BUILDING THE MOLECULAR MACHINERY OF MEMORY: LOCAL PROTEIN SYNTHESIS IN HIPPOCAMPAL NEURONS

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ABSTRACT

Synaptic plasticity is the most widely accepted cellular and molecular model for learning and memory. Although the idea that information is encoding through changes in synaptic strength is simple, the requirement for new protein synthesis to maintain long-lasting forms of plasticity threatens to make this model untenably complex. This complexity arises from the fact that an individual neuron can have thousands of connections, small groups of which change strength independently of others. Since the necessary proteins are likely the effectors of long-term plasticity, non-specific delivery would lead to loss of plasticity-encoded information. Thus it is critical that the effector proteins be faithfully delivered only to the correct sites.

One way to accomplish this task would be to allow the synapses local control over the necessary protein synthesis and delivery. Previous studies have suggested this possibility of dendritic local protein synthesis (LPS). In this thesis we describe the visualization, in real time, of the synthesis of a GFP-based reporter in the dendrites of cultured rat hippocampal neurons. By utilizing a number of physical, optical and molecular manipulations we have insured that the observed synthesis was free of any somatic contribution thereby providing the first definitive evidence for the existence of dendritic LPS in mature vertebrate neurons.

We also describe the regulation of dendritic LPS by two forms of plasticity-inducing stimuli. First, we show that dendritic LPS can be stimulated by brain derived neurotrophic factor (BDNF), a molecule capable of causing persistent synaptic enhancement. Second, we show that a chronic blockade of synaptic activity, which

results in a form of synaptic enhancement termed "disuse hypersensitivity", appears to enhance dendritic LPS.

Finally, we discuss a technique that can facilitate the study of the necessity of dendritic LPS for long-lasting plasticity. By using a "caged" protein synthesis inhibitor, we are able to abolish protein synthesis in a spatially restricted manner. Thus it is now possible to conduct experiments where dendritic LPS is inhibited while somatic synthesis is permitted. If plasticity is not maintained under these conditions, we will have satisfying evidence of the necessity of dendritic LPS for long-lasting plasticity.

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CHAPTER 1

Introduction: Local Protein Synthesis

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Synaptic plasticity and the need for local control

Synaptic plasticity is an attractive cellular correlate of learning and memory. The idea is logical and simple: Information is encoded through the strengthening or weakening of connections between cells based on their history of activity. An abundance of literature exists supporting the link between synaptic plasticity and learning and memory (Castro et al., 1989; Hargreaves et al., 1990; McNaughton et al., 1986; Moser et al., 1998; Tsien et al., 1996). However, the implementation of this model as the basis for learning and memory in the brain may appear problematic. How could an individual neuron hope to maintain control over its thousands of synapses, small groups of which change strength independent of others (Engert and Bonhoeffer, 1997; Schuman and Madison, 1994)? This task appears even more daunting in light of the fact that new proteins are required at the synapses to maintain these changes (Krug et al., 1984; Montarolo et al., 1986; Otani et al., 1989). It is likely critical that the necessary proteins are delivered selectively to those sites that were potentiated or depressed. If the necessary proteins were the effectors of long-term plasticity, then nonspecific delivery would lead to loss of plasticity encoded information. There are several ways in which the cell could effectively supply the proteins needed by its constantly changing array of connections. For example, specificity could be achieved by site-specific transport of proteins, site-specific capture of globally distributed proteins, site-specific modulation of the protein degradation, or the site-specific synthesis of proteins. More than likely, neurons utilize a combination of these mechanisms in maintaining the changes put into place by plasticity-inducing stimuli. However, studies from a variety of systems point to

site specific or local protein synthesis as a promising mechanism to play a significant role in this process (Aakalu et al., 2001; Kang and Schuman, 1996; Martin et al., 1997; Ouyang Y, 1999; Steward et al., 1998). Producing proteins from dendritically localized mRNAs, at or near the synapses where they are needed is an elegant way to achieve the synapse specific delivery of newly synthesized proteins.

This idea of local synthesis, although compelling, evokes several questions: How might such a system have arisen? What molecular machinery exists to support local synthesis? How might local synthesis be regulated to insure the fidelity of the information that was initially encoded?

Local Protein Synthesis in Developmental Systems

Local protein synthesis (LPS) is not unique to neurons. LPS is one of the primary mechanisms that organisms use to determine cell fate and generate differences among cells.

Saccharomyces cerevisiae. The local translation of ASH1 (for asymmetric synthesis of HO) mRNA in the yeast *Saccharomyces cerevisiae* provides a well-detailed example of LPS. There are three different forms of *S. cerevisiae*: the haploid **a**, the haploid α, and the diploid **a**/α. The **a** and α cell types can mate with each other to form a diploid **a**/α cell. Mother cells (haploid yeast that have undergone budding) are capable of switching between the **a** and α forms (mating types), whereas daughter cells (haploid cells formed by budding that have not yet budded themselves) cannot (Nasmyth et al.,

1987; Nasmyth and Shore, 1987; Nasmyth et al., 1987; Schiestl and Wintersberger, 1992).

ASH1p is a transcriptional repressor of *HO* endonuclease, a factor involved in mating-type switching. Since only mother cells can carry out mating-type switching, *ASH1* mRNA is localized to and selectively translated in daughter cells to repress *HO* endonuclease (Bobola et al., 1996; Long et al., 2001; Long et al., 1997; Takizawa et al., 1997).

The localization of the *ASH1* mRNA is mediated by complex of *cis* and *trans*-acting elements (Chartrand et al., 1999). The presence of *cis*-acting localization elements in locally translated mRNA's is quite common (Kloc et al., 2002). There appear to be three basic ways in which these *cis*-acting localization elements perform their function. The first is by providing a binding site for *trans*-acting protein translocation complexes (Yaniv and Yisraeli, 2001). These translocation complexes are often composed of a protein(s) that binds the mRNA, an adaptor protein(s) that mediates binding of the RNA-binding protein to a motor protein, and a motor protein that actively transports the mRNA along the cytoskeleton. The second way in which *cis*-acting localization elements can localize mRNAs is by providing a binding site for RNA-binding proteins to anchor the mRNA to structural elements in specific regions of the cell. The third way *cis*-acting localization elements can localize mRNA species by specifically stabilizing or destabilizing particular mRNAs in particular regions of the cell (Kloc et al., 2002). One or more of these mechanisms could be used to localize a particular mRNA but it is not clear if a single element can perform multiple functions.

Four *cis*-acting elements, each sufficient to localize the transcript to budding daughter cell, have been identified. Three of these elements reside entirely in the coding sequence of *ASH1* and the fourth resides partially in the coding sequence and partially in the 3' untranslated region (UTR). The function of these *cis*-acting elements relies not directly on their sequences but rather on their secondary structure (Chartrand et al., 1999) (Gonzalez et al., 1999). An RNA-binding protein termed She2p is capable of binding all four elements. She2p can itself be bound to a protein She3p that in turn binds to She1p, a type V unconventional myosin. She1 uses the yeast's highly polarized actin cytoskeleton to transport the *ASH1* mRNA/She2p/She3p complex to the daughter cell. She1p, She2p, and She3p are all required for the proper asymmetric expression of the *HO* endonuclease (Chartrand et al., 1999). The means by which LPS is regulated in this system is unclear. However, studies using the *Drosophila* and *Xenopus* oocytes give some insight into area.

***Drosophila* Oocyte.** Just as in *S. cerevisiae*, LPS is used in the *Drosophila* oocyte to generate cellular diversity. In the *Drosophila* oocyte, the translation of certain transcripts is restricted to specific regions of the cell. This serves to create different regions of the cell and later different daughter cells. The local translation of *bicoid* and *nanos* provides an example of this principle.

Bicoid mRNA is translated at the anterior of the oocyte and *nanos* mRNA is translated at the posterior of the oocyte. This results in opposing concentration gradients of their protein products, which helps form the basis for anterior-posterior patterning in

the *Drosophila* embryo (Gavis and Lehmann, 1992; van Eeden and St Johnston, 1999; Wreden et al., 1997; Zhang et al., 2001).

Both these transcripts are transported from the ovarian follicle cells (nurse cells) to the oocyte, but the mechanisms responsible for their localization and translational regulation differs. The *bicoid* mRNA is tethered by stem loop structures formed in its 3'UTR to the microtubules in the anterior region of the oocyte by Swallow and Staufen (Chartrand et al., 2001; Ferrandon et al., 1994; Yaniv and Yisraeli, 2001). There it remains translationally silent until the first division cycle of the cell when its poly(A) tail is extended (Lieberfarb et al., 1996; Salles et al., 1994). This poly(A) extension and subsequent translational activation of *bicoid* is not spatially regulated, and thus its anchoring is crucial.

