Neuromuscular junction maturation defects precede impaired lower motor neuron connectivity in Charcot–Marie–Tooth type 2D mice

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INTRODUCTION

Charcot–Marie–Tooth disease (CMT) is a heterogeneous group of hereditary neuropathies that affect ∼1 in 2500 people (1). Over 40 genetic loci have been linked to CMT, which is characterized by distal motor and sensory dysfunction with progressive muscle atrophy predominantly in the hands and feet (2). CMTs are divided into type 1 forms typified by demyelination and reduced nerve conduction velocity (NCV), type 2 forms that display axon loss and intermediate CMTs that share features of the two.

The aminoacyl-tRNA synthetases (ARSs) are an ancient class of enzymes that charge specific amino acids to their cognate transfer RNAs (tRNAs), thereby ensuring the fidelity of the genetic code during protein translation (3). Mutations in several genes encoding ARSs have been shown to underlie a number of both axonal and intermediate CMTs (4). The first and best characterized of these is axonal CMT type 2D (CMT2D, OMIM ID 601472), which is caused by dominant mutations in GARS (ENSG00000106105) (5–10). CMT2D results in weakness with an emphasis in the distal, upper extremities and usually manifests during the second decade of life. Through different translation start sites, GARS encodes mitochondrial and cytoplasmic isoforms of the highly conserved, non-redundant, homodimeric glycyl-tRNA synthetase (GlyRS), which specifically charges the amino acid glycine...
All disease-associated mutations are located downstream of the mitochondrial-targeting sequence and have been shown to cluster around the dimer interface (15,16). GlyRS has been detected in healthy mouse and human serum (17) and can exist as a monomer that is inactive for aminoacylation (18), indicating that GlyRS may also possess a non-canonical function.

Two mouse models of CMT2D, 
\[ Gars^{Nmf249/+} \] and \[ Gars^{C201R/+} \], share pathological features of the human disease, resulting from dominant amino acid substitutions in \[ Gars \] (ENSMUSG00000029777) equivalent to \[ P234KY \] and \[ C157R \] in humans, respectively (19,20). The more severe \[ Gars^{Nmf249/+} \] mice have a CC-to-AAATA replacement leading to the in-frame substitution of a proline to a lysine and tyrosine at residue 278 (P278KY) (19). At 1 month, these mice display frank denervation in distal muscles and preferential loss of large diameter motor and sensory axons, phenotypes not observed at 1 week (19). Ventral root axons and spinal cord cell bodies remain unperturbed, indicating that the axonopathy progresses in a distal to proximal manner (19). These defects lead to reduced NCV, altered muscle contraction kinetics and overt neuromuscular dysfunction, which precede frequent, genetic background-dependent mortality at 6–8 weeks (19). On a C57BL/6 background, a small percentage of \[ Gars^{Nmf249/+} \] mice (4%) survive for longer but display little progression in phenotype, even up to 1 year, indicating that much of the disease burden is restricted to early life. \[ Gars^{C201R/+} \] mice possess a T-to-C point mutation causing a cysteine-to-arginine alteration at residue 201 (C201R) (20). Similar to the \[ Nmf249 \] heterozygotes, \[ Gars^{C201R/+} \] mice display diminished body weight, mild muscle weakness by 1 month, reduced axon diameters at 3 months and partial loss of innervation at 4 months, but a normal lifespan (20). Together, these two \[ Gars \] alleles represent a spectrum of disease severity and can be used in combination to dissect phenotypes integral to CMT2D pathology.

