Chapter II

Dynamic Visualization of Local Protein Synthesis in Hippocampal Neurons

Summary

Using pharmacological approaches, several recent studies suggest that local protein synthesis is required for synaptic plasticity. Convincing demonstrations of bona fide dendritic protein synthesis in mammalian neurons are rare, however. We developed a protein synthesis reporter in which the coding sequence of green fluorescent protein is flanked by the 5′ and 3′ untranslated regions from CamKIIα, conferring both dendritic mRNA localization and translational regulation. In cultured hippocampal neurons, we show that BDNF, a growth factor involved in synaptic plasticity, stimulates protein synthesis of the reporter in intact, mechanically, or "optically" isolated dendrites. The stimulation of protein synthesis is blocked by anisomycin and not observed in untreated neurons. In addition, dendrites appear to possess translational hot spots, regions near synapses where protein synthesis consistently occurs over time.

Introduction

The discovery that polyribosomes are located near the base of many spines (Steward and Levy, 1982) in the hippocampus suggested the possibility that neuronal proteins can be synthesized in dendrites. In theory, the synthesis of proteins in dendrites provides a mechanism by which synapses can independently control their strength, circumventing the need for precisely addressed protein transport from the soma (Schuman, 1999). In the context of synaptic plasticity, then, the ability to locally synthesize proteins allows synapses to solve the problem of maintaining "specificity" and obtaining the newly

synthesized proteins required for long-term synaptic plasticity (Frey et al., 1988; Kang et al., 1997; Nguyen et al., 1994; Otani et al., 1989; Stanton and Sarvey, 1984).

In the past 5 years, several studies have shown that locally synthesized proteins likely contribute to long-lasting synaptic plasticity (reviewed in Schuman, 1999; Steward and Schuman, 2001; Wells et al., 2000). In hippocampal slices, BDNF-induced synaptic plasticity is blocked by inhibitors of protein synthesis (Kang and Schuman, 1996). In the same study, Schaffer-collateral CA1 synapses that were isolated from their pre- and postsynaptic cell bodies still exhibited protein synthesis-dependent plasticity, suggesting a local, dendritic source of protein synthesis. A similar dependence on dendritic protein synthesis has been observed for metabotropic receptor-induced LTD at Schaffer-collateral CA1 synapses in the hippocampus (Huber et al., 2000). Long-term facilitation induced by 5-HT at cultured sensory motoneuron synapses in Aplysia also shows a requirement for local protein synthesis in the sensory neuron (Casadio et al., 1999; Martin et al., 1997). In addition, 5-HT application to isolated sensory neurites results in new protein synthesis (Martin et al., 1997). Most demonstrations of dendritic protein synthesis have relied on biochemical fractionation techniques to isolate fragments of dendrites and postsynaptic spines (e.g., the synaptoneurosome). In these studies, the incorporation of radiolabeled amino acids into new proteins demonstrated that synthesis can clearly occur in these dendritically derived fractions (Rao and Steward, 1991; Weiler and Greenough, 1991, 1993). The use of a cell culture system in which the cell bodies are separated from the dendrites also showed that isolated dendrites can synthesize proteins (Torre and Steward, 1992) and glycosylate proteins (Torre and Steward, 1996). The drawbacks of the above

techniques include the possibility of contamination by nondendritic fractions, the removal from a physiological context, and the lack of temporal resolution. Here we describe the development of a high-fidelity dendritic protein synthesis reporter and show unequivocally that protein synthesis can be stimulated in dendrites by BDNF, a growth factor involved in synaptic plasticity.

Results

BDNF Stimulates Protein Synthesis of a GFP Reporter in Hippocampal Neurons

In order to examine dendritic protein synthesis dynamically in living neurons, we constructed a green fluorescent protein (GFP) reporter, flanked by the 5′ and 3′ untranslated regions (UTR) from the Ca2+/calmodulin-dependent kinase II-α subunit (CamKIIα) (5′GFP3′). Previous work has shown that the 3′UTR of the CamKIIα mRNA contains information sufficient for its dendritic localization (Mayford et al., 1996; Mori et al., 2000). In initial experiments, the 5′GFP3′ reporter was introduced into cultured neurons using Biolistics. In expressing neurons, GFP was present in the soma and the dendrites, as indicated by immunolabeling for the dendritic marker MAP2 (Figure 2.1A). In most untreated neurons, expression of the reporter was robust in the cell bodies and relatively weak in the associated dendritic processes (Figure 2.1B). We examined whether exposure to BDNF modified the levels and/or pattern of GFP expression in neurons; 6 hr after transfection, dishes were exposed to either BDNF (50 ng/ml) or a control (HBS) solution for 4 hr. Neurons that were exposed to BDNF exhibited an increase in GFP synthesis that was evident in both the cell body and the dendrites (Figure 2.1B). The analysis of total

fluorescence in the dendrites revealed that BDNF-treated neurons had significantly greater quantities of GFP throughout the length of the dendritic process (Figure 2.1C). These experiments demonstrate that BDNF can stimulate protein synthesis in hippocampal neurons but do not indicate the cellular compartment (e.g., dendrites and/or soma) where the synthesis is occurring.

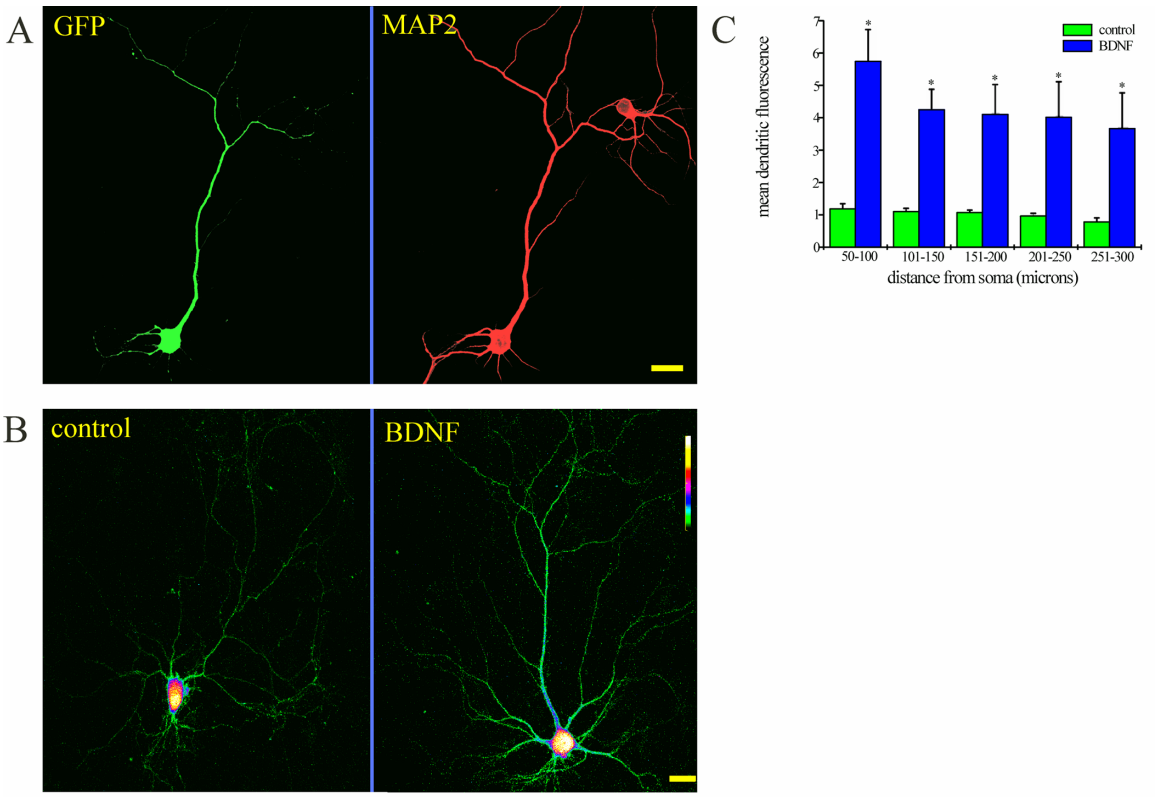

Figure 2.1 BDNF Stimulates Protein Synthesis of the GFP Reporter in Hippocampal Neurons

(A) Shown is a cultured hippocampal neuron expressing the GFP reporter and immunostained for the dendritic marker MAP2. The majority of the GFP signal occurs in the dendrites, as indicated by the coincident MAP2 signal. Scale bar = 15 µm.

(B) Shown are an untreated and BDNF-treated neuron expressing the GFP reporter. The BDNF-treated cell shows enhanced fluorescence in the cell body and dendrites when compared to the untreated cell. Color lookup bar shows that the absence of GFP signal is indicated by black, increasing fluorescence is indicated by transitions to green, blue, red, and yellow, and saturated fluorescence is indicated by white. Scale bar = 15 µm.

