Impaired protein translation in Drosophila models for Charcot–Marie–Tooth neuropathy caused by mutant tRNA synthetases

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Dominant mutations in five tRNA synthetases cause Charcot–Marie–Tooth (CMT) neuropathy, suggesting that altered aminoacylation function underlies the disease. However, previous studies showed that loss of aminoacylation activity is not required to cause CMT. Here we present a Drosophila model for CMT with mutations in glycyl-tRNA synthetase (GARS). Expression of three CMT-mutant GARS proteins induces defects in motor performance and motor and sensory neuron morphology, and shortens lifespan. Mutant GARS proteins display normal subcellular localization but markedly reduce global protein synthesis in motor and sensory neurons, or when ubiquitously expressed in adults, as revealed by FUNCAT and BONCAT. Translational slowdown is not attributable to altered tRNA<sub>Gly</sub> aminoacylation, and cannot be rescued by Drosophila Gars overexpression, indicating a gain-of-toxic-function mechanism. Expression of CMT-mutant tyrosyl-tRNA synthetase also impairs translation, suggesting a common pathogenic mechanism. Finally, genetic reduction of translation is sufficient to induce CMT-like phenotypes, indicating a causal contribution of translational slowdown to CMT.
With an estimated prevalence of 1 in 2,500, Charcot–Marie–Tooth (CMT) peripheral neuropathy is the most common inherited neuromuscular disease. CMT is characterized by progressive distal muscle weakness and wasting, sensory loss, decreased reflexes and foot deformities. These classical symptoms are caused by ‘dying-back’ degeneration of peripheral motor and sensory axons. Traditionally, demyelinating and axonal forms of CMT are distinguished. Demyelinating CMT is characterized by reduced motor nerve conduction velocities (NCVs) due to demyelination. Axonal CMT is characterized by normal or mildly slowed NCVs, reduced compound muscle action potential amplitudes and chronic axonal degeneration and regeneration. More recently, intermediate forms of CMT, characterized by intermediate NCVs and features of both demyelination and axonal degeneration, have been recognized. CMT is not only clinically but also genetically heterogeneous, with >30 causative genes.

Remarkably, dominant mutations in five distinct transfer RNA (tRNA) synthetases give rise to axonal and intermediate forms of CMT: glycyl-tRNA synthetase (GARS), tyrosyl-tRNA synthetase (YARS), alanyl-tRNA synthetase (AARS) and possibly also histidyl-tRNA synthetase (HARS) and methionyl-tRNA synthetase (MARS). tRNA synthetases are ubiquitously expressed enzymes that catalyse the aminoacylation of tRNAs with their cognate amino acids, an essential step in protein translation. All CMT-associated tRNA synthetases are active as homodimers. The association of five different tRNA synthetases with CMT pathogenesis is currently controversial. Nonetheless, several studies have convincingly shown that for GARS, YARS and AARS, some CMT-associated mutations result in loss of aminoacylation function, whereas others do not affect aminoacylation activity.

However, the possibility remains that (aminoacylation-active) mutants display altered subcellular localization, which could lead to defective local protein synthesis, for example, in axonal termini. Indeed, mutant GARS and YARS proteins were reported to be mislocalized in mouse neuroblastoma (N2A), human neuroblastoma (SH-SY5Y) and mouse motor neuron (MN-1) cell lines. In contrast, in mouse models for GARS-associated CMT, subcellular localization of mutant GARS proteins was not altered. Also, AARS and HARS mutant proteins displayed normal subcellular localization in MN-1 cells and Caenorhabditis elegans motor neurons, respectively. Thus, the possible contribution of subcellular mislocalization of mutant tRNA synthetases to CMT pathogenesis is currently controversial.

We previously established a Drosophila model for YARS-associated CMT, which recapitulated several hallmarks of the disease, including progressive motor performance deficits, terminal axonal degeneration and electrophysiological defects. Here we describe the generation and characterization of a Drosophila model for GARS-associated CMT. Although the subcellular localization of CMT-mutant GARS and YARS proteins in our Drosophila CMT models is not altered, direct evaluation of protein translation rates in motor and sensory neurons in vivo shows that CMT-mutant GARS or YARS proteins significantly reduce the levels of newly synthesized proteins. This translational slowdown is not attributable to alteration of tRNA aminoacylation levels, and cannot be rescued by increasing endogenous Drosophila Gars levels, indicating a gain-of-toxic-function mechanism. Importantly, genetic reduction of protein translation is sufficient to induce muscle denervation and sensory neuron morphology defects, suggesting that mutant tRNA synthetase-induced translational slowdown causally contributes to CMT-like phenotypes.

**Results**

**CMT-mutant GARS induces motor defects and reduces lifespan.** To determine whether expression of human GARS can induce dominant phenotypes in Drosophila, we used the UAS/GAL4 system to express full-length wild-type (WT) GARS (GARS_WT) or three CMT-mutant GARS proteins: GARS_E71G, GARS_G240R and GARS_G526R (Fig. 1a). These missense mutations are characterized by strong human genetic evidence for linkage to disease, complete penetrance and they have various effects on in vitro aminoacylation activity (E71G is enzymatically active, G240R and G526R are inactive). To avoid any influence of the genomic environment on transgene expression, UAS-GARS transgenes were targeted to specific chromosomal landing sites.

As a first phenotypic read-out, we determined whether ubiquitous (actin5C-GAL4) or neuron-selective (nsyb-GAL4) expression of mutant GARS would result in developmental lethality. In contrast to GARS_WT, mutant GARS expression induced transgene dosage-dependent developmental lethality, with the phenotypic strength of the mutations ranging from G240R > G526R > E71G (Supplementary Fig. 1a–c).

