The tRNA regulome in neurodevelopmental and neuropsychiatric disease

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Abstract

Transfer (t)RNAs are 70–90 nucleotide small RNAs highly regulated by 43 different types of epitranscriptomic modifications and requiring aminoacylation (‘charging’) for mRNA decoding and protein synthesis. Smaller cleavage products of mature tRNAs, or tRNA fragments, have been linked to a broad variety of non-canonical functions, including translational inhibition and modulation of the immune response. Traditionally, knowledge about tRNA regulation in brain is derived from phenotypic exploration of monogenic neurodevelopmental and neurodegenerative diseases associated with rare mutations in tRNA modification genes. More recent studies point to the previously unrecognized potential of the tRNA regulome to affect memory, synaptic plasticity, and affective states. For example, in mature cortical neurons, cytosine methylation sensitivity of the glycine tRNA family (tRNA Gly) is coupled to glycine biosynthesis and codon-specific alterations in ribosomal translation together with robust changes in cognition and depression-related behaviors. In this Review, we will discuss the emerging knowledge of the neuronal tRNA landscape, with a focus on epitranscriptomic tRNA modifications and downstream molecular pathways affected by alterations in tRNA expression, charging levels, and cleavage while mechanistically linking these pathways to neuropsychiatric disease and provide insight into future areas of study for this field.

INTRODUCTION

Transfer (t)RNAs, essential in all pro- and eukaryotic domains of life, are 70–90 nucleotide RNAs contributing an estimated ~10% of a cell’s RNA pool, transcribed from 596 predicted including 417 ‘high confidence tRNA genes in the human nuclear genome1, covering in toto 61 anticodons decoding 20 canonical amino acids. There is an additional set of 22 mitochondrial tRNAs (mt-tRNAs) assigned to mitochondrial protein synthesis2. tRNAs

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are considered the most ancient molecules containing a genetic code, and molecular replication of proto-tRNAs may have driven evolution of protein translation\(^5\). Canonical tRNA functions include their classical role linking the genetic code to the amino acid sequence during protein synthesis by carrying the cognate amino acid to the growing polypeptide. Non-canonical functions mostly relate to a variety of tRNA fragments (tRFs), or molecules derived from full length tRNAs, recently linked to a surprisingly broad range of biological processes including translational inhibition, gene silencing and retroelement control, regulation of the immune response and cell death, and various others\(^4\).

Characterization of tRNAs, from their biosynthesis, secondary and tertiary structure, and detailed functioning in translation, has been studied over the past decades in various model organisms through elegant lines of work (for excellent and thorough reviews, see ref \(^5\)–\(^10\)). Curiously, while neurodevelopmental and neurological phenotypes often emerge as the primary manifestation of genetic mutations affecting the tRNA regulome\(^11\)–\(^13\), both canonical and non-canonical tRNA functions remain underexplored in the adult brain (for a thorough review of neurodevelopmental disorders associated with tRNA-related mutations, see ref \(^14\)). The purpose of this review is to provide the field with a brief and concise introduction on tRNAs, and then discuss recent discoveries as it pertains to the role of the tRNA regulome in context of the molecular and cellular pathology of neurodevelopmental and adult-onset neuropsychiatric and neurodegenerative disease in the clinical population and using animal models. We will conclude with an outlook on future directions in this promising field.

**tRNA structure and biosynthesis: a brief overview**

The vast majority of nuclear genome-encoded tRNAs, and a subset of mitochondrial tRNAs, are defined by their L-shaped tertiary structures assembled via hydrogen bonds interconnecting the 8 different loops and helices defining the classical ‘clover-like’ secondary structure (Figure 1a–c). These include from 5’ to 3’ the acceptor stem, the dihydrouridine D loop and arm, the anticodon loop and arm, the variable loop, and the TphiC T loop and arm. tRNAs are classified into subgroups including isodecoders, tRNA molecules that share the same anticodon but differ in sequence otherwise, and isoacceptors, which are tRNA molecules that carry the same amino acid but have different anticodons.

tRNA transcription takes place in the nucleolus. Notably, expression of all tRNA genes, except for selenocysteine tRNAs (tRNA\(_{\text{SeC}}\)), is governed by type II internal promoters, RNA Polymerase III (RNAPIII) and the two multi-subunit transcription factors TFIIIB and TFIIIC\(^15\),\(^16\). However, for transcription of tRNA\(_{\text{SeC}}\), RNAPIII is recruited through the internal B-box and an external upstream promoter\(^17\) (reviewed in ref \(^10\)). During normal tRNA transcriptional processes, transcription factor TFIIIC binds to the A- and B-box and the three subunits of TFIIIB are recruited to upstream regions to initiate binding of RNAPIII (reviewed in ref \(^6\)). Immature tRNA transcripts are typically defined by 2–5 nucleotides upstream (‘leader’) and 5–15 nucleotides downstream (‘trailer’), which are cleaved off by RNase P, and Rnase Z respectively, to produce mature tRNA\(^5\). However, a small subset of human tRNAs carry an intron, which is targeted by tRNA splicing endonuclease (TSEN), a tetrameric enzyme comprised of four subunits, TSEN2, TSEN15, TSEN34 and TSEN54\(^18\).
Mutations in each of the 4 TSEN subunits\textsuperscript{19, 20}, and various TSEN-associated proteins such as the CLP1 RNA kinase\textsuperscript{21, 22}, have been linked to various forms of pontocerebellar hypoplasia, a type of autosomal recessive neurological syndrome with early onset, often rapidly progressing neurodegenerative disease. In the nucleus, tRNAs are spliced as described above and then modified with an abundance of post-transcriptional modifications (see Box 2) and some tRNAs are aminoacylated, although most aminoacylation takes place in the cytoplasm after export\textsuperscript{23}. A nuclear surveillance mechanism assures that only correctly processed tRNAs leave the nucleus for functioning in the cytoplasm\textsuperscript{24, 25}, and a bi-directional transport mechanism also exists to import tRNAs back into the nucleus for repair or degradation as well\textsuperscript{26}.

