

Regulation of Protein Levels in Subcellular Domains through mRNA Transport and Localized Translation*

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Localized protein synthesis is increasingly recognized as a means for polarized cells to modulate protein levels in subcellular regions and the distal reaches of their cytoplasm. The axonal and dendritic processes of neurons represent functional domains of cytoplasm that can be separated from their cell body by vast distances. This separation provides a biological setting where the cell uses locally synthesized proteins to both autonomously respond to stimuli and to retrogradely signal the cell body of events occurring in this distal environment. Other cell types undoubtedly take advantage of this localized mechanism, but these have not proven as amenable for isolation of functional subcellular domains. Consequently, neurons have provided an appealing experimental platform for study of mRNA transport and localized protein synthesis. Molecular biology approaches have shown both the population of mRNAs that can localize into axons and dendrites and an unexpectedly complex regulation of their transport into these processes. Several lines of evidence point to similar complexities and specificity for regulation of mRNA translation at subcellular sites. Proteomics studies are beginning to provide a comprehensive view of the protein constituents of subcellular domains in neurons and other cell types. However, these have currently fallen short of dissecting temporal regulation of new protein synthesis in subcellular sites and mechanisms used to ferry mRNAs to these sites. *Molecular & Cellular Proteomics* 9:952–962, 2010.

In eukaryotes, polarized cells have evolved mechanisms for targeting macromolecules to the correct subcellular locales, allowing these cells to establish and maintain the unique domains needed for their specialized functions. Temporally altering the composition of these cellular domains allows dynamic responses to environmental stimuli, such as growth factors for proliferation and differentiation, guidance cues for migration, and peptides and neurotransmitters for cell-cell communication. Many research efforts have focused on how

proteins are delivered to subcellular domains because the proteins provide much of the enzymatic and structural components needed for cellular function. Over the past three decades, transport of mRNAs to subcellular domains with subsequent localized translation has been recognized as a means to regulate local protein composition (1).

Since the first identification of localized mRNAs in ascidian eggs (2), mRNA localization has been suggested as an effective means to target proteins where and when they are needed in the cell. Given that a single mRNA can give rise to multiple copies of a protein, localized protein synthesis effectively provides a means for rapid responses to extracellular stimuli, particularly for cells with large distances separating domains. Newer functional genomics-based studies using high throughput technologies are being applied to address the extent and diversity of mRNA localization. These approaches are establishing a comprehensive view of the overall prevalence and complexity of subcellular protein synthesis. These approaches also emphasize that subcellular targeting of mRNAs is a widespread phenomenon that has far reaching implications for cellular development and function. Transport of mRNAs to subcellular sites may be the norm for most mRNAs rather than an exception seen for a surprising few.

NEURONS AS EXPERIMENTAL PLATFORM FOR ANALYSES OF SUBCELLULAR PROTEIN SYNTHESIS

For neuronal cells, tremendous distances separate subcellular domains from the cell body where most protein synthesis was thought to occur (Fig. 1A). Dendrites, the highly branched efferent processes that receive synaptic input in the nervous system, extend for hundreds of micrometers from the neuronal perikaryon. Early ultrastructural studies in rodent hippocampus pointed to protein synthetic capacity for dendrites because ribosomes were seen concentrated at the base of dendritic spines near the synapse (3). Subsequent studies have shown that protein synthesis in dendrites contributes to the synaptic plasticity that is thought to underlie learning and memory. Indeed, neurons transport mRNAs into and locally translate mRNAs within their dendritic processes in response to transsynaptic stimuli, including various neurotransmitters and trophic factors (4). Although there were a few early examples of neuronal mRNAs in axons (5–7), the presynaptic processes that can extend for thousands to tens of thousands

