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Dynamics of axonal mRNA transport and implications for peripheral nerve regeneration

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Abstract

Locally generating new proteins in subcellular regions provides means to spatially and temporally modify protein content in polarized cells. Recent years have seen resurgence of the concept that axonal processes of neurons can locally synthesize proteins. Experiments from a number of groups have now shown that axonal protein synthesis helps to initiate growth, provides a means to respond to guidance cues, and generates retrograde signaling complexes. Additionally, there is increasing evidence that locally synthesized proteins provide functions beyond injury responses and growth in the mature peripheral nervous system. A key regulatory event in this translational regulation is moving the mRNA templates into the axonal compartment. Transport of mRNAs into axons is a highly regulated and specific process that requires interaction of RNA binding proteins with specific *cis*-elements or structures within the mRNAs. mRNAs are transported in ribonucleoprotein particles that interact with microtubule motor proteins for long-range axonal transport and likely use microfilaments for short-range movement in the axons. The mature axon is able to recruit mRNAs into translation with injury and possibly other stimuli suggesting that mRNAs can be stored in a dormant state in the distal axon until needed. Axotomy triggers a shift in the populations of mRNAs localized to axons indicating a dynamic regulation of the specificity of the axonal transport machinery. In this review, we discuss how axonal mRNA transport and localization are regulated to achieve specific changes in axonal RNA content in response to axonal stimuli.

Keywords

Axon regeneration; axotomy; protein synthesis; translation; RNA transport; ribonucleoprotein particle; trophic factor; axon guidance

Introduction

The peripheral nervous system (PNS) can spontaneously regrow injured axons. This is in contrast to the central nervous system (CNS), where severed axons rarely show significant

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levels of regrowth. PNS neurons have a higher intrinsic growth capacity than CNS neurons, and the environment of the CNS presents molecules that actively inhibit axonal growth, particularly after injury (Benowitz and Yin, 2007). To initiate a regenerative response to injury in the PNS, the neuron must shift its physiology from synaptic transmission and maintenance of structure to growth of the axon. Obviously, shifting to this growth phenotype requires that the remaining proximal axon secure a supply of lipid bilayer for new axoplasmic membranes, organelles for energy and secretion, and proteins to build a new cytoskeleton as the axon regrows. Similar shifts in function of the neuronal processes will be required for CNS neurons when effective therapies are available to better overcome the CNS's non-permissive environment for growth.

Interestingly, work over the past few years indicates that at least some new axonal proteins are derived from localized translation of mRNAs that reside in the axons. This was surprising since the initial studies showing that dendrites of adult hippocampal neurons contain protein synthesis machinery also indicated that ribosomes and RNAs were excluded from the axonal compartment of these mature neurons (Steward and Levy, 1982). Early works from the mid-1960's onwards by Koenig and others suggested that axons could generate some proteins through localized translation (Giuditta, et al., 2002). These early publications did not have the benefit of modern molecular and cellular tools for detecting RNAs and proteins (e.g., polymerase chain reaction [PCR] and transfection of fluorescent reporter constructs), so they could not unequivocally show synthetic activity within the axon. Consequently, there were concerns for glial proteins contaminating these axonal preparations. In support of these early works, studies over the last decade using newer molecular, cellular, and biochemical tools have provided clear proof that axons are capable of synthesizing proteins. This has been the subject of several reviews, including some that detail the controversies underlying earlier observations (Giuditta, et al., 2002; Twiss and van Minnen, 2006). In this work, we focus on how the mRNA templates needed for translating proteins move into the axonal compartment. Rather than non-specific diffusion of mRNAs through the neuronal processes, RNA localization is a highly regulated process that requires mechanisms both for selecting which mRNAs to target into and for actively transporting those mRNAs in distal neuronal processes.

Localized mRNA translation and localized proteolysis provide the axon with means to both spatially and temporally regulate local protein levels. The mechanisms and implications of local translation and proteolysis in axons are discussed in the accompanying paper by Gumy *et al.* (2009). Here, we focus on the mechanisms of axonal mRNA transport. The mRNAs encoding axonally synthesized proteins obviously must be delivered into the axonal compartment for this local translational regulation to occur. The RNA transport machinery itself appears to provide a level of translational control for mRNAs in transit. Thus, RNA transport and localized translational control represent both sequential and convergent mechanisms that the neuron uses to modulate protein levels in distal processes.

