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CMT-associated mutations in glycyl- and tyrosyl-tRNA synthetases exhibit similar pattern of toxicity and share common genetic modifiers in *Drosophila*

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Abstract

Aminoacyl-tRNA synthetases are ubiquitously expressed proteins that charge tRNAs with their cognate amino acids. By ensuring the fidelity of protein synthesis, these enzymes are essential for viability of every cell. Yet, mutations in six tRNA synthetases specifically affect the peripheral nerves and cause Charcot-Marie-Tooth disease (CMT). The CMT-causing mutations in tyrosyl-

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and glycyl-tRNA synthetases (YARS and GARS, respectively) alter the activity of the proteins in a range of ways (some mutations do not impact charging function, while others abrogate it), making a loss of function in tRNA charging unlikely to be the cause of disease pathology. It is currently unknown which cellular mechanisms are triggered by the mutant enzymes and how this leads to neurodegeneration. Here, by expressing two pathogenic mutations (G240R, P234KY) in *Drosophila*, we generated a model for GARS-associated neuropathy. We observed compromised viability, and behavioral, electrophysiological and morphological impairment in flies expressing the cytoplasmic isoform of mutant GARS. Their features recapitulated several hallmarks of CMT pathophysiology and were similar to the phenotypes identified in our previously described *Drosophila* model of YARS-associated neuropathy. Furthermore, CG8316 and CG15599 – genes identified in a retinal degeneration screen to modify mutant *YARS*, also modified the mutant *GARS* phenotypes. Our study presents genetic evidence for common mutant-specific interactions between two CMT-associated aminoacyl-tRNA synthetases, lending support for a shared mechanism responsible for the synthetase-induced peripheral neuropathies.

Keywords

Drosophila; aminoacyl-tRNA synthetase; Charcot-Marie-Tooth disease

Introduction

Charcot-Marie-Tooth disease (CMT), or hereditary motor and sensory neuropathy, is the most common human inherited neuromuscular disorder affecting specifically the peripheral nerves. CMT symptoms include progressive motor impairment, distal muscle weakness and wasting, sensory loss and skeletal deformities. Based on electrophysiological and neuropathological criteria, the disease is classified into demyelinating forms (CMT1), axonal forms (CMT2), and intermediate forms combining features of CMT1 and CMT2 (Pareyson and Marchesi, 2009). There is no cure for any of these CMT phenotypes, posing substantial personal, social and economic burden. Mutations in over 40 genes are known to cause CMT, making it an extremely genetically heterogeneous disorder (<http://www.molgen.vib-ua.be/CMTMutations/>). Notably, the known gene products do not represent a single functional category, but range from molecules regulating and establishing the myelin component of the peripheral nervous system (PNS) to ubiquitously expressed proteins. Intriguingly, genetic defects in six aminoacyl-tRNA synthetases (aaRSs) – GARS (Antonellis et al., 2003), YARS (Jordanova et al., 2006), AARS (Latour et al., 2010), KARS (McLaughlin et al., 2010), HARS (Vester et al., 2013) and MARS (Gonzalez et al., 2013) lead to CMT, highlighting the importance of this protein family in PNS homeostasis. Paradoxically, every cell requires the aaRSs function, as they catalyze the aminoacylation of cognate tRNAs during protein biosynthesis, yet mutations specifically affect the maintenance of peripheral neurons.

We reported that mutations in the tyrosyl-tRNA synthetase (*YARS*) cause dominant intermediate CMT type C (DI-CMTC) (Jordanova et al., 2006) and contributed to the finding that genetic alterations in glycyl-tRNA synthetase (*GARS*) lead to CMT2D and distal spinal muscular atrophy type V (dSMA-V) (Antonellis et al., 2003). To date, four genetic

variants in *YARS* (Jordanova et al., 2006; Hyun et al., 2013) and 13 in *GARS* (referenced in Motley et al., 2010; Lee et al., 2012) have been reported in CMT patients and not in control individuals. In addition to the substitutions observed in humans, two dominant mutations (C201R and P234KY) in *Gars* cause neuropathy phenotypes in mice (Achilli et al., 2009; Seburn et al., 2006). Both holoenzymes function as homodimers. CMT2D mutations cause a range of changes in the ability of the proteins to homodimerize: some mutations completely abolish dimerization, while others have normal or even enhanced dimerization ability (Nangle et al., 2007). DI-CMTC mutations do not significantly affect the dimerization properties of *YARS* (Jordanova et al., 2006). Furthermore, the *YARS* gene encodes for a protein responsible for the charging of tRNA^{TYR} exclusively in the cytoplasm (the *YARS2* paralog is responsible for charging in mitochondria), while the *GARS* gene encodes both cytoplasmic and mitochondrial forms (Ibba and Soll, 2000). We and others extensively studied the canonical function of *YARS* and *GARS* *in vitro* and *in vivo* and demonstrated that several of the mutant proteins retain full aminoacylation activity, thereby excluding a loss-of-function mechanism (Nangle et al., 2007; Seburn et al., 2006; Storkebaum et al., 2009). In addition, studies of the *Gars*^{P234KY} mouse model have made several putative pathomechanisms considerably less likely. These include haploinsufficiency, dominant-negative effects on enzymatic activity, mistranslation, and protein misfolding with resulting aggregation (Motley et al., 2011; Seburn et al., 2006; Stum et al., 2011). Interestingly, the severity of the phenotype in this mouse is unchanged when crossed to the *Wld^S* mutation, suggesting that the mechanism of neurodegeneration is distinct from Wallerian degeneration (Stum et al., 2011). Taken together, this suggests that CMT-causing dominant mutations in *YARS* and *GARS* (and perhaps other *aaRSs*) induce a gain of toxic function or interfere with an unknown function of the wild-type (Wt) protein. There are no clues to what this pathologic function could be and whether it is common to both enzymes.