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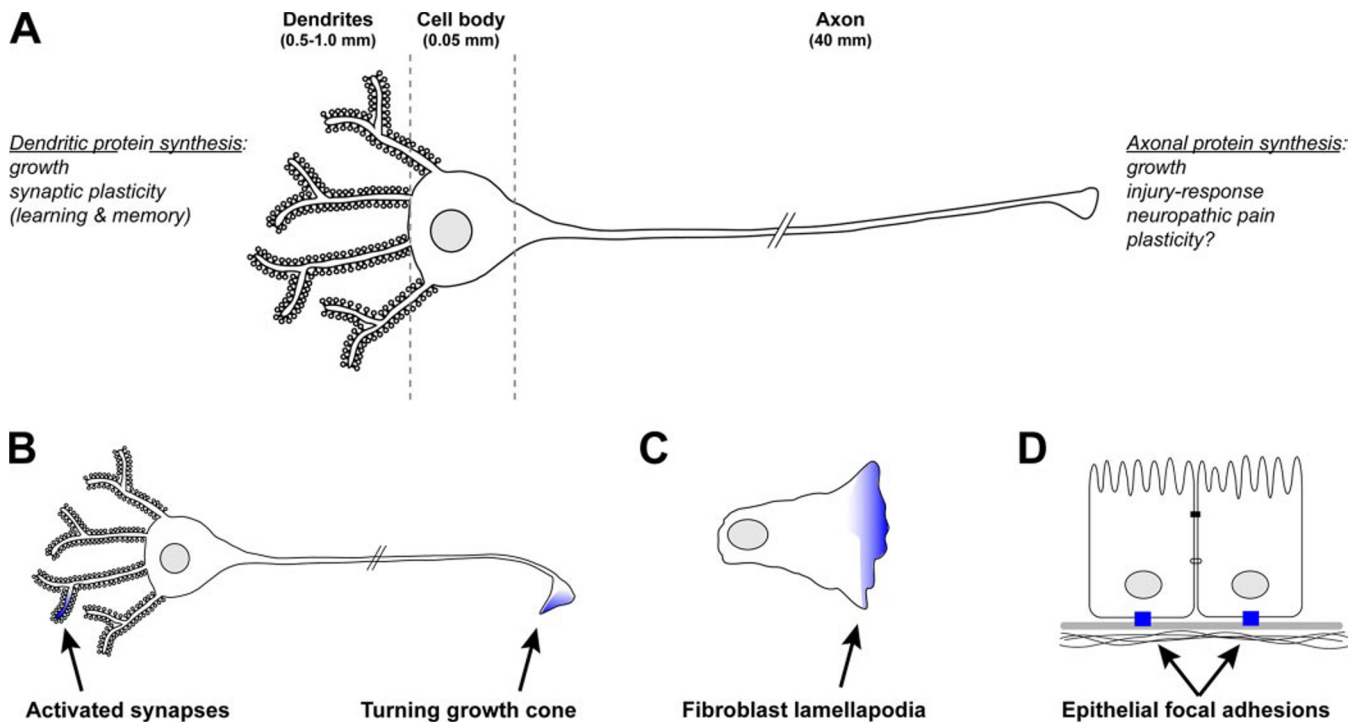


FIG. 1. Local protein translation in subcellular domains. *A*, the neuron is comprised of highly specialized subdomains that extend great distances from the cell body. Local protein synthesis within the dendrites plays a critical role in growth and synaptic plasticity. Within the axon, local protein synthesis is involved in growth, mediating the injury response, regeneration, and neuropathic pain and may play a role in plasticity. *B*, local protein synthesis allows the growth cone to respond autonomously to environmental cues. Attractive turning of the growth cone leads to local translation of mRNAs (blue). In dendrites, translation is activated by transsynaptic stimuli. *C*, in migrating fibroblasts, mRNAs encoding the microfilament regulating complex actin-related protein 2/3 localize to protrusions at the leading migration edge. Similarly, translation of β -actin in this region contributes to cell polarity and subsequent mobility. *D*, in epithelial cells, β -actin mRNA is localized to focal adhesions upon integrin binding with the plasma membrane.

of micrometers from the perikaryon even in the mouse, initial ultrastructural studies did not show any ribosomes in these processes in the central nervous system (3).

Lack of ribosomes was considered a hallmark of axons, but several lines of evidence now indicate that axons of the peripheral nervous system (PNS)¹ contain ribosomes and mRNAs (8). Ultrastructural evidence for ribosomes in myelinated PNS axons has been demonstrated in a few vertebrate organisms (9–13). Early studies with squid giant axon provided a relatively pure preparation of purified axonal contents to show the presence of rRNA, polyadenylated RNAs (likely mRNA), and tRNAs in these invertebrate axons (14–16). With classic biochemical techniques for fractionation of translationally active mRNAs, actively translating mRNAs were identified in purified axoplasm and in presynaptic terminals from the optic lobe of the squid (17–19). Although these findings

were suggested to be a feature of incomplete polarity of these invertebrate neurons, subsequent works in embryonic and adult vertebrate neurons have shown that protein synthesis in axons contributes to both developmental and regenerative axonal growth (20).

As more has been learned of protein synthesis in these neuronal compartments, it has become clear that the neuron provides an excellent experimental platform for analyses of mRNA localization and local protein synthetic mechanisms. The geographic separation of neuronal processes from the cell body provides an experimental model where subcellular domains can be isolated to purity for molecular and biochemical studies. Here, we will summarize some of the recent advances in understanding the complexities in regulation of localized neuronal protein synthesis. We will focus on the axonal compartment here as the link of axonal protein synthesis to growth and injury responses bears similarities with other cellular systems and likely has broad applications to biology and health. However, work on protein synthesis in synapses, which has classically been attributed to the postsynaptic side of the synapse (*i.e.* dendritic), may have more direct implications for disease pathogenesis, and synaptic preparations have been better scrutinized by modern protein chemistry approaches.

¹ The abbreviations used are: PNS, peripheral nervous system; AD, Alzheimer disease; AHA, azido-homoalanine; BDNF, brain-derived neurotrophic factor; PSD, postsynaptic density; RIP, RNA co-immunoprecipitation; RNP, ribonucleoprotein particle; SILAC, stable isotope labeling by amino acids in cell culture; TRAP, targeted translating ribosome affinity purification; ZBP1, zip code-binding protein 1; CREB, cAMP-response element-binding protein; GFP, green fluorescent protein.

