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Kathryn H. Morelli, …, Scott Q. Harper, Robert W. Burgess

Gene therapy approaches are being deployed to treat recessive genetic disorders by restoring the expression of mutated genes. However, the feasibility of these approaches for dominantly inherited diseases — where treatment may require reduction in the expression of a toxic mutant protein resulting from a gain-of-function allele — is unclear. Here we show the efficacy of allele-specific RNAi as a potential therapy for Charcot-Marie-Tooth disease type 2D (CMT2D), caused by dominant mutations in glycyl-tRNA synthetase (GARS). A de novo mutation in GARS was identified in a patient with a severe peripheral neuropathy, and a mouse model precisely recreating the mutation was produced. These mice developed a neuropathy by 3–4 weeks of age, validating the pathogenicity of the mutation. RNAi sequences targeting mutant GARS mRNA, but not wild-type, were optimized and then packaged into AAV9 for in vivo delivery. This almost completely prevented the neuropathy in mice treated at birth. Delaying treatment until after disease onset showed modest benefit, though this effect decreased the longer treatment was delayed. These outcomes were reproduced in a second mouse model of CMT2D using a vector specifically targeting that allele. The effects were dose dependent, and persisted for at least 1 year. Our findings demonstrate the feasibility of AAV9-mediated allele-specific knockdown and provide proof of concept for gene therapy approaches for dominant neuromuscular diseases.

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Allele-specific RNA interference prevents neuropathy in Charcot-Marie-Tooth disease type 2D mouse models

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Gene therapy approaches are being deployed to treat recessive genetic disorders by restoring the expression of mutated genes. However, the feasibility of these approaches for dominantly inherited diseases — where treatment may require reduction in the expression of a toxic mutant protein resulting from a gain-of-function allele — is unclear. Here we show the efficacy of allele-specific RNAi as a potential therapy for Charcot-Marie-Tooth disease type 2D (CMT2D), caused by dominant mutations in glycyl-tRNA synthetase (GARS). A de novo mutation in GARS was identified in a patient with a severe peripheral neuropathy, and a mouse model precisely recreating the mutation was produced. These mice developed a neuropathy by 3–4 weeks of age, validating the pathogenicity of the mutation. RNAi sequences targeting mutant GARS mRNA, but not wild-type, were optimized and then packaged into AAV9 for in vivo delivery. This almost completely prevented the neuropathy in mice treated at birth. Delaying treatment until after disease onset showed modest benefit, though this effect decreased the longer treatment was delayed. These outcomes were reproduced in a second mouse model of CMT2D using a vector specifically targeting that allele. The effects were dose dependent, and persisted for at least 1 year. Our findings demonstrate the feasibility of AAV9-mediated allele-specific knockdown and provide proof of concept for gene therapy approaches for dominant neuromuscular diseases.

Introduction

Personalized medicine seeks to provide optimized treatments for individuals based on the molecular characteristics of their specific disease. For example, cancer treatments are customized to the genetics of the patient and the tumor (1, 2). However, developing personalized treatments for rare Mendelian disorders is challenging, and only about 5% of the more than 6800 recognized rare diseases have approved therapies (https://www.genome.gov/27531963/faq-about-rare-diseases/). Often, even when a disease gene is defined, the pathological impacts of specific genetic lesions may be uncertain or variable. In such cases, animal models recapitulating molecular lesions offer a means for validating the causality of new human mutations, and can serve as surrogates in preclinical therapeutic development (3, 4).

Gene therapy is a potentially powerful approach for treating rare genetic disorders, as it can precisely address the primary cause of these conditions (5). Gene therapy approaches are now in the clinic to treat recessive loss-of-function conditions by restoring the expression of the mutated genes (6, 7). However, the feasibility of gene silencing therapy to treat dominantly inherited, monoallelic mutations — where treatment may require reduction in the expression of a toxic mutant protein resulting from a gain-of-function allele — is unclear. To date, ClinicalTrials.gov lists only 2 phase I/II studies designed to test the safety of vector-expressed inhibitory RNAs in humans, and both were indicated for suppressing chronic hepatitis virus infection (NCT01899092 and NCT02315638). Thus, RNAi-based therapy is still an emerging field.

Here, we combine precision models of a dominant inherited neuropathy — Charcot-Marie-Tooth disease type 2D (MIM #601472), caused by mutations in glycyl-tRNA synthetase (GARS) (MIM #600287) — with allele-specific knockdown by viral vector-delivered gene therapy to demonstrate the effectiveness of this strategy. We designed our treatment for a single patient, but this sets an important precedent, as 90% of inherited peripheral
neuropathies — as well as the majority of familial amyotrophic lateral sclerosis cases — are inherited in an autosomal dominant manner (8, 9).

Charcot-Marie-Tooth disease type 2D (CMT2D) is a progressive, inherited axonal neuropathy caused by dominant mutations in GARS, encoding glycyl-tRNA synthetase (10). Mutations in GARS also cause a purely motor neuropathy, clinically designated as distal spinal muscular atrophy type V, but this is allelic with CMT2D (11). There is no treatment for CMT2D or any other form of inherited peripheral neuropathy. To date, at least 19 individual mutations in GARS have been identified in patients with CMT2D (12), all of which result in single–amino acid changes in different functional domains of GARS (10, 13–16). However, the mechanisms through which mutant forms of GARS cause axon degeneration remain unclear, limiting the development of a small-molecule therapy.

Most disease-associated GARS variants cause impaired enzymatic activity in the charging of glycine onto tRNA in vitro and/or decreased cellular viability in yeast complementation assays, consistent with a loss-of-function effect (17, 18). However, protein-null alleles in mice and humans do not cause dominant neuropathy, ruling out haploinsufficiency and suggesting a dominant-negative (antimorph) mechanism (19–22). Furthermore, transgenic overexpression of wild-type (WT) GARS does not rescue the neuropathy in mouse models, suggesting that mutant forms of GARS adopt a toxic gain-of-function (neomorph) activity that the WT protein cannot outcompete (20). One proposed neomorphic mechanism involves the abnormal binding of mutant GARS to the developmental receptor neuropilin-1 (NRP1). This interaction competes with the normal binding of vascular endothelial growth factor (VEGF), an endogenous ligand of NRP1 (23).

Together, these data support a model in which suppression of the mutant allele of GARS should be of therapeutic benefit, whereas enhancing normal GARS function is ineffective. To achieve this suppression, we developed a gene therapy strategy to reduce the levels of mutant Gars transcripts through allele-specific RNAi, triggered through the delivery of mutant Gars–targeted artificial microRNA (miRNA) expression cassettes packaged within self-complementary adeno-associated viral vectors, serotype 9 (scAAV9).

Results

We sought to demonstrate the therapeutic efficacy of a patient-specific RNAi-based gene therapy in a “humanized” mouse model expressing a patient-specific GARS mutation introduced into the mouse Gars gene. A 13-month-old female presented with impaired motor skills and regressing motor milestones involving both upper and lower extremities. She was able to sit independently, but used her arms to stabilize herself in a sitting position (tripod sitting). Increased lumbar lordosis was also noted at first examination. Dysmorphic features were noted, likely due to generalized muscle atrophy. Extraocular muscle function was normal. Deep tendon reflexes were difficult to obtain or absent, and she showed general, marked decreases in muscle tone, head lag, axillary slippage, mild tongue atrophy, ligamentous laxity in the hands and feet, and excessive retraction of the chest wall. The patient was delivered by C-section at 37 weeks gestation after a pregnancy complicated by hypertension. She required oxygen supplementation and had mild neonatal jaundice, but was discharged after 5 days. Newborn screening was normal, and motor development was probably normal at first, with the ability to reach for objects at 4 months and stand with support at 8 months. There was no history of seizures, and cognitive development was uncompromised.