Some of the *nanos* transcript is localized to the posterior end of the oocyte based on several partially redundant elements in the 3'UTR, all of which are required for optimal localization (Bergsten et al., 2001; Kloc et al., 2002). However, the vast majority of the *nanos* mRNA remains diffusely distributed throughout the oocyte (Bergsten and Gavis, 1999). This is most likely because *nanos* is localized by diffusion from the nurse cells and subsequent capture or sequestration at the posterior of the oocyte (Johnstone and Lasko, 2001). Thus, in order to limit *nanos* translation to the posterior of the oocyte, translation is repressed by a *cis*-acting element in its 3'UTR and *trans*-acting factors that bind to it. One such *trans*-acting factor is known as Smaug. Recent evidence indicates that *nanos* is primarily found associated with ribosomes but is kept translationally inactive by the *trans*-acting translational suppressors bound to the 3'UTR somehow limiting passage of the mRNA through the ribosome (Clark et al., 2000; Markesich et al.,

2000). The translational repressors may accomplish this task by interacting with the coding region of *nanos* or with the ribosome itself (Kloc et al., 2002). Once *nanos* reaches the posterior of the oocyte, Smaug may interact with Oskar, a protein localized to the posterior of the oocyte, to derepress translation of *nanos* (Dahanukar et al., 1999). Oskar may relieve the translational suppression by Smaug by displacing the repressor from the 3'UTR of *nanos*. This idea is supported by a study that indicates that Oskar can bind Smaug's RNA binding domain (Dahanukar et al., 1999). To further insure that translation of *nanos* is properly restricted, all *nanos* mRNA that is not localized to the posterior is rapidly degraded upon fertilization of the oocyte (Bashirullah et al., 1999).

As stated earlier, the local translation of *bicoid* and *nanos* results in opposing concentration gradients of their protein products. Although Bicoid and Nanos have other functions, the primary means by which they establish the anterior-posterior patterning in the *Drosophila* embryo is through their effect on a protein known as Hunchback. Bicoid activates transcription of *hunchback* while Nanos inhibits the translation of the *hunchback* transcript. Thus, this generates a concentration gradient of Hunchback that runs from high in the anterior to low in the posterior of the embryo. The Hunchback gradient in turn regulates the expression of genes responsible for the development of region specific structures (Gilbert, 1997).

***Xenopus* Oocyte.** LPS plays many roles in the development of *Xenopus*. One well-studied example is the regulation of Cyclin B1 expression in the *Xenopus* oocyte by LPS.

The early cleavage divisions of the *Xenopus* embryo depend on *cyclin B1* mRNA being localized to the mitotic spindles of the *Xenopus* oocyte and being kept translationally silent until activated by a progesterone-mediated signal. The translational regulation of *cyclin B1* mRNA is controlled by a cytoplasmic polyadenylation element (CPE) present in its 3'UTR, a CPE binding protein (CPEB), and other *trans*-acting factors that bind to and covalently modify CPEB (Mendez and Richter, 2001; Richter, 2001).

Most mRNAs are fully polyadenylated in the nucleus. The translation of these transcripts begins with the recruitment of the cap-binding protein complex (composed of the cap-binding protein eIF4E, the RNA helicase eIF4A, and the modular scaffold protein eIF4G) to the N^7-methylguanosine cap at the 5' end of the mRNA. The cap-binding complex, with the aid of eIF4B, unwinds the secondary structure of the 5' UTR of the mRNA. This allows the 40S ribosomal subunit access to the mRNA and thus makes possible the assembly of the 43S initiation complex. The 43S initiation complex is composed of the cap-binding complex, eIF3, the ternary complex (eIF2, Met-tRNA and GTP), and the 40S ribosomal subunit. The association of the 43S complex with the mRNA forms the 48S initiation complex (Brown and Schreiber, 1996; Mendez and Richter, 2001).

Prior to the action of progesterone, *cyclin B1* mRNA has a very short poly(A) tail and an unphosphorylated CPEB bound to its 3'UTR. Bound to the CPEB is a protein, Maskin, that also binds to eIF4E (Stebbins-Boaz et al., 1999). This interaction between Maskin and eIF4E may inhibit the assembly of the 48s initiation complex that is needed to start translation. The progesterone-mediated signal causes Eg2 to phosphorylate

CPEB. The phosphorylated CPEB recruits cleavage and polyadenylation specificity factor (CPSF), which in turn recruits poly(A) polymerase (PAP) to extend the poly(A) tail of *cyclin B1* (Groisman et al., 2000; Sheets et al., 1994). During this time Maskin disassociates from eIF4E, which presumably allows the 48s initiation complex to form (Stebbins-Boaz et al., 1999). Although the poly(A) tail extension and Maskin-eIF4E disassociation may be independent events, a model has been proposed for the disassociation to be dependent on the extension. Poly(A)-binding protein (PAPB), which interacts with a component of the 48s initiation complex, may attach to the extended poly(A) tail of *cyclin B1* and help 48s assembly out compete Maskin-eIF4E interactions (Mendez and Richter, 2001) (Figure 1).

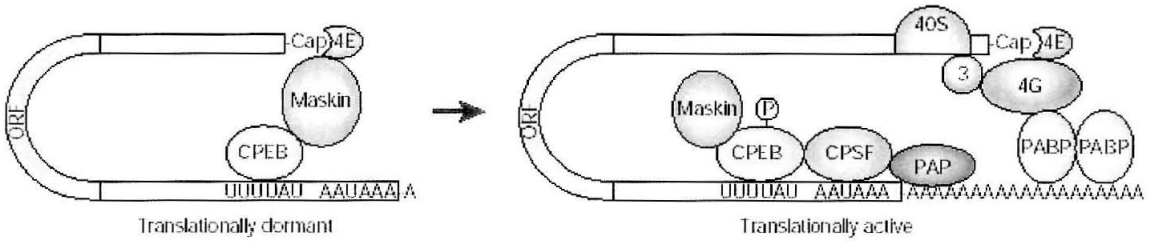

Figure 1. Regulation of synthesis by cytoplasmic polyadenylation. Taken from Mendez and Richter, 2001.

Interestingly, CPEB appears to also mediate the localization of *cyclin B1*. In experiments where a CPEB devoid of its microtubule-binding domain is injected into oocytes, *cyclin B1* mRNA becomes delocalized and the normal cleavage divisions are disrupted (Mendez and Richter, 2001).

Local protein synthesis is also used to create functional microdomains within cells. Studies involving myelinating cells and axonal growth cones provide excellent examples of this use of LPS.

Oligodendrocytes. Myelinating cells, such as oligodendrocytes, produce processes that function as lipid sheaths to electrically insulate axons. They do this by producing a protein called myelin basic protein (MBP) that collapses the oligodendrocyte cell membrane, thus squeezing the cytoplasm out of the region where it is expressed (Kamholz, 1996). The oligodendrocytes use MBP to generate membrane sheaths from its processes to insulate neuronal axons. If MBP were produced in a cell-wide manner, it would be toxic. Therefore, restricted translation of MBP mRNA is carried out in the oligodendrocyte processes (Barbarese et al., 1999; Campagnoni and Macklin, 1988; Campagnoni et al., 1990; Campagnoni et al., 1991; Kalwy and Smith, 1994).

The localization of MBP mRNA is dependent on two separate *cis-acting* elements: the RNA Transport Signal (RTS) and the RNA Localization Region (RLR). Both these elements are located in the MBP mRNA's 3'UTR. The RTS is capable of localizing the transcript to the processes of oligodendrocytes but the RLR is required to localize it to the myelin compartment itself. However, the RLR cannot localize the transcript to the myelin without first being localized to the processes by the RTS (Ainger et al., 1997). The RTS and RLR differ from the multiple *cis*-acting localization signals found in *ASH1* and *nanos* in that they each serve distinct localization functions. They are likely to comprise the most important functional elements of the 3'UTR of MBP mRNA as a transgene containing just the MBP coding sequence, RTS, and RLR is sufficient and

necessary to completely rescue the *shiver* mutation (a deletion of the endogenous MBP gene) (Kimura et al., 1989).

The RTS homology has been found in a number of other mRNAs including MAP2A, ARC, and RC3. The RTS has been shown to interact with heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2). Antisense knockdown of hnRNP A2 abolishes MBP mRNA localization (Ainger et al., 1997). Interestingly, an hnRNP, called Loc 1p, is implicated in Ash1 mRNA localization (Long et al., 2001).

Microdomains are useful not only for avoiding toxicity, but also for allowing distributed control of cellular function. Axonal growth illustrates this principle quite well.

Growth Cones. The growth cone of a developing axon must sort through a dizzying array of attractive and repulsive cues to determine how to reach the appropriate target tissue. To accomplish this task, the growth cone requires specific proteins based on the environmental cues it encounters. However, the growth cone is frequently millimeters away from the soma. To solve this problem, the growth cone might locally synthesize the required proteins when it needs them. Identification of protein synthesis machinery in the growth cone supports the idea of LPS in this system (Kleiman et al., 1994) (Tennyson, 1970).

Growth cones isolated from their somas exhibit protein synthesis-dependent growth cone collapse in response to Sema3A and protein synthesis-dependent chemotropic turning in response to Netrin-1. In fact, exposure of these isolated growth cones to Sema3A or Netrin-1 induces a several fold increase in [^{3}H]-leucine incorporation. This synthesis appears to be initiated through the target of rapamycin-

dependent signaling pathway, but the identity of the synthesized proteins remains unknown (Campbell and Holt, 2001).

Neurotrophin-3 (NT-3) can induce localization of β-Actin mRNA to the growth cone presumably for the purpose of LPS (Zhang et al., 2001; Zhang et al., 1999). *In situ* hybridization experiments demonstrate that this localization is dependent on a *cis-acting* element in the 3'UTR of the transcript and a protein, Zip-code Binding Protein 1, which binds to this element. Treatment with an antisense oligonucleotide to the *cis-acting* element abolishes the growth cone extension normally induced by NT-3 (Zhang et al., 2001). One possible explanation for this effect is that local synthesis of β-Actin mRNA is required for growth cone extension. However, it could be possible that the β-Actin used for growth cone extension is normally supplied by the soma and the antisense oligonucleotide reduces the amount of somatically synthesized β-Actin and thus the amount available at the growth cone.