LOCALIZED PROTEIN SYNTHESIS CONTRIBUTES TO DIRECTIONAL GROWTH OF AXONS

Studies using general protein synthesis inhibitors have pointed to a requirement for localized protein synthesis in response to some guidance cues. Developing axons are guided to their innervation targets by interaction with a variety of attractive and repulsive guidance cues (for a review, see Ref. 21). These soluble and membrane-bound guidance cues drive the turning response of the terminal axon or growth cone. This behavior of the growth cone, which can be separated from its cell body by the large distances outlined above, provides a compelling argument for the utility of local protein synthesis in axons (Fig. 1B). That is, the growth cone must respond to its environment well before any additional supply of proteins could be secured from the cell body through the known rates of anterograde transport ($\leq 5 \mu\text{m/s}$ for vesicular transport and $\leq 8 \text{ mm/day}$ for transport of most cytoskeletal elements). This theory was supported by observations that even after severing from the cell body the distal axon still responds to guidance cues by turning its growth cone toward gradients of Netrin-1 or away from gradients of Semaphorin; this turning response of severed axons is blocked by protein synthesis inhibitors (22). Other guidance cues, such as brain-derived neurotrophic factor (BDNF), Slit2, and engrailed-2, have also been shown to require local protein synthesis to elicit a turning response (22–25). Thus, localized protein synthesis can be required for the distal axon to respond to guidance cues and is autonomous from cell body-derived proteins.

Consistent with the different morphological responses of attraction *versus* repulsion of the growth cone, such localized protein synthetic responses can require translational activation of specific axonal mRNAs. Similar to the motile front of migrating cells, the growth cone is an actin-rich structure, and the actin cytoskeleton appears to be a point of convergence for regulating growth cone turning through localized protein synthesis. Local translation of RhoA mRNA in axons is required for response to Semaphorins (26). RhoA is a small GTPase that can trigger depolymerization of actin microfilaments. Attractive turning of growth cones in response to Netrin-1 and BDNF leads to an asymmetrical translation of β -actin mRNA in the growth cone with a focal concentration of β -actin mRNA in the same region where filamentous actin increases (27, 28). Neurotrophin 3 also increases translation and transport of β -actin mRNA in axons of cortical neurons, and blocking the axonal localization of β -actin mRNA causes growth cone retraction (29, 30). Other effectors of the actin cytoskeleton have also been shown to be locally translated, although it is not known whether they exhibit the same type of asymmetric translation. The actin-depolymerizing protein (also termed cofilin) is increased in a translation-dependent manner in the presence of the guidance cue Slit-2, which leads to growth cone collapse (24). These studies point to a common role for locally synthesized axonal proteins in mod-

ifying the actin cytoskeleton for axonal guidance with attractive cues favoring polymerization and repulsive cues favoring depolymerization. Axonal levels of actin-modifying proteins under basal conditions may also be regulated through localized protein synthesis because axonal translation of *Lymnaea* β -thymosin mRNA, which encodes a small protein that sequesters actin monomers, can modulate basal neurite growth (31).

Regulation of the cytoskeleton is also a feature of localized protein synthesis in non-neuronal cells. Translation of localized β -actin mRNA in migrating fibroblasts contributes to polarity of the motility of these cells (32). mRNAs encoding for the multiprotein actin-related protein 2/3 complex, a complex that regulates microfilament nucleation, similarly localize to protrusions of migrating fibroblasts (Fig. 1C) (33). Subcellular localization of several mRNAs encoding different components of the cytoskeleton and proteins that directly modify cytoskeleton has been detected in numerous cellular systems (34–38). Interestingly, β -actin mRNA is also recruited to focal adhesions upon integrin binding in epithelial cells (Fig. 1D) (39), so localized synthesis of cytoskeletal modifying proteins may be a common means to manipulate subcellular domains.

The above studies point to a level of specificity for localized translational regulation because individual stimuli can trigger local synthesis of proteins that favor depolymerization or polymerization of filamentous actin. Extracellular stimuli can also regulate the local synthesis of non-structural proteins. Cultured neurons that are exposed to constant levels of attractant (Netrin-1 and BDNF) or repellent guidance cues (Semaphorins) undergo adaptation, becoming unresponsive to these stimuli. Resensitization of the axons requires localized mRNA translation, so locally generated proteins allow the axon to regain their initial sensitivity to these stimuli (25, 40). Exactly what protein(s) mediates this process remains unknown, but it is appealing to speculate that the local synthesis of cell surface receptors could resensitize the responsiveness of the axon. There is precedence for localized synthesis of receptor proteins in axons because localized translation of EphA2 mRNA, which encodes a receptor for the ephrin guidance cues, is seen in commissural axons, making a pathfinding decision in spinal cord (41). Although the classic ultrastructurally defined rough endoplasmic reticulum and Golgi apparatus needed for co-translational secretion of proteins have not been seen in axons, recent work indicates that functional equivalents of these organelles exist in the axonal compartment (42). Thus, locally synthesized proteins can be targeted into secretory pathways. Similar outposts of rough endoplasmic reticulum and Golgi have been documented in other cellular systems, suggesting that localized synthesis of secreted and membrane proteins can contribute to responses in other biological systems (for a review, see Ref. 43). Consistent with this notion, localized synthesis of the extracellular matrix receptor component integrin $\alpha 3$ facilitates migration of neoplastic cells (44).

