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On the nature and differential distribution of mRNAs in hippocampal neurites: Implications for neuronal functioning

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ABSTRACT **Neurons are highly polarized cells with a mosaic of cytoplasmic and membrane proteins differentially distributed in axons, dendrites, and somata. In *Drosophila* and *Xenopus*, mRNA localization coupled with local translation is a powerful mechanism by which regionalized domains of surface or cytoplasmic proteins are generated. In neurons, there is substantial ultrastructural evidence positing the presence of protein synthetic machinery in neuronal processes, especially at or near postsynaptic sites. There are, however, remarkably few reports of mRNAs localized to these regions. We now present direct evidence that an unexpectedly large number of mRNAs, including members of the glutamate receptor family, second messenger system, and components of the translational control apparatus, are present in individual processes of hippocampal cells in culture.**

In central nervous system neurons, several lines of evidence suggest that proteins may be synthesized locally at or near postsynaptic sites independent of the cell body. First, ultrastructural studies have revealed the preferential localization of polyribosomes beneath postsynaptic sites and occasionally associated with membrane specializations on dendrites. It has been suggested that these anatomical structures represent the protein synthetic machinery necessary to translate and posttranslationally modify different classes of proteins (1, 2). Second, autoradiographic studies using isolated, nonaxonal growth cones have demonstrated [^{3}H]leucine and [^{3}H]galactose or [^{3}H]fructose incorporation (3, 4). Third, past studies have revealed an energy-dependent mechanism for the selective transport of RNA to somatodendritic regions (5) at a rate comparable to slow axonal transport in the longest dendrites (6). Conspicuously absent, however, is information on the nature and distribution of the RNAs present in somatodendritic domains. *In situ* hybridization (ISH) studies have been successful in identifying curiously few mRNAs in neuronal processes (7–11). We now present direct evidence that a large population of mRNAs coding for both transmembrane receptor and cytosolic proteins is present in hippocampal neurites.

MATERIALS AND METHODS

Hippocampal Cultures. Hippocampi were dissected from embryonic day 20–21 rat fetuses and cultured as described (12, 13). Experiments were performed after 21–28 days in culture. In this study, cell bodies and their neurites were taken from cells cultured from different animals on three different days under similar conditions. During each of these sessions, a sample of culture medium was also aspirated and processed through antisense RNA (aRNA) processing to assess the possible presence of mRNAs in the culture medium from dying cells.

Reverse Northern Blot Analyses. Samples were processed as described (14, 15) with few exceptions. Hybridization signals were normalized to rRNA for each neurite or soma studied.

Differential Display. Reactions were carried out in 25-μl volumes using AmpliWax Gems with an upper-to-lower ratio of 1.5:1. Reaction mixtures contained 200 μM dATP, dGTP, and TTP; 4 μM dCTP; 5 μCi (1 Ci = 37 GBq) of [^{33}P]dCTP (NEN/DuPont); 0.4 μM OPA-5 or other 10-mers (Operon Technologies, Alameda, CA); 0.6 μM oligo A, B, or C; 2.5 mM $MgCl_2$; 1.25 units of AmpliTaq polymerase; and 1 μl of a 1:10 dilution of double-stranded DNA previously processed through a single round of aRNA amplification. Reactions were cycled for 35 rounds at 94°C for 30 sec, 40°C for 90 sec, and 72°C for 45 sec followed with a final 5-min elongation at 72°C in a Biosycler thermocycler.

RESULTS

Isolation of Single Hippocampal Neurites. Isolated hippocampal cells free of overlapping processes from neighboring cells were identified in low-density cultures (12). Under these conditions, neurons grow as isolated cells or in small two- to four-cell groups, either directly on the substrate or on glial cells (17). Neurons were identified by morphological criteria based on a long experience recording synaptic interactions among such neurons. While these cells acquire many physiological and morphological characteristics of mature hippocampal cells, it is not clear whether such neurons in culture demonstrate the same differentiation of axons and dendrites as their counterparts *in situ*. Therefore, we refer to these as either neuronal processes or neurites (18). Individual proximal and distal neurites were harvested by transecting them at varying distances from the cell body and aspirating them into a micropipette containing the reagents necessary for the first step in the aRNA amplification procedure (Fig. 1) and processed through the aRNA amplification procedure as described (14).