Muscle biopsy at 15 months was indicative of neurogenic changes consistent with motor neuronopathy or neuropathy. This included marked atrophy of type I and II fibers with isolated, clustered, and fascicules of hypertrophied type I myofibers. There was no evidence of myofiber necrosis, degeneration, or regeneration, nor of dystrophic or inflammatory myopathy. Electromyography and nerve conduction studies were consistent with motor neuron disease: motor nerve conduction velocities were reduced (26 m/s upper and 15 m/s lower), while sensory examination revealed no deficits, including sensory nerve conduction (2.0 milliseconds latency and 46 μV at her wrist). At 20 months, MRI of the brain and cervical spinal cord were normal, as was an analysis of the cerebrospinal fluid. She did not display evidence of further decline and did not regress in any areas. Indeed, she seemed slightly stronger overall with no problems swallowing or drinking. Cranial nerves were intact and there was no evidence of tongue fasciculation. However, motor examination revealed decreased muscle mass and tone, axillary slippage, flaccid lower limbs, and high-arched feet. She could still sit independently using her upper limbs for support, and displayed head lag and an inability to stand on her own. A diagnosis of neuronopathy was suspected; however, there was no reported family history of this phenotype, and the patient’s nonidentical twin brother was unaffected.

A recessive or dominant de novo inheritance pattern was predicted. Patient DNA was extracted and subjected to both targeted and unbiased screening strategies. This revealed 2 full-length, WT copies of the SMN1 gene and 1 copy of SMN2, ruling out spinal muscular atrophy types I through III. Furthermore, targeted analysis did not reveal mutations in IGHMBP2 or UBE1, ruling out recessive, distal spinal muscular atrophy and X-linked spinal muscular atrophy type II, respectively. Whole-exome sequencing analysis revealed that the patient is heterozygous for a 12-nucleotide deletion in exon 8 of the glycyl-tRNA synthetase (GARS) gene (c.894_905del; GenBank NM_002047.2). This mutation resulted in the deletion of 4 amino acids in the GARS protein (p.Glu299_Gln302del; NP_002038.2), hereafter referred to as ΔETAQ. No other potentially pathogenic variant was identified at another locus that could potentially explain the severity of the neuropathy by a dual molecular diagnosis (24). Neither parent carries the identified GARS mutation, nor does the patient’s twin brother, indicating a de novo mutation. GARS functions to ligate glycine onto cognate tRNA molecules. The ΔETAQ GARS mutation results in the deletion of 4 amino acid residues that are conserved from human to bacteria and that reside within the glycine-binding pocket (Figure 1A and ref. 25).

To determine whether the ΔETAQ GARS mutation affects mRNA expression or stability, we performed RNA-Seq in patient primary dermal fibroblasts. These analyses revealed an even distribution of WT (53.7%) and ΔETAQ (46.3%) RNA-Seq reads, indicating that ΔETAQ GARS does not dramatically affect transcript
levels (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI130600DS1).

To examine GARS protein levels, we performed Western blot analysis on whole-cell lysates from patient cells compared with a control primary dermal fibroblast cell line (i.e., bearing no GARS mutations). These experiments did not reveal an observable difference in total GARS protein levels in the affected fibroblasts compared with the control cell line (Supplemental Figure 1B). We next performed aminocacylation assays and western complementation tests to assess whether ΔETAQ GARS affects the primary activity of the enzyme. Analysis of the initial rate of aminocacylation as a function of the tRNA substrate concentration showed that ΔETAQ GARS retained less than 0.01% aminocacylation activity (Figure 1B and Supplemental Table 1) compared with WT GARS (Supplemental Figure 2). In sum, ΔETAQ showed a severe defect in aminocacylation activity and, at best, a slight aberrant interaction with NRPI.

To definitively validate the pathogenicity of ΔETAQ GARS in vivo, we engineered a mouse model in which the mutation was introduced into the mouse Gars gene (GarsΔETAQ/ΔETAQ) (see Methods). For subsequent preclinical studies, GarsΔETAQ/ΔETAQ mice were crossed to GarsΔhuEx8/huEx8, a second mouse model engineered to harbor a “humanized” WT GARS exon 8 replacement in the mouse gene. The 50 amino acids encoded by exon 8 are 100% identical between mouse and human, although there are silent single-nucleotide differences between the mouse and human Gars/GARS exon 8 that could affect allele specificity of gene silencing. Our attempts to introduce these additional human changes when engineering the ΔETAQ allele were unsuccessful, and only the 12-bp deletion was incorporated.

This breeding produced a cohort of GarsΔETAQ/huEx8 mice with GarsΔhuEx8/ΔhuEx8 littermate controls. Reverse transcriptase PCR using cDNA isolated from sciatic nerve of heterozygous mice revealed coexpression of ΔETAQ and WT Gars (Supplemental Figure 3A). A Western blot analysis of brain homogenates using a polyclonal anti-GARS antibody confirmed that ΔETAQ Gars did not alter GARS protein levels, suggesting that a stable transcript and protein products are produced from the ΔETAQ allele, as in patient fibroblasts (Supplemental Figure 3B).

At 12 weeks of age, GarsΔETAQ/huEx8 and GarsΔhuEx8/littermates were evaluated for features of primary neuropathy, as observed in other mouse models of CMT2D (22, 26). GarsΔETAQ/huEx8 mice displayed overt neuromuscular dysfunction and a reduction in

