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tRNA Dysregulation in Neurodevelopmental and Neurodegenerative Diseases

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Keywords

tRNA, aminoacyl tRNA synthetase, tRNA modification, tRNA splicing, angiogenin, integrated stress response

Abstract

Transfer RNAs (tRNAs) decode messenger RNA codons to peptides at the ribosome. The nuclear genome contains many tRNA genes for each amino acid and even each anticodon. Recent evidence indicates that expression of these tRNAs in neurons is regulated, and they are not functionally redundant. When specific tRNA genes are nonfunctional, this results in an imbalance between codon demand and tRNA availability. Furthermore, tRNAs are spliced, processed, and posttranscriptionally modified. Defects in these processes lead to neurological disorders. Finally, mutations in the aminoacyl tRNA synthetases (aaRSs) also lead to disease. Recessive mutations in several aaRSs cause syndromic disorders, while dominant mutations in a subset of aaRSs lead to peripheral neuropathy, again due to an imbalance between tRNA supply and codon demand. While it is clear that disrupting tRNA biology often leads to neurological disease, additional research is needed to understand the sensitivity of neurons to these changes.

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INTRODUCTION

The central dogma of molecular biology holds that DNA is transcribed into messenger RNA (mRNA), which is translated into proteins. Proteins are polypeptides composed of amino acid building blocks and are the drivers of most cellular functions. Often taken for granted in the translation of mRNA into protein is the role of transfer RNAs (tRNAs), which are small RNA species responsible for decoding mRNA triplet codons into polypeptides. To accomplish this, tRNAs are charged with amino acids at their 3′ ends by aminoacyl tRNA synthetases (aaRSs) (Ibba & Soll 2000, Rubio Gomez & Ibba 2020), and their tRNA anticodon sequences bind to three base pair codons on mRNAs at the ribosome. The specificity of these processes, putting the correct amino acid onto its corresponding anticodon tRNA and the pairing of tRNA anticodons with mRNA codons at the ribosome, is essential to the fidelity of translation and therefore fundamental to all biology. This review examines how a variety of defects in the tRNA life cycle can lead to an array of neurological disorders.

The genetic code is degenerate and most amino acids are encoded by more than one codon, with the exceptions of methionine (AUG) and tryptophan (UGG), which use only one (see **Table 1**). Furthermore, in the human genome there are 429 genes encoding tRNAs [see the genomic tRNA database GtRNAdb (**http://gtrnadb.ucsc.edu**), hg38/GRCh38] despite there being only 61 codons (and three stop codons) in mRNA. Therefore, there are often multiple tRNA genes in the genome for any given tRNA species. tRNAs with different anticodon sequences that are charged with the same amino acid are referred to as isoacceptors. tRNAs with the same anticodon sequence but sequence differences outside the anticodon are referred to as isodecoders. Furthermore, different tRNA genes may give rise to mature tRNAs with identical sequences. The number of isoacceptor tRNAs for each amino acid and the number of genes in the human genome encoding each isodecoder family are shown in **Table 1**. The number of tRNA genes for a given isodecoder family loosely correlates with the frequency of the appearance of its corresponding codon in the genome (**Table 1**). It is notable that for 15 mRNA codons, no tRNA genes with a corresponding anticodon are found in the human genome (**Table 1**). Although incomplete genome annotation may contribute, this is a reproducible phenomenon across genomes (Ehrlich et al. 2021). The

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Table 1 tRNA gene abundance and codon usage

Amino acid	Codon	Anti-codon	Number of genes	Codon usage
Phe	UUU	AAA	0	1.76
	UUC	GAA	10	2.03
Leu	UUA	UAA	4	0.77
	UUG	CAA	6	1.29
	CUU	AAG	9	1.32
	CUC	GAG	0	1.96
	CUA	UAG	3	0.72
	CUG	CAG	9	3.96
Ile	AUU	AAU	15	1.6
	AUC	GAU	3	2.8
	AUA	UAU	5	0.75
Met	AUG	CAU	9i,11 (10i, 13)	2.2
Val	GUU	AAC	9	1.1
	GUC	GAC	0	1.45
	GUA	UAC	5	0.71
	GUG	CAC	13	2.81
Ser	UCU	AGA	9	1.52
	UCC	GGA	0 (1)	1.77
	UCA	UGA	4	1.22
	UCG	CGA	4	0.44
Pro	CCU	AGG	9	1.75
	CCC	GGG	0 (2)	1.98
	CCA	UGG	7	1.69
	CCG	CGG	4	0.69
Thr	ACU	AGU	9	1.31
	ACC	GGU	0	1.89
	ACA	UGU	6	1.51
	ACG	CGU	5	0.61
Ala	GCU	AGC	26	1.84
	GCC	GGC	0	2.77
	GCA	UGC	8	1.58
	GCG	CGC	4	0.74
Tyr	UAU	AUA	0 (1)	1.22
	UAC	GUA	13	1.53
Stop	UAA	NA	NA	0.1
	UAG	NA	NA	0.08
His	CAU	AUG	0	1.09
	CAC	GUG	9	1.51
Gln	CAA	UUG	6	1.23
	CAG	CUG	13	3.42
Asn	AAU	AUU	0 (2)	1.7
	AAC	GUU	25	1.91
Lys	AAA	UUU	12	2.44
	AAG	CUU	15	3.19

(*Continued*)

Table 1 ***(Continued)***

Amino acid	Codon	Anti-codon	Number of genes	Codon usage
Asp	GAU	AUC	0 (1)	2.18
	GAC	GUC	13	2.51
Glu	GAA	UUC	8	2.9
	GAG	CUC	8	3.96
Cys	UGU	ACA	0 (1)	1.06
	UGC	GCA	29	1.26
Selenocysteine	UGA	UCA	1 (2)	
Stop	UGA	NA	NA	0.16
Trp	UGG	CCA	7	1.32
Arg	CGU	ACG	7	0.45
	CGC	GCG	0	1.04
	CGA	UCG	6	0.62
	CGG	CCG	4	1.14
Ser	AGU	ACU	0 (1)	1.21
	AGC	GCU	8	1.95
Arg	AGA	UCU	6	1.22
	AGG	CCU	5	1.2
Gly	GGU	ACC	0	1.08
	GGC	GCC	14	2.22
	GGA	UCC	9	1.65
	GGG	CCC	5	1.65

Data from the human genome (**http://gtrnadb.ucsc.edu**, GRCh38/hg38) show the codon/anticodon pairs for each amino acid as well as stop codons. The number of tRNA genes annotated in the human genome for each anticodon is indicated. Numbers in parentheses indicate the number of genes predicted if all gene models are included rather than just high confidence predictions in which potential pseudogenes and sequences unlikely to fold into functional tRNAs are excluded. Genes for $tRNA^{Met}$ include initiator tRNAs as well as those involved in translation elongation. Codon usage is shown based on the Codon Usage Database (**http://www.kazusa.or.jp/codon/**). Abbreviations: NA, not applicable; tRNA, transfer RNA.

missing anticodons are consistently one of a group of tRNAs in which the first base of the anticodon, corresponding to the more variable third base of the codon, the wobble position, is not represented. The proposed explanation for this is that translational accuracy is actually greater in some cases where non-Watson–Crick base pairing is used, which relies on posttranscriptional modification of the tRNAs to allow a different anticodon tRNA to decode the mRNA (Akashi 1994, Blanchet et al. 2018, Gingold & Pilpel 2011). There is now data in *Escherichia coli* directly supporting this hypothesis (Sun & Zhang 2022). Therefore, both through multicopy genes in the genome and through near-cognate tRNA decoding, tRNA genes have been largely considered functionally redundant. The clear exception to this is the mitochondrial genome, where there are only 22 tRNA genes, one for each amino acid except serine and leucine, which have two genes encoding distinct isoacceptors (Suzuki et al. 2020). Thus, mitochondrial translation provides a system in which tRNA genes are not functionally redundant and in which a single anticodon is able to pair with multiple codons, enabled by a variety of posttranscriptional modifications and by noncanonical base pairing.

Interestingly, some of the same principles demonstrated by mitochondrial tRNAs (mt-tRNAs), such as the importance of base modifications and the lack of functional redundancy, also apply to cytosolic translation mediated by tRNAs encoded in the nuclear genome. Modifications of tRNAs can affect their structure, decoding capacity, and stability. There are many different

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chemical modifications of tRNAs and an increasing list of diseases associated with altered tRNA modification (Chujo & Tomizawa 2021). These alterations can occur through mutations in the tRNAs themselves, which change a modified base, or through mutations in the modifying enzymes. In addition to posttranscriptional chemical modification of the nucleotide bases, tRNAs are subject to a number of other processing steps. In the human genome, 28 tRNAs contain an intron that must be spliced out to produce the mature tRNA sequence. These include all 13 genes encoding the $tRNA^{Tyr}{}_{GUA}$ isodecoder family and all 5 genes encoding the $tRNA^{Ile}{}_{UAU}$ isodecoder family, as well as 5 genes for $tRNA^{Leu}{}_{CAA}$ and 5 for $tRNA^{Arg}{}_{UCU}$ (note that the amino acid encoded by the tRNA is superscripted, and the specific anticodon of the tRNA is subscripted). In addition to the splicing of some transcripts, tRNAs are processed by ribonuclease (RNase) P to remove 5′ leader sequences and by endo- and exonucleases to remove 3′ trailer sequences (Schurer et al. 2001, Xiao et al. 2002), followed by the addition of three bases (CCA) to the 3′ end by a template-independent nucleotidyltransferase to produce the mature tRNA and allow amino acid charging (Hou 2010). Additional RNases such as angiogenin can also cleave tRNAs internally in response to cell stress, and the tRNA fragments produced can have signaling properties (Lyons et al. 2017). Finally, mutations in many aaRSs that charge amino acids onto tRNAs cause disease (Meyer-Schuman & Antonellis 2017, Wei et al. 2019). Many of these mutations are biallelic (recessive), leading to a variety of syndromic clinical presentations, but an interesting subset is monoallelic (dominant) and leads to the specific degeneration of peripheral motor and sensory neurons, suggesting a mechanism distinct from the severe partial loss of tRNA charging activity seen for biallelic mutations.