To circumvent developmental lethality, ubiquitous GARS transgene expression was induced from the adult stage onwards using the GAL80 target system. GARS_WT expression did not alter lifespan, but expression of the three mutant GARS transgenes reduced lifespan, again in a dosage-dependent manner, and with the phenotypic strength ranging from G240R > G526R > E71G (Fig. 1b and Supplementary Fig. 1d). Furthermore, expression of mutant GARS in motor neurons (OK371-GAL4; ref. 21) resulted in motor performance deficits, as determined by an automated negative geotaxis climbing assay (Fig. 1c, and Supplementary Movies 1 and 2). In contrast, GARS_WT did not impair motor performance in a biologically relevant manner (Fig. 1c). Thus, mutant GARS is intrinsically toxic to motor neurons.

**Mutant GARS expression induces neuronal morphology defects.** As peripheral motor and sensory neurons are affected in CMT, we determined whether GARS expression in these neuronal subsets would result in neuronal morphology defects. GARS expression in motor neurons (OK371-GAL4) did not affect the number of motor neuron cell bodies in the ventral nerve cord of third instar larvae (Fig. 2f and Supplementary Fig. 2). However, expression of mutant – but not WT – GARS induced defects in neuromuscular junction (NMJ) morphology, most strikingly the absence of the NMJ innervating muscle 24 in the vast majority of GARS_G240R and GARS_G526R larvae (Fig. 2a–e,g). Remarkably, in second instar larvae muscle 24 was innervated in the vast majority (~85%) of larvae expressing GARS_G240R in motor neurons, but this percentage rapidly declined at later developmental stages (Fig. 2h). This indicates that the lack of muscle 24 innervation is not owing to the fact that the NMJ never developed, but rather attributable to progressive muscle denervation.

We next wanted to evaluate whether NMJs on distal muscles were more severely affected than NMJs on proximal muscles, as is the case in CMT patients. We therefore quantified the synapse length on muscles 8, 21, 4 and 1/9. Muscles 8 and 21 are innervated by the segmental nerve a (SNa) motor nerve that also innervates muscle 24, whereas muscles 4 and 1/9 are innervated by the intersegmental nerve (ISn) motor nerve. This analysis revealed that motor neuron-selective expression (OK371-GAL4) of GARS_G240R or GARS_G526R significantly reduced the synapse size on all muscles analysed (Fig. 2i). Expression of GARS_E71G also reduced synapse size on muscle 1/9, but not on the other muscles. Our analysis further showed that the reduction...
in synapse size was significantly more pronounced on distal muscles (8 and 1/9) as compared with proximal muscles (21 and 4; Fig. 2i). These NMJ morphology defects were independent of the motor neuron driver used, as D42-GAL4 driven expression of GARS_G240R also induced denervation of muscle 24 (Supplementary Fig. 3).

When expressed in class IV multidendritic sensory neurons (ppk-GAL4), well-characterized neurons in the larval body wall with an elaborate dendritic tree22, GARS_WT did not induce morphological defects, but the three mutant GARS proteins all induced a significant reduction of dendritic coverage, whereas cell body morphology was not altered (Fig. 3). In conclusion, expression of CMT-mutant GARS induced morphology defects in the processes of motor and sensory neurons, without affecting the neuronal cell body.

**Mutant GARS and YARS display normal subcellular localization.**

To evaluate whether CMT-associated mutations in tRNA synthetases alter their subcellular localization, we studied the subcellular localization of mutant GARS and YARS proteins in our *Drosophila* CMT models. Transgenic lines for expression of YARS_WT and three CMT-mutant variants (G41R, 153-156delVKQV and E196K; ref. 15) were regenerated using site-specific transgenesis. Immunostaining with antibodies that specifically recognize human GARS or YARS proteins revealed that in third instar larval motor neurons, the WT GARS and YARS proteins are localized throughout the cytoplasm, with homogeneous staining of cell bodies, axons and NMJs (Fig. 4). Interestingly, no differences in subcellular localization could be detected for any of the mutant GARS or YARS proteins (Fig. 4, and Supplementary Figs 4 and 5).

In class IV multidendritic sensory neurons, YARS_WT protein localized to the cell body, axon and the major dendritic branches, but not or to a much lesser extent to smaller dendritic branches.

Importantly, the CMT-mutant YARS proteins displayed a similar subcellular localization pattern (Fig. 4 and Supplementary Fig. 4). Thus, altered subcellular localization of mutant GARS and YARS proteins is not the cause of structural and functional defects in motor and sensory neurons in our *Drosophila* CMT models.

