Published in final edited form as:

Curr Opin Genet Dev. 2011 August ; 21(4): 458–464. doi:10.1016/j.gde.2011.04.004.

Translational regulation in growth cones

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Abstract

Axonal growth cones (GCs) steer in response to extrinsic cues using mechanisms that include local protein synthesis. This adaptive form of gene regulation occurs with spatial precision and depends on subcellular mRNA localisation. Recent genome-wide studies have shown unexpectedly complex and dynamically changing mRNA repertoires in growing axons and GCs. Axonal targeting of some transcripts seems to be highly selective and involves sequence diversity in non-coding regions generated by transcriptional and/or posttranscriptional mechanisms. New evidence reports direct coupling of a guidance receptor to the protein synthesis machinery and other findings demonstrate that some guidance cues can *repress* translation. The recent findings shed further light on the exquisitely regulated process that enables distant cellular compartments to respond to local stimuli.

Introduction

The axonal growth cone (GC) represents a unique signaling compartment, existing for the purpose of guiding an axon to its postsynaptic target. On reaching the target, the GC transforms into the developing axonal arbor with presynaptic terminals. Since the first observation of β -actin mRNA in the GC [1], mounting evidence has shown that this transient structure uses local mRNA translation to respond directionally to stimuli, contributing to its autonomous function [2–10]. For example, the chemotropic responses of GCs to Netrin-1, BDNF, Sema3A and Slit2 require local mRNA translation [4–7,9,11], and axonal mRNA translation is necessary for efficient GC regeneration [12]. A requirement for axonal protein synthesis (PS) for cue-induced responses was not seen in one study [13], although it may have been masked by the high cue concentrations used. Studies in recent years have indicated that remarkable complexity exists in the regulation of the GC's proteome and, moreover, that growing axons have adopted some specialised mechanisms for processing newly synthesised membrane and secreted proteins. Although clear evidence exists that axons can locally synthesise transmembrane and secreted proteins (e.g. snail egg-laying hormone [14], CGRP [15], kappa opioid receptor [16], and EphA2 [3]), puzzlingly, rough ER (RER) and Golgi necessary for the processing and secretion of these types of proteins have rarely been detected ultrastructurally in axons [17*]. A recent study helps to solve this mystery by providing immunocytochemical and functional evidence for RER and Golgi in axons and GCs [17*], and suggests that these trafficking 'outposts' have evolved non-canonical morphology to handle the dynamic demands of growing axons.

The focus of this review is the GC but, due to its small size, most experimental studies use entire axons. Therefore, we will discuss recent results on local translation in both axons and

GCs. It should be kept in mind, however, that the axon shaft and the GC are functionally distinct compartments (e.g. gradient sensing and turning occurs exclusively in GCs) that can employ specific RNA-based mechanisms.

Axonal mRNA repertoire

The number of identified axonally localised mRNAs has grown considerably by the use of more sensitive detection techniques and improved methods for obtaining isolated axons. Recent microarray studies identified around 2000 transcripts in murine retinal axonal GCs [18[•]], primary sensory axons [19[•]], and in cortical and hippocampal neuronal axons [20[•]], and up to 11,000 mRNAs were identified in sympathetic neuronal axons by SAGE analysis [21[•]]. Despite this remarkable complexity, different axonal mRNA repertoires show a surprising similarity as a group, representing 6–10% of the total cellular transcripts and, reassuringly, are composed of functionally similar mRNAs [18[•],19[•],20[•]]. For example, mRNAs encoding proteins involved in PS, molecular transport and mitochondrial maintenance invariably represent major categories in four independent screens using different neurons [18[•],19[•],20[•],21[•]]. Conversely, there are distinct differences that point to cell type-specific roles for particular mRNAs. For example, mRNA encoding *Impa1*, a key enzyme in the inositol cycle, is the most abundant mRNA found in sympathetic axons [21[•]], but it is not reported in other axonal profiling studies. Similarly, CREB mRNA is present in dorsal root ganglion neuronal axons where its translation helps to promote neuronal survival [22] but is absent in sympathetic neuronal axons [21[•]].

