CHAPTER SIX

Local Protein Synthesis at Synapses

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1. INTRODUCTION

It has been 30 years since our discovery of the selective positioning of polyribosomes at spine synapses (Steward and Levy, 1982). In that article, we described what we now call "synapse-associated polyribosome complexes" (SPRCs) and proposed that they allowed local synthesis of key molecules of the synaptic junction, that local synthesis is regulated by synaptic activity, and that local synthesis of key proteins is important for synaptic plasticity. This discovery and accompanying ideas triggered a new line of thinking about how individual synapses could be modified in a protein synthesis-dependent fashion, specifically that synaptic activation could modulate the translation of mRNAs at individual synapses, and the newly synthesized proteins could then act locally to alter synaptic properties. Subsequent studies have confirmed these basic ideas, although the story has become much richer in detail. Findings from many labs have revealed that local protein synthesis plays an important role in synapse construction and plasticity. Although the core ideas about the importance of local protein synthesis in synaptic function are accepted, concepts continue to evolve.

This chapter presents a snapshot of current ideas about protein synthesis at synapses and mechanisms underlying dendritic transport and targeting of mRNA to synapses. Our studies on transport and targeting of mRNA were made possible because of the discovery of a unique immediate early gene (IEG) called activity-regulated cytoskeleton protein (Arc) (Lyford et al., 1995), also known as Arg3.1 (Link et al., 1995). Arc is unique among known IEGs because Arc mRNA is rapidly transported into dendrites and localizes selectively near active synapses (see below). In addition to revealing mechanisms of mRNA trafficking to synapses, studies of Arc have provided important insights into the role of local protein synthesis in synapse function, particularly in the forms of synaptic plasticity that underlie memory storage.

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2.1. SPRCS: ULTRASTRUCTURE OF THE MACHINERY THAT UNDERLIES PROTEIN SYNTHESIS AT SYNAPSES

2.1.1. Overview

Figure 1 illustrates an example of SPRCs at a spine synapse. Our description of SPRCs came at a time when the accepted view was that protein synthesis occurred only in the cell body of neurons. Thus, our proposal for local protein synthesis at synapses was at first controversial. This was despite the fact that ribosomes were known to be present in dendrites, especially proximal dendrites (Peters et al., 1976). The localization of ribosomes at synapses had also been noted (Bodian, 1965; Palacios-Pru et al., 1981). However, the selectivity of the localization of polyribosomes had not been documented—viz., that in mature neurons, the majority of dendritic polyribosomes were selectively localized beneath postsynaptic sites (see below). It was the quantitative documentation of this selective positioning that inspired our hypotheses about the role of local protein synthesis in synaptic function.

Our initial quantitative analysis of SPRCs was carried out on spine synapses on granule cells in the hippocampal dentate gyrus (Steward and Levy, 1982). Later studies documented SPRCs in the subsynaptic cytoplasm of different classes of synapses and in different types of neurons (Steward, 1983). In addition to being localized at spine synapses, polyribosomes are positioned beneath the postsynaptic membrane specialization

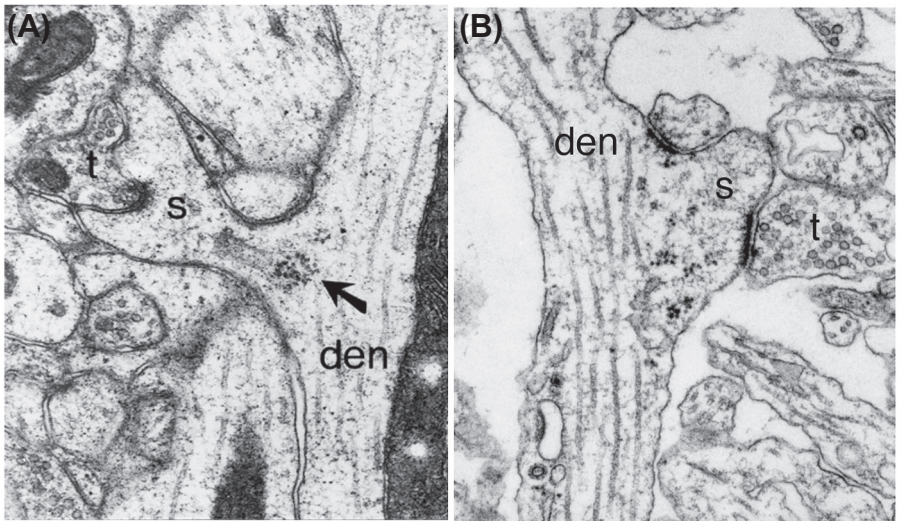

Figure 1 Synapse-associated polyribosome complexes (SPRCs) at spine synapses on the dendrites of CNS neurons. (A) Electron micrograph illustrating clusters of ribosomes (polyribosomes) selectively positioned at the base of a spine on a granule cell in the hippocampal dentate gyrus. (B) Electron micrograph illustrating polyribosomes beneath and within a developing spine. *From Steward and Falk (1986).*

of nonspine synapses including inhibitory synaptic connections on axon initial segments (Steward and Ribak, 1986). Thus, SPRCs are ubiquitous components of the subsynaptic cytoplasm.

Polyribosomes are not seen at every synapse in the mature brain, and even when present, are not seen in every section through the synapse. In fact, it is relatively rare to capture the profile of a spine perfectly in a single electron micrograph such as the one illustrated in Figure 1(A). This may be one reason that the selective localization of polyribosomes at synapses had not been noted prior to our study. Our initial study of serial section reconstructions of dendrites of dentate granule cells in adult rats revealed that about 25% of the spines on mid proximodistal dendrites have underlying polyribosomes (Steward and Levy, 1982). Incidence is higher on proximal dendrites. Other studies involving serial section analyses of pyramidal neurons in the cerebral cortex revealed that 82% of the reconstructed spines had ribosomes in the head, 42% had ribosomes in the neck, and 62% had ribosomes at the base (Spacek and Hartmann, 1983). In cerebellar Purkinje cells, 13% of the spines had ribosomes in the head and 22% had ribosomes at the base. The differences in estimates of incidence may be due in part to the criteria for counting. Spacek and Hartmann counted both singlet ribosomes and polyribosomes, whereas only polyribosomes with at least three singlet ribosomes were counted in Steward and Levy (1982). In any case, it is clear that at any given point in time, some mature synapses lack polyribosomes.