To gain insights into the *aaRS*-associated disease mechanisms we previously developed a *Drosophila* DI-CMTC model, representing the first fly model of an inherited peripheral neuropathy (Storkebaum et al., 2009). We observed signs of motor impairment and electrophysiological dysfunction in *Drosophila*, reproducing the main disease hallmarks. In contrast, mutations in *GARS* that were identified in humans have not yet been successfully studied at the whole-organismal level in an animal model. Therefore, we generated and characterized flies expressing the G240R mutation, which was identified in a large North-American CMT2D family (Antonellis et al., 2003). This substitution was selected because its pathogenicity is supported by strong genetic linkage data and it has among the most severe CMT2D phenotypes. In order to compare our findings with the well-characterized *Gars*^{P234KY} mouse, this variant was also modeled. Importantly, despite targeting neighboring residues, these mutations have different impact on *GARS* aminoacylation activity: G240R abolishes this function, while P234KY has no effect. We also aimed to compare the toxicity triggered by mutations in *GARS* and *YARS* in *Drosophila*. Finally, we have used our fly models to establish the first genetic link between *GARS* and *YARS* in the context of neurodegeneration. While a connection between CMT2D and DI-CMTC has been hypothesized, here we provide experimental evidence that two genes interact in common with CMT-causing variants of both *aaRSs*.

Materials and Methods

Generation of transgenic flies

The BDGP *Drosophila* Gene Collection cDNA clone GH09263 was obtained from the *Drosophila* Genomics Resource Center (Bloomington) and PCR amplified. The forward primer (Aats-Gly-BglIIF) was designed to ligate to the beginning of the cytoplasmic portion of the *Aats-gly* cDNA (after the mitochondrial targeting sequence) and to add a *Bg*III restriction site and a *Drosophila* Kozak consensus sequence upstream of the start site; the reverse primer (Aats-Gly-HA-*Xho*IR) was designed to add a hemagglutinin (HA) epitope tag and a *Xho*I restriction site. The PCR product was isolated using Qiaquick PCR purification kit (Qiagen) and cloned into the pCR2.1-TOPO (Invitrogen) vector using manufacturers protocols. A wild-type TOPO clone was mutagenized with a protocol adapted from the Quickchange Site Directed Mutagenesis kit (Stratagene) using Phusion Taq Polymerase and *Dpn*I (New England Biolabs). Sequencing analysis identified clones with desired mutations. The Wt and mutant constructs were excised from the TOPO vector and purified using the Qiaquick Gel Extraction kit (Qiagen). The inserts were ligated into the pUAST vector (Brand and Perrimon, 1993) using QuickLigase (New England Biolabs). pUAST clones were screened using restriction digestion with *Hind*III. Clones with insert were isolated using an Endo-Free Maxi Kit (Qiagen) and sequence verified. These clones were then sent to BestGene Inc. for injection and balancing. For each of the three constructs several fly lines were established.

Drosophila genetics and behavioral assays

The *UAS-YARS* flies used in the modifier screen were described in (Storkebaum et al., 2009). The *A307-*, *ShakB-* and *c42.2-Gal4* drivers and their expression pattern have been previously reported elsewhere (Allen et al., 1998; Godenschwege et al., 2002; Jacobs et al., 2000). The *nsyb-Gal4* driver line (Pauli et al., 2008) was kindly provided by M. Leyssen and B. Dickson (Research Institute of Molecular Pathology, Vienna, Austria). The *Act5C-Gal4^{strong}* and *Act5C-Gal4^{weak}* driver lines correspond to *P{Act5C-Gal4}25FO1* and *P{Act5C-Gal4}17bFO1* lines from Bloomington *Drosophila* Stock Center (BDSC). We obtained from BDSC also the *P{EPgy2}* and *P{XP}* enhancer promoter (*EP*) lines, as well as the following stocks: *w¹¹¹⁸*; *G1/TM6B*, *UAS-Apoliner* (Bardet et al., 2008), and *GMR-htau.Ex* (referred as *Gl-Tau* in the text). The RNAi lines for CG15599 and CG8316 corresponding to lines 108268 and 26956, respectively, were obtained from Vienna *Drosophila* RNAi Center (VDRC).

All *Drosophila* crosses were performed at 25°C, 12h light/dark cycle, on standard NutriFly medium (Flystuff).

The insertion sites of *P{XP}CG8316^{d01774}*, *P{EPgy2}EY06842* and *P{EPgy2}EY02909* were determined by inverse PCR following the protocol indicated in the Gene Disruption Project (<http://flypush.imgen.bcm.tmc.edu/pscreen/>).

Adult flies' eclosion ratios were determined by the number of *Act5C-Gal4>transgene* versus the number of *Balancer/transgene* flies. Lethality phase determination was done according to the description of Bainbridge and Bownes (1981).

Negative geotaxis was performed on adult age-matched female flies, as described in (Storkebaum et al., 2009). The time needed for the fastest fly from the start of the ascent at the vertical wall to a mark at a height of 82 mm was measured. For each group of 10 flies, the experiment was done 10 times and the average of these 10 walking speeds was calculated. For each genotype, 10 groups of 10 flies were tested.

Electrophysiological recordings from the giant fiber circuit

Intracellular recordings from the TTM of adult male and female flies were obtained as previously described (Allen and Godenschwege, 2010). Briefly, the giant fibers (GFs) were activated with 0.03 ms pulses of 30–60 V using two tungsten electrodes inserted into the brain (Grass S44 stimulator, Grass Instruments). Saline-filled glass electrodes were used for recordings from the TTM and a tungsten electrode in the abdomen served as a ground electrode. The recordings were amplified (Getting 5A amplifier, Getting Instruments) and the signals were stored and analyzed using pCLAMP software (Molecular Devices). For the response latencies, individual stimuli were used to determine the time delay between the stimulation of the GFs and the recording of a response in the TTM. The ability of the GF to TTM pathway to follow high frequency stimulations was assessed with 10 trains of 10 pulses given at 100 Hz with a 1 second interval between the trains. The average following frequencies were calculated as percent responses.