Local protein synthesis in neurons

Dendritic Protein Synthesis Machinery. The initial insight into the possibility of LPS in the mature neuron occurred following the observation of polyribosomes at postsynaptic sites (Steward and Levy, 1982). Since that initial observation, nearly every major type of protein synthesis machinery evidenced in the cell body has also been identified in the dendrites (Gardiol et al., 1999; Steward and Levy, 1982; Tang et al., 2002; Tiedge and Brosius, 1996; Wu et al., 1998) (see table 1).

Table 1. Translational machinery identified in dendrites.

Component	Localization (excluding soma)	Method of Identification
Polyribosomes[1]	Dendritic, Subsynaptic	Immunocytochemistry
Ribosomes[3]	Dendritic, Subsynaptic	Immunocytochemistry
MRNA[*]	Dendritic, Subsynaptic	*in situ* hyb.
tRNA[2]	Dendritic	*in situ* hyb.
Endoplasmic reticulum[3]	Dendritic, Subsynaptic	Immunocytochemistry
Rough ER[2, 3]	Dendritic (close to plama membrane)	Immunocytochemistry
Medial golgi[3]	Dendritic (away from plasma membrane), Subsynaptic	Immunocytochemistry
trans-Golgi[3]	Dendritic (away from plasma membrane), Subsynaptic	Immunocytochemistry
Membrane targeting and insertion signal recognition machinery[2]	Dendritic, no spine associated labeling	Immunocytochemistry
eIF2[3]	Dendritic, Subsynaptic	Immunocytochemistry
CPEB[4]	Dendritic, Subsynaptic	Immunocytochemistry
Components of rapamycin-sensitive signaling pathway[5]	Dendritic, Subsynaptic	Immunocytochemistry

[1]Steward and Levy, 1982
[2]Tiedge and Brosius, 1996
[3]Gargiol et al., 1999
[4]Wu et al., 1998
[5]Tang et al., 2002
[*]Various, See Table 2

This extensive range of synthesis machinery present in the dendrites suggests that a variety of proteins, including transmembrane ones, can be locally synthesized. This idea is supported by the diversity of the mRNA species found in the dendrites, which is discussed in the next section.

The localization of much of this machinery to subsynaptic sites could allow for synapse specific LPS and may indicate that LPS is linked to synaptic activity. These properties would make LPS an attractive way to encode long lasting plasticity.

Dendritic mRNAs. The mRNAs found in the dendrites represent a wide range of potential proteins including ion channels, kinases, cytoskeletal proteins, and transcription

factors (Berry and Brown, 1996; Bian et al., 1996; Burgin et al., 1990; Furuichi et al., 1993; Garner et al., 1988; Gazzaley et al., 1997; Herb et al., 1997; Lim et al., 1999; Link et al., 1995; Lyford et al., 1995; Paradies and Steward, 1997; Prakash et al., 1997; Racca et al., 1997; Watson et al., 1994) (see table 2). As in other systems where local translation occurs, many of these mRNAs contain *cis*-acting elements in their UTRs that control their localization, translation, and stability.

Table 2. Dendritically localized mRNAs. Adapted from Steward and Schuman, 2001.

mRNA	Type of Protein/Protein Function
CaMKIIα[1]	Multifunctional kinase, Ca^{2+} signaling
MAP2[2]	Microtubule associated
Arc[3]	Actin-binding synaptic junction protein
NMDAR1[4]	Receptor
GlyRα[5]	Receptor
Dendrin[6]	Unknown
G-protein γ subunit[7]	Metabotropic receptor signaling
Calmodulin[8]	Ca^{2+} signaling in conjunction with CaMKIIα
Vasopressin[9]	Neuropeptide
Neurofilament protein 68[10]	Neurofilament
InsP3 receptor[11]	Ca^{2+} signaling
L7[12]	Homology to PDGF, oncogene signaling?
PEP19[12]	Ca^{2+} binding
Shank[13]	Scaffolding

[1]Burgin et al., 1990
[2]Garner et al., 1988
[3]Link et al., 1995; Lyford et al., 1995
[4]Gazzaley et al., 1997
[5]Racca et al., 1997
[6]Herb et al., 1997
[7]Watson et al., 1994
[8]Berry and Brown, 1996
[9]Prakash et al., 1997
[10]Paradies and Steward, 1997
[11]Furuichi, 1993
[12]Bian et al., 1996
[13]Lim et al., 1999

The localization and translational control of CaMKIIα and MAP2 have been particularly well studied. CaMKIIα is a calcium-dependent kinase that has been shown

to play an important role in synaptic plasticity as well as learning and memory (Lisman et al., 2002; Silva et al., 1992; Silva et al., 1992). CaMKIIα transcripts can be found constitutively in the proximal through distal regions of dendrites with an even level of abundance (Rongo, 2002). MAP2 is a structural protein whose transcript is also dendritically localized. However, MAP2 mRNA is primarily found in the proximal dendrites (Kindler et al., 2001). It is interesting to note that the 3'UTRs of these two mRNAs are highly conserved across species (see figures 2 and 3). This homology suggests that much of these 3'UTRs play a significant functional role.

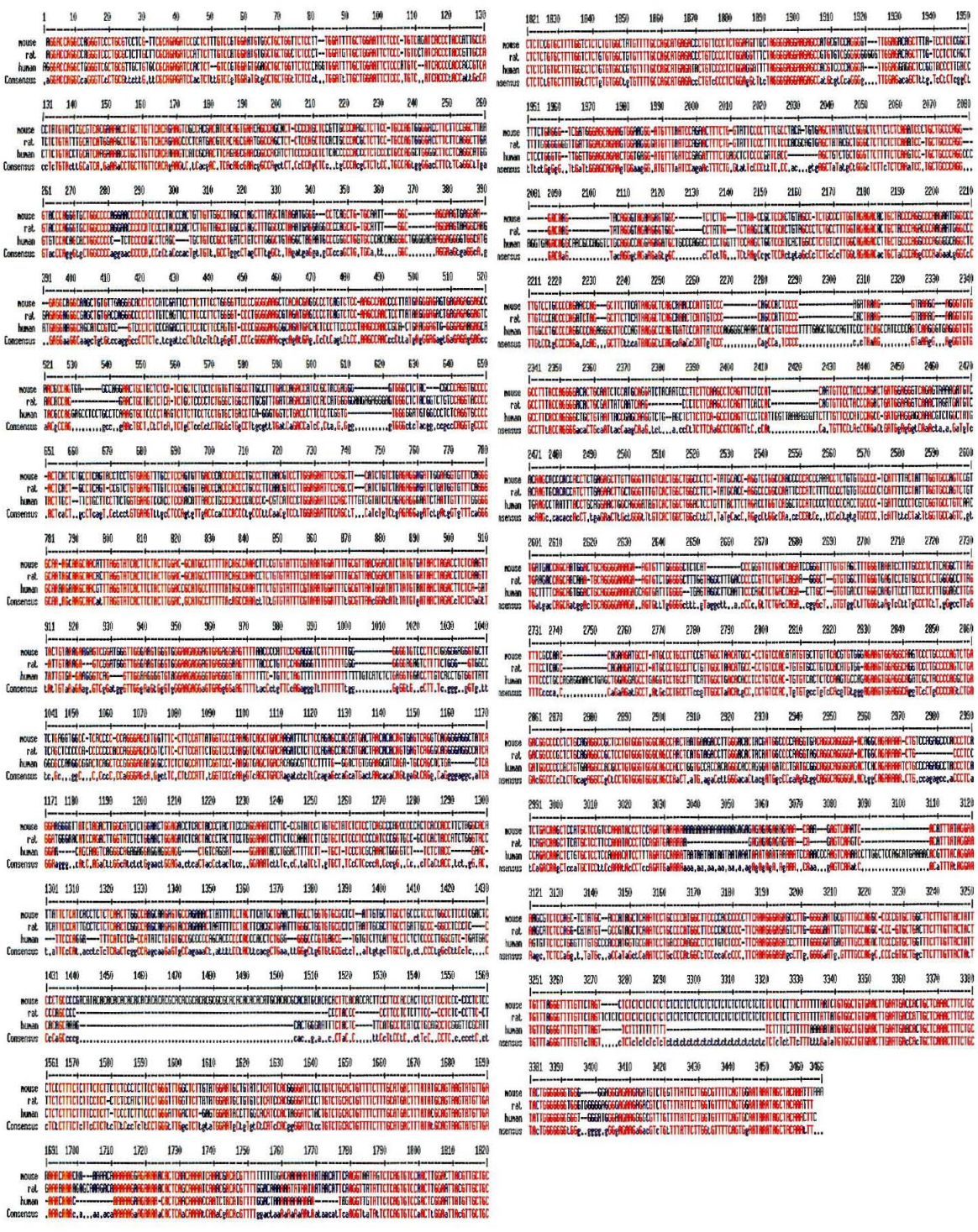

Figure 2. The 3'UTR of CaMKIIα from mouse, rat and human (red = homology for all three species, blue = mismatch between one or more species). Made using ALIGN from Genestream. Accession #'s: mouse (unpublished, sequence provided by M. Mayford, UCSD); rat (AF237778); human (XM_042551).