mRNA localization during development

Localized translation of mRNAs is increasingly recognized to play a vital role in development by contributing to axis formation and body patterning in early embryogenesis (Besse and Ephrussi, 2008; St Johnston, 2005). With little or no active transcription in the oocyte and early embryo, initial development of the organism relies on maternal mRNAs. These maternal mRNAs are translationally activated at specific times and locations during early embryogenesis. The untranslated regions (UTRs) of maternal mRNAs provide an integral role in this post-transcriptional regulation (Mignone, et al., 2002). For example, the localized translation of bicoid and caudal mRNAs helps to determines the anterior-posterior axis of the drosophila embryo (Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987). Asymmetric distribution of bicoid mRNA in an anterior to posterior gradient generates an anterior to

posterior gradient of Bicoid protein, which itself is an RNA binding protein (RBP). Caudal mRNA is uniformly distributed in the embryo. However, the asymmetric distribution of Bicoid protein generates a posterior to anterior gradient of Caudal protein by translationally repressing caudal mRNA in the anterior pole through binding to its 3′UTR (Dubnau and Struhl, 1996; Rivera-Pomar, et al., 1996). Translation of bicoid mRNA is regulated through a cytoplasmic polyadenylation element (CPE) in its 3′UTR, which is recognized by the CPE binding protein (CPEB) (Salles, et al., 1994; St Johnston and Nusslein-Volhard, 1992). Upon phosphorylation of CPEB, poly-A polymerase is recruited to bicoid mRNA and its translation is initiated through recruitment of eIF4E (Mendez and Richter, 2001). Such dynamic interactions between RNA localization, RNA-protein complexes, and translation have now been demonstrated for a number of different developmentally expressed mRNAs (Besse and Ephrussi, 2008; St Johnston, 2005). Interestingly, Lecuyer *et al*. (2007) have shown that over two-thirds of ~3700 *drosophila* mRNAs show distinct patterns of localization during development (Lecuyer, et al., 2007). This suggests that mRNA localization is more common than previously appreciated. As we outline below, it is now clear that neurons also show differential localization of many mRNAs with a surprising complex mRNA population localizing into the axonal compartment.

mRNAs and translational machinery are transported into both dendrites and axons. The first clear indication for localized translation in axons was derived from studies in developing neurons. Cortical neurons undergo a well-defined sequence of progressive polarization in culture where one neurite that is destined to become the axon shows a rapid burst in outgrowth (Craig and Banker, 1994). mRNAs and translational machinery are present in this ‘major neurite’ that will become the axon. With further polarization, this RNA and ribosome localization appears to shift to the developing dendritic processes (Kleiman, et al., 1994). Retrospectively, this shift would be consistent with the now recognized role of dendritically synthesized proteins in synaptic plasticity (Costa-Mattioli, et al., 2009). However, mRNA transport into and localized translation within the developing axon apparently continues beyond this transition, likely at a more selective pace than in the very early axon. Bassell *et al.* (1998) initially showed that localization of mRNAs into axons of developing cortical neuron is selective. β-actin mRNA was shown to concentrate in axonal growth cones of chick cortical neurons, but γ-actin mRNA remained in the neuronal cell body (Bassell, et al., 1998). This has been subsequently documented in mammalian neurons, and it is clear that the localized β-actin mRNA is translated in the axons (Lin and Holt, 2008). Similar differential localization of actin isoforms had been reported in migrating fibroblasts and developing myocytes (Hill and Gunning, 1993; Kislauskis, et al., 1993). In fibroblasts, localized translation of β-actin mRNA is needed for polarity of cell migration (Shestakova, et al., 2001). Drawing analogy to fibroblast migration, this localized translation of β-actin mRNA in the growth cone suggests a role in axonal growth. Independent works from several laboratories have shown that locally synthesized proteins play a role in axon guidance in developing neurons (Lin and Holt, 2008). In addition to ligand dependent translation of β-actin mRNA, translation of RhoA mRNA has also been seen in axons treated with Semphorin 3A (Sema3A) and localized increases in cofilin protein have been shown in growth cones exposed to Slit-2 (Piper, et al., 2006; Wu, et al., 2005). These proteins can each regulate cytoskeletal dynamics, particularly the microfilaments that are needed for growth cone motility. Membrane proteins can also be locally synthesized in axons and inserted into the cell membrane (Brittis et al., 2002; Merianda et al., 2009). By immunolocalization studies, Merianda *et al*. (2009) showed that distal axons contain components of the co-translational machinery for classic membrane protein targeting and/or secretion. This includes components of the signal recognition particle (SRP) and other proteins needed for ribosome docking on the endoplasmic reticulum (ER), membrane and lumenal ER proteins, and components of the cis and trans Golgi apparatus. This detection of ER and Golgi in axons raises the possibility that locally synthesized proteins can undergo both N- and O-glycosylation as has been shown for locally synthesized proteins in dendrites (Torre and Steward, 1996).

It is quite likely that some of the protein synthesis seen in regenerating axons similarly contributes to directional growth of the axons. However, recent data from the Letourneau group challenge the functional consequences of axonal protein synthesis in developing neurons (Roche, et al., 2009). As suggested by Roche et al. (2009), different experimental conditions or metabolic status of neurons used for each of these studies could help to explain these discrepant data. The possibility that the distal axon may have varying needs for localized protein synthesis depending upon its metabolic status or growth state has significant implications for *in vivo* settings, where the axon would encounter a much more complex milieu of extracellular stimuli than in the petri dish. The vast distances separating the distal axon from its cell body *in vivo* could also force even greater reliance on localized protein synthetic mechanisms than can be appreciated with culture preparations.

