Invited Mini Review

Local protein synthesis in neuronal axons: why and how we study

Eunjin Kim & Hosung Jung*

Department of Anatomy, Brain Research Institute, and Brain Korea 21 PLUS Project for Medical Science, Yonsei University College of Medicine, Seoul 120-752, Korea

Adaptive brain function and synaptic plasticity rely on dynamic regulation of local proteome. One way for the neuron to introduce new proteins to the axon terminal is to transport those from the cell body, which had long been thought as the only source of axonal proteins. Another way, which is the topic of this review, is synthesizing proteins on site by local mRNA translation. Recent evidence indicates that the axon stores a reservoir of translationally silent mRNAs and regulates their expression solely by translational control. Different stimuli to axons, such as guidance cues, growth factors, and nerve injury, promote translation of selective mRNAs, a process required for the axon's ability to respond to these cues. One of the critical questions in the field of axonal protein synthesis is how mRNA-specific local translation is regulated by extracellular cues. Here, we review current experimental techniques that can be used to answer this question. Furthermore, we discuss how new technologies can help us understand what biological processes are regulated by axonal protein synthesis *in vivo*. [BMB Reports 2015; 48(3): 139-146]

INTRODUCTION

A typical cell biology textbook published 20 years ago would state that all proteins in the axon of a vertebrate neuron come from the cell body. This 'tenet' of neuronal cell biology was based on thorough transmission electron microscopic observations of the sections from the adult rat brain, where the polyribosomes and rough endoplasmic reticulum were mainly found in neuronal cell bodies (1). In closer investigations, it was found that polyribosomes also localize to the base of den-

dratic spines (2). This finding immediately suggested a novel explanation to a long unanswered question in neuronal cell biology - how a new protein, synthesized in the cell body, is delivered to a specific postsynaptic site in highly complex dendritic arbors. The activated postsynapse can make the required protein on site by local mRNA translation (2). This idea led to a series of important discoveries that have firmly established local protein synthesis as one of key mechanisms underlying activity-dependent structural changes in the synapse (3). In contrast to the dendrite, however, polyribosomes were not readily detected on the other side of the synapse, the axon terminal. Moreover, most of the polyribosomes found in the axon localized to the axon initial segment, which is rather a 'postsynaptic' site for inhibitory synapses than a presynaptic structure (4). This observation led to a speculation that local protein synthesis may occur prevalently in postsynaptic sites, and evidence for protein synthetic machinery in axons in culture (5, 6) and *in vivo* (7) did not draw much attention until the 1990's, perhaps due to this rather strong notion. It was not until the early 2000's when two independent studies convincingly showed that axonal protein synthesis is required for specific biological processes such as chemotropic responses of growth cones (8) and potentiation of neurotransmitter release (9). These discoveries led to a rebirth of the field of axonal protein synthesis. Many follow-up studies have showed that diverse vertebrate neuronal axons are capable of local protein synthesis both during development and in adulthood and have identified several axonally localized mRNAs, whose local translation is required for axon guidance, elongation, survival, and regeneration (references in (10)).

The next goal in the field was to construct a comprehensive catalog of axonally localized mRNAs. Several genome-wide transcriptome analyses were performed aiming to gain insights into the roles that axonal protein synthesis may play, from the list of proteins that can be made from axonal transcriptome (11-15). However, unexpectedly large numbers of mRNAs were found in axons (sometimes thousands), which encode a variety of protein family members (even proteins that one would not normally expect there, such as transcription factors). Intriguingly, the contents of an axonal transcriptome is regulated by aging (15) and extrinsic cues (16), and only a select few of axonally localized mRNAs are translating at any given

*Corresponding author. Tel: +82-2-2228-1651; Fax: +82-2-365-0700; E-mail: hosungjung@yonsei.ac.kr

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time (17). These results suggest that axons store mRNAs encoding proteins that are required in the near future and translate them only when required (Fig. 1).