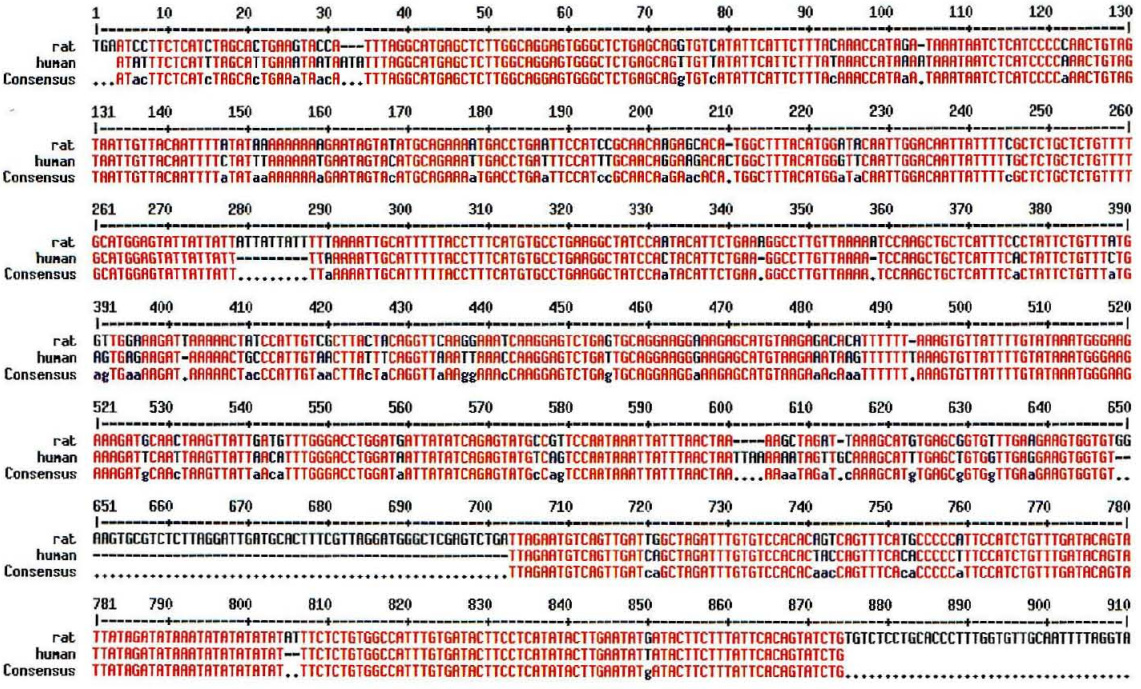

Figure 3. The 3'UTR of MAP2 from rat and human (red = homology, blue = mismatch between species). Made using ALIGN from Genestream. Accession #'s: rat (U30938); human (XM_030840).

The dendritic localization of both these transcripts depends on sequences or secondary structures in their 3' UTRs (Mayford et al., 1996) (Blichenberg et al., 2001; Blichenberg et al., 1999). However, the nature and location of these elements are currently unclear. Mori et al. (Mori et al., 2000) claim to have identified an element in the first 94 base pairs of the rat CaMKIIα 3' UTR that is necessary and sufficient for transcript localization in the dendrites. Blichenberg et al. (Blichenberg et al., 2001) lay the same claim on an element spanning nucleotides 1481 through 2708 of the same 3'UTR. Blichenberg et al. offer differences in cell culture and transfection methodologies as possible explanations for these discrepancies. Indeed, the cells used in Blichenberg et al. were significantly more mature and transfected for a longer time than

those used in Mori et al. However, both regions that were identified may well be sufficient for localization and thus simply represent an example of *cis* targeting element redundancy evidenced in developmental systems. Nevertheless, as these studies utilize only the transcript's UTRs fused to the coding sequence of a reporter molecule, they do clearly show that the dendritic localization does not depend solely on a signal peptide from a partially translated transcript.

The dendritic localization of these transcripts at least in part may be the result of synaptic activity. Mori et al. found that the intact CaMKIIα 3'UTR would not lead to dendritic localization of the mRNA without KCl depolarization of cells, though this conflicts with findings from both Mayford et al. and Blichenberg et al. Additionally, Steward and Worley (Steward et al., 1998) show that in intact hippocampal slices synaptic activation causes no discernable change in the dendritic localization of MAP2 or CaMKIIα mRNAs. These discrepancies may be the result of differences in tonic activity levels in the cell cultures of the different labs. Tongiorgi et al. (Tongiorgi et al., 1997) show increased dendritic localization of TrkB and BDNF mRNAs after KCl depolarization of cells as well. However, both Mori et al. and Tongiorgi et al. fail to distinguish if the increased levels of mRNA found in the dendrites resulted from a cell wide increase of the transcript or from specific dendritic targeting/message stabilization (or both).

The transcript for activity-regulated cytoskeleton-associated protein (*Arc*) has been more clearly demonstrated to localize in the dendrites in an activity-dependent manner. *Arc* is an immediate early gene whose transcription can be induced by neuronal

activity. Steward et al. (Steward et al., 1998) have shown electroconvulsive seizure (ECS) can cause the mRNA to be broadly localized in the dendrites. The selective localization to specific regions of the dendrites can be achieved by selective stimulation. The Arc mRNA that is broadly localized to the dendrites via ECS can be subsequently targeted to activated synapses in a protein synthesis-independent manner. However, this targeting does requires NMDAR activation (Steward and Worley, 2001).

Dendritically targeted mRNAs appear to be transported in granules composed of mRNA, protein, and ribosomes often termed ribonucleoprotein (RNP) complexs. These RNP complexes move at a rate ranging from 4 - 12 μm/min (Kiebler and DesGroseillers, 2000; Knowles et al., 1996; Rook et al., 2000), which is consistent with RNP complex kinetics in developmental and other non-neuronal systems. This RNP complex movement/transport has been shown to be microtubule-dependent (Knowles et al., 1996). RNP complex movement is not unidirectional – several studies utilizing different methods to visualize these complexes (Kiebler et al., 1999; Knowles et al., 1996; Rook et al., 2000) have found that they travel in both the anteriograde and retrograde directions. This bi-directional motion of some RNP complexes can be shifted to a net anteriograde motion by stimuli that elicit synaptic activity (Rook et al., 2000).

The identity of the *trans*-acting factors involved in the dendritic localization of mRNAs is now coming into focus. The localization of some dendritic mRNAs has been shown to utilize proteins that are homologous to those used in developmental systems. In particular, mammalian homologs of Staufen have been identified as potentially important

players in dendritic mRNA localization (Kiebler et al., 1999; Kohrmann et al., 1999; Tang et al., 2001). Kohrmann et al. show that GFP-tagged Staufen homologs can associate with and travel with RNP complexes, and their dendritic localization is dependent on microtubules. Kiebler et al. and Tang et al. have also shown that Staufen and dendritic RNA signal co-localize in the dendrites. Furthermore, Tang et al. have shown that a dominant negative form of Staufen significantly reduces the amount of mRNA found in the dendrites. Together this data indicates that Staufen is likely to be a *trans*-acting factor involved in dendritic localization of mRNA.

Translational Control in Neuronal LPS. In addition to specific transport requirements, if a message is to have a local effect only at its destination — often a distance of several hundred microns from the nucleus — there must be control over translation of the mRNA. Without translational regulation, the protein could be produced *en route* to its destination, thereby negating any specificity that arises as a result of subsequent local synthesis. Thus, it is not surprising that RNP complexes may be translationally incompetent (Krichevsky and Kosik, 2001). Although the exact factors that silence these complexes in mature neuronal systems are not known, biochemical data suggests that KCl depolarization of neurons can cause the RNP complexes to relax their condensed structure to form polysomes (Krichevsky and Kosik, 2001).

The CPE/CPEB system is one of the few known systems of translational regulation of LPS in mature neurons (Richter, 2001). CPEB has been identified at synaptic sites through immunocytochemistry (Wu et al., 1998) and the 3'UTR of CaMKIIα contains two CPEs. Wu et al. have shown that when dark reared rats are

exposed to light, CaMKIIα mRNA's poly(A) tail is extended and that this leads to a significant increase of CaMKIIα protein found in synaptoneurosomes prepared from the visual cortices of these animals (Wu et al., 1998).

Observations by Tang et al. (Tang et al., 2002) suggest that dendritic LPS may also be regulated by the rapamycin sensitive translational signaling pathway. They show through immunostaining that the many of the components of this pathway are present in the dendrites and are synaptically localized to some extent.

Demonstrations of LPS in Mature Neuronal Systems. Although there is much circumstantial evidence supporting the idea of LPS in mature neuronal systems, there have been few convincing demonstrations of its existence. The main failing in most LPS studies in neurons has been the inability to rule out the contribution of non-dendritic synthesis to the measures of LPS that were used.

The first attempt to provide a direct demonstration of neuronal LPS was reported in a study by Feig and Lipton. The authors used [^{3}H]-leucine incorporation to show that new proteins were synthesized in the dendritic regions of hippocampal slice tissue following electrical stimulation. Slices were fixed three minutes post exposure to [^{3}H]-leucine and new synthesis was subsequently visualized via light microscope autoradiography (Feig and Lipton, 1993). The authors make a temporal argument claiming [^{3}H]-leucine incorporation seen in the neuropil of the slice must be due to dendritic LPS, as three minutes was far too short a time for proteins to be synthesized and shipped into the dendrites. This argument may well be valid, but the real failing in this study was the inability to convincingly assign [^{3}H]-leucine incorporation seen in the

neuropil to dendrites. There are somas of interneurons and other cells present in the neuropil and their poor quality images fail to unambiguously exclude these as the actual sites of incorporation.