The fact that polyribosomes are present at some synapses but not others raises the question of whether SPRCs are more or less permanently stationed at particular synapses or whether they shuttle from synapse to synapse. If polyribosomes are permanently stationed, this would imply that local protein synthesis can occur at some synapses but not others. If polyribosomes can move from one synapse for another, then all synapses may have the same capacity for local protein synthesis. It will be of interest to use live-cell imaging techniques to test whether ribosomes can move from one synapse to another.

2.1.2. Selectivity of Polyribosome Localization beneath Synapses

At spine synapses, SPRCs are most often localized at the base of the spine (Figure 1). Selective localization was demonstrated through quantitative analyses of polyribosome distribution within dendrites of granule cells in the dentate gyrus (Steward and Levy, 1982). This study demonstrated that about 10% of the polyribosomes in dendrites were localized beneath or within "identified spine bases" (that is, profiles where neck of the spine could be seen to emerge from the dendrite); about 71% of the polyribosomes in dendrites were localized in "mounds", which serial section reconstruction revealed to be the base of spines that extend out of the plane of the single section. Thus, a reasonable estimate is that about 80% of the polyribosome clusters in dendrites are localized beneath or within spines.

SPRCs are also seen within the spine neck and head, and there is evidence that synaptic activation can cause ribosomes to move from the dendritic shaft into spines. For example, electron microscopic studies following tetanic stimulation of the Schaffer collateral system in hippocampal slices from 15-day-old rats revealed that an increased percentage of spines contained polyribosomes with a corresponding decrease in polyribosomes in dendritic shafts (Ostroff et al., 2002). The likely explanation is activity-dependent translocation of polyribosomes from the shaft into the spine proper. On the other hand, Desmond and Levy (1990) report a decrease in the number of polyribosomes in spines following induction of LTP in the perforant path. It is noteworthy that this occurs at the same time that spine heads undergo striking changes in shape, adopting a highly characteristic chalice-like morphology in which the spine head wraps around the presynaptic terminal (Desmond and Levy, 1983). The interesting observations regarding the disappearance of ribosomes with LTP have never been followed up. One speculation is that the ribosomes become closely associated with the postsynaptic density, which would make them invisible by electron microscopy.

SPRCs located within or near the portal between the spine neck and the shaft of the dendrite are ideally positioned to be influenced by electrical and/or chemical signals from the synapse as well as by events within the dendrite proper. As discussed further below, our recent studies demonstrate that one of the mRNAs that is delivered into dendrites (the mRNA for Arc) also localizes with a high degree of precision at the base of dendritic spines. This implies that the spine base is a specialized cytoplasmic microcompartment where particular mRNAs and protein synthetic machinery dock.

Although most dendritic polyribosomes are localized beneath synapses, a few clusters of ribosomes are localized within the core of the dendritic shaft. Polyribosomes in the dendritic core may be associated with mRNAs that encode proteins that are not destined for synaptic sites that play some other role in dendritic function. This speculation is of particular interest given the diversity of the mRNAs that have been identified in dendrites (see below). Alternatively, clusters of polyribosomes in the core of the dendrite may be in transit from the cell body.

2.1.3. Association between SPRCs and Other Organelles

SPRCs are often associated with membranous organelles. The association can often be seen in single sections (Figure 1), but is more evident when synapses are evaluated using serial sections or with stereoviewing of relatively thick sections (Steward and Reeves, 1988). For example, serial section reconstructions of mid proximodistal dendrites of dentate granule cells and hippocampal pyramidal cells have revealed that about 50% of the polyribosomes are near tubular cisterns. A common configuration is one in which the ribosomes seem to surround a blind end of a cistern. The relationship between the ribosomes and the cisternal membrane is similar to what is seen in rough endoplasmic reticulum (RER), except that the dendritic cisterns are tubular rather than flat like the

RER cell bodies. One obvious hypothesis suggested by these observations is that the SPRC/cisternal complex is a form of RER, which is a potential site for the synthesis of integral membrane proteins or soluble proteins destined for release.

Interestingly, the cisterns with which SPRCs are associated are sometimes connected with a spine apparatus (Steward and Reeves, 1988). Sometimes this connection is prominent; in other cases, the connection is very tenuous. This association invites the speculation that the spine apparatus is involved in some aspects of posttranslational processing of proteins that are synthesized by the SPRCs (more on this below).

2.1.4. SPRCs at Nonspine Synapses

Although there have been no detailed quantitative evaluations of polyribosome distribution in the dendrites of nonspiny neurons, it is clear that the same basic relationships exist as in spiny dendrites. Indeed, the earliest observation of ribosomes at synapses was at nonspine synapses on motoneurons in the spinal cord (Bodian, 1965). Also polyribosomes are present beneath both the asymmetric and the symmetric synapses that are present on dendrites of cortical neurons (Steward et al., 1996). Polyribosomes are also localized beneath GABAergic synapses on axon initial segments (Steward and Ribak, 1986).

2.1.5. Prominence of SPRCs at Developing Synapses

Polyribosomes are especially abundant at developing synapses (Steward and Falk, 1985, 1986, 1991; Figure 1(B)). This suggests that local protein synthesis is especially important as synapses are being constructed. It is not known whether different types of proteins are synthesized at developing vs mature synapses; this question is of considerable importance because many studies seeking to identify mRNAs in dendrites have used young neurons growing in culture (see below).

2.2. PROTEIN SYNTHESIS IN DENDRITES

The demonstration of ribosomes at synapses raised the question of whether there was in fact ongoing protein synthesis. Initially, this was explored using subcellular fractions containing pinched-off dendrites called either synaptodendrosomes or synaptoneurosomes depending on the method of isolation. Synaptodendrosomes are isolated by differential centrifugation in sucrose gradients (Rao and Steward, 1991), whereas synaptoneurosomes are prepared by filtration (Weiler et al., 1991; Weiler and Greenough, 1991). Both types of fractions are enriched in pinched-off nerve terminals that are connected to small pinched-off dendritic fragments and/or spines, which appear as snowman figures at the electron microscopic level. An important limitation is that the fractions can be contaminated by resealed fragments of neuronal and glial cell bodies (Rao and Steward, 1993). Nevertheless, when care is taken to ensure minimal contamination by other elements (Bagni et al., 2000), the preparations can provide information about the

mRNAs that are present in dendrites (Rao and Steward, 1993) and the proteins that are locally synthesized (Rao and Steward, 1991).

Studies involving synaptodendrosomes and synaptoneurosomes have also been useful for assessing how dendritic protein synthesis is influenced by depolarization (high potassium for example) and bath application of neurotransmitters and growth factors (Weiler et al., 1991; Weiler and Greenough, 1991, 1993). Measures include biochemical studies of metabolically labeled proteins (Leski and Steward, 1995, 1996; Villanueva and Steward, 2001a,b) and the degree of ribosome loading onto particular mRNAs (Zalfa et al., 2003).

