Subcellular Compartmentation of Neuronal Protein Synthesis: New Insights into the Biology of the Neuron

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ABSTRACT: During the past few years, it has become well established that the distal structural/functional domains of the neuron contain numerous mRNAs. However, there is a paucity of information on the composition and function of these unique mRNA populations. In this article, we review recent evidence to support the hypothesis that protein synthesis occurs in multiple subcellular compartments in the neuron, to include the axon and presynaptic nerve terminal. The studies we describe use the squid giant axon and photoreceptor neuron as model invertebrate motor and sensory systems, respectively. Initial cell-free translation studies and molecular hybridization analysis established that the giant axon contained a heterogeneous population of polyadenylated mRNAs. The application of differential mRNA display methodology greatly facilitated the isolation and identification of 29 of these mRNAs, which encode cytoskeletal proteins, molecular motors, translation factors, various nuclear-encoded mitochondrial mRNAs, and several novel mRNA species. RT-PCR analysis of RNA from squid brain synaptosomes confirmed the presence of these mRNAs in the presynaptic nerve terminal. The presence of these mRNAs in polysomes purified from the synaptosomal fraction establish that these messengers are actively translated in the terminal. Results of *in vitro* labeling studies demonstrate that a significant fraction of the nuclear-encoded mitochondrial protein derives from the local synthesis in the terminal. This finding calls attention to the intimacy of the relationship that has evolved between the nerve terminal and its energy-generating system. The role that local protein synthesis might play in the mammalian nervous system and in the neuronal response to stress is discussed.

KEYWORDS: protein synthesis; synaptosomes; axons; polyribosomes; novel mRNAs; nuclear-encoded mitochondrial mRNAs; cytoskeleton; squid

INTRODUCTION

Although the majority of neuronal mRNAs are transcribed and translated in the neuronal cell soma, a subset of these gene transcripts is selectively transported to the distal structural/functional domains of the neuron to include the dendrite, axon,

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and presynaptic nerve terminal. Early findings derived from the squid giant axon, used as a model invertebrate motor neuron, indicated that the axon contained as many as 100 to 200 different polyadenylated mRNAs, a value that represented <1 percent of the total mRNAs expressed in the parental cell bodies. Results of quantitative RT-PCR analyses established that the relative abundance of these mRNAs in the axon differed markedly from that in the cell soma, a finding that suggested that these gene transcripts were being differentially transported into the axonal compartment.[1] The subcellular compartmentation of these mRNAs is thought to program the local synthesis of proteins that play a key role in the function of the axon and nerve terminal (for review, see Refs. 2 and 3). The presence of mRNA in the axon and growth cone has been confirmed in vertebrate systems.[4–8] Most recently, the local synthesis of protein has been shown to play a key role in the navigation of the growth cone,[9–11] in axon regeneration,[12] and in the synthesis of membrane receptors used as axon guidance molecules.[13] In invertebrate model systems, there is evidence to indicate that the local synthesis of protein in the presynaptic nerve terminal may be involved in activity-dependent synaptic plasticity such as long-term facilitation.[14,15]

To augment our understanding of the possible function(s) that the local protein synthetic system might play in the adult nervous system, we have used differential mRNA display methodology to more fully characterize the components of the axonal mRNA population. By identifying individual axonal mRNAs, we hoped to generate empirically hypotheses related to the function of this mRNA population. In this article, we provide the initial results of this unbiased screen and review the evidence for the occurrence of local protein synthesis in the axon and presynaptic nerve terminal in the adult nervous system. Based on these findings, it is postulated that local protein synthesis is involved in the maintenance of axonal and nerve terminal architecture, maintains the local transport and mRNA translation systems, and contributes to mitochondrial viability and function.

MATERIALS AND METHODS

Tissue Preparation and RNA Isolation

Squid (*Loligo pealii*) were obtained during the summer at the Marine Biological Laboratory (Woods Hole, MA). The giant axon and the giant fiber lobe (GFL), containing the cell bodies of the giant axon, were hand-dissected from the stellate ganglion and the axoplasm extruded using a Teflon roller.[16] A synaptosomal fraction was obtained from the optic lobe as described by Crispino *et al.*[17] Polysomes were isolated from the optic lobe and synaptosomes, and purified by sucrose density gradient centrifugation as previously described.[18] RNA was isolated from all tissue and cell fractions using TRIZOL reagent (Invitrogen).