Heterozygous deletion of \[ Gars \] using a gene-trap insertion allele (\[ Gars^{XM256/+} \]), causing a 50% reduction in gene expression, has no gross phenotypic effect (19). Moreover, the \[ in vitro \] aminoacylation activity of GlyRS \[ P278KY \] (19) and the charging capacity of brain lysates from \[ Gars^{C201R/+} \] mice are unaffected (20). Lack of congruence between functional capacity and disease severity is corroborated by evidence, suggesting that additional \[ Gars \] mutations differentially affect aminoacylation (18,21). Nevertheless, neither the \[ C201R \] or \[ Nmf249 \] mutations are able to complement \[ XM256 \] loss-of-function as heterozygotes resulting in embryonic lethality, indicating that a secondary GlyRS function could be perturbed (22). Overexpression of human wild-type \[ Gars \] in \[ Gars^{Nmf249/+} \] and \[ Gars^{C201R/+} \] mice has minimal effect on the neuropathy phenotype, whereas severity is dependent on the mutant protein dose, suggesting that CMT2D is caused by a toxic gain-of-function of mutant GlyRS (22). Nevertheless, the cellular and molecular mechanisms linking dominant \[ Gars \] mutations to the selective peripheral nerve pathology in CMT2D remain unresolved (16).

Having a detailed knowledge of the cause and initial downstream mechanisms of neurological diseases is vital to understanding, and ultimately, treating a disease group that currently has very few therapeutic options. The neuromuscular junction (NMJ) appears to be an important site of early pathology in a number of disorders affecting lower motor neurons with synaptic denervation often preceding cell body loss (23–28). Similarly, the neuromuscular synapse appears to degenerate before axons in response to axotomy (29–31) and displays a number of age-related structural alterations in both rodents and humans (32–35). A more comprehensive understanding of the abnormalities occurring through time at the NMJ in \[ Gars \] mice would undoubtedly provide important clues about the pathological events underlying the targeted loss of lower motor neuron connectivity to muscle seen in CMT2D.

Here, we have performed a longitudinal characterization of \[ Gars \] mouse NMJs in the lumbar muscles of the hind-paw and the transversus abdominis (TVA) muscles of the trunk, allowing us to compare distal and proximal pathology. Assessing a number of different parameters, we find that pre- and postsynaptic NMJ development is drastically compromised in the lumbarcs, but not the TVA. This persistent impairment of NMJ maturation is coupled with significant denervation of the lumbar muscles, whereas the TVA, again, remains relatively unaffected. Our work therefore suggests that defective NMJ development provides a substrate for subsequent synaptic degeneration and identifies the neuromuscular synapse as an early, but selective, pathological target in CMT2D mice.

**RESULTS**

**Structural maturation of the pre- and post-synapse is defective in \[ Gars^{C201R/+} \] lumbar NMJs**

Neuromuscular synapses of the extensor digitorum longus (EDL) and tibialis anterior (TA), two distal hind-limb muscles, have previously been examined in 4-month-old \[ Gars^{C201R/+} \] mice (20). Mutant EDL NMJs were indistinguishable from wild-type; however, a significant proportion (~10%) of the \[ Gars^{C201R/+} \] TA NMJs displayed regions in which the nerve terminal no longer directly overlapped the postsynaptic acetylcholine receptors (AChRs), indicative of partial denervation (see Fig. 3A for an example of this). There was no difference between wild-type and mutant mice in the number of motor neurons in the lumbar spinal cord (20). Together, this suggests that the NMJ is a relatively early pathological target in \[ Gars \] mice compared with the cell body, an assertion corroborated by similar evidence from the more severe \[ Nmf249/+ \] model (19).