**Mutant GARS inhibits translation in motor and sensory neurons.**

To directly evaluate the effect of mutant GARS expression on global protein synthesis rates in larval motor neurons *in vivo*, FUNCAT (fluorescent noncanonical amino-acid tagging) and BONCAT (bio-orthogonal noncanonical amino-acid tagging) techniques were used, which rely on incorporation of noncanonical amino acids in newly synthesized proteins23,24. As described in the accompanying paper by Erdmann et al.25, cell-type specificity was achieved by transgenic expression of L262G mutant *Drosophila MASP* with a C-terminal EGFP tag (dMetRS_L262G-enhanced green fluorescent protein (EGFP)). In contrast to endogenous dMetRS, dMetRS_L262G-EGFP can aminoacylate tRNA^Met^ with the noncanonical amino acid azidonorleucine (ANL)26, which is added to the culture medium. FUNCAT involves tagging ANL-labelled proteins with a fluorescent tag by means of [3 + 2] azide-alkyne-cycloaddition (‘click chemistry’)23. The intensity of fluorescent labelling detected by fluorescence microscopy is proportional to the rate of protein synthesis. For BONCAT, proteins are extracted from tissues, a biotin-alkyne affinity tag is added by click chemistry, and biotin-labelled proteins are affinity purified, followed by SDS–polyacrylamide gel electrophoresis (PAGE), blotting and anti-biotin staining to visualize newly synthesized proteins24. Whereas FUNCAT has the advantage of cellular resolution, BONCAT allows for detection of possible genotypic differences in protein synthesis in a wide range of protein sizes.
Figure 2 | Selective mutant GARS expression in motor neurons induces progressive muscle denervation in a proximo–distal gradient. (a–e) NMJs of third instar larvae expressing GARS in motor neurons (OK371-GAL4) were visualized by staining for the postsynaptic marker discs large 1 (dlg1). Arrows indicate the NMJ on muscle 24, which is missing in the vast majority of GARS_G240R and GARS_G526R animals. Scale bar, 50 μm. (f) No differences in motor neuron numbers were found between control and GARS larvae; one-way analysis of variance (ANOVA) with Bonferroni correction; \( P = 0.88; N = 21–33 \). (g) Quantification of the percentage of animals with muscle 24 innervated; \( \chi^2 \)-Test; *** \( P < 1 \times 10^{-8}; N = 26 \). (h) Muscle 24 innervation in OK371-GAL4 > 2 × GARS_G240R larvae at different developmental stages (L2: second instar and L3: third instar). \( N = 51, 42 \) and 30 NMJs; Mann–Whitney U-test; *** \( P < 0.005 \) versus L2. (i) The effect of motor neuron-selective expression of GARS transgenes on synapse length on muscles 21, 8, 4 and 1/9 was quantified and plotted as the percentage change from 2 × GARS_WT. Muscles 8 and 21 are both innervated by the SNa motor nerve, whereas muscles 4 and 1/9 are innervated by the ISN motor nerve. Phenotypic strength ranged from G240R > G526R > E71G, and for all mutants, the phenotypic severity showed a proximo–distal gradient, with distal muscle (8 and 1/9) more severely affected than proximal ones (21 and 4); one-way ANOVA with Bonferroni correction; * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.00005 \); \( N = 12–14 \). Error bars represent s.e.m.

Figure 3 | Selective mutant GARS expression in class IV multidendritic sensory neurons induces dendritic morphology defects. (a–e) Class IV multidendritic sensory neurons in the third instar larval body wall were visualized by ppk-GAL4-driven mCD8-GFP with or without co-expression of GARS. The dendritic tree of individual neurons is delineated. Scale bar, 100 μm. (f) Quantification of the percentage of dendritic coverage; one-way analysis of variance with Bonferroni correction; *** \( P < 1 \times 10^{-5}; N = 15–20 \). Error bars represent s.e.m.
For FUNCAT evaluation of protein synthesis rates in motor neurons, larvae that co-express dMetRSL262G-EGFP and GARS transgenes in motor neurons (OK371-GAL4) were exposed to ANL for 24 h. Whereas expression of GARS_WT did not alter protein synthesis rates (Supplementary Fig. 7a), GARS_G240R and GARS_G526R significantly reduced the levels of newly synthesized proteins, to only 40% of GARS_WT. GARS_E71G did not significantly reduce protein synthesis rates (Fig. 5a–e). BONCAT confirmed reduced protein synthesis rates in GARS_G240R-expressing motor neurons, without obvious alteration of the size distribution of newly synthesized proteins (Fig. 5f,g). Levels of ubiquitinated proteins were not altered in larvae ubiquitously expressing WT or mutant GARS, and autophagy was not induced (Supplementary Fig. 8), suggesting that reduced levels of newly synthesized proteins are not due to enhanced global protein degradation. In fact, the fluorescence intensity of the GFP-LAMP marker was significantly reduced in mutant GARS expressing motor neurons (Supplementary Fig. 8b–g). This may indicate lower levels of autophagy, but may also be due to reduced GFP-LAMP protein levels as a consequence of impaired translation in mutant GARS motor neurons. The latter possibility is in line with the finding that the levels of the mutant GARS transgenic proteins themselves are reduced as compared with GARS_WT, although for GARS_G240R this may in part also be attributable to reduced transcript levels (Supplementary Fig. 9a–d). We further determined that co-expression of GARS_G526R does not significantly alter the levels of dMetRSL262G-EGFP, whereas co-expression of GARS_E71G slightly, but statistically, significantly reduced dMetRSL262G-EGFP protein levels by 18% and GARS_G240R reduced dMetRSL262G-EGFP protein levels by 47% (Supplementary Fig. 9e,f). Taken together, our data indicate that expression of two of three CMT-associated mutant GARS proteins compromises global protein synthesis in motor neurons in vivo.

To evaluate whether mutant GARS would also impair protein translation in sensory neurons, FUNCAT was performed on class II × UAS-GARS_E71G 2 × UAS-GARS_G240R 2 × UAS-GARS_G526R 2 × UAS-GARS_WT and II × UAS-YARS_G41R 2 × UAS-YARS_153-156delVKQV 2 × UAS-YARS_E196K 2 × UAS-YARS_WT Anti-GARS Anti-YARS