(C) Summary data for all untreated (n = 7) and BDNF-treated (n = 10) dendrites. BDNF-treated neurons showed significantly greater fluorescence ($p < 0.01$) in all dendritic compartments (e.g., 50–300 µm from the soma).

Time-Lapse Imaging of BDNF-Stimulated Translation

In an effort to ascertain the source of the increased GFP synthesis apparent in the above experiments, we conducted time-lapse imaging. We monitored the localization and

levels of the GFP reporter over time in individual neurons before and after BDNF treatment. To facilitate the expression of the reporter in a larger population of neurons, we incorporated the reporter construct into a Sindbis virus system (see Experimental Procedures.) We used a destabilized version of GFP, dGFP, in order to decrease the cumulative fluorescence that ultimately contributes to signal saturation. Dishes of cultured hippocampal neurons were infected with Sin-5′dGFP3′; initial images were collected 12 hr after infection, at a time when the fluorescence had reached steady-state levels. Untreated neurons, imaged over a 4 hr period, showed stable or declining fluorescence in the dendrites and cell body over time (Figures 2A and 2B). In contrast, neurons that were treated with BDNF showed increases in GFP fluorescence that were evident within 60 min of BDNF addition (Figures 2A and 2B). BDNF-induced increases in fluorescence were apparent in both the dendritic and somatic compartments. Of particular interest was the observation of increases in fluorescence in remote aspects of the dendrites (see boxed regions in Figure 2.2A); these increases were detected as early as the increases observed in the cell body, consistent with the notion that GFP is synthesized locally. Overall, when the total length of the dendrite was analyzed, we found that only BDNF-treated neurons showed significant increases in dendritic GFP fluorescence; the average increase in fluorescence was roughly 60%. This is likely a very conservative estimate of BDNF's actions since the analysis includes both synaptic and nonsynaptic areas of the dendrite. For example, our analysis of changes at individual “hot spots,” which may correspond to synaptic sites (see below), indicates that BDNF-induced increases in GFP fluorescence ranged from ~1- to 8-fold. Untreated neurons showed no significant increase in dendritic fluorescence when examined over the same time periods (Figure 2.2C). In addition, the

BDNF-induced increases were prevented by coapplication of the protein synthesis inhibitor anisomycin (Figure 2.2C). In BDNF-treated neurons, we also observed, however, what appeared to be the diffusion of GFP from the soma into the dendrite. This observation prevented us from concluding, unambiguously, that all of the increases in dendritic GFP we observed were due to local synthesis.

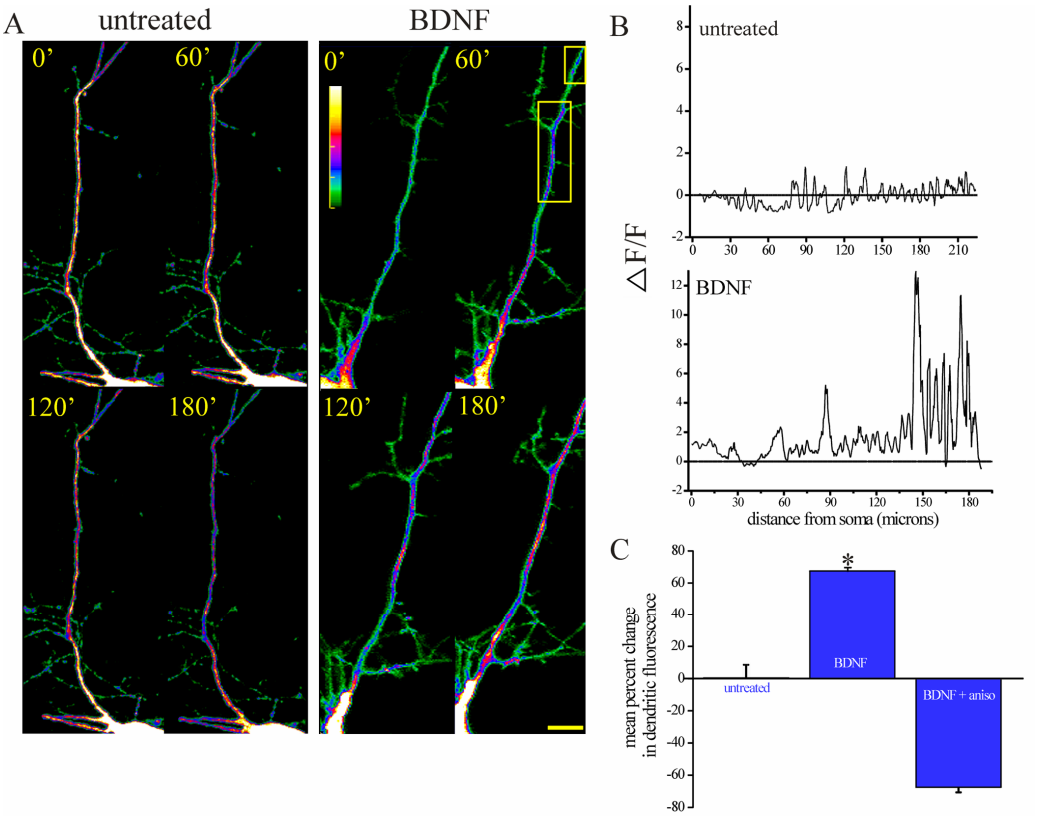

Figure 2.2 Time-Lapse Imaging of BDNF-Stimulated Translation

(A) Repeated images of a control neuron and BDNF-treated neuron. BDNF was added immediately after the 0 min image was acquired. The BDNF-treated neuron showed increased fluorescence in the dendrite whereas the control neuron was relatively stable over time. Scale bar = 15 μm.

(B) Analysis of the individual neurons shown in (A). ΔF/F was calculated using the data from the 0 and 120 min images (see Experimental Procedures).

(C) Summary data for analysis of total dendritic length showing that only dendrites treated with BDNF exhibited significant ($p < 0.01$) increases in fluorescence.

BDNF Stimulates Protein Synthesis in Healthy, Mechanically Isolated Dendrites

In order to remove the cell body as a potential source of GFP signal, we performed dendritic transections in which the dendrites were physically isolated from the cell bodies using a micropipette. Ensuring neuron health and viability following such transections was a major concern. Of approximately 300 transections performed over a 2-year period, only 10 transected neurons fulfilled the health and viability criteria we established for use in experiments (see Experimental Procedures). Technical difficulty aside, the transected dendrite can provide the most unambiguous proof of local protein synthesis. As before, neurons were infected with Sin-5′dGFP3′. Transected dendrites that were not treated with BDNF usually showed declining fluorescence when monitored over time (Figure 2.3). In contrast, transected dendrites treated with BDNF exhibited increases in fluorescence in the isolated dendrites (Figure 2.4). As would be expected, BDNF-induced increases in fluorescence were also observed in the soma and the intact dendrites. The BDNF-induced increases in GFP fluorescence observed in the dendrites were blocked by cotreatment with anisomycin, indicating that the enhanced fluorescence was due to new protein synthesis (Figure 2.5). Plotting the distribution of changes in pixel intensity over time demonstrated that most regions of transected dendrites treated with BDNF showed increases in intensity (Figure 2.5C). In contrast, most regions of untreated dendrites or those treated with anisomycin plus BDNF tended to decrease in intensity. (Note that the small number of pixels that increased in intensity in the presence of anisomycin must represent the redistribution of pixels from adjacent areas of the dendrite or the contribution of synthesized, but not yet fluorescent GFP, e.g., Cubitt et al., 1995.) Taken together, these

data clearly show that BDNF can stimulate protein synthesis in isolated dendrites. The local dendritic protein synthesis we observed was robust and stable over time.

