Strategies for Exploring Local Dendritic Protein Synthesis in Synaptic Plasticity and Memory

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Introduction

Protein biosynthesis plays an essential role in controlling virtually every aspect of eukaryotic cell function. Work over the last several decades in highly tractable model systems, such as yeast, has revealed an intricately complex mechanism for mRNA translation that is subject to powerful regulation by diverse signaling pathways at almost every stage. In more complex biological networks, the functional impact of protein synthesis has also been shown to be under powerful spatial control, particularly in highly polarized cells such as neurons. In these networks, axons and dendrites extend over great distances from the neuronal cell body, suggesting that local translation in these distinct compartments may be critical to maintaining the unique functional specialization of these areas. Indeed, local protein synthesis in axonal growth cones and dendrites is now known to play a diverse repertoire of functional roles, both in the initial wiring of synaptic connectivity during development (Campbell and Holt, 2001; Brittis et al., 2002) and in the maintenance (Sutton et al., 2006) and plasticity (Kang and Schuman, 1996; Huber et al., 2000) of those connections, once formed.

Moreover, evidence continues to link loss of this local translational control with a growing list of human disorders characterized by cognitive dysfunction, including fragile X syndrome and autism (Kelleher and Bear, 2008; Wang et al., 2010). The fact that altered local translational control in neurons is associated with profound cognitive deficits draws an interesting parallel with the well-known role of protein synthesis in the formation of long-term memories (Flexner et al., 1963; Agranoff et al., 1965). However, it has not yet been possible to establish a definitive role for local protein synthesis at synapses in memory processing.

A major hurdle stands in the way of defining the specific roles that local protein synthesis plays in synaptic function and memory: the inherent challenge of specifically manipulating translation efficiency in distinct cellular compartments. This chapter summarizes the main approaches that have been used to tackle this problem. The main purpose of this work is to focus on the techniques themselves and what principles these techniques have served to demonstrate as well as their existing limitations, rather than to summarize an overall picture of the functional capabilities of local translation. To gain a deeper appreciation of the biological roles of compartmentalized protein synthesis in neurons, the reader is directed to several recent reviews (Sutton and Schuman, 2006; Costa-Mattioli et al., 2009; Wang et al., 2010).

Approaches to Visualizing Local Protein Synthesis

Transmission electron microscopy

Visualization methods provided the initial evidence (and for many years, the only evidence) that dendrites are capable of autonomous translational control. Using transmission electron microscopy (TEM), Bodian (1965) observed apparent ribosomes in proximal dendrites of monkey spinal cord neurons, where they were found close to sites of synaptic contact. His observations prompted him to speculate that local translation beneath these synapses might function to establish and modify synaptic connectivity. Several years later, Steward and Levy (1982) demonstrated that polyribosomes could be detected in the distal dendrites of dentate granule cells of the hippocampus, where they tend to cluster beneath sites of excitatory synaptic contact (beneath dendritic spines). Their observation of dendritic polysomes, suggesting active dendritic translation so remote from the neuron cell body, provided much stronger evidence for a role of local translation in regulating synaptic function.

TEM, owing to its unique ability to resolve the fine intracellular structure of neurons, has remained an important tool for understanding local protein synthesis in dendrites and its relationship to synaptic plasticity and memory. For example, Harris and colleagues have used TEM-guided detection of polyribosomes, following synaptic plasticity induction in hippocampal slices, to document a translocation of polyribosomes from dendritic shafts into spines of CA1 pyramidal neurons during long-term potentiation (LTP) (Ostroff et al., 2002). Although this approach is labor-intensive, and before-and-after comparisons in the same preparation are not possible, it does offer the opportunity to delve into questions regarding local translational regulation during memory processing. In a recent study, for example, Ostroff et al. (2010) demonstrated that fear conditioning increases both the number of dendritic polyribosomes and their association with the spine apparatus in lateral amygdala neurons. The observed increase in polyribosomes likely reflects an overall increase in dendritic translation after fear learning, although other interpretations cannot be fully ruled out.

Fluorescent protein–based translation reporters

The major limitation of TEM is that changes in dendritic translation relative to baseline cannot be studied in the same sample. This deficiency prompted the development of methods to dynamically visualize

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local synthesis of proteins in neuronal processes. The first such tools developed used a diffusion-limited fluorescent translation reporter in which a region encoding a destabilized, myristoylated green fluorescent protein (GFP) is flanked by the 5′ and 3′ untranslated regions (UTRs) from αCaMKII mRNA (Aakalu et al., 2001), an mRNA known to be dendritically localized. The half-life of this destabilized GFP is >90 min, and the myristoylation tag has been shown to severely inhibit the free diffusion of the reporter once made (Aakalu et al., 2001).