We therefore decided to investigate neuromuscular pathology of \[ Gars^{C201R/+} \] mice in more detail by performing a longitudinal assessment of NMJ morphology. Time-points of ~2 weeks (post-natal day 15–16, P15-16), 1 and 3 months were chosen to cover a period from early post-natal development to adulthood during which symptoms usually manifest. We began by looking at the deep lumbar muscles between the digits of the hind-paw, which precipitate flexion of the metatarsophalangeal joints contributing to hind-paw claspig (Fig. 1A). Given this function, it is possible that lumbarmuscle dysfunction contributes to the reduced grip strength previously reported in \[ Gars^{C201R/+} \] mice (20). These distal muscles predominantly consist of fast-twitch fibres and are innervated by terminal branches of the tibial nerve (26). The lumbarcs were chosen for analysis because they are thin and flat, permitting visualization of the entire innervation pattern of the muscle in whole-mount preparations, thus obviating the need for sectioning.
We first decided to look at NMJ maturation to determine whether developmental defects contribute to the \textit{Gars} mouse neuromuscular phenotype. As mammalian NMJs develop during the first few post-natal weeks, a number of structural and functional alterations occur (37). At birth, NMJs are innervated by multiple motor axonal inputs (38,39). Through a dynamic, activity-dependent process called synapse elimination, excess branches progressively and asynchronously withdraw from the synapse, such that the majority of mouse NMJs become innervated by a single motor axon within 2 weeks of birth. Occurring at the same time as this pre-synaptic development, the post-synapse transitions from a relatively simple plaque-like structure to a more complex pretzel shape (40).

To view NMJs, pre-synaptic axons and motor neuron terminals were labelled with antibodies against neurofilament (2H3, green) and synaptic vesicle (SV2, green) proteins, and

\begin{figure}[h]
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\caption{\textit{Gars}^{C201R/+} lumbarial NMJs display impaired development of pre- and post-synaptic structures. (A) Schematics of the anatomical locations of the lumbarial and TVA muscles in the mouse. The lumbarial muscles are coloured red, the TVA muscles purple and the main nerve branches of each muscle are depicted in green. Figure adapted from (36). (B and C). Example P15-16 \textit{Gars}^{C201R/+} mouse NMJs with one showing polyinnervation (arrowhead). As is the case for all subsequent figures, nervous tissue is stained green (2H3, neurofilament; SV2, synaptic vesicle 2) and post-synaptic AChRs are stained red (\textalpha-BTX, \textalpha-bungarotoxin). (D) \textit{Gars}^{C201R/+} lumbarial muscles show a significant impairment in the process of synapse elimination as shown by the increased percentage of NMJs with more than one innervating neuron at all time-points. \( P \leq 0.001, \) Kruskal–Wallis test. \( **P \leq 0.01, \) **\( P \leq 0.001, \) Dunn’s multiple comparison test. (E) Mutant NMJs have significantly fewer post-synaptic perforations at P15-16 and 1 month, indicative of hindered progression from the immature plaque to the mature pretzel conformation. \( **P \leq 0.01, \) **\( P \leq 0.001, \) unpaired \( t \)-test with Welch’s Correction. (F) Example endplates from wild-type and mutant mice at P15-16 and 1 month. Note the reduced complexity and size. The arrow highlights a single perforation. A total of 4–13 mice/genotype/time-point were assayed.}
\end{figure}
the post-synaptic AChRs with rhodamine-conjugated α-bungarotoxin (α-BTX, red) (Fig. 1B and C). The percentage of NMJs with more than one axonal input was determined for wild-type and mutant mice at the three time-points (Fig. 1D). As expected, only a small percentage of wild-type NMJs were polyinnervated at P15-16 (3.3 ± 0.7%), which was further reduced at 1 (0.6 ± 0.4%) and 3 months (0.8 ± 0.3%). In contrast, Gars<sup>C201R/+</sup> mice maintained a high percentage of polyinnervation at all time-points (P15-16—20.7 ± 3.5%, P < 0.01; 1 month—16.6 ± 1.2%, P < 0.001; 3 months—6.8 ± 2.4%). This phenotype was most prevalent at P15-16 and slowly declined over time, but persisted to at least 3 months (albeit not quite reaching significance at this final time-point). Nearly all of the polyinnervated NMJs were contacted by two neurons, with a very small proportion being innervated by three.