Dye injections into the giant fiber

The giant fiber dye-injection and imaging methods have previously been described in detail (Boerner and Godenschwege, 2010; Boerner and Godenschwege, 2011). Briefly, the GF axons of dissected central nervous systems were dye-injected within the cervical connective using a glass electrode (80–100 M Ω) filled with tetramethylrhodamine-labeled dextran (Invitrogen) in 2M potassium acetate by passing depolarizing current. The samples fixed in 4% paraformaldehyde were scanned at a resolution of 1024 $\times$ 1024 pixels, 2.5x zoom, and 0.5 μ m step size with a Nikon C1si Fast Spectral Confocal system using a 60x (1.4 NA) oil immersion objective lens. Images were processed using Nikon Elements Advance Research 4.0.

Histological analysis of the giant fiber neurons

The ventral nerve cord of adult female flies was isolated and fixed with 4% PFA in PBS. The *A307-Gal4>UAS-Apoliner>UAS-GARS* flies were visualized without additional staining. The *A307-Gal4>UAS-GARS* flies were incubated with monoclonal anti-HA mouse antibody (1:750, Covance). We used Alexa Fluor® 594 goat anti-mouse secondary antibody (1:1000, Life Technologies) to detect the HA tag. The samples were mounted in 90% glycerol supplemented with 0,5% N-propylgallate. Z-stacks were taken on a Zeiss LSM700 confocal microscope (Carl Zeiss) using a LD LCI 25x (0.8 NA) Plan-Apochromat objective with oil immersion and a 40x (1.3 NA) EC Plan-Neofluar oil immersion objective. Image preparation was performed using ImageJ (Schneider et al., 2012).

Retinal degeneration screen

GMR-Gal4; hYARS_E196K/TM6B or *GMR-Gal4; GARS_P234KY/TM6B* virgins were crossed with *EP* males. In case of *EP* male sterility or lethal *EP*-element insertion, crosses were repeated *vice versa*, using *EP* virgins and *GMR-Gal4; hYARS_E196K/TM6B* males. In F1, female flies heterozygous for *GMR-Gal4>hYARS_E196K* (or *GARS_P234KY>EP* and *GMR-Gal4>TM6B>EP* genotypes were compared with each other. Positive hits were selected when rough eye phenotype was present in flies with the first genotype and was absent in flies with the second genotype, respectively.

Scanning electron microscopy

Adult flies were terminally anesthetized with ether and directly mounted on aluminum stubs (Electron Microscopy Sciences) without any tissue processing steps. After gold sputter coating, the eyes of the flies were imaged using scanning electron microscopy (SEM) with an SEM505 microscope (Philips).

Western blot analysis

Western blot was performed as described in (Storkebaum et al., 2009) on head protein extracts. We used mouse monoclonal primary antibodies against YARS (1:500, Abnova), HA-polypeptide (1:5000, Covance), α -tubulin (1:10000, Abcam) and horseradish peroxidase-labelled secondary antibodies (Jackson ImmunoResearch Laboratories Inc.). A digital 16-bit image of the chemiluminescent signal was acquired with ImageQuantTM LAS 4000 (GE Healthcare Life Sciences). Quantification of the band intensities was performed using ImageJ (Schneider et al., 2012). In brief, after background subtraction of the entire image, the lanes were selected and their intensity profiles were plotted with the ImageJ “Analyze > Gels” commands. On these intensity plots of the lanes, the ImageJ magic wand tool was used to measure the area under the peaks as a quantification of the intensity of the band. The intensity of the GARS-HA bands was normalized to the intensity of the α -tubulin bands.

Statistical analysis

The GraphPad Prism 4 and epiR softwares were used for statistical analyses. One-way ANOVA was used to evaluate the statistical significance of the differences in GARS protein expression levels in different transgenic lines. Z-test for one proportion was performed to compare differences in the observed *Act5C-Gal4>GARS_Wt* vs. *Balancer/GARS_Wt* ratio, compared to the expected 1:1 ratio. Mantel-Haenzsel analysis was performed to compare eclosion ratios of flies carrying the different mutant genotypes against flies carrying the wild type transgene. For both strong and weak *Act5C-Gal4* drivers, odds ratios of *Act5C-Gal4>GARS* vs. *Balancer/GARS* per experiment, as well as pooled odds ratios across experiments were calculated to take into account three independent experiments. Cochran's Q test was used to assess heterogeneity between independent experiments. Unpaired Student's t-test was used to analyze the negative geotaxis data. One-way ANOVA with Bonferroni's Multiple Comparison Test was applied to analyze the GF electrophysiology data. The negative geotaxis and GF electrophysiology data are presented as mean $\pm$ standard error of mean (s.e.m.).

In silico protein analyses

Expression data and functional annotations were retrieved from FlyBase and modENCODE (Roy et al., 2010). ClustalOmega was used for protein sequence alignment (Sievers et al., 2011). For interspecies orthologue detection we used OrthoDB (Waterhouse et al., 2013), InParanoid (Ostlund et al., 2010), and BLAST (Altschul et al., 1990). Conserved domain prediction was done using InterPro and CDD (Marchler-Bauer et al., 2011). Remote homology search was performed with PSI-BLAST (Altschul et al., 1997), HHPred from the MPI Bioinformatics Tool Kit (Biegert et al., 2006) and FUGUE (Shi et al., 2001). PSI-BLAST was run with default settings, except that low complexity regions were filtered, and using 3 and 4 iterations for CG15599 and CG8316, respectively. Secondary structure and functional predictions were performed with the Jpred3 Web service (Cole et al., 2008) on the alignment of the *Drosophila* orthologues, and with ELM (Puntervoll et al., 2003). ELM was also used to check for the presence of peptide motifs and transmembrane domains. Subcellular localization was further predicted using ngLOC (King and Guda, 2007), Euk-mPLoc v.2.0 (Chou and Shen, 2010) and CELLO v.2.5 (Yu et al., 2004). We applied FFPred for predicting the function of proteins independent of homology using a feature-based machine-learning approach (Lobley et al., 2008).