At this time, conducting research on axonal protein synthesis often means asking one or all of the following questions: i) whether a particular protein is locally synthesized in the axon, ii) how the mRNA encoding that specific protein is chosen for translation from thousands of axonally localized mRNAs, and iii) what roles selective translation of that mRNA plays in the axon. Strategies to answer these questions differ depending on whether one has a particular protein of interest (candidate-based approach) or not (unbiased approach), but generally involve a combination of techniques in compartmentalized cell culture, molecular biology, biochemistry, and imaging. These methods can also be applied to study dendritic synthesis, but axons are more amenable for experiments as they are generally longer and therefore easier to isolate from their cell bodies.

UNBIASED SCREENS FOR AXONALLY SYNTHESIZED PROTEINS

The ultimate evidence that a particular protein is locally synthesized in the axon is that its abundance increases in mRNA translation-dependent manner in axons that are physically separated from their cell bodies. In unbiased screens, one selectively labels and/or purifies such proteins and identifies them by mass spectrometry (MS). Extracting proteins from axons with-

Fig. 1. Adaptive gene expression in the axon by local mRNA translation.

out any trace of their cell bodies is critical, because slight contamination of somal proteins will lead to misidentification of axonally synthesized proteins. For this reason, all unbiased screens for axonally synthesized proteins published so far were conducted in cell culture systems, in which cell bodies can be experimentally eliminated from axons (which is often confirmed by absence of genomic DNA in axon lysate). For the same reason, proteins identified in unbiased screens must be validated by independent approaches, which are often imaging-based (see “Confirmation of axonal protein synthesis”). We start this section by introducing most common cell culture platforms used to separate axons from their cell bodies (Fig. 2 and Table 1).

Preparation of ‘axon-only’ culture

Campenot chamber: The Campenot chamber is the first device designed specifically for compartmentalized axon culture (18). It is a scaffold made of Teflon (a material known for non-stick coating of cooking pans), which is attached to a cell culture dish or cover glass via silicone grease (Fig. 2A). Currently,

Fig. 2. Methods to collect pure axons.

Table 1. Comparison of compartmentalized axon culture platforms

Technique	Consistency	Purity	Yield	Selectivity (1)	Fluidic separation	Explant culture?	Accessibility
Campenot chamber	Normal	Good	Good	Good	Good	Yes	Good
Modified Boyden chamber	Good	Normal	Very good	Low	No	Yes	Very good
Microfluidic device	Good	Very good	Moderate	Very good	Very good	With modification	Good
Laser-capture microdissection	Very good	Excellent	Low	Excellent	No	Yes	Low

(1) Whether subcellular compartments within the axon can be selectively collected. Multi-chambered Campenot chambers and microfluidic device may be used to isolate proximal, middle and distal axons, separately Laser-capture microdissection allows selective isolation of minute structures such as the growth cone.

there are many variations of the original three-chamber design, in which the dissociated neurons are plated in the central, or “proximal” compartment. Neuronal cell bodies and proximal neurites remain in the proximal chamber, and only the long neurites (which are likely to be axons) can pass through the silicone grease-culture dish interface under the Teflon divider into the “distal” compartments. With a complete seal, decent fluidic separation between compartments can be made, and axonal growth into the distal compartments is facilitated by selective addition of growth factors, such as nerve growth factor (NGF), to the “distal” compartments. The use of Campenot chambers involves relatively simple procedure (19) with commercially available resources. If a stable and uniform seal is made between the Teflon barrier and culture dish, it is possible to culture a pure population of axons with virtually no trace of cell bodies. In order to make a stable seal, however, the width of the barrier should be relatively large (>1 mm), which makes its use limited to neurons with very long axons. The making of a consistent seal requires training.

Modified Boyden chamber: The Boyden chamber was originally designed to study leukocyte migration, and it is made up of a porous cell culture insert that separates the cell culture dish into two compartments (20) (Fig. 2B). The size of pores can be modified so that only the axons can pass through them and into the distal compartment (approximately $1\ \mu\text{m}$), and the inserts with various pore sizes are commercially available. Neuronal cell bodies are plated on the upper surface of the insert, and axonal growth into the lower compartment is facilitated by selectively coating the lower surface of the insert with extracellular matrix (ECM) molecules (such as laminin) (21). The main advantage of this method is that the culture procedure is straightforward and that tissue explants (such as pieces of the brain and spinal cord) can be used without any modification. After axons grow into the lower “distal” compartment, all the cells in the upper “proximal” compartment can be removed by suction and scraping. With meticulous elimination, one can obtain pure axons with no detectable nuclear DNAs. Two main disadvantages of this method are that there is no fluidic separation between chambers (therefore cell bodies should be physically removed before any chemical stimulation, if axon-specific stimulation is required), and that it is difficult to get clear images of axons grown on a porous insert (plasma membrane can be stained with a fluorescent dye to improve image quality).