Another attempt at demonstrating dendritic LPS by a temporal argument was conducted by Ouyang et al. In this study, levels of CaMKIIα immunostaining were monitored in the dendrites of CA1 pyramidal cells 5 minutes after tetanization of the Schaffer collaterals. An increase in immunostaining was evident up to 200 microns from the soma and this increase was blocked by protein synthesis inhibitors (Ouyang Y, 1999). Given the time frame of the experiment and the distances involved, it is unlikely that CaMKIIα is synthesized in the soma and transported to the sites where the increase in immunostaining signal is seen.

Although this study is fairly convincing, another study of activity-dependent increases of CaMKIIα in the neuropil generated very different results. In this study, Steward and Halpain monitored levels of CaMKIIα immunostaining in the neuropil of the dentate gyrus (DG) following various periods of high frequency stimulation of the pathway from the medial entorhinal cortex. Steward and Halpain found that the synaptically activated region of the neuropil showed protein synthesis-*independent* increases in CaMKIIα immunostaining (Steward and Halpain, 1999). This would indicate the increase in CaMKIIα was due to localization of the protein and not new synthesis. There are many differences between these two experiments that could explain the difference in results including differences in the region of tissue studied (CA1 vs. DG) and differences in the electrophysiological procedures (Steward and Halpain stimulated more intensely and *in vivo*, where Ouyang et al. worked with acute slice

preparations). However, Stewart and Halpain show that CaMKIIα can be localized by activity and this provides the possibility that the increases in CaMKIIα in the synaptically activated neuropil evidenced by Ouyang et al. may in part represent sequestration of preexisting CaMKIIα to activated regions.

More convincing evidence of LPS in mature neurons has been possible in invertebrate systems. This is in part because invertebrate neurons are often hardier than vertebrate CNS neurons, thus allowing for more elaborate preparations. However, invertebrates do not have distinct axons and dendrites, thus studies in invertebrate systems may not provide an accurate model for vertebrate neuronal function. Despite this caveat, two reports of neuronal LPS linked to functional outcomes that have been shown in *Aplysia* neurons bear mention.

The cell culture preparation in both studies is the same (see Figure 4).

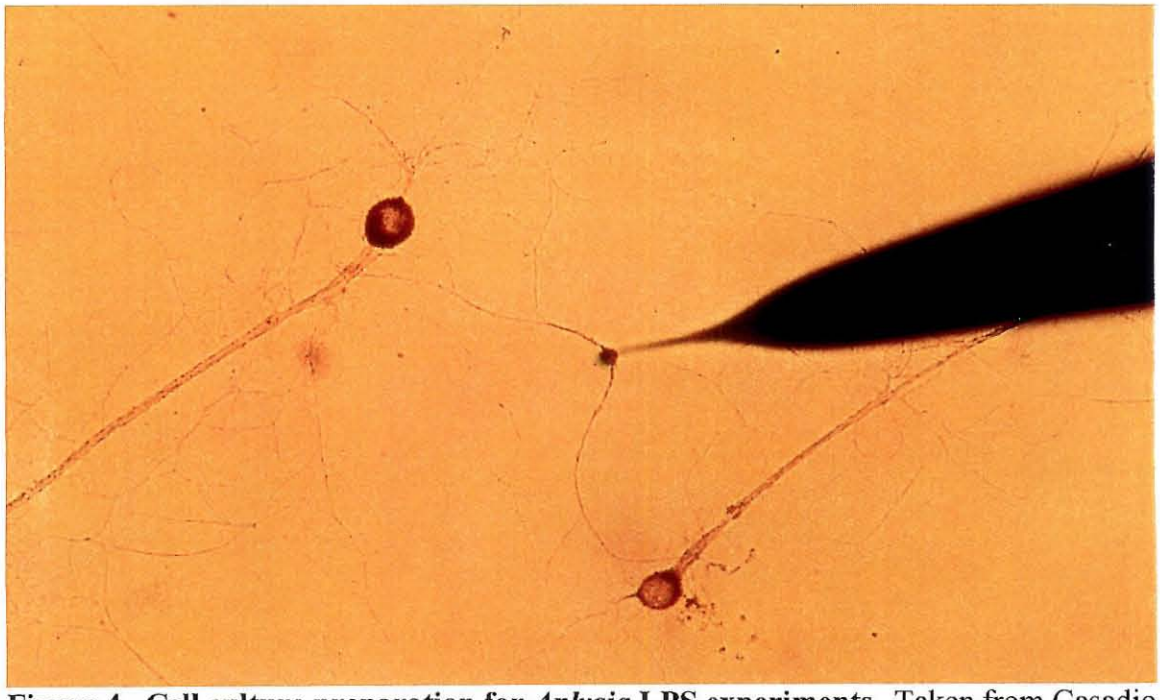

Figure 4. Cell culture preparation for *Aplysia* LPS experiments. Taken from Casadio et al., 1999.

The preparation consists of one bifurcated sensory neuron that synapses on to two different motor neurons.

In the first study, Martin et al. demonstrate that the two different groups of synapses made by the sensory neuron can be independently potentiated to achieve long-term facilitation (LTF). They show that this site-specific LTF requires local synaptic synthesis and that this LTF corresponds with site-specific increases in synaptic varicosities (sites of synaptic contacts) (Martin et al., 1997).

In the second study, Casadio et al. show that a weaker form of cell wide facilitation (induced by stimulating the cell body of the sensory neuron directly, as opposed to stimulating a specific synapse as in Martin et al., 1997) can be converted into a standard site specific LTF with a normally sub-threshold stimulus. This conversion requires rapamycin-sensitive LPS to stabilize both the site-specific increases in synaptic varicosities and the corresponding LTF beyond 24 hours (Casadio A, 1999). Thus it appears that synapses in this system can be initially potentiated specifically or in a cell-wide manner. However, the proteins required for long-term maintenance of this potentiation are likely normally provided by LPS.

Sufficiency and Necessity of LPS to Support Long-Lasting Synaptic Plasticity. Even with a clear demonstration of LPS in a mature vertebrate neuronal system, the question of its ability to support long-lasting synaptic plasticity would remain. The answer to this question came from an elegant series of experiments carried out by Kang and Schuman (Kang and Schuman, 1996). They first demonstrated that neurotrophins were able to induce a long-lasting form of synaptic plasticity that was

sensitive to protein synthesis inhibitors minutes after its initiation. This rapid requirement for protein synthesis made the soma an unlikely source for the proteins required to maintain this plasticity, as the time required to deliver proteins from the soma to the dendrites may be greater than this form of plasticity's insensitivity to synthesis inhibition. Thus, this suggests LPS is required in the dendrites to provide the necessary proteins. Kang and Schuman then showed that dendrites in the CA1 region of the hippocampus that were separated from their somas by a lesion (see figure 5) were able to support this neurotrophin-induced form of long-lasting synaptic plasticity.

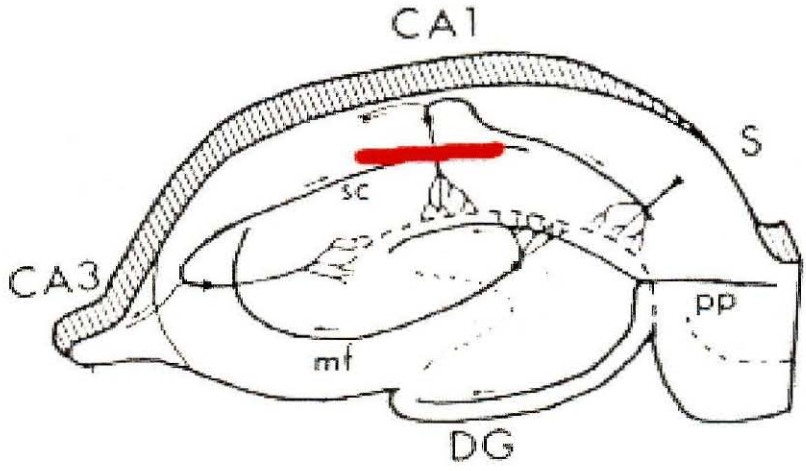

Figure 5. Schematic of experimental preparation used in Kang and Schuman.

However, this long-lasting synaptic plasticity could not be maintained by the transected dendrites when they were treated with the neurotrophin in the presence of a protein synthesis inhibitor. The lesion would have prevented any proteins from being synthesized in the soma and subsequently shipped/diffused into the dendrites, where they are thought to be needed. Thus, LPS in the dendrites was most likely responsible for satisfying the protein synthesis requirement of this long-lasting plasticity. A similar

study was conducted where CA1 neuropil, also isolated from their somas by a lesion, was shown to be able to support a long-lasting form of long-term depression in a protein synthesis-dependent fashion (Huber, 1999). The form of plasticity studied by Huber et al. also had a rapid requirement for protein synthesis as determined through synthesis inhibition. These findings provide compelling evidence of the ability of dendritic LPS to support long-lasting forms of plasticity.

Although the experiments of Kang and Schuman and Huber et al. do suggest the necessity of LPS for maintenance of the specific forms of plasticity that they study, it is neither definitive proof of LPS necessity for their system nor capable of being generalized to other forms of long-lasting plasticity. If they overestimated the time required for protein trafficking from the soma to the dendrites, it may be possible for somatic synthesis to support this form of plasticity in the absence of dendritic LPS. Additionally a temporal argument for LPS necessity likely fails for other forms of plasticity as their periods of synthesis inhibition insensitivity is often much greater (Frey et al., 1988). In order to prove the necessity of dendritic LPS to support long-lasting plasticity, long-lasting plasticity would have to be shown to be abolished when dendritic LPS is inhibited while somatic synthesis is maintained and somatically synthesized proteins are kept unobstructed from their normal transport/diffusion into the dendrites (see figure 6). So far, such an experiment has not been reported.