### Mutations and deletions of tRNA genes

Interestingly, while the nuclear genome-encoded tRNA transcriptome with >400 tRNA genes shows redundant coverage for the large majority of 61 codons (the exception being tRNA\textsuperscript{Sec, UCA} (selenocysteine) which is only recognized by a single copy tRNA gene), expression of individual tRNA genes often shows strong cell- and tissue-specific signatures\textsuperscript{27}, and consequently, deletion of a single nuclear tRNA gene still carries potential for a neurological phenotype if loss of expression in CNS is not compensated by the other isodecoder genes. For example, mice with strain-specific deletion of tRNA-Arg-TCT-4-1, one out of four tRNA\textsuperscript{Arg, UCU} isodecoders in the murine genome, show a ~60% drop of tRNA\textsuperscript{Arg, UCU} levels in brain, resulting in slowing and pausing of the translational process at the site of the AGA codon in brain ribosomes\textsuperscript{28, 29}, which in turn leads to changes in neuronal excitability and seizure threshold in the hippocampus\textsuperscript{28} (Figure 2a) and contributes to neurodegeneration in other brain regions\textsuperscript{29}.

Given the essential role of mitochondria for cellular respiration and general energy homeostasis, and general lack of compensatory capacity among the 22 mt-RNAs, it is unsurprising that mutations affecting the structure of specific mt-tRNAs play a prominent role in disease etiology. To date, structural variations and deletions encompassing human mt-tRNA genes include ~300 pathogenic mutations (out of a total >750 within mtDNA)\textsuperscript{30, 31}. The affected cases often show neurological impairments, which includes the well-studied mt-tRNA\textsuperscript{Leu(UUR)} m.3243 A>G and mt-tRNA\textsuperscript{Lys} m.8344 A > G mutations which broadly destabilize the molecule’s structure and function, thereby negatively impacting mt-protein synthesis for electron transport chains at inner mt membranes and compromising oxidative phosphorylation. Clinical symptoms present either as classical MELAS (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes) and MERRF (myoclonic epilepsy with ragged red fibers) syndromes, or some variation thereof, reflecting various degrees of mitochondrial heteroplasmy among many affected individuals\textsuperscript{32}. Furthermore, many mt-tRNA mutations are associated with increased risk for amyotrophic lateral sclerosis (ALS), or motor neuron disease\textsuperscript{33}, a finding that is in line with the generally increased vulnerability of this neuronal subtype for defects in the RNA processing machinery\textsuperscript{34}.

### Aminoacyl tRNA synthetases

**Monogenic neuropsychiatric disease**—See Box 1 for an overview of tRNA aminoacylation and tRNA synthetase enzymes. Similar to the aforementioned examples

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of genetic mutations in tRNA genes, neurodevelopmental and neurological phenotypes also rank prominently among the various types of clinically significant aaRS mutations (reviewed in ref 36), including some cases carrying lysyl-tRNA synthetase (KARS1), leucyl-tRNA synthetase 2 (LARS2), asparaginyl-tRNA synthetase (NARS1), or valyl-tRNA synthetase (VARS1) mutations who present primarily with neurological and cognitive symptoms including microcephaly, epilepsy, and autism. Furthermore, an increasing number of aaRS mutations are linked to hereditary neuropathies, including Charcot-Marie-Tooth (CMT) disease which can present either as demyelinating disease of peripheral nerves (CMT type I) or as axonopathy (CMT type II). To date, mutations in at least five cytosolic aaRS (AARS1, GARS1, HARS1, YARS1, WARS1) have been associated with autosomal dominant CMT, with additional aaRS linked to CMT in context of compound mutations each affecting one allele. Interestingly, work in CMT mouse models carrying human mutations in cytosolic aaRS, including GARS and YARS, shows that the integrated stress response (ISR) plays a key role in disease pathogenesis (Figure 2b). The ISR is a conserved signaling pathway in mammalian cells aimed towards restoring homeostasis by redirecting the cell’s translational program towards increased expression for regulators of protein synthesis, folding and trafficking. Specifically, genetic ablation or pharmacological inhibition of GCN2 (GENERAL CONTROL NON-DEPRESSIBLE 2), one of four known sensor kinases capable of triggering the ISR in stressed cells, markedly alleviated the neuropathy in the GARS and YARS CMT models (Figure 2b). Further, overexpression of tRNA\textsubscript{Gly} likewise rescued the neuropathy phenotype in this model, showing that tRNA\textsubscript{Gly} sequestration by the mutant GARS is an additional mechanism which ultimately causes the pathophysiology of CMT. This is the second line of evidence demonstrating that lack of availability of a specific tRNA pool for translation can induce hallmarks of the ISR in brain. Additional mechanistic possibilities relating to other aaRS mutations, but unexplored in this model, include defective protein translation, transport and deposition, neomorphic affinity of the mutant protein towards neuropilin receptors at the neuromuscular junction, or even aberrant activation of nuclear transcription factors as shown in a CMT drosophila model modeling tyrosyl-tRNA synthetase (YARS) mutations.

