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.
(11–14). 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\textsuperscript{Nmf249/+} and Gars\textsuperscript{C201R/+}, which share pathological features of the human disease, result from dominant amino acid substitutions in Gars (ENSMUSG00000029777) equivalent to P234KY and C157R in humans, respectively (19,20). The more severe Gars\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{Xm256/+}), causing a 50% reduction in gene expression, has no gross phenotypic effect (19). Moreover, the in vitro aminoacylation activity of GlyRS\textsuperscript{P278KY} (19) and the charging capacity of brain lysates from Gars\textsuperscript{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 nor 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\textsuperscript{Nmf249/+} and Gars\textsuperscript{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 lumbral 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 lumbricals, but not the TVA. This persistent impairment of NMJ maturation is coupled with significant denervation of the lumbral 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\textsuperscript{C201R/+} lumbral 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\textsuperscript{C201R/+} mice (20). Mutant EDL NMJs were indistinguishable from wild-type; however, a significant proportion (\textasciitilde10%) of the Gars\textsuperscript{C201R/+} TA NMJs displayed regions in which the nerve terminal no longer directly overlapped the post-synaptic 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 Gars\textsuperscript{Nmf249/+} model (19).

We therefore decided to investigate neuromuscular pathology of Gars\textsuperscript{C201R/+} mice in more detail by performing a longitudinal assessment of NMJ morphology. Time-points of \textasciitilde2 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 lumbrical muscles between the digits of the hind-paw, during which symptoms usually manifest. We began by looking at the deep lumbrical muscles between the digits of the hind-paw, which precipitate flexion of the metatarsophalangeal joints contributing to hind-paw clasping (Fig. 1A). Given this function, it is possible that lumbral muscle dysfunction contributes to the reduced grip strength previously reported in Gars\textsuperscript{C201R/+} mice (20). These distal muscles predominantly consist of fast-twitch fibres and are innervated by terminal branches of the tibial nerve (26). The lumbricals 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 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...
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 polyin- 
nervation 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 morph- 
ology. 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 matura- 
tion of pre- (Fig. 1B–D) and post-synaptic (Fig. 1E and F) structures 
in lumbrical muscle NMJs.

Transcriptional development of Gars<sup>C201R+</sup> lumbrical 
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 
the NMJ consist of five subunits: two α, one β, one δ, and either 
γ or ε (41,42). Expressed in the embryonic and perinatal 
period, the γ-subunit (encoded by Chrng ENSMUSG0000000 
26253) defines the immature AChR and is exchanged during 
early post-natal development for the ε-subunit (encoded by 
Chrne ENSMUSG00000014609), which characterizes the adult 
AChR. This switch usually occurs in rodents between P5 and P9 
(43–45), altering the AChR gating properties (46,47).

We therefore performed quantitative reverse transcription 
PCR (qPCR) to examine expression of the foetal (γ) and adult 
(ε) AChR subunits (Fig. 2). At P15-16, there was no difference 
in foetal subunit expression between wild-type and mutant 
mice (100.0 ± 19.1% versus 97.9 ± 10.1%), whereas adult 
subunit expression was significantly lower in Gars<sup>C201R+</sup> 
(100.0 ± 4.5% versus 50.8 ± 7.2%, P < 0.05) (Fig. 2A). By 1 
month, adult subunit expression in mutant mice had normalized 
to approximately wild-type levels (100.0 ± 10.3% versus 
121.6 ± 26.1%), whereas expression of the foetal subunit 
remained significantly higher in mutant than wild-type mice 
(100.0 ± 25.9% versus 206.0 ± 30.8%, P < 0.05) (Fig. 2B). 
Together this suggests that there is a delay in the up-regulation 
of adult subunit expression and a defect in the down-regulation 
of foetal subunit expression. It therefore appears that matura- 
tion of the Gars<sup>C201R+</sup> mouse lumbrical NMJ is impaired, as 
determined by the factors mentioned earlier, at both the structural 
(Fig. 1) and transcriptional levels (Fig. 2).