Microfluidic device: Microfluidic device was developed along the original idea of Campenot, but new material of transparent and biologically inert polymer (polydimethylsiloxane, or PDMS) was utilized (22). The PDMS device often contains two symmetrical chambers separated by narrow “microgrooves”, through which only axons can grow. Gently pressing a dry PDMS mold on a dry coverslip or culture dish makes a water-tight seal (Fig. 2C). Plating dissociated neurons in one chamber (“proximal” compartment) over a sufficient period of time allows axons to grow through the microgrooves into the other

chamber (“distal” compartment). The main advantage of this technique is that almost perfect fluidic isolation between the two compartments can be achieved, simply by adjusting the volume of culture medium in each compartment. For example, when more culture medium is added to the proximal compartment, any chemical treated into the distal compartment will remain there because of the negative hydrostatic pressure in the microgrooves (Fig. 2C). PDMS molds with different sizes of microgrooves and chambers are commercially available or can be micro-fabricated. The microgrooves can be made significantly shorter than the barrier of the Campenot chamber (as short as $150\ \mu\text{m}$), and therefore, this method can be used for short axons and even for long dendrites. The transparency of PDMS makes it particularly suitable for live imaging. One disadvantage is that it is more expensive than other methods (unless you have access and expertise in micro-fabrication). Also, it takes some experience to make a good seal between a PDMS mold and ECM molecule-coated coverslip. Neurons that are plated not close enough to the microgrooves will not grow their axons into the distal compartment and therefore will be wasted. Plating the dissociated neurons close to the microgrooves also requires experience. Most commercially available PDMS molds may not be suitable for tissue explant culture, as cells have to be plated in a closed chamber that is covered by $\sim 100\ \mu\text{m}$ -high roof.

Laser-capture microdissection: In laser-capture microdissection (LCM), one can dissect the selected regions from a tissue section or cell culture while directly observing the sample under the microscope (23) (Fig. 2D). To collect axons, individual axons are microdissected from neuron culture grown on an LCM-compatible slide. Neuron culture may be pre-stained with a fluorescent dye to aid visualization. This method provides the most precise way to collect axonal materials and enables subcellular compartment-specific microdissection of the axon (for example, axon shafts versus axon terminals) (15), which is not possible in any other compartmentalized cell culture systems. However, microdissection of individual axons means that one has to collect thousands of axons to obtain enough proteins or mRNAs for analysis. And it is sometimes necessary to fix the sample, because axons in culture are highly dynamic. LCM microscopes are generally expensive.

Selective labeling and identification of axonally synthesized proteins

In order to detect proteins that are locally synthesized in response to extrinsic cues, replicates of somaless axon culture are prepared by one of the methods described in the previous section, leaving one culture untreated and treating others with specific cues. Because severed axons can survive only for several hours in culture, any increase in protein abundance during cue stimulation may only be small and difficult to detect. It is particularly so, when an extrinsic cue promotes the synthesis of a protein that pre-existed in high abundance. Therefore, it is advantageous to analyze newly synthesized proteins, or the *de*

Fig. 3. Methods to identify axonally synthesized proteins.

novo proteome, rather than the steady-state proteome. Two standard biochemical approaches are available for selective labeling of *de novo* proteome: metabolic labeling of protein synthesis and puromycin tagging of nascent polypeptides (Fig. 3 and Table 2).

Metabolic labeling of protein synthesis: Metabolic labeling utilizes tagged amino acids, which live axons can use as building blocks for protein synthesis. Proteins synthesized after the addition of tagged amino acids are thus selectively labeled, which can be visualized, purified, and/or directly identified by MS (Fig. 3 and Table 2). Bioorthogonal noncanonical amino acid tagging (BONCAT) (24) and stable isotope labeling by amino acids in cell culture (SILAC) (25) are well-established techniques.