We then studied post-synaptic development by determining the number of perforations per developing NMJ endplate (Fig. 1E and F). During the transition from a plaque structure to a pretzel-like shape, multiple-folds and perforations in the post-synapse begin to form as the AChRs migrate to closely appose the pre-synaptic input. We scored NMJs as having 0, 1, 2, 3 or >3 perforations at P15-16 and 1 month. The analysis was not performed at 3 months, because it becomes difficult to accurately score perforations in NMJs with complex morphology. We found that Gars<sup>C201R/+</sup> mice had significantly more NMJs without a perforation at P15-16 (49.6 ± 3.1% versus 90.5 ± 2.0%, P < 0.001) and significantly fewer NMJs with more than three perforations per endplate at 1 month (70.5 ± 4.0% versus 28.5 ± 6.1%, P < 0.01), consistent with reduced complexity compared with wild-type mice. Together these results indicate that Gars<sup>C201R/+</sup> mice display impaired maturation of pre- (Fig. 1B–D) and post-synaptic (Fig. 1E and F) structures in lumbrical muscle NMJs.

**Transcriptional development of Gars<sup>C201R/+</sup> lumbral NMJs is also impaired**

In addition to synapse elimination and the increase in post-synaptic complexity, the mammalian NMJ undergoes a process known as AChR subunit switching. AChRs found at synaptic complexity, the mammalian NMJ undergoes a process known as AChR subunit switching. AChRs found at

Figure 2. The transcriptional maturation of Gars<sup>C201R/+</sup> lumbral NMJs is also defective. (A) At P15-16, Gars<sup>C201R/+</sup> lumbricals show a delay in expression of the adult AChR subunit (Chrne) as assessed by qPCR. P = 0.0286, Kruskal–Wallis test. (B) At 1 month, Chrne expression in mutant mice has normalized to wild-type levels, but foetal subunit (Chrng) expression persists. P = 0.109, Kruskal–Wallis test. Gapdh (ENSMUSG00000057666) was used as the reference gene. *P < 0.05, Dunn’s multiple comparison test. A total of 2–6 mice/genotype/time-point were assayed (two wild-type mice were studied at P15-16 and 1 month for the Chrng and Chrne analyses, respectively).
denervated (vacant) $\text{Gars}^{C201R/+}$ NMJs from 1 to 3 months (1.7 ± 0.9% versus 5.8 ± 0.7%, $P < 0.01$ unpaired $t$-test with Welch’s Correction). These longitudinal observations suggest that $\text{Gars}$ mutant mice display a progressive degenerative phenotype, rather than an inability to form specialized neuromuscular connections.

When assessing morphology and denervation at 3 months, we noticed that mutant NMJs appeared to be slightly smaller than wild-type synapses. We therefore measured the surface area enclosed by the perimeter of the post-synaptic staining from maximum intensity Z-stack images taken on a confocal microscope. The difference between wild-type and mutant NMJ areas did not quite reach significance at any time-point measured (P15-16, 166.1 ± 17.3 µm² versus 160.3 ± 5.9 µm²; 1 month—256.3 ± 8.3 µm² versus 232.2 ± 6.5 µm²; 3 months—352.8 ± 9.0 µm² versus 309.6 ± 15.9 µm²); however, the percentage difference between the two genotypes increased with age ($23.5, 29.4, 212.2%$), perhaps indicative of impaired growth over time (Fig. 3D).