Additional mechanistic insight into the effects of tRNA charging on neural function was shown by using sti (sticky) mutant mice, which show degeneration of cerebellar Purkinje neurons over the lifespan leading to tremors and eventual overt ataxia. In sti mice, mischarging of tRNA\textsubscript{Ala} with serine due to loss of function mutation in its cognate tRNA synthetase, alanyl-tRNA synthetase (AlaRS) was causally linked to an accumulation of misfolded proteins in cerebellar neurons and resulted in cell death, demonstrating the potency of even low levels of mischarged tRNAs from one isoacceptor family can affect neuronal function and have potential implications for neurodegenerative disease.

Another notable example for brain-related tRNA-synthetase phenotypes in humans concerns mitochondrial alanyl-tRNA synthetase (AARS2), which could present as leukencephalopathy and, in a subset of female patients, ovarian failure. Remarkably, while age of disease onset varies considerably among cases with AARS2 mutations, some patients present with initial symptoms of clinical depression and frontal lobe dysfunction not before the second or third decade of life, typically heralding rapid neurological deterioration over the next following years. At present, the underlying mechanisms of disease remain
poorly understood. In any case, these examples on the spectrum of AARS2 mutations are an important reminder that even adult-onset psychiatric disease such as depression carries, in rare cases, the potential to represent the initial disease manifestation of a molecular defect impacting some of the most ‘basic’ constituents in the cell’s translational machinery.

**aaRS pathways in psychiatric disease**—Depression (including bipolar illness), schizophrenia, autism, and attention deficit/hyperactivity disorder (ADHD) account for some of the most common psychiatric disorders with complex etiology and share a significant portion of their underlying genetic risk architectures. This raises the question whether the aforementioned, extremely rare cases of tRNA-synthetase mutations initially presenting with depression (AARS2) or autism (KARS1, LARS2, NARS1) should be viewed as ‘private mutations with singular pathophysiology’ and therefore irrelevant as a representative disease mechanism for the broader population of subjects on the autism, or mood and psychosis spectrum. Alternatively, these case reports could be viewed as ‘tip of the iceberg’, pointing towards a previously unrecognized clinical significance of the tRNA regulome for the neurobiology of such types of neuropsychiatric disease. To this end, it is notable that a recent study exploring serum level genetic determinants for 486 metabolites uncovered a link between aminoacyl-tRNA biosynthesis and common risk variants associated with depression and ADHD, and furthermore, glycine, serine and threonine metabolism were linked to common polymorphisms for schizophrenia risk. Furthermore, aminoacyl-tRNA biosynthesis again ranked among the top scoring biological pathways in a study linking common polymorphisms for autism risk to protein interaction networks in fetal cortex. Therefore, a disease-relevant role for regulators of tRNA aminoacylation is supported not only by the rare tRNA-synthetase mutations presenting as monogenic form of major psychiatric disease, but also by the functional analyses of genome-wide association studies that focused on risk alleles in the broader population. In addition to these recent findings from clinical genetics, a recent study exploring metabolomic profiles in rodent models for stress-induced depression listed tRNA charging and amino acid metabolism among the top 5 ranking pathways significantly affected in the depressed brain, together with endocannabinoid signaling, catecholamine biosynthesis, and GABA receptor signaling. We note, however, that such types of gene ontology and pathway-specific enrichment approaches cannot imply causation for tRNA function producing psychiatric outcomes, but nonetheless could serve as starting points to test novel hypotheses exploring a potential link between these regulatory tRNA synthetases and psychiatric disease risk. Therefore, in future studies using animal models, it will be interesting to explore molecular mechanisms and behavioral and neuropsychiatric phenotypes after cell- and circuit-specific genetic ablation or transgenic engineering of specific tRNA synthetases.

**tRNA modifications**

**Mutations in epitranscriptomic regulators**—See Box 2 for a brief overview of tRNA epitranscriptome. To date, mutations in more than 50 enzymes associated with tRNA epitranscriptomic modifications have been linked to human disease. Many of these mutations are responsible for multiorgan syndromes, or ‘RNA modopathies’, with neurodevelopmental defects, neurodegeneration and demyelinating disease prominently represented. These include, in addition to MELAS, MERRF, and related ‘classical’
syndromes presenting with encephalomyelopathy, myoclonic epilepsy and stroke as a result of defective mt-tRNA regulation, an increasing list of gene defects selectively affecting non-mitochondrial tRNAs. A representative example is provided by the ELP family of proteins associated with chromatin remodeling and RNA modifications. For example, ELP1 and ELP2, (Elongator Acetyltransferase Complex Subunit 1 and 2) and convert tRNA uridine to ncm$^5$U, or 5-carbamoylmethyluridine, a type of modification critically important for decoding and translation at sites of some codons with a ‘wobble’ position. While ELP1 and ELP2 mutations are linked to dysautonomia, intellectual disability and autism syndromes, ELP3 is a motor neuron disease/amyotrophic lateral sclerosis (ALS) susceptibility locus by genome-wide association and affects survival in a subset of ALS patients. Of note, ELP3 is essential for converting wobble-positioned uridine to 5-methoxycarbonylmethyl-2-thiouridine (mcm$^5$s$^2$U), and loss of ELP2 function results in general slowing of translation and proteome stress with increased risk for protein aggregation in the affected cells. Another tRNA uridine post-transcriptional modification associated with neurodevelopmental phenotypes is pseudouridinylation, considered the most abundant type of RNA modification, via C-C glycosidic isomerization of uridine. As it pertains to tRNAs, at least two pseudouridine synthases, PUS3 and PUS7, catalyze the isomerization of uridine to pseudouridine. Mutations in PUS3 or PUS7 (independently) are associated with intellectual disability and microcephaly. Further, patients with a PUS7 mutation have impaired tRNA pseudouridinylation and various behavioral deficits including increased aggression, and a PUS7 knockout in Drosophila recapitulated the molecular and behavioral phenotype seen in patients.