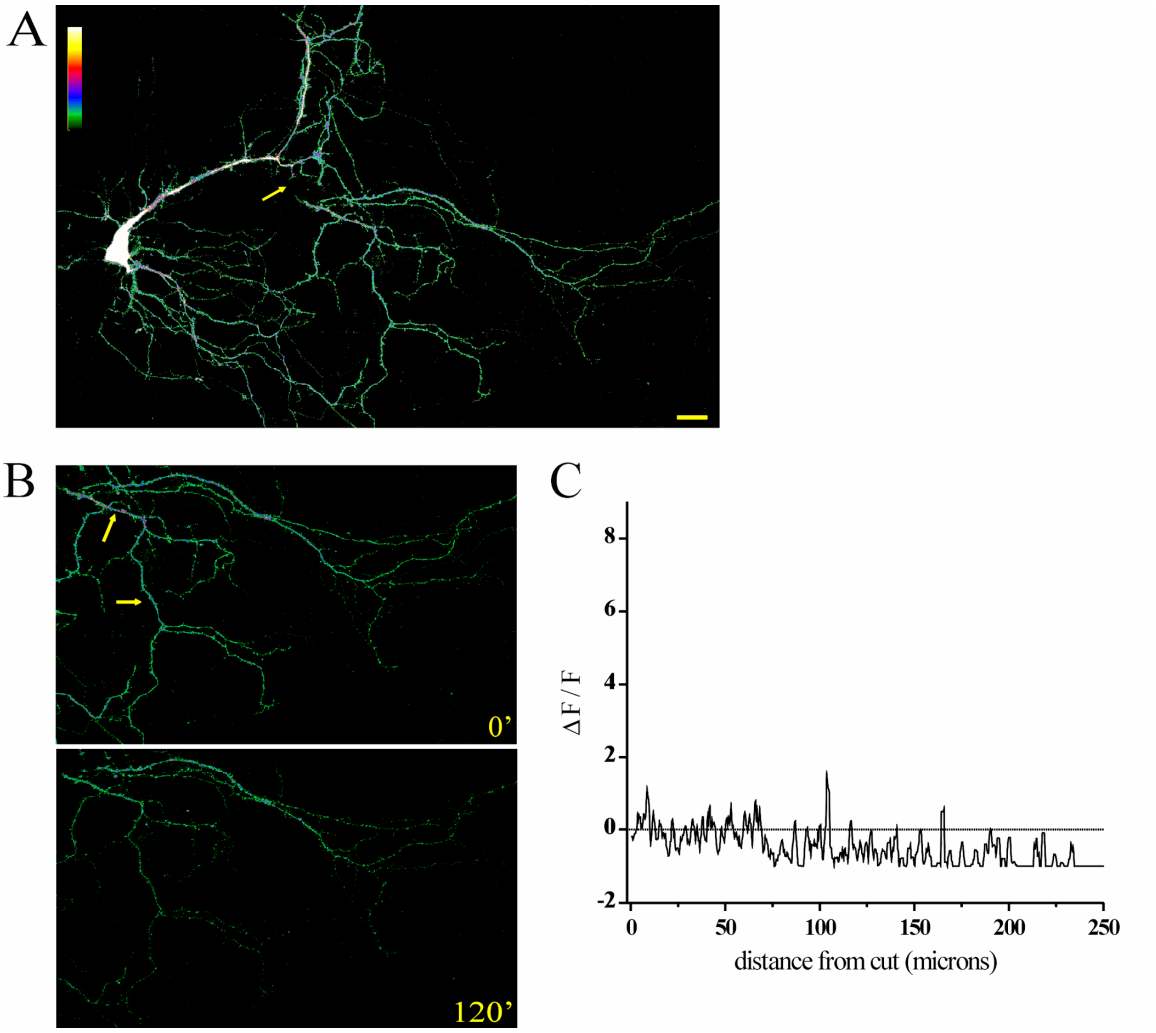

Figure 2.3 Untreated, Transected Dendrites Do Not Show Increases in Protein Synthesis

(A) Image of an infected neuron; arrow points to the region of transection. Scale bar = 15 μm.

(B) Images of the isolated region of the dendrite immediately following transection and 120 min later. The fluorescent signal in the dendrite decreases over time. Arrows point to the dendrite chosen for analysis in (C). The top dendrite was also analyzed and included in the group analysis (Figure 2.5).

(C) Analysis of the transected dendrite shown in (A) and (B). ΔF/F was calculated using the data from the 0 and 120 min images (see Experimental Procedures).

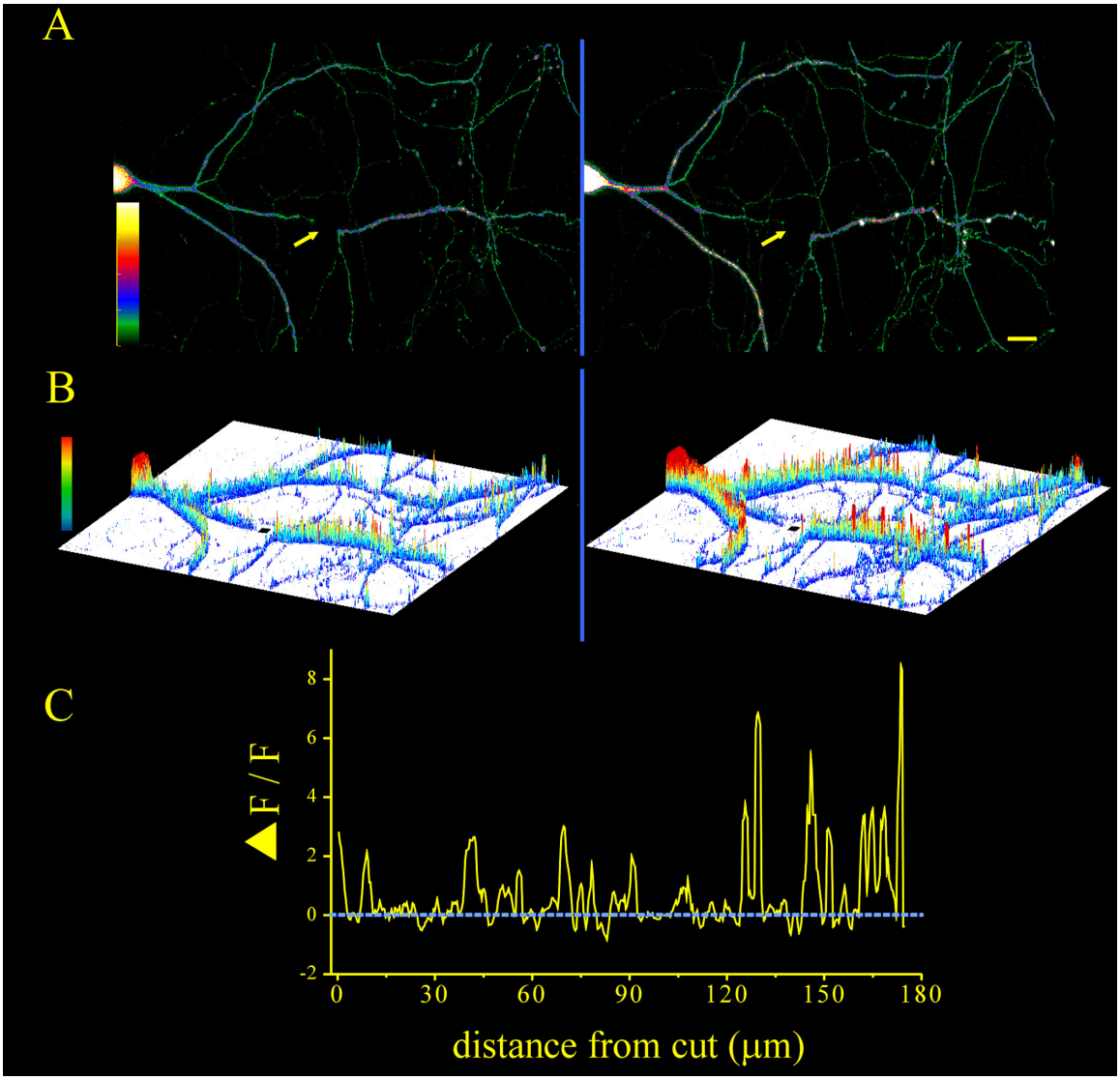

Figure 2.4 BDNF Stimulates Protein Synthesis in Healthy, Mechanically Isolated Dendrites

(A) Images of transected neuron before (left) and 120 min after (right) BDNF treatment; arrow points to the region of transection. The fluorescent signal in the transected dendrite increases following BDNF treatment. Scale bar = 15 μm.

(B) Surface plot of the neuron shown in (A) in which changes in fluorescence are indicated by both changes in color and changes in the height of the pixels shown.

(C) Analysis of the transected dendrite shown in (A) and (B). ΔF/F was calculated using the data from the 0 and 120 min images (see Experimental Procedures).

