Symposium

Presynaptic Protein Synthesis in Brain Function and Disease

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Local protein synthesis in mature brain axons regulates the structure and function of presynaptic boutons by adjusting the presynaptic proteome to local demands. This crucial mechanism underlies experience-dependent modifications of brain circuits, and its dysregulation may contribute to brain disorders, such as autism and intellectual disability. Here, we discuss recent advancements in the axonal transcriptome, axonal RNA localization and translation, and the role of presynaptic local translation in synaptic plasticity and memory.

Introduction

Axons and dendrites form neuronal compartments whose function is determined by the local proteome. Although most studies have focused on local protein synthesis in dendrites, compelling evidence indicates that axons and presynaptic terminals in the mature brain can also undergo local translation (Holt and Schuman, 2013). Axons can span great length, thus imposing a significant challenge for the replenishment and delivery of proteins from the soma to distal axon terminals. One solution to this problem is local protein synthesis, which endows remote neuronal compartments with the ability to rapidly respond and adapt to local cues (Alvarez et al., 2000; Holt and Schuman, 2013). Local translation is also energy efficient as several proteins can be translated from a single mRNA. For many years, it was thought that healthy axons in the adult mammalian brain were not capable of synthesizing proteins, but there is now an abundance of evidence to the contrary (Alvarez et al., 2000; Jung et al., 2012; Biever et al., 2019; Holt et al., 2019).

Local axonal translation is required for the axon to mount injury response and to promote axon growth, maintenance, and survival (Willis and Twiss, 2006; Holt et al., 2019). Using super-resolution and electron microscopy, several studies have revealed translation factors and ribosomes in healthy presynaptic boutons of the mature mammalian brain (Shigeoka et al., 2016; Younts et al., 2016; Scarnati et al., 2018; Hafner et al., 2019; Ostroff et al., 2019). In addition, mRNA and mRNA binding proteins have been observed in adult axons (Baleriola et al., 2014; Shigeoka et al., 2016; Akins et al., 2017; Ostroff et al., 2019; Monday et al., 2022). Growing evidence indicates that presynaptic forms of plasticity require local protein synthesis in presynaptic boutons (Monday et al., 2018; Perrone-Capano et al., 2021). Lastly, dysregulation in axonal mRNA localization and local translation contributes to the pathophysiology of a wide range of brain disorders, including Fragile X Syndrome, Alzheimer's, and motor neuron diseases (Batista and Hengst, 2016; Costa and Willis, 2018; Lin et al., 2021; Perrone-Capano et al., 2021).

Despite the above evidence in support of axonal protein synthesis in the mature brain, several questions remain poorly understood, including the identity of the newly synthesized proteins, the regulatory mechanisms that control axonal translation and local protein abundance, and ultimately, how local translation contributes to structural and functional presynaptic changes to modify behaviors. Below, we summarize recent advancements in the axonal transcriptome in the mature brain; RNA processing, transport, and translation in axons; and local presynaptic protein synthesis in synaptic plasticity and memory (Fig. 1).

Transcriptome of mature CNS axons

Local translation is commonly used in plastic responses that are confined to subcellular compartments, such as the leading edge of a migrating fibroblast and the dendritic spine in synaptic plasticity (Hafner et al., 2019; Das et al., 2021). While local translation in the growth cone has been well established, particularly in response to guidance cues, the understanding of local translation in the mature CNS axons took longer to emerge, partly because of early ultrastructural studies reporting a scarcity of polyribosomes in mature axons (for review, see Kim and Jung, 2020). Recent comprehensive RNA-sequencing studies have cataloged mRNA molecules in CNS neuronal axons cultured to maturity *in vitro* (Maciel et al., 2018; Nijssen et al., 2018); however, questions remain regarding the extent to which cultured neuronal axons faithfully recapitulate axonal behavior *in vivo*.