The degree of NMJ pathology correlates with $\text{Gars}$ mouse model severity

The extent of NMJ denervation in muscles of the hind-leg is more severe in $\text{GarsNmf249/+}$ mice than $\text{GarsC201R/+}$ mice (19,20), although a direct comparison between the same muscles has not been performed previously. We therefore analysed innervation status, occupancy and area of $\text{GarsNmf249/+}$ mouse lumbrical NMJs at 2 months (Fig. 4). $\text{Gars}^{C201R/+}$ and $\text{Gars}^{Nmf249/+}$ were both maintained on a predominantly C57BL/6 background. $\text{Gars}^{Nmf249/+}$ mice display significantly more polyinnervated NMJs than wild-type mice (2.0 ± 1.0% versus 25.8 ± 2.7%, $P < 0.05$), the extent of which supersedes that seen at any time-point in the $\text{C201R}$ allele (P15-16—20.7 ± 3.5%; 1 month—16.6 ± 1.2%; 3 months—6.8 ± 2.4%) (Fig. 4C). $\text{Gars}^{Nmf249/+}$ mice also possessed significantly fewer fully innervated NMJs compared with wild-type mice (99.1 ± 0.3% versus 47.2 ± 3.7%, $P < 0.05$) (Fig. 4D), whereas the percentage of vacant NMJs (17.2 ± 3.5%) was significantly greater than that observed in $\text{Gars}^{C201R/+}$ mice at 1 month (1.7 ± 0.9%, $P < 0.05$ unpaired $t$-test with Welch’s Correction) and 3 months (5.8 ± 0.7%, $P < 0.05$ unpaired $t$-test with Welch’s Correction).

Finally, we measured the area of $\text{GarsNmf249/+}$ NMJs and found that they were significantly smaller than wild-type NMJs (331.8 ± 9.2 µm² versus 205.9 ± 9.0 µm², $P < 0.05$) (Fig. 4E). Once again, the percentage difference between $\text{GarsNmf249/+}$ and wild-type (−37.9%) mice was greater than that seen at any time-point for $\text{Gars}^{C201R/+}$ mice (−3.5, −9.4, −13.6%).

We have thus confirmed both at the structural (Fig. 1) and transcriptional levels (Fig. 2) that $\text{Gars}$ mice display defects in neuromuscular connectivity.
lumbrical NMJ maturation that precede progressive degeneration (Fig. 3) and that the neuromuscular phenotype broadly correlates with severity of the Gars model (Fig. 4).

**Gars**transversus abdominis NMJs remain relatively unperturbed

We fully expected the Gars lumbrical muscles to display denervation defects because CMTs predominantly affect the distal extremities, and various muscles of the hind-leg are affected in the two Gars mouse models. To enable us to compare pathology between a distal and a proximal muscle, and hence comment on the importance of potentially disparate phenotypes, we decided to dissect the TVA of Gars+/+ mice. The TVA is a proximal postural muscle of the anterior abdominal wall (Fig. 1A), which mainly consists of slow-twitch fibres and is innervated by the lower intercostal nerve (26,48). Similar to the lumbrical muscles, the TVA possesses the advantage that it can be dissected and its entire synaptic innervation pattern viewed in whole-mount preparations.

Once again, we assessed maturation and denervation phenotypes at time-points of P15-16, 1 and 3 months (Fig. 5 and Supplementary Material, Fig. S1). At P15-16, similar to the lumbrical muscles, we detected impaired maturation of the pre- and post-synapse in Gars+/+ TVA muscles, as evidenced by the mutants having significantly more polynervated NMJs (1.5 ± 0.8% versus 14.1 ± 7.9%, *P* < 0.05) and significantly more NMJs without a single perforation per endplate (22.5 ± 2.4% versus 49.4 ± 3.9%, *P* < 0.05), respectively (Fig. 5A and B). Nevertheless, the maturation defects were short-lived as there was no difference in polyinnervation at 1 month (0.9 ± 0.6% versus 3.8 ± 1.4%) or 3 months (0.8 ± 0.4% versus 0.7 ± 0.3%), and there was no difference in the percentage of endplates with more than three perforations at 1 month (78.8 ± 4.6% versus 73.3 ± 4.6%). Consistent with this, we observed no difference in the expression levels of the foetal and adult AChR subunits at both P15-16 (Chrng—100.0 ± 33.0% versus 99.4 ± 41.5%; Chrne—100.0 ± 27.3% versus 107.3 ± 18.0%) and 1 month (Chrng—100.0 ± 42.3% versus 131.1 ± 17.0%; Chrne—100.0 ± 14.2% versus 129.8 ± 19.9%) (Fig. 5C and D). It should be noted that at P15-16, one mutant mouse had the highest degree of polyinnervation observed in the whole study (37.5% of NMJs had more than one input), even greater than that seen in any of the GarsNmf249+/+ mice. If this single data point is removed, the result remains significant, but the extent of mutant TVA polyinnervation at P15-16 is drastically reduced (1.5 ± 0.8% versus 6.3 ± 1.9%, *P* < 0.05).