Differential mRNA Display and Dot-Blot Hybridization Analysis

Experiments were performed using the Delta Differential Display Kit (Clontech) following recommended protocols. First-strand cDNA synthesis was conducted using total RNA combined with one of the nine anchored oligo(dT) primers provided

and MMLV reverse transcriptase. [^{32}P]-labeled amplicons were generated from the cDNA by PCR using pairwise combinations of the nine oligo (dT) primers and 10 arbitrary primers, Advantage 2 Polymerase Mix (Clontech). PCR products were displayed by electrophoresis on 5 percent polyacrylamide gels and were visualized by exposure to Biomax MR x-ray film (Kodak). cDNAs preferentially amplified from axoplasmic RNA were excised from the gels and were reamplified using the same primer sets. Reamplified cDNA was ligated into the vector pGEM-T Eazy (Promega) and cloned using standard procedures. Equimolar amounts of cDNA representing each clone was blotted onto a nylon filter support and was hybridized to [^{32}P]-labeled rat brain cDNA in 0.6 M NaCl, 1% SDS, and 100 µg/mL *Escherichia coli* sheared DNA at 55°C for 16 h. The filter blot was washed to a final stringency of 0.5× SSC at 55°C and was exposed to x-ray film overnight.

DNA Sequencing

Sequencing of the cDNA clones was carried out on a PE Applied Biosystems model 310 sequencer using Big Dye Terminator Cycle Sequencing Ready Reaction. To extend the sequence of the primary cDNA clones, we used 5′ and 3′ RACE technology using the First Choice RLM-RACE kit (Ambion). The RACE-extended cDNAs were amplified by PCR. Amplicons were isolated using the QIAquick purification kit (QIAGEN) and were inserted into pGEM-T Eazy vector for sequencing. Comparative sequence analyses were accomplished using the Blast X program to screen GENEBANK and SWISSPRO databases.

RESULTS

Identification of Axonal mRNAs

Previously, we had reported that the squid giant axon contained a diverse population of mRNAs that encoded key elements of the cytoskeleton, axon transport systems, and local energy metabolism.[19–21] To further define the composition of this mRNA population, we used differential mRNA display to compare mRNAs present in the giant axon and its cell bodies located in the GFL. The design of this experiment is outlined in FIGURE 1. PCR amplicons that revealed a higher relative abundance in the axon were excised from the gels and affixed to nylon filter supports. The cDNAs were subsequently screened for evolutionary conservation by dot-blot hybridization using radiolabeled rat brain cDNA as a probe. Approximately 25% to 30% of the squid axoplasmic cDNAs cross-reacted to the rat brain probe under conditions of moderate hybridization stringency. Hence, in principle, these mRNAs could encode proteins that serve a fundamental function in the invertebrate and mammalian nervous systems. Fifty of the cross-reacting cDNAs were cloned and sequenced to establish their identities. The mRNAs could be organized into several broad functional categories to include cytoskeletal proteins, molecular motors and chaperones, ribosomal proteins and translation factors, and several novel mRNAs (TABLE 1). The sequence similarity between the squid sequences and the mammalian homologs ranged from 78% to 93% at the amino acid level.