A neurodegeneration risk gene, TRNA-YW Synthesizing Protein 3 Homolog (TYW3), encodes a protein with an essential role in the biosynthetic pathway of the tricyclic nucleoside and hypermodified purine, wybutosine (yW) at position 37 in phenylalanine tRNA (tRNA$^{\text{Phe}}$; Figure 1b). The yW modification at tRNA$^{\text{Phe}}$ is considered essential for proper codon-anticodon base pairing, and tRNA$^{\text{Phe}}$ lacking yW in yeast (or hydroxywybutosine, OHyW, in mammals) could compromise mRNA decoding because unmodified tRNA$^{\text{Phe}}$ carries a high risk for frameshifting. There is additional complexity, as studies in mouse have shown that proper levels of OHyW$^\text{C37}$-modified tRNA$^{\text{Phe}}$ critically depend on 2'-O-methylation at the neighboring C32 and, critically, G34 via the neurodevelopmental risk gene and RNA methyltransferase, FTSJ1, which is associated with X-linked intellectual disability in humans. Ftsj1 null mutant mice displayed loss of 2'-O-methylguanosine (G34) and 2'-O-methylcytidine (C32) at the tRNA anticodon loop, in addition to the secondary effect of OHyW$^\text{C37}$-modified tRNA$^{\text{Phe}}$, which disrupted translation efficiencies for phenylalanine codons in conjunction with poor memory and increased anxiety in the mutant mice.

RNA editing mechanisms, mainly the deamination of adenosine to inosine, has not historically been denoted as an epitranscriptomic modification, but it is nonetheless a crucial post-transcriptional modification abundant among tRNAs that produces neurological phenotypes when altered. The presence of inosine at the wobble base position of the tRNA anticodon loop (base 34) is essential for proper base pairing and translation (specifically at tRNA$^{\text{ Ala}}_{\text{AGC}}$, tRNA$^{\text{ Pro}}_{\text{AGG}}$, tRNA$^{\text{ Thr}}_{\text{AGT}}$, tRNA$^{\text{ Val}}_{\text{ACC}}$, tRNA$^{\text{ Ser}}_{\text{AGA}}$, tRNA$^{\text{ Arg}}_{\text{ACG}}$, tRNA$^{\text{ Leu}}_{\text{AAG}}$, and tRNA$^{\text{ Ile}}_{\text{AAT}}$) and deamination in human tRNAs is catalyzed by
the ADENOSINE DEAMINASE tRNA-SPECIFIC 2/3 (ADAT2/ADAT3) complex. A valine-to-methionine mutation (V144M) at the ADAT3 locus produces autosomal recessive intellectual disability in addition to microcephaly, epilepsy, brain abnormalities, and behavioral problems including hyperactivity and aggression. Molecular studies confirmed that these patients have deficient wobble inosine levels at multiple tRNA isoacceptors, including tRNA\textsubscript{Val\_AAC} and tRNA\textsubscript{Pro\_AGG}, due to impaired adenosine deaminase activity and impaired interactions with the ADAT2 subunit.