Many axonally localised mRNAs are highly enriched in the axon compared to the cell body, as revealed by comparative bioinformatics analyses, suggesting that anterograde transport, rather than overflow from the cell body, accounts for their axonal localisation [21[•]]. Moreover, using laser capture microdissection to isolate the GC compartment or the axon compartment specifically, Zivraj *et al.* showed that certain mRNAs are enriched in the GC over the axon, suggesting that the GC is a distinct subcellular compartment rather than a simple extension of the axon [18[•]]. Interestingly, GC mRNA repertoires showed functionally relevant developmental changes. For example, mRNAs encoding presynaptic machinery reside in the GCs of target-arriving, but not pathfinding, axons suggesting that the composition of mRNAs changes dynamically to meet the changing demands of the GC [18[•]]. In support of this, presynaptic protein-encoding mRNAs show increased axonal localisation after axotomy in cultured cortical neurons [20[•]]. These results provide a clear example of how axonal PS could be used to regulate context-dependent responses during development and regeneration, in accordance with the notion that axonal PS confers plasticity. A recent study identified over 300 transcripts in uninjured mature CNS neuronal axons [20[•]], lending support to early reports of PS in adult axons [23–26], and suggesting a requirement for PS in fully mature axons.

Mechanisms regulating general translational activity

In addition to altering its mRNA repertoire during development, the GC must possess mechanisms to regulate local PS on a rapid timescale in response to guidance cues. Work in the last two years has begun to reveal how guidance cue receptors are linked to PS machinery in the GC. Most PS-inducing guidance cues identified so far activate various signaling cascades converging on the mTORC1-mediated activation of cap-dependent mRNA translation [9]. Sahin's group recently showed that EphrinA, a non-PS-inducing repulsive guidance cue, represses mTORC1 activity [27[•]]. *tsc2* mutant mice exhibit defects in topographic mapping, similar to *ephrinA* knockout mice, and retinal GCs are less responsive to EphrinA *in vitro*. Furthermore, EphrinA normally increases the activity of Tsc2 through its receptor EphA, resulting in a decrease in downstream mTORC1 activity

and decreased axonal PS (Figure 1b). Previously, Ephrins were shown to induce GC collapse in a PS inhibitor-insensitive manner, and therefore have been regarded as non-PS-inducing cues [28]. This new evidence, however, suggests the interesting possibility that some non-PS-inducing cues may, in fact, repress axonal PS. Intriguingly, Sahin's group also showed that BDNF, an attractive PS-inducing cue, resulted in decreased Tsc2 activity [27**], suggesting the possibility that mTORC1 signaling may be inversely regulated to mediate some attractive versus repulsive responses. Because pathfinding axons receive numerous guidance cues simultaneously *in vivo*, it is conceptually appealing to speculate that multiple gradients of PS-inducing and PS-repressing cues exert concerted actions on the GC, which then integrates these signals to fine-tune local PS.

A more direct link between guidance cue receptors and PS machinery was revealed by Flanagan's group [29**]. They showed that DCC, a Netrin receptor, co-localises with ribosomes at the EM level in axons and GCs of spinal commissural neurons, and provided evidence that DCC and ribosomes form biochemical complexes when co-expressed in cell lines. Intriguingly, DCC appears to interact with translationally inactive PS machinery, as DCC co-purified with ribosomal subunits and monosomes, but not with polysomes. This interaction was negatively regulated by the binding of Netrin-1 to DCC, suggesting an interesting mechanism in which Netrin-1 induces local mRNA translation by releasing ribosomes from DCC (Figure 1a). This study provides not only a novel mechanism for direct coupling of an extracellular cue to PS machinery, but also uncovers a potential mechanism for spatially restricting PS to 'microdomains' within the GC at the site of cue binding. As these experiments were done under conditions in which Netrin-1 is an attractive cue, it will be interesting to know whether DCC-ribosome coupling differs under conditions when Netrin-1 is repulsive. It will be important in future to determine whether other guidance cue receptors interact directly with translational machinery, and specifically whether PS-inhibiting cues such as EphrinA [27**] increase receptor-ribosome association to sequester PS machinery away from mRNAs.

Mechanisms regulating mRNA-specific translation

Different PS-inducing cues regulate translation of distinct sets of mRNAs. For example, attractive cues such as Netrin-1 and BDNF induce β -Actin synthesis [6,7,30], whereas repulsive cues such as Slit2b and Sema3A induce local synthesis of actin depolymerising molecules such as Cofilin and RhoA [5,11]. All of these guidance cues, however, increase general translational activity in the GC. How, then, is mRNA-specific translation achieved? One way would be to control the activity of RNA-binding proteins (RBPs), which regulate a specific subset of mRNAs. Fragile X mental retardation protein (FMRP) is an RBP whose role in dendritic mRNA transport and translation in the context of long-term synaptic plasticity is well characterised [31]. Evidence that FMRP also localises to axons and GCs suggests that it might play a role on the presynaptic side as well [32–35]. Indeed, Li and colleagues report that hippocampal neurons cultured from *fmr1* knockout mice have defects in Sema3A-induced axonal PS and GC collapse response [36*]. They propose that local translation of *map1b* mRNA in axons and GCs via FMRP may mediate Sema3A-induced GC collapse.

