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Local mRNA translation in long-term maintenance of axon health and function

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Distal axons, remote from their cell bodies and nuclei, must survive the lifetime of an organism. Recent studies have provided compelling evidence that proteins are locally synthesized in healthy, mature central nervous system axons and presynaptic terminals *in vivo*. Presynaptic, mitochondrial and ribosomal proteins are locally synthesized in most adult axons of diverse cell types, linking local translation to axon function and survival. Accordingly, inhibiting the intra-axonal translation of key mRNAs or the function of their translational regulators causes dying-back axon degeneration, and human mutations in RNA metabolic pathways are increasingly being associated with neurodegenerative diseases that accompany axon degeneration. Here, we summarize recent relevant findings in a highly simplified 'RNA operon'-based model and discuss open questions and future directions.

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Introduction

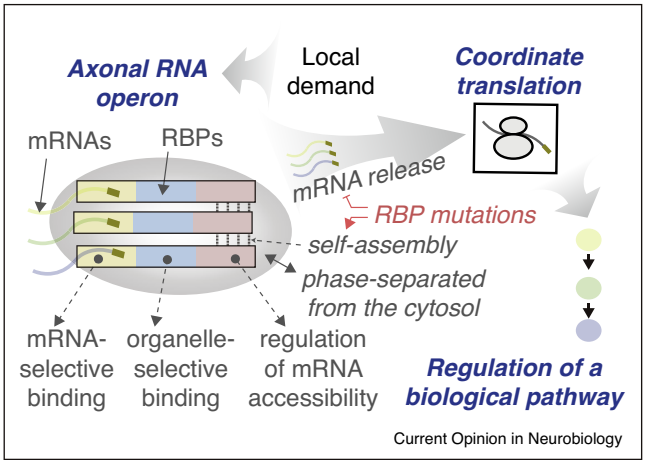
Neurons in our brains live for several decades with a long axon, where proteins have to be replenished throughout the lifetime. Prompt and on-demand protein delivery from the soma to the distal axon is a challenge particularly in the meter-long axons of motor neurons, because it can take over ten days even in a non-stop, full-speed journey (reviewed in Ref. [1]). Nonetheless, anterograde protein transport is a key mechanism and has received well-deserved attention in cell biology and biology of neurodegeneration (reviewed in Ref. [2]). On-site protein synthesis has been regarded as an additional and perhaps exceptional mechanism, operating mainly in the developing and regenerating axons. Indeed, local mRNA translation has been implicated in almost all aspects of axon development, such as axon guidance, elongation, target selection and survival (reviewed in Ref. [3]), and in axon regeneration (reviewed in Ref. [4]). Whether it occurs in healthy, mature axons in the central nervous system was less clear.

In recent years, a surge of compelling evidence suggests that it does happen and may even play important roles in adult axons. In this focused review, we try summarizing these findings with a highly simplified mechanistic model in mind (Figure 1) hoping that obvious open questions may reveal themselves. This model might provide one way to explain why many mutations in RNA metabolism are linked to human neurodegenerative diseases.

Local mRNA translation occurs in healthy adult axons

Overall reluctance to study local translation in adult axons was due, in part, to the scarcity of ultrastructurally identifiable polyribosomes (polysomes), the criteria of which were established in electron microscopy (EM) studies of non-neuronal cells (reviewed in Refs. [1,5]). Indeed, recent evidence using RiboTag mouse [6] suggests that ribosomes in adult axons may take a different form. In this study, epitope-tagged ribosomes (Rpl22-HA) expressed in retinal ganglion cells allowed ultrastructural detection of ribosomes in fully myelinated retinal axon shafts and presynaptic terminals. Interestingly, they were not 'polysomal' and existed in intermittent clusters of monosomes, consistent with earlier observations made in peripheral axons [7]. These ribosomes co-purified with specific mRNAs and 'ran off' the mRNAs when allowed to continue translational elongation, indicating that co-purified mRNAs represent the *in vivo* axonal translatome (i.e. entire set of translated mRNAs). A more recent study generalized this finding [8••]. In this study using vGlut1-EGFP transgenic mouse, 'pre-synaptosomes' could be fluorescently sorted from synaptosomes, which may represent the presynaptic terminals of an 'average' excitatory neuron. There, ribosomes were clearly detected by electron and super-resolution microscopy, again in sparse clusters. Presynaptic ribosomes were slightly away from the active zone, not unlike dendritic ribosomes that translocate from the neck [9] to the spine upon activity [10]. Importantly, metabolic labeling of nascent proteins in culture showed that ~30% of presynaptic terminals are metabolically active under basal conditions, which increases to ~50% after BDNF stimulation. A similar observation was made in auditory cortical neurons [11]. In