Tagged amino acids can be applied to severed axons (which can be cultured for several hours) or to intact axons cultured in a microfluidic device (or a well-prepared Campenot chamber). The latter method has advantages of avoiding the need to cut axons and being able to label axons for longer periods of time (over days). But, there is a risk that tagged amino acids may leak into the proximal compartment, which may label somal proteins that may be transported to axons. This will lead to false identification of axonally synthesized proteins and ruin the entire experiment. The former method can be done in diverse compartmentalized chambers, such as the Boyden chamber that gives a higher yield of axons, but severing axons may initiate unnecessary cellular responses.

BONCAT utilizes amino acid analogs, such as the Met analog azidohomoalanine (AHA), which contains an azide group. Axons are treated with AHA, which is used for protein synthesis, resulting in labeling of *de novo* axonal proteome with the bioorthogonal azide group. These proteins are then covalently linked to an alkyne containing tag, such as fluorescent dyes or biotin, by Click chemistry (24). *De novo* axonal proteomes tagged to fluorescent dyes can be quantitatively analyzed by 2-dimensional differential gel electrophoresis (2D-DIGE) (17), and those tagged to biotin can be directly isolated by streptavidin affinity purification.

Once inside the axon, AHA is first charged to tRNA_{met} by the enzyme Met-tRNA synthetase before being used for protein

Table 2. Comparison of *de novo* proteomic techniques

Technique	Probes	Purification required?	Sensitivity	Labeling duration	Can label from axon lysate? (4)	Can label in vivo axons? (5)	Detect post-translation modification?	Detect regulatory cis-element?
Proteomic approach								
Metabolic labeling								
BONCAT	Detectable amino acid w/ reactive group (1)	Yes (but see ref.26)	Low	minutes	No	No	Yes	No
SILAC	w/ stable isotope (2)	No (3)	Moderate	minutes	No	No	Yes	No
Nascent peptide labeling								
Puromycin tagging	Biotinylated puromycin	Yes (but see ref.26)	Low	0 min	Yes	No	Some (6)	No
Translatomic approach								
Axon-TRAP	Epitope-tagged ribosome	Yes	High	0 min	Yes	Yes	No	Yes

(1) Bioorthogonal noncanonical amino acids, which carry a taggable functional group. (2) Amino acids containing stable isotopes, such as 3H and 13C. (3) SILAC allows direct identification of labeled proteins without additional purification steps. (4) Metabolic labeling approaches require live axons to which probes are treated. (5) TRAP can be used to isolate translating mRNAs from in vivo axons. (6) Puromycin causes premature chain termination and tags elongation-stopped polypeptides. Therefore, post-translational modification that occurs after complete translation may not be detected.

synthesis. AHA charged to tRNA_{met} then incorporates itself into Met residues of nascent peptides during mRNA translation. The formation of AHA-tRNA_{met} takes minutes, which means that there is always a lag between AHA treatment and actual proteome labeling. Axons should be cultured in Met-free culture medium to increase labeling efficiency, because AHA competes with Met for tRNA_{met} and Met-tRNA synthetase.

SILAC utilizes amino acids containing stable isotopes (such as ³H, ¹³C or ¹⁵N), which can be used for protein synthesis. Similarly to BONCAT, SILAC requires time for the probe amino acids to be charged to appropriate tRNAs and specific amino acid-free medium (for example, Lys-free medium to use ¹³C-Lys) to increase labeling efficiency. Incorporation of the “heavy” amino acids (e.g. ¹³C-Lys) causes a predictable mass shift from the normal “light” amino acid (e.g. ¹²C-Lys). Heavy amino acid labeling causes no other chemical or functional changes to the labeled proteins, and therefore SILAC is noninvasive. By contrast, BONCAT may affect function of the protein it labels, because a noncanonical amino acid replaces an endogenous amino acid (e.g. AHA replaces Met). Another advantage of SILAC is that the labeled proteome can be directly identified by MS without any purification. Pre-existing proteome, which is “labeled” by the light isotope, can be distinguished by MS, and normalizing *de novo* proteome to steady state proteome enables quantitative analysis. In contrast, the BONCAT method normally requires purification of labeled proteome before their identification, which reduces the yield of protein recovery and produces biased enrichments of proteins. The recently developed technique for direct identification of biotinylated proteins by MS (26) may increase efficiency and accuracy of BONCAT-based analyses.