Results

Ubiquitous and panneuronal expression of the cytoplasmic form of mutant GARS is toxic in *Drosophila*

As in mammals, the *Drosophila* genome contains a single gene encoding both cytoplasmic and mitochondrial isoforms of glycyl-tRNA synthetase (*Aats-Gly*) (Chihara et al., 2007). The *Drosophila* Aats-Gly (referred to as GARS in the text) is 60% identical and 75% similar to the human (GlyRS) and mouse (Gars) orthologues. The *GARS* mutations evaluated in this study affect conserved amino acid residues (Supplemental Fig. 1). We targeted the expression of cytoplasmic forms of *Wt* and mutant *GARS* transgenes to different cells and tissues in *Drosophila* using the Gal4-UAS system (Brand and Perrimon, 1993). Transgenic lines with comparable expression levels were used in the experiments (Supplemental Fig. 2A and 2B).

Initially, we tested the effect of *GARS* mutations at the whole organism level, by ubiquitously expressing the transgenes with two *Act5C-Gal4* drivers (strong and weak). *UAS-GARS* males were crossed with *Act5C-Gal4/Balancer* virgins and we counted the number of flies in the F1 generation that eclosed with the two possible genotypes (*Act5C-Gal4>transgene* and *Balancer/transgene*). The expected ratio of eclosure for these genotypes is 1:1. *Act5C^{strong}-Gal4>GARS_Wt* expression mildly affected the eclosion ratio, while *Act5C^{weak}-Gal4>GARS_Wt* ubiquitous overexpression had no effect on viability, and flies eclosed at the expected ratio (Fig. 1A and Supplemental Table 1). Strong *GARS_G240R* expression severely impaired the fly development at late pupal stage (P15) and yielded only 3% adult escapers, while weak expression had no detrimental effect. Importantly, both strong and weak ubiquitous expression of *GARS_P234KY* induced lethality in the first instar larval stage and no adult *Act5C-Gal4>GARS_P234KY* flies emerged (Fig. 1A and Supplemental Table 1). The observed phenotypes were confirmed with 2 independent

transgenic lines of each genotype. This data suggests that mutant GARS is toxic when expressed ubiquitously in *Drosophila*

We then sought to evaluate putative toxic effects induced by specific expression of *GARS* mutations in the nervous system of *Drosophila*, using the *nsyb-Gal4* driver. The *nsyb-Gal4>GARS_P234KY* flies reached late stage of pupal development (P15), but were unable to execute the pupal to adult ecdysis. *Nsyb-Gal4*-driven expression of *GARS_G240R* and *GARS_Wt* yielded viable adult flies, whose motor performance was scored in a negative geotaxis assay. We could not detect significant differences in climbing ability of 10 day-old flies. However, progressive motor impairment of *GARS_G240R* compared to *GARS_Wt* animals was observed upon ageing, at day 20 and 30 (Fig. 1B). This suggests that while wild-type *GARS* is not toxic in the fly nervous system, *GARS_G240R* and *GARS_P234KY* are both harmful to neurons. In particular, *GARS_G240R* is neurotoxic in an age-dependent manner, suggesting that this is a faithful model of CMT2D.

Mutations in *GARS* affect functionality and morphology of the giant fiber system in *Drosophila*

To circumvent the observed lethality associated with *GARS_P234KY* and to test the effect of both mutations, we restricted their expression to the neurons of the giant fiber circuit in adult flies. The appropriately named giant axons of the circuit have already proven useful in modeling CMT in *Drosophila*, as we demonstrated progressive impairment of synaptic strength and reliability when expressing mutant YARS proteins (Storkebaum et al., 2009). Similarly, we assessed the impact of *GARS* mutations on GF function upon *A307-Gal4* expression in the GFs and their postsynaptic target neurons throughout their development. We recorded the response latency (RL) and the ability to follow 100 Hz repetitive stimulations of the GF-tergotrochanteral motoneuron-tergotrochanteral muscle (GF-TTMn-TTM) circuit (Fig. 2A and 2B), as a measure for synaptic strength and reliability of the GF-TTMn connection (Allen and Godenschwege, 2010). Upon GF stimulation in the brain, we observed a mild but significant RL increase in the *GARS_G240R* flies (~1 ms) when compared to controls (*GARS_Wt* and *A307-Gal4/+*) (~0.8 ms) (Fig. 2C). Unlike the control flies, the GF-TTMn synaptic reliability decreased in one day old *GARS_G240R* expressing flies (Fig. 2D), and did not progress in 8 and 16 day old flies (data not shown). In contrast, in 40% of the recordings in *GARS_P234KY* flies, the TTM response was completely absent when the GF was stimulated, suggesting that GF stimulation did not evoke a postsynaptic potential in the TTMn or that the synaptic strength was below the threshold to trigger an action potential. However, in the remaining 60% of the recordings the RL was severely increased to ~1.8 ms (Fig. 2A and 2C), which associated with significantly reduced ability of the GF-TTMn-TTM pathway to follow high frequency one-to-one stimulations already one day after eclosion (Fig. 2B and 2D).

Furthermore, we expressed the *GARS_P234KY* transgene presynaptically, after the GF to TTMn synaptic contact had been established (*c42.2-Gal4*), as well as postsynaptically in the TTMn throughout motor neuron development (*ShakB-Gal4*). Although neither expression affected the RL significantly (Fig. 2A and 2E), the ability of the GF-TTMn to TTM pathway to follow repetitive stimulations was significantly reduced to ~50% when *GARS_P234KY*

was expressed with either Gal4-line (Fig. 2B and 2F). This suggests that *GARS_P234KY* has a disruptive effect in the GF and the TTMn during development, but also on the synapse function after its development.