NMJ denervation succeeds the dysfunctional developmental

To determine whether the defective synaptic maturation pre- 
cedes a denervation phenotype, we assessed NMJ innervation 
status at the same three time-points (Fig. 3). NMJs were scored 
as being fully innervated (full overlap between the pre-synaptic 
nerve terminal and the post-synaptic endplate), partially 
inervated (regions of the post-synapse lacking neuronal input) 
or vacant (no neurofilament overlaying the endplate) (Fig. 3A 
and B). At P15-16, there was no difference in the percentage 
of fully occupied NMJs between wild-type and Gars<sup>C201R+</sup> 
mice (96.0 ± 1.6% versus 96.5 ± 1.3%) (Fig. 3C). However, 
at 1 month, we saw significantly fewer fully innervated NMJs 
in mutant muscle (98.8 ± 0.7% versus 68.5 ± 5.9%, P < 0.05), 
which became progressively worse by 3 months (99.2 ± 0.5% versus 46.6 ± 2.1%, P < 0.01) (Fig. 3C). There 
was also a significant increase in the percentage of completely

Figure 2. The transcriptional maturation of Gars<sup>C201R+</sup> lumbrical 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) $Gars^{C201R/+}$ 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 $Gars$ 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 μm$^2$ versus 160.3 ± 5.9 μm$^2$; 1 month—256.3 ± 8.3 μm$^2$ versus 232.2 ± 6.5 μm$^2$; 3 months—352.8 ± 9.0 μm$^2$ versus 309.6 ± 15.9 μm$^2$); 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 $Gars$ mouse model severity

The extent of NMJ denervation in muscles of the hind-leg is more severe in $Gars^{Nmf249/+}$ mice than $Gars^{C201R/+}$ 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 $Gars^{Nmf249/+}$ mouse lumbral NMJs at 2 months (Fig. 4). $Gars^{C201R/+}$ and $Gars^{Nmf249/+}$ were both maintained on a predominantly C57BL/6 background. $Gars^{Nmf249/+}$ 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 $C201R$ allele (P15-16—20.7 ± 3.5%; 1 month—16.6 ± 1.2%; 3 months—6.8 ± 2.4%) (Fig. 4C). $Gars^{Nmf249/+}$ 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 $Gars^{C201R/+}$ 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 $Gars^{Nmf249/+}$ NMJs and found that they were significantly smaller than wild-type NMJs (331.8 ± 9.2 μm$^2$ versus 205.9 ± 9.0 μm$^2$, $P < 0.05$) (Fig. 4E).

We have thus confirmed both at the structural (Fig. 1) and transcriptional levels (Fig. 2) that $Gars$ mice display defects in

![Figure 3](https://academic.oup.com/hmg/article-abstract/23/10/2639/61452) by guest on 24 January 2019
lumbrical NMJ maturation that precede progressive degeneration (Fig. 3) and that the neuromuscular phenotype broadly correlates with severity of the Gars model (Fig. 4).

GarsC201R/+ 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 GarsC201R/+ 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 GarsC201R/+ TVA muscles, as evidenced by the mutants having significantly more polyinnervated NMJs (1.5 ± 0.8% versus 14.1 ± 7.9%, P < 0.05) and significantly more NMJs without a single perforation per end-plate (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 GarsC201R/+ 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%),

Figure 4. Gars mouse lumbrical NMJ defects correlate with mutant severity. (A and B) Example lumbrical muscle NMJs from 2-month-old wild-type and GarsNmf249/+ mice. A denervated (arrowhead) and polyinnervated (arrow) NMJ can be seen in the mutant muscle. Differences in post-synaptic complexity and size are evident between genotypes. Neuronal atrophy can also be seen in the mutant mouse. (C–E) At 2 months, the more severe mutant mouse, GarsNmf249/+ displays significantly more NMJs that are polyinnervated (C, P = 0.0159), denervated (D, P = 0.0175) and smaller (E, P = 0.0159) than wild-type mice. The degree of GarsNmf249/+ polyinnervation is greater than that seen in GarsC201R/+ mice at any time-point. Similarly, denervation and the reduction in NMJ size are also more severe. *P < 0.05, Mann–Whitney U test. A total of 4–5 mice/genotype/time-point were assayed.
indicating that \textit{GarsC201R/} 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 lumbrical muscles found no significant difference between wild-type and \textit{GarsC201R/} mice at any time-point (P15-16—388.5 ± 13.5 \( \mu \text{m}^2 \) versus 394.1 ± 19.4 \( \mu \text{m}^2 \); 1 month—563.6 ± 22.0 \( \mu \text{m}^2 \) versus 481.0 ± 36.9 \( \mu \text{m}^2 \); 3 months—985.9 ± 56.4 \( \mu \text{m}^2 \) versus 715.6 ± 29.0 \( \mu \text{m}^2 \)). 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 \((P = 0.0357, \text{Mann–Whitney } U \text{ test})\). 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{GarsC201R/} mice.