The best known example of an RBP that regulates mRNA translation in axons is zipcode-binding protein (ZBP), which controls the transport, stability, and translation of β -actin mRNA by directly binding to a *cis*-element in the 3'-UTR. The *zipcode*, a 54-nt segment in the 3'-UTR, is necessary and sufficient for local translation of β -actin mRNA and GC turning in response to guidance cues such as Netrin-1 and BDNF [6,7]. Interestingly, the core sequence of the *zipcode* that directly participates in ZBP binding is present in other mRNAs, such as Arp2/3, which are also found enriched in the axon and are functionally

related to β -Actin [37]. Bassell's group recently uncovered a mechanism by which the attractive guidance cue, BDNF, regulates mRNA-specific translation by altering the function of ZBP1 [38*] (Figure 2b). They showed that BDNF activates a cascade of signaling events leading to Src-mediated phosphorylation of ZBP1 at Y396. When this phosphorylation was blocked by overexpression of a nonphosphorylatable version of ZBP1 (Y396F), both BDNF-induced β -actin mRNA translation and GC turning responses were attenuated. Therefore, it could be speculated that different guidance cues activate a distinct set of RBPs, which then bind a cohort of functionally related mRNAs to co-ordinately regulate their translation. RBP-mRNA binding may induce a conformational change conducive to the translation of a given mRNA, as was recently shown to be the case for ZBP1- β -actin mRNA interaction [39] (Figure 2b).

Another recent example of mRNA-specific regulation by RBPs in axons comes from Okano and colleagues, who reported a novel function of the RBP Musashi1 (Msi1) to control the translation of *robo3* mRNA [40*]. They observed that precerebellar inferior olivary neurons in *msi1* knockout mice showed a midline-crossing defect similar to *robo3* knockout mice [41]. Furthermore, they reported that Msi1 positively regulates Robo3 expression under normal circumstances by binding to and increasing the translation of *robo3* mRNA. Interestingly, the *cis*-element responsible for this regulation resides in the protein coding sequence rather than in the predicted Msi1-binding motif in the 3'-UTR. Msi1 binding to this motif is likely to displace unknown translational repressors because the RNA-binding domain of Msi1 alone functions as a weak activator rather than a dominant negative. Previously, Msi1 was shown to *inhibit* translation of other target mRNAs such as *m-numb* [42], providing an example of an RBP that can both enhance and repress mRNA translation depending on the *cis*-element. This could represent a particularly efficient mechanism for a single RBP to control diverse groups of mRNAs, and it will be interesting to determine whether mRNAs encoding proteins with antagonistic functions could be inversely regulated by a single RBP in this manner.

A better understanding of axonal mRNA translation awaits molecular identification of additional RNA regulatory elements. Novel *cis*-elements have been identified in recent years. Riccio and colleagues showed that *impa1* mRNA is transported into axons in response to NGF stimulation and that this axonal targeting is mediated by a newly identified 150-nt sequence in the 3'-UTR [21**]. Like the *zipcode*, this sequence is necessary and sufficient for axonal *impa1* mRNA transport and axonal survival, although the responsible RBP is not known. Interestingly, different species of *impa1* mRNA are generated from alternative transcriptional initiation and termination, generating NGF-responsive and NGF-nonresponsive species of mRNAs (Figure 2a). Considering most mRNAs are produced with diverse UTRs by differential transcriptional and/or posttranscriptional regulation, regulating UTRs would be an efficient way to control axonal mRNA translation without altering protein structures [43]. It is also plausible that similar mechanisms are used to control the responsiveness of mRNAs to microRNA regulation as microRNA-mediated translational inhibition and disinhibition continue to be identified as common mechanisms to control mRNA-specific translation in neuronal processes [44].