Ionotropic Glutamate Receptor mRNA Expression. The population of the mRNAs in neurites was assessed initially by mRNA expression profiling. Southern blots containing cloned cDNAs encoding members of the ionotropic glutamate receptor family were probed with radiolabeled aRNA from individual neurites or cell bodies. The glutamate receptors, classified into *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA; GluR1–4), and kainate (GluR5–7) subtypes, were the focus of this analysis as they are the primary mediators of excitatory synaptic transmission in the brain (19). Moreover, their overt role in the biochemical events associated with excitotoxicity (20) and long-term potentiation (21, 22) makes their RNAs intriguing candidates for local protein synthesis. All neurites

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Abbreviations: aRNA, antisense RNA; ISH, *in situ* hybridization; GFAP, glial fibrillary acidic protein; DD, differential display; FPP, farnesyl diphosphate; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate.

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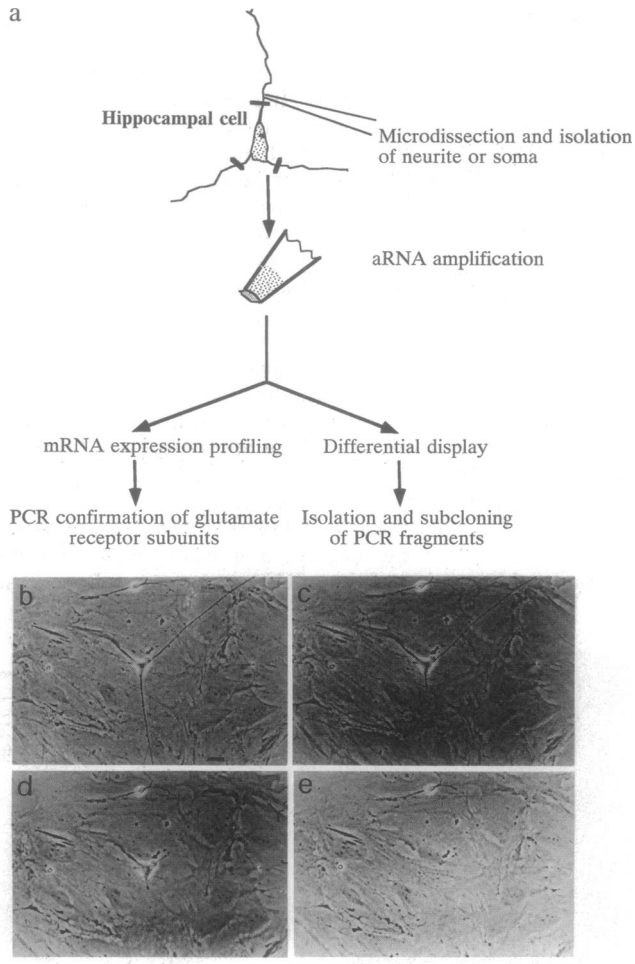
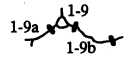
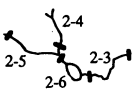
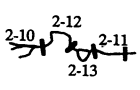
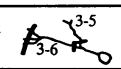


FIG. 1. (*a*) Schematic representation of the techniques used in this study. (*b–e*) Phase-contrast photomicrographs of a typical hippocampal cell used for this study. These panels illustrate a cell prior to transection and isolation of any neurites (*b*), followed by the serial dissection of its neurites (*c* and *d*). Processes were physically isolated by transection of the neurite with the tip of a glass micropipette attached to a micromanipulator. Generally, there was a small (30 sec to 3 min) lag time between isolations during which the cell or neurite rarely showed morphological signs of cell distress (i.e., swelling). (*e*) Note the undisturbed glial cell layer following aspiration of individual neurites and the soma. (Bar = 25 μm).

(19/19 from nine cells) expressed GluR1, GluR2, GluR4, and NMDAR1 mRNA. In a majority of neurites, GluR3 (15/19), GluR5 (14/19), GluR7 (14/19), and NR2b (10/19) mRNA expression was observed, whereas expression for GluR6 mRNA was detectable in approximately one-half of the neurites (9/19) studied. The presence of several of these subunits was confirmed by subunit-specific PCR (data not shown). In contrast, only one neurite showed detectable hybridization signals for NR2a and NR2c mRNA.