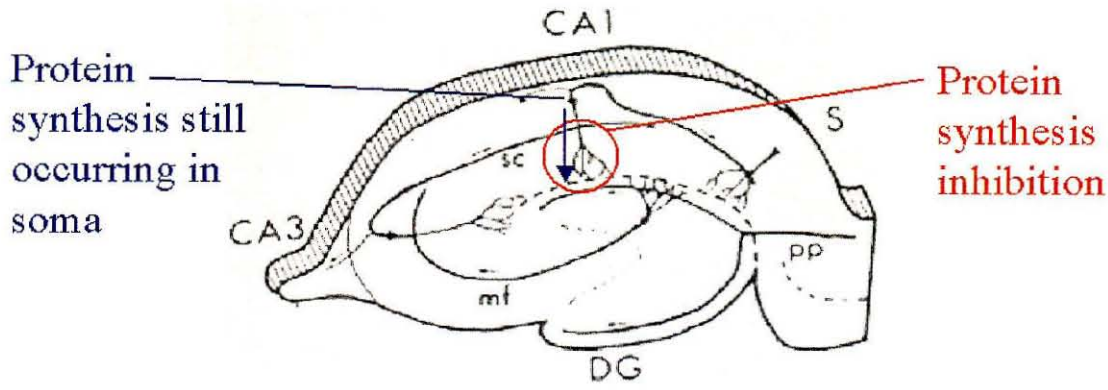

Figure 6. Experimental setup for dendritic LPS necessity experiment.

Overview of Thesis

The second chapter in this thesis describes a series of studies that provide the first clear demonstration of dendritic LPS in living vertebrate neuronal tissue. Theses studies avoid the confounding effects of dendritic targeting of the reporter molecule by using GFP, and not a native dendritic protein. Furthermore, by using a reporter construct with the full 5' and 3' UTRs of CaMKIIα flanking the GFP coding sequence, this work was able to show that this LPS was under the regulation of plasticity inducing stimuli. Finally, the high resolution images used in these studies revealed that there were "hot spots" of LPS in the dendrites–and that these "hot spots" were often associated with synaptic markers and protein synthesis machinery.

The third chapter details an attempted study of the role of endogenous synaptic activity and dendritic LPS. What is actually observed in this study is more likely the ability of a disuse hypersensitivity (a form of synaptic plasticity) inducing environment to stimulate dendritic LPS.

The fourth chapter discusses the development of a technique capable of exploring the necessity of LPS in long-lasting forms of plasticity. This technique utilizes a photoactivatable compound to inhibit protein synthesis in a spatially restricted manner. Thus, this technique would allow for the realization of the experimental paradigm illustrated by Figure 5. This will likely be an important development, as it will allow those studying LPS in neurons to move beyond what has largely been a descriptive effort. This technique will enable research to elucidate the role of LPS in synaptic plasticity.

The fifth and final chapter discusses the current state and future directions of the study of LPS in neurons as well as some commentary on the clinical relevance of this area of investigation.

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CHAPTER 2

Dynamic Visualization of Local Protein Synthesis in Hippocampal Neurons

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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 CAMKII-α, 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, 1999a). 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, 1999a; 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 Ca^{2+}/calmodulin-dependent kinase II-α subunit (CAMKII-α) (5'GFP3'). Previous work has shown that the 3'UTR of the CAMKII-α 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 1A). In most untreated neurons, expression of the reporter was robust in the cell bodies and relatively weak in the associated dendritic processes (Figure 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 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 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.

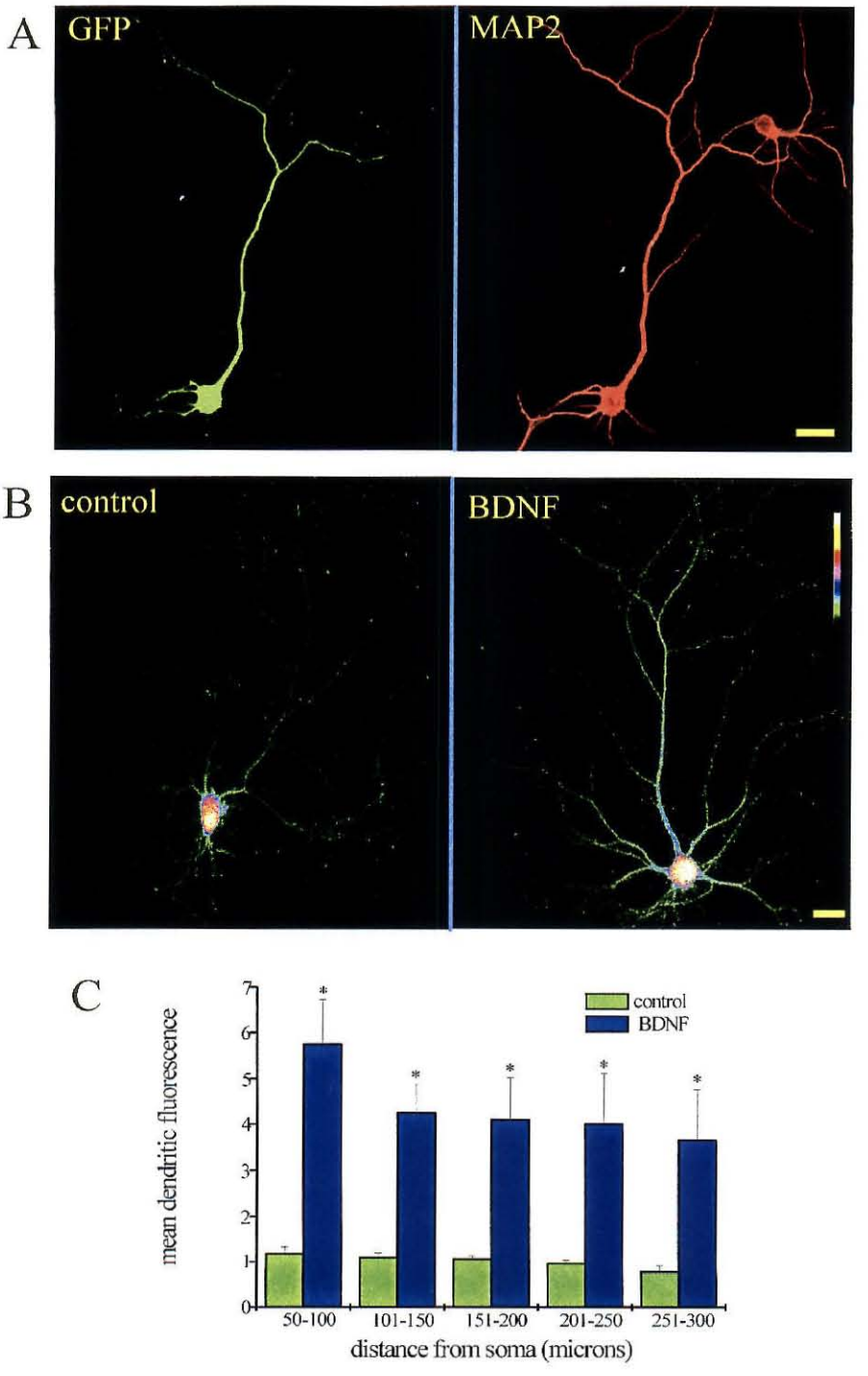

Figure 1. BDNF stimulates protein synthesis of the GFP reporter in hippocampal neurons. A. Shown is a cultured hippocampal neuron expressing the GFP reporter and immunostained for the dendritic marker MAP2. The majority of the GFP signal occurs in the dendrite, as indicated by the coincident MAP2 signal. 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 look-up 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. 0-300 microns 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 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 2C). In addition, the BDNF-induced increases were prevented by coapplication of the protein synthesis inhibitor anisomycin (Figure 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.