Experimental evidence indicates that an adult-specific transcriptome in mice is shaped by selective mRNA transport and

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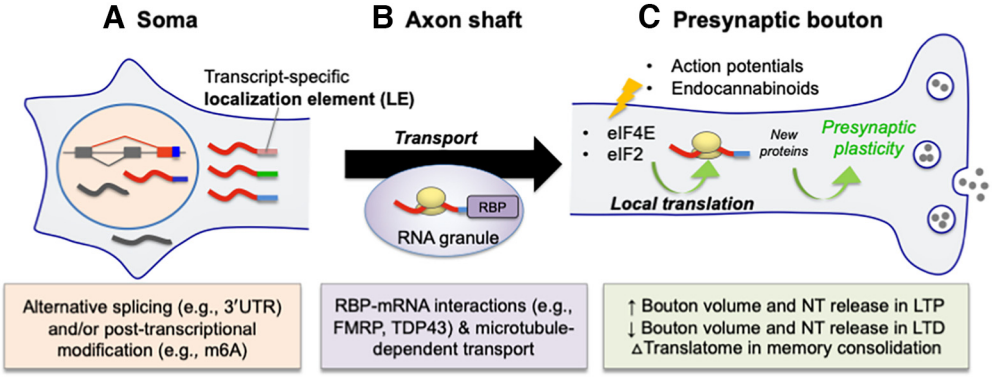

Figure 1. Axonal mRNA transport and presynaptic translation. ***A***, Axon LEs. LEs may be mRNA-intrinsic (i.e., sequence or structure) or may involve modified bases, such as m6A. ***B***, Axonal transport. RBPs bind to LEs and form RNA granules, which travel along the microtubule to presynaptic boutons in a translationally inactive state. FMRP and TDP-43 are among those that play crucial roles in the axonal transportation of mRNAs in mature CNS axons. ***C***, Transcript-selective local translation. Depending on the nature of the stimulus, such as presynaptic activity (e.g., action potentials) or receptor activation (e.g., via endocannabinoids), specific mRNAs are released from RNA granules and translated on-site. Translational regulation often involves phosphorylation of eIF4E-binding proteins (cap-dependent translation) or eIF2α (cellular stress-responsive translation). Presynaptic translation is required for structural and functional plasticity in presynaptic forms LTP and LTD associated with increased and decreased neurotransmitter (NT) release, respectively. Different sets of mRNAs are translated during memory formation and consolidation.

decay (Jung et al., 2023). The axon transcriptome capture (axon-TC) method was developed to isolate mRNAs present in the axon terminals of a well-defined CNS neuronal subtype, retinal ganglion cells, enabling assessment of both the quantity and diversity of RNA in their axons. Surprisingly, a direct comparison of axon-TC between young and old axons revealed comparable levels of RNA allocated to the axon terminal during both developmental and adult stages, constituting ~4% of the total RNA in the cell body. Moreover, a significant shift in the mRNA repertoire occurred following synaptic maturation, ~2 weeks after birth, with a relatively stable composition observed in adulthood for at least 6 months after birth. These findings suggest the existence of an axonal transcriptome specifically tailored to the adult stage.

The distinct mRNA profiles observed in developing and mature axons imply that older mRNAs undergo turnover to accommodate new mRNAs. By using a pulse-and-chase approach in combination with axon-TC, it was possible to estimate the relative turnover rates of mRNA species. This approach revealed that certain mRNAs are removed from axons at a faster rate compared with others. An intriguing finding was that the susceptibility to decay of an mRNA directly correlates with the degree of its association with the ribosome, as measured by axon–translating ribosome affinity purification (TRAP) (Shigeoka et al., 2016). This observation suggests a simple yet efficient mechanism to allow space for new mRNAs by coupling mRNA translation to decay.

To transport specific mRNAs to axon terminals, RNA-binding proteins (RBPs) must recognize and present their target mRNAs to molecular motors (Turner-Bridger et al., 2020). Comparing mRNA abundance between the axon (by axon-TC) and the cell body (by FACS of somata) revealed information about mRNA-selective axonal enrichment. Notably, the mRNAs known to interact with Fragile X messenger ribonucleoprotein (FMRP) and TAR DNA-binding protein 43 (TDP-43), strongly associated with neurodevelopmental (Bagni and Zukin, 2019) and neurodegenerative (Tziortzouda et al., 2021) diseases, respectively, were found to be highly enriched in axon terminals (see below). This observation suggests that these RBPs may play a critical role in maintaining the adult-specific axonal transcriptome. Moreover, it raises the possibility that defects in this process could be a potential risk factor for developing neurodevelopmental and neurodegenerative diseases.