The functional roles of axonally synthesized proteins are not limited to growth, since NGF-dependent translation of CREB mRNA in sympathetic axons appears to contribute to neuronal survival (Cox, et al., 2008). Moreover, axonal translation of the nuclear-encoded mitochondrial protein COX IV contributes to energy homeostasis in sympathetic axons (Aschrafi, et al., 2008). It is quite likely that we will see additional roles for axonally synthesized proteins as knowledge and interest in these mechanisms evolve. Indeed, a recent RNA profiling study of axons isolated from cultured rodent hippocampal neurons suggests that hundreds of different proteins could be synthesized locally in the axonal compartment (Taylor, et al., 2009). Our own profiling studies of axons from cultures of adult dorsal root ganglion (DRG) neurons show over 200 different mRNAs in the axonal compartment (Willis, et al., 2007). We used lower density arrays and more conservative presence calling in this study than Taylor et al. (2009) used; thus, it is likely that the adult DRG axons contain even more mRNAs than reported. Considering the complexity of mRNA populations in axons, the functional consequences of axonal protein synthesis, both in developing and mature neurons, will undoubtedly be much more diverse than indicated by the current literature.

mRNAs are transported as RNA-protein complexes

Axonal localization of mRNAs, or transport, plays a key role in regulation of localized protein synthesis. Mechanisms have evolved for cells to recognize which mRNAs are to be targeted for subcellular localization. Specific *trans*-acting factors, RBPs, recognize *cis*-elements in the mRNA sequences to regulate the localization and translation of the target mRNA (Bassell and Kelic, 2004; Martin and Ephrussi, 2009). These *cis*-elements are largely composed of secondary structures (*i.e.*, stems, loops, and bulges) rather than being identified by their sequence; this has complicated identification of localizing elements in mRNAs (Jambhekar and Derisi, 2007). In Xenopus oocytes, the steps for tagging an mRNA for localization can begin in the nucleus, possibly through co-transcriptional mechanisms (Kress, et al., 2004). Nuclear events, in particular RNA binding protein interaction with nascent transcripts that localize to dendrites and spinal axons, have also recently been demonstrated for neuronal mRNAs (di Penta, et al., 2009). The non-translated regions of mRNAs play a critical role in their post-transcriptional regulation, including their subcellular localization. Sequence elements that are needed to localize mRNAs have been largely linked to their 3′ untranslated regions (3′UTR). For example, an approximately 55 nucleotide element in the 3′UTR of β-actin mRNA, referred to as the ‘zip-code,’ is necessary for localizing this mRNA in fibroblasts, axons, and dendrites (Kislauskis, et al., 1994; Tiruchinapalli, et al., 2003; Zhang, et al., 2001). The zip-code binding protein 1 (ZBP1) recognizes and binds to this 3′UTR element; this RNA-protein interaction is essential for localization of β-actin mRNA (Farina, et al., 2003; Ross, et al., 1997; Zhang, et al., 2001). Interestingly, ZBP1 binding also inhibits translation of β-actin mRNA while bound, so the transported β-actin is translationally suppressed. Phosphorylation of ZBP-1 on tyrosine residues by a Src-family kinase(s) triggers dissociation of this RNA-protein complex with derepression of β-actin mRNA translation

(Huttelmaier, et al., 2005). Most mRNAs that are locally transported remain repressed as parts of ribonucleoprotein complexes (RNPs) until a triggering signal arrives (Martin and Ephrussi, 2009; Wells, 2006). This provides both spatial and temporal control of localized protein expression, by preventing translation of the mRNA in incorrect locales and until an appropriate stimulus is provided.

The CPEB protein, which contributes to translational control of maternal mRNAs in early embryogenesis, also plays a role in regulating subcellular protein synthesis in neurons. This is best characterized for dendrites, where CPE within 3′UTR of dendritic mRNAs contributes both to localization and translational control (Huang, et al., 2003; Huang, et al., 2006). However, a CPE sequence within the ephrin receptor EphA2 mRNA 3′UTR is required for upregulation of chick EphA2 protein in distal commissural axons that have reached or crossed the midline floor plate (Brittis et al., 2002). There are at least four different CPEB isoforms (Richter, 2007). Although potential roles for these CPEB proteins in axonal mRNA localization have not been as firmly established as in dendrites, a very recent study in *Xenopus* points to a role for cytoplasmic polyadenylation in retinal axon development. Interestingly, RNA-protein pull down experiments suggest that CPE-binding proteins other than CPEB1 regulate this developmental cytoplasmic polyadenylation (Lin, et al., 2009). In a similar vein, but likely with differing mechanisms of regulation and mRNA targets, the fragile X mental retardation protein (FMRP) that is known to regulate translation in dendrites was recently shown to play a role in mRNA transport (Dictenberg, et al., 2008; Estes, et al., 2008). FMRP has also been detected in adult PNS axons (Murashov, et al., 2007) and recent work from the Fallon lab suggests that FMRP and related proteins (FXR1 and FXR2) localize to presynaptic nerve endings in some CNS neuronal populations (Christie, et al., 2009). There will undoubtedly be more overlap between dendritic and axonal mRNA localization mechanisms as knowledge is gleaned of both processes. It also should be noted that *cis*-elements for localization have also been demonstrated 5′UTRs (Bi, et al., 2007; Muslimov, et al., 2004), and it is likely that coding sequences cannot be ignored in considering potential regulatory regions of individual mRNAs.