Thus, multiple tRNA processing, splicing, modification, maturation, and charging steps affect tRNA function, and as anticipated, defects in these processes can lead to dysfunction because a large number of tRNAs are potentially impacted, overriding functional redundancy among the tRNAs even within isoacceptor or isodecoder families. Similar to mt-tRNAs, emerging evidence indicates that nuclear-encoded cytosolic tRNAs are also nonredundant. Surprisingly, the lack of redundancy manifested in specific neuronal populations in the mouse brain (Ishimura et al. 2014). This observation suggests cell-type specificity to tRNA gene expression and raises several important issues that need to be resolved. First, the control of tRNA gene expression is not well understood, and the mechanisms leading to cell-type-specific tRNA gene expression need to be elucidated. Second, the pool of tRNAs expressed in a given tissue and especially in a given cell type is often not known. This is due in part to technical challenges in high-throughput tRNA sequencing and is exacerbated by the sequence similarity of the tRNAs, making the genomic origin of a tRNA difficult to determine. Finally, the available pool of cytosolic tRNAs must be balanced with the codon demand of the transcriptome, but how this is achieved is unclear. Here the term codon demand encompasses the frequency with which a specific codon appears in transcripts in a cell, weighted by the abundance of that transcript, with additional considerations such as whether the transcript is actively translated and the rate of translation necessary to assure proper protein folding and translation elongation without ribosome collisions or stalls. This essentially equates to the frequency at which a charged tRNA from a given pool with the same anticodon sequence, an isodecoder family, has to be recruited to a ribosome, and this may be cell-type dependent given differences in the transcriptomes, proteomes, and metabolic states of different cells.

Here we review recent advances in how altered tRNA biology leads to disease, particularly neurological disorders. We describe the evidence that tRNAs are not functionally redundant and provide examples where defects in tRNA splicing, processing, and modification lead to disease. We also describe disorders associated with mutations in aaRSs and discuss pathogenic mechanisms for both biallelic and monoallelic mutations. Finally, we highlight challenges and areas requiring more

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research to understand how altered tRNA biology leads to disease and why the nervous system seems to be particularly sensitive to defects in translation.

GENETICS OF tRNAs AND FUNCTIONAL REDUNDANCY

The large number of tRNA genes in mammalian genomes implies functional redundancy, but it is becoming clear this is not necessarily the case. In this regard, nuclear tRNA genes may share properties with tRNAs encoded by the mitochondrial genome. Although tRNAs encoded in the nuclear genome of mammals, notably $tRNA^{Gln}$, can be imported into mitochondria, the mitochondrial genome of most mammals encodes a minimal but complete set of tRNAs for mitochondrial translation (Rubio et al. 2008, Schneider 2011). Consistent with single copy genes lacking functional redundancy, mutations in mt-tRNA genes invariably lead to disease, and pathogenic mutations in all 22 mt-tRNAs have been identified (Ruiz-Pesini et al. 2007). In humans, the most frequent diseases associated with mutations in mt-tRNA genes are mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) and myoclonic epilepsy associated with ragged-red fibers (MERRF). MELAS is a syndromic disorder, but many of the most severe outcomes are neurological, including seizures, hemiparesis, and cortical blindness. Mutations in at least six mt-tRNAs can lead to MELAS, including mt-$tRNA^{Leu1}$ (Goto et al. 1990; Kobayashi et al. 1990, 1991), mt-$tRNA^{Glu}$ (Bataillard et al. 2001), mt-$tRNA^{His}$ (Melone et al. 2004, Taylor et al. 2004), mt-$tRNA^{Lys}$ (Silvestri et al. 1992, Zeviani et al. 1993), mt-$tRNA^{Cys}$ (Manfredi et al. 1996, Santorelli et al. 1997), mt-$tRNA^{Ser1}$ (Nakamura et al. 1995), and mt-$tRNA^{Ser2}$ (Wong et al. 2006), with the most common mutation being c.3243A>G in mt-$tRNA^{Leu1}$. Similarly, MERRF is associated with mutations in mt-$tRNA^{Lys}$ (Shoffner et al. 1990, Yoneda et al. 1990), mt-$tRNA^{Leu1}$ (Moraes et al. 1993), mt-$tRNA^{His}$ (Melone et al. 2004, Taylor et al. 2004), mt-$tRNA^{Phe}$ (Mancuso et al. 2004), mt-$tRNA^{Ser1}$ (Nakamura et al. 1995), and mt-$tRNA^{Ser2}$ (Wong et al. 2006). As the name implies, the prominent features of MERRF are epilepsy and myopathy, with ragged-red muscle fibers evident in histopathology. The most common mutation associated with MERRF is c.8344 A>G in mt-$tRNA^{Lys}$. The genetic overlap of MELAS and MERRF is evident, and the same mutation in the same family can lead to both diagnoses, and many patients share signs of both diseases. This is consistent with the general variability of mitochondrial disease presentation, and the possibility of variability in mitochondrial genomes within cells and between patients (heteroplasmy).

In contrast to the large number of mt-tRNAs associated with disease, there are no human diseases known to be associated with mutations in nuclear-encoded cytosolic tRNAs, with one atypical exception. A homozygous mutation in the tRNA responsible for selenocysteine incorporation ($tRNA^{Sec}$) was identified in a young male with abdominal pain, weakness, fatigue, thyroid dysfunction, and decreased plasma selenium levels, symptoms consistent with other mutations involved in selenoprotein synthesis (Schoenmakers et al. 2016). Selenocysteine incorporation into proteins has several unique features. First, the amino acid is synthesized on $tRNA^{Sec}$ in a multistep process: Serine aminoacyl tRNA synthetase (encoded by *SARS1*) charges serine onto $tRNA^{Sec}$, and the serine is then converted to selenocysteine (Labunskyy et al. 2014). Second, $tRNA^{Sec}$ is a single copy gene in the nuclear genome (Schoenmakers et al. 2016). Finally, the codon for the incorporation of selenocysteine is UGA, which is typically a stop codon. The $tRNA^{Sec}$ anticodon (UCA) recognizes early stop UGA codons in transcripts encoding selenoproteins when *cis*-acting stem-loop structures in the 3′ untranslated region and associated proteins are present (Labunskyy et al. 2014). Incorporation of selenocysteine at these early stop codons prevents nonsense mediated decay of mRNAs encoding selenoproteins (Shetty & Copeland 2015). Complete removal of $tRNA^{Sec}$ from the mouse genome results in embryonic lethality (Bosl et al. 1997). Thus, given the peculiarities of selenocysteine in translation, the mutation in $tRNA^{Sec}$ is an interesting but atypical case.

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At face value, the lack of disease-causing mutations in typical nuclear tRNA genes is consistent with functional redundancy among isodecoder tRNAs. It is worth noting that the number of genes for isodecoder tRNAs present in genomes decreases in simpler organisms to the point where yeast has very little redundancy (Orellana et al. 2022). Therefore, in simpler organisms, mutations in tRNA genes with phenotypes may be more likely to manifest, but the number of genes for isodecoders is consistently high across vertebrates (Goodenbour & Pan 2006). Humans show copy number variation in tRNA genes, including deletions of a $tRNA^{Lys}$-CUU gene on chromosome 11, which may have implications for the translation of proinsulin, which is rich in AAG codons, and therefore type 2 diabetes (Iben & Maraia 2014), but no firm disease associations with cytosolic tRNAs are known. The sequence of nuclear tRNA genes shows higher than expected variability, but most of this is in the sequences immediately upstream and downstream, and the core sequence of the mature tRNAs is strongly conserved, suggesting evolutionary pressure despite being multicopy (Thornlow et al. 2018).

The expression of tRNA genes is clearly regulated (Pan 2018). Some human tissues have higher tRNA expression levels than others, and interestingly, the brain is narrowly behind only the spleen for the highest levels of cytosolic tRNAs and is highest for the levels of mt-tRNAs (Dittmar et al. 2006). The expression of individual tRNA genes can be examined by chromatin immunoprecipitation using RNA polymerase III (PolIII) antibodies to determine where in the genome tRNA transcription occurs. In one such study comparing cell lines in vitro as well as tumors versus control tissues in vivo, a complementary set of tRNA genes was expressed in proliferating versus differentiating cells (Gingold et al. 2014). Similarly, the tRNA pool was dynamically regulated in developing mouse tissue to maintain a match between available anticodons and the codons of the mRNA transcriptome (Schmitt et al. 2014). In mice, different tissues have different PolIII occupancy, indicating that different tRNA genes are being expressed, but tissues were highly correlated when results were collapsed to the level of isodecoder and isoacceptor families (Kutter et al. 2011). Therefore, not all tRNA genes are expressed equally by all cells at all times, and the expression of these genes is spatially and temporally regulated. Whether tRNA gene expression ever becomes restricted in a given cell or cell type to an extent that results in nonredundancy (an essential dependence on a single tRNA gene within an isodecoder family) is not resolved by these genomic studies.

The strongest evidence indicating that cell-type-specific tRNA gene expression actually leads to nonredundancy comes from mice. The commonly used wild-type mouse strain, C57BL/6J, carries a C50T mutation in *n-Tr20*, encoding $tRNA^{Arg}_{UCU}$ (Ishimura et al. 2014). This mutation in *n-Tr20* results in ribosome stalling at the cognate AGA codons, altered synaptic transmission, reduced seizure susceptibility, and activation of the integrated stress response (ISR) (Kapur et al. 2020). Loss of *n-Tr20* function also suppressed mTor signaling, resulting in reduced levels of phosphorylated p70-S6Kinase and 4E-BP, which may further inhibit translation initiation (Kapur et al. 2020). There are four other $tRNA^{Arg}_{UCU}$ isodecoder genes in the mouse genome, but loss of *n-Tr20* results in a 60% reduction in $tRNA^{Arg}_{UCU}$ levels in the mouse brain, indicating it is the predominantly expressed gene in the nervous system for this isodecoder family (Ishimura et al. 2014). Importantly, deletion of a highly expressed $tRNA^{Ile}_{UAU}$ (*n-Ti17*) also triggered ISR activation and suppression of mTor signaling, indicating that inhibition of translation initiation is a conserved response to tRNA loss and that this gene is also limiting in the brain in this family of four isodecoders (Kapur et al. 2020). The simultaneous inactivation of *n-Tr20* and the ribosome rescue factor *Gtpbp1* or *Gtpbp2* exacerbated ribosome stalling at AGA codons and triggered the degeneration of cerebellar, hippocampal, cortical, and retinal neurons. This indicates that loss of *n-Tr20* function induces ribosome stalling, consistent with ISR activation, but these stalls are normally mostly resolved by *Gtpbp1* or *Gtpbp2* (Ishimura et al. 2014, Terrey et al. 2020). The ribosome

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stalling in *n-Tr20* mutant mice triggers GCN2-mediated ISR activation, which increases eIF2α phosphorylation and ATF4 target gene expression, and this progresses to neurodegeneration if the ribosome rescue factors are also deleted (Ishimura et al. 2016, Kapur et al. 2020, Terrey et al. 2020).