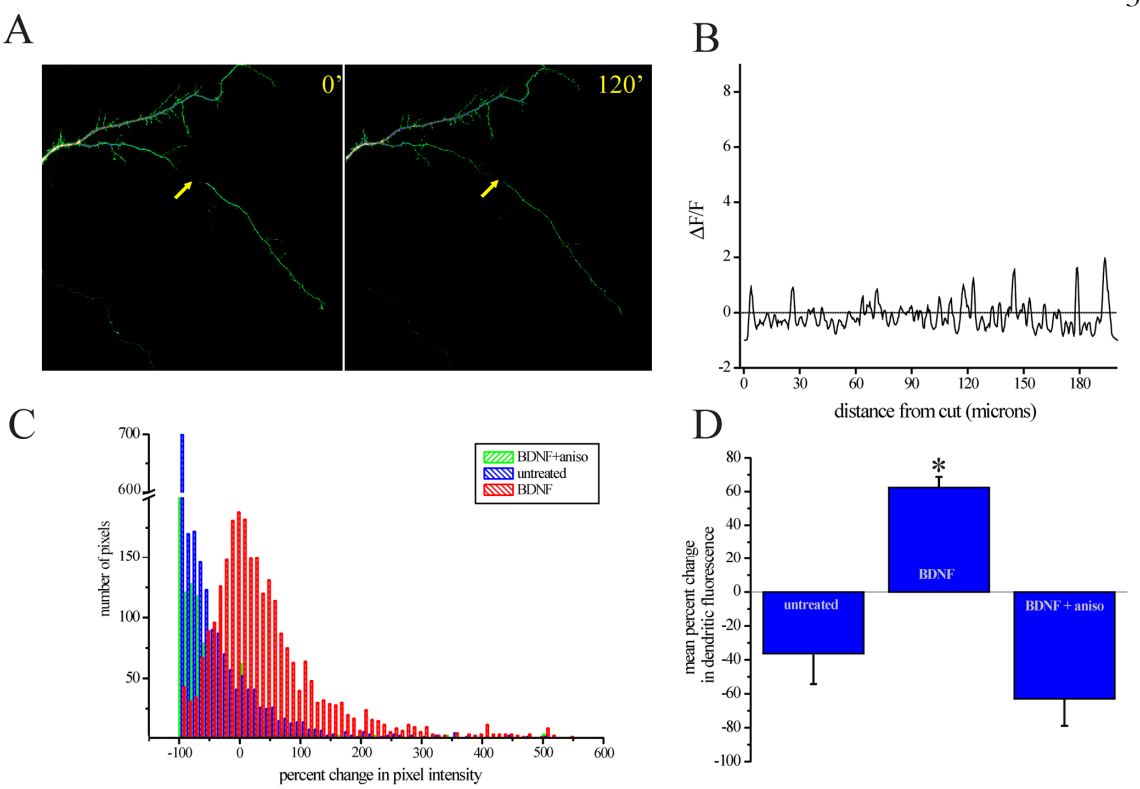

Figure 2.5 Anisomycin Prevents BDNF-Induced Increases in Protein Synthesis in Transected Dendrites

(A) Images of a transected dendrite before and 120 min after coapplication of BDNF and anisomycin; arrow points to the region of transection. The fluorescent signal in the transected dendrite decreased over time. Arrows indicate the site of transection. Scale bar = 15 μm.

(B) Analysis of the transected dendrite shown in (A). ΔF/F was calculated using the data from the 0 and 120 min images (see Experimental Procedures).

(C) Summary histogram indicating the pixel intensity distributions for all untreated, BDNF-treated, and BDNF plus anisomycin-treated transected dendrites.

(D) Summary diagram indicating the mean percent change in pixel intensity for the three groups. Only the BDNF-treated dendrites showed a significant increase in fluorescence intensity over time ($p \leq 0.01$). N (cells, dendrites) for each group are as follows: untreated (3, 4); BDNF (4, 5); BDNF + aniso (3, 4).

A Membrane-Anchored GFP Reporter Exhibits Extremely Limited Diffusion

Because of technical difficulty and the potential for compromising long-term neuronal health, the transection experiments are not a viable option for extensive explorations of local protein synthesis. We reasoned that modifications to the protein synthesis reporter that limited its diffusion would also decrease the potential contribution of somatically synthesized GFP to the signal observed in dendrites. Toward this end, we conferred membrane localization to the reporter by adding a myristoylation consensus sequence (Patrick et al., 1999) at the N terminus of the GFP molecule and expressed this construct, Sin-5′myrdGFP3′, in neurons using Sindbis virus. We compared the diffusion of Sin-5′dGFP3′ and Sin-5′myr dGFP3′ by conducting FRAP (fluorescence recovery after photobleaching) experiments in dendrites. Note that the recovery of reporter fluorescence in dendrites following photobleaching is due to both diffusion from the adjacent (nonbleached) compartment as well as new synthesis of the reporter in the bleached domain. To monitor the contribution of diffusion exclusively, we included anisomycin in the bath. In the nonmembrane anchored version of the reporter (Sin-5′dGFP3′), there was substantial recovery of fluorescence in the bleached dendrite within 60 min (Figure 2.6A). We found that the addition of the myr sequence, however, severely retarded the diffusion of the modified (Sin-5′myrdGFP3′) reporter (Figure 2.6A). Negligible recovery from the photobleached state was observed in the 120 min following the photobleaching episode. These data indicate that the myristoylated reporter exhibits limited diffusion (see Experimental Procedures), suggesting that it can be used to faithfully report local protein synthesis in intact dendrites.

BDNF Stimulates Protein Synthesis in Healthy, "Optically Isolated" Dendrites

We next used the diffusion-restricted reporter (Sin-5′myrdGFP3′) in combination with photobleaching to examine dendritic protein synthesis in intact neurons. In these experiments, we continuously photobleached the cell body in order to abolish the contribution of somatically synthesized GFP to the dendritic signal; in this way we "optically isolated" the dendrites of interest. The continuous photobleaching of the soma did not compromise neuronal health: propidium iodide labeling of bleached cells revealed no incorporation of the dye (see Experimental Procedures). When we analyzed untreated, optically isolated dendrites, we found that the fluorescence of the reporter decreased over time at most dendritic sites (Figures 6B and 6C). We occasionally observed small (e.g., 0- to 5-fold) fluorescence increases at some sites. These small increases in signal reflect either the redistribution of GFP from adjacent dendritic sites or bona fide new protein synthesis. The fact that both untreated and anisomycin-treated dendrites showed similar average fluorescence change profiles (Figure 2.8) suggests that most of these small increases reflect redistribution from adjacent portions of the dendrite.

In contrast to untreated neurons, the addition of BDNF to optically isolated dendrites resulted in a robust stimulation of protein synthesis. As shown in Figure 2.7, increases in reporter translation ranging from 1- to 17-fold were observed at many sites along optically isolated dendrites. Sites of decreased fluorescence were not common in BDNF-treated dendrites. The coapplication of anisomycin completely prevented the BDNF-induced increases in GFP fluorescence, confirming that the observed effects of BDNF were due to new protein synthesis (Figure 2.8). Dendrites that were treated with

anisomycin alone or anisomycin plus BDNF usually showed decreases in fluorescence along the length of the dendrite interspersed with very small increases that likely represented redistribution of GFP molecules from adjacent regions of the dendrites (Figure 2.8).

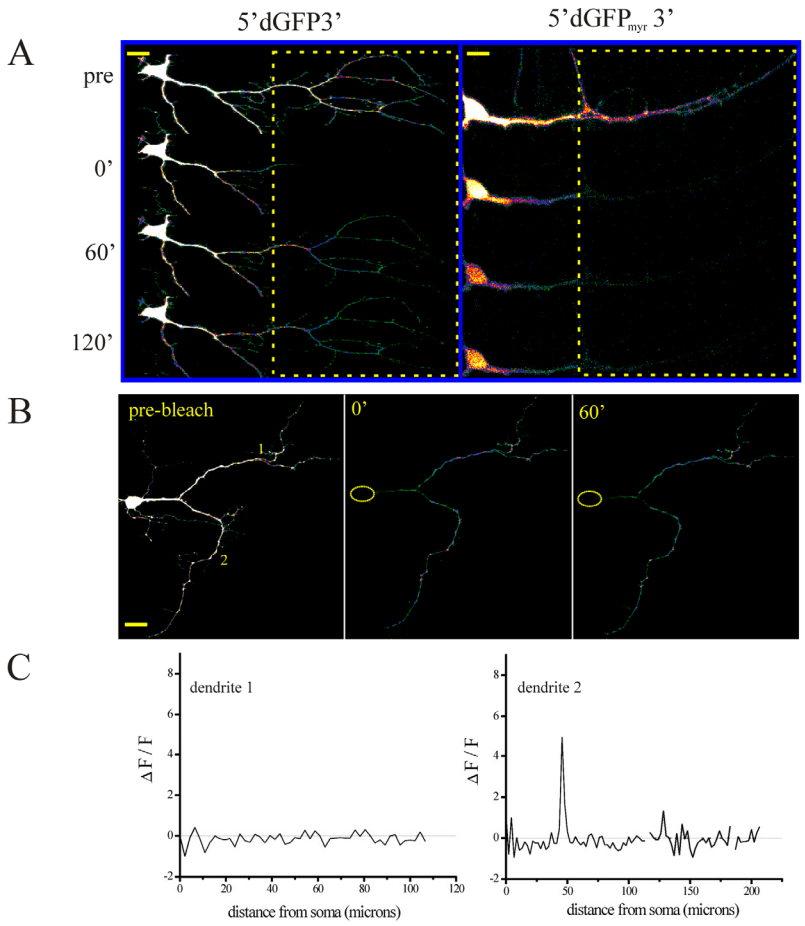

Figure 2.6 A Membrane-Anchored GFP Reporter Exhibits Limited Diffusion

(A) Shown are two neurons infected with either 5′dGFP3′ or the membrane-anchored 5′$_{myr}$dGFP3′. Neurons were treated with anisomycin for 1 hr prior to the initiation of photobleaching (boxed region shows bleached area). FRAP was monitored in each neuron over time. The neuron infected with the diffusible reporter (5′dGFP3′) showed significant recovery of fluorescence within 60 min of the photobleaching. In contrast, the myristoylated reporter showed negligible recovery within 2 hr following photobleaching. Scale bars: 5′dGFP3′, 15 μm; 5′$_{myr}$dGFP3′, 10 μm.