Despite that we now know of many different axonally localized mRNAs, discovery of RNA binding proteins that are responsible for their axonal localization has been slow. In motor axons, β-actin mRNA has been shown to interact with hnRNP R, hnRNP Q, and SMN (Rossoll, et al., 2002). κ-opioid receptor mRNA, which localizes in sensory axons, is within an RNP that includes a coatamer binding protein (Copb1), Grb7, and an Elav protein (HuR) (Bi, et al., 2007; Tsai, et al., 2006). Tau-containing RNPs isolated from the neuronal-like P19 cell line also contain an Elav protein (HuD) and IMP1 (the human ortholog of ZBP1) (Atlas, et al., 2004). There undoubtedly will be significant overlap in transport RNP composition, with individual RBPs capable of binding many different mRNAs. For example, HuD mentioned above is known to bind and stabilize GAP-43 mRNA (Beckel-Mitchener, et al., 2002), and GAP-43 mRNA has been shown to localize into growing neurites of PC12 cells (Smith, et al., 2004) and axons of cultured adult sensory neurons (Willis, et al., 2007). Pull-downs of IMP1 from lysates of non-neuronal cells have also detected a surprisingly complex population of different mRNAs (Jonson, et al., 2007). Considering that the axon will present an enriched subcellular fraction compared to whole cell lysates as used for the IMP1 studies, the axonal RNPs and RBP targets may be somewhat less complex. However, one must also consider that with a biochemical approach, a mixed population of different RNPs will be present; cellular approaches will be needed to fully dissect the composition of individual transport RNPs. For example, the Sossin group identified multiple DEAD box RBPs in purified RNPs from hippocampal neurons, but they were able to define different RNPs in dendrites by co-expressing epitope tagged versions of these DEAD box proteins (Elvira, et al., 2006; Miller, et al., 2009). As discussed below, injury generates a shift in both transport and localized translation of mRNAs in PNS axons. Activity of individual RBPs, both in their affinity for different mRNAs and in their interaction with motor proteins for transport, and transitions in RNP

composition may be key mechanisms that the adult neuron utilizes to target mRNAs for axonal transport under different physiological conditions.

Interaction of the SMN protein with β-actin mRNA in motor axons importantly raises a link between motor neuron disease and axonal RNA transport. Loss of SMN protein results in spinal muscular atrophy (SMA) with degeneration of spinal motor neurons that can begin in early infancy (Lefebvre, et al., 1995). Animal models of SMA have correlated decreases in axonal β-actin mRNA with depletion of SMN protein (McWhorter, et al., 2003; Rossoll, et al., 2003; Rossoll, et al., 2002). Axonal localization of the mRNA encoding the voltage-gated calcium channel $Ca_V2.2$ is similarly decreased in neurons cultured from SMA mice (Jablonka, et al., 2007). Consistent with this, the depletion of $Ca_V2.2$ mRNA from SMA axons was associated with decreased Ca^{2+} signaling in growth cones (Jablonka, et al., 2007). Thus, SMN can modify the axonal levels of multiple mRNAs similar to the ability of IMP1 to bind many different mRNAs (Jonson, et al., 2007). Two other RBPs have intriguingly been implicated in adult motor neuron disease (Kwiatkowski, et al., 2009; Sreedharan, et al., 2008; Vance, et al., 2009). Although it is not clear if TDP-43 and FUS/TLS play a role in axonal mRNA localization and/or translation, it is tempting to speculate that alterations in mRNA localization may also contribute to degeneration of mature motor neurons.

Axonal mRNA localization in adult neurons

Just as new understanding of the roles of axonal protein synthesis in developing neurons has come to light, studies over the past few years have also pointed to the potential for translation occurring in axons of adult neurons. Early ultrastructural observations demonstrated ribosome like particles in myelinated PNS axons (Zelena, 1970; Zelena, 1972). Koenig and colleagues went on to use electron spectroscopic imaging to show that high phosphorus signals seen in vertebrate axons, which likely represent rRNA, are concentrated in plaque-like domains along the periphery of the axoplasm (Koenig and Martin, 1996; Koenig, et al., 2000). Furthermore, application of pharmacological inhibitors to injured peripheral nerves that had been depleted of glial cells suggested that axonal ribosomes have access to mRNA templates necessary for generating new proteins locally (Gaete, et al., 1998). Which proteins might be generated in these axons remained a mystery, and potential contributions of proteins synthesized in residual Schwann cells could not be completely excluded in these animal studies. However, the Fainzilber lab clearly showed that adult mammalian PNS axons contain mRNA that is locally translated into protein. Using isolated segments of rat sciatic nerve, Hanz *et al.* (2003) detected an injury-induced accumulation of new Importin-β1 protein in the axons (Hanz, et al., 2003). Importantly, these authors used *in situ* hybridization to prove that the encoding mRNA resides in the axon and isolated preparations of axons from cultured neurons to show synthesis of Importin-β1 protein directly within axons ((Rishal and Fainzilber, 2009). Considering the possibility of RNA localization in mature axons, this work from Hanz *et al.* (2003) as well as subsequent papers on vimentin and RanBP1 mRNAs (Perlson, et al., 2005; Yudin, et al., 2008) indicate that mRNAs are indeed transported into mature axons (Figure 1A).