Figure 4 | Mutant and WT GARS and YARS proteins display similar subcellular localization in motor and sensory neurons. (a–h) GARS immunostaining reveals the subcellular localization of GARS proteins at third instar larval NMJs (a–d) and motor neuron cell bodies (e–h) upon expression in motor neurons (OK371-GAL4). (i–t) Subcellular localization of YARS proteins at third instar larval NMJs (i–l, nsyb-GAL4), motor neuron cell bodies (m–p, nsyb-GAL4) and class IV multidendritic sensory neurons (q–t, ppk-GAL4), as revealed by YARS immunostaining. Scale bars, 20 µm (a–p) and 100 µm (q–t).
Ubiquitous mutant GARS expression inhibits translation. To evaluate whether the translation impairment induced by mutant GARS expression is specific to larval motor and sensory neurons or also occurs in adult flies upon ubiquitous mutant GARS expression, expression of GARS transgenes was induced from the adult stage onwards using the GAL80 target system, and protein translation was evaluated by 35S-methionine incorporation as an independent method that does not depend on ANL incorporation. 35S-methionine incorporation was also measured in age-matched uninduced control flies of the same genotype, and the ratio of induced to uninduced 35S-methionine incorporation was determined for each sample contained equal total protein concentrations. Part of the samples was used for NeutrAvidin affinity purification and subsequent western blotting using anti-biotin antibodies detected biotinylated proteins before (B) and after (A) purification. The full-length blot is shown in Supplementary Fig. 16a. Quantification of BONCAT signal intensity after affinity purification. Averages ± s.e.m. relative to GARS_WT (100%); Mann–Whitney U-test; ***P < 0.001; N = 9–12. (b) Levels of 35S-methionine incorporation in proteins of flies expressing GARS transgenes from the adult stage onwards. 35S-methionine incorporation was determined 4 days after transgene induction and normalized to total protein content. 35S-methionine incorporation was also measured in age-matched uninduced control flies of the same genotype, and the ratio of induced to uninduced 35S-methionine incorporation is shown as percentage of 2 × GARS_WT. Ubiquitous expression of each of the mutant GARS transgenes significantly reduced 35S-methionine incorporation by ∼50%. Welch’s analysis of variance (Dunnett’s T3 post hoc test); ***P < 0.005; N = 10–12. Ubiquitous mutant GARS expression inhibits translation. To evaluate whether the translation impairment induced by mutant GARS expression is specific to larval motor and sensory neurons or also occurs in adult flies upon ubiquitous mutant GARS expression, expression of GARS transgenes was induced from the adult stage onwards using the GAL80 target system, and protein translation was evaluated by 35S-methionine incorporation as an independent method that does not depend on ANL incorporation catalysed by dMetRS1262G-EGFP and click chemistry used in FANCAT/BONCAT approaches. Four days after transgene induction, flies were transferred to 35S-methionine-containing IV multidendritic sensory neurons. Again, expression of GARS_WT did not alter translation rates (Supplementary Fig. 7b), but expression of any of the mutant GARS proteins reduced the levels of newly synthesized proteins by ∼30–50% (Fig. 5h). A control experiment showed that dMetRS1262G-EGFP was not toxic to larval motor and sensory neurons, as co-expression of dMetRS1262G-EGFP did not enhance NMJ or dendritic morphology defects induced by mutant GARS expression (Supplementary Fig. 10). Thus, in contrast to motor neurons, the GARS_E71G mutant significantly impaired translation in sensory neurons.
food for 24 h, and 35S-methionine incorporation in proteins was evaluated (see Methods section for details). These experiments revealed that expression of mutant GARS proteins reduced 35S-methionine incorporation by ≈50%, even when normalized to total protein content and to uninduced control flies of the same genotype (Fig. 5i). These results were confirmed by a BONCAT experiment in which azidohomoalanine (AHA) was fed to flies of the same genotypes. AHA is a noncanonical amino acid that is used by endogenous MetRS to aminoacylate tRNA^Met (refs 24,27). Whereas AHA incorporation in newly synthesized proteins was clearly detectable in control and 2×GARS WT flies, ubiquitous expression of mutant GARS diminished BONCAT labelling (Supplementary Fig. 11). These data indicate that mutant GARS expression markedly impairs protein translation, even when expressed ubiquitously in adult flies.

Mutant YARS inhibits translation in motor and sensory neurons.

We next wanted to evaluate whether translational defects can also be induced by expression of CMT-mutant YARS. FUNCAT experiments revealed that expression of any of the three YARS mutants induced translational slowdown, both in motor and in sensory neurons (Fig. 5j,k and Supplementary Fig. 7c,d). These data suggest that impaired protein translation may be a common pathogenic mechanism for CMT associated with mutations in tRNA synthetases.

Mutant GARS does not reduce overall tRNA^Gly aminoacylation.

We next wanted to determine whether mutant GARS-induced translational slowdown could be attributed to defective tRNA^Gly aminoacylation, which might be caused by a dominant-negative effect of mutant GARS on endogenous dGars aminoacylation activity, possibly by the formation of aminoacylation-inactive heterodimers between mutant GARS and dGars. To determine whether heterodimers between dGars and human GARS are formed, we used bacterial artificial chromosome (BAC) recombineering to introduce a C-terminal EGFP tag in the Drosophila gars gene and used this construct to generate a dGars:EGFP bacterial artificial chromosome (BAC) transgenic fly line. When expressing GARS transgenes panneuronally (nsyb-GAL4) in the dGars:EGFP background, co-immunoprecipitation revealed that dGars:GARS heterodimers are indeed formed in neurons in vivo (Supplementary Fig. 12). We subsequently used an in vitro aminoacylation assay to determine the overall tRNA^Gly aminoacylation activity of protein extracts from larvae expressing GARS transgenes ubiquitously in an otherwise WT Drosophila gars background (Fig. 6a). Not surprisingly, GARS WT and GARS_E71G expression markedly increased tRNA^Gly aminoacylation activity over non-transgenic control levels. Unexpectedly, GARS_G240R also increased aminoacylation activity, albeit to a lesser extent, indicating that this mutant displays residual activity when overexpressed in Drosophila. GARS_G526R did not significantly alter tRNA^Gly aminoacylation activity. Thus, expression of mutant GARS increased overall tRNA^Gly aminoacylation activity corresponding to the charging activity of each mutant. Importantly, none of the mutant GARS variants led to decreased tRNA^Gly aminoacylation activity, whereas knockdown of endogenous dGars by either of three independent transgenic RNA interference (RNAi) lines decreased tRNA^Gly aminoacylation activity (Supplementary Fig. 13a). These data argue against a dominant-negative effect of mutant GARS on dGars aminoacylation activity.