Taken together, the results with mutations in both *n-Tr20* and *n-Ti17* indicate that these tRNA genes are preferentially expressed in the nervous system and that their loss does result in changes in cell physiology and cell stress. These changes only result in frank neurodegeneration if ribosome rescue factors such as *Gtpbp1* or *Gtpbp2* are also compromised, but the other isodecoder $tRNA^{Arg}{}_{UCU}$ and $tRNA^{Ile}{}_{UAU}$ genes in the mouse genome do not fully compensate for the loss of the predominantly expressed genes. The lack of functional redundancy is largely attributable to the tissue specificity of the tRNA gene expression. Additional studies will be needed to see if the nervous system is particularly limited in terms of which tRNA genes it expresses, perhaps explaining some of the reasons mutations affecting tRNAs and the components of the translation machinery often have neurological presentations, or whether other tissues would be similarly compromised if a different subset of tRNA genes were mutated.

DEFECTIVE tRNA METABOLISM IN NEUROLOGICAL DISEASE

Pontocerebellar hypoplasia (PCH) refers to a group of rare, autosomal recessive neurodegenerative disorders, mainly with prenatal onset and typically fatal in infancy or childhood (van Dijk et al. 2018). Patients display severe underdevelopment (hypoplasia) and progressive atrophy of various brain regions, in particular the cerebellum and pons. This results in microcephaly, severe psychomotor retardation, and intellectual disability (van Dijk et al. 2018). Currently, biallelic mutations in 26 genes have been associated with PCH. Six of these genes are involved in tRNA metabolism (*TSEN54*, *TSEN2*, *TSEN34*, *TSEN15*, and *CLP1*) or tRNA aminoacylation (*RARS2*).

Biallelic mutations in each of the four subunits of the tRNA-splicing endonuclease (TSEN) complex cause PCH (Breuss et al. 2016, Budde et al. 2008). TSEN catalyzes intron excision of intron-containing tRNAs (Paushkin et al. 2004) (**Figure 1**). Of the intron-containing tRNA genes in the human genome, $tRNA^{Tyr}{}_{GUA}$ (13 genes) and $tRNA^{Ile}{}_{UAU}$ (5 genes) all contain introns, and 5 of the 6 genes each for $tRNA^{Arg}{}_{UCU}$ and $tRNA^{Leu}{}_{CAA}$ contain introns, so redundancy with non-intron-containing tRNA genes is limited at best. PCH-associated TSEN mutations include mostly missense but also splice site and nonsense mutations, suggesting a partial-loss-of-function mechanism (Breuss et al. 2016, Budde et al. 2008). Both in patient-derived fibroblasts (Sekulovski et al. 2021) and in vitro (Breuss et al. 2016), TSEN–PCH mutations substantially reduce precursor tRNA (pre-tRNA) cleavage activity, leading to the accumulation of pre-tRNAs (Sekulovski et al. 2021). Beyond tRNA splicing, the TSEN complex may also be involved in an mRNA decay pathway, as yeast TSEN cleaves a specific subset of mRNAs (Hurtig et al. 2021). If so, TSEN–PCH mutations may cause a defect in the mRNA cleavage function of TSEN. Thus, it remains to be established whether excessive pre-tRNA accumulation or other molecular mechanisms underlie TSEN-associated PCH.

Homozygosity for an R140H missense mutation in cleavage factor polynucleotide kinase subunit 1 (CLP1) also causes PCH (Karaca et al. 2014, Schaffer et al. 2014). CLP1 is an RNA kinase involved in tRNA, mRNA, and small interfering RNA maturation (de Vries et al. 2000, Weitzer & Martinez 2007). In pre-tRNA splicing, CLP1 acts downstream of the TSEN complex to phosphorylate the 5′ OH of the 3′ tRNA exon and the tRNA intron (**Figure 1**). CLP1-mediated phosphorylation of the tRNA 3′ exon prevents ligation of the 5′ and 3′ exons by the canonical tRNA ligase RTCB to produce the mature tRNA. Thus, CLP1 is a negative regulator of the direct ligation pathway in animal cells (Hayne et al. 2020). tRNA intron phosphorylation by CLP1 precludes circularization of the tRNA intron. Consistently, knockdown of the *Drosophila CLP1* ortholog *cbc* promotes the biogenesis of mature tRNAs and circularized tRNA introns (tricRNAs)

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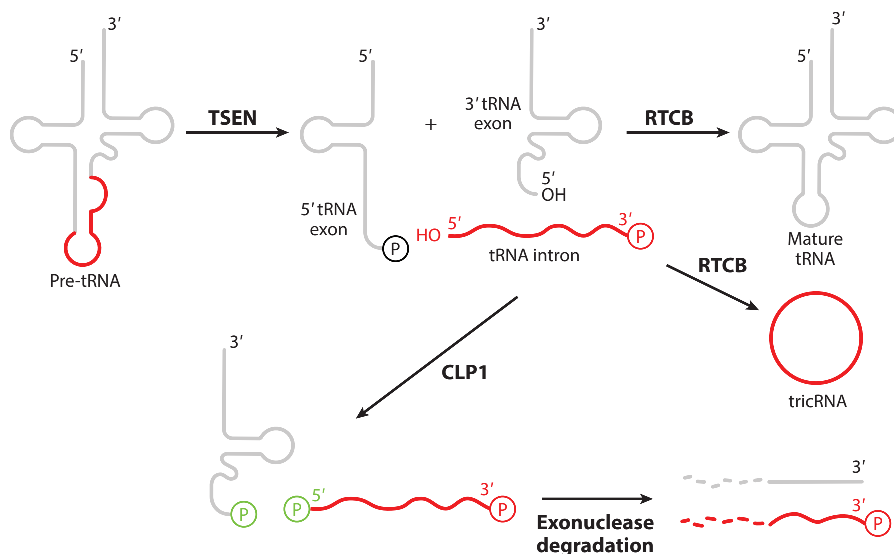

Figure 1

Activities of the TSEN complex and CLP1. The TSEN complex splices introns from pre-tRNAs, producing the 5′ exon, the 3′ exon, and the intron. The tRNA ligase RTCB then produces the mature tRNA from the 5′ and 3′ exons. Decreased TSEN activity leads to an increase in pre-tRNA levels. CLP1 phosphorylates the 5′ end of both the 3′ tRNA exon and the spliced intron. Decreased CLP1 activity leads to increased circularization of the introns by RTCB to produce tricRNAs, and it also decreases exonuclease-mediated degradation of the intron and the 3′ tRNA exon. Abbreviations: pre-tRNA, precursor tRNA; RTCB, RNA 2′,3′-cyclic phosphate and 5′ OH ligase; tricRNAs, circularized tRNA introns; TSEN, tRNA-splicing endonuclease.

(Hayne et al. 2020). Furthermore, CLP1-mediated phosphorylation of tRNA fragments promotes their exonuclease-mediated degradation (Hayne et al. 2020).

The R140H mutation reduces CLP1 kinase activity (Karaca et al. 2014, Morisaki et al. 2021, Schaffer et al. 2014), leading to the accumulation of tRNA fragments in patient cells (Karaca et al. 2014) and brain tissue of *Clp1*$^{R140H/R140H}$ knock-in mice (Monaghan et al. 2021, Morisaki et al. 2021). Consistent with reduced CLP1 kinase activity as a possible pathogenic mechanism in PCH, mice expressing a kinase-dead CLP1 display PCH-like phenotypes and lower motor neuron disease (Hanada et al. 2013, Karaca et al. 2014), similar to *Clp1*$^{R140H/R140H}$ mice (Monaghan et al. 2021), as well as the accumulation of tRNA fragments (Hanada et al. 2013). These tRNA fragments sensitize cells to oxidative stress–induced p53 activation and p53-dependent cell death (Hanada et al. 2013, Schaffer et al. 2014). Consistently, loss of p53 function rescues motor neuron disease in kinase-dead CLP1 mice (Hanada et al. 2013).

Interestingly, CLP1 associates with the TSEN complex (Hayne et al. 2020, van Dijk et al. 2018), and the R140H mutation results in the loss of CLP1 interaction with TSEN (Karaca et al. 2014), destabilization of the TSEN complex (Schaffer et al. 2014), largely reduced pre-tRNA cleavage activity (Karaca et al. 2014), and accumulation of pre-tRNA in patient-derived cells and *Clp1*$^{R140H/R140H}$ mice (Monaghan et al. 2021, Morisaki et al. 2021, Schaffer et al. 2014). Finally, CLP1 also associates with the mRNA cleavage and polyadenylation machinery (de Vries et al. 2000). In the spinal cord of *Clp1*$^{R140H/R140H}$ mice, poly(A) site usage shifted from proximal to distal sites for a subset of mRNAs, correlating with a lower expression level for most of these genes (Monaghan et al. 2021). Thus, impaired mRNA 3′ processing may also contribute to PCH

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pathogenesis. Future research will need to disentangle whether and to what extent the accumulation of pre-tRNAs and/or tRNA fragments (including tricRNAs) and mRNA 3′ processing defects contribute to CLP1-PCH pathogenesis.