One explanation for protein synthesis in regenerating axons is that adult neurons recapitulate translational control mechanisms used during development of the nervous system. Earlier work from Zheng *et al.* (2001) had shown that axons of adult DRGs, which were cultured after an *in vivo* ‘conditioning crush’ lesion, showed robust protein synthesis even when severed from their cell body (Zheng, et al., 2001). In subsequent studies, Willis *et al.* (2005) resolved over 100 axonally synthesized proteins from these DRG cultures by two dimensional gel electrophoresis (Willis, et al., 2005). Mass spectroscopy of the most abundant ‘spots’ from these gels of metabolically labeled axons included proteins whose axonal synthesis had been established in developing neurons as well as many that were previously not known to be synthesized locally. Interestingly, axons from the injury-conditioned neurons showed

significantly more protein synthesis than did those axons from naïve neurons (Willis, et al., 2005). This lends credence to the notion that injury of adult neurons reactivates a developmental mechanism.

An important consideration for any shift in capacity for axonal protein synthesis upon transition to a growth phenotype is that should be accompanied by a dramatic change in delivery of mRNAs into the axonal compartment (Figure 1). Taylor *et al.* (2009) recently tried to directly address differences in RNA content in regenerating *vs.* mature axons using hippocampal neurons grown in microfluidic chambers, which allow physical separation of axonal and dendritic processes. Consistent with an injury-induced shift in RNA transport, 866 mRNAs showed significantly different levels between the mature and regenerating axons. Importantly, this was roughly an even split between increased and decreased transport into the regenerating axons; thus, axonal injury triggers a specific change in transported mRNA populations rather than a global upregulation of axonal mRNA transport (Taylor, et al., 2009). As noted below, stimulation of axons with tropic agents similarly shows specific alterations in axonal mRNA populations that are matched to the ligand (Willis, et al., 2007). PNS neurons surely undergo a shift in axonal mRNA localization with injury. In support of this idea, Toth *et al.* (2009) recently showed that axonal localization of CGRP mRNA in DRG neurons is dramatically increased by axotomy despite an apparent decline in CGRP transcription in the cell body (Toth, et al., 2009). RNA profiling of axons isolated from DRG neurons cultured from adult rats also show a shift in RNA content after in vivo injury conditioning (DE Willis and JL Twiss, unpublished observations). Recent work from Vogelaar et al. (2009) indicates that localized translation of β-actin mRNA changes with developmental stage of the neuron but its transport rate apparently remains unchanged (Vogelaar, et al., 2009). Thus, axonal mRNA transport and translation are uniquely regulated depending upon the needs of the axon, with individual mRNAs showing specific alterations, rather than being an all or none phenomenon. Future studies will need to address these specificity mechanisms of axonal mRNA transport.

A key question for understanding regulation of axonal RNA transport in mature neurons is whether injury reactivates a developmental mechanism that is kept dormant or does injury merely instruct the neuron to send different mRNAs into the axonal compartment. The latter possibility implies that adult neurons could utilize axonal protein synthesis for functions other than injury response and growth. Processes of sensory neurons from the invertebrate *Aplysia* show activity-dependent translation (Martin, et al., 1997). This response to long-term facilitation in the *Aplysia* neurons bears some resemblance to mammalian dendritic protein synthesis that is seen with long-term potentiation (Costa-Mattioli, et al., 2009). Neurites from invertebrate sensory neurons have some dendritic features, so their activity-dependent mRNA localization could represent a dendritic character of these incompletely polarized neurons (Martin, et al., 2000). Importantly, recent work from the Hunt lab provides evidence for activity-dependent translation in adult PNS sensory axons of mammals. In this study, activation of translation factors was seen in cutaneous sensory axons after injection of capsaicin into the footpad; rapamycin prevented both this translation factor activation in the axons and subsequent generation of neuropathic pain (Jimenez-Diaz, et al., 2008). Although these authors did not determine which proteins might be generated in these PNS axons, this work clearly shows that stimuli beyond injury can trigger protein synthesis in mature axons. Mutations of glycyl and tyrosyl tRNA synthetases (GARS and YARS, respectively) have been demonstrated in an axonal form of Charcot-Marie Tooth PNS neuropathy (Antonellis, et al., 2006; James, et al., 2006; Jordanova, et al., 2006; Nangle, et al., 2007; Seburn, et al., 2006). Interestingly, both GARS and YARS proteins localize to axons and expressing the mutant GARS decreased localized protein synthesis in *Drosophila* and murine neurons (Chihara, et al., 2007; Jordanova, et al., 2006). Since localized protein synthesis would obviously require charged tRNAs, the effects of mutating GARS and YARS suggest that ongoing protein synthesis in adult PNS axons may help to maintain axon integrity. Further, these observations support the hypothesis