Mutant GARS does not alter tRNA^Gly aminoacylation in vivo.

The possibility remains that in vivo, mutant GARS could bind tRNA^Gly while failing to aminoacylate it, or aminoacylate it at a much slower rate. This could potentially reduce levels of aminoacylated tRNA^Gly under a critical threshold and slow down protein synthesis. To evaluate this possibility, the ratio of aminoacylated versus non-aminoacylated tRNA^Gly was determined in larvae ubiquitously expressing GARS transgenes in a WT Drosophila gars background. Acid urea PAGE and northern blotting revealed that the steady-state in vivo aminoacylation levels of the two cytoplasmic tRNA^Gly present in Drosophila (Supplementary Fig. 13b) were not altered by expression of WT or mutant GARS (Fig. 6b,c). As a control, steady-state aminoacylation levels were determined for one of the tRNA^Ala isoacceptors and for cytoplasmic initiator tRNA^Met, and no changes were found (Fig. 6b,c and Supplementary Fig. 13c). These data indicate that CMT-mutant GARS expression does not interfere with aminoacylation of tRNA^Gly, excluding this mechanism as a cause for reduced protein synthesis.

Inhibiting translation is sufficient to induce CMT phenotypes.

To investigate whether the translational slowdown induced by mutant tRNA synthetases could causally contribute to CMT-like phenotypes, we genetically reduced global protein translation in motor neurons by expressing constitutively active variants of the Drosophila eukaryotic initiation factor 4E (eIF4E)-binding protein (d4E-BP). eIF4E is the cap-binding protein, which mediates binding of eIF4F to the 5’-cap structure of messenger RNAs, a rate-limiting step in translation initiation29. The activity of eIF4E is inhibited by 4E-BP, which sequesters eIF4E from the eIF4F complex. 4E-BP is regulated by phosphorylation, causing its release from eIF4E and relieving its inhibitory effect on translation29. To genetically inhibit protein translation, we used two constitutively active variants of d4E-BP: d4E-BP T37/46A (d4E-BP^TA), which can no longer be phosphorylated on T37 and T46 (ref. 30), and a d4E-BP variant in which M59 and K60 are changed to L (d4E-BP^LL), increasing dE4F4E binding by 3.4-fold (ref. 31).

FUNCAT revealed that expression of these d4E-BP variants in motor neurons reduced translation rates by ~40% (Fig. 6e) and resulted in muscle 24 denervation in the vast majority of larvae (Fig. 6f). Furthermore, expression of constitutively active d4E-BP in class IV multidendritic sensory neurons reduced translation rates by ~60–80% (Fig. 6g) and induced severe morphology defects, with reduction of dendritic coverage by ~40% (Fig. 6h and Supplementary Fig. 14). Thus, inhibition of global protein synthesis in motor and sensory neurons is sufficient to induce CMT-like phenotypes, suggesting a causal contribution of translational slowdown to mutant tRNA synthetase-induced phenotypes.

Discussion

In this study, we evaluated the effect of CMT-mutant GARS and YARS expression on protein translation in Drosophila CMT
models. For that purpose, we generated a Drosophila model for GARS-associated CMT by expressing human GARS in an otherwise WT Drosophila gars background. Expression of mutant—but not WT—GARS recapitulated several hallmarks of the human disease, including motor performance deficits, neuronal morphology defects and reduced lifespan. Motor neuron-selective mutant GARS expression was sufficient to induce phenotypes, indicating that mutant GARS is intrinsically toxic to motor neurons. NMJ and sensory neuron dendritic morphology defects were found, but neuronal cell bodies remained intact. Interestingly, NMJs on distal muscles were more severely affected than NMJs on proximal muscles, and muscle denervation was progressive over larval life, indicative of a degenerative event. This is reminiscent of human CMT2 and the GarsP234KY/+ mouse model for CMT2D (ref. 13), where progressive NMJ denervation and terminal axonal degeneration are observed, with distal muscles most severely affected, and without loss of axons in ventral roots or death of cell bodies in the spinal cord. Overall, the phenotypes are similar to a Drosophila YARS model15 and a recently reported dGars model16, indicating that CMT-mutant forms of YARS and GARS both induce dominant phenotypes in Drosophila.

Initially, a (partial) loss of aminoacylation activity was suggested as the underlying mechanism for GARS and YARS-associated CMT5,6, but more recently it became apparent that loss of aminoacylation activity is not required to cause the disease, as some CMT-associated mutations in GARS12–14, YARS15,33 and AARS8 do not affect enzymatic activity. Consistent herewith, the strength of phenotypes induced by CMT-mutant GARS and YARS proteins in Drosophila does not correlate with aminoacylation activity, as GARS mutants with loss of aminoacylation activity (G240R and G526R) displayed the strongest phenotypes, whereas for YARS, the aminoacylation-active E196K mutant induced the strongest phenotypes15.