A more extensive examination of reverse Northern blots with scanning densitometry analyses revealed distinct relative variations in glutamate receptor mRNA expression (Table 1). Although neither NMDA nor non-NMDA receptor mRNAs displayed any general trend restricting specific subunits to different cellular locations, there were clear differences in the relative levels of glutamate receptor mRNAs expressed between a neuronal process or processes from the same cell and its cell body. In a number of cells, the qualitative expression patterns were similar (Table 1, cells a_1 and a_2), yet the relative intensity of the hybridization signal was more profound for specific subunits. For example, relative levels of NMDAR1 and GluR5 mRNA were clearly elevated in HP 1-9a (the apical neurite) versus HP 1-9b (the basal neurite) or the soma. This tendency was complemented in other cells, which displayed a more differentiated qualitative pattern of glutamate receptor mRNA expression (Table 1, cells b_1 and b_2). In one striking example, HP 3-5, an isolated branch segment of a single neuronal process, exhibited NR2a and NR2c while its other branch segment did not. In rare instances—for example, GluR6 in cell b_1—mRNA expression was highly enriched in neuronal processes. In the course of these studies, other nonglutamate receptor mRNAs were restricted to the soma (data not shown). In the framework of a local protein synthesis hypothesis, the observation of different qualitative patterns of glutamate receptor mRNA expression and different relative levels of the glutamate receptors being expressed allows for a variety of combinations of receptors at individual synapses. Clearly, a differentiated pattern of mRNA expression (Table 1, cells b_1 and b_2) may contribute greatly to the physiological identity of a synapse, as the partitioning of specific mRNAs would presumably delimit the expression of glutamate receptors, if translated, to a given area. No less significant is the finding that differences in the relative levels of glutamate receptor mRNA expression (Table 1) may impart different neuronal processes from the same cell, as well as different segments of the *same* process, with varying capabilities to rapidly sup-

Table 1. Relative levels of glutamate receptor mRNA expression

Representative line drawing of cell analyzed	Sample analyzed	NMDA receptor subunits				AMPA receptor subunits				Kainate receptor subunits		
		R1	R2a	R2b	R2c	GluR1	GluR2	GluR3	GluR4	GluR5	GluR6	GluR7
a_1 	HP 1-9a	++++	−	−	−	++	++	++	++	+++	−	−
	HP 1-9b	++	−	−	−	+++	+	+	+	+	−	−
	HP 1-9 (soma)	++	−	−	−	+++	++	++	++	+	−	−
a_2 	HP 2-3	+	−	+	−	++++	++	++	++++	+	+	+
	HP 2-4	+	−	+	−	+++	++	++	+	++	+++	++
	HP 2-5	+++	−	++	−	++++	+++	++	++++	++	+++	++
	HP 2-6 (soma)	++++	−	+	−	++++	+++	++	++++	++	+	+
b_1 	HP 2-10	+	−	−	−	++	+	−	+	−	+	+
	HP 2-12	+	−	++	−	+++	++	+	++	+	+	+
	HP 2-11	++++	−	+	−	++++	++	+	+++	−	++	+
	HP 2-13 (soma)	++	−	+	−	++++	++	++	+++	++	−	+
b_2 	HP 3-5	++	++	++	+	+++	++	+++	+++	++	+	+
	HP 3-6	+++	−	+++	−	++++	++++	++++	++++	+	−	−

Four sets of neurites with (a_1, a_2, and b_1) or without (b_2) their soma are shown; soma for cell b_2 was lost during aRNA processing. Dark bars perpendicular to neurites in representative line drawings, not drawn to scale, represent approximate transection points of the isolated neurite. Generally, proximal neurites were transected 10–20 μm from the cell body. Distal transections (see b_1 and b_2) varied in distance from the cell soma. Plus signs denote the increasing intensity of a hybridization signal. A minus sign denotes a lack of a hybridization signal.

plement focal segments of the surface membrane with specific receptor complexes. In this scenario, the integrated signal at a synapse may be determined by the stoichiometry of specific membrane receptors that respond to an environmental stimulus.

Recent studies using ISH (23) and Northern blot analyses of synaptosomal RNA fractions (24) with the AMPA and kainate-sensitive receptor subunits failed to reveal these mRNAs at dendritic locations. The differences between these reports and our studies may be due to a number of factors. The microdissection of individual neurites is more anatomically precise than the synaptosomal preparation and in combination with the aRNA amplification scheme permits a more sensitive detection system than ISH (25). In addition, the low abundance of these mRNAs may have limited their detectability with ISH or Northern blot analysis. Alternatively, recent reports suggest that RNA may interact with numerous proteins in a ribonucleoprotein complex. RNA–protein interactions (26, 27), as well as secondary RNA structure (28), may impede the hybridization of the ISH probe. Differences in cell culturing methods also may be responsible for the contrasting results.