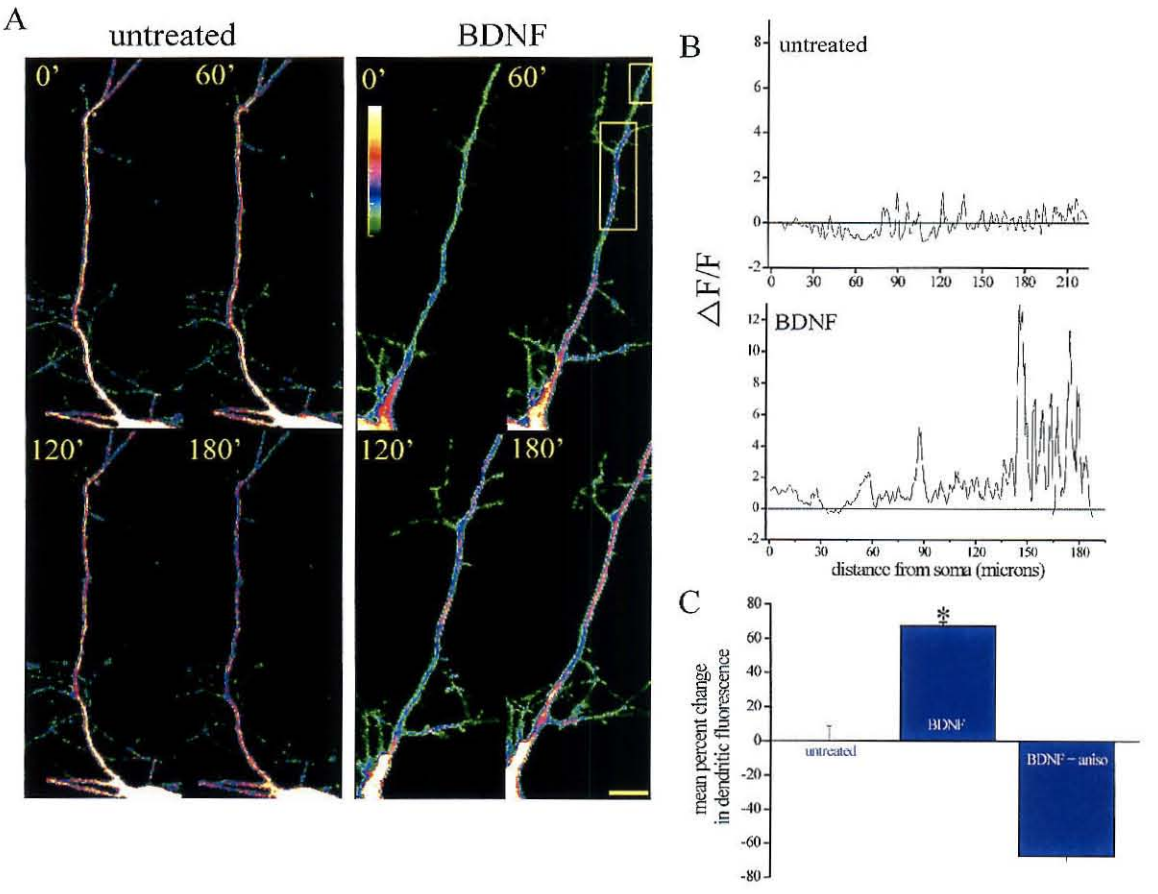

Figure 2. Time-lapse imaging of BDNF-stimulated translation. A. Repeated images of a control neuron and BDNF-treated neuron. BDNF was added immediately after the 0' image was acquired. The BDNF-treated neuron showed increased fluorescence in the dendrite whereas the control neuron was relatively stable 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' images (see methods). C. Summary data for analysis of total dendritic length showing that only dendrites treated with BDNF exhibited significant ($p < 0.01$) increases in fluorescence. Data courtesy of W. B. Smith.

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

two-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 3). In contrast, transected dendrites treated with BDNF exhibited increases in fluorescence in the isolated dendrites (Figure 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 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 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.

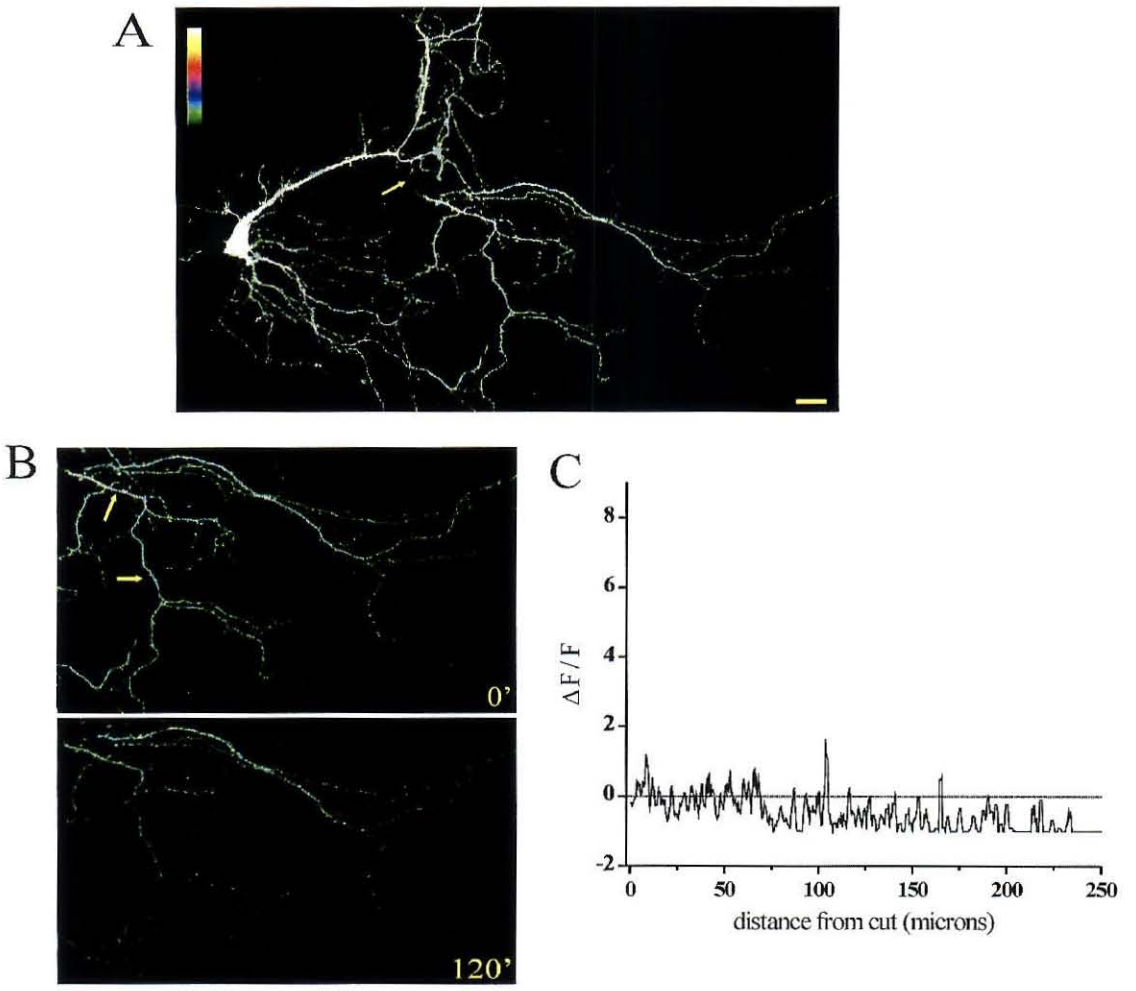

Figure 3. Untreated, transected dendrites do not show increases in protein synthesis. A. Image of an infected neuron; arrow points to the region of transection. Scale bar = 15 μm. B. Images of the isolated region of the dendrite immediately following transection and 120 minutes later. The fluorescent signal in the dendrite decreases over time. Arrows point to the dendrite chosen for analysis in C. C. Analysis of the transected dendrite shown in A and B. ΔF/F was calculated using the data from the 0 and 120' images (see methods). Data courtesy of W. B. Smith.

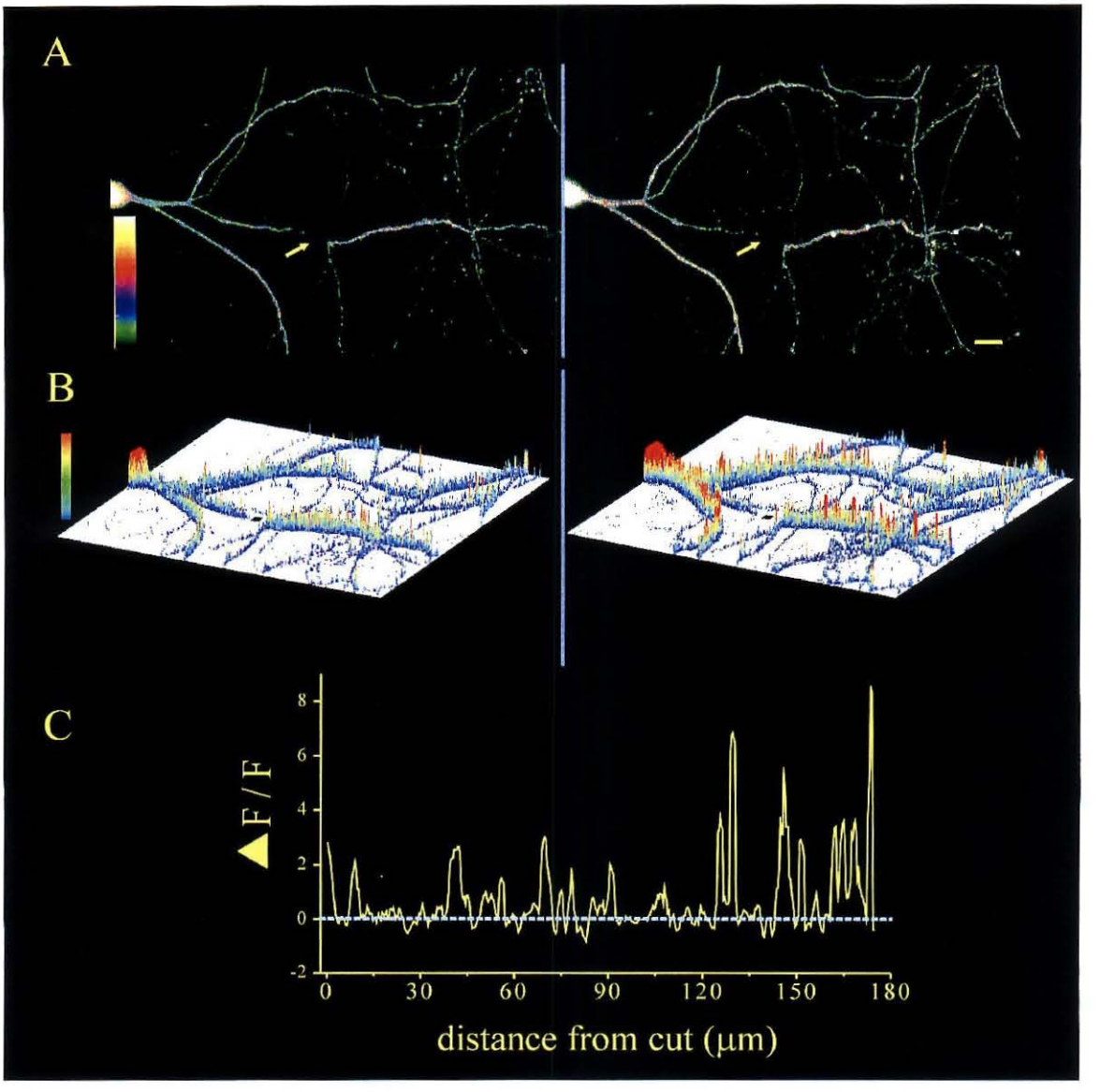

Figure 4. BDNF stimulates protein synthesis in healthy, mechanically-isolated dendrites. A. Images of transected neuron before and 120 minutes after BDNF treatment; arrow points to the region of transection. The fluorescent signal in the transected dendrite increases following BDNF treatment. Scale bar = 15 μm. B. X-Y-Z 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' images (see methods). Data courtesy of W. B. Smith.