However, subcellular mislocation of mutant tRNA synthetases could still give rise to defects in local protein synthesis. In spite of previous reports of mutant GARS and YARS mislocalization in several neuronal cell lines6,12,14, we could not detect any alteration of subcellular localization of mutant GARS or YARS proteins in motor and sensory neurons in vivo. Possibly, these seemingly contradicting results can be explained by the fact that previous studies used tagged GARS or YARS proteins transiently expressed in cultured neuronal cell lines, whereas in the current study the distribution of untagged GARS and YARS proteins was evaluated in vivo, in motor and sensory neurons with established NMJs and dendrite projections, respectively. Our localization studies demonstrate that, at least in our Drosophila CMT models, subcellular mislocalization is not the cause of CMT-related phenotypes. This is in accordance with previous reports of unaltered subcellular localization of CMT-mutant GARS16, AARS8 and HARS9 proteins. Therefore, defects in local protein synthesis due to subcellular mislocalization of CMT-mutant tRNA synthetases seem unlikely.

We nevertheless decided to evaluate the effect of mutant GARS and YARS expression on global protein translation in motor and sensory neurons in vivo. To do so, the previously described FUNCAT and BONCAT methodologies23,24 were adapted to allow for cell-type-specific evaluation of protein translation rates in vivo, as described in the accompanying paper by Erdmann et al. Remarkably, in motor neurons, expression of GARS_G240R and G526R effectively reduced protein translation rates in larval motor neurons, as determined by FUNCAT; Mann–Whitney U-test; ***P < 0.001 versus control; NS, not significant; N = 10–12.

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However, subcellular mislocation of mutant tRNA synthetases could still give rise to defects in local protein synthesis. In spite of previous reports of mutant GARS and YARS mislocalization in several neuronal cell lines6,12,14, we could not detect any alteration of subcellular localization of mutant GARS or YARS proteins in motor and sensory neurons in vivo. Possibly, these seemingly contradicting results can be explained by the fact that previous studies used tagged GARS or YARS proteins transiently expressed in cultured neuronal cell lines, whereas in the current study the distribution of untagged GARS and YARS proteins was evaluated in vivo, in motor and sensory neurons with established NMJs and dendrite projections, respectively. Our localization studies demonstrate that, at least in our Drosophila CMT models, subcellular mislocalization is not the cause of CMT-related phenotypes. This is in accordance with previous reports of unaltered subcellular localization of CMT-mutant GARS16, AARS8 and HARS9 proteins. Therefore, defects in local protein synthesis due to subcellular mislocalization of CMT-mutant tRNA synthetases seem unlikely.

We nevertheless decided to evaluate the effect of mutant GARS and YARS expression on global protein translation in motor and sensory neurons in vivo. To do so, the previously described FUNCAT and BONCAT methodologies23,24 were adapted to allow for cell-type-specific evaluation of protein translation rates in vivo, as described in the accompanying paper by Erdmann et al. Remarkably, in motor neurons, expression of GARS_G240R and G526R effectively reduced protein translation rates in larval motor neurons, as determined by FUNCAT; Mann–Whitney U-test; ***P < 0.001 versus control; NS, not significant; N = 10–12.
and GARS_G526R reduced the levels of newly synthesized proteins by ~60%, whereas GARS_E71G expression did not significantly reduce translation rates. In contrast, in class IV multidendritic sensory neurons, all three CMT-mutant GARS proteins significantly impaired translation. In this context, it is particularly interesting to note that GARS_E71G induced dendritic morphology defects in sensory neurons, but did not induce muscle denervation upon expression in motor neurons, suggesting a positive correlation between impaired translation and neuronal morphology defects. A causal relationship between translational slowdown and CMT-related phenotypes is further indicated by the fact that suppression of translation by expression of constitutively active forms of d4E-BP was sufficient to induce muscle denervation and sensory neuron morphology defects. Translation deficits induced by mutant GARS were not specific to motor and sensory neurons, as ubiquitous expression of mutant GARS from the adult stage onwards also strongly inhibited translation, as shown by 35S-methionine incorporation and AHA-BONCAT. This is also important from a methodological perspective, as 35S-methionine incorporation does not depend on ANL incorporation catalysed by dMetRS1262-GFP and click chemistry, thus further validating the FUNCAT/BONCAT methodology. Also, whereas co-expression of GARS_G526R did not significantly alter dMetRS1262-GFP protein levels, co-expression of GARS_E71G reduced dMetRS1262-GFP levels by ~20% and GARS_G240R reduced dMetRS1262-GFP levels by ~50%. In case the level of dMetRS1262-GFP would be limiting for ANL incorporation, this may amplify the effect of GARS_E71G and GARS_G240R on ANL incorporation, which could lead to overestimation of the magnitude of translational inhibition. Finally, the fact that not only mutant GARS but also CMT-mutant YARS reduced protein synthesis rates in motor and sensory neurons suggests defective protein synthesis as a common pathogenic mechanism underlying mutant tRNA synthetase-associated CMT.

Several lines of evidence indicate that the slowdown of protein synthesis induced by mutant GARS expression is not due to defective tRNA Gly aminoacylation. First, in an *in vitro* aminoacylation assay using larval extracts, mutant GARS expression did not reduce the overall tRNA Gly aminoacylation activity, that is, the combined activity of endogenous dGars and transgenic GARS. Second, the *in vivo* ratio of glycylated versus nonglycylated tRNA Gly was not altered, with a ratio of >90% in all conditions. As the glycylated tRNA Gly is continuously used for translation, resulting in deacylation, a ratio of >90% can be considered as fully aminoacylated. Moreover, overall tRNA Gly levels were indistinguishable between genotypes. Finally, co-overexpression of WT dGars markedly increased overall tRNA Gly aminoacylation activity in larval extracts, but failed to rescue reduced translation rates induced by GARS_G240R. Thus, translation defects are not due to a dominant-negative effect of mutant GARS on dGars, but rather attributable to a gain-of-toxic-function mechanism, consistent with previous findings in CMT2D mouse models.34