A homozygous intronic mutation in *RARS2*—encoding the mitochondrial form of arginyl-tRNA synthetase—leads to an abnormally short RARS2 transcript due to exon 2 skipping and causes severe infantile encephalopathy associated with PCH (Edvardson et al. 2007). In patient fibroblasts, the total and aminoacylated mtRNA^{Arg} levels were reduced, which presumably results in a mitochondrial translation defect that may explain the multiple mitochondrial respiratory chain defects (Edvardson et al. 2007).

tRNA MODIFICATIONS AND DISEASE

tRNAs are heavily modified posttranscriptionally, and alterations in tRNA modifications are increasingly associated with disease. As summarized in recent reviews, there are at least 43 distinct chemical modifications to tRNAs in humans, and each cytosolic tRNA carries an average of 13 modifications per molecule (Chujo & Tomizawa 2021, Pan 2018). Although mt-tRNAs are typically less modified than cytosolic tRNAs, 18 different chemical modifications can be found at 137 different sites on human mt-tRNAs (Suzuki et al. 2020). Over 70 enzymes can modify tRNAs, and mutations in over 50 of these can lead to disease, with more than half presenting as neurological disorders (Chujo & Tomizawa 2021).

The major functions of tRNA modifications are to enhance decoding, stability/degradation, and the local structure of the tRNA stem-loops as well as the global structure (Chujo & Tomizawa 2021), which is L-shaped and required for the tRNA to be charged and to participate in translation (Ishitani et al. 2003, Kim et al. 1974, Robertus et al. 1974) (**Figure 2**). In some cases, changes to a specific tRNA affect its modification and therefore its function, and in other cases, changes to modifying enzymes more broadly impact a population of tRNAs, leading to widespread dysfunction. Examples of diseases arising from each of these mechanisms are provided below.

As mentioned, the most common mutation associated with MELAS is an A>G mutation at position 3243 in the mitochondrial genome (Goto et al. 1990, Yoneda et al. 1990). The consequence of this is a change at U34 in mt-tRNA^{Leu1}. This is the first position of the anticodon, which pairs with the wobble position of leucine codons, and in the presence of the mutation, it is no longer able to be modified to 5-taurinomethyl-uridine (Yasukawa et al. 2000). This results in decreased efficiency of UUG decoding (Kirino et al. 2004). The complex 1 protein NADH dehydrogenase 6 (ND6) is particularly rich in UUG codons and is decreased in abundance as a result (**Figure 2*b***). Interestingly, mutations in ND6 itself can also cause MELAS, supporting the idea that this translation deficit explains why the A3243G mutation is deleterious (Ravn et al. 2001). A change in the modification status of the tRNA is also involved in the mt-tRNA^{Lys} 8344 mutation that causes MERRF (Kirino & Suzuki 2005, Richter et al. 2018). Although this is an example from mitochondrial translation where tRNAs are nonredundant, it provides a proof of principle for a change in a tRNA causing a change in a modification site, resulting in changes in translation efficiency for a key protein with an mRNA rich in the relevant codon. As the cell-type-specific expression of nuclear tRNA genes becomes better defined, similar changes in the cellular proteome related to codon usage may emerge.

The mutation in the selenocysteine tRNA mentioned previously may also act through a change in tRNA modification (Schoenmakers et al. 2016). The mutation identified is a C65>G that leads to decreased levels of tRNA^{Sec}, even in heterozygous carriers. However, tRNA^{Sec} also exists in two pools with either 5-methylcarbonylmethyluridine (mcm^{5}U) or 5-methylcarbonylmethyl-2-*O*-methyluridine (mcm5Um) at position 34. The C65>G mutation leads to a fivefold decrease

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in the mcm5Um form with no decrease in charging of serine, suggesting that the overall tRNA structure is intact. A patient with this mutation showed a preservation of so-called housekeeping selenoproteins but a decrease in stress-related selenoproteins (Schoenmakers et al. 2016). This selective effect on selenoprotein translation is also seen in mice engineered to lack the modification

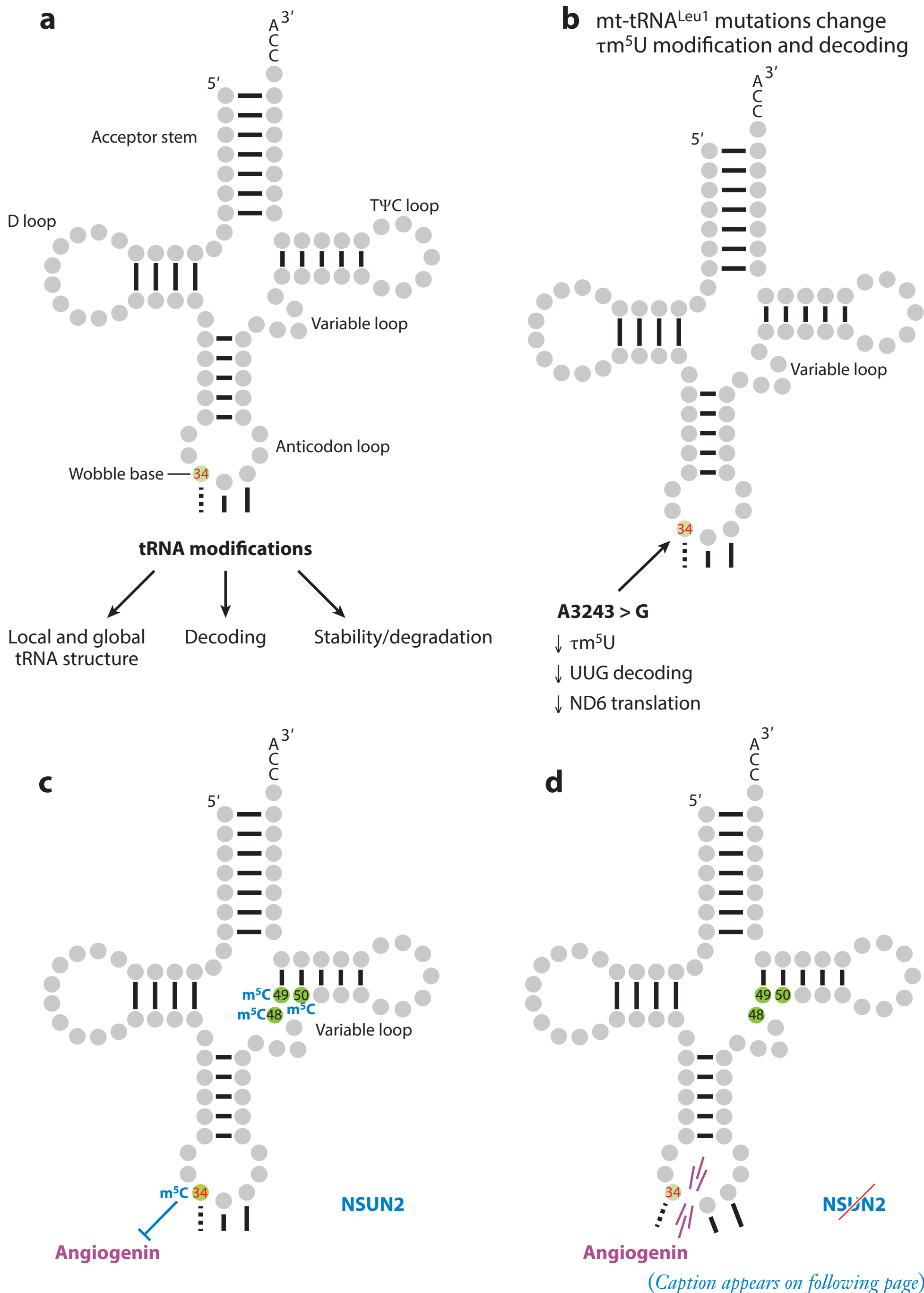

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Figure 2 (*Figure appears on preceding page*)

Effects of posttranscriptional modification of tRNAs. (*a*) tRNA modifications can influence the local stem-loop structure and global tRNA structure, the decoding ability of the tRNA, and the stability and turnover of the tRNA. The first position of the anticodon (34, *green*) pairs with the wobble position of the codon. (*b*) The MELAS-associated A3243>G mutation in mt-tRNA^{Leu1} results in a decrease in the 5-taurinomethyl-uridine modification at position 34, which decreases UUG decoding and translation of the complex 1 protein ND6. (*c*) The activity of NSUN2 deposits 5-methyl-cytosines at positions 34, 48, 49, and 50 on tRNAs (*green bases*). This modification protects the tRNAs from cleavage by the RNase angiogenin. (*d*) In the absence of NSUN2-mediated modification, the tRNAs are cleaved by angiogenin, which cuts in the anticodon loop. Abbreviations: MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; mt-tRNA, mitochondrial tRNA; RNase, ribonuclease; tRNAs, transfer RNAs.

at position 34, suggesting that at least some of the effect of the mutation may be through changes in tRNA^{Sec} modification (Carlson et al. 2007, 2009).

An example of a mutation in a modifying enzyme leading to neurological disease is NSUN2. NSUN2 produces 5-methyl-cytosine (m^{5}C) modifications at multiple sites on tRNAs (positions 34, 48, 49, and 50), an activity shared by DMNT2 (Tuorto et al. 2012). The m^{5}C confers protection against cleavage by angiogenin, which has RNase activity and cleaves tRNAs in their anticodon loop (see below for a discussion of this activity under conditions of cell stress and in neurological disease) (**Figure 2*c***). In the absence of NSUN2 activity, tRNAs are susceptible to cleavage by angiogenin and tRNA fragments accumulate, inducing cell stress pathways (Blanco et al. 2014). In humans, recessive mutations in NSUN2 result in intellectual developmental disorder 5 [Mendelian Inheritance in Man (MIM) 611091], characterized by intellectual disability with other neurological symptoms including microcephaly, delayed speech, and behavioral abnormalities (Abbasi-Moheb et al. 2012, Khan et al. 2012, Martinez et al. 2012). In both knockout mice and NSUN2 patients, the predominant effects of loss of NSUN2 function are neurological, despite widespread expression in other tissues. Again, the sensitivity of the nervous system to changes in tRNA metabolism is clear but unexplained.

