Dominant, toxic gain-of-function mutations in gars lead to non-cell autonomous neuropathology

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Abstract
Charcot–Marie–Tooth (CMT) neuropathies are collectively the most common hereditary neurological condition and a major health burden for society. Dominant mutations in the gene GARS, encoding the ubiquitous enzyme, glycyl-tRNA synthetase (GlyRS), cause peripheral nerve degeneration and lead to CMT disease type 2D. This genetic disorder exemplifies a recurring motif in neurodegeneration, whereby mutations in essential, widely expressed genes have selective deleterious consequences for the nervous system. Here, using novel Drosophila models, we show a potential solution to this phenomenon. Ubiquitous expression of mutant GlyRS leads to motor deficits, progressive neuromuscular junction (NMJ) denervation and pre-synaptic build-up of mutant GlyRS. Intriguingly, neuronal toxicity is, at least in part, non-cell autonomous, as expression of mutant GlyRS in mesoderm or muscle alone results in similar pathology. This mutant GlyRS toxic gain-of-function, which is WHEP domain-dependent, coincides with abnormal NMJ assembly, leading to synaptic degeneration, and, ultimately, reduced viability. Our findings suggest that mutant GlyRS gains access to ectopic sub-compartments of the motor neuron, providing a possible explanation for the selective neuropathology caused by mutations in a widely expressed gene.

Introduction
Mutations in several aminoacyl-tRNA synthetase (ARS) genes have been linked to different forms of Charcot–Marie–Tooth (CMT) disease (1–6), a heterogeneous set of conditions characterized by progressive distal muscle wasting, weakness and sensory dysfunction (7). Dominant mutations in GARS (ENSG00000106105) cause CMT type 2D (CMT2D, OMIM ID 601472) (1). GARS encodes the non-redundant, homodimeric enzyme, glycyl-tRNA synthetase (GlyRS), which covalently links the amino acid glycine to its cognate tRNA, making it essential for protein translation fidelity. Two GARS translational start sites result in the production of mitochondrial and cytoplasmic GlyRS isoforms. GlyRS possesses three functional domains common to both isoforms: a highly conserved N-terminal WHEP-TRS domain of unclear function, a catalytic core, and a C-terminal anticodon-binding domain.

Two mouse models of CMT2D, Gars¹⁹⁴⁸/+, and Gars¹²⁰⁸/+, result from dominant amino acid substitutions and display features akin to the human condition, including muscle weakness and peripheral axon degeneration (8,9). Mouse phenotypes do not correlate with aminoacylation function (8–11), a 50%
reduction in Gars mRNA levels results in no overt phenotype (8), and GARS mutations have differential effects on GlyRS dimerization (11); however, defects in the canonical function of GlyRS have not been completely ruled out as a possible contributing factor in the human disease (12). Interestingly, neuromuscular junction (NMJ) maturation defects precede lower motor neuron connectivity abnormalities in mice, indicating that defective synapse development may play a role in pathology (13). Furthermore, overexpression of wild-type human GARS does not rescue CMT2D mice, suggesting a toxic gain-of-function of mutant GlyRS as the disease trigger (14). Consistent with this, a number of CMT2D-associated GARS mutations have been shown to cause the same conformational opening of the protein thereby exposing a consensus neomorphic area with potential for aberrant protein interactions (15).

The Drosophila melanogaster gars gene (also known as acts-gly) shares 60% homology with human GARS, and most patient mutations affect amino acids conserved in the mutations were shown to affect branching of olfactory bulb and type interactions, which may contribute to the pathology of cell autonomous neuronal toxicity of mutant GlyRS (18). We shares 60% homology with human GARS. Subsequent experiments in Drosophila have specifically looked at cell autonomous neuronal toxicity of mutant GlyRS (18). We now describe a new Drosophila model expressing a dominant disease allele that we use to identify novel features of the GlyRS toxic gain-of-function mechanism, and in particular inter-cell type interactions, which may contribute to the pathology of CMT2D.

Results
Non-neuronal gars$^{P234KY}$ expression leads to motor deficits and reduced lifespan

All CMT2D-associated mutations are located downstream of the S4 amino acid mitochondrial targeting sequence, clustering at the dimer interface (11,15,19,20). We therefore created UAS constructs to express Drosophila wild-type and mutant cytoplasmic, not mitochondrial, gars, focusing on the P234KY mutation—the equivalent Drosophila and human mutations corresponding to P278KY in the Gars$^{Nmp249/+}$ mouse. This residue is in ‘kissing contact’ with residues I280 and C201 mutated in human disease and the second gars mouse model, respectively (15).