Figure 1



Axonal RNA operon model.
See text (Section 'Translationally co-regulated mRNAs serve common functions: axonal RNA operons') for details.

this study, an epitope-tagged ribosomal protein (EYFP-Rpl10A) gene, which was previously used to label axonal ribosomes of upper motor neurons [12], was lentivirally expressed to tag ribosomes in auditory axon terminals in the amygdala. The mRNAs bound to these axonal ribosomes changed after associative fear learning, suggesting stimulus-dependent axonal mRNA translation. Again, polysomes were only occasionally detected in these axons. These studies strongly suggest that axonal mRNA translation occurs in adult axons *in vivo*, perhaps using monosomes as previously proposed [1] and supported by a recent study [13].

Protein synthetic machinery may take different forms in axons

Another puzzling ultrastructural feature of axons is that they appear to lack the Golgi apparatus. For this reason, whether axonally synthesized proteins can be delivered to the plasma membrane has been controversial, despite the molecular evidence that axons contain transcripts encoding a variety of transmembrane and secretory proteins and the functional evidence that axons locally synthesize cell surface receptors such as EphA2 and κ-opioid receptors (reviewed in Refs. [14,15]). A recent study shed light upon this puzzle, by providing evidence that isolated peripheral sensory axons can deliver subunits of the voltage-gated sodium channel from the axonal smooth endoplasmic reticulum (ER) to the plasma membrane [16•]. This local route may involve 'mixed-identity organelles' budding off from the axonal 'smooth' ER, which possess the functional and biochemical properties of lysosomes and endosomes (reviewed in Ref. [15]). Indeed, recent evidence suggests that axonally synthesized transmembrane proteins do bypass the Golgi and retain 'immature' glycosylation patterns (reviewed in Refs. [14,15]). Therefore, like mitochondria, whose morphology is net-like in the soma and rod-like in the axon (reviewed in Ref. [17]), protein synthetic and processing machinery may be subcellularly specialized. In this sense, it is worthwhile to mention that translational mechanisms were studied mainly in the soma and therefore may have differences in the axon. For example, cap-independent translation may play particularly important roles [18], although cap-dependent mechanisms also operate [19,20]. Phosphorylation of eIF2α, which generally decreases global translational activity and increases translation of specific mRNAs including *Atf4* (reviewed in Ref. [21] and found in axons [22]), may upregulate global translation [23]. Intra-axonal translation may occur at highly confined domains, such as axon branching points [24], the surface of late endosomes [25], and the cytoplasmic domains of cell surface receptors [26,27•].

Translationally co-regulated mRNAs serve common functions: axonal RNA operons

An important finding is that blocking translation of one gene produces specific phenotypes, such as cue-induced steering and collapse of the growth cone, and elongation and survival of axons (reviewed in Ref. [3]), suggesting that cue-induced, mRNA-selective translation takes place to subserve a specific function. Consistently, metabolic labeling of nascent proteins in severed *Xenopus* retinal axons in culture shows stimulus-dependent proteome signatures [28••]. To explain this mRNA-selective translation in axons, a model [29] — sometimes entailing coordinated regulation of multiple transcripts in an 'operon' — has emerged [30,31].