Another way an RBP can control mRNA translation is by regulating the length of its poly(A) tail. Cytoplasmic polyadenylation element binding proteins (CPEBs) control translation through this mechanism by directly binding to the CPE present in 3'-UTRs. Two recent papers provide evidence that this mechanism is indeed used to regulate mRNA translation in the axon. The first paper showed that Sema3A-induced local PS involves CPEB function, and that the translation of CPEB-regulated mRNAs is required for Sema3A-induced GC collapse in *Xenopus* retinal axons [45*]. Moreover, inhibiting the function of multiple CPEBs (e.g. CPEB1–4) by over-expressing a dominant negative mutant (i.e. RNA-

binding domain alone) of CPEB1 (CPEB1-RBD) disrupted axonal growth *in vivo*. This is likely mediated by CPEBs other than CPEB1 (e.g. CPEB2–4), because knocking down CPEB1 itself did not interfere with axonal growth. Evidence for how CPEB function is controlled by guidance cues comes from Wells and colleagues [46*], who showed that NT3 treatment of cultured rat hippocampal neurons activates local translation of β -catenin mRNA in the GC. As was shown in *Xenopus*, CPEB1-RBD was used to interfere with CPE-mediated mRNA regulation, and its overexpression disrupted NT3-induced β -catenin synthesis in the GC as well as axonal outgrowth (and branching). Furthermore, NT3 induces a rise in intracellular Ca^{2+} by the activation of IP3 receptors, which then activates CamKII to phosphorylate and activate CPEB1, providing a mechanistic link between cue binding and mRNA translation in the GC (Figure 2b).

As next generation sequencing is becoming the most powerful tool to analyse mRNA diversity [47], we expect more complete information on the UTRs of axonally localised mRNAs will emerge in the near future, helping us to identify more axon-resident RBPs and leading us to a better understanding of how those RBPs regulate mRNA-specific translation in axons and GCs. Remarkably, Eberwine's group recently reported that the targeting of certain dendritically localised mRNAs is dependent on a sequence within retained introns, rather than the 3'-UTR [48], indicating that unbiased cataloguing of axonally localised mRNAs may be needed to uncover regulatory mechanisms that include cytoplasmic splicing (Figure 2b).

Future prospects

Functional studies have so far concentrated on only a handful of axonal mRNAs yet thousands of axonal mRNAs have now been identified. This presents a new challenge commonly encountered in the post-genomic era: how to determine the most functionally relevant candidates? Moreover, advances in next generation RNA sequencing (RNA-seq) technology will likely add even more to this ever-growing list of axonal mRNAs. Careful characterization of dynamic spatiotemporal changes in repertoires (e.g. young versus old; axon versus GC) will be one way to gain insight into the functional aspects of axonal PS. On the other hand, the growing lists compiled from the work of different groups will allow for systematic bioinformatics analysis and identification of common elements from these mRNAs that may help uncover novel RBPs and regulatory mechanisms. Sensitive RNA-Seq technologies may eventually allow profiling of single axons and GCs enabling the question to be addressed of whether mRNA diversity is equally represented across an axonal population, or whether it reflects diversity between individual axons of a given subtype.

In terms of GC signaling, it will be important to test whether guidance receptors other than DCC [29**], such as Neuropilin and Robo, are also coupled to the translation machinery in order to determine whether this represents a general mechanism of stimulus-induced translation regulation. Future studies should explore the interplay between factors that activate (e.g. Sema3A) versus those that repress (e.g. EphrinA) local PS to build a better understanding of how the GC integrates guidance signals at the level of translation. Finally, it will prove critical in the next few years to determine what role axonal PS plays *in vivo*. Developing new strategies to inhibit translation of selected mRNAs in axons without affecting the cell bodies *in vivo* will be an important challenge that may be met with photo-inducible technologies [49].

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Figure 1.

Mechanisms regulating overall translational activity. **(a)** Under attractive conditions, Netrin binding causes release of ribosome subunits from DCC receptors, allowing assembly of polysomes and localized translation necessary for an attractive GC response. **(b)** In response to repulsive EphrinA, EphA receptor activation increases Tsc2 activity via decreased phosphorylation at the ERK1/2 site, inhibiting mTORC1 signaling and downstream PS to mediate a repulsive collapse response. Diagram of repulsive turning is speculatively based on demonstrated collapse response.

Figure 2.

Mechanisms regulating mRNA-specific translation. **(a)** In the nucleus, diverse species of mRNAs encoding the same protein can be generated by using different transcription initiation/termination sites (1) [21^{**}] or by alternative splicing (2). RBPs (green oval) regulate axonal transport of their target mRNAs by binding to specific *cis*-elements (green rectangle). **(b)** In the GC, cue-RBP-mRNA specificity regulates mRNA-specific translation. BDNF induces Src-mediated phosphorylation of ZBP1 [38] activating translation of β -actin mRNA, at least in part by RNA looping [39], and GC turning. NT3 induces CamKII-mediated phosphorylation of CPEB1, which activates translation of β -catenin mRNA by cytoplasmic polyadenylation [46^{*}], and axon elongation and branching [45^{*},46^{*}].