This translation reporter has been used to examine both activity-dependent (Sutton et al., 2004) and neuromodulatory control (Aakalu et al., 2001; Smith et al., 2004) of dendritic protein synthesis in hippocampal neurons. In addition, transgenic expression of a similar reporter (yellow fluorescent protein [YFP] fused to the 3′ UTR of CaMKII) allowed Ashraf et al. (2006) to examine changes in dendritic reporter repression in defined glomeruli of the *Drosophila* antennal lobe after an associative olfactory conditioning task. Importantly, this particular translation reporter served as proof-of-principle for the design strategy itself, which encouraged the development of new reporters with improved characteristics.

One of the major improvements in this class of translation reporter has come from replacing a generic fluorescent protein with either photoconvertible fluorescent proteins (e.g., Kaede or Dendra) or epitope tags that bind spectrally distinct fluorescent dyes with high affinity (e.g., FlAsH/ReAsH). These modifications make it possible to distinguish newly synthesized reporter from preexisting fluorescent signal. Also, they allow for a more precise "dating" of new reporter signal without the need for photobleaching existing signal. These newer reporter systems have been used to examine local synthesis of sensorin at *Aplysia* synapses (Wang et al., 2009) and local dendritic synthesis of AMPA receptor subunits GluA1 and GluA2 (Ju et al., 2004) as well as Kv1.1 voltage-gated potassium channels (Raab-Graham et al., 2006).

TimeSTAMP

Recently, Tsien and colleagues introduced a novel strategy for monitoring new synthesis of candidate proteins (Lin et al., 2008). This approach, termed time-specific tagging for the age measurement of proteins (TimeSTAMP), utilizes fusion of the hepatitis C virus (HCV) protease upstream of its cognate recognition sites surrounding an epitope/fluorescent protein tag introduced into a protein of interest. The resulting default *cis* cleavage by HCV protease results in constitutive removal of the visualization tag upon synthesis. Hence, under basal conditions, newly synthesized proteins are not fluorescent. By coupling this strategy with a small-molecule HCV protease inhibitor (to prevent proteolysis of the fluorescent tag), new synthesis of the resulting protein can be visualized with little or no background from preexisting protein. Applying this twofold strategy, Lin et al. (2008) were able to demonstrate that newly synthesized recombinant PSD95 and CaMKII could be visualized in cultured neurons and intact fly brains, respectively. Moreover, the shift in molecular weight that protease cleavage confers provides a useful signature for distinguishing newly synthesized and preexisting proteins in Western blots, illustrating that the usefulness of TimeSTAMP extends beyond visualization.

F2P fluorescence labeling

The reporter strategies outlined above all require a candidate-based approach. Thus, these reporters are overexpressed in neurons and are not likely to reveal endogenous translational control principles influenced by mRNA availability, which could be circumvented by overexpression. A related limitation is that these candidate-based reporters do not yield a complete picture of local translation beyond the specific candidate in question.

Novel reporter systems developed during the last few years have used a modified design that fluorescently labels endogenous proteins as they are synthesized. The first of these, a fluorescein-conjugated derivative of puromycin (F2P), exploits the fact that puromycin is a tRNA-mimetic that becomes incorporated into elongating polypeptides during protein synthesis. Smith et al. (2004) utilized bath and focal application of F2P to dendrites in order to demonstrate that dopamine D_1/D_5 receptor activation potently drives overall protein synthesis in dendrites. Importantly, F2P fluorescence was substantially reduced (though not eliminated entirely) by co-applying protein synthesis inhibitors. This effect indicates that the majority of detected signal reflected F2P integration into endogenous proteins. However, because F2P is intrinsically fluorescent, conditions need to be optimized in order to minimize the influence of unincorporated F2P. Moreover, although it is a useful reporter strategy, the impact of F2P incorporation on endogenous protein function is difficult to gauge, making it less desirable for functional studies.

FUNCAT

A slightly different approach, recently developed by Dietrich and colleagues (2010), uses an inventive

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strategy of allowing neurons to take up noncanonical amino acids with novel chemical properties, which are then exploited to label newly synthesized proteins that have incorporated them. This strategy, termed fluorescence noncanonical amino acid tagging (FUNCAT), has been successfully used for a variety of tasks: to visualize dendritic protein synthesis, to monitor the fate of proteins synthesized in distinct cellular compartments, and to visualize the mobility of newly synthesized cell surface proteins. A major advantage of FUNCAT is its ability to generate views of the complete translational response a particular set of conditions induces; a disadvantage is that it yields little information about the new synthesis of specific proteins. Hence, newer technologies such as TimeSTAMP and FUNCAT are complementary techniques and should be chosen based on the specific experimental question in mind.

