow, but progressive disorder associated with axonal dysfunction and the deterioration of axonal connections and communication with muscles.
- Clinical symptoms include muscle weakness, hammer toes, high arches, and the loss of balance, coordination, and sensation that progresses to the distal extremities.
- Subtype CMT2E is an autosomal dominant axonopathy caused by mutations in the *NEFL gene* that encodes for the intermediate filament protein, neurofilament light (NF-L).
- NF-L is one of five subunits (NF-H, NF-M, NF-L, peripherin, and a-internexin) that composes neurofilaments and contributes to the cytoskeleton of axons.
- Disruptions in neurofilament formation impair axonal assembly, maintenance, and integrity.
- Different *NEFL* mutations result in variable times of onset and typically lead to paresis and atrophy of the muscles in the distal lower limbs, loss of fine motor skills, and gait abnormalities.
- Currently, there are no FDA-approved treatments for CMT2.
- We are directing focus toward the human mutation E396K (E397K), a point mutation in the rod domain of NF-L that alters amino acid 396 from glutamic acid to lysine.
- In this study, we use a newly derived E396K mouse model in which the corresponding mutation was generated in the mouse *NEFL* gene.

What is CMT2?

CMT2 Subtype	Affected Gene
CMT2A	*MFN2*
CMT2B	*RAB7*
CMT2C	*TRPV4*
CMT2D	*GARS*
CMT2E	***NEFL***
CMT2F	*HSPB1*
CMT2I	*MPZ*
CMT2K	*GDAP1*
CMT2L	*HSPB8*
CMT2M	*DNM2*
CMT2P	*LRSAM1*
CMT2S	*IGHMBP2*
CMT2T	*MME*
CMT2 (unassigned)	*MT-ATP6*

Adapted from *Charcot-Marie-Tooth News*, 2021

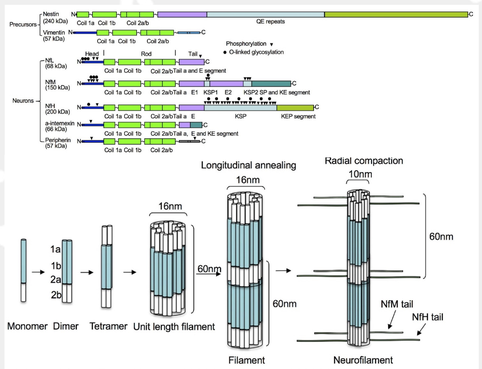
Figure 1. CMT2 subtypes and the associated gene. Mutations in *NEFL* result in CMT2E.



Adapted from *Am J Hum Genet* 2014; 95(5): 590-601

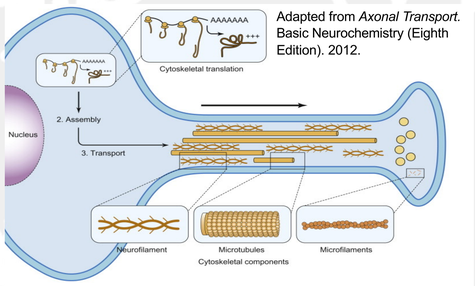
Figure 2. Clinical symptoms of patients with CMT2E. (A) Muscular atrophy of the lower legs, (B) hand contractures, (C) hand flattening, (D) high arches, (E) trombone-shaped tongue, (F) muscular atrophy of the hand.

What are neurofilaments?



Adapted from *Front Neurosci* 2021; 15: Article 689938

Figure 3. *NEFL* encodes for the protein NF-L. NF-L monomers combine to form coiled-coil dimers that form filaments that then twist to create neurofilaments.



Adapted from *Axonal Transport.* Basic Neurochemistry (Eighth Edition). 2012.

Figure 4. Neurofilaments, along with microtubules and microfilaments compose the neuronal cytoskeleton. They support the growth and maintenance of the axon.

OBJECTIVE

- The objective of this study is to characterize the newly-derived *NEFL* E396K model and develop a therapeutic strategy for the treatment of CMT2E.

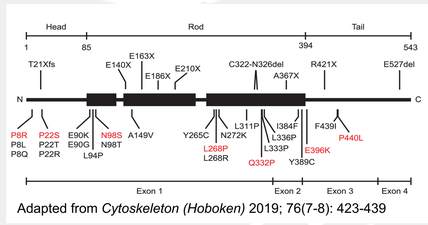
GENERATION OF *NEFL* E396K MOUSE

Glu-Lys-Gln-Leu-Gln-Glu-Leu-Glu-Asp-Lys

G to A

Glu-Lys-Gln-Leu-Gln-Lys-Leu-Glu-Asp-Lys

Figure 5. *NEFL*$^{+/E396K}$ and *NEFL*$^{E396K/E396K}$ (AMC100 mice) were generated at the MU-MMRRC core using CRISPR technology. An orthologous mutation was created in the mouse *NEFL* gene that corresponds to human *NEFL*^{E396K}, making a Glutamic Acid (**G**AA) to a Lysine (**A**AA).