Other studies demonstrated local protein synthesis by dendrites that had been mechanically separated from their cell bodies. One approach was to grow neurons on a sandwich made up of a millipore filter attached to a glass cover slip with Matrigel (Torre and Steward, 1992). Neurons growing on the filter extend their axons and dendrites into the Matrigel. The filter with cell bodies can then be pulled off, leaving the separated dendrites in the Matrigel. Dendrites isolated in this way exhibited local incorporation of radiolabeled amino acids into protein.

More recent studies used unique fluorescent reporters to demonstrate both the sites and dynamics of local protein synthesis in the dendrites of living neurons in culture (Aakalu et al., 2001). These experiments convincingly settle the question of whether dendrites are capable of local protein synthesis. Experiments involving mechanical separation of dendrites from cell bodies in hippocampal slices also provided strong evidence that dendritic protein synthesis is critical for changes in synaptic efficacy (Aakalu et al., 2001; Huber et al., 2000; Kang and Schuman, 1996) and for a review, see Steward and Schuman (2001).

2.3. POSTTRANSLATIONAL PROCESSING WITHIN DENDRITES

Evidence that components of the endoplasmic reticulum (ER) and Golgi complex are present in dendrites has come from studies that assessed the distribution of protein markers and enzyme activities characteristic of the RER and Golgi apparatus (GA). One approach has been to pulse-label neurons with the sugar precursors which are the substrates of various glycosyl transferases that are present in the RER and GA and assess sites of incorporation using autoradiography (Torre and Steward, 1996). This study revealed both mannosyl transferase as well as higher order (Golgi-like) glycosyltransferase activity in the dendrites of hippocampal neurons grown in culture. Immunocytochemistry further revealed that proteins that are considered markers of the endomembrane system are present in dendrites, including ribophorin I, which is a marker for the RER (Torre and Steward, 1996).

Subsequent studies used electron microscopic immunocytochemistry to assess how the different processing enzymes map onto membranous organelles at the spine. The membranous cisterns near spine synapses stain for Sec6Ialpha protein complex, which is part of the machinery for translocation of proteins through the RER during their synthesis (Pierce et al., 2000). The cisterns exhibiting labeling have the same appearance as the cisterns that represent the membranous component of SPRCs.

Immunostaining for the ribosomal protein S3 revealed labeling over the same membranous cisterns that were labeled for Sec6Ialpha. A follow-up study (Pierce et al., 2001) further mapped the distribution of key enzymes, showing that immunostaining for TGN38 and ERGIC53/58 was present in the dendritic shaft, membranous organelles at the base of spines stained for Rab6, spine apparatuses stained for mannosidase II and Giantin, and immunostaining for RAB1b was present in spine heads (Figure 2).

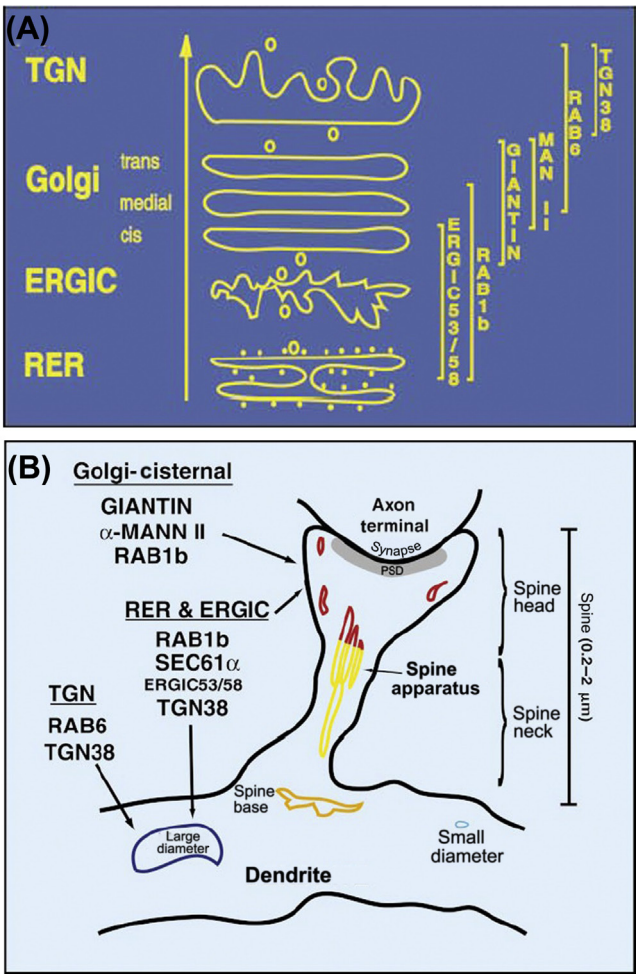

Figure 2 Schematic illustration of the distribution of elements of the endoplasmic reticulum (ER) and golgi apparatus at spine synapses. (A) Distribution of different proteins involved in posttranslational processing of proteins. RER, rough endoplasmic reticulum; ERGIC, endoplasmic reticulum to Golgi intermediate compartment; TGN, trans-Golgi network. (B) Schematic of the distribution of protein biosynthetic compartments in dendrites based on immunostaining. Early secretory pathway components (RER and ERGIC) are localized throughout spines and within dendrites. Middle compartments (Golgi cisternae) are primarily localized in spine heads. Late Golgi and trans-Golgi network (TGN) are predominantly found in dendrites. *From Pierce et al. (2001).*

These data indicate that all the machinery that is necessary to glycosylate newly synthesized protein is present at spines.

An interesting twist is that live-cell imaging studies reveal that Golgi vesicles in dendrites are mobile (Horton and Ehlers, 2003). Live-cell imaging using a green fluorescent protein (GFP)-tagged temperature-sensitive vesicular stomatitis virus glycoprotein (VSVG), and ER and Golgi apparatus markers, documented the presence of functional ER, post-ER carriers, Golgi Apparatus, and post-Golgi apparatus trafficking in dendrites. VSVG–GFP moves from ER elements in dendrites into tuberovesicular structures that move bidirectionally in dendrites, fusing with stationary structures that stained for Golgi apparatus markers (GM130). The accumulation of VSVG–GFP into tuberovesicular structures occurred at defined immobile foci that were positive for Sec13, a marker of ER exit sites in other cells. These data suggest the interesting possibility that elements of the Golgi can shuttle from spine to spine, sometimes moving tens of micrometers.