Limitations of current visualization methods

One important limitation of visualization methods, as utilized, is that they provide information that is correlative in nature. Thus, although these methods fill a critical gap in our understanding of activity-dependent and neuromodulatory-dependent control of local translation, they do not reveal whether such local synthetic events actually contribute to changes in synaptic function. Below, I describe alternative approaches that have been applied specifically with this limitation in mind.

In vitro Approaches to Link Local Protein Synthesis with Synaptic Plasticity

Physical isolation methods

Physically separating the neuronal processes from their somata by way of surgical cuts is one of the more definitive methods for ensuring that the source of proteins required for synaptic plasticity derives from a local dendritic pool. This method has been used in cultured neurons (Aakalu et al., 2001; Ju et al., 2004) but is particularly useful in hippocampal slices. In the hippocampus, pyramidal neurons cluster in a defined layer and extend apical dendrites in a characteristic orientation through the laminar regions of synaptic neuropil. Surgically isolating dendritic lamina from parent cell bodies is thus easily accomplished using a dissection microscope, and synaptic responses from these isolated dendrites can be measured by taking field potential recordings with an extracellular electrode.

Kang and Schuman (1996) first applied this approach to the question of protein synthesis–dependent forms of synaptic plasticity. They found that isolated CA1 dendrites could still support translation-dependent synaptic potentiation induced by brain-derived neurotrophic factor (BDNF). Using a similar surgical technique, Huber and colleagues (2000) later demonstrated that long-term depression (LTD) induced by activation of group I metabotropic glutamate receptors (mGluR-LTD) also requires local dendritic protein synthesis. Other protein synthesis–dependent forms of synaptic plasticity have been similarly revealed using isolated dendritic laminae (Sutton and Schuman, 2006), and this preparation has been used in RNA profiling studies to estimate the population of mRNAs localized to dendrites (Zhong et al., 2006).

One important caveat with surgical isolation in hippocampal slices is that the dendritic lamina still contain interspersed interneurons and astrocytes. These particles could participate in the induction of synaptic plasticity (Stellwagen and Malenka, 2006), and their gene products are likely to be detected in RNA profiling studies. Hence, on its own, surgical isolation leaves open the possibility that protein synthesis in these cells, and not the dendrites of pyramidal neurons, is contributing to the observed outcome. Huber and colleagues (2000) nicely ruled out this possibility experimentally by demonstrating that translation in the pyramidal neurons is required during mGluR-LTD. Still, the "contaminating" influence of nonpyramidal cells in isolated dendritic lamina remains a limitation of the surgical isolation approach overall.

Synapse-enriched biochemical fractions

The use of biochemical fractions enriched in synaptic structures has remained a popular method for examining local translational regulation at synapses. Several variants of these fractions are routinely used, including synaptosomes, synaptoneurosomes, and synaptodendrosomes. Of these, synaptoneurosomes have been the most commonly employed. These structures contain presynaptic terminals and resealed postsynaptic compartments that remain physically associated and thus have a characteristic "snowman" appearance in TEM micrographs (Muddashetty et al., 2007). These fractions have been used to estimate synaptic mRNA content (Chicurel et al., 1993) and broad translational activation at synapses (Weiler and Greenough, 1993; Takei et al., 2004) as well as to monitor the *de novo* synthesis of specific proteins such as the fragile X mental retardation protein (FMRP) (Weiler et al., 1997); GluA1 (Muddashetty et al., 2007; Aoto et al., 2008); Arc (Yin et al., 2002; Waung et al., 2008); and many others.

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A major advantage of biochemical fraction preparation is that it allows one to apply conventional biochemical techniques (e.g., Western blotting, metabolic labeling) and more recently developed proteomic approaches to the investigation of local translational control at synapses. Indeed, a promising future application of these preparations will be to use them in conjunction with newly developed methods of purifying newly synthesized protein pools (e.g., BONCAT [bio-orthogonal noncanonical amino acid tagging]) (Dietrich et al., 2006) in order to estimate the complement of proteins that may be locally synthesized at synapses. However, this approach also has several limitations, including concerns over the "purity" of synaptic components in these fractions and the fact that, once isolated, these fractions do not capture potentially important trafficking events to and from synaptic compartments that might powerfully regulate protein synthesis at synapses (Ostroff et al., 2002).