Figure 1. In vitro characterization of ΔETAQ mutation. (A) The position and evolutionary conservation of the ΔETAQ (red) and P234KY (green) GARS mutations. (B) Initial aminocacylation rates (pmol/min) of WT (black), P234KY (green), and ΔETAQ (blue) GARS were plotted against tRNA concentrations and fit to the Michaelis-Menten equation. (C) Representative cultures of yeast strains lacking GRS1 to test for growth in the presence of each mutation (ΔETAQ or P234KY) modeled in the human GARS open reading frame. (D) WT, P234KY, or ΔETAQ GARS was expressed (with a V5 tag) and tested by immunoprecipitation with an anti-NRP1 antibody to detect aberrant interactions. Western blots were performed with anti-NRP1 and anti-V5 antibodies. Immunoprecipitation (IP), negative control (IgG), and input experiments are indicated.
The velocities (NCVs) were reduced by 62%, falling from 35 ± 6.29 m/s in control animals to 13.5 ± 4.1 m/s (P = 0.0002) in the sciatic nerve in mutant mice (Figure 2F). This decrease was consistent with other mouse Gars neuropathy models and with some patients with GARS-mediated peripheral neuropathy (CMT2D). There was a concomitant disruption of neuromuscular junctions (NMJs) in distal muscles. While postsynaptic receptor fields of NMJs in the plantaris muscle were fully occupied by motor nerve terminals in GarshuEx8/+ controls, GarsΔETAQ/huEx8 mice were significantly lighter, weighing 19 ± 1.9 g (P = 0.0006, n = 8), compared with GarshuEx8/+ controls, which weighed 27.4 ± 4.84 g (n = 7). Gross motor performance in GarsΔETAQ/huEx8 mice was quantified using a wire hang test. While GarshuEx8/+ mice averaged 55 ± 9.57 seconds before letting go, GarsΔETAQ/huEx8 mice (n = 8) fell after only 17.3 ± 11.3 seconds. Myelinated axon number in the motor branch of the femoral nerve was reduced by 21% from 551 ± 45 axons in littermate controls to 438 ± 92 axons in GarsΔETAQ/huEx8 (n = 6 mice per genotype). Axon diameters were reduced, as shown in a cumulative histogram (P < 0.0001, Kolmogorov-Smirnov test, average diameter 1.6 ± 0.8 μm, n = 6), in comparison with GarshuEx8/+ littermates (3.3 ± 2.198 μm, n = 6). Representative images of femoral motor nerve cross sections. Nerve conduction velocity (NCV) was reduced from 35 ± 6.29 m/s in littermate controls to 13.5 ± 4.1 m/s in GarsΔETAQ/huEx8 mice (P = 0.0002, n = 6 GarsΔETAQ/huEx8, n = 7 GarshuEx8/+). Neuromuscular junctions (NMJs) from the plantaris muscle showed partial innervation and denervation, scored based on the overlap between pre- and postsynaptic staining. Differences in body weight, grip strength, conduction, and axon number between genotypes were statistically evaluated using a 2-way Student’s t-test; axon diameter was evaluated by a Kolmogorov-Smirnov test. Significant difference in overall percentage NMJ innervation was determined by 2-way ANOVA with Tukey’s honestly significant difference (HSD) post hoc comparisons. For all analyses, *P < 0.05, ***P < 0.001, ****P < 0.0001 represent post hoc significance between genotypes. Values are mean ± SD. Scale bars: 100 μm (E); 50 μm (H, I).
Using RNAi would be efficacious, we first engineered a miRNA
genic (22, 26, 29, 30). ETAQ
of CMT2D, confirming that the Δ
peripheral neuropathy similar to other established mouse models
were (Figure 2, G–I). Defects in NMJ innervation were evident at
6 weeks of age, and examples of fully innervated, partially inner-
mice (Figure 2, G–I). Defects in NMJ innervation were evident at
in control littermates, 60% ± 14.2% of NMJs were partially occu-
ery adeno-associated viral vector
was transcribed and then processed by the RNAses DROSHA and Dicer and the nuclear export
factor Exportin-5 (Exp5). The mature antisense strand (red line) incorporates into the RNA-induced silencing
complex (RISC) to elicit sequence-specific degradation of the mutant Gars mRNA. (B) MiRNAs were tested
in vitro by cotransfection of HEK293 cells with U6-miGars, or control, plasmid miRNA and a dual-luciferase
reporter plasmid containing 1 of 4 target genes cloned into the 3′-UTR of Renilla luciferase. The values are
reported as mean ratios ± SEM. (C) The sequence of the guide strand of the lead mi.ETAQ, now referred
to as mi.ΔETAQ (Figure 3, B and C) was cloned into a self-complementary adeno-associated viral vector
serotype 9; scAAV9) for in vivo delivery (Figure 3B and Supplemental Figure 5).
To establish the proof of principle of this approach in vivo, we
tested whether the reduction of mutant Gars expression before dis-
ease onset could prevent the neuropathy in GarsΔETAQ/huEx8 mice. A dose of approximately 2.6 × 10^{11} viral
genomes (vg) of scAAV9.mi.ΔETAQ or scAAV9.mi.LacZ (expressing a
control miRNA targeting the E. coli LacZ gene) was delivered with
an intracerebroventricular (i.c.v.) injection at postnatal day 0–1 (P0–P1) to GarsΔETAQ/huEx8 and litter-

termate control (GarshuEx8/huEx8) pups. All mice were evaluated
for established signs of neuropathy at 4 weeks of age, approxi-
ately 1.5 weeks after the initial onset of overt signs of neurop-
athy. GarsΔETAQ/huEx8 mice treated with scAAV9.mi.ΔETAQ showed
improvement in a wire hang test of grip strength, increased
muscle-to-body-weight (MW/BW) ratios, and improved sciatic
nerve conduction velocity (NCV) compared with control-treated
ΔETAQ mice (Figure 4, A–C). Examination of cross sections of
the motor branch of the femoral nerve revealed that scAAV9.
mi.ΔETAQ treatment prevented the axon loss and lessened the
decrease in axon diameters observed in untreated and scAAV9,
mi.LacZ-treated ΔETAQ mice (Figure 4, D–F). Injection with scAAV9.mi.LacZ (or scAAV9.mi.LacZ) did not cause adverse effects in control mice. Collectively, these data provide the proof of concept that allele-specific knockdown using virally delivered RNAi may be an effective approach for treating CMT2D.

We next delivered scAAV9.mi.ΔETAQ to cohorts of both early-symptomatic (5-week-old) and late-symptomatic (9-week-old) GarsΔETAQ/huEx8 mice and littermate controls via a single intrathecal injection into the lumbar spinal cord. When left untreated, 5-week-old early-symptomatic GarsΔETAQ/huEx8 mice undergo active axon loss, while axon loss slows and muscle atrophy accelerates in 9-week-old late-symptomatic ΔETAQ mice.

The scAAV9.mi.ΔETAQ-treated early-symptomatic GarsΔETAQ/huEx8 mice displayed enhanced grip strength and increases in body weight starting at about 5 weeks after treatment compared with untreated controls (Figure 5A). When analyzed for primary signs of neuropathy at 7 weeks after treatment, early-symptomatic GarsΔETAQ/huEx8 mice also exhibited significant increases in MW/BW ratios, NCV, and NMJ innervation and a reduction in axon loss but no improvement in axon size compared with untreated GarsΔETAQ/huEx8 mice (Figure 5, B–D and F). When treated with scAAV9.mi.ΔETAQ at 9 weeks of age, GarsΔETAQ/huEx8 mice gained weight and displayed enhanced grip strength starting at 5–7 weeks after treatment (Figure 5A). While MW/BW ratios were not improved and axon loss and atrophy were not prevented, scAAV9.mi.ΔETAQ did improve NCV and NMJ innervation (Figure 5, B, E, and F). Analysis of mRNA from sensory dorsal root ganglia (DRGs), which are also transduced by scAAV9, via pyrosequencing indicated that mutant GarsΔETAQ mRNA levels were significantly reduced in scAAV9.mi.ΔETAQ-treated mice (Supplemental Table 2).

We also evaluated possible off-target effects of the RNAi. In addition to prediction programs, we also empirically tested for gene expression changes and found 325 genes that were altered in their expression with an FDR less than 0.05 based on RNA-Seq of patient fibroblasts transfected with the mi.ΔETAQ construct in comparison with eGFP transfection as a control. The physiological significance of these changes is unknown, and the transcriptome of fibroblasts will be only partially shared with that of neurons. To evaluate possible adverse effects in vivo, WT mice were treated with the same dose of vector by intrathecal injection (30 seven-week-old female mice comprising 3 groups: 10 mi.ΔETAQ treated, 10 mi.LacZ treated, and 10 untreated). No adverse effects other than a mild increase in germinal center activity in the spleen were seen 10 weeks after treatment after full necropsy and evaluation by a veterinary pathologist blinded to treatment.