RNA processing, transport, and translation in axons

In neurons, mRNAs translocate to dendrites and axons where they are rapidly translated in response to extracellular stimuli (Willis et al., 2007; Tushev et al., 2018; Andreassi et al., 2021). A vast recent literature has shown that local protein synthesis contributes to synaptic development and plasticity in dendrites (Perez et al., 2021), to axon extension during development (Shigeoka et al., 2016; Andreassi et al., 2021; Luisier et al., 2022), and to nerve regeneration after injury (Terenzio et al., 2018). Exciting technology advances recently allowed deep sequencing of increasingly small amount of RNA, revealing that transcripts localized in dendrites, axons, or cell bodies only partially overlap (Perez et al., 2021). Importantly, they also differ depending on the cell type and the developmental stage. Much is known regarding "what" is localized peripherally and "when" the transport takes place during the lifespan of the neuron. However, "how" transcripts are sorted in the cell bodies to be targeted to either dendrites or axons and the regulatory events involved in these critical decisions remains largely unknown.

At least two mechanisms must be considered regarding how neurons select transcripts to be transported peripherally. The first mechanism is intrinsic and depends on the RNA sequence and how transcripts fold into their secondary and tertiary structure. The RNA three-dimensional structure is instrumental in mediating the affinity for specific RBPs that will determine the fate of the transcript in terms of both localization and translation (Gebauer et al., 2021; Schieweck et al., 2021). Although elements essential for the peripheral targeting can be found anywhere along the transcript, most localization elements (LEs) identified so far are found within the 3′ untranslated regions (3′ UTRs) (Andreassi and Riccio, 2009). Following the discovery of the first LE in the 3′ UTR of β-actin (Kislauskis et al., 1994), the search for additional elements revealed that the RNA sequences showed little resemblance. Interestingly, RNA transcripts that are functionally related may be cotransported by sharing the interaction with RBP complexes. In sensory neurons, for example, the Splicing factor Poly-glutamine Rich RBP is assembled with several transcripts that are cotransported in axons where they promote axon survival (Cosker et al., 2016). An in-depth analysis of the predicted RBP binding proteome in sympathetic neuron axons revealed that RBPs responsible for mRNA

transport are specifically assembled in the cell bodies in response to neurotrophic factors (Luisier et al., 2022). Localized transcripts in both axons and dendrites express longer 3′ UTRs perhaps because they include multiple LEs (Andreassi et al., 2010; Tushev et al., 2018; Arora et al., 2022). The highest regulatory potential for peripheral localization is often found within the [−200:−50] nt region preceding the 3′ end, where RBPs promote the expression of isoforms with long 3′ UTRs transported in axons. Once they have reached distal axons, the same RBP complexes mediate mRNA processing that results in 3′ UTR cleavage (Luisier et al., 2022). This interesting phenomenon is observed in sympathetic neuron axons and probably common to most cell types (Mercer et al., 2011) and generates mRNA transcripts expressing shorter 3′ UTRs that are more efficiently translated, and 3′ UTR-derived noncoding RNAs with yet unknown functions (Andreassi et al., 2021). Thus, mRNA transcripts targeted peripherally to axons or dendrites may harbor coding and noncoding functions, switching between states depending on the developmental stage or the extracellular stimuli.

The second mechanism that contributes to the sorting of transcripts for peripheral localization involves post-transcriptional modifications of the RNA (Zaccara et al., 2019). Over 100 types of RNA epigenetic modifications have been identified so far, although the biological significance for most of them remains unclear (Kan et al., 2022). N6-methyl-adenosine (m6A) is the most common and abundant RNA modification and depends on the combined action of "writers" (enzymes that adds the methyl group cotranscriptionally), "erasers" (enzymes such as FTO and ALKBH5, that remove the methyl group and can be found also in dendrites and axons) (Yu et al., 2018; Martinez De La Cruz et al., 2021), and "readers" (RBPs that recognize the modified nucleotide) (Tzelepis et al., 2019). Several studies have shown that m6A is highly enriched in the mouse and human brain, with an enrichment of m6A modifications observed around the stop codon and the 3′ UTRs (Meyer et al., 2012), suggesting that the methylation state of the 3′ UTR may determine the ability to be transported peripherally. The RNA demethylases FTO and ALKB5 colocalize with methylated transcripts in axons of sympathetic and sensory neurons (Yu et al., 2018; Martinez De La Cruz et al., 2021), suggesting that m6A may influence the ability of transcripts to interact with specific RBPs responsible for repressing translation during the active transport. m6A enrichment is observed around the predicted 3′ UTR cleavage site of axonal transcripts (Luisier et al., 2022), opening the intriguing possibility of a crosstalk between two highly regulated mRNA post-transcriptional modifications that may determine both peripheral localization and the ability to function in a coding or noncoding manner.