(B) Time-lapse images of a 5′$_{myr}$dGFP3′-expressing neuron that was subjected to somatic photobleaching for the duration of the experiment. The prebleached neuron is shown at the right and two consecutive time points following photobleaching are shown in the middle and left. In this untreated neuron there was an overall decline in dendritic fluorescence during the experiment. Scale bar = 15 μm.

(C) Profile of fluorescence changes between 0 and 60 min for dendrites 1 and 2 (labeled in [B]). In the profiles shown, the mean change in fluorescence between t = 0 and t = 60 was −15.4% and −12.3% for dendrite 1 and 2, respectively.

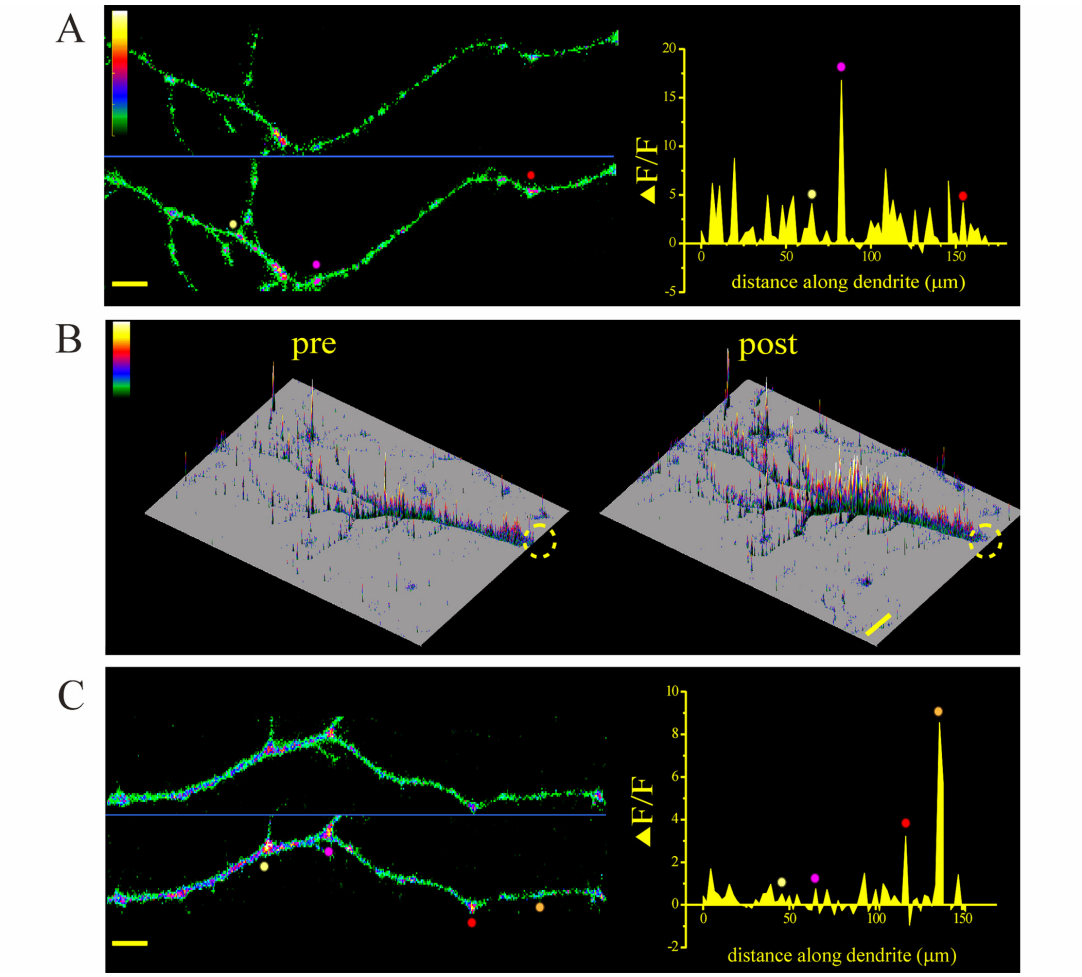

Figure 2.7 BDNF Stimulates Protein Synthesis in Healthy, "Optically Isolated" Dendrites

(A) Shown is BDNF-induced enhancement of GFP translation in an optically isolated dendrite at two consecutive time points. The profile of fluorescence change between the two time points shown is plotted on the right. Individual hot spots are identified by colored circles on the image and the corresponding profile. Scale bar = 5 μm.

(B) A surface plot of a different neuron in which the dendrites were optically isolated. The region of the bleached soma is shown by the dashed circle. The effects of BDNF are evident in comparing the dendritic fluorescence in the pre and post images. Scale bar = 15 μm.

(C) An isolated dendritic segment from the neuron shown in (B) at two different time points. The profile of fluorescence change between the two time points shown is plotted on the right. Individual hot spots are identified by colored circles on the image and the corresponding profile. Scale bar = 5 μm.

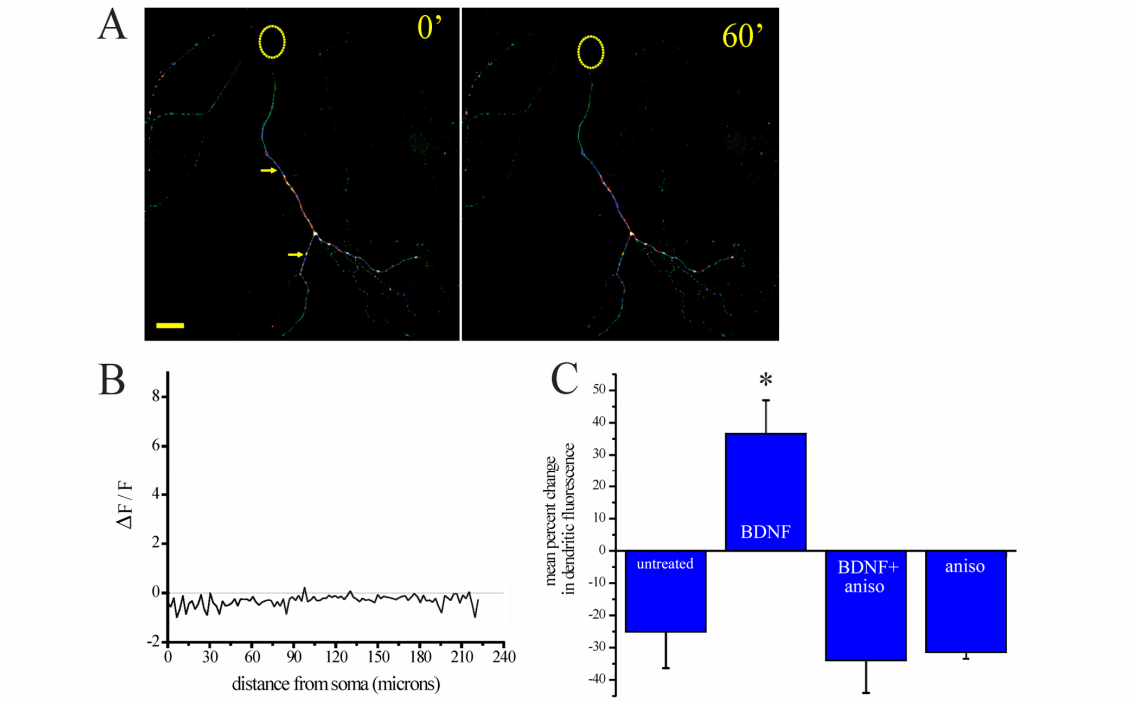

Figure 2.8 Anisomycin Blocks BDNF-Induced Increases in Protein Synthesis in Optically Isolated Dendrites

(A) Time-lapse images of an optically isolated dendrite that was treated with BDNF plus anisomycin. The optically isolated dendrites are shown at two consecutive time points. There was an overall decline in dendritic fluorescence during the experiment. The area occupied by the photobleached soma is shown by the yellow circle. Scale bar = 15 μm.