m\textsuperscript{5}C in cognitive and affective states—The role of epitranscriptomic modifications on tRNAs in brain has only recently become an emerging aspect of neuroscience, and of the 43 tRNA epitranscriptomic modifications identified thus far, only 4 of these modification types (m\textsuperscript{5}C, m\textsuperscript{1}A, 2'-O-methylation, OHyW; Figure 1a–c) have been investigated in the context of neurological function and neuropsychiatric disease phenotypes. Cytosines targeted for cytosine-5 methylation (m\textsuperscript{5}C) are mostly positioned in the anticodon stem and variable loop of the majority of tRNA molecules. In mammals, there are currently four known enzymes with RNA cytosine methyltransferase (RNA-MTase) activity directed against tRNAs, including three members of the NOL1/NOP2/SUN DOMAIN family of proteins: NSUN3, which methylates only mt-tRNA\textsuperscript{Met} at the wobble base cytosine 34, NSUN6, which mediates m\textsuperscript{5}C methylation at only cytosine 72 of tRNA\textsuperscript{Cys} and tRNA\textsuperscript{Thr}, and NSUN2, which mediates m\textsuperscript{5}C methylation at 4–5 cytosines positioned in the variable loop on >75% of actively transcribed mammalian nuclear-encoded tRNAs and mitochondrial tRNAs. The third enzyme, DNA methyltransferase 2 (DNMT2, also known as TRDMT1) mediates m\textsuperscript{5}C methylation at a single cytosine positioned in the anticodon loop of tRNA\textsuperscript{Asp}, tRNA\textsuperscript{Gly}, and tRNA\textsuperscript{Val} (note that DNMT2, contrary to its nomenclature, is solely an RNA-MTase and lacks activity towards DNA). In humans, there have been no noted DNMT2 or NSUN6 mutations leading to aberrant neurological phenotypes, but both NSUN3\textsuperscript{74, 79} and NSUN2\textsuperscript{80–83} loss of function variants are associated with neurological dysfunction. Patients with a mutation in NSUN3 display early-onset mitochondrial encephalopathy and seizures, likely due to impaired methylation at mt-tRNA\textsuperscript{Met}. Heterozygous NSUN2 loss of function mutations are associated with neurodevelopmental phenotypes, with patients showing intellectual disability (ID), facial dysmorphism and distal myopathy. Studies in fruit flies and mice further confirmed the importance of Nsun2 for normal brain function and behavior. For example, knock-down of drosophila dNsun2 was associated with impaired short-term memory after aversive olfactory conditioning, a phenotype that was rescued by pan-neuronal expression of dNsun2. Likewise, mice with Nsun2 germline deletion demonstrated various impairments in locomotor activity and behavior together with reduced brain size due to excessive cell death and impaired migration of upper-layer cortical neurons in the prenatal brain (Figure 2c). This neurodevelopmental phenotype occurred in context of increased endoribonuclease (including ANG)-mediated tRNA fragmentation with excessive production of S' tRNA halves and shorter derivatives, which in turn led to an overall decrease in protein synthesis. tRNA fragments reportedly interfere with ribosomal assembly and alter the dynamics of charged (amino acid loaded) full length RNAs, and inhibit of cap-dependent protein translation, among other mechanisms. Given these destabilizing effects of tRNA m\textsuperscript{5}C hypomethylation, a subset of (full-length) tRNAs including a subset of the glycine tRNA family (tRNA\textsuperscript{Gly}) showed a marked
depletion in Nsun2-deficient tissues including skin, liver, and most strikingly, in adult cerebral cortex with conditional neuron-specific Nsun2 ablation, with significant deficits in 4/4 tRNA\textsubscript{Gly} \textsuperscript{GCC}, 2/4 tRNA\textsubscript{Gly} \textsuperscript{CCC} and 1/1 tRNA\textsubscript{Gly} \textsuperscript{UCC} isodecoders. This selectively vulnerability of tRNA\textsubscript{Gly} remains poorly understood, because various other isoacceptor families also became hypomethylated but maintained normal levels of expression in the Nsun2-deficient cortex. Regardless, tRNA abundance is a key determinant for translational elongation rates during protein biogenesis, including codon-specific variabilities in ribosome speed along mRNAs, and indeed, the adult Nsun2-deficient cortex showed disruption of translational elongation specifically at the site of the Gly codons (Figure 2d). This resulted in downregulation of Gly-rich synaptic signaling proteins and defective excitatory neurotransmission in the prefrontal cortex (PFC), in conjunction with impaired contextual fear memory and other behavioral alterations (Figure 2d). Strikingly, however, bi-directional changes in Nsun2 tRNA methyltransferase activity in adult PFC neurons were associated with directly opposing changes in behavioral despair paradigms and differential effects on anxiety-related behaviors, with neuronal Nsun2-deficiency presenting as anxiolytic and anti-depressant like phenotypes while transgenic overexpression of neuronal Nsun2 resulted in pro-depressant-like behaviors. These studies then strongly suggest that proper regulation of tRNA cytosine methylation remains critically important for cortical brain function and behavior beyond the developmental period. Likewise, other telencephalic brain regions depend on Nsun2 for normal function. For example, a global loss of Nsun2 produced deficits in NMDAR-dependent long-term potentiation (LTP) but enhanced basal synaptic transmission in hippocampal area CA1 (Figure 2c).

While it is tempting to speculate that these behavioral and physiological alterations are directly related to changes in codon-specific elongation rates during ribosomal protein synthesis, it is noteworthy that in the Nsun2 mutant model in adult cerebral cortex, additional molecular adaptations downstream of tRNA hypomethylation and loss of tRNA\textsubscript{Gly} may have contributed to some of alterations in synaptic plasticity and complex behaviors in mutant mice. For example, there was a massive 2.46-fold increase of glycine amino acid levels in bulk extract from the Nsun2-deficient cortex while levels for the remaining 19 canonical amino acids were indistinguishable from wildtype cortex, suggesting that the loss of Gly tRNA and the corresponding drop in translational efficiencies of Gly-rich neuronal proteins triggers a highly specific increase in cortical glycine levels (Figure 2d). Therefore, these data, as an emerging hypothesis, would suggest that mature cortical neurons, when sensing codon-specific disruptions in tRNA supply, could respond by adjusting metabolic and biosynthetic pathways regulating the cellular pool of the cognate amino acids. These data also emphasize remarkably that a very selective deficit in one isoacceptor family (Gly), due to impaired cytosine methylation, has the capacity to alter both the proteome and amino acid levels to produce a strong behavioral phenotype. This extreme specificity of the tRNA regulome has been shown previously, and underscores the crucial impact of individual tRNA loss in the mammalian brain. Therefore, in addition to the long-established glycine neurophysiology via NMDA receptor binding sites and glycinergic receptors in subtelencephalic areas, we suggest a third glycinergic pathways that could critically regulate adult brain function and complex behaviors by way of tRNA\textsubscript{Gly} cytosine methylation. Because pharmacological alterations in glycinergic signaling have shown
promise in alleviating behavioral deficits in humans\textsuperscript{95} and animals\textsuperscript{96}, the molecular, cellular and behavioral phenotypes of mice with neuron-specific changes in \textit{Nsun2} expression and activity could broadly align with the therapeutic promise of glycinergic pathways for neuropsychiatric disease.