Local presynaptic protein synthesis in synaptic plasticity

Presynaptic plasticity is generally defined as activity-dependent modulation of neurotransmitter release, which can occur over different timescales. Presynaptic LTP and LTD typically persist for hours and are widely expressed in the CNS by both excitatory and inhibitory synapses (Monday et al., 2018). Presynaptic structural changes in response to experience and activity are commonly associated with altered synaptic strength (Gogolla et al., 2007; Sigrist and Schmitz, 2011; Monday and Castillo, 2017). While presynaptic and postsynaptic forms of long-term plasticity can coexist at the same synapse, long-term presynaptic plasticity can contribute uniquely to circuit computations by providing a way to modify short-term synaptic dynamics (e.g., by changing the probability of neurotransmitter release) (Costa et al., 2017; Monday et al., 2018). Moreover, presynaptic long-term plasticity has been implicated in diverse forms of learning, and its dysregulation participates in several neuropsychiatric conditions, including schizophrenia, autism, intellectual disabilities, neurodegenerative diseases, and drug abuse (Monday et al., 2018). Despite significant advancements in the molecular basis of neurotransmission, exactly how transmitter release is modified in a long-term manner remains largely unclear. However, growing evidence indicates that functional and structural presynaptic long-term plasticity requires local protein synthesis in presynaptic boutons (Monday et al., 2018; Perrone-Capano et al., 2021).

A good example of a ubiquitous form of long-lasting depression of neurotransmitter release is endocannabinoid-mediated LTD. Here, endocannabinoids are released on activity and travel in a retrograde manner to bind presynaptic Type 1 cannabinoid (CB1) receptors, resulting in LTD at both excitatory and inhibitory synapses (Castillo et al., 2012). The need for local presynaptic protein synthesis in plasticity was first demonstrated at the endocannabinoid-mediated LTD at hippocampal inhibitory synapses (Younts et al., 2016). This plasticity requires rapid translation (<30 min) and is associated with protein synthesis-dependent reduction in presynaptic bouton volume (Monday et al., 2020). In addition, CB1 activation increases ribosomal proteins and initiation factors but decreases levels of proteins involved in regulating the actin cytoskeleton, such as Arp2/3, and presynaptic release (Monday et al., 2020). Local presynaptic translation has also been demonstrated at the mossy fiber to CA3 pyramidal cell synapse (MF-CA3) (Monday et al., 2022), a model synapse that expresses robust functional and structural presynaptic plasticity and conveys the main excitatory input to the hippocampus proper (Nicoll and Schmitz, 2005). MF boutons contain ribosomes and locally synthesize β-actin following LTP induction. Moreover, increasing *in vitro* or *in vivo* MF activity enhances the protein synthesis in MFs, and MF-LTP is associated with a translation-dependent enlargement of MF boutons (Monday et al., 2022). Together, these studies demonstrate that presynaptic structural and functional changes at both inhibitory and excitatory synapses rely on local protein synthesis.

Regulators of protein synthesis, such as the mRNA-binding protein and translation repressor FMRP (Darnell and Klann, 2013), have been observed in axons (Akins et al., 2017) and presynaptic terminals (Mitchell et al., 2023), including the MF bouton (Christie et al., 2009). A loss-of-function mutation of the *FMR1* gene that encodes FMRP causes Fragile X Syndrome, the most frequently inherited form of intellectual disability and the most prevalent monogenic cause of autism (Bassell and Warren, 2008; Bagni and Zukin, 2019). Under basal conditions, FMRP binds ribosomes and mRNAs to form an FMRP granule; and in this bound state, mRNA translation is repressed (Bauer et al., 2023). Activity leads to FMRP dephosphorylation, which triggers granule disassembly and de-repression of translation. FMRP regulates up to 30% of the presynaptic proteome, including scaffolding proteins, synaptic vesicle proteins, and voltage-gated calcium channels (Klemmer et al., 2011; Darnell and Klann, 2013). Remarkably, MF-LTP involves dephosphorylation-dependent disassembly of FMRP granules in MF boutons and translational activation, and loss of presynaptic FMRP impairs structural and functional MF-LTP and increases the local protein synthesis in the MF track (Monday et al., 2022). These findings indicate that presynaptic protein synthesis is