Adapted from *Cytoskeleton (Hoboken)* 2019; 76(7-8): 423-439

Figure 6. NF-L protein including some CMT2E mutations. The E396K mutation is located within the rod domain of NF-L.

ASSAYS FOR CHARACTERIZING THE *NEFL* E396K MUTATION

- The *NEFL*^{E396K} and wildtype mice were evaluated on the following criteria for future comparison to age-matched wildtype mice in order to characterize the phenotype of the heterozygous and homozygous mutant strains:
  - Lifespan
  - Weight
  - Hindlimb splay scores
  - Rotarod scores
  - Dowel rod scores
  - Gait and motor coordination

Figure 7. Rotarod test is used to assess motor performance and coordination. The initial speed was set at 4 rpm and accelerated and accelerated to 40 rpm over 300 s.

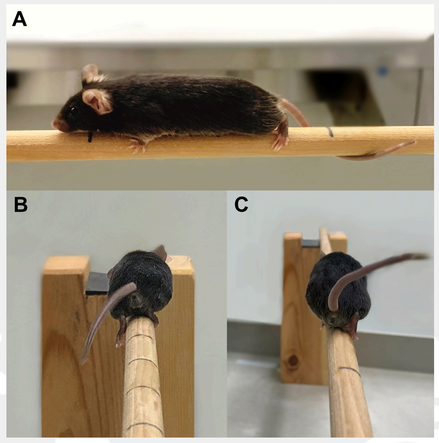


Figure 8. The dowel rod test is used to assess coordination and balance. Mice are assessed for tail grabs (A), foot slips (B), falls, and the time to transverse the rod. (C) serves as a comparison for the foot slip seen in (B).

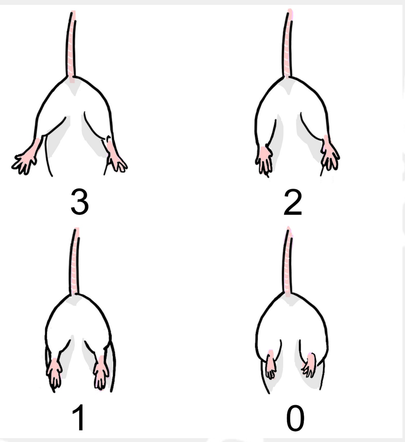


Figure 9. The hindlimb splay assay evaluates hindlimb strength. Mice are grasped by the base of their tail and observed for the splay of the hindlimbs. When limbs are splayed outward, the animal receives a score of 3. When one or both hindlimbs are less than 50% retracted toward the body, they are scored a 2. Mice with greater than 50% retraction receive a 1, and mice with complete retraction are given a 0.

RESULTS

Data collection for all assays began on post-natal day 108. All statistical significance determined by two-way ANOVA followed by a Tukey post-hoc test for pairwise comparisons. Error bars represent standard error of the mean. * = p <0.05.

Rotarod Time

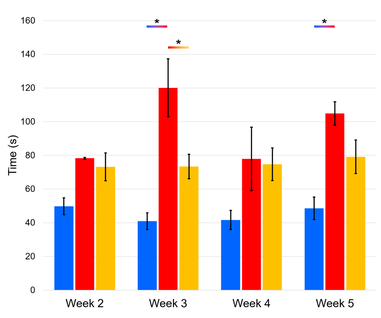


Figure 10. *NEFL*$^{E396K/E396K}$ mice show reduced time on the rotarod wheel compared to *NEFL*$^{+/E396K}$ mutant mice and wildtype controls.

Comparison	P Value
NEFL$^{E396K/E396K}$ vs. *NEFL*$^{+/E396K}$	0.0000001897*
NEFL$^{E396K/E396K}$ vs. WT	0.000006682*
NEFL$^{+/E396K}$ vs. WT	0.02283*

Dowel Rod Time

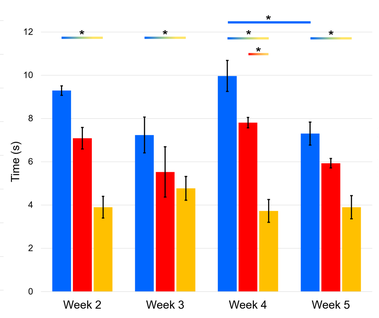


Figure 11. *NEFL*$^{E396K/E396K}$ and *NEFL*$^{+/E396K}$ mice take longer to cross the dowel rod than wildtype controls.

Comparison	P Value
NEFL$^{E396K/E396K}$ vs. *NEFL*$^{+/E396K}$	0.001696*
NEFL$^{E396K/E396K}$ vs. WT	0*
NEFL$^{+/E396K}$ vs. WT	0.00002485*

Weight

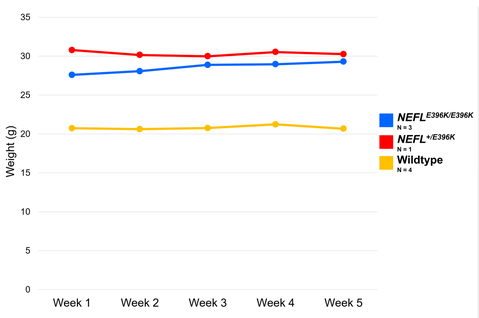


Figure 12. *NEFL* E396K mutant mice show increased weight as compared to controls.