A key concept in this model is that mRNAs encoding proteins with related functions bear a common '*cis*-element' and are co-stored in a translationally repressed state (Figure 1). A *trans*-acting factor, for example, an RNA-binding protein (RBP) or a microRNA, binds to and prevents the translation of the mRNAs. This group of mRNAs, or 'RNA operon', may interact with others to form a higher-order structure called 'RNA regulon' [31]. When a particular signal is received, a specific RNA operon releases its target mRNAs, allowing their translation. Functionally, axonal RNA operons should contain 1) transcript-selective RNA binding element, 2) a localization element, and 3) a regulatory element that regulates localization and/or translation. Biochemically, they may simply be 'neuronal RNA granules' [32], whose RNA-binding activity can be modulated.

A good example is 'SFPQ RNA operon' that localizes to and supports the function of axonal mitochondria in peripheral sensory axons [33]. SFPQ (splicing factor proline and glutamine rich) is an RBP that can bind to *bcl2l2* and *lmnb2* mRNAs encoding mitochondrially localized regulators of axon survival, and to a kinesin motor. SFPQ-containing granules deliver both mRNAs to distal axons in a single packet, and release them upon

neurotrophin stimulation. Decreasing SFPQ expression leads to decreased *bcl2l2* and *lmnb2* mRNA abundance in axons, leading to a loss of response to neurotrophin. Other RBPs, such as hnRNPs [34•] and FMRP [35], localize to axons, suggesting that more axonal RNA operons and regulons await discovery. Soma-restricted RBPs such as Pum2 keep their target mRNAs away from the axon and indirectly shape the axonal transcriptome [36]. RBP modification (e.g. phosphorylation) is a well-studied mechanism that may underlie operon-selective translational activation (reviewed in Ref. [3]).

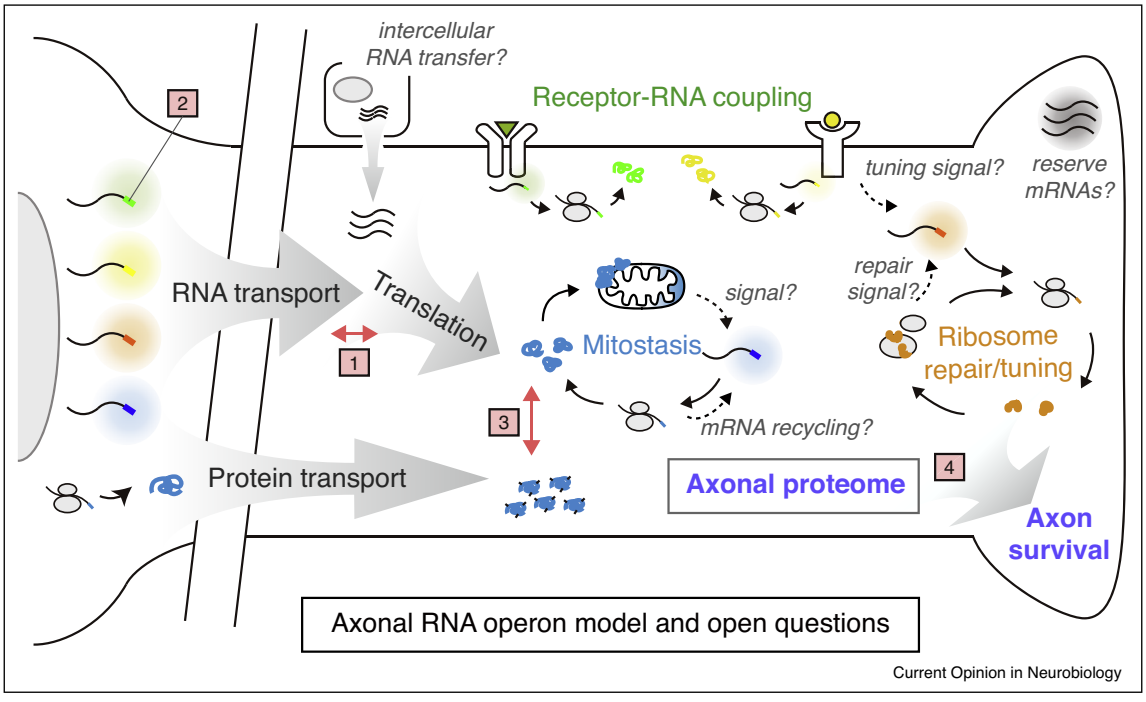
An intriguing idea is to include transmembrane receptors in RNA operons (Figure 2). Extending the original finding that DCC forms a complex with translation-stalled ribosomes and releases them upon ligand binding [26], a recent study generalizes this idea [27•]. The receptors for guidance cues Netrin-1 and Sema3A, which elicit opposite steering responses in the growth cone, bind to ribosome-containing RNA granules with unique protein and mRNA signatures. DCC and Nrp1 make complexes with hnRNPA2/B1 and Staufen1 with respective target mRNAs, respectively, at least in a neuronal cell line, and these interactions are weakened by ligand binding in cultured *Xenopus* retinal axons. An emerging model, conceptually similar to G-protein-coupled receptor activation, is that ligand binding leads to a conformational change of its receptor and subsequent release of mRNAs from the receptor-docked RNA granules for immediate translation, upon and at the site of signal reception. Release of mRNAs might also involve direct modification of mRNAs, for example, cleavage of 3′-UTR [37].