The expression of several other cDNAs was assessed with reverse Northern blot analysis to further determine the biochemical makeup of the neuronal processes as well as the possibility of glial cell contamination. Previous reports (13) have demonstrated the dendritic localization of the α subunit of the Ca^{2+}/calmodulin-dependent protein kinase (CaMK II) mRNA. In every case examined (19/19), mRNA expression in distal and proximal segments of isolated neurites was strongly positive for CaMK II. Additionally, previous studies have shown that the aRNA procedure may amplify some rRNAs due to the A-T content of the primary sequence (14). Using a cloned rRNA cDNA (14), rRNA expression was assessed simultaneously with the other previously mentioned mRNAs. rRNA expression was observed in each of the neurites studied. These data are consistent with a biochemical makeup reminiscent of a "dendrite-like" neurite. Finally, the contamination of neurites with surrounding glia or astroglial processes was assessed by glial fibrillary acidic protein (GFAP) mRNA. With one exception, neurite and soma preparations were free of GFAP mRNA (19/20). The single neurite that showed GFAP expression was excluded from further analysis. The expression of GFAP mRNA in glia from these cultures was shown in an unrelated set of experiments in which glia ($n = 2$) were aspirated into a micropipette and processed through the aRNA amplification scheme and reverse Northern blotting. The media controls showed that the constituents of the media were not amplifiable. *In toto*, these data demonstrate that the mRNA expression profiles are specific for messages seen in single hippocampal neurites and free of contaminating RNA.

Differential Distribution of mRNAs. To investigate the complexity of the mRNA population in neurites, we used a PCR-based assay, differential display (DD; ref. 29). DD of mRNAs from one cell is shown in Fig. 2. In these experiments, a single 10-mer (OPA-5; 5′-AGGGGTCTTG-3′), which serves as the 5′ primer, and a panel of modified polythymidine primers containing a two-base extension are used to amplify specific populations of the polyadenylylated RNA pool. It is apparent from the complement of DD profiles that neurites exhibit a large number of mRNA species. While it does not appear that any single primer combination yields a greater number of PCR products, it does appear that some neurites express a greater number of products with certain primer combinations (data not shown). This sometimes results in a cell process exhibiting more bands in the autoradiogram than its corresponding cell body. Indeed, a full complement of modified anchor primers in combination with OPA-5 supports the notion that a large population of translatable mRNAs are present in neurites (data not shown). Second, the patterns of PCR products conclusively show that mRNAs are differentially distributed. For example, for a typical cell in which proximal (HP2-10) and distal (HP2-12) segments of the same process, in addition to a separate process (HP2-11), are transected and isolated, there are transcripts that migrate at similar molecular sizes between individual neurites. While some products are found concomitantly in one or more neurites (closed arrowheads) and between neurites and their corresponding cell bodies (open arrowheads), there are numerous transcripts that are selectively enriched in individual neurites or segments of individual neurites (arrows). Frequently, there are differences in the intensity of the autoradiograph signal for bands migrating at the same molecular weight whether from neurite or soma preparations. However, the intensity differences do not correspond to the absolute levels of mRNA present from the banding patterns, because DD is not a quantitative assay. In a biological context, the unexpected presence of a panoply of mRNAs strengthens the possibility that synapses may develop, at least to some degree, a mosaic of proteins by regulating the position of the mRNA in a neuronal process.