In order to determine the GF morphology in *GARS_P234KY* mutants and *GARS_Wt* control animals, fluorescent dye was injected into the GF axons. In comparison to *GARS_Wt* controls, the synaptic terminal of *A307-Gal4>GARS_P234KY* flies was abnormally thin and the axon as well as the synaptic terminal contained prominent vacuoles (Fig. 2G). These anatomical phenotypes correlated with the physiological defects noted earlier, and were reminiscent to the ones observed in the mutant *YARS*-expressing animals (Storkebaum et al., 2009).

The electrophysiological and axon-terminal abnormalities were not due to apoptotic cell death, as evaluated by the subcellular localization of the *in vivo* caspase reporter Apoliner (Bardet et al., 2008) (Fig. 2H). This is in line with our findings that despite the severe functional and morphological synaptic defects, the neurons of the GF circuit were present in all analyzed animals. Consistent with findings in the *Gars^{P234KY}* mice, no mislocalization or aggregation of *GARS_P234KY* was observed and the mutant protein had a comparable distribution with *GARS_Wt* in the GF and TTMn (Fig. 2I).

GARS and YARS share common genetic modifiers in Drosophila

We have previously shown that retinal expression of *YARS_E196K* (*GMR-Gal4>YARS_E196K*) induced a rough eye phenotype in a dose-dependent manner (Storkebaum et al., 2009). Here, we observed that also *GMR*-targeted expression of *GARS_P234KY* causes a mild rough eye phenotype (Fig. 3D). Retinal expression of *GARS_Wt* (Fig. 3E) and *GARS_G240R* (data not shown) did not affect the ommatidial organization.

We made use of the sensitized background induced by one copy of *YARS_E196K* to perform a gain-of-function screen for genetic enhancers of the retinal degeneration (Rorth, 1996). Flies expressing *YARS_E196K* transgene in the eye (*GMR-Gal4; YARS_E196K/TM6B*) were crossed to flies carrying enhancer promoter *P*-element insertions (*EPs*), targeting genes on the *Drosophila* X-chromosome (Bellen et al., 2004; Thibault et al., 2004) and enhancement of the rough eye phenotype was scored in the female animals of F1-progeny, which were heterozygous for the *EP*-insertion. Out of 614 *EPs* screened, we encountered a phenotype on their own in 2.93% and an absence of interaction in 96.74% of them. We identified two lines (*P{XP}CG8316^{d01774}* and *P{EPgy2}EY06842*, referred as *d01774* and *EY06842* in the text) that specifically enhanced the *YARS_E196K*-associated rough eye phenotype (Fig. 3I and 3O). Expression of *d01774* alone had mild effect on the ommatidial organization (Fig. 3H), which was not further modified upon co-expression of *YARS_Wt* (Fig. 3J). *GMR-Gal4*-driven expression of *EY06842* did not exhibit phenotype *per se*, nor when co-expressed with *YARS_Wt* (Fig. 3N and 3P).

We further tested whether the mutant *YARS*-specific modifiers alter the *GARS_P234KY* induced eye phenotype. We co-expressed *GARS_P234KY* with *d01774* or *EY06842* and we observed a strong enhancement in both cases (Fig. 3K and 3Q). The co-expression of

GARS_Wt with the modifiers did not exhibit any phenotype, confirming the mutant-specificity of the interactions (Fig. 3L and 3R).

We aimed to identify the genes targeted by the *d01774* and *EY06842* elements. The insertion site of all *EP* lines used in our screen has previously been molecularly mapped (Bellen et al., 2004; Thibault et al., 2004). *EY06842* was annotated as inserted in CG43132 (*Drosophila* genome release 5.33) and ~1kb upstream from CG15599; the insertion of *d01774* was in the 3rd exon of CG8316. We have remapped the insertion sites of both *EP*-elements and confirmed their molecular coordinates and orientation (Supplemental Fig. 3A and 3B). *EY06842* could either disrupt the CG43132 gene, or promote transcription of the neighboring CG15599 gene. An independent *EP*-element inserted in CG43132 (*P{EPgy2}EY02909*) in the 5'-3' orientation, whose topology was re-confirmed (data not shown), did not interact with *YARS_E196K* and *GARS_P234KY* alleles. This excluded the possibility that alteration of CG43132 expression by the *EP*-element is causing the rough eye enhancement. Furthermore, by co-expressing an RNAi transgene against CG15599 (*GMR-Gal4>UAS-YARS_E196K>EY06842>RNAi^{CG15599}*), the rough eye phenotype was fully suppressed (Fig. 3S), confirming the modifier identity. Similarly, downregulation of CG8316 transcript using RNAi reverted the rough eye phenotype of *GMR-Gal4>UAS-YARS_E196K>d01774* flies and proved that the gene targeted by *d01774* is CG8316 (Fig. 3M). Notably, the YARS protein levels were not changed in the background of *d01774* or *RNAi^{CG8316}*, indicating that the modification effect was not due to altered *YARS_E196K* expression (Supplemental Fig. 2C).

Furthermore, we overexpressed *d01774* and *EY06842* in the retina of two fly neurodegenerative models (*Gl-Tau* and *Gl¹*) (Jackson et al., 2002; Fan and Ready, 1997). Pathological alterations in Microtubule-associated protein tau are implicated in several brain neurodegenerative disorders, including Alzheimer's disease and frontotemporal dementia (Spillantini and Goedert, 2013). Mutations in the human ortholog of *Gl¹* (Dynactin 1) are associated with motoneuron disorders like distal hereditary motor neuropathy with vocal paresis (Puls et al., 2003) and amyotrophic lateral sclerosis (Munch et al., 2004; Munch et al., 2005). The rough eye phenotypes of *Gl-Tau* and *Gl¹* flies were not enhanced by *GMR-Gal4* induced expression of *d01774* or *EY06842*, emphasizing their specific interaction with mutant forms of *YARS/GARS*, rather than enhancing additive effects unrelated to the aaRS pathomechanism (Supplemental Fig. 3C–3K).