2.4. DENDRITIC mRNAs

The discovery of polyribosomes beneath synapses naturally raised the question of what mRNAs were present in dendrites. The development of cloning techniques and in situ hybridization in the late 1980s made it possible to address this question directly. Many early studies revealed that most mRNAs expressed by neurons were largely restricted to the region of the neuronal cell body, but a few mRNAs turned up that were present in dendrites at high levels.

The first two mRNAs that were found to be localized in dendrites by in situ hybridization were the mRNA encoding the high-molecular-weight microtubule-associated protein MAP2 (Garner et al., 1988) and the mRNA encoding the a-subunit of calcium/calmodulin-dependent protein kinase II (CaMKII) (Burgin et al., 1990). These mRNAs are present at high levels within the dendrites of forebrain neurons. Subsequently, a different set of mRNAs was found to be present at high levels in the dendrites of cerebellar Purkinje cells including the mRNA encoding the type I inositol trisphosphate receptor (InsP3 receptor) (Furuichi et al., 1993), and the mRNAs for proteins called L7 and PEP19 (Bian et al., 1996). The mRNA for the InsP3 receptor is present at the highest level in Purkinje cells, but is also present in dendrites of forebrain neurons. L7 and PEP19 are expressed at high levels in Purkinje cells, but not in most other neurons.

Later studies revealed other transcripts that are easily detectable by in situ hybridization in dendrites of neurons in vivo including the postsynaptic scaffold protein shank (Bockers et al., 2004; Kindler et al., 2004), dendrin (Herb et al., 1997), and elongation factor 1 alpha (Huang et al., 2005). When induced by activity, Arc mRNA is present at high levels in dendrites (more on this below), and BDNF mRNA appears in dendrites following seizures that lead to epilepsy (Tongiorgi et al., 2004). In addition to the

mRNAs that are present in dendrites at high levels, there is increasing evidence that a large number of different mRNAs are present in dendrites at lower levels. This story line began with studies involving isolation and amplification of mRNAs from dendrites of young neurons growing in culture (Miyashiro et al., 1994). Many mRNAs were identified, but there were caveats because mRNAs were harvested from very young neurons growing in culture and there were no follow-up in situ hybridization analyses to verify dendritic localization in neurons in the CNS.

The question of how many mRNAs are in dendrites has been readdressed in recent studies that included many elegant controls (Cajigas et al., 2012). In this study, mRNA was isolated from dendritic laminae of hippocampal slices from adult rats and analyzed by deep sequencing. Because dendritic laminae of the hippocampus contain the cell bodies of glial cells and many types of interneurons, mRNAs expressed by glia and inhibitory interneurons were subtracted out. Even after the subtraction, 2550 candidate dendritic mRNAs were identified. High-resolution in situ hybridization analyses of a subset of the mRNAs revealed that a majority of mRNAs could in fact be detected in dendrites or axons. Interestingly, the mRNAs for a large proportion of known synaptic molecules were localized in dendrites including signaling molecules, scaffolds, and receptors.

One noteworthy finding was that the abundance of the different dendritically localized transcripts varied over a threefold range. The significance of having thousands of different mRNAs in dendrites remains to be explored. All proteins turn over, and synaptic synapses turn over with half-lives that are probably in the range of hours to days. The accepted view has been that most synaptic proteins are synthesized in the cell body and transported to synapses except for the few whose mRNAs are present in dendrites in abundance. The presence of thousands of different mRNAs in dendrites raises the alternative possibility that many synaptic proteins could be replaced via local synthesis. This idea raises many questions, however. If there are only a few copies of a particular transcript in the dendrites of a neuron, how could such a small complement replace proteins at the thousands of synapses on a dendrite? What determines where mRNAs will be translated, especially those present at very low abundance? Do the protein constituents of multiprotein signaling complexes turn over independently, and if so, how does a newly synthesized protein get assembled into a complex? If relative levels of the different mRNAs are the main determinant of the levels of synthesis of different proteins, can the nonabundant transcripts mediate synthesis of a meaningful percent of the total amount needed for ongoing turnover? During periods of synapse modification, which of the thousands of different mRNAs are actually needed?

Finally, there is the issue of the stoichiometric relationships between mRNAs and the translational machinery. The number of ribosomes may be rate limiting for translation (Schuman et al., 2006). At any given point in time, a single ribosome can only associate with a single mRNA, so even if each ribosome at a synapse was engaged with a

different mRNA, only a limited number of different proteins could be produced at one time. If there are thousands of different transcripts available for translation, how are particular mRNAs selected for translation initiation? It remains possible that even though the mRNAs for many proteins are detectable in dendrites, it could still be that most of their protein products are synthesized in the cell body. Clearly, these new findings raise a myriad of new questions about how mRNA translation at synapses is regulated both during normal turnover and during periods of synapse modification.

2.5. DENDRITIC TRANSPORT OF RIBOSOMES AND mRNA

2.5.1. Overview

How are ribosomes and mRNAs delivered into dendrites? This question was initially addressed by assessing by pulse-labeling neurons in low-density cultures with 3H-uridine and then assessing the distribution of newly synthesized RNA with autoradiography (Davis et al., 1987, 1990). One hour after exposing neurons to 3H-uridine, newly synthesized RNA is restricted to the nucleus. Over the course of several hours, labeled RNA migrated into dendrites, reaching the distal tips of dendrites within about 12 h. Using mathematical modeling, the average rate of transport of labeled RNA was calculated to be 11–12 μm/h (Davis et al., 1990). Rates of transport were somewhat faster in longer dendrites. This study also documented transport of labeled RNA into axons, albeit at lower levels than dendrites.

When pulse-labeling neurons in culture with 3H-uridine, about 80% of the label incorporated into newly synthesized RNA is actually into ribosomal RNA (unpublished observations). Thus, in retrospect, it is likely that the slow transport documented by autoradiography mainly involved the transport of ribosomes. This is especially likely because subsequent studies revealed that mRNAs are transported much more rapidly (see below).

2.5.2. Dendritic Transport of mRNA; Lessons Learned from Studies of Arc

The initial hypotheses about protein synthesis at synapses focused on the translation of mRNAs that were present constitutively in dendrites. A new dimension of the story emerged with the discovery of a dendritic mRNA that is expressed as an IEG. Arc-associated protein (activity-regulated cytoskeleton-associated protein) (Lyford et al., 1995), also known as Arg3.1 (Link et al., 1995), is expressed at low levels under resting conditions (Figure 3(A) and (D)), but its transcription is strongly induced by physiological activity. For example, different types of sensory stimulation induce Arc in the activated portions of the sensory cortex, and learning experiences induce Arc in different neuron types depending on the nature of the experience. Seizures or intense synaptic activation sufficient to activate NMDA receptors trigger robust induction. For example, a single electroconvulsive seizure (ECS) activates Arc transcription in large numbers of forebrain neurons (Figure 3(B) and (E)).

