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An assessment of mechanisms underlying peripheral axonal degeneration caused by aminoacyl-tRNA synthetase mutations

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ABSTRACT

Mutations in glycyl-, tyrosyl-, and alanyl-tRNA synthetases (*GARS*, *YARS* and *AARS* respectively) cause autosomal dominant Charcot–Marie–Tooth disease, and mutations in *Gars* cause a similar peripheral neuropathy in mice. Aminoacyl-tRNA synthetases (ARSs) charge amino acids onto their cognate tRNAs during translation; however, the pathological mechanism(s) of ARS mutations remains unclear. To address this, we tested possible mechanisms using mouse models. First, amino acid mischarging was discounted by examining the recessive "*sticky*" mutation in alanyl-tRNA synthetase (*Aars*^{sti}), which causes cerebellar neurodegeneration through a failure to efficiently correct mischarging of tRNA^{Ala}. *Aars*$^{sti/sti}$ mice do not have peripheral neuropathy, and they share no phenotypic features with the *Gars* mutant mice. Next, we determined that the Wallerian Degeneration Slow (*Wlds*) mutation did not alter the *Gars* phenotype. Therefore, no evidence for misfolding of GARS itself or other proteins was found. Similarly, there were no indications of general insufficiencies in protein synthesis caused by *Gars* mutations based on yeast complementation assays. Mutant GARS localized differently than wild type GARS in transfected cells, but a similar distribution was not observed in motor neurons derived from wild type mouse ES cells, and there was no evidence for abnormal GARS distribution in mouse tissue. Both GARS and YARS proteins were present in sciatic axons and Schwann cells from *Gars* mutant and control mice, consistent with a direct role for tRNA synthetases in peripheral nerves. Unless defects in translation are in some way restricted to peripheral axons, as suggested by the axonal localization of GARS and YARS, we conclude that mutations in tRNA synthetases are not causing peripheral neuropathy through amino acid mischarging or through a defect in their known function in translation.

Introduction

Mutations in three genes encoding aminoacyl-tRNA synthetases (ARSs) have recently been associated with dominant peripheral neuropathies (Charcot–Marie–Tooth disease) (Antonellis et al., 2003; Jordanova et al., 2006; Latour et al., 2010). The extent to which these conditions share common pathogenic mechanisms and what these mechanisms may be remain unclear. We therefore compared possible pathogenic mechanisms in mice with mutations in two ARS genes, and examined other phenotypes related to the known function of these enzymes.

The canonical activity of ARSs is to charge amino acids onto their cognate tRNAs so they can function in translation (Ibba and Soll, 2004; Schimmel, 2008). As such, these enzymes are responsible for maintaining the fidelity of the genetic code. The mammalian nuclear genome contains 37 ARS genes, generally with two genes encoding an enzyme for each amino acid — one encoding the cytosolic enzyme, the other encoding the enzyme that functions in mitochondrial protein synthesis (Antonellis and Green, 2008). In some cases, a single gene encodes both the cytosolic and mitochondrial forms of the protein by using alternative start codons, as is the case for glycyl-tRNA synthetase (*Gars*, or GARS for the protein). Thus, each ARS gene is essential for its nonredundant role in translation.

Human mutations in mitochondrial aspartyl-tRNA synthetase (*DARS2*) and mitochondrial arginyl-tRNA synthetase (*RARS2*) cause leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation (LBSL), and pontocerebellar hypoplasia and

Abbreviations: ARS, aminoacyl-tRNA synthetase; GARS, glycyl-tRNA synthetase; YARS, tyrosyl-tRNA synthetase; AARS, alanyl-tRNA synthetase; CMT, Charcot–Marie–Tooth disease; *Wlds*, Wallerian Degeneration Slow; HSMN, hereditary sensory and motor neuropathy; DI-CMTC, dominant intermediate Charcot–Marie–Tooth type C; CMT2D, Charcot–Marie–Tooth type 2D; CAST, Castaneus; YFP, yellow fluorescent protein; WT, wild type; ES, embryonic stem cell; P#, postnatal age in days.