We then calculated occupancy percentages for the TVA muscles of wild-type and Gars+/+ mice (Fig. 5E). Contrasting with the lumbrical muscles, we saw no difference in the percentage of fully innervated NMJs at any time-point (P15-16—99.6 ± 0.3% versus 99.4 ± 0.6%; 1 month—99.7 ± 0.3% versus 98.3 ± 1.1%; 3 months—98.3 ± 0.4% versus 97.8 ± 0.5%).
indicating that \textit{Gars}^{C201R/+} lower motor neuron connectivity remains unaffected in the TVA up to at least 3 months of age.

We also measured TVA NMJ area (Fig. 5F) and similar to the lumbral muscles found no significant difference between wild-type and \textit{Gars}^{C201R/+} mice at any time-point (P15-16—388.5 ± 13.5 μm² versus 394.1 ± 19.4 μm²; 1 month—563.6 ± 22.0 μm² versus 481.0 ± 36.9 μm²; 3 months—985.9 ± 56.4 μm² versus 715.6 ± 29.0 μm²). However, the percentage difference between wild-type and mutant TVA NMJ areas increased with time (+1.5, −14.7, −27.4%), and when the data generated at 3 months were analysed with a single statistical test, the difference between wild-type and mutant was statistically significant (P = 0.0357, Mann–Whitney U test). These observations suggest that mutant NMJs display defective growth and that this is unlikely to be due to denervation, but rather the smaller overall size of the \textit{Gars}^{C201R/+} mice.

\textit{Gars}^{Nmf249/+} TVA NMJs also show normal development and connectivity

To complete our study, we analysed NMJs of the TVA muscle in \textit{Gars}^{Nmf249/+} mice at the same time-point as the lumbral muscle NMJs (Fig. 6 and Supplementary Material, Fig. S2). Consistent with the \textit{Gars}^{C201R/+} TVA phenotype, we saw no difference in the percentage of polyinnervated NMJs (1.1 ± 1.3% versus 2.5 ± 1.7%) (Fig. 6A) or fully innervated endplates.
DISCUSSION

The peripheral neuropathy CMT2D results from dominant mutations in the GARS gene, which encodes the essential housekeeping protein GlyRS (5). CMT2D typifies a number of neuromuscular conditions where dysfunction of a widely and constitutively expressed gene results in a specific detrimental effect on the motor neurons (23,49–53). Despite having identified the disease-causing genes for many neuromuscular disorders, there remains a dearth of information on the molecular mechanisms underlying motor neuron vulnerability. Nevertheless, a number of studies have identified the specialized interface between lower motor neurons and muscle fibres as an early and important site of neuropathology (23–28). In the current study, we therefore performed a longitudinal examination of NMJs in wild-type and Gars mice to better understand the series of pathological events leading to axonal loss in CMT2D.

As CMT2D patients display selective muscle weakness (6–10), we decided to analyse the NMJs in two muscles from distinct anatomical locations in the hope that we could discern pertinent features of neuropathology. We dissected the distal lumbrical muscles of the hind-feet and the proximal TVA muscles of the trunk (Fig. 1A) (26) and viewed the NMJs at different time-points spanning the asymptomatic perinatal period to the loss in synaptic connectivity, because the enduring developmental phenotypes are only observed in the denervation-susceptible lumbrical muscles. Nonetheless, the small delay in

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Animals were retrospectively genotyped using previously published protocols (19,20). All reagents were obtained from Sigma–Aldrich unless otherwise stated.