a highly regulated process that can be altered in some neurodevelopmental disorders.

Cell type-specific and local translation in emotional memory

Early studies demonstrating the requirement of *de novo* protein synthesis for long-term memory (LTM) consolidation primarily used pharmacological inhibitors, such as puromycin, anisomycin, and cycloheximide (Davis and Squire, 1984), which target translation elongation. Beginning in the 2000s, investigation of the role of translation initiation, the rate-limiting step in protein synthesis, in memory began to intensify. Most of these studies focused on regulation of two translation initiation factors: eukaryotic translation initiation factor 2α (eIF2α) and eukaryotic translation initiation factor 4E (eIF4E) (Costa-Mattioli et al., 2009; Richter and Klann, 2009).

Phosphorylation of eIF2α is a key regulatory control point in translation initiation. eIF2 binds Met-tRNA$_i^{Met}$ and GTP to form the stable 43S preinitiation complex. eIF2B promotes exchange of GDP for GTP, which is required for new rounds of translation. Phosphorylation of eIF2α on serine 51 (Ser51) transforms eIF2 to a competitive inhibitor of eIF2B, thereby blocking GDP/GTP-exchange to inhibit general translation, while simultaneously increasing the translation of mRNAs with upstream open reading frames in their 5′ UTRs (Pestova and Hellen, 2003; Sonenberg and Dever, 2003; Pestova et al., 2007). Numerous studies have shown that tight regulation of eIF2α is required to trigger the formation of LTM. For example, depending on the training paradigm, constitutive genetic deletion of several eIF2α kinases, including the double-stranded (ds) RNA activated protein kinase (PKR), can promote memory formation (Costa-Mattioli et al., 2005; Zhu et al., 2011). Similar findings were observed with knockin phospho-mutant eIF2α mice (Costa-Mattioli et al., 2007). Thus, regulation of eIF2α phosphorylation is a critical requirement for LTM.

Mechanistic target of rapamycin complex 1 (mTORC1) stimulates cap-dependent translation initiation via phosphorylation of eIF4E-binding proteins (4E-BPs) and p70 S6 kinase 1 (S6K1) (Richter and Klann, 2009). Briefly, mTORC1 binds both 4E-BP2 (the brain-enriched isoform) and S6K1. eIF4E, which binds to the 5′ methylated-cap of mRNAs, binds to 4E-BP2; unphosphorylated 4E-BP2 binds tightly to eIF4E, whereas 4E-BP2 phosphorylated by mTORC1 does not, thereby permitting the eIF4F complex (eIF4E+ eIF4G+eIF4A1) to form so that translation initiation can proceed. mTORC1 also impacts translation by phosphorylating S6K1, which then phosphorylates targets that promote cap-dependent translation. mTORC1 signaling is required for multiple forms of LTM, including associative threat memory (for reviews, see Hoeffer and Klann, 2010; Costa-Mattioli and Monteggia, 2013). Notably, the mTORC1 effectors eIF4E and S6K1 have specific roles in associative threat memory consolidation, reconsolidation, and extinction (Hoeffer et al., 2011; Huynh et al., 2014, 2018).

Although eIF2- and eIF4E-dependent translational control are fundamental mediators of memory, little is known about their role in local translation during memory consolidation. It was shown that polyribosomes accumulate in lateral amygdala dendritic spines during threat memory consolidation (Ostroff et al., 2010) that was blocked by preventing eIF4E–eIF4G interactions (Ostroff et al., 2017), suggesting that local eIF4E-dependent translation is involved in learning. Regarding axonal translation, TRAP was used to determine whether translation occurs in cortical axons in the amygdala following learning. Combining TRAP with threat conditioning, it was shown that over 1200 mRNAs in the axonal translatome were regulated during memory consolidation (Ostroff et al., 2019). These results demonstrate that axonal translation occurs during memory formation and support the idea that local translation is involved in memory consolidation. However, it remains to be determined whether local axonal translation is causal with respect to threat memory consolidation.