To mimic the dominant GARS mutations that cause CMT2D, and to better understand the selective neuronal pathology seen in the disease, we expressed the cytoplasmic isoform of wild-type (UAS-gars$^{WT}$) which produces GlyRS$^{WT}$ and mutant (UAS-gars$^{P234KY}$) which produces GlyRS$^{P234KY}$ Drosophila gars transgenes using the UAS-GAL4 system (18). Two different constructs were used for both the wild-type (UAS-gars$^{WT}$-1 and UAS-gars$^{WT}$-2) and the mutant (UAS-gars$^{P234KY}$-1 and UAS-gars$^{P234KY}$-2) transgenes, with UAS-gars$^{WT}$-1 and UAS-gars$^{P234KY}$-1 being the same as those used by Ermanoska et al. (18), and UAS-gars$^{WT}$-2 and UAS-gars$^{P234KY}$-2 being novel to this work. UAS-gars$^{WT}$-1 and UAS-gars$^{P234KY}$-1 were previously shown to be expressed at equivalent levels and both proteins localized to the cytoplasm within the neuronal populations studied (18). Here, we firstly analysed the localization of the GlyRS proteins expressed from our new UAS-gars$^{WT}$-2 (Fig. 1A) and UAS-gars$^{P234KY}$-2 (Fig. 1B) constructs, when driven ubiquitously (1032-GAL4). Both GlyRS$^{WT}$ and GlyRS$^{P234KY}$ localized in a similar cytoplasmic diffuse pattern in the cell bodies of larval central nervous system neurons (Fig. 1A and B). Both proteins were also observed in the axonal bundles that contain afferent motor and sensory neurons ensheathed in glia (Fig. 1A and B), as well as the neuropil region of axons and dendrites, which was labelled with bruchpilot (Brp, Fig. 1A and B). We also found analogous localization patterns when UAS-gars$^{WT}$-1 and UAS-gars$^{P234KY}$-1 were used (data not shown), as previously described (18). As GlyRS was observed in the axonal bundles, we expressed both GlyRS$^{WT}$ and GlyRS$^{P234KY}$ using a neuronal-specific driver (elav-GAL4) to determine whether GlyRS reaches the most distal region of the motor neuron, the NMJ (Fig. 1C and D). Both GlyRS$^{WT}$ and GlyRS$^{P234KY}$ did not become enriched at the larval NMJ, suggesting that neurally expressed GlyRS does not reach the peripheral synapses of the neuromuscular system.

We next looked to identify any toxic effects of wild-type or mutant GlyRS. To this end, we analysed lethality and motor function deterioration, both of which are common features of Drosophila models of neurodegenerative diseases (21–23). Previous experiments have shown that ubiquitous expression of gars$^{P234KY}$ leads to very early lethality, whereas neuronal expression causes late pupal lethality (18). This suggests that, in addition to neuronal involvement, toxicity may manifest or be contributed from tissues beyond the nervous system. We therefore analysed tissue-specific lethality and motor function deterioration (Fig. 1E–I). To take advantage of temperature-dependent GAL4 activity (24) and thus express transgenes at different levels, we performed experiments at different temperatures; we cultured flies at both 20°C (Fig. 1E) and 25°C (Fig. 1F), which correspond to low and high transgene expression levels, respectively. Concurrent with the previous study, we demonstrated that ubiquitous expression of gars$^{P234KY}$-1 and gars$^{P234KY}$-2, using the moderately expressing 1032-GAL4 driver, leads to early lethality at both 20°C (Fig. 1E) and 25°C (Fig. 1F), with the majority of flies dying during the pupal stages (designated White Pupa and Pupa). Ubiquitous expression of both wild-type constructs (UAS-gars$^{WT}$-1 and UAS-gars$^{WT}$-2) had no overt effect when compared with control (1032-GAL4; UAS-CDS::GFP), at either temperature (Fig. 1E and F). Tissue-specific GlyRS$^{P234KY}$ toxicity was then analysed by expressing gars$^{P234KY}$ constructs in the mesoderm (how$^{2+48}$-GAL4, called how-GAL4 henceforth), muscle (MHC-GAL4) or neurons (elav-GAL4). Mesodermal and muscle expression of either gars$^{P234KY}$ construct reduced survival, with mesodermal expression leading to earlier lethality than muscle at both temperatures. Surprisingly, given the previous study (18), gars$^{P234KY}$ expression in the nervous system caused only a modest affect at 20°C, whereas leading to more pronounced lethality at 25°C (Fig. 1F). This result was confirmed using both versions on the UAS-gars$^{P234KY}$ transgene. This discrepancy is likely due to the different expression levels of the GAL4 promoters used in the two studies.

A number of flies ubiquitously expressing gars$^{P234KY}$ that reached the pupal stage at 25°C failed to eclose from the pupal case (Fig. 1G). In addition, the few successfully eclosed flies failed to expand their wings, mirroring defects that have been previously observed in classical neurodegenerative models that display axonal transport and neuronal viability defects (25,26).

We next looked at how the P234KY mutation affects larval motor function by assessing the peristaltic waves of muscle contraction (Fig. 1H and I). When larvae were reared at 20°C, reduced muscle contractions were detected upon ubiquitous, mesodermal and muscle expression of both GlyRS$^{P234KY}$-1 and GlyRS$^{P234KY}$-2 (Fig. 1H). At 25°C, when transgenes were expressed at higher levels, muscle contraction defects were also now observed upon neuronal expression of gars$^{P234KY}$, although not to the same extent as defects caused by mesodermal and muscle expression.
These results suggest that multiple tissues may be involved in the selective neuropathy caused by mutant GlyRS.

To confirm the specificity of the toxicity to mutant gars, we tested an additional, CMT2D-associated GlyRS mutant in the muscular system. Importantly, the second gars mutation, G240R, was shown to exert the same pathological effects as P234KY when expressed ubiquitously, in the mesoderm, or in muscle (Supplementary Material, Fig. S1A).

These findings reveal a clear dominant, toxic gain-of-function for mutant but not wild-type, GlyRS that is mediated, in part, through tissues beyond the nervous system. Moreover, the severity of pathology was modulated by temperature, suggesting the importance of mutant GlyRS levels. In this study, we now aim to characterize these non-cell autonomous defects to understand how mutant gars expressed in non-neuronal cell types may contribute to the selective deterioration of the nervous system.