An initial database search revealed that the common *YARS* and *GARS* modifiers have low to moderate expression throughout the developmental stages of *Drosophila* and interestingly, both genes share similar expression dynamics (modENCODE expression cluster 21) (Roy et al., 2010). They encode orphan proteins with unknown molecular function that have orthologues only within the *Drosophila* genus (12 and 5 orthologues for CG8316 and CG15599, respectively) (Supplemental table 2A). Neither a conventional BLAST, nor remote homology searches could identify other convincing orthologues outside the genus, as the hits had a very low sequence identity and were very diverse in function (Supplemental table 2B). Domain analysis detected hits to part of cyclin dependent kinase 7 (CDK7) and Med15, but these are likely artefactual as they are not conserved even in the orthologues of the modifiers in other *Drosophila* species (Supplemental figure 4). The crystal structure of

both proteins or any close orthologues is unknown. In the N-terminal part of CG8316 a coiled-coil structural motif is predicted, while the rest of the protein is predicted to be largely disordered (Fig. 4A). The CG15599 protein is largely disordered; secondary structures are predicted mainly near the C-terminus (Fig. 4B). Feature-based function prediction indicated that both proteins are likely involved in the regulation of transcription and cytoskeletal protein binding (Supplemental Table 3). Furthermore, both proteins contain predicted nuclear localization sequence (NLS) motifs, CG15599 has a predicted nuclear export sequence (NES) motif (Fig. 4A and 4B), and their nuclear localization is computationally predicted by three different programs (Supplemental Table 4). Thus, extensive data mining could not identify vertebrate orthologues, however it provided potentially useful predictions as to the structure and function of CG8316 and CG15599.

Discussion

Glycyl-tRNA synthetase is the most studied of the aaRSs involved in CMT, however molecular and cellular mechanisms linking this and the other aminoacyl-tRNA synthetases to PNS degeneration remain unclear (Motley et al., 2010). Mechanistic insights into CMT2D pathology came from cellular and yeast studies (Antonellis et al., 2006; Nangle et al., 2007), as well as 2 mouse models having motor and sensory deficits (Achilli et al., 2009; Seburn et al., 2006). Two CMT2D mutations (E71G and L129P) were examined in fruit flies, but no phenotypes could be detected even in aged flies (Chihara et al., 2007). Therefore, our study reports the successful generation of a CMT2D *Drosophila* model, where the effect of two *GARS* mutations was studied at behavioral, electrophysiological and morphological levels, and upon ubiquitous or spacio-temporally restricted expression. In addition to the patient mutation G240R, we studied the P234KY mutation in the fly for three reasons. First, we opted to compare mutations affecting neighboring residues in the catalytic domain of GARS, with divergent effects on charging function (fully active and completely inactive, respectively). Second, we can benefit from the advantages offered by *Drosophila* as a model organism to gain mechanistic insights and identify therapeutic targets, which can be further validated in the existing mouse model. Finally, the phenotype of *Gars*^{P234KY} mouse is arguably more severe than any of the human disease phenotypes and most likely to have deleterious effects *in vivo*.

Unlike the YARS-encoded protein which charges tRNA only in the cytoplasm, GARS encodes both the cytoplasmic and mitochondrial isoforms of glycyl-tRNA synthetase. In order to compare the effects induced by both synthetases, we expressed only the cytoplasmic form of GARS in the flies. Based on our experience with the DI-CMTC model (Storkebaum et al., 2009), we tested the Wt and mutant animals in the same experimental paradigms. Similar to *YARS_E196K* (Storkebaum et al., 2009), ubiquitous expression of *GARS_P234KY* severely affected the fly fitness with no adults emerging. Since both mutations do not impair the aminoacylation activity of the respective proteins and they are overexpressed on the background of active endogeneous enzymes, these results reconfirm that loss of canonical activity is not a prerequisite for aaRSs toxicity. Ubiquitous expression of the enzymatically inactive GARS_G240R protein had a dosage-dependent effect on the preadult lethality, as has been observed for the YARS mutants with reduced or fully abolished aminoacylation activity (YARS_156VKQV159 and YARS_G41R) (Jordanova et al., 2006; Storkebaum et

al., 2009). The neurotoxic effect of *GARS_P234KY* expression was further demonstrated at the panneuronal level, where again no adult flies emerged. Panneuronal expression of *GARS_G240R* rendered flies exhibiting progressive motor impairment. These findings further underscore the important role of GARS for normal neuronal function, which is abolished to a different degree by the two mutations.

When targeted to the giant fiber circuit, the *GARS_G240R* flies only mildly impaired the strength and reliability of the GF-TTMn synapse. Contrastingly, expression of *GARS_P234KY* during GF development fully disrupted, or severely weakened, the function of the GF to TTMn connection, which was associated with increased RL and a compromised ability to follow high frequency stimulations, similar to DI-CMTC flies (Storkebaum et al., 2009). Consistent with the electrophysiological phenotype, the synaptic terminal of the GF in *GARS_P234KY* animals was abnormally thin. This suggests that *GARS_P234KY* interferes with the development of the giant axon terminal. In addition, we observed numerous large vacuoles in the axon and synaptic terminal of *GARS_P234KY* but not *GARS_Wt* animals. Although the nature of the vacuoles remains to be determined, they may be indicative of degenerative phenotype. This is supported by the finding that expression of *GARS_P234KY* after the GF-TTMn connection was established, also disrupted the function of the GF synapse. In addition, expression of *GARS_P234KY* in the TTMn affected the function of the GF-TTMn connection although much less severe, suggesting that mutant GARS has also a toxic effect in the motoneuron. Therefore, it seems plausible that *GARS_P234KY* interferes with neuronal differentiation and maintenance of the electrical-chemical GF synapse presynaptically and postsynaptically, but the exact role of GARS in these processes remains to be elucidated. Finally, the anatomical and functional GF phenotypes are similar to those previously described for the expression of DI-CMTC mutations in *YARS* (Storkebaum et al., 2009), suggesting that mutant *YARS* and *GARS* disrupt neuronal development, maintenance and function via similar mechanisms.