TABLE I
Profiling of neuronal subdomain mRNA populations

Experimental system	Subcellular domain	Method for RNA analyses	mRNA profile
Squid giant axon	Extruded axoplasm (14)	Kinetic analyses of DNA-RNA hybrids	~200 different mRNAs, identity unknown
Sensory neurons (<i>Aplysia</i>)	Dissected neurites (54)	cDNA library sequencing	198 different mRNA species
Sensory neurons (rodent)	Isolated axons (modified Boyden chamber) (56)	DNA array (absolute hybridization)	>200 different mRNA species (with transcripts identified)
Hippocampal neurons (rodent)	Isolated axonal processes (microfluidic chamber) (58)	DNA array (hybridization with advanced presence-calling algorithms)	≥800 different mRNA species (with transcripts identified)
Hippocampal neurons (rodent)	Isolated dendrites (modified Boyden chamber) (110)	DNA arrays (differential hybridization, dendrite vs. cell body)	>100 mRNAs enriched in dendritic processes
Rodent brain	Synaptosome fractionation plus polysome sedimentations (79)	DNA arrays (differential hybridization)	≥50 mRNAs enriched in heavy polysome fraction (likely within RNA granules)
Human brain (control and incipient AD)	Synaptosome fractionation from post-mortem prefrontal cortex (80)	DNA arrays (differential hybridization)	>400 mRNAs with different levels in control vs. AD at $p < 0.01$
Fibroblasts (human)	Isolated pseudopodia (59)	DNA array (differential hybridization)	≥50 mRNAs with enrichment upon lysophosphatidic acid vs. fibronectin treatment

LOCALLY SYNTHESIZED PROTEINS CAN GENERATE LONG DISTANCE SIGNALING

In addition to growth cone guidance, mRNA translation in axons is known to facilitate regenerative responses, both locally in the injured axon and distally in the cell body. Intra-axonal translation and proteolysis are needed to locally initiate growth cone formation after axotomy (45). In mature PNS axons, axonally synthesized proteins generate a retrograde signaling complex that is critical for an injury response in the cell body (46). Translation products of importin β 1 and RanBP1 mRNAs allow formation of an importin α/β heterodimer that retrogradely transports signaling proteins to the cell body through interaction with dynein (47, 48). A proteolytic fragment of locally translated vimentin mRNA further provides a scaffold to couple activated mitogen-activated protein kinase to importin α/β complex (49). The sum of these distally placed protein synthetic events signals the cell body that its axon has been injured and initiates a new program of gene expression to support regeneration.

Neurotransmitters have also been shown to trigger retrograde transport of importins in dendrites of cultured cortical neurons, but it is not entirely clear whether this is through localized translation of importin mRNAs (50). Synaptic plasticity-invoking stimuli were recently shown to trigger retrograde transport of CREB2, a transcriptionally repressing member of the CREB family, from distal processes through importins, but this is independent of translation (51). However, translation of another member of the CREB family (*i.e.* "CREB" or CREB1) was detected in axons of neonatal sympathetic neurons (52). In this system, nerve growth factor stimulates retrograde transport of the axonally synthesized CREB, which seems to be required for nerve growth factor-dependent sur-

vival in these neurons. It is quite possible that distinct proteins are needed for retrograde signaling, formation of a growth cone, and turning of a growth cone; this would imply that localized translation of different mRNAs is specifically coupled to the metabolic and functional needs of the axon.

NEURONAL PROCESSES CONTAIN UP TO HUNDREDS OF DIFFERENT mRNAs

The studies outlined above point to the importance of localized protein synthesis in axonal responses to extracellular stimuli and injury, but these analyses have largely been limited to individual mRNAs and proteins. With the ability to isolate neuronal processes to purity combined with sensitivity provided by amplifying nucleic acids, we now know that hundreds of individual mRNAs are transported into distal neuronal processes (Table I). Early studies in the squid giant axon estimated that up to 200 different polypeptides could be encoded by the localized mRNAs based on a differential RNA-DNA hybridization approach (53). Analyses of sequences in a cDNA library generated from neurites of *Aplysia* sensory neurons also supported this level of complexity for localized mRNAs (54). We have used a modified Boyden chamber for isolating axonal processes from cultured sensory neurons (55). By hybridization to DNA arrays, we identified a similar number of mRNAs in the axons of vertebrate sensory neurons using very conservative measures for presence calling (56). These sensory neurons are unique in that they only extend axonal processes in culture, making their isolation from cell body and non-neuronal cells much easier than in neurons with both dendrites and axons. Taylor *et al.* (57) developed a microfluidic culture device to effectively separate distal axons from dendrites of mammalian central nervous

system neurons. Using this approach, they recently provided a sum of several hundred mRNAs in hippocampal axons (58).