Surprisingly, the DNA sequence analysis also revealed four messages for nuclear-

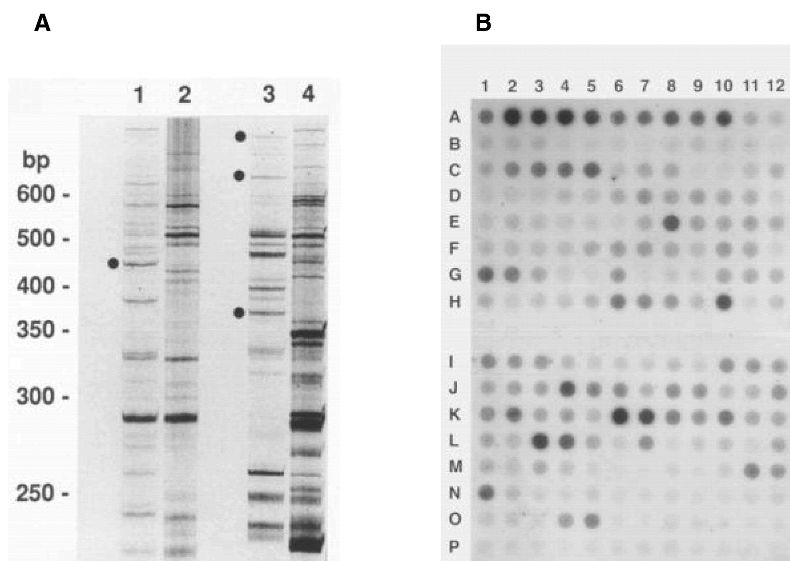

FIGURE 1. Experimental design used in the identification of axonal mRNAs. (**A**) Differential mRNA display. Total RNA from the giant axon (lanes 1 and 3) and the parental cell soma located in the GFL (lanes 2 and 4) were reverse transcribed into cDNA and fragments amplified by PCR, using a set of random oligonucleotide primers as described in MATERIALS AND METHODS. Amplicons from the axon and GFL were then displayed on urea-polyacrylamide sequencing gels. Darkened circles indicate amplicons preferentially expressed in the axon that were selected for further analysis. (**B**) Cross-species hybridization analysis. Selected axonal cDNAs were affixed to nylon filter supports and were hybridized to radiolabeled rat brain cDNAs as described in MATERIALS AND METHODS. A subset of clones that manifested evolutionary sequence conservation ($n = 50$) was selected for nucleic acid sequencing. The identity of these clones was established by comparative sequence analysis; the results are summarized in TABLE 1.

encoded mitochondrial proteins: COX17, cytochrome oxidase subunit Vb, dihydrolipoamide dehydrogenase, and CoQ7. In addition, the mRNA for HSP70, a chaperone known to facilitate the transport of cytosolically synthesized protein into the mitochondrion, was also delineated.

Nerve Terminals Contain a Heterogeneous mRNA Population

To test the hypothesis that the presynaptic nerve terminal contains mRNAs, we used a synaptosomal preparation obtained from the squid optic lobe. This preparation is comprised predominantly of the large nerve terminals (1–3 μm in diameter) of the retinal photoreceptor neurons.[17] In this experiment, total RNA was isolated from the synaptosomal fraction, and the presence of mRNA was determined by RT-PCR, using gene-specific primer sets for β-actin, β-tubulin, synapsin, neurofilament protein, COX17, and CoQ7. A primer set for the squid sodium channel, an mRNA shown previously to be abundant in the optic lobe but absent from the axon,

TABLE 1. mRNAs identified by cDNA analysis in the squid giant axon

• Cytoskeletal proteins	• Nuclear-encoded mitochondrial proteins
β-actin	Cytochrome oxidase assembly protein COX 17
α- and β-tubulin	Ubiquinone biosynthesis protein CoQ7
β-spectrin	Cytochrome oxidase subunit Vb
Neurofilament proteins	Dihydrolipoamide dehydrogenase
• Molecular motors	• Mitochondrial-encoded proteins
Kinesin	NADH dehydrogenase
MAP HI	Cytochrome C oxidase
• Ribosome-associated proteins	Cytochrome B
S5	ATP synthetase
L7A, L8, L9, L22	• Other proteins
Enlongation factor 2	Heat shock protein 70
• Metabolic enzymes	Ubiquitin
Enolase	• Novel proteins
Nucleoside diphosphate kinase	pA6
Fructose PTS enzyme II	pA127
	pA134

was used as an internal control.[20,21] The results of a typical experiment are shown in FIGURE 2. Amplicons for COX17 and CoQ7 were generated from microsomal and synaptosomal RNA, indicating that mRNAs for these proteins were present in the perikaryon and in the nerve terminal. In contrast, RNA for the sodium channel was largely absent in the synaptosomal fraction, indicating that the signals obtained for the nuclear-encoded mitochondrial mRNAs were not derived from microsomal contamination. The data obtained for tubulin mRNA support this conclusion (see below). Identical results were obtained for actin, neurofilament protein, and synapsin (data not shown). Amplicons for tubulin mRNA were not detected in synaptosomal RNA, although they were highly abundant in the microsomal fraction. This finding suggests that not all mRNAs located in the axon are present in the nerve terminal.

To determine if mRNAs were being translated in the nerve terminal, polysomes were isolated from synaptosomes and were subsequently displayed on linear sucrose density gradients. RT-PCR, using a battery of gene-specific primers, was conducted on the RNA isolated from the monosome and polysome fractions. The results of a typical experiment are shown in FIGURE 3. Here, CoQ7 sequences were readily amplified from the polysome fraction, providing strong evidence that this message was actively translated in the nerve terminal. The absence of amplicons generated from the sodium channel primers establishes that the synaptosomal polysome fraction is free of microsomal contamination. Identical results were obtained for COX17,[3] HSP70,[22] actin, and for the novel mRNAs pA6 and pA134 (data not shown).