**Figure 1.** Dominant, toxic effects caused by GlyRS <sup>P234KY</sup> lead to non-cell autonomous longevity and motor defects. (A) GlyRS<sup>WT</sup> and (B) GlyRS<sup>P234KY</sup> localization in the central nervous system of larvae ubiquitously (1032-GAL4) expressing UAS-gars<sup>Wt</sup>_2 and UAS-gars<sup>P234KY</sup>_2, respectively. (C) GlyRS<sup>WT</sup> and (D) GlyRS<sup>P234KY</sup> localization at the NMJ in larvae neuronally expressing UAS-gars<sup>Wt</sup>_2 and UAS-gars<sup>P234KY</sup>_2, respectively. Note that GlyRS expressed in neurons does not reach the peripheral synapse. (E and F) GlyRS<sup>P234KY</sup> toxicity was analysed by expressing gars<sup>P234KY</sup> ubiquitously (1032-GAL4), in mesoderm (hsc-GAL4), muscle (MHC-GAL4) or neurons (elav-GAL4) at 20°C (E) and 25°C (F). Transgene expression is lower at 20 than 25°C. Flies ubiquitously expressing gars<sup>WT</sup> (blue bars) and CD8::GFP (red bars) were used as controls. (G) Mutant gars<sup>P234KY</sup> flies reach the pupal stage, but fail to eclose and often become trapped in their pupal cases (arrow). The few flies that escape and reach adulthood display wing expansion defects (arrow head). (H) Ubiquitous, mesodermal and muscle expression of gars<sup>P234KY</sup> caused a significant loss of muscle contractions at 20°C. (I) Ubiquitous, mesodermal, muscle and neuronal driven gars<sup>P234KY</sup> expression also reduced larval muscle contractions at 25°C. For all graphs, *P < 0.05; **P < 0.01; ***P < 0.001 Dunn’s multiple comparison test. For survival studies, 25 flies per genotype were scored over four independent experiments. For muscle contraction studies, at least 15 larvae were analysed per genotype. Scale bars = 10 μm.
circumstantial synaptic specializations called boutons, which form structures like beads on a string, where both pre- and post-synaptic regions closely overlap (Fig. 2A). Larvae ubiquitously expressing *gars*Δ*WHEP-P234KY* displayed reduced bouton number and increased bouton size when compared with wild-type and *gars*WT-overexpressing flies (Fig. 2B and C). No gross changes in muscle size or structure were observed in any of the genotypes analysed (Supplementary Material, Fig. S2). Again, tissue-specific GlyRSΔ*WHEP* toxicity was analysed by expressing *gars*Δ*WHEP-P234KY* in mesoderm (how-GAL4), muscle (MHC-GAL4) or neurons (elav-GAL4), whereas flies ubiquitously expressing *gars*WT were used as controls. Bouton number defects were also observed in larvae with mesoderm and muscle-driven GlyRSΔ*WHEP*, whereas neuronal *gars*Δ*WHEP-P234KY* expression caused no overt synaptic phenotype (Fig. 2B and C).

**Muscle *gars*P234KY expression causes early denervation defects**

As the synaptic structure was affected in *gars* mutant flies, we next looked to see if axonal and synaptic degenerative defects presented at the late L3 larval stage. CMT2D patients display muscle weakness and axonal degeneration (27). In Drosophila, denervation of the synapse can be analysed by detecting the loss or reduction of pre-synaptic antigens such as the active zone protein Brp (28). In ubiquitous, mesodermal and muscle *gars*P234KY-expressing larvae, we found synaptic Brp intensity was reduced compared with control (Fig. 3A and C). This was not seen when *gars*WT was overexpressed (Fig. 3A and C). It is worth noting that HRP immunoreactivity was reduced (without detergent) (Fig. 4). The relative florescence of GlyRS at the synapse was measured and normalized to the background intensity (Fig. 4G). When *gars*P234KY was ubiquitously expressed, GlyRSΔ*WHEP-P234KY* was found to broadly cover the neuromuscular synapse in puncta that were mainly closely associated with the HRP-labelled pre-synaptic membrane (Fig. 4A and B). Signal was not detectable in controls or when *gars*WT was expressed (Fig. 4C). This was again seen when GlyRSΔ*WHEP-P234KY* was driven independently in the mesoderm (Fig. 4D) or in the muscle (Fig. 4E). These experiments suggest that GlyRSΔ*WHEP*, but not GlyRSWT, is secreted into the extracellular milieu at the NMJ and is able to localize to the outside of the pre-synaptic membrane. In addition, neuron-expressed GlyRSΔ*WHEP-P234KY* (elav-GAL4) did not appear at the pre-synapse (Fig. 4F), suggesting that the synaptic build-up of mutant GlyRS is due to a non-cell autonomous mechanism. Ubiquitous, mesodermal and muscle-expressed mutant GlyRS localized to the NMJ when using both UAS-*gars*Δ*WHEP-P234KY* and UAS-*gars*Δ*WHEP-P234KY* constructs (Fig. 4G), whereas muscle-expressed GlyRSWT did not (Supplementary Material, Fig. S1B).

**GlyRS toxicity and synapse binding, but not secretion, are dependent on the WHEP domain**

GlyRS was recently shown to be secreted by macrophages and to be present in human and mouse serum, serving as a defence against ERK-activated tumour formation (31). Several other ARS proteins are also known to be secreted (32). The capacity of mesoderm- and muscle-expressed GlyRSΔ*P234KY*, through secretion, to exert non-cell autonomous effects on the nervous system appears to be an integral component to the pathology we have observed. A candidate determinant for GlyRS secretion is the N-terminal WHEP domain, which currently has no known function in GlyRS and is dispensable for aminoacylation activity (20). We therefore investigated the importance of the WHEP domain for GlyRS toxicity by generating a WHEP-deletion fly. Intriguingly, removal of the WHEP domain from GlyRSΔ*P234KY* (GlyRSΔ*WHEP-P234KY*) abrogated the fly viability (Fig. 5A) and NMJ defects (data not shown) caused by GlyRSΔ*P234KY* (Fig. 5A). Furthermore, build-up of GlyRSΔ*WHEP-P234KY* was not found at the NMJ, suggesting that synaptic mutant GlyRS accumulation is integral to pathology (Fig. 5B). To show that our transgenes were successfully expressed and to assess protein localization, we used UAS ‘flip-out’ clones expressing UAS-*gars*Δ*P234KY* and UAS-*gars*Δ*WHEP-P234KY*. Both GlyRSΔ*P234KY* and GlyRSΔ*WHEP-P234KY* were expressed and localized in the cytoplasm of disc clones (Supplementary Material, Fig. S3A), indicating that deletion of the WHEP domain is unlikely to affect protein stability. This is corroborated by previous observations that deletion of the WHEP domain does not affect the
canonical function of GlyRS and does not prevent the crystallization of the truncated protein (20). Finally, to determine whether WHEP deletion restricts GlyRS secretion and is the principal cause of the abolished mutant pathology, we expressed GARSWT and GARS234KY with and without the WHEP domain in human kidney (HEK293) and muscle-like (TE671) cells. Contrary to our starting hypothesis that the WHEP domain may be a determinant of secretion, we found that its deletion did not affect secretion.