We confirmed the efficacy of allele-specific knockdown in a second mouse model of CMT2D, GarsP278KY/+ (22). A miRNA
shuttle targeting the mouse P278KY allele was optimized as before and packaged into scAAV9 (Supplemental Figure 6). As with the ΔETAQ vector, improvements were observed in neonatal, early- and late-symptomatic GarsP278KY/+ mice treated with mi.ΔETAQ, identified in a patient with an early-onset motor neuropathy. Although not unprecedented, the severity and onset of this case are atypical for CMT2D (14). Biochemical characterization of the mutation revealed a loss of activity in the charging of glycine onto tRNA_Gly, consistent with other pathogenic alleles of GARS (18). However, in contrast to other pathogenic alleles (23), the mutant protein does not strongly bind to NRP1. Given the atypical clinical presentation and biochemical properties of the ΔETAQ variant, we confirmed its pathogenicity by introducing the same change into the mouse Gars gene. Mice carrying this dominant mutation developed a severe, early-onset peripheral neuropathy, confirming that it is indeed disease-causing. We next developed an allele-specific-knockdown vector to target mutant Gars transcripts while leaving the WT Gars mRNA intact. RNAi sequences were optimized in vitro and cloned into a miR-30 shuttle construct driven by a U6 promoter, which was packaged into scAAV9 for in vivo delivery. Treatment of the mouse model at birth by i.v. injection was able to largely prevent the onset of disease, reduced NCV, axon loss, or axon atrophy (Figure 7, C–G). Thus, consistent with the perdurance of AA9, a single dose at birth prevented neuropathy for at least 1 year.

The outcomes in post-onset studies in both genotypes were variable, likely because of the challenges in intrathecal delivery in mice. However, the knockdown efficacy of mutant Gars transcripts within DRGs (Supplemental Tables 2–4) correlated with therapeutic outcomes within both post-onset studies (Supplemental Figure 9, A, B, E, and F). This correlation was stronger with mRNA isolated from DRGs than when outcomes were compared with mutant Gars mRNA levels in liver, another tissue transduced by scAAV9 (Supplemental Figure 9, C, D, G, and H). This is consistent with the benefit coming from the transduction of cells with direct delivery to the nervous system and not from transduction of peripheral organs.

**Discussion**

In this study we examined a new de novo variant of GARS, ΔETAQ, identified in a patient with an early-onset motor neuropathy. Although not unprecedented, the severity and onset of this case are atypical for CMT2D (14). Biochemical characterization of the mutation revealed a loss of activity in the charging of glycine onto tRNA_Gly, consistent with other pathogenic alleles of GARS (18). However, in contrast to other pathogenic alleles (23), the mutant protein does not strongly bind to NRP1. Given the atypical clinical presentation and biochemical properties of the ΔETAQ variant, we confirmed its pathogenicity by introducing the same change into the mouse Gars gene. Mice carrying this dominant mutation developed a severe, early-onset peripheral neuropathy, confirming that it is indeed disease-causing. We next developed an allele-specific-knockdown vector to target mutant Gars transcripts while leaving the WT Gars mRNA intact. RNAi sequences were optimized in vitro and cloned into a miR-30 shuttle construct driven by a U6 promoter, which was packaged into scAAV9 for in vivo delivery. Treatment of the mouse model at birth by i.v. injection was able to largely prevent the onset of disease, reduced NCV, axon loss, or axon atrophy (Figure 7, C–G). Thus, consistent with the perdurance of AA9, a single dose at birth prevented neuropathy for at least 1 year.

The improvement in neuropathy phenotypes correlated with the degree of knockdown of the mutant Gars mRNA. Thus, our findings support the feasibility of virally delivered RNAi for allele-specific knockdown as a treatment strategy for a dominant neuropathy caused by mutations in an essential gene.
Figure 6. Reduction of mutant Gars by RNAi prevents neuropathy in Gars<sup>P278KY/+</sup> mice. (A and B) Neonatal scAAV9.mi.P278KY treatment prevented deficits in gross motor performance quantified at 4 weeks of age by the wire hang test (P = 0.0001) (A) and reductions in MW/BW ratios (P = 0.0463) (B) in comparison with untreated and vehicle-treated P278KY mice. (C) NCVs were also significantly improved (P ≤ 0.0001) in treated P278KY mice. (D) Quantification of axon number within cross sections of the motor branch of the femoral nerve showed that while axon number was reduced by 17% in control-treated P278KY mice, axon counts in scAAV9.mi.P278KY-treated P278KY mice (589 ± 15 axons) were similar to those in untreated control littermates (600 ± 11 axons). (E) scAAV9.mi.P278KY treatment also restored the presence of large-diameter axons; average axon diameter was 1.98 ± 4.47 μm in control-treated P278KY mice, 2.71 ± 3.71 μm in scAAV9.mi.P278KY-treated P278KY mice, and 3.84 ± 3.74 μm in untreated Gars<sup>+/+</sup> mice. (F) Representative images of cross sections of the motor branch of the femoral nerve isolated from untreated Gars<sup>+/+</sup>, Gars<sup>P278KY/+</sup>, and scAAV9.mi.P278KY-treated Gars<sup>P278KY/+</sup> mice. (G) Representative images of NMJ morphology isolated from plantaris muscle. (H) While over 70% of the NMJs were partially or completely denervated in control-treated Gars<sup>P278KY/+</sup> mice by 4 weeks of age, less than 30% of NMJs showed any degree of denervation in scAAV9.mi.P278KY-treated Gars<sup>P278KY/+</sup> mice. Numbers for all outcome measures: untreated Gars<sup>+/+</sup>, n = 5; control-treated Gars<sup>+/+</sup>, n = 4; scAAV9.mi.P278KY-treated Gars<sup>+/+</sup>, n = 8; untreated Gars<sup>P278KY/+</sup>, n = 6; control-treated Gars<sup>P278KY/+</sup>, n = 5; scAAV9.mi.P278KY-treated Gars<sup>P278KY/+</sup>, n = 7. Significance in A–D and H was determined by 2-way ANOVA with Tukey’s HSD post hoc comparisons. Significant changes in axon diameter (E) were determined with a Kolmogorov-Smirnov test. *P < 0.05, ****P < 0.0001 represent post hoc significance between mi.LacZ-treated and scAAV9.mi.P278KY-treated Gars<sup>P278KY/+</sup> mice. Values are mean ± SD. All scale bars: 100 μm.
The disease mechanism through which mutant forms of GARS cause CMT2D is not clear. Variants of GARS that result in neuropathy impact the enzymatic activity of the protein to varying degrees. Since alleles that do not produce a mutant protein do not cause a dominant disease in mice or humans, a loss-of-function mechanism underlying the neuropathy would have to be through a dominant-negative mechanism. Alternatively, neomorphic gain-of-function activities are consistent with findings in mouse and Drosophila models (20, 32). Mutant forms of GARS bind to NRP1, an important neurodevelopmental receptor that transduces both VEGF and semaphorin signals, whereas WT GARS does not bind NRP1 (23). This aberrant interaction interferes with VEGF binding, possibly constituting a pathogenic neomorphic activity of the mutant protein. However, our finding that the ΔETAQ form of GARS does not have a strong interaction with NRP1 indicates that while this mechanism may still contribute to the disease, it is not essential for pathogenicity. Importantly, our allele-specific knockdown approach applies equally well to a dominant-negative or a neomorphic mechanism.