MONOALLELIC MUTATIONS IN ANGIOGENIN TRIGGER MOTOR NEURON DISEASE

Mature tRNAs can be cleaved by endoribonucleases to produce tRNA-derived small RNA fragments (Su et al. 2020). Here, we focus on angiogenin-mediated tRNA cleavage because monoallelic missense mutations in the angiogenin (*ANG*) gene are associated with both sporadic and familial forms of amyotrophic lateral sclerosis (ALS) (Greenway et al. 2006, van Es et al. 2011). ALS is an adult-onset disease characterized by progressive degeneration of motor neurons, leading to muscle weakness and wasting, and ultimately complete paralysis and death (Taylor et al. 2016).

Angiogenin was first identified as a potent angiogenic factor secreted from a human adenocarcinoma cell line (Fett et al. 1985). Angiogenin is a member of the pancreatic RNase A family and contains a nuclear localization signal (Lyons et al. 2017). Plexin-B2 is the functional cell surface receptor for angiogenin in endothelial cells and neurons (Yu et al. 2017). Angiogenin binding to Plexin-B2 triggers angiogenin internalization by clathrin-mediated endocytosis and translocation of angiogenin to the nucleus, where angiogenin accumulates in nucleoli and stimulates ribosomal RNA expression (Moroianu & Riordan 1994, Xu et al. 2002). Remarkably, both nuclear translocation and ribonucleolytic activity of angiogenin are required for its angiogenic activity (Hu 1998, Shapiro & Vallee 1989).

In unstressed cells, angiogenin is bound by its inhibitor RNH1. Various types of stress induce the dissociation of angiogenin from RNH1, leading to angiogenin-mediated cleavage of

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tRNAs in their anticodon loop (Yamasaki et al. 2009). This results in the accumulation of 5′ and 3′ tRNA-derived stress-induced small RNAs (tiRNAs), also referred to as 5′ and 3′ tRNA halves (Yamasaki et al. 2009). A subset of 5′ tiRNAs inhibit mRNA translation independent of eIF2α phosphorylation by displacing the eIF4F complex from mRNAs (Ivanov et al. 2011). Angiogenin-induced tiRNAs also inhibit apoptosis through interaction with released cytochrome *c*, thus preventing apoptosome formation and reducing downstream caspase-3 activity (Saikia et al. 2014). Interestingly, tRNA modifications modulate angiogenin-induced tRNA cleavage: tRNA methylation by DNMT2 or NSUN2, or queuosine modification, protects tRNAs against angiogenin cleavage (Blanco et al. 2014, Schaefer et al. 2010, Wang et al. 2018) (**Figure 2**), while demethylation by ALKBH3 renders tRNAs more sensitive to angiogenin cleavage (Chen et al. 2019).

ALS-associated *ANG* mutations result in (partial) loss of either ribonucleolytic activity or nuclear translocation, or both (Thiyagarajan et al. 2012, Wu et al. 2007). Consistently, ALS-mutant angiogenin is unable to induce angiogenesis (Wu et al. 2007). Furthermore, recombinant angiogenin supplementation or wild-type angiogenin expression protects cultured motor neurons from stress-induced cell death in a phosphatidylinositol 3-kinase/Akt-dependent manner and stimulates neurite outgrowth. In contrast, ALS-mutant angiogenin is still internalized in motor neurons, but angiogenin-mediated neuroprotection is compromised (Kieran et al. 2008, Sebastia et al. 2009, Subramanian et al. 2008, Thiyagarajan et al. 2012). Importantly, treatment of the SOD1^{G93A} mouse model of ALS with intraperitoneal (IP) injection of human angiogenin, even when started at age of onset, mitigated motor deficits, increased life span, restored the disease-associated decrease in phosphatidylinositol 3-kinase/Akt signaling, and protected against motor neuron loss and vascular network regression (Crivello et al. 2018, Kieran et al. 2008). IP-injected angiogenin localized to spinal cord astrocytes and endothelial cells (Crivello et al. 2018). Thus, it is currently unclear whether angiogenin-induced therapeutic outcomes are mediated via the vasculature or via neuroprotective effects on motor neurons, either directly or indirectly through effects on astrocytes. Similarly, given that *ANG* is expressed in both motor neurons and endothelial cells (Greenway et al. 2006, Wu et al. 2007), it remains to be established whether *ANG* mutations cause ALS through vascular defects or compromised neuroprotection, or a combination of both. Moreover, whether defective angiogenin-mediated tRNA cleavage causally contributes to ALS pathogenesis is an open question. Finally, the therapeutic potential of recombinant angiogenin administration should be evaluated in additional SOD1-ALS mouse models and in mouse models of other genetic forms of ALS.

MUTATIONS IN AMINOACYL tRNA SYNTHETASE GENES LEAD TO NEUROLOGICAL DISEASE

aaRSs are the enzymes that covalently link amino acids to their corresponding (or cognate) tRNA. aaRSs catalyze tRNA aminoacylation in a two-step reaction: The cognate amino acid is first activated with ATP, leading to the formation of an aminoacyl-adenylate intermediate; next, the activated amino acid is transferred to the cognate tRNA, yielding the aminoacyl-tRNA (**Figure 3*a***) (Ibba & Soll 2000, Rubio Gomez & Ibba 2020). In total, 19 cytoplasmic and 19 mitochondrial aaRSs mediate tRNA aminoacylation, one for each amino acid used in protein biosynthesis, with the exception of the bifunctional cytoplasmic glutamyl-prolyl tRNA synthetase. For the glycyl and lysyl tRNA synthetase, the cytoplasmic and mitochondrial isoforms are encoded by the same gene and produced through the use of alternative translation start sites and alternative splicing, respectively (Tolkunova et al. 2000, Turner et al. 2000). aaRSs are divided in two structural classes (I and II) based on the architecture of their catalytic core domains (Ibba

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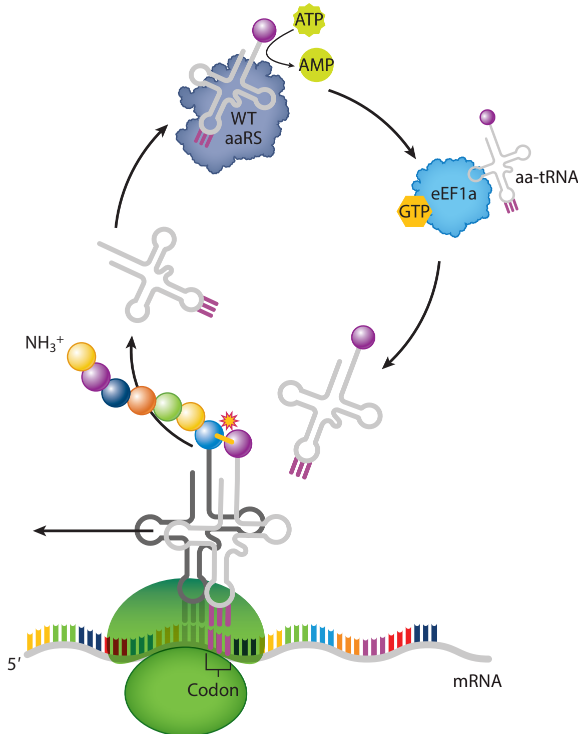
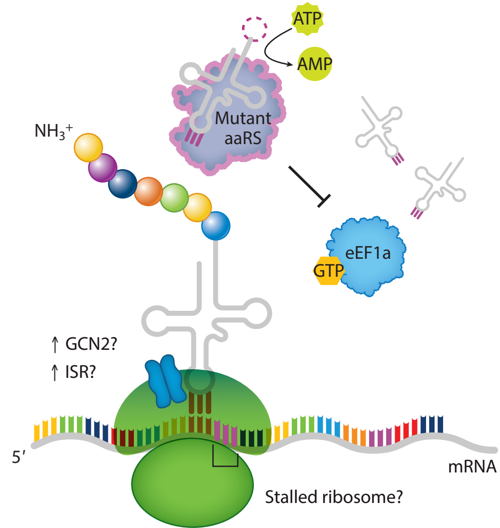
& Soll 2000, Rubio Gomez & Ibba 2020). All aaRSs harbor a catalytic domain and an anticodon binding domain, the latter mediating specific binding to the cognate tRNA. Many aaRSs further contain a dimerization or multimerization domain, and some aaRSs contain an editing domain, which allows hydrolysis of misacetylated tRNAs to prevent amino acid misincorporation into nascent proteins (Ibba & Soll 2000, Rubio Gomez & Ibba 2020).





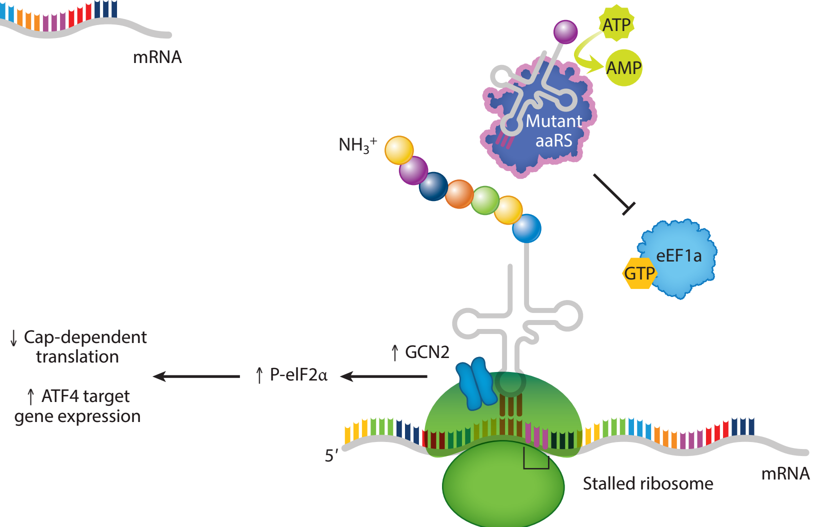


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Figure 3 (*Figure appears on preceding page*)

tRNA charging and mutations in aaRSs. (*a*) In normal tRNA charging, the aaRS binds its substrate tRNA and, in a two-step reaction involving an aminoacyl-adenylate intermediate, charges the amino acid onto the 3′ end of the tRNA. The charged tRNA is then transferred to eEF1a, which takes the charged tRNA to the ribosome for translation elongation. Following peptide synthesis, the uncharged tRNA is recycled back to the aaRS for the next round of charging. (*b*) In recessive loss-of-function mutations in aaRS genes, there is a failure to charge the amino acid onto the tRNA. This presumably leads to ribosome stalling and activation of the ISR through the sensor kinase GCN2, but that has not been examined in patients. (*c*) In dominant aaRS mutations that lead to peripheral neuropathy, the mutant aaRS binds the tRNA but fails to release it, leading to sequestration of the tRNA on the mutant protein. The lack of substrate for the remaining WT aaRS results in ribosome stalling and activation of the ISR through GCN2. GCN2 phosphorylates eIF2a, resulting in the suppression of cap-dependent translation and the expression of stress response genes mediated by the transcription factor ATF4. Abbreviations: aaRSs, aminoacyl tRNA synthetases; aa-tRNA, aminoacylated tRNA; ISR, integrated stress response; mRNA, messenger RNA; tRNA, transfer RNA; WT, wild type. Figure adapted from Hines et al. (2022a) (CC BY 4.0).