To determine whether local axonal translation is required for LTM, the development of chemogenetic and optogenetic tools to manipulate axonal protein synthesis will be necessary. Historically, studies examining the requirement of translation initiation for LTM have used either pharmacological or genetic approaches, each of which has merits and shortcomings. Pharmacological approaches provide temporal control, which permitted the determination that *de novo* translation is required for LTM consolidation but lack cell type specificity. Genetic approaches can provide cell type specificity but typically lack fine temporal control required for studies of memory consolidation (Shrestha and Klann, 2022). For example, cell type-specific and inducible knockdown of eIF4E was used to identify cell types in the amygdala that require cap-dependent translation for associative threat memory (Shrestha et al., 2020a,b) and safety memory (Shrestha et al., 2020b). However, the knockdown of eIF4E in these studies used a tet-inducible system, which has temporal control that is inadequate to determine whether eIF4E was necessary for acquisition, consolidation, or retrieval of LTM. The development of a chemogenetic knockin mouse line harboring an inducible PKR (iPKR) allowed for the demonstration that eIF2-dependent translation in excitatory neurons in the lateral amygdala and somatostatin-expressing inhibitory neurons in the centrolateral amygdala is required for the consolidation of threat memory (Shrestha et al., 2020a,b). These findings indicate that *de novo* translation in distinct subpopulations of lateral amygdala and centrolateral amygdala neurons is required for LTM consolidation. Moreover, the iPKR knockin mice could be used to determine whether the learning-induced local axonal translation that occurs in cortical axons in the amygdala (Ostroff et al., 2019) is required for the consolidation of auditory threat memory.

Concluding remarks and future directions

Local presynaptic translation in mature mammalian axons regulates the local proteome and plays essential roles in synaptic plasticity and memory. Although axons contain thousands of mRNAs, it will be important to know what determines the translation of specific mRNAs but not others in response to local cues and how this translation is regulated. Because a CNS axon can establish multiple synaptic contacts with heterogeneous properties, it will be crucial to know the mechanisms underlying mRNA sorting in axons, as well as the mRNA distribution in more refined subcellular compartments, such as the presynaptic bouton versus the axon shaft. There is a need for tools that could be used *in vivo* to visualize axonal translation in real time and to manipulate presynaptic protein synthesis and degradation. As the dynamic transport of mRNAs finely regulates the site of their translation (Sahoo et al., 2018), imaging-based approaches that allow simultaneous monitoring of different mRNA species under physiological conditions will provide deeper insights into the mRNA-based molecular processes occurring at these sites. Selective blockade of axonal protein synthesis *in vivo*, either with the iPKR system or new chemogenetic and optogenetic tools,

would allow us to unequivocally demonstrate a role for local translation in axon survival, presynaptic plasticity, and memory.

A better understanding of the roles of newly synthesized synaptic proteins in axons is needed to establish a clear molecular link between local translation and structural and functional changes at the synapse. A critical knowledge gap concerns the identity of the locally synthesized proteins that enable the long-term strengthening and weakening of neurotransmitter release. Exploring the transcriptomes of single presynaptic terminals offers a promising avenue to unravel the heterogeneity and functional diversity of individual presynapses within a neural circuit. A recurring and puzzling finding is that mRNAs found in axon terminals generally encode already abundant proteins, such as ribosomal and cytoskeletal proteins. Proteomic studies of the difference between locally synthesized and preexisting proteins may shed light on the roles played by proteins synthesized on-site (Holt et al., 2019). By addressing these and other missing links, we will better understand RNA localization and its implications for brain function and diseases. Lastly, in addition to protein synthesis, the presynaptic proteome is also controlled by protein degradation (Giandomenico et al., 2022). Much work is required to determine the local cues and mechanisms implicated in axonal protein degradation and its contribution to neurodegenerative and neurodevelopmental disorders.

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