Our results also serve as a preclinical demonstration of the feasibility of RNAi-mediated allele-specific knockdown as a therapeutic strategy. Pre-onset treatments were able to almost completely prevent the neuropathy. This provides strong proof-of-concept data for the effectiveness of knocking down mutant GARS. Post-onset treatment still provided benefit, though this was less complete and diminished the longer treatment was withheld. The post-onset treatment may stop or slow the progression of axon loss. This is best demonstrated by the reduction in axon loss seen in ΔETAQ mice treated at 5 weeks of age compared with untreated mutant mice (Figure 5E), an age by which most of the axons have already degen-
erated. Even with axon loss, some of the residual benefit may come from improved synaptic transmission at NMJs, consistent with the improved innervation and with previous findings that synaptic transmission is progressively impaired in Gars mutant mice (28).

Another important consideration for the translational potential of this approach is whether we can achieve sufficient allelic specificity with single-base pair mutations. The mutations that we targeted here differed from WT sequence by 5 (P278KY) or 12 (ΔETAQ) bp, making them relatively good targets for specific RNAi sequences. Allele specificity of RNAi for single-nucleotide differences will depend on surrounding local sequence, but is precluded, and efficiency can be improved by addition of additional mismatches to the RNAi sequence (33–35). Therefore, a similar allele-specific knockdown strategy may be effective for additional alleles of GARS. An alternative strategy that would apply equally to any allele of GARS would be to knock down all GARS transcripts, mutant and WT, using a nonspecific GARS RNAi, and, in the same vector, provide an RNAi-resistant WT cDNA. This strategy has been used for α-antitrypsin (36). Other strategies, such as allele-specific antisense oligonucleotides or allele-specific CRISPR/Cas9 gene editing, could also be used to silence mutant GARS alleles while preserving WT GARS, but would require additional development.

The reduced efficacy of the treatment after the onset of neuropathy will be complicated to dissect. It is possible that the intrathecal delivery or reduced transduction efficiency of adult peripheral neurons contributed to the reduced efficacy. Intrathecal delivery was chosen as it is the route of delivery in gene therapy trials for related disorders (6). In our study, the degree of improvement in outcomes such as NCV correlated with the extent of the knockdown of mutant Gars relative to WT Gars transcripts assayed by PCR in sensory DRGs (Supplemental Figures 7 and 8). We chose to assay DRGs because they are efficiently transduced and the sensory neurons comprise a large proportion of the tissue mass, providing the best opportunity to assess the efficiency of the knockdown. However, this analysis does not resolve this question on a cell-by-cell basis, nor does it address knockdown in motor neurons. The biodistribution of AAV9 in mice and other animals has been previously reported (37–39), and therefore, we did not repeat those analyses here. However, in pilot studies using AAV9-GFP, we saw transduction in spinal cord and DRGs that closely matched published results.

Despite the unknowns regarding transduction efficiency and the extent of the knockdown, the benefit observed with post-onset delivery is encouraging. However, it is notable that we also did not see regeneration. The axon numbers did not increase when treatment was delivered after axon loss. Similarly, at NMJs, there was no evidence of collateral sprouting and reinnervation of denervated NMJs by the remaining axons. It is unclear whether this is due to insufficient knockdown of the mutant mRNA, or whether the axon degeneration is irreversible. The fact that the results were so similar in both mouse models despite the knockdown of the P278KY allele being so efficient in vitro may suggest that the degeneration is irreversible under normal circumstances. Therefore, the benefit of treatment after the onset of symptoms may be limited to slowing or stopping progression, and may need to be combined with other strategies to promote regeneration to achieve a better recovery of function.

In summary, these studies demonstrate how precision animal models can be used for testing personalized therapies for rare and orphan diseases, and provide important proof of concept for RNAi-based gene therapy for this dominant disease. This approach could be applied to other, related disorders including other dominantly inherited peripheral neuropathies or motor neuron diseases.

**Methods**

**Clinical evaluation and mutation analysis.** The proband was clinically evaluated at Texas Children's Hospital (Houston, Texas, USA). Diagnostic, whole-exome sequencing (XomeDxPlus) was performed by GeneDx (Gaithersburg, Maryland, USA). For allele-specific Sanger sequencing, we first isolated DNA from patient-derived primary fibroblasts. Cells were treated with trypsin according to the Wizard Genomic DNA Purification Kit (Promega) protocol. PCR amplification was performed to obtain a 381-bp region including GARS exon 8 using PCR SuperMix (Thermo Fisher Scientific). PCR products were cloned with the TOPO TA Cloning Kit (Thermo Fisher Scientific). Plasmid DNA from 6 isolated colonies was purified and Sanger-sequenced using the PCR primers: forward 5′-GCATTGCCAAGTAGTACTGC-3′ and reverse 5′-CCTGACTCTGATCAGTCCAGTC-3′.

**GARS expression studies**

For RNA expression studies, RNA was isolated from patient fibroblasts using the RNasy Mini Kit (Qiagen). cDNA was generated from 1 μg of RNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). The resulting cDNA was used to amplify a 224-bp product including the ΔETAQ GARS mutation. The reaction was column purified and checked for quality via gel electrophoresis. For next-generation sequencing, the product was digested and "tagmented" using Tn5 transposase. The library was amplified by PCR using Kapa HiFi DNA polymerase and Illumina-compatible indexing primers. Final library fragment size and purity were determined via gel electrophoresis, and fragments were column purified and sequenced on the Illumina MiSeq with paired 150-bp reads. Overlapping reads were merged using PEAR (version 0.9.6) and aligned using BWA MEM (version 0.7.12) to custom references containing the WT exon 7/exon 8 junction or the ΔETAQ-containing equivalent. A custom Python script (https://github.com/kitzmanlab/gars_burgess_2019) was used to count reads with higher-scoring alignment to each junction. Uninformative reads (e.g., those not spanning the mutation) were disregarded.

For fibroblast protein expression studies, proteins were isolated in 1 mL cell lysis buffer (990 μL RIPA Lysis Buffer [Thermo Fisher Scientific] plus 10 μL 100× Halt Protease Inhibitor [Thermo Fisher Scientific]) and quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Protein samples were digested with trypsin and analyzed via Western blot. Sample input was 1× SDS sample buffer (Thermo Fisher Scientific) plus 5 μL β-mercaptoethanol and boiled at 99°C for 10 minutes, then separated on precast 4%–20% Tris-glycine gels (Thermo Fisher Scientific), transferred onto a PVDF membrane, and probed with the respective primary antibody at the following dilutions in blocking solution: anti-GARS 1:1000, described in ref. 17; anti-NRP1 (Abcam, EPR3113) 1:1000; and anti-actin (Sigma-Aldrich, A5060) 1:5000. Mem-
branes were then rinsed 3 times in 1x TBST to remove unbound antibody and incubated with the respective HRP-conjugated secondary antibody at 1:10,000. Membranes were rinsed in 1x TBST and exposed using SuperSignal West Dura substrate and enhancer (Thermo Fisher Scientific).