Puromycin labeling of nascent polypeptides: Puromycin tagging approach utilizes puromycin derivatives, which cause premature translation termination by incorporating themselves into the C terminus of nascent polypeptides. Therefore, this method differs from metabolic labeling approaches in the sense that it takes a snapshot of axonal protein synthesis at the time of puromycin treatment. Puromycin treated to axons lysate, as well as live axons, causes translation termination, and thus this approach does not require live axons. Instead, axon lysate can be obtained from intact neuron culture in the presence of emetine (which is an inhibitor of translation elongation, but unlike cycloheximide does not inhibit puromycin incorporation) (27). Biotinylated puromycin is added to axon lysate, labeling each translation-stalled, nascent polypeptide with a single puromycin tag at its carboxy terminus. These peptides are affinity-purified using streptavidin and identified by MS, but this may be combined with a recent technique to directly identify biotinylated proteins (26).

Selective isolation of axonally translating mRNAs

Most proteomic approaches utilize MS for protein identification. MS-based identification is less sensitive than DNA-based detection technologies, such as deep sequencing, main-

ly because proteins cannot be amplified or fully sequenced. Therefore, although analyzing proteins is the most accurate way to study axonal protein synthesis, the use of nucleic acid-based technologies has its own merits. Nucleic acid-based approaches utilize the same principle of puromycin tagging. Information on *de novo* proteome is obtained from translation-stalled, ribosome-mRNA complexes. Instead of analyzing nascent polypeptides, however, this approach utilizes highly sensitive DNA-based technologies to get the sequence information of translating mRNAs. Ribosome-mRNA complexes can be purified either by the traditional polysome fractionation technique or ribosome immunoprecipitation. The particular strength of the latter approach, which is known as translating ribosome affinity purification (TRAP), is that a tagged ribosomal protein can be genetically encoded, enabling cell type-specific ribosome tagging (28).

Axon-TRAP: Like the puromycin tagging approach, TRAP takes a snapshot of axonal protein synthesis from axon lysate isolated from intact neurons. One advantage of this technique over biochemical approaches is that mRNAs can be identified by the RNA sequencing technology. This is not only more sensitive in identifying axonally synthesized proteins than MS, but it also has potential to identify regulatory *cis*-element in untranslated regions (UTRs). The second advantage, which may have a stronger impact, is that it can be used to study axonal translation *in vivo*. For example, the tagged ribosome can be selectively expressed in particular neurons, whose cell bodies are localized to one part of the brain (*in vivo* “proximal” compartment) and axons are localized to another part of the brain (*in vivo* “distal” compartment in culture). Although *in vivo* “distal” compartment may contain other cells in addition to the axons of interest, these cells do not express the tagged ribosome. Therefore, axonal ribosomes can be selectively retrieved from the lysate of the *in vivo* “distal” compartment, by affinity-purification. In this way, the mRNAs translating in axons of particular neurons can be isolated from an intact brain. The plausibility of this technique, axon-TRAP, has been confirmed recently, although it was used to measure translation of a few candidate mRNAs (17). It will be possible to perform RNA sequencing analysis of axon-TRAPed mRNAs in the future, which will reveal genome-wide information on the entire set of axonally translating mRNAs (translatome). One drawback of this technique is that ribosome immunoprecipitation does not distinguish ribosome-bound, translationally inactive mRNAs (which are often in RNA granules) from actively translating mRNAs. Combining axon-TRAP with ribosome run-off (29) or ribosome profiling (30) will enable us to perform a more accurate survey of axonal protein synthesis *in vivo*.

CONFIRMATION OF AXONAL PROTEIN SYNTHESIS

Regardless of whether one has found candidate mRNAs from unbiased screens or reasoning, their axonal synthesis must be experimentally validated. Because it is difficult to obtain

enough axonal proteins for standard biochemical assays such as Western blotting, researchers often take a reasonable alternative, imaging based approaches, to measure local synthesis of a specific protein.