Connection to neurodegenerative diseases: when axonal RNA operons go wrong

What is consistent in 'adult' axonal transcriptome/translatome studies is that common functions are highly represented (Table 1). First, mRNAs encoding 'synaptic' proteins are enriched [8••] and translated [6] in axons *in vivo*. Inhibiting translation in the brain slice that contains the calyx of Held synapse, in which presynaptic ribosomes are clearly identifiable, causes a presynaptic phenotype [38••]. Secondly, mRNAs encoding 'mitochondrial' proteins are enriched, whose local translation is required for axon survival [33,39]. Finally, mRNAs encoding 'ribosomal' proteins are abundant. Of course, axonal translatome data are collective, and translation of these mRNA is likely to be dynamically regulated in individual axons. Such a regulation is highly relevant in understanding axon maintenance and degeneration, which are the topics of this and following sections.

The idea that dysfunctional axonal mRNA translation might be a common cause of neurodegenerative diseases is not new (for example, see Ref. [5]). Many neurodegenerative diseases are thought to start with metabolic failure at the presynaptic terminal, which then progresses

Figure 2



Working model and open questions.
See text for details. See section 'Open questions' for boxed numbers. Dashed arrows are speculation of this review.

Table 1

Recent genome-wide screening studies on axonal mRNA localization and translation, relevant in understanding healthy and diseased adult axons

Biomolecule	Neuron	*In vivo*	Axon purification	Detection	Enriched function	Ref.
RNAs (steady-state)	**Spinal motor** (rat embryonic culture)	–	Boyden chamber	RNA-seq	Mitochondrial Ribosomal	[50]
RNAs (steady-state)	**Spinal motor** (hiSPC-derived)	–	Boyden chamber	RNA-seq	Mitochondrial	[48]
RNAs (steady-state)	**Spinal motor** (m/hESC-derived)	–	Microfluidic device	RNA-seq	Mitochondrial	[49]
Nascent proteins	**Retinal** (*Xenopus* embryonic culture)	–	Boyden chamber	LC–MS/MS (axotomy - SILAC)	Cytoskeletal Ribosomal Signaling	[28••]
RNAs/proteins (steady-state)	**Cortical callosal** (EGFP-transfected perinatal mouse)	Yes	Fluorescence sorting of growth cone	RNA-seq or LC–MS/MS	Ribosomal Mitochondrial	[54•]
RNAs (steady-state)	**Cortical excitatory** (vGlut1::EGFP adult mouse)	Yes (adult)	Fluorescence sorting of synaptosome	RNA-seq	Mitochondrial Ribosomal Synaptic	[8••]
Ribosome-bound mRNAs	**Retinal** (embryonic and adult RiboTag; αCre mouse)	Yes (adult)	Anti-HA TRAP from superior colliculus	RNA-seq	Mitochondrial Ribosomal Synaptic	[6]
Ribosome-bound mRNAs	**Cortical auditory** (adult rat with lentivirally delivered EYFP-Rpl10A)	Yes (adult)	Anti-EGFP TRAP from amygdala	RNA-seq	Mitochondrial Ribosomal Synaptic	[11]