Expression construct development
All GARS expression constructs were generated using Gateway cloning (Invitrogen). The human GARS open reading frame was amplified from human cdNA using primers with the attB1 (forward, GARS ORF F = ATGGACGGCGGGGGGCTGAGG) and attB2 (reverse, GARS ORF R = TCAATTCCTGATTGCTCT) Gateway sequences. Entry clones were generated by recombination of PCR-purified amplicons into the pDONR221 vector using BP clonase. Individual WT entry clones were confirmed by sequencing. Oligonucleotides containing sequences corresponding to each GARS mutation studied were generated, and mutagenesis was performed using the Quick-Change II XL Site-Directed Mutagenesis Kit (Stratagene). Individual clones were sequenced to confirm each mutation and the absence of errors. Validated entry clones were recombined into the appropriate Gateway-compatible vector using LR clonase: pET-21a(+) for aminocacylation assays and pTM3xFLAG for coimmunoprecipitation assays. For yeast complementation assays, human GARS was expressed from the pY11 expression construct containing ΔMTSΔWHEP GARS; the pY11 constructs were a gift from Chin-I Chien and Chien-Chia Wang (National Central University, Taoyuan City, Taiwan).

Aminocacylation assays
WT and mutant GARS proteins were expressed in E. coli with a C-terminal His tag and purified with nickel affinity resins (Novagen). The T7 transcript of human tRNA\(^{\text{Glu}}\) (CCC, anticodon) was prepared and purified as previously described (40), heat-denatured at 85°C for 3 minutes, and annealed at 37°C for 20 minutes before use. Steady-state aminocacylation assays were monitored at 37°C in 50 mM HEPES (pH 7.5), 20 mM KCl, 10 mM MgCl\(_2\), 4 mM DTT, 2 mM ATP, and 50 μM \(^3\)H-glycine (PerkinElmer) at a specific activity of 16,500 dpm/pmol. The reaction was initiated by mixing of GARS enzyme (20 nM for WT enzyme and 600 nM for the ΔETAQ and P234KY mutants) with varying concentrations of tRNA (0.3–20 μM). Aliquots of a reaction mixture were spotted on filter paper, quenched by 5% trichloroacetic acid, washed, dried, and measured for radioactivity using a liquid scintillation counter (LS6000SC, Beckman Coulter Inc.). The amount of radioactivity retained on filter pads was corrected for quenching effects to determine the amount of synthesis of Gly-tRNA\(^{\text{Glu}}\). Steady-state kinetics was determined by fitting of the initial rate of aminocacylation as a function of tRNA concentration to the Michaelis-Menten equation (41).

Yeast complementation assays
Yeast complementation assays were carried out as previously described (17,18). At least 2 colonies per transformation were assayed, and each transformation was repeated at least twice.

MNI transfections for immunoprecipitation
MNI cells were grown at 37°C in 5% CO\(_2\) and standard growth medium (DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 50 μg/mL of streptomycin [Invitrogen]). We obtained mouse MNI cells in 2004 from Kurt Fischbeck (NIH/National Institute of Neurological Disorders and Stroke, Bethesda, Maryland, USA), who obtained them directly from the laboratory that generated these cells (H. Kim, University of Chicago, Chicago, Illinois, USA) (42). Cells were transfected using Lipofectamine 2000 (Invitrogen) with 15 μg of a plasmid to express WT or mutant GARS pTM3xFLAG per 1 T-175 flask. Cells were incubated for 48 hours at 37°C in 5% CO\(_2\). Cells were then harvested using 0.25% trypsin (Invitrogen), centrifuged at 805 g for 2 minutes, and washed twice with 1x PBS.

Coimmunoprecipitation analyses
Twenty-five microliters of Dynabeads Protein G Magnetic beads (Invitrogen) were used for each cell lysate from one T-175 flask. Beads were washed twice in 1 mL of wash solution (0.5% BSA and 0.1% Triton X-100 in PBS) and resuspended in 1 mL of wash solution. Two micrograms anti-FLAG antibody (Sigma-Aldrich, clone M2) was added and incubated at 4°C with gentle shaking overnight. Transfected cells were resuspended in 1.5 mL lysis buffer (20 mM Tris-HCl [pH 7.5], 2.5 mM MgCl\(_2\), 300 mM KCl, 0.1% NP-40, 1 mM DTT, 0.2 mM PMSF, 1x Halt Protease Inhibitor Cocktail [EDTA free] [Thermo Fisher Scientific]) and incubated with gentle rocking at 4°C for 90 minutes. Cell lysates were centrifuged at 17,800 × g for 5 minutes at 4°C. The supernatant was removed and used as the input for the immunoprecipitation (IP) reaction. Protein was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), and 1 mg of protein was used as input. Expression of 3xFLAG-tagged protein in input samples was confirmed by anti-FLAG M2 Western blotting. The αFLAG-conjugated magnetic beads were washed twice with 500 μL of lysis buffer, resuspended in 25 μL lysis buffer, and added to the protein sample. After 2 hours of incubation at 4°C with gentle shaking, beads were magnetically isolated, washed 5 times with 1 mL of buffer (20 mM Tris-HCl [pH 7.5], 2.5 mM MgCl\(_2\), 300 mM KCl, 0.1% NP-40, 1 mM DTT), and resuspended in 30–50 μL of wash buffer per 25 μL of starting beads. Two hundred micrograms per milliliter of 3xFLAG peptide was added to each sample and incubated at 4°C with gentle shaking for 3 hours to elute the FLAG-tagged protein.

Mass spectrometry and bioinformatics
For mass spectrometry at the Fred Hutchinson Cancer Institute, IP products were separated on a denaturing 4%–20% Tris-glycine gel and silver-stained using standard protocols. Destained gel slices were used for in-gel digestion with an ice-cold solution of 12.5 ng/μL trypsin in 50 mM ammonium bicarbonate on ice for 1 hour. The trypsin solution was removed and replaced with 50 mM ammonium bicarbonate, and digestion was carried out overnight at 37°C. Peptides were extracted by the addition of 0.1% trifluoroacetic acid and light vortexing for 30 minutes followed by addition of an equal volume of 100% acetonitrile and light vortexing for an additional 30 minutes. Desalted samples were brought up in 10 μL of 2% acetonitrile in 0.1% formic acid, and 8 μL was analyzed by liquid chromatography/electrospray ionization tandem mass spectrometry (MS/MS) using an Easy-nLC 1000 (Thermo Fisher Scientific) HPLC system coupled to a hybrid Orbitrap Elite (Thermo Fisher Scientific) as previously described (43). The Orbitrap Elite instrument was operated in the data-dependent mode, switching automatically between mass spectrometric survey scans in the Orbitrap with MS/MS spectra acquisition in the linear ion trap. The 15 most intense ions from the Fourier transform full scan were selected.
for fragmentation in the linear trap by collision-induced dissociation with normalized collision energy of 35%. Selected ions were dynamically excluded for 30 seconds with a list size of 500 and exclusion mass width of ±10 ppm.