The enzymatically active *GARS_P234KY* protein induced more severe defects in all paradigms. This nicely correlates with what is described in mice. The *Gars^{P234KY}*, when compared to *Gars^{C201R}* mouse, has more severe neuronal defects, and exhibit decreased life span and body mass (Motley et al., 2010). The deleteriousness of this particular mutation is therefore well conserved between flies and mice, highlighting the complementarities of both models.

Furthermore, we detected similar patterns of toxicity in the CMT2D and DI-CMTC flies, suggesting that the nervous system of *Drosophila* is susceptible to the effects induced by two CMT-associated aaRSs. Noteworthy, expression of the cytoplasmic isoform of mutant GARS was sufficient to induce phenotypes. This suggests that neurodegeneration occurring in both CMT subtypes can be triggered by pathways outside the mitochondria. One approach to identify those pathways is by identifying the interacting partners of the mutant enzymes. Since our fly models faithfully recapitulate the disease pathophysiology, they could be used for further interaction studies.

The identification of genetic modifiers of CMT-associated genes has not previously been performed in *Drosophila*. Here, in the context of our DI-CMTC and CMT2D models we

initiated an unbiased pilot screen using the rough eye phenotype common to mutant alleles of both aaRSs. Our genetic screen identified CG8316 and CG15599 as shared mutant-specific enhancers. Further studies of the modifiers were compromised by their unknown molecular function and lack of orthologues outside the *Drosophila* genus. Extensive *in silico* domain and structural analysis on these orphan proteins could not provide direct insight into their function. Coiled-coil structures, as present in CG8316, are found in proteins with very diverse functions. Unstructured regions, as annotated in CG8316 and CG15599, are also not immediately functionally informative. However, a feature-based machine-learning approach could assign regulation of gene expression and interaction with cytoskeletal proteins as common functions for CG8316 and CG15599. Both modifier proteins were also predicted to be located in the nucleus by several computational methods. Interestingly, many aaRSs (including YARS) are detected in the nucleus of eukaryotic cells (Nathanson and Deutscher, 2000; Popenko et al., 1994; Fu et al., 2012). Their currently known nuclear functions are related to quality control of tRNA nuclear export and transcriptional regulation of protein-coding and ribosomal genes (Grosshans et al., 2000; Ko et al., 2000; Lund and Dahlberg, 1998; Sarkar et al., 1999; Yannay-Cohen et al., 2009). The predicted functions of the modifiers could point to a putative impairment of these biological processes in the aaRSs-related neurodegeneration, however additional studies are necessary to confirm this possibility. Furthermore, identification of more genetic modifiers will enrich the aaRSs interaction network and will provide supporting evidence for the abovementioned or new molecular pathways to be tested experimentally in neuron-specific functional assays.

Our results represent the first genetic evidence that two genes interact in common with CMT-causing alleles of both *GARS* and *YARS*. In the context of a large-scale screening strategy in *Drosophila*, these findings are encouraging as we anticipate being able to pinpoint additional commonalities between the YARS- and GARS-related toxicity. We hypothesize that the targets of these mutant proteins will likely be shared, and analyzing two disease models will help eliminate targets that are not central to their PNS pathogenesis. In the context of CMT disease, putative common pathomechanisms would unravel avenues for targeted drug design and therapy for both CMT forms and possibly other aaRS-related neuropathies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- First animal model of a *GARS* mutation causing peripheral neuropathy in humans
- CMT mutations in *GARS* and *YARS* induce comparable phenotypes in *Drosophila*
- The cytoplasmic isoform of GARS is sufficient to trigger neurotoxicity in flies
- CMT mutations in *YARS* and *GARS* have common genetic modifiers in *Drosophila*

Fig. 1. Expression of mutant *GARS* in *Drosophila* causes pre-adult lethality and induces motor impairment

A) Ubiquitous expression of *GARS_Wt* has mild (with *Act5C^{strong}-Gal4*) or no effect (with *Act5C^{weak}-Gal4*) on the expected 1:1 adult flies' eclosion ratio. *GARS_G240R* expression has a dosage-dependent effect on the adult offspring eclosion, while *GARS_P234KY* expression has detrimental effect upon both strong and weak ubiquitous expression. On the Y-axis the ratio of genotypes eclosing per cross is indicated. Dashed line marks the expected 1:1 genotypes' eclosion ratio. ***, $P < 0.001$; ns – non-significant were determined after Mantel-Haenzel analysis which compares the odds ratios (*Act5C-Gal4>transgene* vs. *Balancer/transgene*) of flies with ubiquitous expression of wild type and mutant *GARS* flies of three independent experiments.

B) Panneuronal expression of *GARS_G240R* with *nsyb-Gal4* renders adult flies, which exhibit progressive motor impairment as evaluated in a negative geotaxis assay. The Y-axis indicates the time needed for the fastest fly to climb to a height of 82mm. *, $P < 0.05$ and **, $P < 0.01$, was determined after unpaired Student's t-test.

Fig. 2. Electrophysiological and morphological impairment of the GF-TTMn-TTM circuit in mutant *GARS* expressing flies is not associated with apoptotic cell death or *GARS* mislocalization and aggregation

A) Sample traces of recordings from TTM upon GF stimulation (black arrow) in the brain. Control animals have response latency ~0.8 ms (dashed line), while A307-Gal4>*GARS_P234KY* flies have significantly increased response latency. The response latency of *ShakB-Gal4*>*GARS_P234KY* animals is not affected and is similar to controls. Asterisk indicates the recorded response.