Microperfusion techniques

In recent years, spatially restricted microperfusion approaches have been powerful tools for linking local protein synthesis in dendrites with the induction of specific synaptic adaptations associated with altered neurotransmission. The first successful application of this general approach to studying compartmentalized protein synthesis during synaptic plasticity used the sensory neuron to motor neuron (SN-MN) synapse of *Aplysia* as a model system.

Martin and colleagues (1997) took advantage of a cell culture system in which a single SN makes separate synaptic contacts with two MNs, thus allowing for treatments to be selectively restricted to one SN-MN contact. Using local pressure ejection of serotonin (5-HT) from a micropipette, coupled with fast-bulk perfusion for rapid clearance, this group showed that local application of 5-HT to the site of synaptic contact induced both short-term facilitation (STF) and long-term facilitation (LTF) of synapses at those inputs without altering SN-MN function at the other SN-MN contact. Importantly, including protein synthesis inhibitors in the puffer pipette completely prevented LTF induced by repeated 5-HT pulses but without altering STF induced by a single 5-HT pulse. These results clearly demonstrated a crucial role for local protein synthesis in the SN terminal in inducing long-lasting synaptic facilitation. This unique SN-MN culture system has been a powerful tool for both identifying locally translated proteins that contribute to LTF (Wang et al., 2009) and defining how these products confer unique functional abilities on the synapses that synthesize them (Casadio et al., 1999; Si et al., 2003; Miniaci et al., 2008).

Local microperfusion has also been adapted to study the functional impact local translation has in mammalian neurons. Typically, this approach is carried out in monolayers of neurons in primary culture, which affords the ability of targeting treatments to specific regions of a neuron of interest through a dual micropipette perfusion system. In hippocampal neurons, dendrites are highly sensitive to mechanical effects (M. A. Sutton, unpublished observations), making pressure ejection through a "puffer" pipette a less desirable strategy. Instead, a delivery micropipette is fastened to allow the perfusate to escape under conditions of very-low-positive pressure, and a nearby suction pipette is positioned to draw a stream of perfusate across areas of interest and to remove the perfusate from the bath. This dual micropipette system is also typically used with a bulk flow perfusion oriented away from the neuron of interest. A fluorescent dye is included in the delivery pipette to monitor the size and stability of the perfusion region over the course of an experiment.

Although it is challenging to maintain a stable local perfusion region over the course of 1-2 h, several groups have successfully applied this approach to examine local regulation of protein synthesis reporters (Smith et al., 2004; Sutton et al., 2007; Dietrich et al., 2010); study the expression of target proteins thought to be locally synthesized (Sutton et al., 2006; Waung et al., 2008); and for other applications (Ibata et al., 2008). When used to locally deliver protein synthesis inhibitors, the primary advantage of local microperfusion is that the contribution of local translation can be assessed in intact neurons in which a somatic source of proteins is still available. Hence, by targeting protein synthesis inhibitors to either the dendrites or cell body (Dietrich et al., 2010), unique roles for translation in each compartment can be assessed under the same set of experimental conditions.

A particularly promising extension of the local microperfusion approach has come with the implementation of microfluidic chambers that can be used to maintain different neuronal compartments in distinct microenvironments. In a recent paper, Taylor and colleagues (2010) designed a novel microfluidic chamber with a local perfusion channel running perpendicular to the microgrooves in which dendrites and axons are localized but cell bodies are not. In order to validate their design, Taylor et al. were able to use these chambers to locally apply glutamate to synaptic regions in different patterns and to monitor cAMP response element-binding protein (CREB) phosphorylation in the cell soma.

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Microfluidic chambers remove many of the technical challenges associated with local microperfusion; thus, in principle, they allow for more extended, spatially restricted treatment applications as well as greater stability of the local perfusion area. These chambers are likely to be a valuable resource when studying local translation at synapses.

Optical approaches

Optical approaches offer perhaps the most exciting opportunities for designing novel methods of manipulating protein synthesis on fine spatial scales. One strategy typified by such approaches is to couple the use of caged protein synthesis inhibitors with focal ultraviolet (UV) light exposure to inhibit protein synthesis in a spatially tunable fashion.