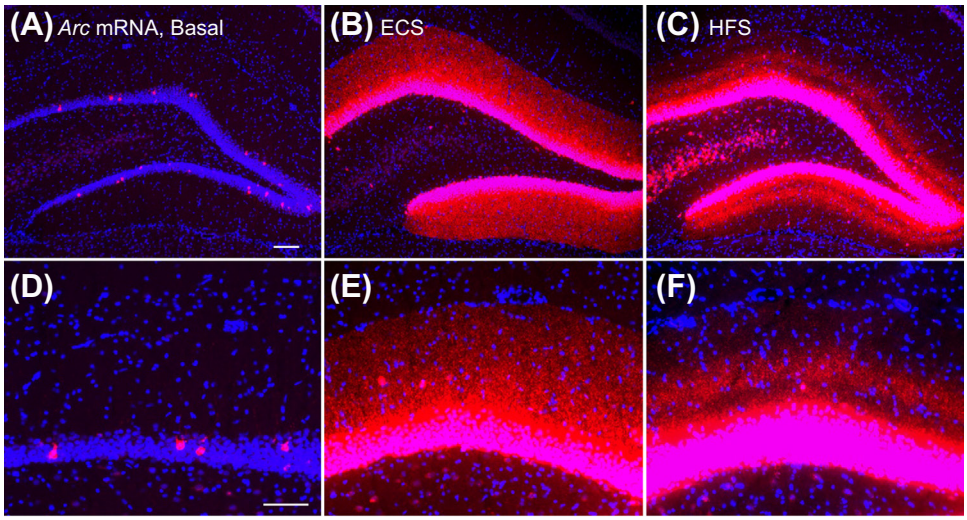

Figure 3 Activity-dependent induction and Arc mRNA and localization near active synapses as revealed by fluorescent In situ hybridization (FISH). (A) Arc mRNA is expressed at low levels under basal conditions. The image illustrates the distribution of Arc mRNA (red) on the nonstimulated side of an anesthetized rat. (B) Induction of Arc expression after an electroconvulsive seizure. Note that Arc is expressed by large numbers of dentate granule cells and Arc mRNA is transported throughout dendrites. (C) Selective targeting of Arc mRNA to active synapses. The panel illustrates the selective localization of Arc mRNA in the middle molecular layer of the dentate gyrus after high-frequency stimulation of the medial perforant path. (D, E, and F) High-magnification views of the fields shown in A, B, and C, respectively. Selective targeting of Arc mRNA to the activated portions of dendrites was initially described by Steward et al. (1998).

Because Arc is expressed as an IEG, it is possible to follow the life history of Arc mRNA from its transcription in the nucleus to its delivery into dendrites using in situ hybridization. Studies using high-resolution fluorescent in situ hybridization (FISH) reveal that a discrete behavioral experience induces Arc within minutes. Studies using a probe that recognizes the intron that is spliced out revealed two discrete foci of unspliced Arc mRNA in the nucleus of neurons in the CA1 region of the hippocampus 5 min after introducing a rat to a novel spatial environment. These represent transcription foci. Studies using probes for mature Arc mRNA revealed that newly synthesized Arc mRNA appears in the cytoplasm within 30 min (Guzowski et al., 1999, 2005). In CA1 neurons, epochs of transcription induced by experience are brief, lasting as little as 16 min, and levels of Arc mRNA then decrease rapidly. After ECS, Arc is activated in many forebrain neurons, but in most neurons, transcription turns off within a few minutes. An exception is the dentate gyrus, where transcription continues at high levels for hours in dentate granule cells (Lyford et al., 1995; Wallace et al., 1998).

2.5.3. Rapid Dendritic Transport of Arc mRNA

Once induced, Arc mRNA is rapidly transported throughout dendrites at a rate consistent with rapid microtubule-based transport. The original estimates of the rate of movement of Arc mRNA into dendrites were derived by inducing Arc expression with an ECS, and assessing the distribution of Arc mRNA over time using in situ hybridization (Wallace et al., 1998). These experiments indicated that Arc mRNA moved from the nucleus to the distal tips of dendrites of dentate granule cells (about 300 μm) within 60 min, indicating a minimal rate of 300 μm/h. It was recognized that this was an underestimate, however, because of the time required for Arc mRNA to be transcribed and undergo nucleocytoplasmic transport (approximately 20–30 min, see above). This study also revealed that Arc mRNA was delivered into dendrites in the presence of protein synthesis inhibitors, indicating that delivery into dendrites was determined by sequences within the Arc mRNA itself and not by the encoded protein. As discussed further below, the presence of the 3′-UTR of Arc in fusion transcripts is sufficient to lead to dendritic transport. Thus, signals that mark Arc mRNA for dendritic delivery are localized in the 3′-UTR, as is also true for most other mRNAs that are localized in different cell types.

2.5.4. Multiple Rates of Transport of Arc mRNA-Containing Particles

To directly visualize mRNA transport in living neurons, we used an exogenous expression approach (Dynes and Steward, 2007). Constructs containing the 3′-UTR of Arc mRNA and a sequence that binds the bacterial protein MS2 (Arc/MS2 mRNA) were coexpressed with transcripts encoding fusion proteins of MS2 and GFP. When expressed by neurons in culture, Arc/MS2 mRNA binds MS2/GFP protein, tagging the mRNA. The mRNA is visible in the cytoplasm in discrete fluorescent particles that extend throughout dendrites (Figure 4(A)).

Live-cell imaging of Arc/MS2 mRNA particle movement in unstimulated, cortical neurons in vitro revealed bidirectional dendritic transport at a wide range of velocities ranging from <1 μm/s to about 70 μm/s Figure 4(B) illustrates a kymograph of a single dendrite imaged for 500 s in which particles can be seen to move at different rates. In this segment, most particles move from proximal to distal, although long bouts of retrograde movement were seen in other segments. The fast and slow movements occurred at rates that are consistent with microtubule and actin/myosin-based transport, respectively, but there was also a distribution of velocities that were intermediate between what is considered typical for microtubule-based and actin/myosin-based mechanisms. At the fastest rate, Arc mRNA could move from the nucleus to a synapse hundreds of micrometers distant within minutes. Taken together, these results mean that synaptic activity can trigger transcription and newly synthesized gene transcripts can reach active synapses within minutes, which could allow translation-dependent synaptic modifications to occur much faster than previously imagined.