For mass spectrometry at the University of Michigan (Ann Arbor, Michigan, USA), IP products were concentrated using TCA precipitation. Two microliters of 2% sodium deoxycholate was added to 200 μL of IP product and incubated on ice for 30 minutes. Twenty microliters of 100% (wt/vol) TCA was added (brining TCA to ~10%) and incubated on ice for 1 hour. The product was then centrifuged at maximum speed at 4°C for 10 minutes, and the pellet was washed in 500 μL of acetone and incubated on ice for 10 minutes. The acetone solution was centrifuged at maximum speed at 4°C for 10 minutes. The resulting pellet was resuspended in 30 μL of 20 mM HEPEs and 8 M urea (pH 8.0). Three independently generated IP products for each WT, ΔETAQ, and an untagged WT GARS sample were analyzed. MS/MS was performed using the Orbitrap Fusion Mass Spectrometer (Thermo Fisher Scientific).

For all mass spectrometry data analyses, the open-source proteomics search engine X! Tandem (The Global Proteome Machine Organization) was used to match mass spectrometry data with peptide spectra. Peptide Prophet was used to validate peptide assignments (43), and Protein Prophet grouped peptides into proteins (44). ABACUS extracted spectral counts for quantitative analysis (45). Finally, interactions were scored to remove background, and comparisons of interacting proteins were performed to calculate the relative fold changes for interactions between WT and ΔETAQ GARS.

Targeted assessment of NRPI-GARS interactions
NSC-34 cells (ATCC) were grown to 70% confluence before transfection. Human WT, P234KY, or ΔETAQ GARS cDNAs were subcloned into the pcDNA6 plasmid in-frame with a V5 tag. Transfections were performed using Lipofectamine 2000 (Invitrogen). Thirty-six hours after transfection, cells were washed twice in PBS, scraped into PBS, pelleted, and resuspended in Pierce IP Lysis Buffer (Thermo Fisher Scientific) for 30 minutes and centrifuged for 7 minutes at 12,000 g; the insoluble fraction was discarded. Protein G beads (Invitrogen) were preincubated with anti-NRPI antibody (Abcam, EPR3113) or rabbit IgG (Cell Signaling Technology, 2729) for 30 minutes and then mixed with the cell lysates for overnight. Beads were then washed 3 times with buffer (100 mM NaCl, 50 mM Tris [pH 7.5], 0.1% Triton X-100, 5% glycerol). The immunoprecipitates were fractionated by 4%-12% Bis-Tris-Plus SDS-PAGE gels (Invitrogen) and transferred to PVDF membranes. Membranes were blocked for 1 hour with Tris-buffered saline with Tween-20 (TBST) containing 5% nonfat dry milk. Proteins were detected using mouse monoclonal V5 antibody (Invitrogen, R960-25). NRPI was detected using the same antibody for communoprecipitation. After incubation with primary antibodies, membranes were washed and incubated with Horseradish-conjugated anti-mouse or anti-rabbit secondary antibodies (Cell Signaling Technology, 7076, 7074, respectively) followed by detection using ECL substrate (Thermo Fisher Scientific), and exposed using the FluorChem M imager (ProteinSimple).

Cloning of allele-specific, mutant GARS-targeted miRNAs
All design rules for artificial miRNAs are previously described (46), including: 22-nucleotide mature miRNA length, antisense complementarity to the target (Gars; GARS), less than 60% GC content of the mature duplex, and guide-strand biasing, such that the last 4 nucleotides of the antisense 5’ end were A:U rich, and the last 4 nucleotides of the antisense 3’ end were G:C rich. Mutant GARS/GARS-targeting miRNA constructs were designed with the seed match region targeting the differing nucleotides present in the mutant Gars alleles and intentional mismatches with WT GARS/Gars. MiRNAs were cloned into a U6T6 expression vector (31). After in vitro testing, lead candidate U6 miRNAs were cloned into a self-complementary proviral AAV plasmid containing a CMV-driven eGFP reporter. scAAV9 viruses were generated and titered by the Viral Vector Core at The Research Institute at Nationwide Children’s Hospital (Columbus, Ohio, USA).

Luciferase assays
The dual-luciferase plasmids were created in the Psicheck2 vector (Promega), with firefly luciferase serving as a control, and the various Gars target regions cloned downstream of the Renilla luciferase stop codon. HEK293 cells were cotransfected (Lipofectamine 2000, Invitrogen) with the appropriate reporter and an individual U6 miRNA expression plasmid in a 1:5 molar ratio. GARS silencing was determined 24 hours after transfection, using the Dual-Luciferase Reporter Assay System (Promega). Triplicate data were averaged, and knockdown significance was analyzed by 2-way ANOVA. Results are presented as the mean ratio of Renilla to firefly ± SEM.

Mice
Mice were housed in the research animal facility at The Jackson Laboratory and provided with food and water ad libitum. Gars (CAST; B6-Gars<sup>ΔhEx8</sup>/Rwb; referred to as Gars<sup>ΔhEx8</sup>−/−) are previously described (22). The official strain designations of the newly engineered mouse models are B6;FVB-Gars<sup>ΔhEx8</sup>/em1Rwb<sup>+</sup>/Rwb (referred to as Gars<sup>ΔhEx8</sup>−/−) and B6;FVB-Gars<sup>ΔhEx8</sup>/em2Rwb<sup>+</sup>/Rwb (referred to as Gars<sup>ΔhEx8</sup>−/−). Experimental cohorts used for direct comparisons consisted of littermates.

Generation of Gars<sup>ΔhEx8</sup>−/− and Gars<sup>ΔhEx8</sup>−/− models with CRISPR/Cas9 genome-editing technology
Donor constructs. For Gars<sup>ΔhEx8A</sup>−/−, the mouse exon 8 sequence was replaced with a donor vector containing the human exon 8 sequence. The donor was a 10-kb sequence containing a 2.8-kb 5’ arm of homology and a 7-kb 3’ arm of homology isolated from a C57BL/6J BAC library flanking the human exon 8 sequence. For Gars<sup>ΔhEx8A</sup>−/−, the donor construct consisted of an single-stranded oligonucleotide sequence spanning the first 52 bases of mouse exon 8 with short arms of homology (see below for sequence) containing a 12-base deletion (bases 12–23 of exon 8).

Microinjection. Preparation and microinjection of CRISPR/Cas9 reagents were performed as previously described (47). All components, including Cas9 mRNA (100 ng/μL; either TriLink or synthesized by in vitro transcription), sgRNA, guides 144 and 1340 (50 ng/μL; guide sequence below), and each donor vector (20 ng/μL plasmid DNA or 100 ng/μL single-stranded donor oligonucleotides), were injected into the male pronucleus and cytoplasm of about 300 zygotes at the pronuclei stage. All zygotes were isolated from superovulated FVB/NJ (JAX stock 001600, The Jackson Laboratory) females mated with C57BL/6N (JAX stock 005304) males. Then, groups of 15–25 blastocysts were transferred into the uterus of pseudopregnant females.
Gars were used to detect the 12-bp deletion in bases 13–24 in ΔΔGTGGTCA with subsequent Sanger sequencing with ETAQF0_R: TACAACAGAAACAAACTATT TTACTGTG and subsequent generations. Primers ΔETAQ_F: GGCCATAAGCATA-miluminescence Substrate (PerkinElmer).