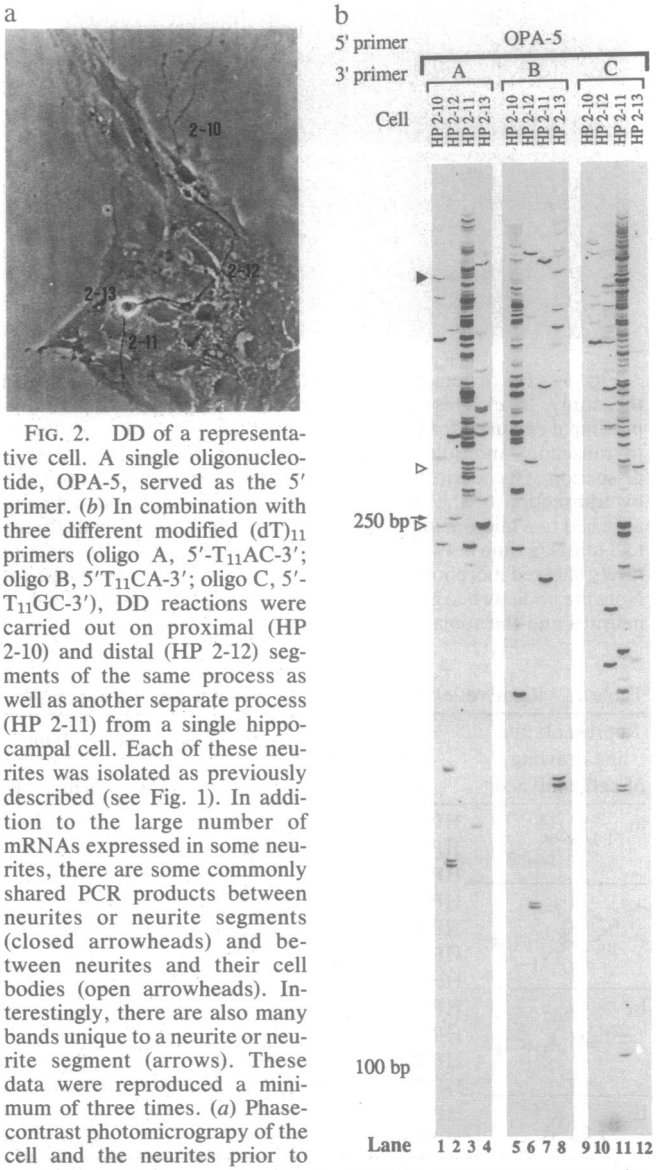


FIG. 2. DD of a representative cell. A single oligonucleotide, OPA-5, served as the 5′ primer. (*b*) In combination with three different modified $(dT)_{11}$ primers (oligo A, 5′-T_{11}AC-3′; oligo B, 5′T_{11}CA-3′; oligo C, 5′-T_{11}GC-3′), DD reactions were carried out on proximal (HP 2-10) and distal (HP 2-12) segments of the same process as well as another separate process (HP 2-11) from a single hippocampal cell. Each of these neurites was isolated as previously described (see Fig. 1). In addition to the large number of mRNAs expressed in some neurites, there are some commonly shared PCR products between neurites or neurite segments (closed arrowheads) and between neurites and their cell bodies (open arrowheads). Interestingly, there are also many bands unique to a neurite or neurite segment (arrows). These data were reproduced a minimum of three times. (*a*) Phase-contrast photomicrograpy of the cell and the neurites prior to isolation. Dark bars perpendicular to each process represent the approximate transection point. Adjacent to each neurite or neurite segment is the nomenclature associated with that particular region of the neuron.

As a result of the assemblage of metabolic and signaling pathways conserved in most cell types, it is reasonable to presume that a proportion of bands would be common between cell bodies. Our data are in general agreement with this prediction in spite of the heterogeneity of hippocampal cells in culture as roughly one-third or more of the PCR products between different cell body preparations migrate at similar molecular weights (Fig. 3*a*). Since differential display is not quantitative, some bands may be missed due to differences in the relative abundance of these mRNAs between the cell bodies. The presence and absence of bands does, however, imply a difference in abundance of the corresponding mRNA between the cell bodies. This is not surprising since cells not only differ in the specific mRNAs that are transcribed but also in levels of mRNAs common to these cells.

Concurrent with the experiments enumerated above, a number of intensive controls were undertaken to minimize the possibility of false positives and to ensure reproducibility. In each set of experiments, the DD was repeated a minimum of three times. Each produced the same banding pattern. As additional controls, process cDNAs (HP 2-10) were PCR-amplified exclusively with the 5′ or 3′ primer (Fig. 3*b*). Although the use of the 5′ primer by itself yields a banding pattern, it is abolished to a large extent when both 5′ and 3′ primers are used in combination. For example, compare the banding pattern generated in Fig. 3*b* with the 5′ primer versus the banding pattern of HP 2-10 in Fig. 3*b*, lane 1, when both primers are used. This banding pattern results from the polymorphic nature of this primer and its ability to hybridize to multiple sites within the same cDNA for a fraction of the cDNA population. These bands still correspond to mRNAs that are present in the RNA population. When there is no cDNA template in the presence of both primers, the reactions do not exhibit any banding pattern (Fig. 3*b*, no DNA lane). In the reverse situation when DD reactions are performed in the presence of only template (i.e., no primers), no banding pattern is observed.