Taken together, these studies paint a picture of the neuronal process as an elaborate cellular subdomain with a complex population of mRNAs available for localized translation. Dendritic mRNA populations are likely to be similarly complex. Subdomains of other cellular types have not been as easily studied as neurons. However, a recent differential analysis of protrusions from mammalian fibroblasts showed at least 50 distinct mRNAs (59). Importantly, the local transcriptomes in both neurons and other cell types encode proteins with diverse cellular functions including metabolism and protein synthesis. This could provide these distal cellular regions with a degree of functional autonomy from the more proximal regions of the cell cytoplasm and organelles.

REGULATING SPECIFICITY OF SUBCELLULAR PROTEIN SYNTHESIS

mRNA profiles of subcellular sites have proven informative to show what proteins can be produced locally, but these studies do not address how and when translation of these mRNAs occurs. Transport of mRNAs to subcellular sites, with extracellular stimuli modifying the population of localized mRNAs, is one means to regulate specificity of translation (60). However, once localized, individual mRNAs are also subjected to translational regulation in response to specific stimuli. An example of this is seen with translation of axonal importin β 1, vimentin, and RanBP1 mRNAs; the rapid appearance of these proteins in the axons with injury argues that their mRNAs are locally stored in a translationally dormant state until needed. Despite evidence for local translational regulation, the vast majority of analyses of axons and dendrites have been limited to individual proteins rather than the needed systematic approach that proteomics studies would provide.

Nonetheless, analyzing the proteome of neuronal processes has given insight into protein composition of these subdomains. Recent proteomics analysis of neurites fractionated from differentiated NIE-115 neuroblastoma cells mapped the spatial relationship of nearly 5000 proteins for neurites *versus* cell body (61). This study revealed networks of signaling proteins involved in neuritogenesis, cytoskeletal organization, axonal guidance, and cell polarity. Subsequent analysis of growth cones and membrane fractions from growth cones isolated from developing rat forebrain further established the proteomes of these regions of growing axons (62). Synaptosomes, an enriched subcellular fraction isolated from mature brain that contains both presynaptic and postsynaptic membrane-bound compartments, have given new insight into the synaptic proteome (63, 64). Even further interrogation of the synapse has been provided by purification of the postsynaptic density (PSD), an electron-dense postsynaptic region involved in afferent signaling (65). Several presynaptic proteins are also included in these PSD isolates, and correlation profiling has recently been used to show that scaffolding proteins

and cytoskeletal proteins are highly enriched in the PSD compared with mitochondrial proteins and transporters (66). Mass spectrometry of co-immunoprecipitating synaptosome proteins has provided clues into temporal and spatial patterns of protein-protein interactions in the synapse and may also help to distinguish pre- and postsynaptic components (67). Because isolated axons, growth cones, and synaptosomes all have protein synthetic activity, at least some of the proteins identified in these preparations are generated locally. For the PSD, ribosomes are concentrated further beneath the synapse, but some of the proteins within the PSD are locally synthesized in the dendrite.

Although the studies outlined above do not differentiate between locally synthesized and cell body-synthesized proteins, they have been informative by identifying proteins that are highly enriched in these compartments. Several locally synthesized proteins have been identified in synaptosomes from squid optic lobe and in isolated axons from adult rodents through isotopic metabolic labeling, but the requirement for two-dimensional electrophoretic separation limited these studies to the most abundant locally synthesized proteins (68, 69). As noted above, both pre- and postsynaptic processes contain hundreds of different mRNAs (see Table I), so localized translation likely contributes to enrichment of many proteins in presynaptic and postsynaptic compartments, and this would not be detected by standard isotopic labeling approaches. Future integration of proteomics data with transcriptomics work from subcellular compartments will further point to the potential for localized synthesis modulating protein levels, but better methodologies are needed to enrich for locally synthesized proteins prior to mass spectrometry.

Both the synapse and growth cones are dynamic structures where protein levels and activities are rapidly altered by extracellular stimuli. Protein activity is often regulated by post-translational modifications, and phosphorylation of PSD proteins has been implicated in their function, localization, and interactions with other proteins (70). Phosphorylation can also directly modulate activity of protein synthesis machinery, including localized machinery, by activating and inactivating signaling networks that converge on translation factors and ribosomal proteins (71). Identification of phosphorylated peptides has been technically difficult given the relative low abundance of these peptides within the larger PSD proteome. Affinity chromatography has been used to enrich phosphopeptides from PSD fractions prior to mass spectrometry (72, 73), and analyzing different regions of the mouse brain has provided a unique view of protein and phosphoprotein levels in brain structures with distinct functions (74).