Dowel Rod Foot Slips

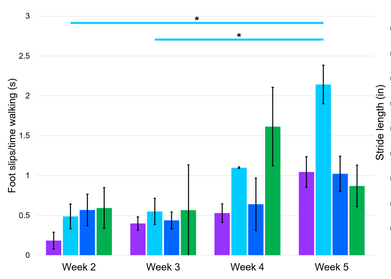


Figure 13. Number of foot slips per time walking on the dowel rod. This is the result of the average of 2 trials.

Comparison	P Value
NEFL$^{E396K/E396K}$ vs. *NEFL*$^{E396K/E396K}$	0.04946*

Comparison	P Value
Week 2 vs. Week 5	0.002332*
Week 3 vs. Week 5	0.003236*

Dowel Rod Stride Length

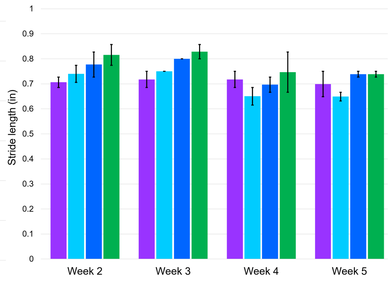


Figure 14. Stride length while walking on the dowel rod. This is the result of the average of 2 trials.

Comparison	P Value
NEFL$^{E396K/E396K}$ vs. *NEFL*$^{+/E396K}$	0.01864*

Dowel Rod Tail Grabs

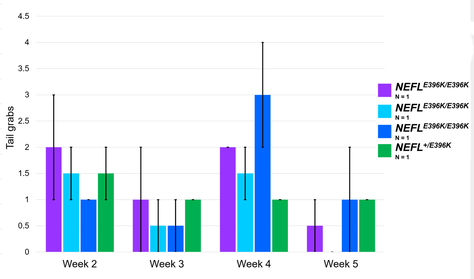


Figure 15. Number of tail grabs during the dowel rod test. This is the result of the average of 2 trials.

THERAPEUTIC DEVELOPMENT

- CMT2E is predominantly a result of autosomal dominant mutations distributed throughout human *NEFL*.
- The focus of our studies is the E396K mutation, but our therapeutic strategy could be applied to any dominant mutation.

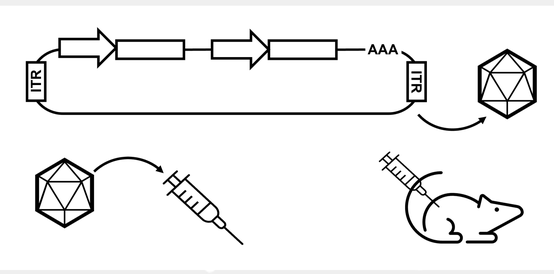


Figure 16. The construct made for AAV9 delivery is transfected to generate viral particles.

Steps of construct delivery and action:

1. Subcutaneous delivery of ssAAV virus to *NEFL* E396K mutant mice at post-natal day 2.
2. The construct is designed to reduce the expression of *NEFL* in the mouse while providing normal NF-L protein.
3. Look for modification of mutant phenotype and molecular changes.

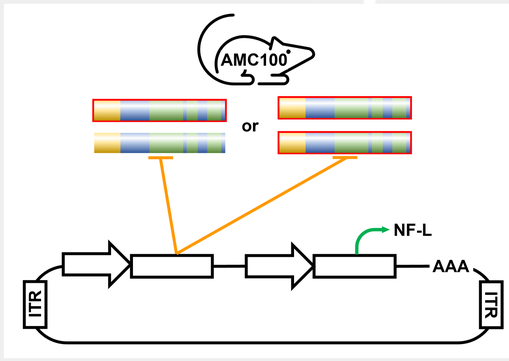


Figure 17. The construct inhibits expression of the wildtype, mouse mutant, and human mutant copies of *NEFL* and will "rescue"/provide normal NF-L protein.

CONCLUSIONS

- Our findings suggest that the dowel rod and rotarod assays, and weight can be used to evaluate disease progression in *NEFL*$^{E396K/E396K}$ and *NEFL*$^{+/E396K}$ mice.
- Additional phenotypes of *NEFL*$^{E396K/E396K}$ and *NEFL*$^{+/E396K}$ mice include a slight tremor and abnormal gait.
- *NEFL*$^{E396K/E396K}$ and *NEFL*$^{+/E396K}$ mice are useful models of CMT2E for the assessment of disease progression and the efficacy of therapeutic treatments.

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