that mature axons contain mRNAs that can be recruited into translation, with the localized environment of the axon likely determining when to translate specific mRNAs. Consistent with stimulus-dependent translation in axons, work from the Fainzilber lab argues that generation of retrograde signaling complexes through localized translation in axons is Ca^{2+}-dependent (Hanz, et al., 2003; Perlson, et al., 2005; Yudin, et al., 2008). Axoplasmic Ca^{2+} also is key for the asymmetric translation of β-actin mRNA in growth cones exposed to gradients of BDNF (Yao, et al., 2006). It is noteworthy that other guidance and growth regulating cues including netrin-1, myelin-associated glycoprotein (MAG), chondroitin sulfate proteogylcans, and neurotrophins can by modulate axonal Ca^{2+} levels (Zheng and Poo, 2007).

The possibility that axonal mRNAs can be recruited into action by specific stimuli argues that mRNAs can be stored locally in the nerve. Although not well defined in neurons, stress granules and processing bodies ('P-bodies') have been characterized in other cellular systems as sites to sequester mRNAs (Anderson and Kedersha, 2006). Stress granules are more typically considered as organelles for storing unused mRNAs during periods of cellular stress. P-bodies provide transient storage of unutilized mRNAs but they also contain machinery needed for RNA decay, including components of the RNA interference (RNAi) machinery (Parker and Sheth, 2007). Stress granules have been visualized in dendrites and axons of cultured neurons (Tsai, et al., 2009; Vessey, et al., 2006), and P-bodies have been demonstrated in dendrites (Cougot, et al., 2008; Zeitelhofer, et al., 2008). Although P-bodies have not yet been reported in axons, RNAi machinery that localizes to P-bodies has been reported in axons of PNS neurons (Hengst, et al., 2006; Murashov, et al., 2007). Recent analysis of dendritic RNA granules suggests transport RNPs can dock with P-bodies in dendrites (Zeitelhofer, et al., 2008). Transport RNPs, P bodies, and stress granules are dynamic organelles, likely with active exchange of components between them. Interactions between these organelles would effectively provide the axon with a means for both short-term and long-term storage of mRNAs. PNS neurons may also be able to recruit new protein synthesis machinery into their distal axons since Court *et al.* (2008) recently showed that the distal axon can obtain ribosomes from adjacent Schwann cells (Court, et al., 2008). Transfer of protein and even small RNAs from glia to axons has been documented previously (Eyman, et al., 2007; Sheller, et al., 1995; Tytell and Lasek, 1984). Ironically, early observations of transfer of glial-derived proteins to axons contributed to the dogma that axons are not capable of synthesizing proteins (Twiss and Fainzilber, 2009). This combination of recruiting ribosomes from adjacent cells and mRNAs from axonal storage organelles would give the distal axon a more immediate means to respond to stimuli than could be provided by anterograde transport of proteins from the cell body.

Stimulus dependent regulation of axonal mRNA localization

In addition to regulating translation of mRNAs in axons, tropic stimuli can also modify the populations of mRNAs in axons by regulating anterograde movement of mRNAs. Analogous to the injury-induced transition in axonal mRNA transport, modulating axonal mRNA levels by tropic stimulation would provide the neuron with a means to alter protein content over longer term than by merely triggering the translation of resident stored mRNAs. Many of the tropic agents that regulate axonal mRNA transport have been implicated in axonal regeneration. Zhang *et al*. (1999) initially showed that axonal transport of β-actin mRNA is increased by bath application of neurotrophins to cultured chick cortical neurons, stimuli that also increase neurite outgrowth from these neurons (Zhang, et al., 1999). Inhibition of protein kinase A (PKA) blocked this increased axonal RNA localization, indicating that neurotrophin-dependent activation of PKA instructs the neuron to send more β-actin mRNA into axons. Such instructive events are not limited to the neurotrophins or even to growth-promoting stimuli. Sasaki *et al.* (2001) showed Sema3A, which causes generalized growth cone retraction when bath applied to DRG neurons, triggers an increase in both anterograde and retrograde transport in axons through a signaling cascade that requires activation of cyclin dependent kinase 5 and Fyn kinase

(Sasaki, et al., 2002). Subsequent studies from this group indicated that localized activation of protein synthesis by Sema3A was needed for this increase in axonal transport (Li, et al., 2004). Although neither of these studies addressed the transported cargo, Willis et al. (2007) clearly showed that neurotrophins can specifically regulate transport of mRNAs into axons of adult DRG neurons, with individual mRNAs showing increased or decreased transport. Interestingly, application of negative growth cues to these same DRG neurons triggers transport of different mRNAs or causes an opposite regulation of mRNAs that the neurotrophins regulated (Figure 1B–C). For example, axonal transport of β-actin mRNA is increased by NGF, BDNF, and NT3 but decreased by MAG and Sema3A (Willis, et al., 2007). Thus, there is remarkable specificity in the axonal mRNA transport mechanisms, allowing localized mRNA populations (and proteins generated) to be matched to the growth needs of the axon.