Together, the above and other studies are beginning to establish a comprehensive view of protein constituents and basal post-translational modifications in functional subdomains of the neuron. Quantitative mass spectrometry approaches have shown changes in synaptosome and PSD proteins and phosphoproteins in biological models of disease

and injury processes including Alzheimer disease, Down syndrome, fragile X mental retardation, and Wallerian degeneration (75–78). These studies point to potential pathophysiology for neuronal subdomain dysfunction. However, they fall short of determining the dynamic regulation of proteins that may occur through changes in protein synthesis. As noted above, alterations in axonal mRNA levels have been shown to occur in response to extracellular stimulation (56), and electroconvulsive shock of rodents was also shown to change mRNA composition of synaptosomes (79). These dynamic transitions in axonal mRNA levels would have direct impact on what proteins can be produced locally.

Alterations in localized mRNA populations may have a role in disease pathogenesis. Interestingly, mRNA profiles in synaptosomes were very recently shown to be altered in the incipient stages of Alzheimer disease (AD) (80). By *in vivo* imaging techniques using localized reporter mRNAs, translation in dendrites was shown to be altered in AD model mice directly adjacent to amyloid plaques that are seen in AD (81). Decreased axonal transport of β -actin mRNA has also been demonstrated in spinal muscular atrophy (82). Although one can generate a compelling argument for analyses of dendritic and axonal mRNA profiles and translation in other neurological diseases, our knowledge of how and when translation is regulated is limited and cries out for more sensitive means to systematically and temporally analyze new protein synthesis. Although the stable isotope labeling by amino acids in cell culture (SILAC) technique was used to quantify synaptosome proteins in the fragile X neurons mentioned above, this required steady-state labeling so that changes in protein synthesis elicited by the absence of dendritic fragile X mental retardation protein were not addressed (77). Therefore, new labeling and enrichment methods are needed to analyze localized protein synthesis. Currently available labeling and tagging methods for mass spectrometry are summarized in Table II. Although RNA-based methods have the advantage of amplification of protein analysis, it is important to note that eukaryotes use organelle-like structures, stress granules, and processing bodies to segregate mRNAs for storage and degradation (83). Axonal importin β 1, RanBP1, and vimentin are prime examples of localized mRNAs that are sequestered until needed. Thus, an increase in mRNA localization does not always equate to temporal regulation of translation (Table II).

METHODS FOR UNBIASED ANALYSES OF LOCALIZED TRANSLATIONAL REGULATION

Local protein synthesis in intact and severed axons has largely been detected through incorporation of radioactively labeled amino acids. Isotopic labeling with [^{35}S]methionine has been used to demonstrate stimulus-dependent changes in protein synthesis in axons (84). However, systematic proteomics analysis of locally synthesized axonal proteins has been an unmet challenge considering the excessively limited

amounts of proteins synthesized locally. Metabolic labeling of axons indicated that ~5% of total cellular protein synthesis occurs in these distal processes (85). Thus, at least 95% of protein isolated from neuronal processes and other subcellular compartments has been transported into these processes rather than synthesized *de novo*. In our hands, isolated axons only remain visibly intact for about 6 h after severing from the cell body (55). Thus, this relatively meager fraction of protein is further limited by the survival of isolated subcellular domains. Considering that newly synthesized proteins can only be labeled over a time period of a few hours and they represent only a small fraction of the proteins present in the processes, proteomics approaches for identifying and quantifying locally synthesized proteins will require significant enrichment similar to the approaches used for phosphoproteomes. A newly developed methionine analog, azido-homoalanine (AHA), may prove amenable to this because AHA-labeled proteins can be covalently tagged with epitopes for purification through a copper-catalyzed azide-alkyne ligation (86). Dieterich *et al.* (87) found that a 1–2-h labeling period generates sufficient protein for affinity purification and mass spectrometry using non-fractionated cells (*i.e.* whole cell lysates rather than subcellular domains). AHA has recently been used with similar labeling coupled with fluorescent tagging to visualize protein synthesis in axonal growth cones (88). Nonetheless, RNA-based approaches remain appealing with the ability to exponentially amplify signals through RT-PCR.

Translational activity of individual mRNAs can be addressed through testing for their association with ribosomes. This classic ultracentrifugation technique, or polysome fractionation, has been used to show translation of mRNAs in squid axoplasm, synaptosome preparations, and regenerating axons by showing that the transcripts are associated with ribosomes (19, 55, 89). Zheng *et al.* (55) further used an RNA co-immunoprecipitation (RIP) by using the Y10B monoclonal antibody to 28 S rRNA and axonal lysates prepared under conditions that maintain ribosome subunit interactions. Dissociation of 80 S ribosome particles by application of EDTA or puromycin prior to fractionation by centrifugation of RIP provides a control for these analyses. Combining polysome fractionation techniques with RT-PCR and DNA microarray or deep sequencing technologies could be used to quantitatively address the relative translation state of localized mRNAs in cell culture preparations. The deep sequencing approach was recently used to quantify translation of mRNAs in *Saccharomyces cerevisiae* by comparing ribosome bound to total mRNA isolates (90).