If not attributable to altered tRNA aminoacylation, what could then be the molecular mechanism by which mutant tRNA synthetases inhibit translation? One possibility is that mutant tRNA synthetases may suppress protein synthesis by acting on upstream regulators of translation. Alternatively, mutant tRNA synthetases may act on immediate effectors of translation initiation or elongation, thereby more directly interfering with translation. A key upstream regulatory pathway to control translation levels involves phosphorylation of eIF2z, which occurs in response to cellular stress and results in downregulation of global translation. Elevated eIF2z phosphorylation has previously been implicated in both Alzheimer’s and prion diseases. eIF2z phosphorylation is elevated in brains of Alzheimer’s disease (AD) patients and in AD mouse models37–40, and genetic deletion or haploinsufficiency of the eIF2z kinase PERK prevented enhanced eIF2z phosphorylation and deficits in protein synthesis, as well as synaptic plasticity defects, memory deficits and cholinergic neurodegeneration37,38. Similarly, deletion of GCN2, another eIF2z kinase, also prevented impairment of synaptic plasticity and defects in spatial memory in AD model mice.47 In prion-diseased mice, accumulation of prion protein during prion replication induced the unfolded protein response and caused persistent repression of global protein synthesis by phospho-eIF2z. Overexpression of GADD34, a specific phospho-eIF2z phosphatase, reduced phospho-eIF2z levels, restored translation rates, reduced synaptic deficits and neuronal loss, and significantly increased survival.41 In line with a central role of phospho-eIF2z in prion disease, treatment of prion-infected mice with a specific PERK kinase inhibitor restored global protein synthesis and abrogated development of clinical prion disease in mice, with neuroprotection observed throughout the brain.42 However, in spite of the central role of elevated eIF2z phosphorylation in AD and prion disease, ubiquitous expression of mutant GARS transgenes in *Drosophila* larvae did not significantly alter eIF2z phosphorylation (Supplementary Fig. 15). These data suggest that decreased translation induced by mutant GARS expression is not simply secondary to cellular stress. Further studies are needed to evaluate whether mutant GARS could interfere with other upstream regulatory pathways of translation.

Direct interference with translation initiation was previously implicated in autism. A human genetic study indicated that increased eIF4E expression may contribute to autism, and in transgenic mice, increasing the levels of eIF4E or deletion of 4E-BP2 results in exaggerated cap-dependent translation and autistic-like behaviours, accompanied by synaptic pathophysiology. These defects could be corrected by pharmacological inhibition of eIF4E activity.44,45

An attractive hypothesis is that mutant tRNA synthetases may hamper translation elongation, as an appropriate supply of charged aminocyl-tRNAs to the ribosome is essential for efficient translation elongation. It is tempting to speculate that CMT-mutant tRNA synthetases may cause defects downstream of tRNA aminoacylation, such as defective transfer of aminocylated tRNAs to eEF1A, the elongation factor that transports charged tRNAs to the ribosome. That such a defect could lead to neurodegeneration is illustrated by the recent finding that in Gtpbp2 mutant mice, loss of a central nervous system (CNS)-specific tRNA, called n-Tr20, causes ribosome stalling at AGA codons and consequent neurodegeneration.46 Furthermore, the spontaneous mouse mutant ‘wasted’ exhibits severe muscle wasting and neurodegeneration due to loss of eEF1A2, an isoform of eEF1A that is selectively expressed in neurons and muscle from around 2 weeks of age onwards.47–50 Thus, the wasted phenotype may be caused by inadequate delivery of charged tRNAs to the ribosome.

Mutant tRNA synthetases might also impede translation elongation by a molecular mechanism independent of tRNA supply to the ribosome. In this respect, the findings by Chen et al.51 that the fragile X mental retardation protein inhibits translation by binding directly to the L5 protein on the 80S ribosome are of interest. fragile X mental retardation protein binds within the intersubunit space of the ribosome, such that it would preclude the binding of tRNA and translation elongation factors to the ribosome, thus causing ribosome stalling.51,52

In conclusion, our findings may unite two current viewpoints in the field that seemed to contradict each other: on the one hand, the fact that five different tRNA synthetases have been implicated...
as a genetic cause of CMT strongly suggests that alteration of a function that all these enzymes have in common is the cause of the disease. The only common function known to date is aminoacylation. On the other hand, several studies have convincingly shown that some disease-causing mutations do not affect aminoacylation activity. Based on our findings, it seems reasonable to postulate that CMT-associated mutations in tRNA synthetases may all interfere with protein translation and to evaluate whether impaired protein synthesis is also found in CMT mouse models and patients. Such insights may lay the foundation for novel therapeutic approaches for this incurable disease.

Methods

Generation of transgenic flies. Full-length human GARS and YARS complementary DNA was purchased from OriGene (Rockville, MD, USA), clones RC208252 and RC204590, respectively. Mutations were introduced by site-directed PCR mutagenesis and GARS and YARS complementary DNA with or without mutations was subcloned into the pUAST-AttB transformation vector. All constructs were sequence verified and injected into Drosophila embryos by Genetix (Houston, TX, USA). Genomic landing sites VK37 and VK31 were used for transgenesis. The flies were crossed onto a conventional sugar-based diet. The latter two antibodies specifically react with the dGars gene and were obtained from BACPAC Resources Center (Oakland, CA, USA). We modified the dGars gene according to Ejsmont et al.13 to introduce a C-terminal 2× Ty1-EGF-P3×FLAG-tag and used the modified BAC for embryo injection. The BAC transgene was inserted into the VK22 genomic landing site.