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DISEASES ASSOCIATED WITH BIALLELIC MUTATIONS IN aaRS GENES

Biallelic mutations in cytoplasmic or mitochondrial aaRS genes are associated with a broad variety of diseases, often involving multiple organ systems and frequently including neurological symptoms (Turvey et al. 2022). Moreover, for some aaRSs, different biallelic mutations in the same aaRS can give rise to distinct diseases. For instance, biallelic mutations in *MARS1* have been associated with hereditary spastic paraplegia (Novarino et al. 2014), lung disease (pulmonary alveolar proteinosis) (Hadchouel et al. 2015), developmental delay and spasticity (Okamoto et al. 2022), and a multi-organ phenotype (van Meel et al. 2013). Furthermore, biallelic *AARS2* mutations have been identified in patients with hypertrophic cardiomyopathy (Gotz et al. 2011), sometimes with additional muscle and neurological features (Taylor et al. 2014), and in patients with leukoencephalopathy with signs of neurologic deterioration consisting of ataxia, spasticity, cognitive decline, and frontal lobe dysfunction, but no cardiomyopathy (Dallabona et al. 2014).

A substantial reduction in aminoacylation of the cognate tRNA likely underlies (most) biallelic aaRS-associated diseases (**Figure 3*b***). Although reduced charging activity has only been tested for a subset of these mutations, arguments in favor of such a partial loss-of-function mechanism include the recessive inheritance pattern and the fact that in compound heterozygous patients a missense mutation frequently occurs in combination with a presumable null allele (e.g., frameshift, stop-gain, or start codon mutations). Furthermore, missense mutations often affect aminoacylation activity (Kopajtich et al. 2016, Kuo et al. 2019, McLaughlin et al. 2010, Oprescu et al. 2017, Scheper et al. 2007, Simons et al. 2015, van Meel et al. 2013, Williams et al. 2019, Zhang et al. 2014) and/or aaRS protein abundance (Antonellis et al. 2018, Bayat et al. 2012, Botta et al. 2021, Diodato et al. 2014, Galatolo et al. 2020, Mendes et al. 2018, Nafisinia et al. 2017, Nakayama et al. 2017, Ravel et al. 2021, Riley et al. 2010, Sasarman et al. 2012, Simon et al. 2015, Theil et al. 2019, Wang et al. 2020, Webb et al. 2015, Wortmann et al. 2017). The effects of diminished aaRS aminoacylation activity on aminoacylated cognate tRNA levels and on mRNA translation have been investigated for relatively few biallelic aaRS-associated diseases. Reduced levels of the aminoacylated cognate mt-tRNAs were found in cells of patients with biallelic *RARS2* (Edvardson et al. 2007), *WARS2* (Wortmann et al. 2017), and *NARS2* mutations (Simon et al. 2015, Vanlander et al. 2015). Reduced aminoacylated cognate tRNA levels are expected to hamper mRNA translation, which was confirmed in the mitochondria of myoblasts from patients with biallelic *YARS2* mutations (Riley et al. 2010, Sasarman et al. 2012), in the skeletal muscle of an infant with biallelic *AARS2* mutations (Calvo et al. 2012), and in fibroblasts from a patient with biallelic *WARS2* mutations (Theisen et al. 2017). Also, biallelic *MARS2* mutations resulted in a reduced rate of mitochondrial protein synthesis (Bayat et al. 2012, Webb et al. 2015). A similar concept applies to biallelic mutations in cytoplasmic aaRSs. For instance, biallelic *NARS1* mutations reduced global

protein synthesis in patient-derived induced pluripotent stem cells (Wang et al. 2020). Given that some of the subunits of the respiratory chain complexes are encoded by the mitochondrial genome, defective mitochondrial mRNA translation may result in impaired oxidative phosphorylation, which has been documented for several mitochondrial aaRS-associated diseases (Bayat et al. 2012, Calvo et al. 2012, Diodato et al. 2014, Riley et al. 2010, Simon et al. 2015, Steenweg et al. 2012, Taylor et al. 2014, Theisen et al. 2017, Vanlander et al. 2015, Webb et al. 2015).

The reason why biallelic mutations in different aaRS genes result in a surprising variety of phenotypes, and why even different mutations in the same aaRS gene can cause disparate diseases, is currently enigmatic. Apart from a potential influence of the patient genetic background on disease manifestations and severity, the expression level of aaRSs may vary between tissues and cell types, rendering cells with relatively low aaRS expression levels particularly sensitive to partial loss of aminoacylation activity. This is compounded by our poor understanding of tRNA availability and translational demand in different tissue and cell types. Also, different mutations may impact aminoacylation activity to varying degrees, and mutations that reduce activity more dramatically may impact a broader range of tissues and cell types, and therefore disease characteristics and severity. In addition, the codon demand for the affected tRNA may also vary between cell types, rendering cells with high codon demand for the affected tRNA more sensitive. Finally, in addition to diminished tRNA aminoacylation activity, alternative pathogenic mechanisms may be involved in some biallelic aaRS-associated diseases. Future research will need to address these outstanding questions.

MONOALLELIC MUTATIONS IN aaRS GENES CAUSE PERIPHERAL NEUROPATHY

In contrast to the broad range of clinical phenotypes associated with biallelic mutations in aaRS genes, monoallelic mutations in seven distinct cytoplasmic aaRS genes give rise to peripheral neuropathy (PN): *GARS1* (Antonellis et al. 2003), *YARS1* (Jordanova et al. 2006), *AARS1* (Latour et al. 2010), *HARS1* (Safka Brozkova et al. 2015), *WARS1* (Tsai et al. 2017), *MARS1* (Sagi-Dain et al. 2018), and *SARS1* (He et al. 2023). Most monoallelic aaRS mutations have been linked to hereditary motor and sensory neuropathy, also known as Charcot-Marie-Tooth (CMT) disease. CMT is characterized by length-dependent degeneration of peripheral motor and sensory axons, leading to progressive muscle weakness and wasting, and sensory dysfunction (Pareyson & Marchesi 2009, Rossor et al. 2013). CMT is clinically heterogeneous and includes demyelinating forms (CMT1), primarily characterized by demyelination, axonal forms (CMT2), primarily characterized by axonal degeneration, and intermediate forms, characterized by features of both demyelination and axonal degeneration (Pareyson & Marchesi 2009, Rossor et al. 2013). Almost all CMT-associated monoallelic aaRS mutations are linked to axonal or intermediate forms of CMT. A fraction of monoallelic mutations in *GARS1*, *AARS1*, *HARS1*, and *WARS1* cause distal hereditary motor neuropathy (dHMN), a related disease characterized by selective degeneration of peripheral motor axons but lacking sensory involvement (Frasquet & Sevilla 2022). PN is also genetically heterogeneous, with more than 120 genes linked to CMT and dHMN (Laura et al. 2019). Cytoplasmic aaRSs constitute the largest protein family implicated in CMT (Kuo & Antonellis 2020, Storkebaum 2016, Wei et al. 2019).

It became evident that a simple loss of aminoacylation activity (haploinsufficiency) is not the pathogenic mechanism underlying PN associated with monoallelic aaRS mutations (PN-aaRS) (Storkebaum 2016, Wei et al. 2019). Indeed, although many PN-aaRS mutations reduce or abolish aminoacylation activity, some mutations do not affect aminoacylation activity, including GlyRS-E71G (Antonellis et al. 2006, Nangle et al. 2007), TyrRS-E196K (Froelich & First 2011,

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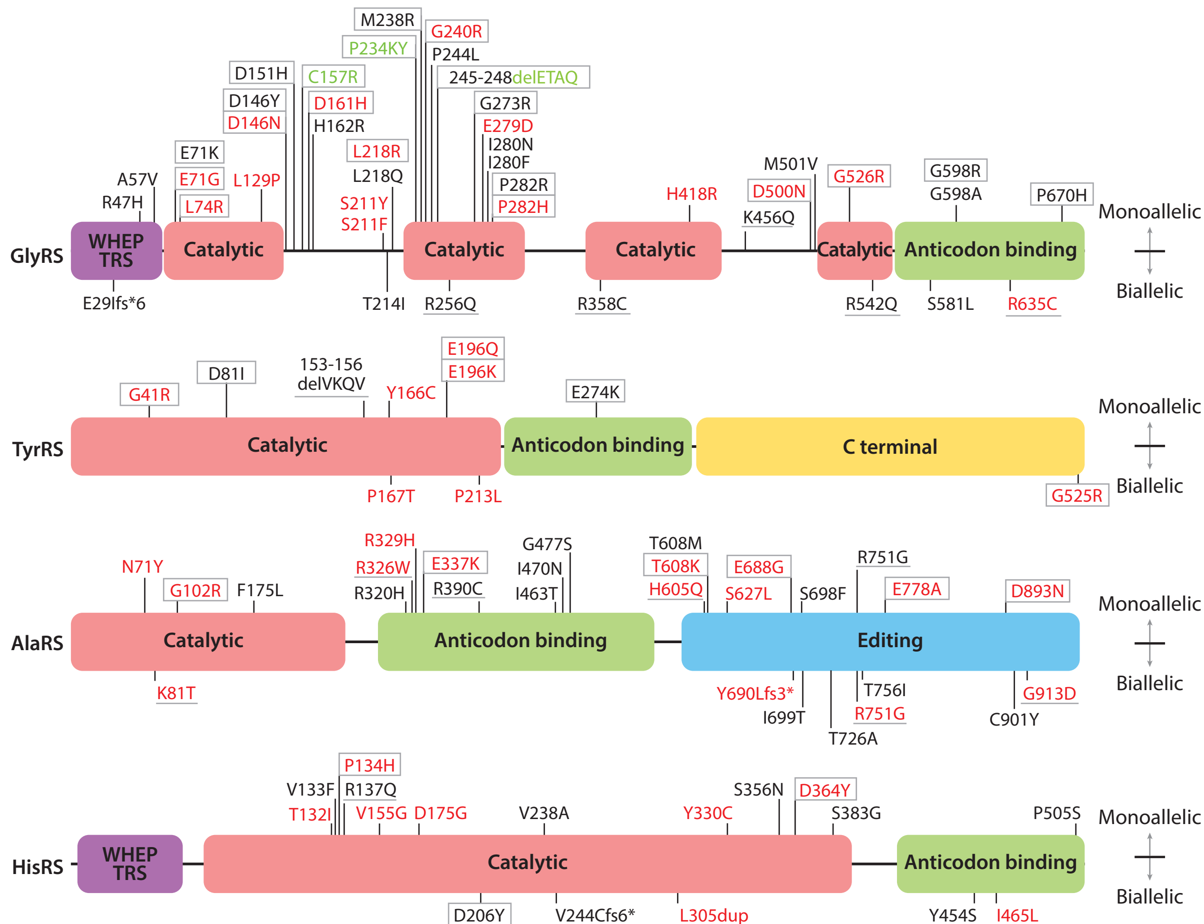