Alterations in axonal protein synthesis are also spatially regulated such that the region of the process where the stimulus is located is where changes in protein synthesis occur (Lin and Holt, 2008). This matching of extracellular stimulus to intra-axonal events is also linked to RNA localization. Application of gradients of Netrin-1 or BDNF to developing axons results in an asymmetric localization of β-actin mRNA within the growth cone, concentrating the mRNA along the side of the growth cone receives the greatest concentration of ligand and where new β-actin protein is generated (Leung, et al., 2006; Yao, et al., 2006). This concentration of β-actin is microfilament-dependent, so it likely represents a relocalization of β-actin mRNA than had already been transported into the distal axon (Leung, et al., 2006; Yao, et al., 2006). For long-range movement of mRNAs in axons (and dendrites), microtubule-based transport rather than microfilament-based transport is utilized. Long-range microtubule-based transport is also seen in response to localized sources of ligands. Using a reporter for β-actin mRNA localization and translation, Willis *et al*. (2007) showed that neurotrophin-dependent increase in β-actin mRNA transport requires retrograde signaling to the cell body. Curiously, decreases in axonal mRNA localization can occur without signaling to the cell body (Willis, et al., 2007). Thus, the axon uses both interactions with the cell body and autonomous events within the axoplasm to modulate its mRNA levels. These changes in axonal mRNA localization occurred despite transcriptional inhibition, indicating that distal axonal signals can act upon existing pool of mRNAs (Willis, et al., 2007). However, some changes in RNA transport clearly require new gene expression. For example, axotomy triggers transcription of β-thymosin and fasciculin in *Aplysia* sensory neurons, and the newly transcribed mRNAs are transported into the injured axons (Colby, et al., 2005). Activity-dependent localization of Arc in dendrites also occurs through transcription of new Arc mRNA (Steward and Worley, 2001). New gene expression may also affect the localization of RBPs to modify mRNA localization. In oligodendrocytes, cellular stress triggers redistribution of existing Staufen to the cell body, but newly transcribed Staufen is targeted to the distal processes of these glial cells (Thomas, et al., 2005). The interplay between transcription, mRNA localization, and localized protein synthesis needs to be experimentally tested. Convergence of these pathways could have profound effects on injury responses of PNS neurons where many injury-induced and regeneration-induced transcriptional events have been defined.

Perspectives for regeneration

Mechanistically, proteins can be supplied to distal axons by transporting polypeptides or the encoding mRNAs. While only a fraction of axonal proteins are derived by transporting and translating their encoding mRNAs within the axons, functional studies indicate that these locally generated proteins can contribute to growth, support local energy requirements, and provide means to retrogradely signal to the cell body. Recent RNA profiling studies of axons indicate that a very diverse population of proteins can be generated locally (Taylor, et al., 2009; Willis, et al., 2007). Additional studies are clearly needed to uncover what these locally synthesized proteins are doing in the axonal compartment. A key issue for future studies is to

move experimental models to *in vivo* settings. The vast majority of work on RNA localization has been performed with cultured neurons; modulation of RNA transport and localized translation may be quite different *in vivo*. However, work from the Fainzilber, Hunt, and Zochodne groups clearly shows functionality of axonally synthesized proteins *in vivo* (Hanz, et al., 2003; Jimenez-Diaz, et al., 2008; Perlson, et al., 2005; Toth, et al., 2009; Yudin and Fainzilber, 2009).

Transport of mRNAs into the axonal compartment is a highly regulated mechanism. It is obvious from RNA profiling studies that not all mRNAs localize into axons. Recent studies on ligand and stimulus dependent modulation of axonal mRNA transport have revealed that only some of the localizing mRNAs show regulation of their transport into axons with an individual stimulus (e.g., injury, tropic ligands). mRNA interactions with RBPs are needed for transport, and these interactions likely provide this level of specificity to the localization mechanisms both for determining what mRNAs localize and for when to localize individual mRNAs into the axonal compartment. There undoubtedly will be overlap in transport mechanisms with several mRNAs sharing a single RBP. Signaling pathways converging upon shared transport mechanisms could provide a means for commonly regulating groups of mRNAs. Knowledge of what RBPs are needed for localization, how activity of these RBPs is regulated, and what sequence structures these RBPs recognize is limiting progress in understanding the dynamics of axonal mRNA transport. Given the shifts in mRNA localization that occur with injury and with tropic stimulation, determination of RBP content and regulation may provide new directions for modulating axonal growth.