Adult offspring frequency and lifespan analysis. For determination of adult offspring frequencies, the number of adult flies eclosing was counted for each genotypic group within batches of 10 flies per food vial. The number of dead flies was counted across at least 10 flies per genotype were used. Crosses were cultured at 18°C with a 12-h light/dark cycle. Female flies were collected within 48 h after eclosion and larval CNS was dissected in 4 mM ANL-containing food. 120 h AEL, larval CNS was dissected in ice-cold HL3 solution. For FUNCAT in class IV multidendritic sensory neurons, larvae were transferred to 4 mM ANL-containing medium at 72 h AEL, and larval CNS filets were prepared and stained with primary antibodies against GFP (Invitrogen, A6455, 1/1,000) and elav (Developmental Studies Hybridoma Bank, cat. no. AS153) at 25°C. After fixation, newly synthesized proteins were tagged using a fluorescent TAMRA tag as described in the accompanying paper by Erdmann et al.40 Fluorescence intensities were quantified using ImageJ software. For image acquisition, identical confocal settings were used for all CNSs. The LAMP-GFP staining intensity was measured with Fiji by surrounding the cells and measuring the mean intensity of all of the pixels within the framed area. Ten cells per brain (one motor neuron cluster) were quantified and the average value of those cells was taken as one data point.

Analysis of protein translation rates (FUNCAT and BONCAT). For FUNCAT analysis of protein translation rates in motor neurons, 2-hour egg collections were performed and animals were raised on Ice-Mix Drosophila medium (Fisher Scientific, cat. no. AS553) at 25°C in a 12-h light/dark cycle. Early egg-laying (EEL) larvae were transferred to 4 mM ANL-containing medium. 120 h AEL, larval CNS was dissected in ice-cold HL3 solution. For FUNCAT in class IV multidendritic sensory neurons, larvae were transferred to 4 mM ANL-containing medium at 72 h AEL, and larval CNS filets were prepared and stained with ice-cold HL3 solution at 120 h AEL. After CNS fixation, newly synthesized proteins were tagged using a fluorescent TAMRA tag as described in the accompanying paper by Erdmann et al.40 For image acquisition, identical confocal settings were used for all CNSs. Fluorescence intensities were quantified using ImageJ software.

For BONCAT, 2-hour egg collections were performed and eggs were directly transferred to 4 mM ANL-containing food. 120 h AEL, larval CNS was dissected in ice-cold HL3 solution and collected in PBS (pH 7.8) supplemented with protease inhibitor without EDTA. Tagging of newly synthesized proteins was performed by incubating total protein extracts from larval brains with a biotin-alkyne affinity tag, as described in the accompanying paper by Erdmann et al. Total protein concentrations were determined using the amidoblack protein assay. Samples were diluted so that each sample contained equal total protein concentrations. A fraction of each sample was affinity purified using NeutrAvidin agarose. Before purification and after purification fractions were used for western blotting to estimate the amounts of newly synthesized proteins. This was done according to Dietrich et al.41 using an antibody against biotin (Bethyl). Silver gel staining was used to confirm equal amounts of protein in all lanes. AHA-BONCAT was performed as described in the accompanying paper by Erdmann et al.40

Quantification of protein synthesis rate was performed using dot blot analysis, using the same elute fractions (after purification) as for western blot analysis. Eluates were applied onto a nylon membrane (Hybond-C, Duren, Germany) and transferred to the membrane. The membrane was incubated with the polyclonal rabbit anti-biotin antibody (1:10,000, Bethyl) described in ref. 27. Subsequently, the membrane was
probed with a fluorescent antibody (donkey anti-rabbit-680; 1:15,000; PerkinElmer) using T4-PNK (New England Biolabs) at a molar ratio of 3:1. Non-incorporated γ-[32P]-ATP was removed by G-25 Sephadex chromatography (MicroSpin G-25 columns; GE Healthcare). Oligonucleotide sequences were used: 5'-TCTACCACTGAAACCC GTAGC-3' for tRNA54-25, 5'-TAACACTAACACCGAGCAGC-3' for tRNA58-25, 5'-CAAGAGCAAGAGCCGACCT-3' for tRNA52-25 and 5'-TGGGATT GCGGGTATCGA-3' for tRNA41. Prehybridization T was 42°C, hybridization T was 45°C and 2 x saline-sodium citrate buffer (SCC) was used for wash steps at room temperature following hybridization.

Statistical analysis. For lifespan analysis, log-rank test was used to test for statistical significance. Statistical analyses were used to analyze offering frequency data and frequency of innervation of larval muscle. For analysis of motor performance, the Mann–Whitney U-test was used to compare climbing speed of individual flies per genotype and per run. As all flies were tested in five subsequent runs, five P values were generated per genotype. These P values were combined using the Fisher’s combined probability test. For analysis of NMJ development, the Mann–Whitney U-test was used. For analysis of motor neuron number, synapse length, dendritic coverage of class IV MD neurons, GARS protein levels and GARS messenger RNA levels, one-way analysis of variance with Bonferroni correction was used as data displayed normal distribution and equal variance. Levels of 2-3S-methionine incorporation and 2-3S-methionine fluorescence intensity were analysed by Welch’s analysis of variance (Dunnnett’s T3 post hoc test) as data displayed normal distribution but unequal variance. FUNCAT fluorescence intensities, ΔMetRS28S-EF2G protein expression levels and phospho-EF2 protein levels relative to total EF2 levels were analysed using Mann–Whitney U-test. For BONCAT quantification, unpaired t-test with Welch’s correction (two-tailed) was used.

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S.N. and E.S. performed the research; U.L.R., U.T., R.W.B. and D.D. contributed reagents; S.N., J.B., C.K., H.A., X.-L.Y., D.D. and E.S. analysed data; all authors commented on the paper; S.N. and J.B. made figures; and E.S. wrote the paper.

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Corrigendum: Impaired protein translation in *Drosophila* models for Charcot-Marie-Tooth neuropathy caused by mutant tRNA synthetases

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