Figure 4

Mono- and biallelic mutations in cytoplasmic aaRSs. Shown is a schematic representation of four of the seven aaRSs that have been associated with peripheral neuropathy: GlyRS, TyrRS, AlaRS, and HisRS. These four aaRSs have the highest number of peripheral neuropathy-associated mutations. Protein functional domains are color coded. Monoallelic mutations associated with peripheral neuropathy are shown above the respective aaRSs and biallelic mutations are shown below. Only mutations that were reported as (probably or likely) pathogenic are shown; variants of unknown significance or likely benign variants are not shown. Mutations that segregate with disease in a family (strongest genetic evidence for disease causality) are labeled in red, mutations found in single patients are in black, and mutations in PN-aaRS mouse models are in green. Mutations that increase the charge of the aaRS (add positive charge) are boxed, while mutations that reduce the charge are underlined. For GlyRS, the positions of the mutations refer to the cytoplasmic isoform. Abbreviations: aaRSs, aminoacyl tRNA synthetases; PN-aaRS, peripheral neuropathy associated with monoallelic aaRS mutations. Figure adapted from Storkebaum (2016) (CC BY 4.0).

Storkebaum et al. 2009), AlaRS-E337K (Weterman et al. 2018), and AlaRS-E688G (Sun et al. 2021). This indicates that loss of aminoacylation activity is not required to cause PN. Furthermore, PN-aaRS mutations include exclusively missense mutations and two small in-frame deletions (**Figure 4**), and no frameshift, stop-gain, start codon mutations, or large deletions (presumable null alleles) have been identified. In addition, the parents of patients with biallelic aaRS mutations do not develop PN. These carriers are heterozygous for aaRS mutations, including frameshift (Galatolo et al. 2020, Nakayama et al. 2017, Oprescu et al. 2017) and start codon mutations (Lin et al. 2022). This human genetic evidence indicates that heterozygous loss of

aminoacylation activity is not sufficient to cause PN-aaRS, thus arguing against haploinsufficiency as a pathogenic mechanism underlying PN-aaRS. Consistently, mice and flies heterozygous for full loss-of-function alleles of *Gars1* or *Yars1* do not develop PN (Hines et al. 2022b, Seburn et al. 2006, Storkebaum et al. 2009).

Rather, evidence from animal models indicates that a gain-of-toxic-function mechanism underlies PN-aaRS. Firstly, overexpression of PN-mutant human TyrRS or GlyRS in *Drosophila* with intact endogenous *TyrRS* and *GlyRS* genes induces PN phenotypes (Niehues et al. 2015, Storkebaum et al. 2009). Secondly, in mouse models of CMT associated with mutations in GlyRS (CMT-GlyRS), which harbor heterozygous C201R or P278KY mutations in the endogenous *Gars1* gene, transgenic overexpression of wild-type (WT) human GlyRS did not rescue PN phenotypes (Motley et al. 2011). This also constitutes an argument against a dominant negative mechanism. Indeed, given that five of the PN-associated aaRSs, including GlyRS, function as an obligatory homodimer, in which tRNA binds across the subunits, a monoallelic aaRS mutation may reduce aminoacylation activity by up to 75% due to enzymatic inactivity of both heterodimers (consisting of a PN mutant and a WT subunit) and mutant homodimers (Storkebaum 2016). A further argument against a dominant negative mechanism is that overexpression of CMT-mutant GlyRS, including aminoacylation-inactive variants, in *Drosophila* did not reduce overall aminoacylation activity (Niehues et al. 2015). Thirdly, mice homozygous or compound heterozygous for PN-causing *Gars1* mutations, and simultaneously transgenic for WT human GlyRS, displayed more severe PN phenotypes than heterozygous mice, supporting a dose-dependent gain-of-toxic-function mechanism (Motley et al. 2011).

What could be the molecular mechanism underlying a gain-of-toxic-function scenario? A number of neomorphic molecular interactions of PN-mutant aaRSs, in particular GlyRS, have been reported. Several PN mutations substantially increase binding of GlyRS to the neuronal surface receptor neuropilin 1 (Nrp1) (He et al. 2015). Thus, extracellular—putatively secreted—PN-mutant GlyRS may bind Nrp1 expressed on peripheral motor and sensory axons, and GlyRS-Nrp1 binding was shown to interfere with binding of the main vascular endothelial growth factor isoform ($VEGF_{165}$) to Nrp1 (He et al. 2015). Because Nrp1 acts as a coreceptor for VEGF receptor 2, GlyRS-Nrp1 binding may diminish $VEGF_{165}$-Nrp1-VEGFR2 neurotrophic signaling (Lange et al. 2016). In support of this mechanism, increasing $VEGF_{165}$ expression in skeletal muscle moderately improved PN in CMT-GlyRS mice (He et al. 2015). However, at least one severe, early-onset PN-causing GlyRS variant does not show increased binding to Nrp1 (Morelli et al. 2019), and the therapeutic effect induced by increased muscle $VEGF_{165}$ levels may primarily be due to the neuroprotective effect of this neurotrophic factor. Indeed, increasing $VEGF_{165}$ levels also induces therapeutic effects in rodent models of *SOD1*-associated ALS (Azzouz et al. 2004, Storkebaum et al. 2005). Interestingly, three PN-causing variants in the catalytic domain of AlaRS also gained the ability to interact with Nrp1; however, three AlaRS variants in the editing and C-Ala domain did not (Sun et al. 2021). Thus, for some PN-aaRS variants, aberrant interaction with Nrp1 may contribute to PN, but additional pathogenic mechanisms must exist.

In contrast to WT GlyRS, several PN-GlyRS variants were shown to aberrantly interact with HDAC6, leading to enhanced HDAC6 deacetylation activity, reduced α-tubulin acetylation in motor and sensory axons, and impaired axonal transport. Treatment with the HDAC6 inhibitor Tubastatin A restored α-tubulin acetylation, rescued axonal transport defects, and modestly improved the motor performance of CMT-GlyRS mouse models (Benoy et al. 2018, Mo et al. 2018). Furthermore, a number of PN-GlyRS variants, but not WT GlyRS, were shown to interact with Trk receptors and activate Trk signaling, resulting in altered proportions of sensory neuron subtypes in dorsal root ganglia and multiple sensory behavior defects in CMT-GlyRS mouse models (Sleigh et al. 2017).

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Although these gain-of-toxic-function mechanisms may explain aspects of the PN phenotype for some mutants, they do not constitute a unified pathogenic mechanism underlying PN-aaRS. Given that a gain-of-toxic-function mechanism may involve mutation-induced novel molecular interactions that may interfere with any molecular or cellular pathway, an important discovery was that each of six different PN-mutant GlyRS or TyrRS variants—including two aminoacylation-active variants—dramatically inhibits protein synthesis in *Drosophila* motor and sensory neurons (Niehues et al. 2015). These studies indicate that inhibition of protein synthesis may constitute a unified pathogenic mechanism underlying PN-aaRS. The molecular mechanism underlying the inhibition of protein synthesis was recently elucidated for PN-GlyRS. Each of six PN-mutant GlyRS variants evaluated was able to bind $tRNA^{Gly}$ but failed to release it (**Figure 3*c***) (Zuko et al. 2021). This $tRNA^{Gly}$ sequestration depleted the cellular $tRNA^{Gly}$ pool below a critical threshold, leaving insufficient $tRNA^{Gly}$ substrate for GlyRS derived from the WT allele. This in turn led to an insufficient supply of glycyl-$tRNA^{Gly}$ to the ribosome and ribosome stalling on glycine codons (Zuko et al. 2021). Stalled ribosomes were shown to activate the eIF2α kinase GCN2, leading to ISR activation selectively in affected motor and sensory neurons (Spaulding et al. 2021). GCN2-mediated ISR activation promotes axonal degeneration, as genetic inactivation or pharmacological inhibition of GCN2 substantially rescued PN phenotypes of CMT-GlyRS mouse models (Spaulding et al. 2021). Consistent with a key contribution of ribosome stalling to the pathogenic mechanism, loss of function of the ribosome rescue factor *Gtpbp2* significantly aggravated PN phenotypes in CMT-GlyRS model mice (Zuko et al. 2021). Strikingly, transgenic $tRNA^{Gly}$ overexpression substantially rescued PN phenotypes and inhibition of protein synthesis in PN-GlyRS *Drosophila* models, and it fully prevented ISR activation and PN in two CMT-GlyRS mouse models (Zuko et al. 2021). The latter finding identifies $tRNA^{Gly}$ sequestration by mutant GlyRS variants as the molecular root cause of PN-GlyRS, and this indicates that elevating $tRNA^{Gly}$ levels or inhibiting GCN2 and the ISR may constitute novel therapeutic approaches for this genetic form of PN.