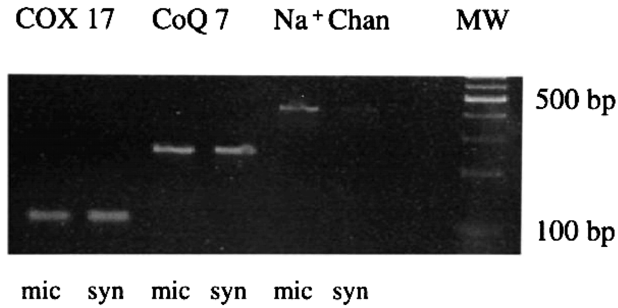

FIGURE 2. Synaptosomes contain a diverse population of mRNAs. RT-PCR analysis was performed on total RNA from the synaptosomal (syn) and microsomal (mic) fractions from the squid optic lobe. Note the appearance of COX17 and CoQ7 amplicons in the synaptosomal fraction and the relative lack of a PCR product for the sodium channel. MW, molecular weight ladder. Reproduced from Gioio *et al.*[18]

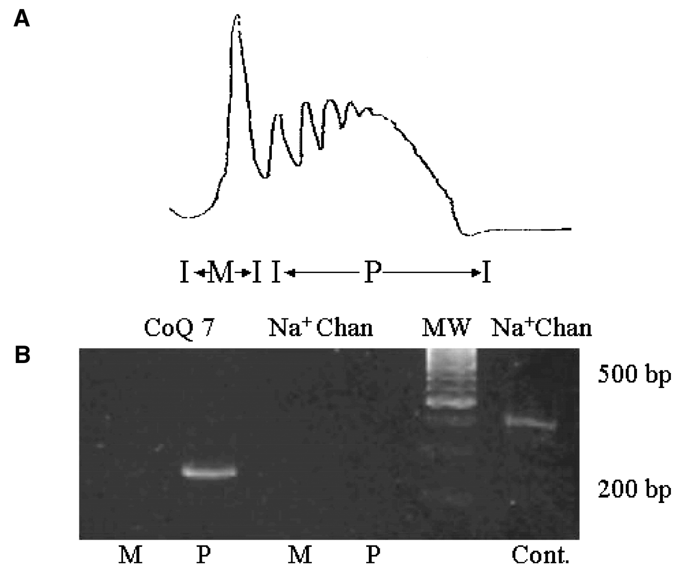

FIGURE 3. mRNAs are actively translated in the synaptosome. Polysomes were prepared from squid optic lobe synaptosomes as described in MATERIALS AND METHODS. (**A**) Polysomes were displayed on linear sucrose density gradients, and the gradients were divided into monosome (M) and polysome (P) fractions. UV absorbance of RNA was monitored continuously at 254 nm. (**B**) RT-PCR analysis of monosome and polysome fractions using gene-specific primer sets for CoQ7 and the sodium channel. bp, base-pairs. Reproduced from Gioio *et al.*[18]

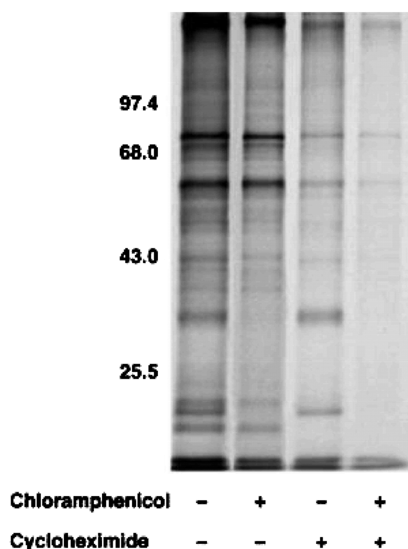

FIGURE 4. Synthesis of nuclear-encoded mitochondrial proteins in synaptosomes. Synaptosomes were incubated in artificial seawater containing radiolabeled methionine in the presence (+) or absence (–) of chloramphenicol and/or cycloheximide. Mitochondria were subsequently isolated by density gradient centrifugation, and proteins were fractionated by 10% SDS-PAGE (10 μg total protein/lane). Arrows indicate chloramphenicol sensitive translation products (i.e., endogenous mitochondrial protein synthesis). Reproduced from Gioio *et al*.[18]