retrogradely—a process known as dying-back degeneration (reviewed in Refs. [40,41]). To our knowledge, the first evidence implicating local translation in dying-back degeneration was that inhibiting translation of *lmnb2* mRNA only in axons leads to age-dependent, soma-independent axon degeneration in *Xenopus* [39]. Sustained local translation of *lmnb2* and other related mRNAs, perhaps from SFPQ granules [33], may be required to maintain mitochondrial integrity and axon health (reviewed in Ref. [17]). A defect in this process may lead to accumulation of damaged mitochondria, metabolic failure, and initiation of the axon destruction pathway. The axon terminal may be particularly vulnerable, because the reliance on local protein synthesis is higher than proximal neurites. In this sense, it is noteworthy that Nmnat2, a labile protein that should be maintained at a certain level to suppress Sarm1-dependent axon destruction pathway (reviewed in Ref. [40]), is locally translated in mature axons *in vivo* [6]. What regulates translation of these mRNAs in healthy axons is unknown. Exhausted mitochondria may send an SOS to axonal mitochondrial operons (Figure 2), which may sit on mitochondrion-docked late endosomes [25], as cytoplasmic translation can be modulated by mitochondria in yeast [42].

Biochemical studies on RBPs revealed an important mechanistic clue that is highly relevant to neurodegenerative diseases [43]. Many RBPs show propensity to self-assemble into gel and phase-separate from surrounding liquid via the prion-like domain (or low complexity sequence, or intrinsically disordered region) (reviewed in Ref. [44]) (Figure 1). This phase separation can be regulated by biological or physical cues (e.g. phosphorylation or temperature), making it a novel mechanism for mRNA-selective translational inhibition and activation. Recent evidence suggests that this operates in axons, as phase separation of FUS-containing granules are regulated by methylation and chaperon binding in *Xenopus* retinal axons [45•]. If so, any mutation that makes an RBP to gelate stronger might gradually shift the phase to irreversible gelation, preventing translation of target mRNAs. Again, axon terminals will be particularly vulnerable. A recent study using a mouse with a humanized mutation supports this idea [46••]. When the endogenous *FUS* allele is replaced with a human mutation linked to ALS/FTD (amyotrophic lateral sclerosis/frontotemporal dementia), decreased axonal protein synthesis is the first phenotype, which appears well before synaptic dysfunction, somal death and behavioral deficits. Importantly, it occurs without any defects in nuclear FUS functions, suggesting that dysfunctional axonal protein synthesis is key to pathogenesis.

A recent study by Liao and colleagues revealed an intriguing mechanism by which RNA granules 'hitchhike' lysosomes for axonal transport [47••]. Annexin 11, which contains an N-terminal prion-like domain that phase-separates and binds to RNA granules and a C-terminal phosphoinositide binding domain that binds to lysosomes in Ca^{2+}-dependent manner, acts as a reversible molecular tether. Intriguingly, ALS/FTD-linked mutations in *annexin 11* impairs its tethering function and axonal transport of RNA granules, supporting the

notion that axonal RNA transport and local translation are required for long-term axon survival. Considering high abundance and diversity of axonal mRNAs in human motor neurons [48], it will be interesting to see whether other ALS-linked mutations affect axonal mRNA transport and/or translation. Of interest, introducing ALS-linked mutant genes, such as *Sod1G93A* [49] and *Tdp43A315T* [49,50], changes axonal mRNA repertoires in cultured motor neurons.