A limitation for much of the mRNA profiling work outlined above is that it is limited to preparations of cultured neurons. A recently developed method, termed “targeted translating ribosome affinity purification” (TRAP) (91, 92), holds promise for quantifying mRNA-polysome association in subcellular domains from complex *in vivo* preparations. Heintz and co-workers (91, 92) developed transgenic mice that express en-

TABLE II

Non-biased approaches to analyze local protein synthesis

2D, two-dimensional; iTRAQ, isotope tags for relative and absolute quantification; BONCAT, bioorthogonal noncanonical amino acid tagging.

	Advantages	Disadvantages
RNA-based analyses		
Microarray hybridization (56, 58)	Widely available; can be non-biased (at least for genome-wide analyses)	Only addresses mRNA levels not translation; background hybridization signals complicate presence calling; limited to expression of specific target genes
Deep sequencing (“454 sequencing”) (90)	Non-biased; can allow identification of small, non-coding RNAs; eases presence calling (RNA is either there or not there)	Only addresses mRNA levels, not translation; sensitivity for subcellular domains remains undetermined; expense
Polysomal RNA fractionation (18, 55, 89)	Identifies translationally active mRNAs; can be applied to small samples by using microultracentrifugation	Fractionation can be difficult to normalize; cannot distinguish mRNAs with stalled ribosomes; must confirm with microarrays or 454 sequencing
TRAP (91, 92)	Identifies translationally active mRNAs; cell-specific mRNA profiling; potential to isolate translationally active mRNAs from <i>in vivo</i> preparations	Sensitivity for subcellular domains remains undetermined; cannot distinguish mRNAs with stalled ribosomes; need to generate/obtain transgenic mouse for <i>in vivo</i> study
Protein-based approaches		
Classic isotopic labeling (e.g. [³⁵ S]methionine) (68, 69)	Identifies translation products from small quantities of starting materials; potential to scale up by adding more isotope	Limited to most abundant proteins; requires 2D gel-based fractionations to separate labeled proteins; poor sensitivity for small quantities
SILAC (77)	Quantitation of synthesized proteins; steady-state labeling useful for studying post-translational modifications (e.g. phosphopeptides)	Requires steady-state labeling (labeling period of days); limited number of samples can be compared at one time
Non-isotopic labeling (AHA; BONCAT) (87, 88)	Reported high level of incorporation with short labeling times (2 h); allows for affinity enrichment of labeled proteins	Sensitivity is unproven for subcellular samples; no quantitation possible unless coupled with additional methods (e.g. iTRAQ)
ICAT (65, 66)	Allows for relative quantitation; useful for less complex samples (<i>i.e.</i> subdomains) and small amount of material	Cannot distinguish between locally and non-locally synthesized proteins; targets only cysteine-containing peptides leading to under-representation of some proteins
iTRAQ (74, 111)	Relative quantitation of protein levels between samples; multiplex analyses (8-plex); affinity enrichment of tagged proteins	Cannot distinguish between locally and non-locally synthesized proteins; every sample must be screened by tandem MS, increasing analysis time and amount of sample needed

hanced GFP-tagged ribosomal protein L10a (GFP-L10a) using bacterial artificial chromosomes containing regulatory elements for gene expression to provide transgene expression that is remarkably restricted to cell types and subtypes. For example, neuronal subtype-specific expression was achieved for pyramidal neuron subtypes in different layers of the cerebral cortex, regions that could never be completely separated by manual dissection or culture. Taking advantage of the GFP-tagged ribosomes, cell type-specific ribosome-mRNA complexes could be immunoprecipitated from whole brain lysates using anti-GFP antibodies. With amplification of the mRNAs followed by DNA microarray hybridization, a translational profile of individual cell populations can be determined (91, 92).

Combining TRAP with established and developing techniques for isolating the axonal subdomain would be a pow-

erful tool for profiling the population of translationally active axonal mRNAs. Recently developed methods for isolation of axoplasm from peripheral nerve, where axons are widely separated from cell body and other neurons, offer a particularly appealing approach for quantifying *in vivo* axonal mRNA translation (93). A drawback for both TRAP and the Y10B RIP approaches is that a fraction of the purified mRNA could represent transcripts with stalled ribosomes. However, this should be a relatively minor fraction that may be separated by their relative levels compared with other mRNAs. Additionally, the deep sequencing approach brings the potential for “footprinting” mRNA sequences by identifying sites that are protected from nuclease digestion through binding by protein or other nuclease-resistant macromolecules. Ingolia *et al.* (90) successfully used this approach to gauge translational efficiency for yeast mRNAs, and translational efficiency can be

extrapolated to give an indication of true polysome association of the mRNAs.