\textit{GarsNmf249/} TVA NMJs also show normal development and connectivity

To complete our study, we analysed NMJs of the TVA muscle in \textit{GarsNmf249/} mice at the same time-point as the lumbrical muscle NMJs (Fig. 6 and Supplementary Material, Fig. S2). Consistent with the \textit{GarsC201R/} 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.
(100.0 ± 0.0% versus 98.4 ± 1.6%) (Fig. 6B). Similarly, there was a 34.7% reduction in the area of mutant TVA NMJs (778.0 ± 32.1 μm² versus 507.8 ± 16.3 μm², \( P < 0.05 \)) (Fig. 6C).

Through this work on the TVA in both Gars alleles, we have determined that different muscles show selective susceptibility to disruption of lower motor neuron connectivity to muscle and that NMJ maturation defects precede denervation.

**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 symptomatic young adulthood.

We began by assessing the morphological and transcriptional development of lumbral NMJs. We found that Gars>C201R/+ mice display significantly impaired development of both pre- and post-synaptic structures (Fig. 1). The process of synapse elimination, which is usually complete by ~2 weeks post-birth in wild-type animals, showed significant defect at 2 weeks and 1 month and persisted to at least 3 months, albeit to a lesser extent (Fig. 1B–D). Mirroring this, the transition of the postsynapse from a plaque to a pretzel shape was also delayed in Gars>C201R/+ mice (Fig. 1E and F). Similarly, the transcriptional maturation was impaired as evidenced by the defective expression of AChR subunit genes (Fig. 2).

After confirming the presence of mutant NMJ maturation defects, we then showed that they preceded a progressive, age-dependent loss of lower motor neuron connectivity (Fig. 3). No denervation was seen at P15-16, but by 1 month, the percentage of fully innervated NMJs was significantly lower in Gars>Nmf249/+ mice compared with wild-type mice, which worsened by 3 months. These results indicate that mutant motor neurons are able to successfully target and contact lumbral muscle fibres, but are unable to maintain all of their synaptic connections in post-natal life. The importance and general applicability to CMT2D of these synaptic phenotypes was subsequently confirmed in the more severe Gars>Nmf249/+ mouse (Fig. 4). Defects in NMJ maturation and connectivity were more exaggerated in Gars>Nmf249/+ mice, indicating that the neuromuscular pathology correlates with CMT2D mouse model severity.

We then examined the TVA muscle of wild-type and mutant mice at the same longitudinal time-points. We observed in Gars>C201R/+ mice that at P15-16, this proximal muscle showed only minor deficiencies in synapse elimination and post-synaptic AChR cluster migration that were both corrected by 1 month (Fig. 5A and B). Moreover, AChR subunit gene expression was unaffected (Fig. 5C and D). The TVA therefore appears to display only a short-lived defect in NMJ maturation, which is less pronounced than that observed in the mutant lumbral muscles. Meanwhile, we found no difference in the innervation status between wild-type and mutant TVA muscles up to 3 months (Fig. 5E). Once again, we confirmed the validity of this phenotype in the severe Gars>Nmf249/+ mouse (Fig. 6). Together, these observations indicate that there is little pathology in these proximal muscles compared with the distal lumbral muscles, analogous to the pattern of muscle weakness seen in human CMT2D patients (6–10). These experiments also suggest that the persistent defect in NMJ maturation is linked to the loss in synaptic connectivity, because the enduring developmental phenotypes are only observed in the denervation-susceptible lumbral muscles. Nonetheless, the small delay in...
pre- and post-synaptic NMJ development observed in GarsC201R/+ TNV 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 transection 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.

MATERIALS AND METHODS

Animals

GarsC201R/+ mice and GarsNm1249/+ mice were maintained as heterozygote breeding pairs each on a predominantly C57BL/6 background as previously described (19,20,65). GarsC201R/+ 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. GarsNm1249/+ 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 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) paraformaldehyde (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 antimouse, 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, afferent nerve was 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 polyinnervation, 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 postsynaptic 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: Chrnε—forward 5′ GCA GCT TTT ACC GAG AAT GG 3′ and reverse 5′ CGT CAG TTT CTC CAG CAC 3′; Chrnβ—forward 5′ GGC AGA AAT GCA CAG TGG 3′ and reverse 5′ CAG GTA CTG GAT GAG TGG 3′; Gapdh—forward 5′ TGT GTC CGT CGT GGA TCT GA
3′ and reverse 5′ CCT GCT TCA 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: \((10^{(-1/slope)} - 1) \times 100\). 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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