Figure 3. garsP234KY larvae display NMJ denervation and axonal defects. (A) Representative NMJs from late L3 larvae ubiquitously (1032-GAL4) expressing garsWT and garsP234KY. (B) Representative axonal segment pictures taken of the transverse nerve (TN) over muscles 6 and 7 in abdominal segment A2 in late L3 control, garsWT (1032-GAL4), garsP234KY (1032-GAL4) and garsP234KY mesodermal (how-GAL4) larvae. (C) Quantification of the normalized Brp fluorescence at the NMJ showing ubiquitous, mesodermal and muscle garsP234KY expression leads to reduced Brp signal. (D) Quantification of the number of Brp-positive foci in the TN showing that ubiquitous, mesodermal and muscle garsP234KY expression leads to increased Brp accumulation. For all graphs, *P < 0.05, **P < 0.01, ***P < 0.001 Bonferroni’s/Dunn’s multiple comparison test. At least 20 NMJs and axons were analysed per genotype. Scale bars = 10 µm.
of eGFP- or V5-tagged versions of the protein (Fig. 5C, Supplementary Material, Fig. S4A). To confirm that the secretion of GlyRS proteins was not simply a product of the overexpression system, we demonstrated that eGFP-tagged survival motor neuron (Smn) protein is not secreted under similar conditions in both HEK293 (Supplementary Material, Fig. S4B) and TE671 cells (data not shown). The higher expression of Smn-eGFP than GlyRS-eGFP makes this result all the more convincing. Smn was chosen as a control, because, like GlyRS, it is expressed both in the nucleus and cytoplasm (33), it has been identified in axonal projections (10,34), and its dysfunction is associated with a disorder that affects the lower motor neurons (spinal muscular atrophy) (34,35). The similar subcellular localization patterns of eGFP-tagged GlyRS and Smn were confirmed by immunofluorescence (Supplementary Material, Fig. S4C). Altogether, this work indicates that the GlyRS WHEP domain, although not required for secretion, nevertheless is integral to pre-synaptic accumulation of muscle-expressed mutant GlyRS.

Discussion

This work reveals a novel mechanism for the pathology associated with the peripheral neuropathy CMT2D, and identifies a complex series of events leading to the characteristic selective neuromuscular toxicity. CMT2D, like many other neuromuscular diseases, is caused by mutations in a widely and constitutively required gene, yet pathology is strictly limited to the neuromuscular system. Despite having identified the disease-causing genes for many motor neuropathies, there remains a dearth of information on the molecular mechanisms underlying selective motor neuron vulnerability (36,37). Here, we show two unexpected acquired functions of mutant GlyRS in a Drosophila model, wherein ubiquitous expression of mutant gars leads to specific, progressive NMJ degeneration. First, we show that pathology has a non-cell autonomous contribution—mutant GlyRS exerts significant neuronal defects when expressed just in muscle (Figs 1–4). This non-neuronally derived mutant GlyRS causes a loss of motility (Fig. 1H and I), synaptic degeneration (Figs 2B, C and 3A, C), and, ultimately, a reduction in lifespan (Fig. 1E and F). Second, we find mutant GlyRS associated with the neuronal membrane at the synapse when expressed ubiquitously or in muscle, but not when expressed in the neuron (Fig. 4). Wild-type GlyRS was not observed to accumulate at the NMJ; however, we show that both wild-type and mutant GlyRS proteins are secreted (Fig. 5). This suggests that CMT2D-associated mutations in GlyRS, including P234KY, may cause the selective neuropathy by either (i) a toxic gain of neuronal binding function or (ii) a notable increase in neuronal binding affinity. It has been shown that tRNA synthetases, including GlyRS, are secreted and have non-canonical functions (12,31). For example, GlyRS is secreted by macrophages in response to serum-circulating FAS ligand (31), whereas tryptophanyl-tRNA synthetase (WARS) is secreted by vascular endothelial cells (12). It is worth noting that muscle cells are required to secrete a number of factors to maintain
synapse integrity and growth (38,39). It is therefore possible that wild-type GlyRS possesses a second non-canonical function specific to the NMJ that is dependent upon sub-synaptic secretion. Mutant gars expression leads to synaptic maturation abnormalities that present as decreased bouton number and increased bouton size (Fig. 2). Although not yet observed in patients, NMJ maturation defects precede lower motor neuron degeneration in Gars mice (13), indicating that defective synapse development may play an important role in pathology. In addition to the synaptic developmental defects, we observed a potential deficit in axonal transport (Fig. 3B and D). Although not yet assessed in CMT2D, axonal transport defects have been reported in a range of CMT2 models (10,35–42) and may play a part in numerous other disorders affecting the peripheral nerves (43–48). We show that mutant gars expression causes regions of the synapse to degenerate at late larval stages, as identified by parts of the NMJ lacking post-synaptic anchoring (Figs 2 and 3).