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multiple mitochondrial respiratory-chain defects, respectively (Edvardson et al., 2007; Scheper et al., 2007). In both cases, the mutations are in the genes encoding the mitochondrial forms of the enzymes, and the disease phenotypes may be a reflection of mitochondrial dysfunction arising from impaired protein synthesis.

A recessive mutation in the cytosolic form of alanyl-tRNA synthetase (the *sticky* mutation, *Aars*^{sti}) causes a neurological phenotype in mice through a different mechanism (Lee et al., 2006). This mutation is a single amino acid change in the editing domain of AARS (A734E), causing a subtle but significant decrease in the ability of AARS to proofread mischarged tRNA^{Ala}. The most common mischarging of this tRNA is expected to be with glycine or serine, given the molecular dimensions of the enzyme's active site and data from editing deficient bacterial alanyl-tRNA synthetase (Beebe et al., 2008; Guo et al., 2009). The *Aars*^{sti} homozygous mice have degeneration of Purkinje cells that contain ubiquitin inclusions as well as other non-neuronal phenotypes such as trichodystrophy. Protein misfolding is caused by a low level of amino acid misincorporation in place of alanine residues across the entire proteome, resulting in neurodegeneration.

In addition, three human peripheral neuropathies, Charcot–Marie–Tooth type 2D (CMT2D), Dominant Intermediate Charcot–Marie–Tooth type C (DI-CMTC), and a third recently characterized form of CMT2 are caused by mutations in glycyl-tRNA synthetase (*GARS*), tyrosyl-tRNA synthetase (*YARS*), and alanyl-tRNA synthetase (*AARS*) respectively (Antonellis et al., 2003; Jordanova et al., 2006; Latour et al., 2010). Interestingly, mutations in *DARS2* also cause an axonal neuropathy in some patients (Isohanni et al., 2010), and a patient with mutations in lysyl-tRNA synthetase (*KARS*) has also recently been identified (McLaughlin et al., 2010). This patient carries compound heterozygous mutations including an amino acid substitution (L133H) that causes a severe reduction in tRNA charging activity, and a frame shift (Y173SerfsX7) that causes a presumed null allele. Severe neurological and behavior phenotypes were present, including an axonal neuropathy. While there may be pathological gain-of-function effects of the point mutation, a loss-of-function of KARS activity seems likely to be contributing to the severity of the phenotype.

The mutations in *YARS* that cause DI-CMTC are proposed to be partial loss-of-function or dominant-negative changes, based on yeast complementation studies and altered subcellular localization of the mutant protein in transfected cells (Jordanova et al., 2006). Studies in *Drosophila* also indicate that expression of mutant forms of YARS specifically in neurons results in axonal defects, but mammalian models of DI-CMTC are not yet available (Storkebaum et al., 2009). Mutant forms of the GARS protein also have altered distribution, but there is a less clear correlation between retained enzymatic activity and the disease phenotype (Antonellis et al., 2006; Nangle et al., 2007). Mice with a spontaneous, dominant mutation in *Gars* (*Gars*$^{Nmf249/+}$) develop a peripheral neuropathy very similar to human CMT2D (Seburn et al., 2006). This phenotype is not evident in mice heterozygous for a loss-of-function gene trap allele that decreases expression of *Gars* at the RNA level with a corresponding reduction in enzyme activity in tissue homogenates. Similar but milder neuromuscular dysfunction is also seen in second, dominant *Gars* allele (*Gars*^{C201R}) (Achilli et al., 2009). These results suggest that the disease phenotype of CMT2D requires the expression of the mutant protein and this interpretation is supported by studies of protein expression in patient cell lines (Antonellis et al., 2006). The neuropathy mutation in *AARS* affects a residue (R329H) critical for binding and aminoacylation of tRNA^{Ala} based on studies of the bacterial protein, but how this mutation affects the function of the mammalian protein has not been tested and a mischarging mechanism analogous to the *Aars*^{sti} mouse has been suggested (Latour et al., 2010).