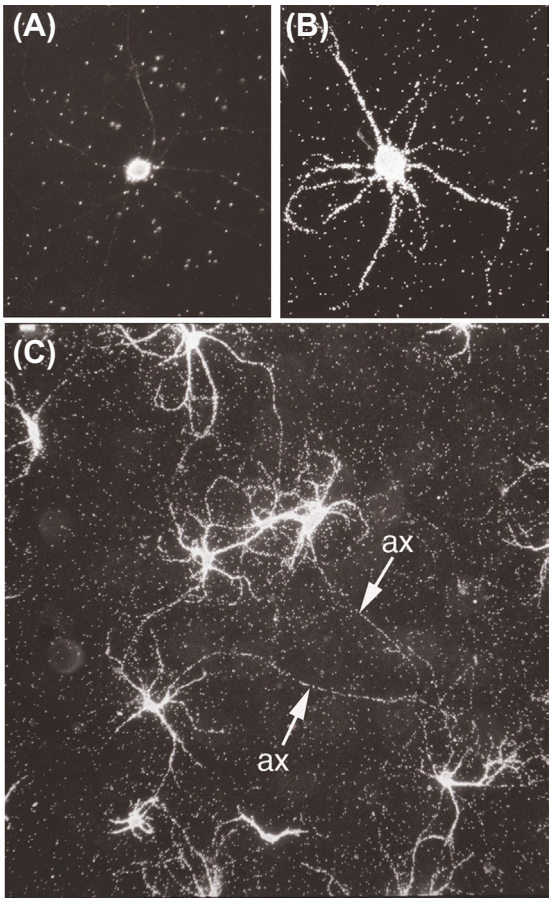

Figure 4 Slow dendritic transport of RNA. Hippocampal neurons in low-density culture were pulse-labeled with 3H-uridine for 1 h and then prepared for autoradiography at different times after the pulse. (A) At the end of the 1 h pulse, newly synthesized RNA has not yet left the nucleus. (B) After 12 h, labeled RNA extends throughout the dendrites. (C) A few axons (ax) also exhibit labeling, indicating RNA transport into axons, albeit at a lower level than into dendrites. *From Davis et al. (1990).*

Other studies have reported transport rates for other mRNAs or mRNA-binding proteins. The mean rate and maximal instantaneous velocity of Arc/MS2 is about 4–6 fold faster than reported for complexes containing CaMKII mRNA (Rook et al., 2000) and 4–6 fold slower than for the actin mRNA-binding protein ZBP1 (Tiruchinapalli et al., 2003). However, transport rates for Arc are not markedly different from those for complexes containing the mRNA-binding proteins Staufen (Kohrmann et al., 1999), Fragile-X Mental Retardation Protein (FMRP) (De Diego Otero et al., 2002), Cytoplasmic Polyadenylation Element Binding Protein (CPEB) (Huang et al., 2003), and RNA particles of unknown type labeled with syto 14 (Knowles et al., 1996). It is noteworthy

that complexes that contain KIF5A and Pur ? also move more slowly (maximum of 7.4 μm/min) and for shorter distances (maximum of 8 μm) (Kanai et al., 2004) than the Arc mRNA containing particles. Possibly, the KIF5A motor may be used by Arc mRNA particles for the slow, local phase of movement (see below). It should be noted, however, that differences in experimental parameters (such as neuron type and maturity, image acquisition rate, and incubation temperature) make direct comparisons of data between studies problematic. Also, given the wide range of transport velocities, measures of central tendency (either mean or median) may not be very meaningful.

In our studies (Dynes and Steward, 2007), transcripts lacking the 3′-UTR of Arc were not efficiently delivered into dendrites, indicating that the presence of the 3′-UTR was sufficient for targeting fusion transcripts for dendritic delivery. Studies involving transfection of parts of the 3′-UTR followed by in situ hybridization indicate that two regions mapping between bases 2162–2513 and 2518–2889 are sufficient to confer some degree of dendritic localization (Kobayashi et al., 2005). This information has not yet been helpful in identifying unique mRNA-binding proteins involved in selective localization.

2.5.5. Behavior as Particles Stop Moving Suggests Docking

We noted that some Arc/MS2 particles moved at a characteristic rate and suddenly stopped during the recording period. Many of the particles that stopped moving at their characteristic velocity exhibited low velocity movements just before stopping completely. One of these is noted by the star in Figure (B) and another involving a particle moving in the retrograde direction is shown in Figure (A). Nearly half of these movements were in the opposite direction from the preceding bouts. These reverse slow movements were progressive, and thus are difficult to explain as being due to random dissociations from the transport machinery. None of the particles that reversed direction before stopping resumed rapid transport during the rest of the imaging session. This behavior suggests some final targeting based on local cues. An obvious speculation is that the particles stopped at synapses, but this was not assessed directly in our initial study.

The question of whether Arc/MS2 mRNA docked at synapses was assessed in a follow-up study involving somewhat older (17–20 div) neurons with well-developed dendritic spines (Dynes and Steward, 2012). In this experiment, Arc/MS2 particles were imaged 16–26 h after cotransfection when most particles are stationary. Arc/MS2 particles that remained stationary over the recording interval were identified by averaging consecutive images. Remarkably, many stationary particles were parked precisely at the base of dendritic spines (Figure 5). To quantify this association, we determined the position of every Arc/MS2 mRNA particle and spine base in a sample of dendritic segments with a combined length of 479 μm. This analysis revealed that 69/216 spines (32%) had an Arc/MS2 mRNA particle at or near the spine base (defined as within 0.35 μm), while 71/216 (33%) had no visible particle within 1 mm of the spine base.