(B) Profile of fluorescence changes between 0 and 60 min for dendrite indicated by the arrow in (A). The mean change in fluorescence between t = 0 and t = 60 was −25.7%.

(C) Summary diagram indicating the mean percent change in pixel intensity for all untreated, BDNF-, and BDNF plus anisomycin-, and anisomycin-treated optically isolated dendrites. Only the BDNF-treated dendrites showed a significant increase in fluorescence intensity over time ($p \leq 0.01$). N (cells, dendrites) for each group are as follows: untreated (4, 6); BDNF (5, 8); BDNF + aniso (4, 5); aniso (3, 5).

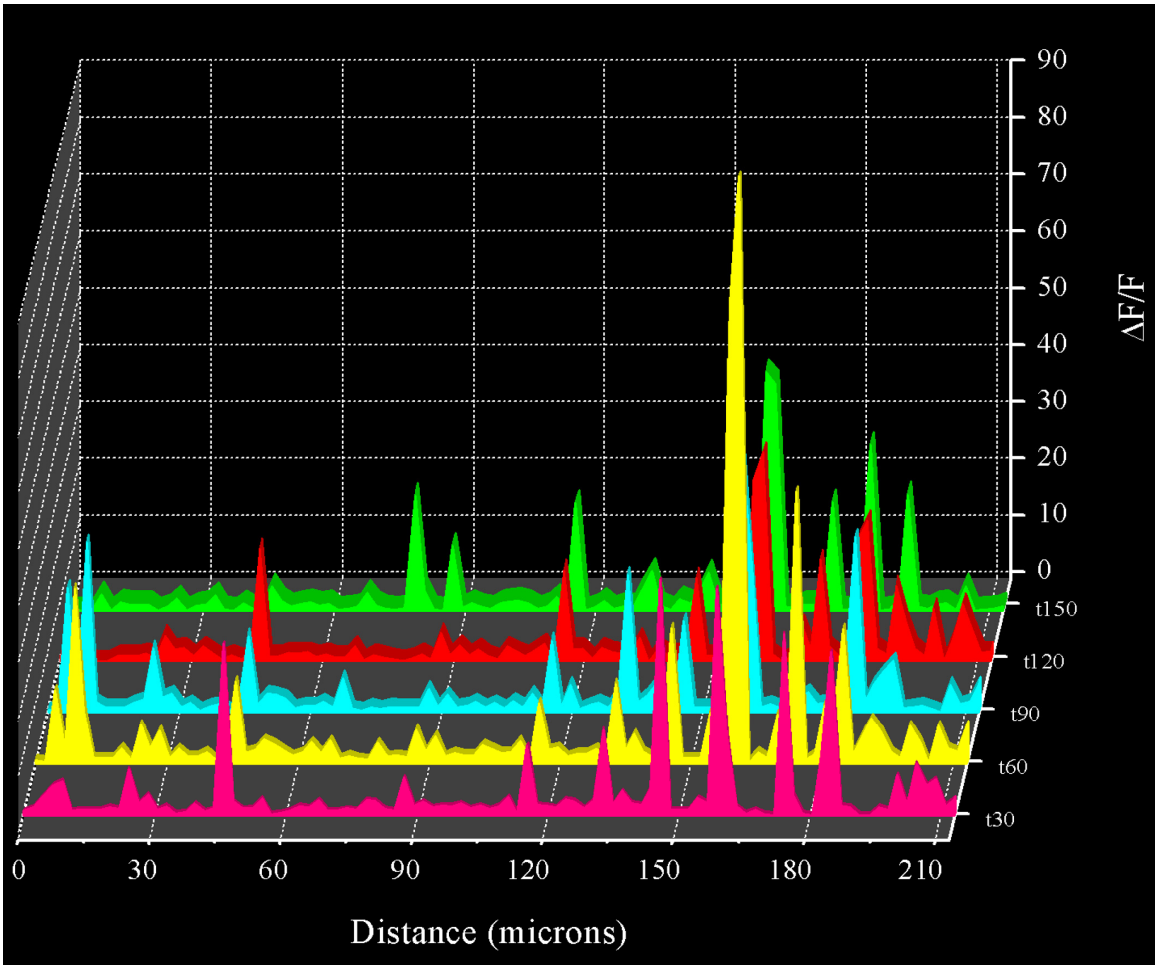

Figure 2.9 GFP Reporter Signals Are Spatially and Temporally Persistent

Shown are the ΔF/F profiles for a single optically isolated dendrite at several time points (t30 – t150 minutes). The increased GFP signal is concentrated at relatively stable sites along the length of the dendrites. These sites also appear to be temporally stable over a 2 hr time period.

The Protein Synthesis Reporter Is Concentrated near Sites of Translation and Synapses

Repeated imaging of optically isolated dendrites allowed us to examine the location of GFP signals over time. When the fluorescence intensity profiles derived from time-lapse imaging of an individual dendrite were plotted together, it became clear that the GFP signals appeared to be spatially concentrated at hot spots that were stationary over time

(Figure 2.9). The increases and occasional decreases in GFP signal that were observed over time tended to appear at the same locations along the dendrite. We next examined whether these reporter hot spots were in the vicinity of synaptic sites and/or sites of protein translation. We immunolabeled 5′myrdGFP3′-expressing cells with an antibody to the postsynaptic marker PSD-95, the presynaptic marker synapsin I, or rRNA (Y10B; Koenig et al., 2000; Lerner et al., 1981). We found that the GFP hot spots often were near ribosomes or synaptic regions as indicated by the proximity of the PSD-95, synapsin, or Y10B signal to GFP (Figure 2.10). The colabeling for PSD-95 also revealed that the myristoylated reporter appeared not to enter synaptic spines to an appreciable extent and appeared more concentrated in dendritic shafts. As such, we did not expect to observe strict "colocalization" of the GFP signal with the synaptic markers. We did observe, however, that GFP was, much more often than not, in the vicinity of ribosomes and synapses. To quantify this relationship, we calculated the mean fluorescence for each signal across the dendritic width (thus obtaining mean fluorescence values for the entire length of each dendrite), and calculated the pairwise cross-correlation of GFP and PSD-95, synapsin, or Y10B. A cross-correlation measures the spatial coincidence of the two signals, with the lag value representing the distance one signal must be shifted in order to spatially correlate with the other signal. Analysis of the GFP/Y10B, GFP/synapsin, or GFP/PSD-95 (data not shown) signals revealed a significant cross-correlation between the two signals (Figure 2.10). The peaks at zero lag for both the GFP/Y10B and GFP/synapsin analysis (Figures 10F and 10G) indicate that the two signals are highly correlated. The observation that locally synthesized GFP is concentrated in the vicinity of ribosomes and synapses suggests that there are local hot spots of translation that are near synaptic sites.

These data are predicted to some extent by previous anatomical observations of ribosomes at or near the bases of dendritic spines (Steward and Levy, 1982). The spatially and temporally stable sites of translation that we have identified provide evidence for a central tenet of the local protein synthesis hypothesis—the notion that locally synthesized proteins might be selectively made available to their associated synapses, thus providing a mechanism for synapse specificity (Schuman, 1999a; Steward, 1997; Steward and Schuman, 2001). Whether these observations hold true for other types of locally synthesized proteins (e.g., nonmyristoylated) is an important issue for future studies.