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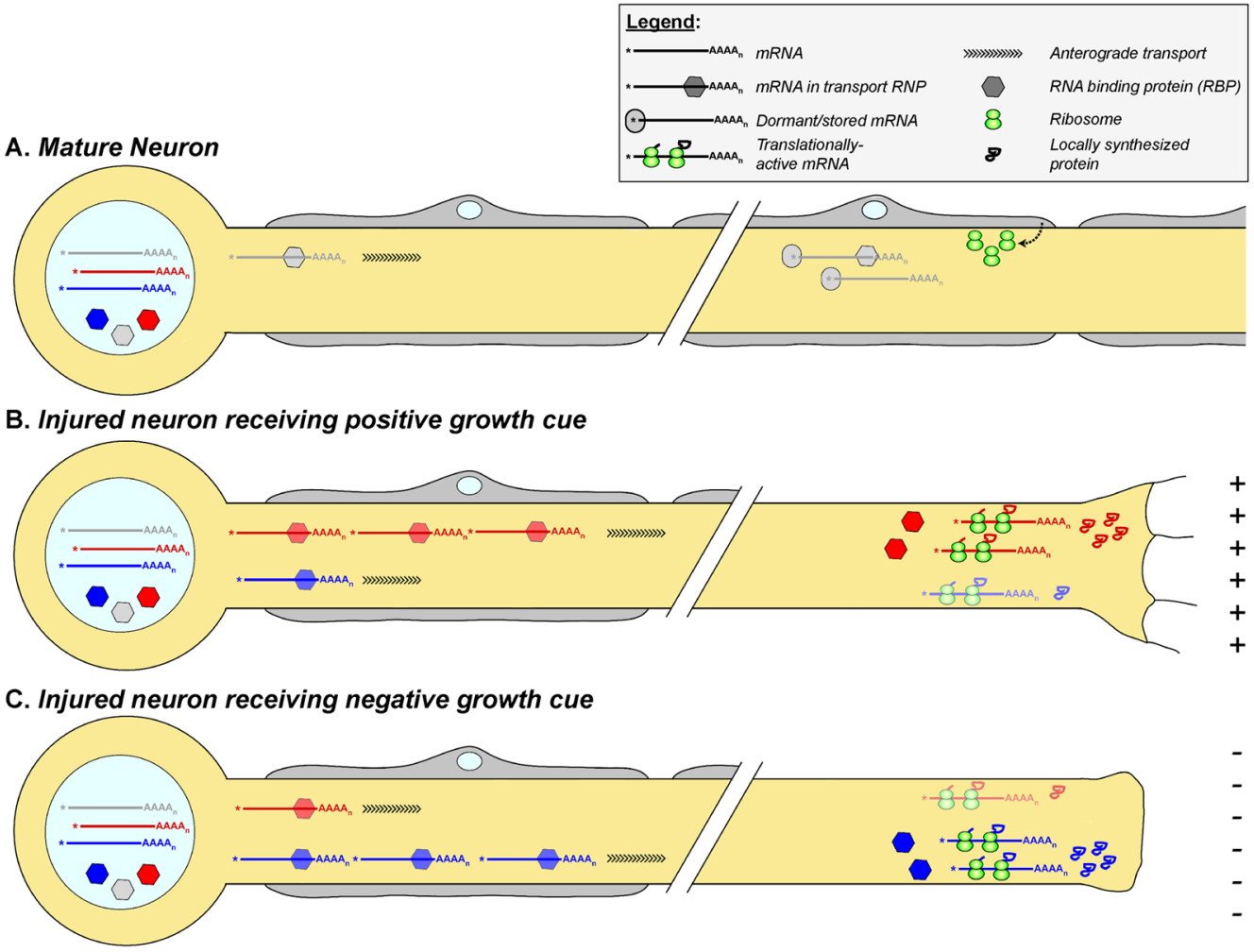

Figure 1. Transport of mRNAs into axons

Schematic of a PNS neuron with an axon surrounded by its myelinated sheath is shown in panels A–C with sources of mRNAs and ribosomes as indicated by the arrows. As discussed in the text, several lines of evidence support the hypothesis that the mature PNS axon contains mRNAs, some of which are likely stored in a dormant state until needed (panel **A**). Injury of the axon recruits dormant mRNAs into translation helping to initiate the regenerative response (Gumy and Fawcett, 2009; Rishal and Fainzilber, 2009). Injury results in a shift in populations of mRNAs delivered into the axonal compartment; this is likely a combination of shifting localization of existing mRNAs as well as injury-induced transcriptional events introducing new mRNAs (and possibly RNA binding proteins [RBPs]) into the neuron for localization. Additionally, the regrowing axon encounters both positive and negative growth cues as it regenerates into the distal nerve stump that has undergone Wallerian degeneration. These tropic and trophic stimuli modify the transport of individual mRNAs by generating retrograde signals that converge on the RBPs and transport machinery. The specificity of this RNA transport is likely generated by RBPs that are specific for individual classes of mRNAs (panels **B–C**). In these schematics, individual classes of mRNAs and their RBPs are color-coded for transport stimulus: grey = constitutively transported, red = transported in response to positive growth cues, and blue = transported in response to negative growth cues.