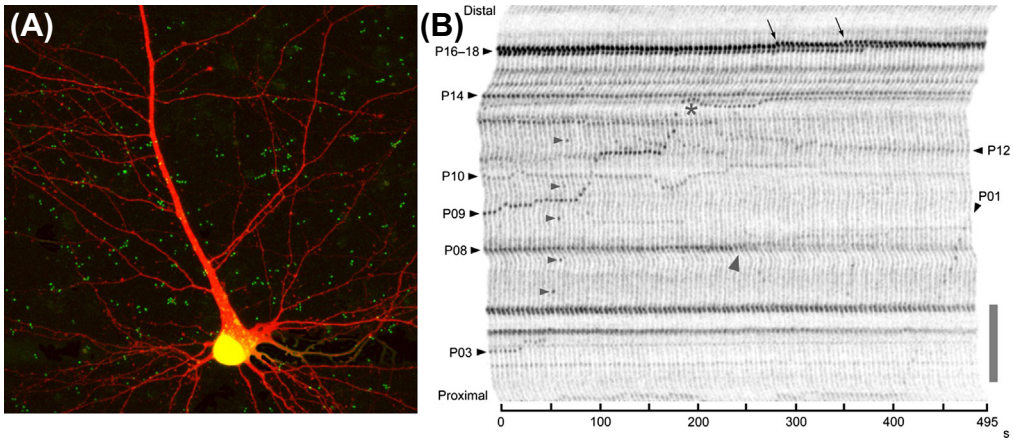

Figure 5 Rapid dendritic transport of Arc/MS2 mRNA. (A) The image illustrates a neuron in culture that had been biolistically transfected with constructs containing the 3′-UTR of Arc mRNA and a sequence that binds the bacterial protein MS2 (Arc/MS2 mRNA) and transcripts encoding fusion proteins of MS2 and GFP. The Arc/MS2 construct also contained the coding sequence for dsRed. Red fluorescence is present throughout the cell body, axons, and dendrites of transfected neurons. Arc/MS2 mRNA tagged with MS2/GFP protein is present in particles in the cell body and dendrites (yellow fluorescence). Larger green particles are the beads that were biolistically delivered to the cultures. (B) The image illustrates a kymograph of a single dendritic segment that was imaged for 500 s in which particles can be seen to move at different rates. Particles are numbered from 01 to 18. Particle #3 exhibits the fastest rate of movement approaching 70 μm/s. Particle number 09 exhibits slow movements just before stopping completely (asterisk). Large arrow head illustrates a particle that undergoes fragmentation. *From Dynes and Steward (2007).*

Conversely, of 235 particles analyzed, 71 (30%) were positioned within 0.35 μm of a spine base. This is likely to underestimate the number of particles at spines because some particles might be under spines that extend perpendicular to the dish, which may be invisible (Figure 6).

Although Arc/MS2 particles clearly localize with a high degree of precision at the base of spines, the results do not prove that the final movements of Arc/MS2 particles in the live-cell imaging experiments actually indicate docking at synapses. Also it remains to be established whether native Arc mRNA is transported in the same way as Arc/MS2. Fluorescent in situ hybridization analyses reveal that newly synthesized native Arc mRNA is present in particles that appear to be roughly the same size as Arc/MS2 particles (Figure 3). Nevertheless, native Arc mRNA undergoes nuclear processing including splicing, which fusion transcripts expressed by transfected DNAs do not, and proteins of the splice junction complex remain bound to the mRNA as it moves into dendrites (Giorgi et al., 2007). If proteins involved in nuclear processing are important for proper localization, then the movement and localization of fusion transcripts may not fully recapitulate what actually occurs with native mRNAs.

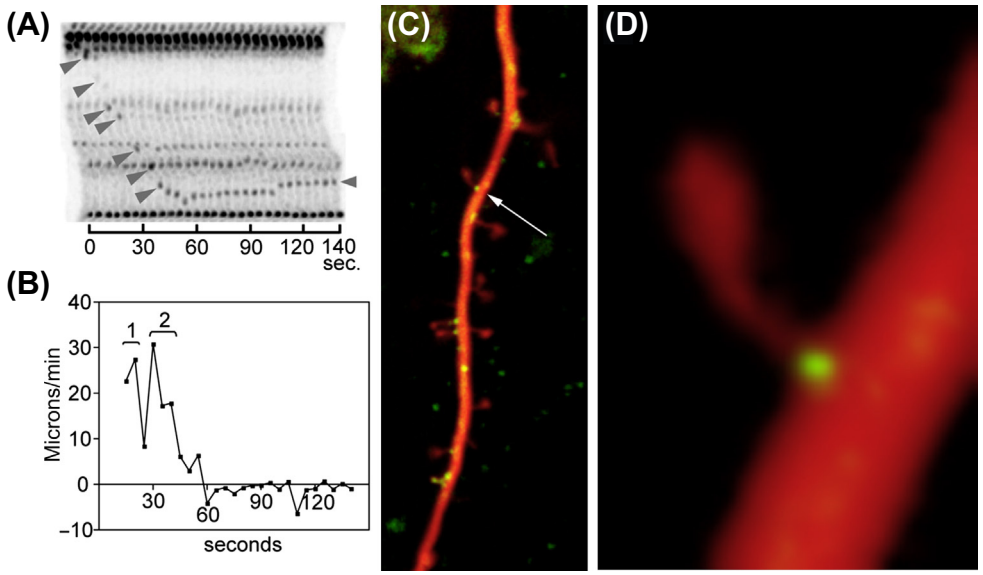

Figure 6 Precise localization of Arc/MS2 mRNA at the base of dendritic spines. (A) The kymograph illustrates the stopping behavior of a particle moving at a steady rate in a retrograde direction. Note slow movements in the reverse direction just before stopping completely. (B) Illustration of the instantaneous velocity of the article, determined by the distance moved from one image to the next. (C) Image of a dendrite in which immobile particles are identified by image averaging over time. (D) Higher power view of an Arc/MS2 particle parked at the base of a dendritic spine. The image was smoothed by Gaussian blurring. *A and B are from Dynes and Steward (2007). C and D are from Dynes and Steward (2012).*

2.5.6. Localization of Arc mRNA near Active Synaptic Sites

A striking feature of Arc mRNA is that it localizes selectively near synapses that have been recently activated. This was demonstrated in studies involving high-frequency stimulation of the pathway from the entorhinal cortex to the dentate gyrus (perforant path) in anesthetized rats (Steward et al., 1998). The perforant path terminates in a topographically organized fashion along the dendrites of dentate granule cells; projections from the medial entorhinal cortex terminate in the middle molecular layer on mid proximodistal dendrites and projections from the lateral entorhinal cortex terminate on distal dendrites (Steward, 1976). Delivery of HFS that induces perforant path LTP (8-pulse trains at 400 Hz at 1/10 s intervals) also induces Arc transcription in dentate granule cells. If stimulation is continued as Arc mRNA moves into dendrites, the newly synthesized mRNA localizes selectively in a discrete band in the activated portion of the dendrite (Steward et al., 1998). This selective localization is in striking contrast to the uniform distribution of Arc mRNA throughout dendrites after a seizure (Figure 3). Especially noteworthy is that if synapses on mid proximodistal dendrites are active as Arc mRNA moves into dendrites, Arc mRNA never

reaches more distal dendritic laminae. This suggests that there is some active docking mechanism that captures Arc mRNA that is in transit.