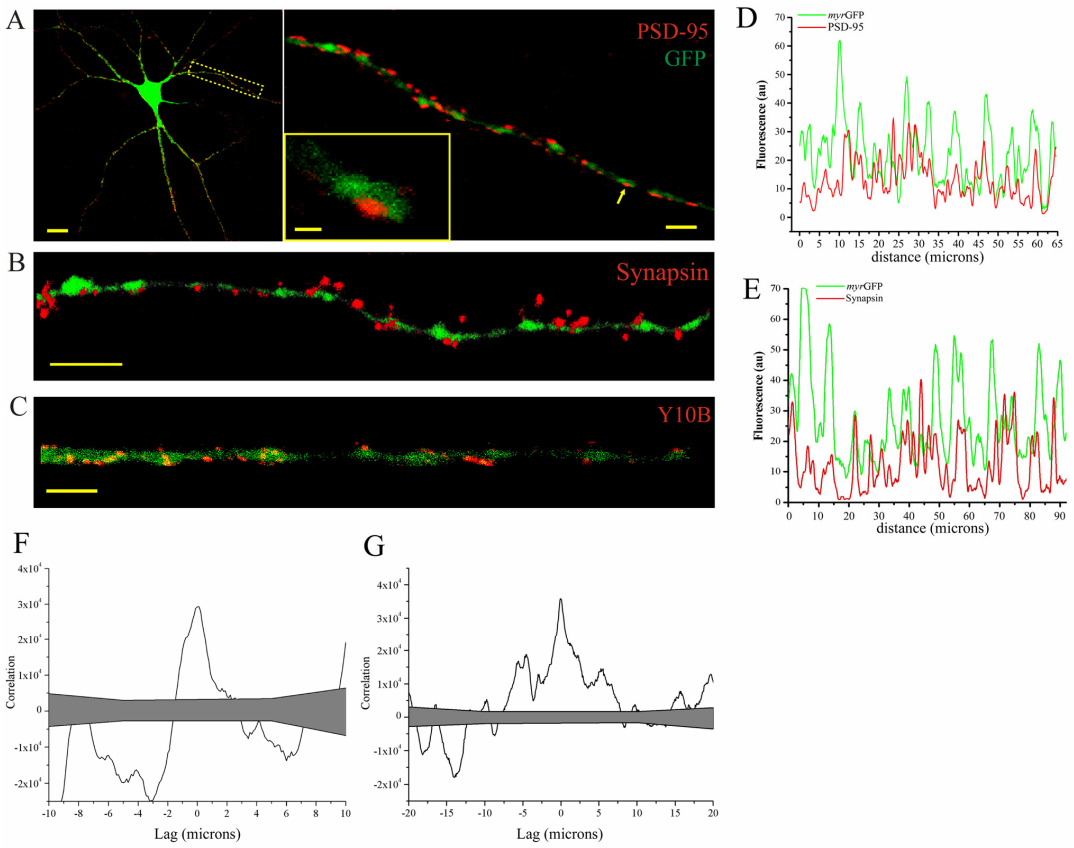

Figure 2.10 GFP Reporter Signals Colocalize with Ribosomes and Synaptic Markers

(A) Low- (left) and high-power (right) images of a GFP reporter-expressing neuron immunostained with an antibody against the synaptic marker PSD-95. The inset shows the GFP signal that forms a cloud around the punctate PSD-95 signal. Scale bars = 15, 5, and 1 μm, for low-, high-power, and inset images, respectively.

(B) High-power image of a GFP reporter-expressing neuron immunostained with an antibody against the presynaptic protein synapsin I, showing that the GFP signal is often in the vicinity of the presynaptic marker.

(C) High-power image of a GFP reporter-expressing neuron immunostained with an antibody against the ribosomal marker Y10B.

(D) Fluorescence intensity plots for the GFP and PSD-95 signals of the dendrite shown in (A). The mean fluorescence along the width of the dendrite was calculated.

(E) Fluorescence intensity plots for the GFP and synapsin signals of the dendrite shown in (B). The mean fluorescence along the width of the dendrite was calculated.

(F) The cross-correlation functions for GFP and synapsin is shown for the dendrite in (B). The shaded area indicates the results of 100 cross-correlations computed on randomized versions of the data. The upper and lower bounds of the shaded area define the 95% confidence interval.

(G) The cross-correlation functions for GFP and Y10B is shown for the dendrite in (C). The shaded area indicates the results of 100 cross-correlations computed on randomized versions of the data. The upper and lower bounds of the shaded area define the 95% confidence interval.

Discussion

We have described the visualization of dendritic protein synthesis in mature cultured hippocampal neurons. We report a robust stimulation of local protein synthesis by the growth factor BDNF. In the last 5 years, several studies have used clever applications of protein synthesis inhibitors to demonstrate roles for locally synthesized proteins in different forms of synaptic plasticity (Kang and Schuman, 1996; Martin et al., 1997; Casadio et al., 1999; Huber et al., 2000). In Aplysia sensory neurons, serotonin application to isolated neurites results in detectable protein synthesis (Casadio et al., 1999). Direct demonstrations of protein synthesis in mature mammalian dendrites are scarce, however. Using radiolabeling, Feig and Lipton (1993) showed that newly synthesized proteins could be detected in dendrites of hippocampal slices—the timing was such that the cell body was unlikely to be the source of protein synthesis. Nevertheless, the difficulty associated with the radiolabeling procedure as well as the troublesome identification of dendritic compartments limits the appeal of this approach. In contrast, the approach we have developed allows the visualization of dendritic protein synthesis in living neurons over time. Ultimately, we combined the use of a membrane-anchored, destabilized GFP with somatic photobleaching to be sure that reporter signals observed in the dendrite were synthesized in the dendrite. Given the limited diffusion of the myristoylated dGFP (e.g., Figure 2.6), a case could certainly be made for using the myristoylated reporter alone (without somatic photobleaching) in future investigations of dendritic protein synthesis in slice preparations as well as in vivo.

The BDNF-induced increases in GFP fluorescence we observed were completely blocked by anisomycin. Since BDNF does not slow the degradation rate of the GFP reporter (our unpublished data), these data indicate that the fluorescence increases were due to new protein synthesis. In addition, BDNF-induced dendritic protein synthesis was not accompanied by any obvious or systematic changes in cell morphology. We observed increases in GFP reporter within 45–60 min of BDNF application (e.g., Figure 2.2). Preceding its ability to fluoresce, GFP possesses posttranslational requirements for cyclization and oxidation (Cubitt et al., 1995). As such, GFP is not an optimal reporter for addressing how quickly protein synthesis can occur in dendrites. Previous studies using developing neurons reported that a combination of BDNF and NT-3 (Crino and Eberwine, 1996) or a metabotropic receptor agonist (Kacharmina et al., 2000) could stimulate translation of a myc epitope in transected growth cones between 1 and 4 hr after transfection. In addition to participating in synaptic plasticity, a role for BDNF-stimulated dendritic protein synthesis might also be imagined in other contexts where BDNF clearly plays an important neurotrophic role in development and the morphology of neurons (McAllister et al., 1999; Schuman, 1999b).

The regulated synthesis of our reporter may mimic the translation of endogenous CamKIIα since our reporter contains both the 5′ and 3′UTR from the CamKIIα gene. Indeed, a stimulation of dendritic CamKIIα translation by LTP has been suggested by immunohistochemical studies (Ouyang et al., 1997, 1999). The 5′UTR may contain translational regulatory elements: we noticed that GFP fluorescence in neurons transfected with a construct lacking the 5′UTR (GFP3′) appeared to be greater than that observed in

cells expressing a construct containing both the 5′ and 3′UTR (data not shown). The 3′UTR was included primarily to confer dendritic localization of the GFP mRNA (Mayford et al., 1996; Mori et al., 2000), although this was likely not necessary in the present study given the abundance of the mRNA produced by viral infection. The 3′UTR of CamKIIα also contains elements for regulation of translation, namely the CPE (cytoplasmic polyadenylation element) and polyadenylation signal (Wu et al., 1998). We have not addressed whether the BDNF-stimulated translation we observe requires these elements, although others have clearly shown regulation of CamKIIα translation via these and other regulatory elements (Sheetz et al., 2000; Wu et al., 1998).

In addition to its effects on hippocampal slices, BDNF can also facilitate synaptic transmission in cultured hippocampal neurons (Levine et al., 1995; Li et al., 1998a, 1998b). Both pre- and postsynaptic (Levine et al., 1997) mechanisms have been proposed for BDNF's actions on synaptic transmission. In the present experiments, the stimulation of protein synthesis could be due to activation of postsynaptic TrkB receptors and subsequent stimulation of local translation machinery, potentially through the rapamycin-sensitive m-TOR kinase pathway (e.g., Brown and Schreiber, 1996; Casadio et al., 1999). Alternatively, BDNF could enhance glutamate release from presynaptic terminals (e.g., Li et al., 1998a, 1998b), which could then stimulate postsynaptic protein synthesis through glutamate receptor signaling.

In sum, these data clearly show that dendrites of mammalian neurons can synthesize proteins. The demonstration that dendrites that are in a synaptic network can

synthesize proteins provides support for the idea that locally synthesized proteins contribute to synaptic function. The observation that there are spatially and temporally consistent hot spots for translation suggests that local synthesis might play a role in maintaining the specificity of synaptic connections.