There are several possible mechanisms by which ARS mutations could cause disease (Motley et al., 2010). For example, disease-causing mutations could change the structure of the active site of the tRNA synthetase, allowing misincorporation of amino acids, analogous to the editing domain mutation in *Aars*^{sti}. Indeed, GARS does not have an editing domain because its active site is normally conformationally constrained to accept only glycine, the smallest amino acid (Arnez et al., 1999; Logan et al., 1995). A crystal structure of the human GARS protein is solved, and some mutations are predicted to influence the conformation of the active site, but many are not, although this has not been experimentally tested (Xie et al., 2007).

The ARS phenotypes could also arise from defects in protein synthesis caused by insufficient enzyme activity. In some *GARS* mutations, enzyme activity assayed *in vitro* is reduced, and in other cases it is not (Antonellis et al., 2006; Nangle et al., 2007; Seburn et al., 2006). Even in cases where enzymatic activity is retained, activity within cells could be compromised if the enzymes are not properly localized and therefore not available to participate in translation (Antonellis et al., 2006; Nangle et al., 2007). Furthermore, the mutant proteins could result in toxic aggregates, as noted for other neurodegenerative conditions, or they could be assuming a novel pathological function. However, the incidence of three ARS genes causing peripheral neuropathy suggests a common mechanism related to their shared function in translation.

To test these possible mechanisms, we have compared the phenotypes and pathogenic changes in the mouse *Aars*^{sti} and *Gars*^{Nmf249} mutations and examined GARS localization and the *in vivo* ability of the mutant protein to sustain protein synthesis. We conclude that *Aars*^{sti} and *Gars*^{Nmf249} are indeed distinct phenotypes, affecting different cell populations through different mechanisms, making it unlikely that the human *GARS*, *YARS* and *AARS* mutations cause amino acid misincorporation. Furthermore, if the *Gars*$^{Nmf249/+}$ phenotype arises from deficits in protein synthesis, this is unlikely to be a general phenomenon, but may represent dysfunction specifically in axons based on analyses of cell morphology, activity and localization. Consistent with this, the GARS and YARS proteins are found in peripheral axons and Schwann cells, suggesting a direct role for the protein in peripheral nerves.

Results

Peripheral neuropathy versus Purkinje cell loss in Aars$^{sti/sti}$ *and Gars*$^{Nmf249/+}$ *mice*

To compare the pathogenic mechanisms of mutations in *Aars* and *Gars* and to determine if mischarging of tRNA^{Ala} can lead to peripheral neuropathy, we first compared the phenotypes in cell populations affected by each mouse mutation. As previously described, *Gars*$^{Nmf249/+}$ mice have overt neuromuscular dysfunction and peripheral neuropathy beginning at 2–3 weeks of age (Seburn et al., 2006). In contrast, *Aars*$^{sti/sti}$ mice have ataxia and cerebellar Purkinje cell death caused by protein misfolding, but peripheral axons were not examined, although dominant mutations in human AARS were recently shown to cause an axonal Charcot–Marie–Tooth disease (Latour et al., 2010; Lee et al., 2006). We therefore examined the femoral nerves and neuromuscular junctions of *Aars*$^{sti/sti}$ mice to look for signs of peripheral neuropathy. There was no decrease in axon number in the motor branch of the femoral nerve, even at 14 months of age (Figs. 1A–C, 540 ± 40, 521 ± 36 and 590 ± 54 in control, young *Aars*$^{sti/sti}$, and old *Aars*$^{sti/sti}$ mice respectively). Similarly, there was no defect in NMJ morphology or motor nerve terminal occupancy in the gastrocnemius, with the nerve terminal completely overlapping the field of postsynaptic acetylcholine receptors (Figs. 1D,E). The apparent decrease in overall cross-sectional area of the nerve (Figs. 1A,B) is consistent with a significant, uniform decrease in axon diameter, but no change in the G-ratio (a comparison of axon diameter to myelin thickness). The decreased nerve diameters correlate with a general decrease in body size (at least 20% decrease in body weight) in the *Aars*$^{sti/sti}$ mice. Therefore, the *Aars*$^{sti/sti}$ mice do not develop a peripheral neuropathy phenotype, even at ages when the loss of Purkinje cells in the cerebellum is nearly complete.