2.5.7. Mechanisms Underlying the Localization of Arc mRNA near Active Synapses

One clue to the localization mechanism is that simple induction of LTP, which can be achieved by delivering thirty 400 Hz trains at 1/10 s intervals, induces Arc transcription, but when Arc mRNA reaches the dendrites 30–45 min later, there is little evidence for selective localization in the activated lamina. Instead, the mRNA is delivered throughout dendrites including the distal regions beyond the zone of synapses that had been active. There are two possible explanations for the failure to localize after simple LTP induction: (1) localization may require stimulation over long periods of time; (2) the signals generated by activity may be relatively short lived. Evidence against the first possibility came from experiments in which Arc expression is induced by an ECS, time was allowed for the mRNA to migrate into dendrites, and then stimulation was delivered to activate synapses terminating on particular dendritic domains. In this paradigm, 15 min of synaptic activation led to a selective localization of Arc mRNA in the activated dendritic lamina (Steward and Worley, 2001). Because 15 min of stimulation is sufficient to generate a localized distribution, the likely explanation of the failure of Arc mRNA to localize in a simple LTP paradigm is that by the time the Arc mRNA that is induced by the stimulation reaches the dendrite, the signal for localization has dissipated.

To define other properties of the localization mechanism, we used the paradigm described above, in which Arc is first induced by a generalized seizure and then targeted by synaptic stimulation (Steward and Worley, 2001). This approach was essential because some of the same intracellular signals are involved in both induction of transcription and localization. We first demonstrated that localization of Arc mRNA at active synapses was blocked by local infusion of NMDA receptor antagonists (Steward and Worley, 2001). Subsequent studies revealed that stimulation sufficient to cause localization triggers striking activation of the MAP kinase pathway as revealed using phosphospecific antibodies for ERK1/2 (Huang et al., 2007). Moreover, pharmacological blockade of ERK activation with U0126 completely blocked the localization of Arc mRNA that would otherwise occur. Also, stimulation that induced localization triggers polymerization of actin at active synapses and blockade of actin polymerization also blocks the localization of Arc mRNA that would otherwise occur during synaptic activation. These findings indicate that localization is driven by NMDA receptor activation and requires activation of MAP kinase and polymerization of actin at active synapses (Huang et al., 2007).

There is evidence that synaptic activation can cause mRNAs to move from the dendritic shaft into spines. For example, in one study, perforant path LTP was induced in unanesthetized adult rats, and subcellular fractionation techniques were then used to isolate synaptoneurosomes (Havik et al., 2003). Real-time PCR was then used to

determine the levels of the mRNAs for alpha-CaMKII and Arc in the synaptoneurosomes and also in total homogenates. It was found that levels of aCaMKII mRNA increased about 2.5-fold in the synaptoneurosomes after inducing LTP, whereas there was no change in aCaMKII mRNA levels in total homogenates. These findings suggest that aCaMKII mRNA translocates into spines as a result of stimulation that induces perforant path LTP. There were also increases in the levels of Arc mRNA in synaptoneurosomes, but these were accompanied by increases in Arc mRNA levels in the total homogenates. Thus, the increases in synaptoneurosomes could reflect the overall increase in Arc mRNA levels, and not necessarily translocation of Arc mRNA into spines.

Although Arc mRNA is targeted to regions of dendrites contacted by active synapses, it remains to be established whether the mRNA localizes specifically at active synapses or spines. This is an important issue because of recent evidence that Arc protein plays a role in receptor endocytosis leading to synaptic depression. Induction of LTP increases synaptic efficacy, so if Arc mRNA localizes at active synapses, it would be expected that Arc protein might be delivered preferentially to the potentiated synapses. Alternatively, it is conceivable that Arc mRNA localizes selectively at inactive synapses on dendritic segments that are strongly activated by other synapses. Clearly, there is still much to learn about the mechanisms and functional significance of the remarkable localization of Arc mRNA induced by synaptic stimulation.

2.5.8. Multiple Patterns of mRNA Localization within Dendrites

It is common to talk of mRNAs as being dendritically localized or restricted to the region of the cell body, but there are actually a number of different localization patterns. For example, although the mRNAs for both MAP2 and the a-subunit of CaMKII are present in the dendrites of cortical neurons, hippocampal pyramidal cells, and dentate granule cells, the subcellular distributions of the two mRNAs are quite different (Paradies and Steward, 1997). The mRNA for aCaMKII is present at moderately high levels throughout the neuropil layers. In contrast, the levels of MAP2 mRNA are highest in the portion of the neuropil that contains proximal dendrites. Levels of labeling fall off dramatically in portions of the neuropil that contain distal dendrites.

Of the other dendritic RNAs, the mRNA for Den is present throughout dendrites; Arc (when induced) comes to have a distribution within granule cells of the dentate gyrus that is similar to aCaMKII unless there is ongoing synaptic activity. The RNA pol III transcript BC1 has a distribution that is similar to MAP2 in hippocampal neurons but is expressed at low levels by dentate granule cells. Some other mRNAs that are localized primarily in cell bodies also extend slightly into proximal dendrites. The mRNAs that are present in the dendrites of Purkinje cells also exhibit somewhat different patterns of distribution. The mRNA for the InsP3 receptor is present throughout dendrites but is concentrated in the proximal one-third of the dendrite. L7 mRNA appears to be more uniformly distributed in Purkinje cell dendrites (Bian et al., 1996). The fact that

there are a number of different subcellular distribution patterns of the mRNAs localized in dendrites suggests that there will also be different localization signals that mark the mRNAs for dendritic delivery.

3. CONCLUSIONS

The goal of this chapter was to provide a snapshot of current ideas about the protein synthetic machinery that is present at postsynaptic sites on CNS neurons—machinery which provides the basis for gene expression at the synapse. Despite 30 years of research, there are still a number of unresolved questions. To note just a few are the following:

1. What are the address markers that control the dendritic distribution of different mRNAs?
2. Do ribosomes and mRNAs shuttle from one synapse to another so that all synapses are capable of local synthesis of proteins?
3. How is the translation of different mRNAs regulated? Is the synthesis of different proteins determined by the abundance of the different mRNAs or is there selection based on signals from the synapse?
4. What is the mechanism that mediates docking of mRNAs at spine bases, and how is their subsequent movement within the spine controlled?

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