\textbf{m1A in neurodegeneration}—1-methyladenosine (m\textsuperscript{1}A) is another epitranscriptomic modification that has been identified within mammalian nuclear-encoded tRNAs at site 9 (m\textsuperscript{1}A\textsubscript{9}), 14 (m\textsuperscript{1}A\textsubscript{14}), and 58 (m\textsuperscript{1}A\textsubscript{58}), and both m\textsuperscript{1}A\textsubscript{9} and m\textsuperscript{1}A\textsubscript{58} are likewise found in mitochondrial tRNAs\textsuperscript{97, 98} (Figure 1c). Distribution of m\textsuperscript{1}A sites in nuclear-encoded vs. mt-tRNAs differs, such that m\textsuperscript{1}A\textsubscript{58} is present in almost all nuclear-encoded tRNAs and in about a quarter of mt-tRNA isodecoders\textsuperscript{99, 100} but m\textsuperscript{1}A\textsubscript{9} is less common in nuclear-encoded tRNAs (thus far only Asp tRNA has m\textsuperscript{1}A\textsubscript{9} \textsuperscript{99, 100}) while three quarters of mt-tRNAs contain m\textsuperscript{1}A\textsubscript{9}. In a mouse model of AD, 5XFAD (5X Familial Alzheimer’s Disease) mutant mice, which express molecular hallmarks of AD pathogenesis such as amyloid deposition and gliosis\textsuperscript{101}, exhibited a decrease in m\textsuperscript{1}A writers (TRMT10C, HSD17B10 and TRMT61A) and hypomethylation of m\textsuperscript{1}A\textsubscript{9} and m\textsuperscript{1}A\textsubscript{58} at both nuclear-encoded and mitochondrial tRNAs in the cortex, however, mature tRNA expression was only depleted in hypomethylated mitochondrial tRNAs\textsuperscript{102}. This depletion may be due to diminished stability of tRNA secondary structure, a known effect of m\textsuperscript{1}A\textsubscript{9} loss in mt-tRNA\textsuperscript{102}, while the lack of depletion in hypomethylated nuclear-encoded tRNA expression may be due to the overwhelming presence of m\textsuperscript{1}A\textsubscript{58}, which is mainly involved in recruitment of tRNAs to polysomes to promote translation elongation\textsuperscript{98}. Additionally, another AD model of tauopathy using \textit{Drosophila} demonstrated that loss of the multiple enzymes involved in m\textsuperscript{1}A deposition on mitochondrial tRNAs exacerbated the AD-like phenotype\textsuperscript{102}. One of the enzymes mechanistically linked to AD pathology, in both the \textit{Drosophila} and mouse models, is TRMT10C, which fascinatingly is also significantly decreased in post-mortem dorsolateral prefrontal cortex (DLPFC) of human AD subjects\textsuperscript{102, 103}. Further, another post-mortem study identified hypermethylation of m\textsuperscript{1}A\textsubscript{9} in the cerebellum of AD subjects\textsuperscript{104}, suggesting a brain region-specificity to the mitochondrial tRNA epitranscriptome. It is still unclear how mitochondrial function is altered by the depletion of m\textsuperscript{1}A enzymes and hypomethylated mt-tRNAs, but the probable outcome of mitochondrial tRNA loss would likely be on the level of translation efficiency and protein expression, which should be a target of future studies. Interestingly, recent evidence suggests that m\textsuperscript{1}A plays a role in normal learning processes unrelated to diseased brain function. Global m\textsuperscript{1}A levels in tRNAs are increased following non-associative learning in the cerebral ganglia of \textit{Aplysia californica}\textsuperscript{105}. Individual methylated adenosine residues and tRNA species were not identified in this global approach, but these findings suggest that m\textsuperscript{1}A levels could promote synthesis of learning-related proteins.

\textbf{tRNA fragments}

\textbf{Neurological insult and inflammation}—It is now generally accepted that tRNA fragments (tRFs), the majority of which encompasses 18–40bp either 5’ or 3’ from the anticodon (tRF-5 and tRF-3 respectively) do not reflect non-specific degradation but instead are highly regulated, fulfilling distinct biological functions\textsuperscript{4} (Figure 1a; reviewed in ref \textsuperscript{6, 106, 107}). For example, tRNA half-molecules, generated from anticodon loop
cleavage by a subset of endoribonucleases, including *RNASE5 (ANGIOGENIN)*, are upregulated under conditions such as oxidative or hyperosmotic stress, nutrient deprivation, and inflammation\(^{108}\), an effect that is preceeded by conformational changes and tRNA unfolding\(^{109}\). Likewise, the interferon-stimulated nucleases *RNASEL* and *SLFN11* and *SLFN13* show cleavage activity directed towards a limited set of tRNAs when activated in response to exposures such as double-stranded RNA, DNA damage, or viruses (incl. HIV)\(^{110–112}\). According to studies in peripheral systems, tRNA halves, and some of the other types of tRNA fragments, exert repressive effects on global protein translation, most likely due to interference with ribosomal assembly and dynamics of charged (amino acid loaded) full length RNAs\(^4,86\), and by inhibition of cap-dependent protein translation\(^87\).

Another potential mechanism in which tRNA cleavage products may alter downstream processes in brain is through binding to target mRNAs to influence gene expression in a microRNA-like manner, and a recent report showed that this may be a regulatory mechanism of neuronal function in the primate hippocampus, where 5’ tRFs are highly expressed\(^{113}\).