Muscle preparation and immunohistochemistry
Mice were sacrificed, muscles dissected, and NMJs immunohistochemically labelled and observed as previously described (26). Muscles were dissected in cold 1× phosphate-buffered saline (PBS) and fixed in freshly prepared PBS containing 4% (w/v) parafomaldehyde (PFA, Electron Microscopy Sciences) for 10 min. Muscles were permeabilized with 2% (v/v) Triton X-100 in PBS for 30 min, blocked in 4% (w/v) bovine serum albumin and 1% (v/v) Triton X-100 in PBS for 30 min and then incubated overnight at 4°C in blocking solution with primary antibodies against neurofilament (2H3, 1/50, DSHB) and synaptic vesicles (SV2, 1/100, DSHB). PBS was used to wash the muscles three times for a total of 1.5 h before 2-h incubation with AlexaFluor 488 secondary antibody (goat anti-mouse, 1/250, Invitrogen) and 1.5 μg/ml tetramethylrhodamine α-BTX (Cambridge Bioscience) in PBS. Finally, muscles were washed three times in PBS for 30 min and mounted in Mowiol on glass slides with coverslips for subsequent imaging.

NMJ imaging and analysis
NMJs were imaged using a Zeiss LSM 510 META laser scanning microscope or a Zeiss Axioplan 2 microscope. For all images, nervous tissue is labelled green (2H3 and SV2) and post-synaptic AChRs red (α-BTX). Only NMJs with clearly visible pre-synaptic axons and terminals were scored. The first to fourth deep lumbrical muscles and the TVA muscles were dissected and scored: ≥ 40 NMJs were scored per mouse for polynervation, perforation and occupancy counts and ≥ 15 for NMJ area measurements. At each time-point, ≥3 mice per genotype were scored. For perforation counts, NMJs with no perforations and more than three perforations were used for statistical testing at P15-16 and 1 month, respectively. For occupancy counts, NMJs were categorized as previously described (66). To measure the NMJ area, the perimeter circumscribing the post-synaptic staining from a maximum intensity Z-stack image (1-μm intervals) was drawn by hand and the enclosed surface area measured using ImageJ as performed previously (67).

RNA extraction and qPCR
Freshly dissected muscles were snap-frozen in liquid nitrogen, before thawing and extraction of RNA using an RNeasy Mini Kit (QIAGEN). To each 30 μl SuperScript III (Invitrogen) reverse transcription reaction, 150–500 ng of RNA was added. TVA cDNA was diluted 1/10 in water, whereas lumbrical cDNA was pre-amplified in a 50 μl reaction using a Taqman Preamp Master Mix Kit (Invitrogen) and then diluted 1/25 in water. Two microliters of cDNA was used per 20-μl qPCR using SYBR Green (Applied Biosystems) and a StepOnePlus real-time PCR machine (Applied Biosystems). Primer sequences: Chrne—forward 5′ GCA GCT TTT ACC GAG AAT GG 3′ and reverse 5′ CGT CAG TTT CTC CAG GAC C 3′; Chrne—forward 5′ GGC AGA AAT GCA CAG TGG 3′ and reverse 5′ CAG GTA CTT GAT GAT TGG 3′; Gapdh—forward 5′ TGT GTC CGT CGT GGA TCT GA

MATERIALS AND METHODS
Animals
Gars
C201R/+
 mice and Gars
Nmfb249/+
 mice were maintained as heterozygote breeding pairs each on a predominantly C57BL/6 background as previously described (19,20,65). Gars
C201R/+ handling and experiments were performed in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act (1986) and approved by the University of Oxford Ethical Review Panel. Gars
Nmfb249/+ mouse husbandry and procedures were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by The Jackson Laboratory Animal Care and Use Committee. Animals were retrospectively genotyped using previously

pre- and post-synaptic NMJ development observed in Gars
C201R/+ TVA muscle at 2 weeks (Fig. 5A and B) suggests that there may be a subtle systemic developmental component to CMT2D.