Our study demonstrates a potential role for non-cell autonomous mechanisms in CMT2D, a topic that has recently gathered interest in a number of other nervous system disorders, including the motor neuron disease amyotrophic lateral sclerosis (11). Previous work has shown that mutant GlyRS causes cell autonomous neuronal pathology in a Drosophila model (18). In that study, overexpression of mutant gars exclusively in neurons resulted in both electrophysiological and morphological defects. It is likely that both neuronal and non-neuronal mechanisms contribute to CMT2D neuropathology; and it will therefore be of great interest to analyse the interaction between these modular pathological mechanisms in future studies.

Throughout evolution, ARSs have acquired domains and motifs that are not required for the canonical aminoacylation function of the proteins (49). One such domain is the helix–turn–helix motif known as the WHEP domain, which has been shown to be involved in several non-canonical ARS functions (50,51). For example, removal of the WARS WHEP domain promotes angiostatic growth through interactions with VE-cadherin (51), whereas a Glu–Pro dual activity tRNA synthetase (52) translationally represses a number of mRNAs by WHEP domain-mediated binding to transcript 3′UTRs (20,24). We show that removal of the WHEP domain from GlyRSP234KY prevents the build-up of mutant protein at the NMJ and abrogates the longevity defects (Fig. 5A and B), suggesting that the WHEP domain is required for synaptic mutant GlyRS accumulation and toxicity. As WHEP domain removal did not affect secretion in vitro (Fig. 5C, Supplementary Material, Fig. S4), this suggests that the domain perhaps determines binding affinity of mutant GlyRS to the pre-synapse.

In summary, we show that, mediated by the WHEP domain, mutant GlyRS escapes muscle cells to accumulate at the pre-synapse.
synaptic membrane, before internalization into ectopic regions of the motor neuron. In parallel, we see synaptic maturation defects that ultimately lead to NMJ degeneration, similar to what is observed in mice (13), and progressive neuromuscular and survival defects. Our findings provide novel insight into the selective effect on the neuromuscular system of mutations in a widely expressed protein.

**Materials and Methods**

**General methods**

Reagents were obtained from Sigma-Aldrich unless otherwise stated. All Drosophila stocks were cultured on standard molasses/maize meal and agar medium in plastic vials or bottles at 25°C. Coding sequences of D. garsWT, garsP234K, garsG240R and garsIWHEP-P234K were amplified using primers containing KpnI sites, sub-cloned into pUAST and injected into embryos. We wish to thank the following for stocks/reagents: 1032–GAL4, ActSC–GAL4, elav–GAL4 and hour–GAL4 (BDSC, Indiana University), BG487–GAL4 and C57–GAL4 (Vivian Budnik), nrv2–GAL4 (Paul Salavaterra), 69B–GAL4 (Clive Wilson), and UAS–garsWT.1 and UAS–garsP234K.2 (Albena Jordanova).

**Drosophila behavioural assays**

Adult viability assays were conducted by crossing GAL4 driver stocks to lines harbouring gars overexpression or to w1118 for controls. First instar larvae for each genotype were counted and placed on apple juice plates with yeast, and the development of the larvae was noted (53). Fresh yeast was added daily. Survivors were counted each day. Twenty-five flies per genotype were scored over four independent experiments. Measurement of motor function involved placing individual age-matched larvae scored over four independent experiments. Measurement of background over the whole synapse (marked by HRP staining), again using op-ting at the synapse, average fluo-rescence intensity in the muscle. Boutons, which were identified by intense DLG staining, were measured per NMJ. All digital analyses and fluorescence measurements were performed using ImageJ. For BRP and HA staining at the synapse, average fluorescence intensity was analysed over the whole synapse (marked by HRP staining), again using optical sections of 0.2 μm. For HA staining, this was normalized to the background fluorescence intensity in the muscle.

**Clonal analysis**

All UAS ‘flip-out’ clones were generated using the inducible driver: HsFLP, UAS–GFPnls UAS–Dcr2; tub>Gal80–GAL4/SMS, CyO-TM6, Tb. Virgin females of this genotype were crossed to males with UAS-shRNA or UAS–ORFs for clonal knockdown or overexpression, respectively. Progeny were heat-shocked at 24 h for imaginal tissue. Larvae were aged until third instar and non-TM6B (non-tubby) animals were selected. GAL4-expressing clones were identified by the presence of nuclear GFP marker.

**Cell culture**

Human HEK293 (human embryonic kidney) and TE671 (muscle-like) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/l d-glucose and pyruvate (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen). Cells were kept at 37°C in a 5% (v/v) CO₂ humidified atmosphere and sub-cultured every 4–6 days when 70–90% confluent.

**Cell secretion assays and western blotting**

HEK293 cells were plated at 2.5 × 10⁵ cells per well on poly-D-lysine (BD Biosciences), and TE671 cells were plated at 1.0 × 10⁵ on Geltrex (Invitrogen). 6 ± 0.5 h post-plating, HEK293 cells were transfected with 500 ng DNA using 1 μl Lipofectamine 2000 Transfection Reagent (Invitrogen), as previously described (43). TE671 cells were transfected/magnetofected with 1 μg DNA using a combination of 1 μl Lipofectamine 2000 and 0.5 μl NeuroMag beads (Oz Biosciences), with an initial incubation step of 20 min at 37°C on a magnetic plate (Oz Biosciences). At 15–18 h later, cell media was discarded and the cells carefully washed twice with 0.2 ml pre-warmed phosphate-buffered saline. Cells were then incubated at 37°C for 6 h in 0.5 ml serum-free DMEM. The media from 4–6 wells/condition were spun at 1500 × g for 4 min and the supernatant re-spun at 4000 × g for 10 min. The media were then concentrated using VivAspin columns (MWCO 10 kDa, Sartorius) at 4000 × g for 18–28 min. A 1.5% (v/v) protease inhibitor cocktail was added to the concentrated media before cell protein extraction and western blotting, as previously described (43). The following antibodies were used for western blotting: anti-β-actin (Cell Signaling Technology, 8H10D10, 1/5000), anti–β-tubulin (Abcam, ab15568, 1/5000), anti–HA (Roche, 1-867-423, 1/1000), anti-GFP (Abcam, ab290, 1/5000) and anti-V5 (Invitrogen, R960-25, 1/5000). Cells were stained with anti-GFP at 1/1000 as previously described (43).