Further support for tRFs as dynamic modulators of brain function was evidenced in studies of neurological insults such as ischemic stroke. Up-regulation of 5’ tRNA halves has been reported in a rat model for acute ischemia, with dramatic multi-fold increases in tRNA\(_{\text{Gly}}\) and tRNA\(_{\text{Val}}\) isodecoder levels in the rodent brain lasting for several days post-stroke\(^{114}\). Increased tRNA fragment abundance post-stroke is also reflected in peripheral blood from stroke patients, during which time an increase in tRFs is concomitant with a decrease in microRNA levels\(^{115}\). Fascinatingly, the increased tRFs include those derived from tRNA\(_{\text{Gly}}\) and function via microRNA-like mechanisms to target anti-inflammatory cholinergic signaling arising from the peripheral nervous system\(^{115}\). While this study did not directly investigate brain levels of tRFs and microRNAs, the finding that peripheral tRFs may be involved in homeostasis of inflammation following brain injury is promising for diagnostic purposes and treatment of various types of neurological trauma and disease.

**Neurodegeneration**—As discussed previously, an overall increase in 5’ tRF including 5’ tRNA halves in embryonic brain of *Nsun2* RNA-MTase null mutant mice was associated with broad downregulation of protein translation, contributing to reduced brain size, postsynaptic defects in neuronal signaling and behavioral deficits in adulthood\(^{116}\) (Figure 2c). In addition to these neurodevelopmental phenotypes associated with alterations in tRNA fragmentation and processing, abnormal expression of 5’ and 3’ tRFs have been reported in animal models of neurodegenerative conditions\(^{117}\), and in clinical samples from humans with Parkinson’s disease\(^{118}\) and Alzheimer’s Disease (AD)\(^{119}\). For example, post-mortem hippocampi from AD patients showed increased expression of the endoribonuclease and tRNA digestive enzyme, *ANGIOGENIN (ANG)* and 5’ tRNA fragments from various isooacceptor families\(^{119}\), which may be related to the aforementioned pathology in the developing brain\(^{77}\) due to decreased Nsun2 levels. However, the disease-relevance of tRFs and their potential sequelae, such as disruption of protein synthesis, remain completely unexplored in the context of chronic or acute neurodegeneration. Interestingly, however, mutations and sequence variants in *ANG* have been linked to some case with Parkinson’s\(^s\)\(^{120}\) and other neurodegenerative conditions, including amyotrophic lateral sclerosis/motor neuron disease (ALS/MND)\(^{121}\) and Alzheimer’s\(^s\)\(^{122}\). However, ANG, which participates...
in cell-to-cell communication via secretion and binding to its cognate receptor, plexin-B2, is highly pleiotropic with the potential to repress (by cytoplasmic digestion of full length tRNAs into tRFs) or stimulate (by activating ribosomal gene expression in the nucleus) translation, respectively. Therefore, additional work will be required to draw a mechanistic link between specific ANG mutations and neurodegenerative disease.

Conclusions and future perspectives

Multiple paths of converging evidence (i.e., tRNA gene mutations, tRNA charging, tRNA epitranscriptome, and tRFs) suggest that various aspects of tRNA function and metabolism are implicated in aspects of neurodevelopmental, neuropsychiatric, and neurodegenerative disorders, but we have only just begun to unravel the functions of the tRNA regulome in brain function and behavior. Previous work has shown that the tRNA pool is modified in response to mRNA abundance across development to enable efficient codon-anticodon pairings during translation, but it is still unknown whether tRNA abundance can shift dynamically in brain during times of enhanced or depleted translation (i.e. learning processes or diseased neurons, respectively). For example, are certain pools of tRNAs activity-dependent or cell type-specific in brain, and is this regulated by epitranscriptomic modifications? The remaining questions and opportunities for exploration are endless, and answering the complex questions about the tRNA regulome will require an integrative toolbox of molecular techniques ranging from the individual tRNA gene and its epitranscriptome, translational efficiency and protein readout, and ultimately behavioral and phenotypic outcomes relating to psychopathology.

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Box 1: tRNA aminoacylation via tRNA synthetase enzymes – an overview

Among the various types of post-transcriptional tRNA modification, aminoacylation, or the esterification of a tRNA to its cognate amino acid according to the genetic code, is accomplished by the group of enzymes known as the aminoacyl-tRNA-synthetases (aaRS), the only enzymes capable of implementing the genetic code. Therefore, in order to minimize translation errors, the aaRS enzymes are associated with two extremely delicate challenges – recognition of the tRNA molecule with a correct anticodon, and recognition of the correct amino acid to deposit onto the tRNA acceptor stem, characterized by its CCA leader sequence\textsuperscript{125}. The aaRS gene family is encoded in the nuclear genome, with separate isoforms for cytosolic and mitochondrial aaRS\textsuperscript{35}. There are two exceptions, with cytosolic and mitochondrial forms of glycyl-tRNA synthetase (GARS) and lysyl-tRNA synthetase (KARS) encoded by the same genes albeit via alternative transcripts, with cytosolic GARS1 lacking the mitochondrial signal (MTS) specific to GARS2\textsuperscript{126}. Furthermore, owing the lack of mitochondrial glutamyl-tRNA synthetase (QARS), conjugation of glutamic acid to its tRNA is mediated via amidotransferase activity\textsuperscript{127}. 
Box 2: The tRNA epitranscriptome