MECHANISTIC INSIGHTS INTO mRNA TRANSPORT

mRNAs are actively transported to subcellular sites as RNA-protein complexes, or RNPs, that interact with motor proteins (94). For example, subcellular localization of β -actin mRNA requires binding of zip code-binding protein 1 (ZBP1) to its 3'-untranslated region localization element (zip code) (95). Studies in *Xenopus* oocytes argue that initial mRNA-protein interactions for localizing mRNAs occur in the nucleus with remodeling of the transport RNP as it enters the cytoplasm and then engages with motor proteins (96). Addition and exchange of protein constituents are the basis of this remodeling. Such remodeling has also been seen for mammalian β -actin mRNA where the transcript is initially bound by the nuclear protein KH-type splicing regulatory protein (termed ZBP2 in chick); this initial binding by KH-type splicing regulatory protein provides cooperative binding for ZBP1 that is needed for subcellular localization of β -actin mRNA (97). Interestingly, ZBP1 serves as a translational suppressor while bound to mRNA; affinity of ZBP1 for mRNA is decreased by Src kinase-dependent phosphorylation, resulting in translational derepression of β -actin mRNA (98). Translational repression within transport RNPs is now a common theme in RNA localization with post-translational modifications frequently serving as the basis to alter RNA-binding protein activity (99).

Despite remarkably increased knowledge of the subcellular transcriptome, a huge gap in knowledge remains when it comes to proteins responsible for their transport. Attempts to isolate transport RNPs from whole cell lysates have generated new insight into composition of these complexes through proteomics and ribonomics studies (100–102). Cell biology-based approaches show that these isolates likely contain multiple different RNP populations when individual neuronal processes are queried (103). Thus, how specificity is achieved for mRNA transport still remains to be solved. Applying proteomics to RNPs from subcellular regions, such as axons or dendrites, will help to enrich for transported RNPs and potentially provide a more pure population for study. The yield of proteins from isolated axonal and dendritic RNPs will be even more challenging than the RNA profiling studies, but the increasing sensitivity of proteomics instrumentation offers hope for meeting this challenge in the near future.

Studies of single RNA-binding proteins have shown that most can bind to a surprisingly large number of different mRNAs. Immunoprecipitates of the human ortholog of ZBP1, termed IMP1 (insulin-like protein 2 mRNA-binding protein), were shown to contain >350 different mRNAs in HEK293 cells (104). Similarly, for the neuronal RNA binding protein HuD, that recognizes AU-rich elements and stabilizes bound mRNAs, several hundred mRNA targets were identified by array profiling (105). It is not always clear whether such inter-

actions are due to direct protein recognition of RNA motifs, protein-protein interactions, or nonspecific binding from *in vitro* preparations. Addition of *in vivo* cross-linking to RIP followed by nuclease digestion (*i.e.* cross-linking with co-immunoprecipitation (106)) has advantages of avoiding both protein-protein interactions and nonspecific RNA-protein interactions that can occur in cell lysates (107). Deep sequencing of RNAs isolated through cross-linking and immunoprecipitation brings the potential to identify RNA motifs that are directly targeted by RNA-binding proteins because the protected regions are indicative of protein binding sites (108, 109). Considering that many RNA motifs for localization are determined by secondary structure that have proven difficult to analyze through bioinformatics approaches (for a review, see Ref. 60), large scale analyses of RNA-protein interaction sites through cross-linking and immunoprecipitation will likely uncover functional RNA motifs that have been unrecognized to date. It is not clear whether these RNA co-immunoprecipitation approaches are of sufficient sensitivity to distinguish where the mRNA-protein interactions occur within the cell; *i.e.* will they be of sufficient sensitivity for the low amounts of protein and RNA isolated from subcellular compartments like axons and dendrites? Additionally, proteins indirectly binding to mRNAs through protein-protein interactions likely contribute to specificity for when individual mRNAs are transported. So for dissecting the transported RNP composition, both the target mRNAs and the protein constituents will need to be considered; this will require seeking new means to integrate high sensitivity ribonomics and proteomics data sets.

CONCLUSIONS

Polarized cells spatially regulate mRNA translation by delivering transcripts and protein synthesis machinery to subcellular regions. Molecular biology approaches have been used to dissect this spatial regulation by providing profiles of localized mRNAs, and cell biology approaches have been used to both detect localized synthetic machinery and show its functionality. These same approaches indicate that localized protein synthesis is temporally regulated both in neurons and other cell types. A key question for understanding the biology of localized protein synthesis is how specificity is achieved. Several lines of evidence point to specific translation of different mRNAs in response different stimuli. Cell biology and biochemical approaches thus far have been limited to the study of individual proteins or mRNAs. Enhanced sensitivity of mass spectrometry, new approaches to enrich metabolically labeled proteins, and continued effort by cell biologists to isolate subcellular domains should provide means to systematically profile localized protein synthesis and mRNA transport (see Table II). This will be key in any future approaches to therapeutically modulate axonal and dendritic protein synthesis. The roles of axonal protein synthesis in regeneration and dendritic (and possibly axonal) protein synthesis in neuronal function are appealing targets

for intervention. Understanding how specificity is achieved in neuronal processes may also lead to new developments for general cell migration where translational control can have biomedical implications for metastasis of cancer cells (44).

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