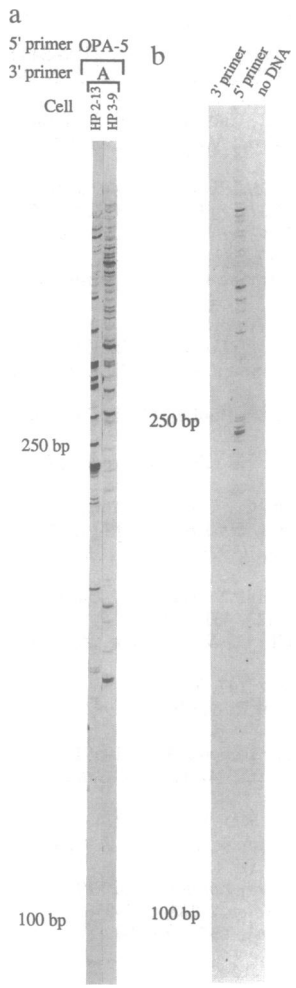


FIG. 3. DD experimental controls. (*a*) Comparison of banding patterns exhibited by cell bodies. Despite the heterogeneity of cells in culture, a reasonable presumption is that the banding patterns from different cell bodies would exhibit several bands migrating at the same molecular weight. DD of two cell bodies (HP 2-13 and HP 3-9) with primary combination OPA-5 and 3′-oligonucleotide A illustrates that a large number of bands are common between these cell bodies. (*b*) As controls, process cDNA template (HP 2-10) was PCR-amplified in the presence of either the 5′ primer (OPA-5) or 3′ primer (oligonucleotide A) at previously described molar concentrations (see *Methods*).

Identity of Neurite cDNA Clones. The PCR-based nature of the DD assay enabled us to clone 40 of these individual bands from multiple processes that are selectively enriched in neurites. Isolated PCR bands, ranging in size from 75 bases to >1 kb, were reamplified and cloned into the pT7 or PCR-Script vectors using conventional techniques. Two clones (98% over 107 bp) corresponding to the farnesyl diphosphate (FPP) synthase mRNA are found in two separate distal processes (HP 2-10 and HP 3-5). As part of the isoprene biosynthetic pathway, FPP synthase generates the farnesyl moiety ultimately transferred to the COOH-terminal CaaX (a = aliphatic) motif of mammalian ras proteins (30).

While two other cDNAs had significant sequence similarity with mRNAs for the γ subunit of the interleukin 2 receptor and the tumor necrosis factor-inducible protein A20, the remainder of the cDNA clones have little sequence similarity with any published sequences. However, it is important to note that differential display favors the amplification of the 3′ end of the mRNA. Thus, the cloned cDNAs likely represent the 3′ untranslated region of particular mRNAs. Unfortunately, the 3′ untranslated sequences for cloned cDNAs are often not included in the data base citations.

DISCUSSION

Previous reports have shown that subregions of the dendritic arbor can be populated by functionally and anatomically distinct synaptic connections (31). During synapse construction, maintenance, and remodeling, it is likely that proteins are selectively transported to these microdomains (32). As a result, the exquisite specificity of the nervous system is established and modified, at least in part, by these protein-targeting mechanisms. In the present study, we provide direct evidence that significantly advances the biological importance of a complementary mechanism for the generation of this diversity whereby local protein synthesis may contribute newly made proteins to specific dendritic regions.

There are a number of attractive features afforded by a local protein synthesis model. For example, at selected regions of the dendrite, stimulus-induced synthesis of glutamate receptors from their corresponding mRNAs may occur to maintain local levels of receptor in the face of protein turnover or increase the amount of functional receptor to a defined region of synapses. This does not preclude the transport of glutamate receptors from the cell body as one mechanism for generating receptor protein for use in processes (Fig. 4*a*). As a single mRNA template can be translated many times (essentially amplifying the amount of protein product), a local protein synthesis model provides a rapid and flexible model for supplying surface or other cytosolic proteins to discrete domains as a consequence of the biological activity during synaptogenesis or synapse elimination.