Superimposed on the regulatory layers governing tRNA expression and processing are at least 43 additional epitranscriptomic modifications\(^{54}\) (reviewed in ref\(^6\),\(^{128, 129}\)). Types of covalent tRNA modifications include methylation of various C, N and O atoms in adenine, guanine and cytosine residues, as well as pseudouridylation, thiolation and taurinomethylation of uracil residues, in addition to ‘hypermodified’ purines carrying very large organic molecules tethered to a guanine or adenine backbone\(^{54, 130}\). For each of these modifications, the exact nucleotide position varies between tRNA molecules, thereby adding additional complexity, and the complete set of modifications for the pool of nuclear genome-derived tRNAs remains unknown. However, for the subset of the 22 human mt-tRNAs, 18 different types of covalent modifications targeting a total of 137 nucleotide positions have been reported\(^{31}\). Many of these site- and residue-specific tRNA modifications appear to be critical for proper 3D-folding of the individual tRNA molecules and therefore essential to protect the tRNA from excessive degradation. Furthermore, epitranscriptomic modifications of residues in proximity to, or directly at the ‘wobble’ position often are essential for translational fidelity in the ribosomal tunnel, defined by the correct pairing of the tRNA’s anticodon with the proper codon on the mRNA molecule.
Box 3: Methodological considerations for tRNA studies in brain

Taken together, the aforementioned studies emphasize the importance of assessing the tRNA regulome as a layer of complexity previously overlooked in the context of neuropsychiatric disorders, but there are important methodological considerations that must be considered for future research. Primarily, the intricacy of tRNA secondary structure and abundance of post-transcriptional modifications across the majority of tRNA species creates challenges for measuring tRNA abundance with next-generation sequencing methods, such as inefficient reverse transcription and adapter ligation that may bias a dataset toward certain tRNA families. For this reason, it is crucial that specialized tRNA sequencing methods are used to eliminate bias, such as demethylation tRNA sequencing (DM-tRNA-seq)\textsuperscript{131} or Y-shaped Adapter MAture tRNA sequencing (YAMATseq)\textsuperscript{132}, instead of obtaining data from traditional RNAseq datasets. Additionally, new techniques such as Ribo-tRNA-seq\textsuperscript{133} have been developed to better capture the function of tRNAs within the ribosome during translation, although have yet to be used in the context of brain function. While these types of experiments would prove fruitful for the field and provide novel insight into translational mechanisms of an altered neuronal tRNA regulome, limitations such as large input requirements for these advanced techniques make this a challenge for future studies.
**Figure 1. tRNA structure and modifications linked to neuropsychiatric disease models.**

**a)** Schematic of tRNA structure and m^5^C modifications on tRNA^Gly^. tRNA schematic including clover-leaf secondary structure and anti-codon loop that pairs with corresponding codons on the mRNA strand during translation. Note cytosine positions within the anticodon arm and variable loop on tRNA^Gly^ that are methylated by Nsun2 and Dnmt2 in mouse brain\(^77,90\). Intact tRNAs are cleaved during various stress conditions by Dicer or Angiogenin to create tRNA halves or tRNA fragments originating from the 5’ or 3’ end, which have been implicated themselves in various cellular processes\(^77\).

**b)** Schematic of tRNA^Phe^ 2’-O-methylation and wybutosine modifications and site-specific methyltransferases identified in human cells\(^68\). In panels a and b, dark gray colored circles represent bases with other known tRNA chemical modifications\(^134\).

**c)** Schematic of tRNA m^1^A modifications and site-specific methyltransferases identified in human cells\(^100,102\).

AA= Amino acid.
Figure 2. Evidence from rodent studies of the altered tRNA regulome and downstream consequences for neuronal function and neuropsychiatric disease-related behavioral outcomes.

a) Mutation C50T at Arg-TCT-4-1 (also known as n-Tr20) naturally occurs in B6J mice, producing decreased tRNA$^{\text{Arg}}_{\text{UCU}}$ expression$^{28, 29}$. As a result, translation is impaired at AGA codons, and the integrated stress response is activated, accompanied by decreased mTOR signaling and increases in amino acids Gly and Ser. These changes lead to changes in synaptic transmission and seizure threshold in B6J mice$^{28}$.

b) In a rodent model of Charcot-Marie-Tooth disease type 2D (CMT2D), mice carry a gain of function mutation
in the *Gars* gene, in which GARS sequesters tRNA\textsuperscript{Gly} resulting in loss of available tRNA\textsuperscript{Gly} for translation\textsuperscript{44}. Mechanistic studies showed that phenotypic outcomes are caused by loss of functional tRNA\textsuperscript{Gly} produces stalling of Gly codons in the ribosome during translation and initiation of the integrated stress response (ISR), including phosphorylation of Eif2a and increased transcription of ATF4 target genes, via activation of GCN2\textsuperscript{42, 44}. 

c) Germline deletion of the tRNA MTase Nsun2 in mice completely eliminated Nsun2-mediated tRNA m\textsuperscript{5}C in embryonic brain resulting in an abundance of 5' tRNA fragments via Angiogenin-mediated cleavage and activated cellular stress pathways\textsuperscript{77}. KO brains had decreased global protein synthesis but also decreases in Post-synaptic density 95 (PSD-95) and synaptic puncta\textsuperscript{77}, likely contributing to adult impairments in hippocampal LTP\textsuperscript{94} and altered behavioral phenotypes\textsuperscript{77}. 

d) Mice with conditional deletion of *Nsun2* in *Camk2a*-expressing neurons likewise had decreased tRNA m\textsuperscript{5}C but showed a selective deficit in full length tRNA\textsuperscript{Gly} which was associated with ribosomal slowing at Gly codons and decreased expression of Gly-rich synaptic proteins, producing impaired neurotransmission\textsuperscript{90}. Coupled with the dramatic increase in Gly amino acid, these molecular changes contribute to phenotypes relating to anxiety, depression, and cognition\textsuperscript{90}. 

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