**DNA vector creation**

The cytoplasmic isoform of human GARS was cloned into the pcDNA6.2/C-EmGFP-DEST and pcDNA6.2/V5-DEST vectors (Invitrogen) to express GlyR5 with a C-terminal eGFP or V5 tag, respectively. Smn with a C-terminal eGFP tag was expressed from pcEGFP-N1 (43). To create the GARS P234K and ΔWHEP (residues 71–126) mutation constructs, a Q5 Site-Directed Mutagenesis Kit (New England Biolabs) was used, as per the manufacturer’s instructions, with the following primers: WT-P234KY_F 5’–TTT CAT TGG GAA ATA TGG AGG AAA CAT GC_3’ , WT-P234KY_R 5’–GTC TTT AAC ATT AAG TTA AAA GAC_3’ , ddWHEP_F 5’–ACC CGT AAG AGG AGG TTT TTC_3’ and ddWHEP_R 5’–TGC TAG CCT CAG AGG TGC_3’.

**Statistical analysis**

When normally distributed, datasets were statistically analysed using either an unpaired t-test with Welch’s correction or a one-way analysis of variance with Bonferroni’s multiple comparison test. If the data did not pass normality testing, the non-parametric Mann–Whitney U test or Kruskal–Wallis test was used.
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 unless otherwise stated.

**Supplementary Material**
Supplementary Material is available at HMG online.

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**References**
1. Antonellis, A., Ellsworth, R.E., Sambuughin, N., Puls, I., Abel, A., Lee-Lin, S.Q., Jordanova, A., Kremensky, I., Christodoulou, K., Middleton, L.T. et al. (2003) Glycyl tRNA synthetase mutations in Charcot-Marie-Tooth disease type 2D and distal spinal muscular atrophy type V. *Am. J. Hum. Genet.*, 72, 1293–1299.
2. Jordanova, A., Irobi, J., Thomas, F.P., Van Dijck, P., Meerschaert, K., Dewil, M., Dierick, I., Jacobs, A., De Vriendt, E., Guerguetitcheva, V. et al. (2006) Disrupted function and axonal distribution of mutant tyrosyl-tRNA synthetase in dominant intermediate Charcot-Marie-Tooth neuropathy. *Nat. Genet.*, 38, 197–202.
3. Latour, P., Thauvin-Robinet, C., Baudelet-Mery, C., Soichot, P., Cusin, V., Faivre, L., Locatelli, M.C., Mayencon, M., Sarcey, A., Broussolle, E. et al. (2010) A major determinant for binding and aminocacylation of tRNA(Ala) in cytoplasmic Alanyl-tRNA synthetase is mutated in dominant axonal Charcot-Marie-Tooth disease. *Am. J. Hum. Genet.*, 86, 77–82.
4. McLaughlin, H.M., Sakaguchi, R., Liu, C., Igarashi, T., Pehlivan, D., Chu, K., Iyer, R., Cruz, P., Cherukuri, P.F., Hansen, N.F. et al. (2010) Compound heterozygosity for loss-of-function lysyl-tRNA synthetase mutations in a patient with peripheral neuropathy. *Am. J. Hum. Genet.*, 87, 560–566.
5. Gonzalez, M., McLaughlin, H., Houliden, H., Guo, M., Yo-Tsen, L., Hadjivasiliou, M., Speziani, F., Yang, X.L., Antonellis, A., Reilly, M.M. et al. (2013) Exome sequencing identifies a significant variant in methionyl-tRNA synthetase (MARS) in a family with late-onset CMT2J. *Neurol. Neurosurg. Psychiatry*, 84, 1247–1249.
6. Vester, A., Velez-Ruiz, G., McLaughlin, H.M., Lupski, J.R., Talbot, K., Vance, J.M., Zuchner, S., Roda, R.H., Fischbeck, K.H., Biesecker, L.G. et al. (2013) A loss-of-function variant in the human histidyl-tRNA synthetase (HARS) gene is neurotrophic in vivo. *Hum. Mutat.*, 34, 191–199.
7. Reilly, M.M., Murphy, S.M. and Laura, M. (2011) Charcot-Marie-Tooth disease. *J. Periphr. Nerv. Syst.*, 16, 1–14.
8. Seburn, K.L., Nangle, L.A., Cox, G.A., Schimmel, P. and Burgess, R.W. (2006) An active dominant mutation of glycyl-tRNA synthetase causes neuropathy in a Charcot-Marie-Tooth 2D mouse model. *Neuron*, 51, 715–726.
9. Achilli, F., Bros-Facer, V., Williams, H.P., Banks, G.T., AlQatari, M., Chia, R., Tucci, V., Groves, M., Nickols, C.D., Seburn, K.L. et al. (2009) An ENU-induced mutation in mouse glycyl-tRNA synthetase (GARS) causes peripheral sensory and motor phenotypes creating a model of Charcot-Marie-Tooth type 2D peripheral neuropathy. *Dis. Model. Mech.*, 2, 359–373.
10. Stum, M., McLaughlin, H.M., Kleinbrink, E.L., Miers, K.E., Ackerman, S.L., Seburn, K.L., Antonellis, A. and Burgess, R.W. (2011) An assessment of mechanisms underlying peripheral axonal degeneration caused by aminocyl-tRNA synthetase mutations. *Mol. Cell. Neurosci.*, 46, 432–443.
11. Nangle, L.A., Zhang, W., Xie, W., Yang, X.L. and Schimmel, P. (2007) Charcot-Marie-Tooth disease–associated mutant tRNA synthetases linked to altered dimer interface and neurite distribution defect. *Proc. Natl Acad. Sci. U.S.A.*, 104, 11239–11244.
12. Griffin, L.B., Sakaguchi, R., McGuigan, D., Gonzalez, M.A., Searby, C., Zuchner, S., Hou, Y.M. and Antonellis, A. (2014) Impaired function is a common feature of neuropathy-associated glycyl-tRNA synthetase mutations. *Hum. Mutat.*, 11, 1363–1371.
13. Sleigh, J.N., Grice, S.J., Burgess, R.W., Talbot, K. and Cader, M.Z. (2014) Neuromuscular junction maturation defects precede impaired lower motor neuron connectivity in Charcot-Marie-Tooth type 2D mice. *Hum. Mol. Genet.*, 23, 2639–2650.
14. Motley, W.W., Seburn, K.L., Nawaz, M.H., Miers, K.E., Cheng, J., Antonellis, A., Green, E.D., Talbot, K., Yang, X.L., Fischbeck, K.H. et al. (2011) Charcot-Marie-Tooth-linked mutant GARS is toxic to peripheral neurons independent of wild-type GARS levels. *PloS Genet.*, 7, e1002399.
15. He, W., Zhang, H.M., Cheong, Y.E., Guo, M., Marshall, A.G. and Yang, X.L. (2011) Dispersed disease-causing neomorphic mutations in a single protein promote the same localized conformational opening. *Proc. Natl Acad. Sci. U.S.A.*, 108, 12307–12312.
16. Motley, W.W., Talbot, K. and Fischbeck, K.H. (2010) GARS axonopathy: not every neuron’s cup of tRNA. *Trends Neurosci.*, 33, 59–66.
17. Chihara, T., Luginbuhl, D. and Luo, L. (2007) Cytoplasmic and mitochondrial protein translation in axonal and dendritic terminal arborization. *Nat. Neurosci.*, 10, 828–837.
18. Ermanoska, B., Motley, W.W., Leita-Goncalves, R., Asselbergh, B., Lee, L.H., De Rijk, P., Sleeers, K., Ooms, T., Godenschwege, T.A., Timmerman, V. et al. (2014) CMT-associated mutations in glycyl- and tyrosyl-tRNA synthetases exhibit similar pattern of toxicity and share common genetic modifiers in Drosophila. *Neurobiol. Dis.*, 68, 180–189.
19. Cader, M.Z., Ren, J., James, P.A., Bird, L.E., Talbot, K. and Stammers, D.K. (2007) Crystal structure of human wildtype and S581L-mutant glycyl-tRNA synthetase, an enzyme underly- ing distal spinal muscular atrophy. *FEBS Lett.*, 581, 2959–2964.
20. Xie, W., Nangle, L.A., Zhang, W., Schimmel, P. and Yang, X.L. (2007) Long-range structural effects of a Charcot-Marie-Tooth...
On 30 July 2018