Nuclear-Encoded Mitochondrial Proteins Are Synthesized in the Nerve Terminal

Based on the above findings, we evaluated the hypothesis that mitochondria derived some of their protein from local synthesis in the terminal. Toward this end, synaptosomes from the optic lobe were incubated in artificial sea water in the presence of [^{35}S]methionine and lysed by osmotic shock, and the mitochondria isolated by differential centrifugation. The antibiotics chloramphenicol and cycloheximide were used to discriminate between endogenous mitochondrial synthesis and cytoplasmic translation activity. Results of biochemical analysis indicated that approximately 20% to 25% of the total translational activity of the synaptosome was associated with the mitochondrial fraction and that the majority (~80%) of the newly synthesized mitochondrial protein derived from cytosolic polysomes.[18]

Comparison of the electrophoretic banding patterns of synaptosomal protein synthesized in the presence of cycloheximide or chloramphenicol establish that mitochondria obtain protein from endogenous and cytosolic polysomes (FIG. 4). For example, in the presence of chloramphenicol, at least two major bands (22 and 32 kD; FIG. 4, arrows) were eliminated, whereas the banding pattern of the majority of the newly synthesized mitochondrial proteins remained unchanged. In contrast, cycloheximide inhibited the synthesis of the majority of protein without diminishing the intensity of the 22- and 32-kD bands. These findings demonstrate that the nerve terminal synthesizes a wide variety of mitochondrial proteins.

DISCUSSION

We have provided several lines of evidence to support the hypothesis that protein synthesis occurs in discrete subcellular locations in the neuron. Although the evidence

reviewed here was derived from invertebrate motor and sensory neurons, similar findings have been reported in the mammalian nervous system.[8,12,23] These reports highlight the general applicability of the data generated from invertebrate model systems.

To gain insight into the function(s) of the local protein synthetic system, we initiated a characterization of the constituents of the mRNA populations present in the axon and nerve terminal, using differential mRNA display as an unbiased experimental approach. The 29 mRNAs identified to date could be grouped into several broad functional categories (see TABLE 1). This classification suggests that local protein synthesis could subserve the maintenance/remodeling of the cytoarchitecture of the axon and terminal and could maintain the local axon transport and mRNA translation systems.

Perhaps the most intriguing result of this investigation was the finding that the axon and terminal contained several nuclear-encoded mRNAs for mitochondrial proteins and molecular chaperones (e.g., HSP70) that facilitate the transport of cytosolically synthesized proteins into the mitochondrion. The radiolabeling experiments conducted in the synaptosomal preparation indicate that the majority of mitochondrial-associated proteins are synthesized locally and that 20% to 25% of the protein synthesized in the terminal is destined for this organelle. These findings call attention to the intimacy of the relationship that exists between the nerve terminal and its energy-generating system.

In view of the fact that the axonal mRNA population has been little studied, it is not surprising to encounter several novel mRNAs. Our initial biochemical and immunohistochemical findings on the novel pA6 mRNA indicate that it encodes a small, highly basic, mitochondrial-associated protein that is preferentially expressed in neurons of the squid and rodent nervous systems.[24] Preliminary data acquired on pA134 suggest that this mRNA codes for a 34-kD PTB-binding protein that might play a role in vesicular transport/recycling or receptor trafficking. Last, pA127 manifests significant sequence similarity to a selenoprotein, a family of recently discovered proteins that are thought to be involved in protection from oxidative damage. The expression and function of each of these novel mRNAs and their cognate protein are under investigation.

Results of a recent independent proteomics study of local protein synthesis in the synaptosome revealed the *de novo* synthesis of about 80 different proteins, as judged by two-dimensional polyacrylamide gel electrophoresis.[22] Consistent with the findings obtained at the mRNA level, mass spectrometric analysis of the newly synthesized protein established the presence of several cytosolic enzymes, cytoskeletal proteins, molecular chaperones, and nuclear-encoded mitochondrial proteins. In addition, the synthesis of a number of novel proteins was detected. Taken together, the results of these studies demonstrate that the presynaptic nerve terminals of squid photoreceptor neurons synthesize a wide variety of proteins involved in synaptic function and establish that the squid optic lobe synaptosomal preparation will serve as a rich resource for the identification of novel gene products.