Quantitative immunofluorescence (QIF)

In QIF, the relative amount of a protein is measured by quantitative immunolabeling of fixed neurons, followed by quantitative fluorescent microscopy. Net immunofluorescence in the axon (i.e. fluorescent intensity in axon subtracted by background fluorescent intensity) represents the relative amount of the protein, which the antibody recognizes. To detect axonal protein synthesis, somaless axons are treated with an extrinsic cue or vehicle, and the abundance of a specific protein in axons is compared between groups. An increase in protein abundance, which can be blocked by translation inhibitors, indicates the axonal synthesis of that protein (8).

Live imaging of translational reporter fluorescence

It is well accepted that most *cis*-elements regulating translation of an mRNA reside in UTRs, particularly in the 3'-UTR. By expressing a chimeric mRNA consisting of the protein-coding sequence of a fluorescent protein and the UTRs of the gene of interest, translation of a particular mRNA can be visualized in real time. Membrane-targeted, destabilized fluorescent proteins are common reporter proteins, because they are not diffusible (therefore any fluorescence results from the local source) and are short-lived (therefore any fluorescence results from *de novo* protein synthesis) (31). Use of photoconvertible fluorescent *de novo* (such as Kaede and Dendra) (32) or fluorescent recovery after photobleaching (FRAP) (31) are some of the alternatives to measure translation by imaging.

FUTURE DIRECTIONS

Although axons contain hundreds or thousands of different mRNAs, inhibiting cue-induced translation of one or a few mRNAs almost completely blocks the axon's ability to respond to that cue (references in (33)), suggesting that cue-induced translation of selective mRNAs mediates specific biological processes. In this sense, an important question is how the different extrinsic cues activate translation of specific mRNAs. The first step in answering this question will be systematically analyzing extrinsic cue/target mRNA relationships. Axon-TRAP from axons treated with different cues would provide valuable information, which will lead to discoveries of new *cis*- and *trans*-acting elements that regulate message-specific translation in the axon.

Most of the unbiased screens for axonally synthesized proteins have been performed in cultured axons, mainly because it was impossible to selectively label or purify proteins from axons *in vivo*, which are normally intertwined with other cells. In this sense, the second important question is which proteins are synthesized in axons *in vivo*. Currently, axon-TRAP is the

only technology that allows us to identify axonally synthesized proteins *in vivo*. Although translatoome gives accurate information on *de novo* proteome, it does not tell us what happens after new proteins are made. For example, it has been suggested that axonally synthesized β -actin proteins differ from their pre-existing counterparts only by post-translational modification (34, 35), and this difference is key to newly made β -actin's ability to steer growing axons. Therefore, it will be important to develop new technologies that will enable direct and selective labeling of axonal proteome *in vivo*. A promising progress in this direction was recently made in *Drosophila* (36), and it will be exciting to see this technique applied to vertebrates.

Answering the first two questions will increase the number of mRNAs whose axonal translation may play key roles *in vivo*. For the clear understanding of their function, technologies need to be developed, which can selectively interfere with mRNA translation in axons, while leaving translation in the cell body intact. One promising strategy to decrease global translation activity selectively in the axon is chemically-inducible, genetically-encoded translational inhibitors (37, 38). Analogous to axon-TRAP, dormant inhibitor proteins could be expressed in specific neurons, whose cell bodies are localized to one part of the brain (*in vivo* "proximal" compartment). These inhibitor proteins could then be selectively activated in the axons of these neurons, by treating chemicals that induce inhibitor activity specifically to the other part of the brain, where their axon terminals are localized (*in vivo* "distal" compartment). To inhibit axonal translation of specific mRNAs, photoactivatable, caged antisense oligonucleotide (39) could be used, which can be delivered to specific neurons and uncaged selectively in the axon. An alternative approach is gene targeting of mRNA localization elements. For example, one can knock out the axon-specific splice variant, which contains a specific 3'-UTR, while leaving the entire protein coding region intact (40). This approach, however, requires the detailed knowledge on localization *cis*-elements of the target mRNA.

Recent development in DNA and protein detection technologies and new cell culture platform has made significant contribution to the field of axonal protein synthesis. Rapidly adopting and improving new technologies in diverse disciplines will be required to answer these remaining questions and pinpoint the biological significance of axonal protein synthesis *in vivo*.

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