The segregation of mRNAs to processes may provide a mechanism for ensuring the correct functional positioning of the corresponding protein. In this regard, it is intriguing to speculate upon the finding of FPP synthase mRNA (Fig. 4*b*). In response to the proper cellular signals, translational repression of the mRNA is relieved. Recent data with *nanos* RNA support the concept that the mRNA primary sequence contains elements that repress translation (probably via the action of RNA-binding proteins) until the proper cellular signals are received (33). Upon translation, FPP synthase may be transported directly to the cell soma where the mammalian ras proteins may be alkylated with the FPP synthase product, C_{15}-FPP. Alternatively, isoprenylation of ras proteins may occur locally in the neurite. Physiologically,

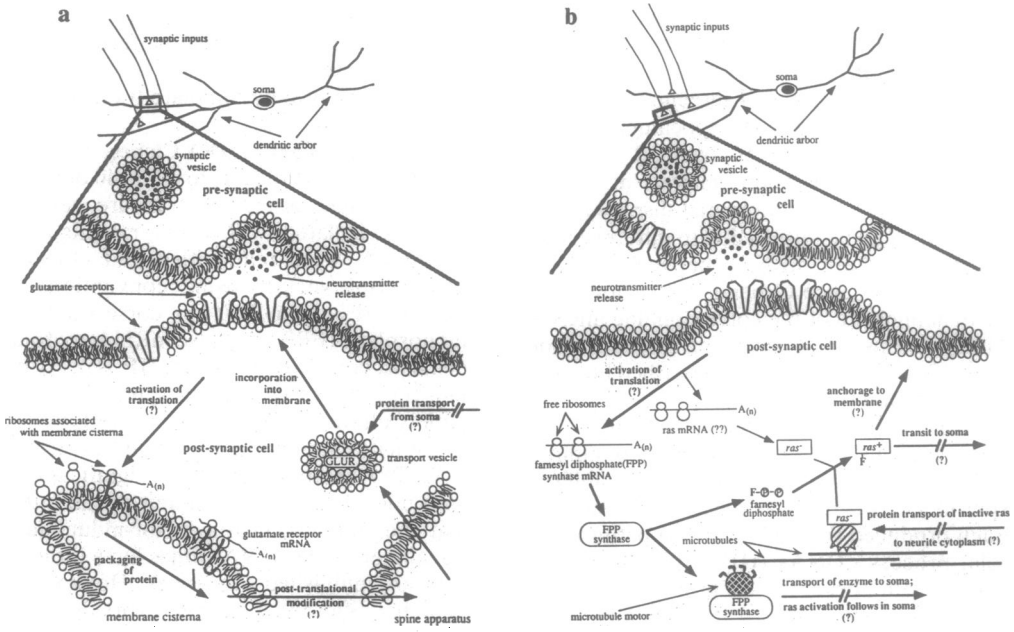


FIG. 4. Models implicating mRNA targeting and local protein synthesis mechanisms in neuronal functioning. (*a*) Schematic of the potential for steady-state maintenance or enrichment of glutamate receptors at synaptic sites. (*b*) Schematic of a mechanism for the generation of a nuclear response to synaptic signaling. It should be noted that in this model mammalian ras mRNA has not yet been characterized in the process and ras proteins may come from local protein synthesis or transport from the cell body. Although these models are shown for different dendritic regions, they may be present within the same region or in areas lacking synaptic input. Various assumptions, which seem most likely based upon current knowledge (highlighted with a question mark), are made for the transport mechanisms within the postsynaptic cell.

the activation of this second messenger cascade at different cellular sites may be part of an integrative mechanism whereby a strong stimulus or set of stimuli activate multiple synapses to elicit a nuclear response. This type of cooperation and association is well-established in other models of plasticity (35, 36).

While the present study addresses the identity and distribution of some of the mRNAs in processes, we have not investigated the complex mechanisms (2, 33) by which mRNAs may be transported to these cellular regions. In *Xenopus* oocytes, a localization signal present in the 3′ untranslated region of the mRNA sequence is integrally involved in directing Vg1 RNA to the vegetal hemisphere (37) and, in *Drosophila*, retaining bicoid RNA at the anterior tip (16). Similarly, in myocytes, α- and β-actin mRNAs are sorted to different cytoplasmic compartments by a localization element present in the 3′ untranslated region (34). Whether or not this is the case with any of the RNAs found in hippocampal neurites remains to be proven. By sequencing cDNA clones corresponding to full-length RNAs, it may be possible to address what role, if any, the primary sequence and/or secondary structural characteristics of the mRNA play as recognition elements in its targeting and transport.

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