25. Fossgreen, A., Bruckner, B., Czech, C., Masters, C.L., Gunawardena, S. and Goldstein, L.S. (2001) Disruption of flightless models of neuromuscular disease. Ann. NY Acad. Sci., 1184, e1–20.

24. Duffy, J.B. (2002) GAL4 system in Drosophila: a fly geneticist’s Swiss army knife. Genesis, 34, 1–15.

23. Lloyd, T.E. and Taylor, J.P. (2010) Flightless models of neuromuscular disease. Human Molecular Genetics, 2015, Vol. 24, No. 15

22. Grice, S.J., Sleight, J.N., Liu, J.L. and Sattelle, D.B. (2011) Invertebrate models of spinal muscular atrophy: insights into mechanisms and potential therapeutics. BioEssays, 33, 956–965.

21. Ambegaokar, S.S., Roy, B. and Jackson, G.R. (2010) Neurodegenerative models in Drosophila: polyglutamine disorders, Parkinson disease, and amyotrophic lateral sclerosis. Neurobiol. Dis., 40, 29–39.

20. Beggs, J.L., Zamba-Papanicolaou, E., Gottlieb, B., Antonellis, A., Sambuughin, N., Christodoulou, K., Davis, G.W. (2011) Glial-derived prodegenerative signaling functions. Proc. Natl Acad. Sci. U.S.A., 107, 20525–20528.

19. Harris, M.B., Ori-McKenney, K.M., Scoto, M., Tuck, E.P., Bell, S., Ma, D., Masl, A., Allred, P., Al-Lozi, M., Reilly, M.M. et al. (2012) Mutations in the tail domain of DYN1C1 cause dominant spinal muscular atrophy. Mn. Med., 968–974.

18. Korkut, C., Li, Y., Koles, K., Brewer, C., Ashley, J., Yoshihara, M. and Budnik, V. (2013) Regulation of postsynaptic retrograde signaling by presynaptic exosome release. Aueron, 77, 1039–1046.

17. Zhang, K., Fishel Ben Kenan, R., Osakada, Y., Xu, W., Sinit, R., Chen, L., Zhao, X., Chen, J.Y., Cui, B. and Wu, C. (2013) Defective axonal transport of Rab7 GTPase results in dysregulated trophic signaling. J. Neurosci., 33, 7451–7462.