The beneficial effects of inhibiting GCN2 in CMT-GlyRS mouse models stand in contrast to results in the *n-Tr20*$^{-/-}$;*Gtpbp2*$^{-/-}$ mice mentioned previously. In that background, the additional loss of *Gcn2* exacerbated neurodegeneration, indicating that GCN2-mediated ISR activation promotes neuronal survival in that mouse model (Ishimura et al. 2016, Terrey et al. 2020), while it promotes axonal neuropathy in the CMT-GlyRS mice (Spaulding et al. 2021). The reason for this discrepancy remains to be elucidated.

These novel insights raise a number of questions that remain to be addressed. Firstly, do all PN-GlyRS variants cause disease through $tRNA^{Gly}$ sequestration? Or do some PN-GlyRS variants act via an alternative mechanism, possibly a dominant negative mechanism? Although the initial molecular mechanism is different, a dominant negative scenario may also lead to insufficient glycyl-$tRNA^{Gly}$ supply to the ribosome, ribosome stalling on glycine codons, and GCN2-mediated ISR activation. Also, for diseases caused by biallelic aaRS mutations, it remains to be investigated whether ribosome stalling on cognate codons and ISR activation are implicated (**Figure 3*b***). Secondly, do mutations in the six other PN-associated aaRSs also involve sequestration of their cognate tRNA? An intriguing hint comes from assessing the effect of PN-associated mutations on the net charge of aaRS proteins. Of the 35 PN-GlyRS variants, 20 increase positive charge (57%), 14 do not alter protein charge (40%), and only a single PN-linked mutation increases negative charge (**Figure 4**). In comparison, of the six GARS1 mutations linked to biallelic diseases, two do not alter protein charge and four add negative charge. The addition of positive charge may promote tRNA sequestration, as tRNA is negatively charged. Of note is that, of the six PN-GlyRS variants evaluated, two did not alter protein charge yet they displayed clear $tRNA^{Gly}$ sequestration (Zuko et al. 2021). Thus, the addition of positive charge is not strictly

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required to induce tRNA sequestration. Remarkably, five out of seven PN-TyrRS variants increase positive charge (71%), suggesting that $tRNA^{Tyr}$ sequestration may underlie PN-TyrRS. Consistent with this, mice with a patient-associated CMT mutation introduced into the *Yars1* (TyrRS) gene have a mild neuropathy, but they show gene expression changes indicative of ISR activation that are highly reminiscent of those found in the CMT-GlyRS mice (Hines et al. 2022b, Spaulding et al. 2021). In contrast, for PN-AlaRS and PN-HisRS, only 30% and 17% of the variants add positive charge, respectively, suggesting the potential involvement of alternative mechanisms. Finally, given that aaRSs are ubiquitously expressed, the reason for the cell-type-specific nature of PN-aaRS is currently elusive.

IMBALANCE BETWEEN mRNA CODON DEMAND AND tRNA SUPPLY TRIGGERS NEUROLOGICAL DISEASE

In addition to $tRNA^{Gly}$ sequestration in PN-GlyRS and *n-Tr20* mutation in mice, insufficient tRNA supply to the ribosome has been implicated in other neurological and neurodegenerative conditions. Selective inactivation of *Nsun2* in excitatory neurons in the mouse forebrain caused a relatively selective reduction of $tRNA^{Gly}$ expression. This resulted in increased ribosomal dwell times at glycine codons, decreased expression of glycine-rich proteins, and triggered synaptic transmission defects, cognitive deficits, and depressive behavior (Blaze et al. 2021). The Elongator complex is required for the modification of wobble uridines (U_{34}) in 11 tRNAs, which is thought to enhance the efficiency and fidelity of translation (Hershberg & Petrov 2008, Plotkin & Kudla 2011). Conditional inactivation of Elp3, the enzymatic core of the Elongator complex, in the mouse forebrain impaired tRNA U_{34} modification and elicited ribosome pausing specifically on codons read by U_{34}-containing tRNAs (Laguesse et al. 2015). This triggered PERK-mediated ISR activation, which led to defective neurogenesis in the developing cortex, resulting in microcephaly (Laguesse et al. 2015). In a cellular model of Huntington's disease, translation of expanded CAG repeats in mutant Huntingtin exon 1 led to depletion of glutaminyl-$tRNA^{Gln}{}_{CUG}$ that decodes CAG (Girstmair et al. 2013). This resulted in translational frameshifting and the generation of various polyQ-polyA hybrid proteins, which may nucleate polyQ protein aggregates. The frequency of frameshifting increased proportionally to the CAG repeat length and in response to decreasing $tRNA^{Gln}{}_{CUG}$ levels or increasing $tRNA^{Ala}{}_{UGC}$ levels (which decodes the GCA codon in the −1 reading frame) (Girstmair et al. 2013).

CHALLENGES

The example of monoallelic aaRS mutations causing PN highlights current challenges in research on tRNA-related diseases, particularly in understanding their propensity to affect the nervous system. Following the mechanistic model in which mutant tRNA synthetases bind and sequester their cognate tRNAs, leading to ribosome stalling and activation of the ISR (Spaulding et al. 2021, Zuko et al. 2021), the tRNA substrates for those synthetases that lead to neuropathy must in some way become limiting. This may simply be due to expression levels of the tRNAs in the relevant sensory and motor neurons, and not all isoacceptors need to be limiting; presumably having just one isoacceptor class being sequestered could lead to ribosome stalling at that specific codon. However, sequencing methods to identify and quantify tRNAs are technically challenging, requiring multiple demethylation steps to remove modifications and allow reverse transcription and library construction (Zheng et al. 2015). New sequencing approaches offer the promise of more robust reverse transcription as well as the ability to detect aminoacylation and modification status (e.g., see Behrens et al. 2021, Pinkard et al. 2020), and they even include direct sequencing using nanopore technology (Thomas et al. 2021). However, these methods are still challenging

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to apply at the level of discrete cell types, particularly in the brain where isolating specific cell types by sorting is difficult, let alone at the level of individual cells. Therefore, a challenge is to quantitatively determine the tRNA pools expressed in different neuronal cell types.

A related challenge is to determine the posttranscriptional modifications on the tRNAs in these cell types. An intriguing explanation for the specificity of monoallelic aaRS mutations for motor and sensory neuropathy would be a specific modification that only occurs, or never occurs, in these cell types. However, unless the tRNA pools in specific cell types are defined by a technology that simultaneously identifies modifications, describing modifications seems premature until the expressed tRNA pools are defined. Some progress may be gained by gene expression analysis and an evaluation of the modifying enzymes expressed in given cell types, but the list of modifying enzymes is not complete, and the presence of the enzyme does not equate to the presence of the modification, particularly given the dynamic nature of these processes.

How the expression of tRNAs is regulated is also unclear. Presumably, they are subject to epigenetics and chromatin accessibility to allow PolIII transcription, but whether there are other factors that influence their abundance and tissue-specific expression is unclear. The unique cell biology of neurons may also be making them more susceptible to perturbations in translation and tRNA function. Neurons are highly polarized cells with large somata and dendritic compartments and long axons, and translation occurs locally within these different compartments (Holt et al. 2019, Spaulding & Burgess 2017). While the mRNAs and locally translated proteins are being investigated (Glock et al. 2021, Koppel & Fainzilber 2018), the distribution of tRNAs within neurons has not been explored in detail. Finally, the balance of available tRNAs and codon demand in the translating mRNA pool must be maintained. Precisely how this is achieved is unclear, and precisely how codon demand can be quantified when the transcriptome, proteome, translation rate of individual proteins and metabolic state of the cell must all be considered is also unclear. For example, cystic fibrosis transmembrane conductance regulator (CFTR) is inherently unstable, and the speed of translation elongation, which is influenced by codon usage, can have major impacts on protein folding and membrane insertion (Rauscher et al. 2021, Ward & Kopito 1994). Faster is not necessarily better. Therefore, while this balancing of codon demand and anticodon availability may be obvious at a superficial level, it is extremely complex in practical reality.

Addressing these challenges involves (*a*) defining tRNA pools in specific cell types and individual cells, (*b*) defining tRNA modifications within those pools, (*c*) understanding how tRNA expression is regulated, (*d*) determining how tRNAs are spatially distributed within complex cells like neurons, and (*e*) determining how codon demand and anticodon availability are balanced, as well as the consequences of imbalance. These challenges are increasingly tractable as technologies to profile tRNA expression and modifications improve. However, it is also safe to assume that as these questions are answered, new questions and details regarding the fundamental function of tRNAs will emerge.

CONCLUSIONS

As we have outlined, tRNAs play a central role in human health and disease. Dysfunction arising from mutations in mitochondrial and possibly nuclear tRNA genes; from defects in splicing, modification, and cleavage of tRNAs; and from changes in amino acid charging onto tRNAs and their availability for translation at the ribosome can all lead to disease in humans. These diseases often result in neurological deficits, and the reasons behind the sensitivity of the nervous system to defects in tRNA biology and translation are poorly understood. To address this basic question, and to devise treatments for these disorders, a better understanding of the tRNA pools in given cell types is needed, as well as a better understanding of the complex relationship between the

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pool of available tRNA anticodons and the codon demand of translation in the cell. Emerging technologies will help address these questions, but the unusual cell biology of neurons and the context-dependent and dynamic nature of protein synthesis ensure that additional, currently unappreciated, details will need to be resolved before we have a full understanding of translation in neurons.

DISCLOSURE STATEMENT

E.S. is listed as inventor on an international patent (WO2021158100A1) on "tRNA Overexpression as a Therapeutic Approach for CMT Neuropathy Associated with Mutations in tRNA Synthetases." R.W.B. is listed as an inventor on a patent application (17/288,178) to inhibit the integrated stress response as a treatment for tRNA synthetase-associated forms of Charcot-Marie-Tooth and has served as a paid consultant for Roche. He also has unpaid positions on the scientific boards of the Charcot-Marie-Tooth Association and the Hereditary Neuropathy Foundation.

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