Goard et al. (2005) developed the first of such compounds: dimethoxynitrobenzyl (DMNB)–Anisomycin and bromohydroxycoumarin (Bhc)–Anisomycin. They demonstrated (with Bhc-Anisomycin) that UV light exposure could impart spatially restricted inhibition of protein synthesis. More recently, Woolley and colleagues (Sadovski et al., 2010) greatly expanded the repertoire of photoreleasable translation inhibitors by introducing a diethylaminocoumarin (DEAC) caged version of anisomycin (DEAC-Anisomycin) as well as caged 4E-BP peptides and caged rapamycin. These new caged compounds will allow not only for spatially targeted inhibition of general protein synthesis (with caged anisomycin) but also for more selective inhibition of cap-dependent translation (with caged 4E-BP) and mammalian target of rapamycin complex 1 (mTORC1)–driven protein synthesis (caged rapamycin).

Because optical methods permit precise spatial and temporal control, these compounds have obvious potential for inhibiting protein synthesis with high spatial resolution (potentially, at single synapses). However, the selective disruption of dendritic protein synthesis has yet to be shown with such caged inhibitors, although the effectiveness of microperfusing such inhibitors suggests that these are almost certainly effective for this purpose. Another limitation of optical approaches is that repeated UV light exposure has potential secondary consequences for neuronal physiology. This problem can be circumvented using two-photon excitation to photorelease, as shown by Goard et al. (2005). In addition, the DEAC-Anisomycin caged compound developed by Sadovski et al. (2010) has longer wavelength absorption and can be effectively uncaged with 405 nm light. This characteristic is predicted to have fewer secondary consequences and to make this tool useful to a wider community, given that 405 nm laser lines are now common on many commercial microscope systems.

In vivo Approaches to Link Local Protein Synthesis with Memory Processing

Given that many enduring forms of synaptic plasticity critically depend on local protein synthesis in the dendritic compartment, similar compartmentalized synthesis likely plays a fundamental role in memory processing. However, testing this general hypothesis has proven extremely difficult owing to the technical challenges of manipulating translation in a compartment-specific fashion *in vivo*. At this point, we still lack the appropriate tools to accomplish this goal, but there are indications that it is feasible in the long run.

One strategy to address this question was illustrated by Miller et al. (2002), who generated mutant mice in which the native 3′UTR of αCaMKII mRNA had been replaced with the 3′UTR of bovine growth hormone to prevent its dendritic localization. Whereas dendritic αCaMKII mRNA was completely abolished, the somatic αCaMKII mRNA pool was only slightly decreased relative to wild-type mice. These mice exhibited deficits in protein synthesis–dependent forms of LTP as well as memory in hippocampal-dependent learning tasks, suggesting a potential role for dendritic αCaMKII synthesis in LTP and learning.

Recently, a similar strategy was adopted for excluding BDNF mRNA from dendrites (An et al., 2008). BDNF mRNA transcripts contain one of two 3′UTRs: The expression of the short UTR is restricted to cell bodies, whereas the long UTR is trafficked to dendrites. An et al. utilized a mouse mutant in which the long 3′UTR is truncated; they found little BDNF mRNA in dendrites and that, despite normal overall levels of BDNF protein, dendritic BDNF levels were substantially diminished. Similar to the αCaMKII 3′UTR mutants (Miller et al., 2002), these animals showed deficits in protein synthesis–dependent forms of LTP, as well as altered spine morphology in CA1 pyramidal neurons.

Together, these studies suggest a potential role for dendritic protein synthesis in regulating synaptic plasticity and memory. However, because the modified mRNAs in these studies were present throughout development, it was not possible to rule out the notion that the constitutive loss of these proteins at synapses, rather than *de novo* synthesis, accounted for the deficits. Although the specific

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implications of these findings with respect to *de novo* dendritic synthesis of αCaMKII and BDNF remain uncertain, these studies are important because they make the initial inroads towards defining a role for local dendritic translation in long-term memory formation. Perhaps by combining this general strategy with conditional genetic, chemical genetic, or optogenetic tools, it may be possible to disentangle the roles of local *de novo* synthesis from basal depletion of dendritic protein expression.

Perspective

Our understanding of local translation in neurons has closely followed technical innovations that have provided new access for investigating questions about the activity-dependent control of dendritic protein synthesis and its impact on synaptic function in different contexts. We have learned a great deal in recent years about activity-dependent control of protein synthesis in dendrites and axons, as well as how such local translation contributes to synapse formation, maintenance, and plasticity. However, our ability to address some of the larger questions surrounding this field, such as what the compartmentalized roles of translation play in learning and memory, remain severely limited. A major challenge going forward is to develop innovative approaches, particularly *in vivo* strategies, to begin to address this fundamental question.

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