16. Misko, A., Jiang, S., Wexorzewiska, I., Milbrandt, J. and Baloh, R.H. (2010) Mitofusin 2 is necessary for transport of axonal mitochondria and interacts with the Miro/Milton complex. J. Neurosci., 30, 4232–4240.

15. d’Ydewalle, C., Krishnan, J., Chiheb, D.M., Van Damme, P., Irobi, J., Kozikowski, A.P., Vanden Berghe, P., Timmerman, V., Robberecht, W. and Van Den Bosch, L. (2011) HDAC6 inhibitors reverse axonal loss in a mouse model of mutant HSPB1-induced Charcot-Marie-Tooth disease. Nat. Med., 17, 968–974.

14. Sasaki, S., Maruyama, S., Yamane, K., Sakuma, H. and Takeishi, M. (1989) Swellings of proximal axons in a case of motor neuron disease. Ann. Neurol., 25, 520–522.

13. Billsland, L.G., Sahai, E., Kelly, G., Golding, M., Greensmith, L. and Schiavo, G. (2010) Deficits in axonal transport precede ALS symptoms in vivo. Proc Natl Acad Sci. U.S.A., 107, 20525–20528.

12. Lo, W.S., Gardiner, E., Xu, Z., Lau, C.F., Wang, F., Zhou, J.J., Mendle, J.D., Nangle, L.A., Chiang, K.P., Yang, X.L. et al. (2014) Human tRNA synthetase catalytic nulls with diverse functions. Science, 345, 328–332.

11. Lefebvre, S., Burglen, L., Reboulet, S., Clermont, O., Burlet, P., Viollet, L., Benchou, B., Cruaud, C., Millasseau, P., Zeviani, M. et al. (1995) Identification and characterization of a spinal muscular atrophy-determining gene. Cell, 80, 155–165.

10. Sleight, J.N., Gillingwater, T.H. and Talbot, K. (2011) The contribution of mouse models to understanding the pathogenesis of spinal muscular atrophy. Dis. Model. Mech., 4, 457–467.

9. Bouhy, D. and Timmerman, V. (2013) Animal models and therapeutic prospects for Charcot-Marie-Tooth disease. Annu. Rev. Neurosci., 36, 371–393.

8. Pedersen, B.K. (2013) Muscle as a secretory organ. Compr. Physiol., 3, 1337–1362.

7. Korkut, C., Li, Y., Koles, K., Brewer, C., Ashley, J., Yoshihara, M. and Budnik, V. (2013) Regulation of postsynaptic retrograde signaling by presynaptic exosome release. Neuron, 77, 1039–1046.

6. Zhang, K., Fishel Ben Kenan, R., Osakada, Y., Xu, W., Sinit, R., Chen, L., Zhao, X., Chen, J.Y., Cui, B. and Wu, C. (2013) Defective axonal transport of Rab7 GTPase results in dysregulated trophic signaling. J. Neurosci., 33, 7451–7462.

5. Misko, A., Jiang, S., Wexorzewiska, I., Milbrandt, J. and Baloh, R.H. (2010) Mitofusin 2 is necessary for transport of axonal mitochondria and interacts with the Miro/Milton complex. J. Neurosci., 30, 4232–4240.

4. d’Ydewalle, C., Krishnan, J., Chiheb, D.M., Van Damme, P., Irobi, J., Kozikowski, A.P., Vanden Berghe, P., Timmerman, V., Robberecht, W. and Van Den Bosch, L. (2011) HDAC6 inhibitors reverse axonal loss in a mouse model of mutant HSPB1-induced Charcot-Marie-Tooth disease. Nat. Med., 17, 968–974.

3. Sasaki, S., Maruyama, S., Yamane, K., Sakuma, H. and Takeishi, M. (1989) Swellings of proximal axons in a case of motor neuron disease. Ann. Neurol., 25, 520–522.

2. Billsland, L.G., Sahai, E., Kelly, G., Golding, M., Greensmith, L. and Schiavo, G. (2010) Deficits in axonal transport precede ALS symptoms in vivo. Proc Natl Acad Sci. U.S.A., 107, 20525–20528.

1. Lo, W.S., Gardiner, E., Xu, Z., Lau, C.F., Wang, F., Zhou, J.J., Mendle, J.D., Nangle, L.A., Chiang, K.P., Yang, X.L. et al. (2014) Human tRNA synthetase catalytic nulls with diverse functions. Science, 345, 328–332.

0. Guo, M. and Schimmell, P. (2013) Essential nontranslational functions of tRNA synthetases. Nat. Chem. Biol., 9, 145–153.

- 1. Tzima, E., Reader, J.S., Irani-Tehrani, M., Ewalt, K.L., Schwartz, M.A. and Schimmell, P. (2005) VE-cadherin links tRNA synthetase cytokine to anti-angiogenic function. J. Biol. Chem., 280, 2405–2408.

- 2. Klueg, K.M., Aldor, D., Muskavitch, M.A. and Duffy, J.B. (2002) Creation of a GAL4/UAS-coupled inducible gene expression system for use in Drosophila cultured cell lines. Genesis, 34, 119–122.

- 3. Grice, S.J. and Liu, J.L. (2011) Survival motor neuron protein regulates stem cell division, proliferation, and differentiation in Drosophila. PLoS Genet., 7, e1002030.

- 4. Brent, J.R., Werner, K.M. and McCabe, B.D. (2009) Drosophila larval NMJ dissection. J. Vis. Exp., 24, e1107.

- 5. Grice, S.J., Liu, J.L. and Webber, C. (2015) Synergistic interactions between Drosophila orthologues of genes spanned by de novo human CNVs support multiple-hit models of autism. PLoS Genet., 11, e1004998.