The process of synapse elimination shares morphological and physiological parallels with the synaptic response to axotomy and loss of lower motor neuron connectivity at the NMJ observed in a number of neurological conditions (54,55). All three processes result in the asynchronous retraction of nerve terminals from the motor endplate over a period of days to weeks. It has therefore been postulated that pathological NMJ denervation may represent misregulated synapse elimination pathways, whereby overactive neuronal retraction mechanisms precipitate uncontrolled, aberrant axonal die-back (54). As we observed a significantly higher percentage of polyinnervated NMJs (i.e. incomplete synapse elimination) in the Gars mice, the denervation is unlikely to be a product of up-regulated elimination pathways. Nevertheless, it has long been recognized that polynervation persists in response to early post-natal insult in rodent models (56), whereas rat peripheral nerve transaction experiments suggest that polynervation may be a limiting factor in restoration of function (57,58). It is therefore possible that the defective NMJ maturation, evident in pre-symptomatic Gars mutant mice, is indeed contributing to later onset muscle weakness and denervation (20). The process of synapse elimination is drastically influenced by nerve–muscle electrical activity (56,59–62). Furthermore, synaptic neurotransmission abnormalities have frequently been reported to precede morphological changes at the NMJ during the processes of synapse elimination and denervation in disease (54,63). It would therefore be interesting to perform electrophysiological studies on the Gars mutant mice, in order to determine whether defects in synaptic transmission underlie the maturation and denervation defects. Determining whether microtubule-stabilizing compounds, which have been shown to reduce polynervation (58,64), provide any benefit to CMT2D mice would also be of interest.

In summary, we have shown that persistent maturation defects selectively precede progressive, age-dependent loss of lower motor neuron connectivity at the NMJ in mutant Gars mice. This work highlights the neuromuscular synapse as an early and important site of pathology in CMT2D and indicates that further study of the electrophysiological properties of the NMJ are required in order to improve our understanding of the disease aetiology and progression.

DNA extraction and qPCR
Freshly dissected muscles were snap-frozen in liquid nitrogen, before thawing and extraction of RNA using an RNeasy Mini Kit (QIAGEN). To each 30 μl SuperScript III (Invitrogen) reverse transcription reaction, 150–500 ng of RNA was added. TVA cDNA was diluted 1/10 in water, whereas lumbrical cDNA was pre-amplified in a 50 μl reaction using a Taqman Preamp Master Mix Kit (Invitrogen) and then diluted 1/25 in water. Two microliters of cDNA was used per 20-μl qPCR using SYBR Green (Applied Biosystems) and a StepOnePlus real-time PCR machine (Applied Biosystems). Primer sequences: Chrne—forward 5′ GCA GCT TTT ACC GAG AAT GG 3′ and reverse 5′ CGT CAG TTT CTC CAG GAC C 3′; Chrne—forward 5′ GGC AGA AAT GCA CAG TGG 3′ and reverse 5′ CAG GTA CTT GAT GAT TGG 3′; Gapdh—forward 5′ TGT GTC CGT CGT GGA TCT GA

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3’ and reverse 5’ CCT GCT TCA CCA CCT TCT TGA 3’. Cycling conditions: 95°C for 10 min, 40 × [95°C for 15 s, 60°C for 1 min], 95°C for 15 s. Three technical replicates per reaction were performed, and primers were used at 200 nm. Primer efficiencies were estimated by performing qPCR on serial dilutions of at least two independent cDNA samples, plotting Ct values on the Y-axis against log-transformed cDNA inputs, and using the slope of the line of regression in the following equation: \(10^{-(1/slope)} - 1\) × 100. Relative gene expression was calculated using the comparative Ct (ΔΔCt) method and Gapdh as the reference gene as previously described (68).

Statistical analysis
When normally distributed, data were statistically analysed using an unpaired t-test with Welch’s Correction or a one-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test. If the data did not pass normality testing, a Mann–Whitney U test or a Kruskal–Wallis test with Dunn’s multiple comparison test was used. GraphPad Prism 5 software was used for all statistical analyses. Means ± SEM are plotted for all graphs.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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