Experimental Procedures

Cultured Neurons

Dissociated hippocampal neuron cultures are prepared from postnatal 2- and 3-day rat pups as described (Banker and Goslin, 1990). Neurons are plated at a density of 15,000–45,000 cells/cm2 onto poly-L-lysine and laminin-coated coverslips. The cultures are maintained and allowed to mature in growth medium (Neurobasal-A supplemented with B27 and Gluta MAX-1) for 14–21 days before use. In Biolistic experiments, dissociated P2 hippocampal neurons were transfected with the pcDNA3.1-5′GFP3′ construct according to the manufacturer's protocol (Bio-Rad). DNA-gold complexes were generated with the following parameters: 50 μg plasmid DNA, 17 mg 1.6 μm diameter gold particles, and 0.01% PVP. In viral infection experiments, dissociated P2 hippocampal neurons were infected for 12 hr in growth medium containing the Sindbis virus of choice. Six hours post initial transfection or 10–12 hr post initial infection, growth medium was removed and replaced with HEPES-buffered solution (HBS) (Malgaroli and Tsien, 1992; without glycine or picrotoxin) for imaging. All neurons used in our experiments had a pyramidal neuron-like morphology with one or two major dendrites emanating from the cell body. For immunolabeling, neurons were fixed at room temperature with 4%

paraformaldehyde for 20 min. Fixed cultures were then treated sequentially with PBS, PBT (1× PBS, 2 mg/mL BSA, 0.1% Triton X-100), preblock (20% normal goat serum in PBT), primary Ab in preblock at 4°C overnight, preblock, Cy3-conjugated secondary Ab in preblock, preblock, and PBS. Immunostained specimens were imaged in PBS. The sources of the antibodies were as follows: MAP2 (Chemicon), PSD-95 (Upstate Biotechnology), Y10B (generous gift from Jeff Twiss, UCLA), synapsin I (Novus).

Constructs

pcDNA3.1-5′dGFP3′: The CamKIIα 3′UTR sequence obtained from plasmid (Mayford et al., 1996) was PCR amplified (forward primer: 5′-ttatatttgcggccgcggtcgctac-cattaccagtt-3′; reverse primer: 5′-ggcgctctctcgagtttaaatttgtagct-3′) and cloned into the NotI and XhoI sites of the pcDNA3.1 vector (Invitrogen). The resulting vector was then cleaved with BamHI and NotI for insertion of the destabilized EGFP ORF (from pd2EGFP, Clontech). The CamKIIα 5′UTR was released from a plasmid (obtained from J. Fallon) and inserted at the HindIII-BamHI sites, yielding pcDNA-5′dGFP3′. pSinRep5-5′dGFP3′: The 5′dGFP3′ fragment was released with PmeI-ApaI (blunted) and ligated into pSinRep5 (Invitrogen). pcDNA3.1-5′myrdGFP3′: The d2EGFP ORF (from pd2EGFP, Clontech) was PCR amplified (forward primer: 5′-cgactctagagtgagcaagggcgaggagctg-3′; reverse primer: 5′-tctagagtcgcggccgcatctacaca-3′), digested, and inserted into the XbaI-NotI sites of pBSK. To generate the myristoylation signal, two oligos corresponding to the N-terminal 10 amino acids of p10 were annealed (myr1: 5′-gatccatgggcacggtgctgtccctgtctcccagct-3′; myr2: 5′-ctagagctgggagacagggacagcaccgtgcccatg-3′), digested, and inserted into the BamHI-XbaI sites of pBSK-d2EGFP. The myrdGFP was subcloned into the BamHI-NotI sites of

pcDNA3.1-5′dGFP3′. pSinRep5-5′myrdGFP3′: The 5′myr3 dGFP3′ fragment was released with PmeI-ApaI and subcloned into the StuI-ApaI sites of pSinRep5 (Invitrogen). Sindbis viroids were produced according to the Experimental Procedures provided by Invitrogen. Contrary to observations in other cell types, it appears that single-stranded RNA viruses of the α family do not shut down protein synthesis in neurons (K. Lundstrom, personal communication, Hoffmann-LaRoche, Basel, Switzerland).

Microscopy

Confocal images were acquired in 0.3 μm sections; image analysis was conducted on z-compressed image stacks that contained the entire neuron of interest. GFP was excited at 488 nm and emitted light was collected between 510–550 nm. Images were acquired with parameters that maximized the dynamic range of pixel intensity for the dendritic signal. Using these parameters, the cell body fluorescence intensity was necessarily, occasionally, saturated. In all experiments, identical acquisition parameters and settings were used for both control and BDNF-treated dendrites on a given experimental day. In time-lapse experiments, the cultured neurons were maintained in an incubator (36.5°C) between image acquisition episodes. Images were acquired at room temperature. The acquisition of images at individual time points took less than 3 min.

Transections

Cells were preincubated in HBS for 2 hr before the start of transection experiments. Transection was carried out as follows: GFP-expressing neurons were oriented such that the dendritic segment to be cut was in line with the long axis of the microelectrode. The

glass microelectrode was then carefully lowered onto the dendrite until a spot of no GFP signal was seen, indicating that the dendrite had been pinched at that point. After allowing the microelectrode to rest in this position for 1-2 min, the electrode was slowly moved down vertically, allowing the tip to flex and push the proximal aspect of the dendrite toward the soma and away from the more distal dendrites. Complete transection of the dendrite as well as the integrity of the dendritic arbor was verified by DIC images. In order to be included in experiments, transected neurons were required to meet the following criteria: (1) both the transected process and the neuron from which it was cut must remain morphologically intact and healthy for the duration of the experiment; (2) no signs of varicosity formation or blebbing; (3) some detectable fluorescence signal must be observed in the transected process 2 hr post transection.

Photobleaching

In FRAP experiments, an infected neuron was selected and a dendrite from that neuron was scanned for 1 hr with a 488 nm, 5 mW laser. Complete volumetric data of the dendrite to be studied were acquired at regular intervals before and after the bleaching. In optical isolation experiments, an infected neuron was selected, and its soma was continuously scanned to photobleach the somatic GFP. Propidium iodide (PI) exclusion experiments were conducted to insure neuronal viability during optical isolation experiments. After ~90 min somatic photobleaching, the cell was stained with PI solution (50 μg/ml). The cell was then assessed for PI staining. Positive control experiments utilizing glutamate-induced toxicity (250 μM) were also carried out. No PI staining was evident after somatic photobleaching whereas the glutamate-induced toxicity in the same

cells led to strong PI staining. Pilot experiments indicated that complete somatic photobleaching was obtained within 120 min of the initiation of photobleaching. In all experiments, data analysis began with this (complete somatic photobleaching) time point and hence was designated as t = 0. Using data from FRAP experiments, we estimate the membrane tethered reporter's diffusion coefficient to be $1 \times 10{-}8$ $cm^{2/s}$, which is only slightly greater than the diffusion coefficient of rhodopsin ($\sim 5 \times 10{-}9$ $cm^{2/s}$) (Wey et al., 1981) and glycine receptors ($\sim 1 \times 10{-}9$ $cm^{2/s}$) (Srinivasan et al., 1990). This difference is expected since rhodopsin and glycine receptors are integral membrane proteins that may be bound to elements in the cytoskeleton and therefore would be more diffusion limited than a myristoylated protein. This estimate of the reporter's diffusion coefficient may include the simultaneous effects of degradation on the reporter. Indeed, there are examples of FRAP experiments where the rate of degradation of the reporter exceeded the rate of diffusion of the reporter.

Analysis

To analyze the GFP of individual dendrites, we calculated the mean pixel intensity for each dendrite along its length (NIH Image, or ImageJ), thus controlling for changes in the width of the dendrite. In time-lapse experiments, we calculated a normalized difference score, $\Delta F/F(y - x/x)$, that indicates the change in dendritic fluorescence as a function of time and, when appropriate, treatment with BDNF. In plotting $\Delta F/F$, the data were binned into 1–2 μm sections. In regular time-lapse and dendritic transection experiments, x was

the baseline (first) fluorescence measurement and y was the brightest time point following the baseline (typically 120 min). In "optical isolation" experiments, x was chosen as the lowest time point following somatic photobleaching and y was usually taken 60 min later. All untreated cells were "yoked" to experimental cells. That is, they were infected at the same time, imaged at equivalent time points, and the analysis was calculated using the same time points as their BDNF-treated "sister" neurons. For analysis of colocalization, horizontal dendritic segments were analyzed by obtaining the mean fluorescence signal across the width of the dendritic segment. A cross-correlation was calculated for the myrdGFP and PSD-95, synapsin, or Y10B: the mean fluorescence across the width of a dendritic segment was calculated, generating a one-dimensional representation of the relative amplitudes of the red and green signals. A cross-correlation was calculated on these two data sets. To calculate the significance of the cross-correlation, one hundred cross-correlations of the randomized data were performed to yield a 95% confidence level. In other experiments, Student's t tests were performed to assess statistical differences between groups. We chose for analysis the brightest (usually principal) dendrite from each neuron in each group. When a single neuron possessed two bright, principal dendrites, both were used in the analysis.