One of the central tenants in neuroscience has been that protein synthesis occurs exclusively in the neuronal cell soma and that the molecules required for the growth, maintenance, repair, and plasticity of the distal domains of the neuron are provided by slow anterograde axonal transport. Transported proteins are subsequently targeted to their sites of utilization by undetermined intracellular mechanisms. However,

this somal-dominant, proteocentric view has not adequately addressed several fundamental issues in neuronal cell biology. For example, there is the issue pertaining to the considerable lag times encountered in the stimulus-induced synthesis of protein and the delivery of the translation products to their respective target sites. In view of the half-lives of the proteins being conveyed by slow transport and the distances to be transited, it is unclear how much of the transported material reaches its intended destination. To illustrate the magnitude of the problem, at the rate of slow transport, protein would require years to reach their final destinations in many large mammalian motor or sensory neurons. Consistent with this point, it has been reported that <3% of the protein transported in the ciliary ganglion reaches the nerve terminal (for detailed discussion, see Ref. 2). In addition, the mechanism(s) requisite for the delivery of the transported proteins to their sites of function is unclear. This proves to be a vexing issue in neurons that manifest a highly ramified dendritic arborization. Neurons that have terminals far removed from the cell soma, collateral axons, or a highly branched terminal field present similar conceptual difficulties. Hence, there exist significant gaps in our fundamental understanding of neuronal function, at least from a cellular and molecular point of view.

An alternative model of neuronal protein synthesis is illustrated in FIGURE 5. A key feature of this conceptualization is the selective transport of stable messenger ribonucleoprotein complexes (mRNPs) to the various structural/functional domains of the neuron. In contrast to protein transport, mRNPs are conveyed to these sites

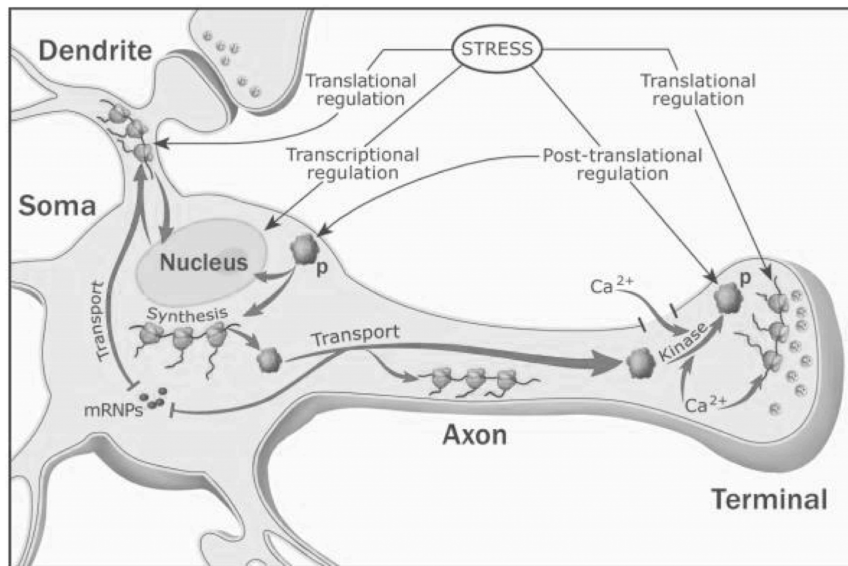

FIGURE 5. Model of neuronal protein synthesis and its response to stress. Protein synthesis occurs in multiple compartments within the neuron to include the dendrite, axon, and nerve terminal. Key features of the model include the rapid and selective transport of stable messenger ribonucleoprotein complexes (mRNPs) to the neuronal periphery and the local regulation of mRNA translation in response to neuronal activity.

rapidly via a kinesin-dependent, microtubule-based transport system. It is envisaged that axon and dendritic targeting information would reside in cis-acting regulatory elements present in the 3′ untranslated region (i.e., "zip codes") or in the secondary structure of the region (see for example, Ref. 25). A second desirable feature of the model is the allowance for the activity-dependent synthesis of significant amounts of protein directly at the target site. Oligodendrocytes use the same mechanisms in the transport and synthesis of myelin basic protein in the cytoplasmic processes actively involved in myelination.[26]

In the dendrite, the local synthesis of protein plays a key role in LTP/LTD, memory, and learning (for review, see Refs. 27 and 28) and can be altered by stress.[29] Last, the localization of CREB mRNA in the dendrite[30] raises the possibility of creating a positive feed-back system involving the local synthesis of transcription factors and their retrograde transport to the nucleus.

In conclusion, the features delineated in this model of neuronal protein synthesis would greatly augment the speed, malleability, specificity and selectivity of the cell's response to alterations in neuronal activity or its homeostatic milieu.

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