Axonal synthesis of ribosomal proteins

A finding that has been generally overlooked is that mRNAs encoding ribosomal proteins are abundant in axons. In fact, they are not only abundant, but also enriched in axons [51–53], growth cones [53,54•] and presynaptic terminals [8••], and they are axonally translated [6,28••]. One potential consequence is that axonally synthesized ribosomal proteins join axonal ribosomes, a possibility supported by a recent study [55]. The ribosome is an expensive organelle, whose biogenesis involves all three RNA polymerases and complex RNA modifications that begin in the nucleolus [56]—the structure that axons clearly lack. Considering that adult axons may synthesize proteins for years, damaged ribosomes might be better repaired than scrapped (Figure 2). A major repair, involving multiple ribosomal proteins, is a possibility not to be dismissed, because the mitochondrial ribosome, whose biogenesis is also highly complex, is indeed locally assembled (i.e. in the axonal mitochondria) using nuclear-encoded genes for mitochondrial ribosomal proteins and RNA modifying enzymes [57].

Another intriguing possibility is that axonal ribosomes can be 'tuned' for specific mRNAs using locally synthesized ribosomal proteins, extending the idea of subcellular ribosome heterogeneity (reviewed in Ref. [58]). Indeed, distinct ribosomal proteins are locally synthesized in axons treated with different cues [28••] and might bind ribosomes [55], although whether they join same or different ribosomes is not known. However, the existence of heterogenous ribosomes tuned to specific mRNAs, perhaps docked to different receptors, is a tantalizing idea worth serious investigation. Ribosomes could also be tuned by RBPs such as SMN, which primes ribosomes to mRNAs required for motor neuron function and stability [59].

Open questions

In short, mature axons coordinately translate locally stored mRNAs to support synaptic function and long-term survival. Mutations that perturb this process are linked to neurodegenerative diseases in human, which can be explained by the 'axonal RNA operon' model. We end this review with open questions (Figure 2).

mRNA transport versus translation

Most studies looked at either the transcriptome or translatome to understand axonal mRNA translation. To delineate mechanisms based on mRNA transport and translation, dynamics of axonal transcriptomes *in vivo* should be determined and compared with translatomes and/or nascent proteomes. New knowledge that can be gained from this approach includes the possibility of mRNA recycling, which should be economical for sustained translation; the existence of translationally dormant mRNAs, which might play a coding-independent role [60]; and the possibility of inter-cellular mRNA transfer, which was previously proposed [61] and may utilize cell-penetrating RNA-binding homeobox proteins [62] or Arc capsids [63].

Axon targeting *cis*-elements

Motif analyses in axonal mRNAs have not produced easily interpretable results, although mRNA localization is key to protein localization [64]. Rules governing RNA localization should be discovered, which may lie among alternatively spliced 3′-UTRs [65]. Post-transcriptional RNA modification is an interesting possibility, as synaptosomal mRNAs are highly N6-methyladenosine (m6A)-modified [66] and m6A modification regulates local translation in axons [67•,68•]. Other modifications, such as poly(A) addition/removal, 3′-end uridylation and pseudourydylation, are also interesting possibilities.

Proteomics of axonally synthesized proteins

Axonally synthesized proteins may have different properties compared to those transported from the soma, such as post-translational modification (proposed in Ref. [30]) and different C-terminal amino acids encoded in alternatively spliced last exons [6,69••]. Quantitative analysis of contribution of axonally synthesized proteins to the axonal proteome *in vivo* is also lacking. Engineered genetic code-based techniques that allow cell type-specific metabolic labeling of nascent proteins [70,71] *in vivo* show a promising direction.

Local translation in axon survival

Quantitative analysis of local protein synthesis in healthy, mature axons compared to developing axons will help us better understand its contribution to axon maintenance. An unequivocal test on the requirement of local mRNA translation on long-term axon survival in the mammalian central nervous system is missing, which may require new methods to inhibit translation only in axons *in vivo*.

Together, the results of the above experiments will add important details to the model and may shed new light on the causes and therapeutic strategies of neurodegenerative diseases.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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