Secondary antibodies (PerkinElmer, NEF812, NEF822001EA) diluted in blocking solution at 4°C. After three 10-minute washes in TBST, the blots were incubated with the appropriate HRP-conjugated anti-GARS (rabbit; Abcam, ab42905; 1:1000 dilution) and anti-NeuN (mouse monoclonal; Cell Signaling Technology, E4M5P; 1:1000).

Western blot analysis. Protein lysates were resolved on MiniPROTEAN 4%–15% Tris-glycine gels (Bio-Rad) and transferred to an Invitronol & Immobilon-P PVDF membrane for Western blot analysis. Membranes were blocked with 5% skim milk in TBST (1x Tris-buffered saline, 0.1% Tween-20), and incubated overnight with anti-GARS (rabbit; Abcam, ab42905; 1:1000 dilution) and anti-NeuN (mouse monoclonal; Cell Signaling Technology, E4M5P; 1:1000) diluted in blocking solution at 4°C. After 30-minute washes in TBST, the blots were incubated with the appropriate HRP-conjugated secondary antibodies (PerkinElmer, NEF812, NEF822001EA) diluted in blocking solution. After three 10-minute washes in TBST, the blots were developed using Western Lightening Plus-ECL, Enhanced Chemiluminescence Substrate (PerkinElmer).

Assessment of axonal neuropathy in all mouse models
Grip strength was evaluated by wire hang test (20, 26) to evaluate gross muscle strength and endurance. Nerve conduction studies, motor nerve histology and analysis, NMJ immunofluorescence and analysis, and body weight evaluation were completed as previously described (20, 30). These studies were not formally blinded. First, the Gars mice have a visible phenotype that makes blinding with respect to genotype difficult, although mice treated at birth were often rescued to an extent that made them difficult to distinguish from control mice. The primary evaluator of the in vivo mouse studies was also the only person proficient at the intrathecal and i.c.v. injections at the time. Therefore, although records of which mice were treated or untreated were not immediately at hand during the analysis, this information was available to the tester.

Neonatal delivery of scAAV9.mi.P278KY and scAAV9.mi.ΔETAQ
Before all injections of mice at P0–P1, all pups were anesthetized via cryoanesthesia as previously described (48). Then, all i.c.v. injections were performed using a Hamilton syringe (catalog 65460_03) with a 32-gauge needle and escalating doses of scAAV9.mi.P278KY as stated in Supplemental Tables 2 and 3 or 2.6 × 10^11 DNAse-1–resistant particles (DRPS) per mouse of mi.ΔETAQ (about 2–10 μL) diluted in sterile PBS. All gene therapy vectors were injected in to the lateral ventricles by positioning of the needle directly lateral to the sagittal suture and rostral to the neonatal coronal suture. For i.v. injections, all cryoanesthetized mice were injected with 1 × 10^11 DRPS per mouse directly into the superficial temporal vein in a caudal orientation with the use of a Hamilton syringe (catalog 7655-01) with a 32-gauge needle.

Intrathecal delivery of gene therapy constructs to post-onset mice
With the use of a Hamilton syringe (catalog 7655-01) with a 32-gauge needle, all adult post-onset mice were injected with approximately 1 × 10^11 DRPS per mouse of scAAV9.mi.P278KY or scAAV9.mi.ΔETAQ diluted into sterile PBS (~10 μL) via an intrathecal injection by lumbar puncture. Here, all mice were anesthetized with isoflurane and received an injection of the proper vector into the L6 spinous process with the use of a Hamilton syringe with a 32-gauge needle. Each vector was slowly injected and the needle left in place for 5–10 seconds before withdrawal.

Quantification of allele-specific expression
Whole liver and lumbar DRG samples were isolated from animals immediately after they were euthanized by cervical dislocation. The tissues were frozen in liquid nitrogen and stored at −80°C. Samples were homogenized using a mortar and pestle followed by a Dounce homogenizer in 1% NP-40 in PBS supplemented with Protease Inhibitor Cocktail Tablets (Roche), then centrifuged at 14,000 g twice for 5 minutes at 4°C. Cleared homogenates were then sonicated at 4°C and centrifuged again at 14,000 g for 5 minutes. Twenty micrograms of protein was then analyzed by immunoblot.

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Whole liver and lumbar DRG samples were isolated from animals immediately after they were euthanized by cervical dislocation. The tissues were frozen in liquid nitrogen and stored at −80°C. Samples were homogenized using a mortar and pestle followed by a Dounce homogenizer, and RNA was isolated from liver using Trizol Reagent (catalog 15596018, Thermo Fisher Scientific) and DRGs using either an RNeasy Mini Kit (catalog 74104 and 74106, Qiagen) or a mirVana miRNA Isolation Kit (catalog AM1560, Thermo Fisher Scientific). All RNA samples were reverse transcribed using SuperScript III First-Strand Synthesis System (catalog 18080051). To quantify allele-specific expression of WT and mutant GARS, EpigenDX performed pyrosequencing on the PSQ96 HS System (Qiagen) following the manufacturer’s instructions, using custom assays.

Statistics
Statistical tests were performed using GraphPad Prism 7 software. A 2-tailed Student’s t test or 1-way or 2-way ANOVA followed by Tukey’s honestly significant difference (HSD) post hoc comparisons test (as indicated in the figure legends) was used to determine significant differences between treatment groups and/or genotypes for axon counts, conduction velocity, grip strength, and body weight. Axon diameters were compared using nonparametric Kolmogorov-Smirnov 2-sample and Shapiro-Wilk normality tests. NMJ innervation status between genotypes and categories (fully innervated, partially innervated, and denervated) was evaluated using a Hamilton syringe (catalog 7655-01) with a 32-gauge needle.
with a 2-way ANOVA followed by Tukey’s HSD post hoc comparisons test. A P value less than 0.05 was considered significant.

Study approval

Clinical procedures and sequencing that went beyond standard-of-care clinical evaluation and diagnostics were performed under the Institutional Review Board–approved protocol H-29697, Genome Sequencing to Elucidate the Causes and Mechanisms of Mendelian Genetic Disorders, Baylor College of Medicine, Houston, Texas, USA. Clinical data were obtained after written informed consent from the proband’s parents. All mouse husbandry and experimental procedures were conducted according to the NIH Guide for the Care and Use of Laboratory Animals (National Academies Press, 2011) and were reviewed and approved by the Animal Care and Use Committee of The Jackson Laboratory, Bar Harbor, Maine, under Animal Use Summary 1026, “The formation and maintenance of neuronal synapses and circuits.”

Author contributions

RWB, SQH, and AA contributed to experimental design, data interpretation, and manuscript preparation, with input from all authors. In vivo studies were completed by KHM; additional in vivo studies were conducted by TJH and ELS. All miGARS constructs were designed by SQH and constructed and tested for efficacy in vitro by NKP. AMF assisted with viral vector production. RNA isolation and cDNA synthesis from mouse tissue were completed by SGK, NKP, LMW, and KHM. Neurophilin IP was performed by NW and XLY. Enzyme kinetics were evaluated by RT and YMH. Yeast complementation studies and evaluation of human ΔETAQ expression were done by LBG and SNO. Patient clinical, pathological, and genomic evaluations were done by AN, JRL, PM, IJB, JOK, DM, and RMS.

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