B) Sample traces of recordings from TTM upon applying train of 10 stimuli at 100 Hz. Control animals respond (asterisk) one-to-one to each applied stimulus, while *A307-Gal4>GARS_P234KY* flies have severely impaired ability to follow high frequency stimulations. The *ShakB-Gal4> GARS_P234KY* animals respond to ~50% of the applied stimuli.

C) Average response latency of wild type and *GARS* mutant flies upon *A307-Gal4* expression. Expression of *GARS_G240R* and *GARS_P234KY* in the GF circuit throughout development significantly increases the response latency in comparison to control flies (*A307-Gal4/+*). In contrast, expression of *GARS_Wt* has no effect on the response latency.

D) Average following frequency at 100 Hz of wild type and *GARS* mutant flies upon *A307-Gal4* expression. Expression of *GARS_G240R* and *GARS_P234KY* significantly decreases the ability to follow stimuli at 100 Hz when compared to control flies or flies expressing *GARS_Wt*.

A minimum of ten one day old flies (20 TTMs) were tested for each genotype in (C) and (D). *, $P<0.05$; **, $P<0.01$ and ***, $P<0.001$, one-way ANOVA with Bonferonni Multiple Comparison Test.

E) Presynaptic (*c42.2-Gal4*) expression after GF synapse formation or postsynaptic (*ShakB-Gal4*) expression throughout development of *GARS_P234KY* flies does not significantly affect the response latency when compared to control flies (*Gal4-drivers/+*) or flies expressing the transgene with the *A307-Gal4* driver. *A307-Gal4* data are reused from panel C of this figure.

F) The ability to follow stimuli at 100 Hz significantly decreases when *GARS_P234KY* is expressed with *A307-Gal4*, *c42.2-Gal4* and *ShakB-Gal4* drivers, compared to control flies (*Gal4-drivers/+*). A minimum of five one day old flies (10 TTMs) were tested for each genotype in (E) and (F). *, $P<0.05$; **, $P<0.01$ and ***, $P<0.001$, one-way ANOVA with Bonferonni Multiple Comparison Test. *A307-Gal4* data are reused from panel D of this figure.

G) Giant fiber morphology in wild type and mutant *GARS* flies. Dye injected in the *A307-Gal4>GARS_Wt* GF axon is homogenously distributed through the axon and synaptic terminal. In contrast to *A307-Gal4>GARS_Wt* flies, the GF terminal of *GARS_P234KY* flies is thinner and vacuolar structures (arrows) are observed throughout the axon and synaptic terminal. Scale bar in (G) is 20 μm .

H) TTMn cell of a ten-day old female flies expressing Apoliner (*Apo*) under the control of *A307-Gal4* driver. The eGFP and mRFP fluorescence signals are shown independently and in overlay. Assessment of caspase-induced cleavage of the Apoliner reporter does not reveal apoptosis in the TTMn cell bodies of *GARS_Wt* and *GARS_P234KY* flies, as the eGFP signal does not translocate to the TTMn nucleus, but co-localizes with the mRFP signal. eGFP – enhanced green fluorescent protein; mRFP – monomeric red fluorescent protein. Scale bar is 20 μm .

I) GARS-HA immunofluorescence pattern in the giant fiber axons, giant fiber-TTMn synapsing area and TTMn cell bodies upon *A307-Gal4* driven expression of *GARS_Wt* and *GARS_P234KY*. Both wild type and mutant flies have a comparable distribution pattern of GARS-HA along the GF axon and in the TTMn cell body, without any visible protein aggregation. Scale bar is respectively 20 μm and 10 μm for the GF and TTMn images.

Fig. 3. YARS and GARS mutant-specific interactions in the adult fly retina

Control flies (*GMR-Gal4/+*) (A) or flies expressing *YARS_E196K* (B), *YARS_Wt* (C) and *GARS_Wt* (E) in the retina do not exhibit rough eye phenotype. *GMR-Gal4>GARS_P234KY* expression renders mild rough eye phenotype (D). *GMR* targeted expression of *d01774* mildly affects the retina on its own (H), or when co-expressed with *Wt YARS* and *GARS* forms (J and L, respectively). In contrast, it provokes a strong rough eye phenotype when co-expressed with *YARS_E196K* and *GARS_P234KY* (I and K). Retinal expression of *EY06842* *per se* does not show a phenotype (N), or in *Wt YARS* and *GARS* background (P and R, respectively), but gives a strong interaction when co-expressed with mutant *YARS* and *GARS* (O and Q, respectively). Retinal expression of *RNAi^{CG8316}* and *RNAi^{CG15599}* on their own does not give a phenotype (F and G, respectively). Retinal co-expression of *d01774*, *RNAi^{CG8316}* and *YARS_E196K* fully recovers normal ommatidial organization (M), proving that *d01774* is mis-expressing the *CG8316* gene. Likewise, simultaneous expression of *EY06842*, *RNAi^{CG15599}* and *YARS_E196K* restores the normal morphology of the fly eye (S). Representative SEM micrographs at lower and higher magnification are presented. Scale bars in (A) are 50 μ m.

Fig. 4. Schematic representation and disorder propensities of CG8316 (A) and CG15599 (B)
 The Y-axis represents the disorder tendency values per amino acid residue as calculated by the IUPred program run on the ELM server. The X-axis depicts the number of amino acid residues. The dashed line at 0.5 of Y-axis indicates the threshold value for disordered/structured residues. Scores above 0.5 correspond to the disordered residues/regions, whereas scores below 0.5 indicate residues/regions predicted to be ordered. Black boxes in (A) and (B) indicate nuclear localization signal motifs (NLS), patterned box - nuclear export signal motif (NES), and CC - coiled-coil domain as predicted by ELM. CG8316 has two predicted overlapping NLS motifs with probability scores $p = 0.00072$ and $p = 0.0013$ respectively. CG15599 has NES ($p = 7.62e^{-04}$) and NLS ($p = 1,276e^{-03}$) motifs.