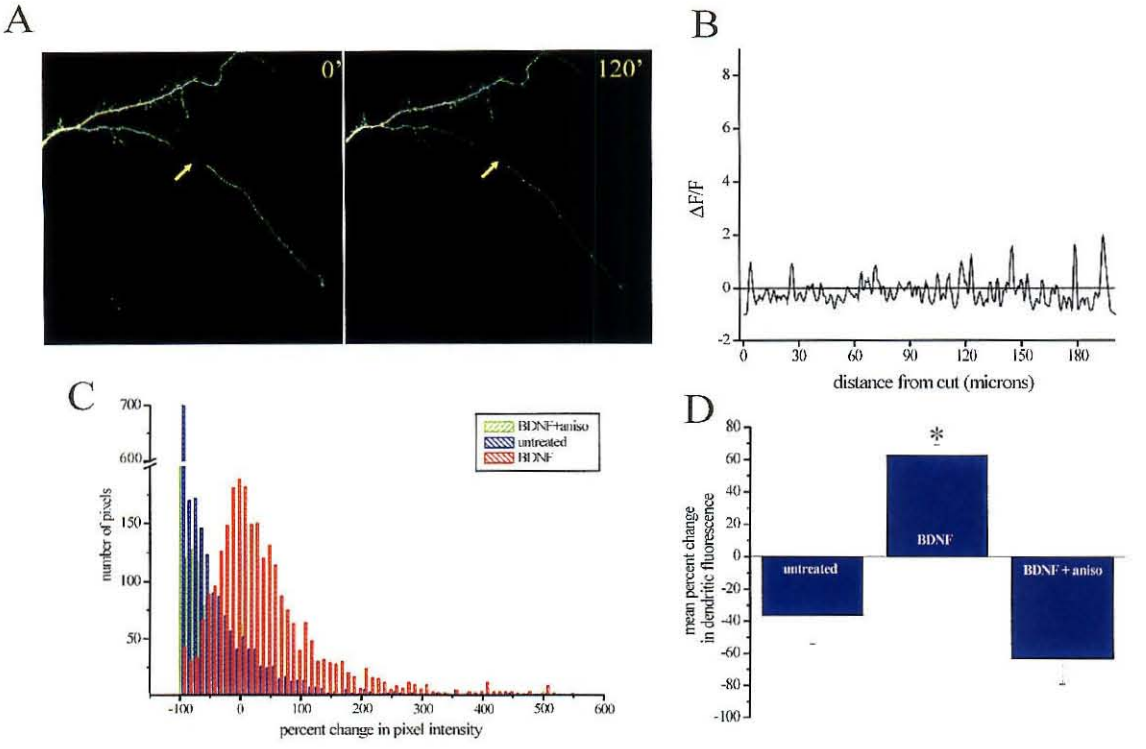

Figure 5. Anisomycin prevents BDNF-induced increases in protein synthesis in transected dendrites. A. Images of transected dendrite before and 120 minutes after co-application of BDNF and anisomycin; arrow points to the region of transection. The fluorescent signal in the transected dendrite decreased over time. 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' images (see methods). C. Summary histogram indicating the pixel intensity distributions for all untreated, BDNF-treated, and BDNF + 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 signficant increase in fluorescence intensity over time ($p < 0.01$). Data courtesy of W. B. Smith.

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'$_{myr}$dGFP3', 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 6A). We found that the addition of the myr sequence, however, severely retarded the diffusion of the modified (Sin-5'$_{myr}$dGFP3') reporter (Figure 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.

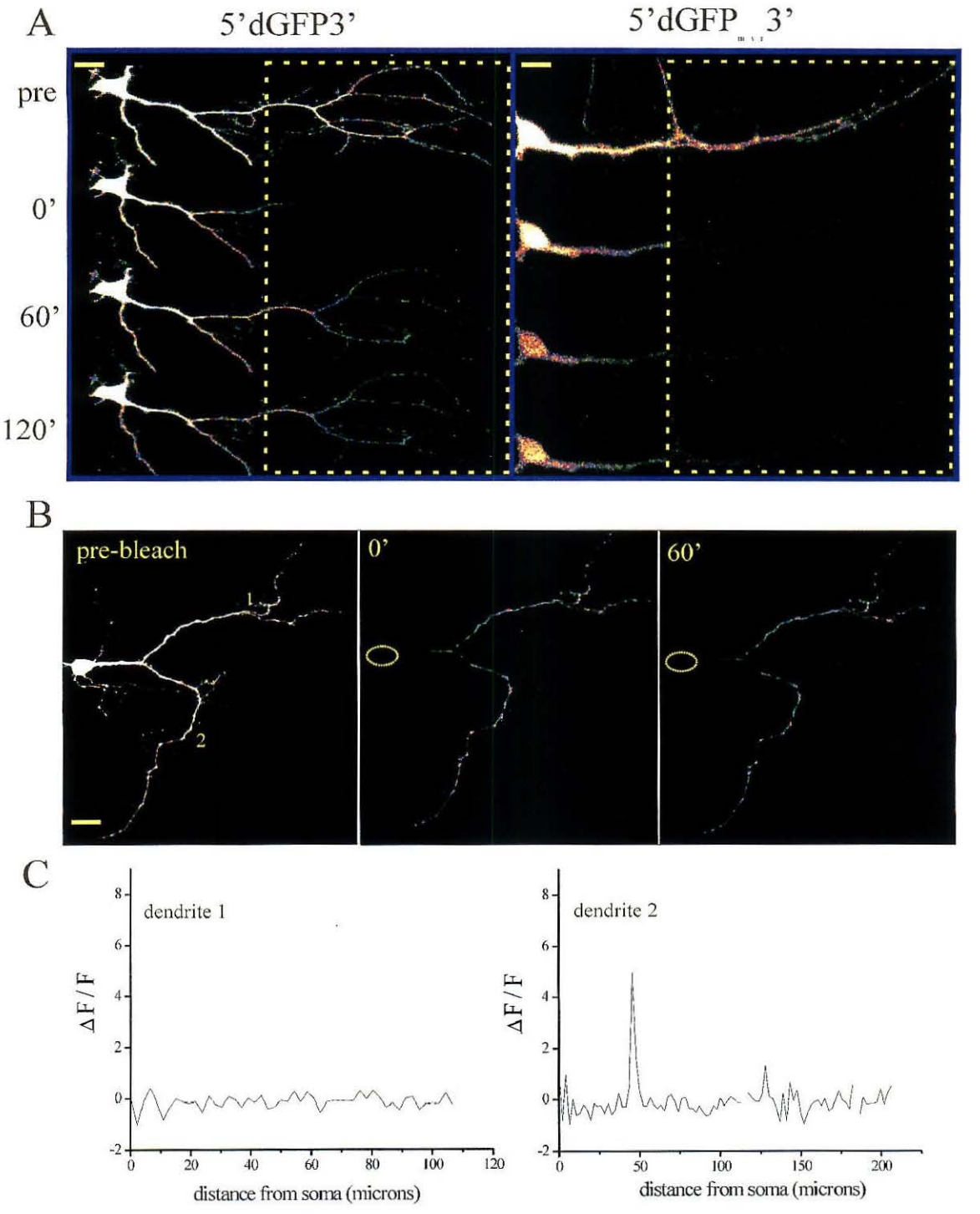

Figure 6. A membrane-anchored GFP reporter exhibits extremely limited diffusion. A. Shown are two neurons infected with either 5'dGFP3' or the membrane anchored 5'dGFP$_{myr}$3'. Neurons were treated with anisomycin for 1 hour prior to the initiation of photobleaching (boxed region shows bleached area). FRAP was monitored in each

neuron over time. The neuron infected with the diffusible reporter (5'dGFP3') showed significant recovery of fluorescence within 60 minutes of the photobleaching. In contrast, the myristoylated reporter showed negligible recovery within 2 hours following photobleaching. Scale bars: 5'dGFP3' : 15 μm; 5'dGFP$_{myr}$3': 10 μm. B. Timelapse images of a 5'dGFP$_{myr}$3'-expressing neuron that was subjected to somatic photobleaching for the duration of the experiment. The pre-bleached 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 minutes 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.

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

We next used the diffusion-restricted reporter (Sin-5'$_{myr}$dGFP3') 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 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 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 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 8).

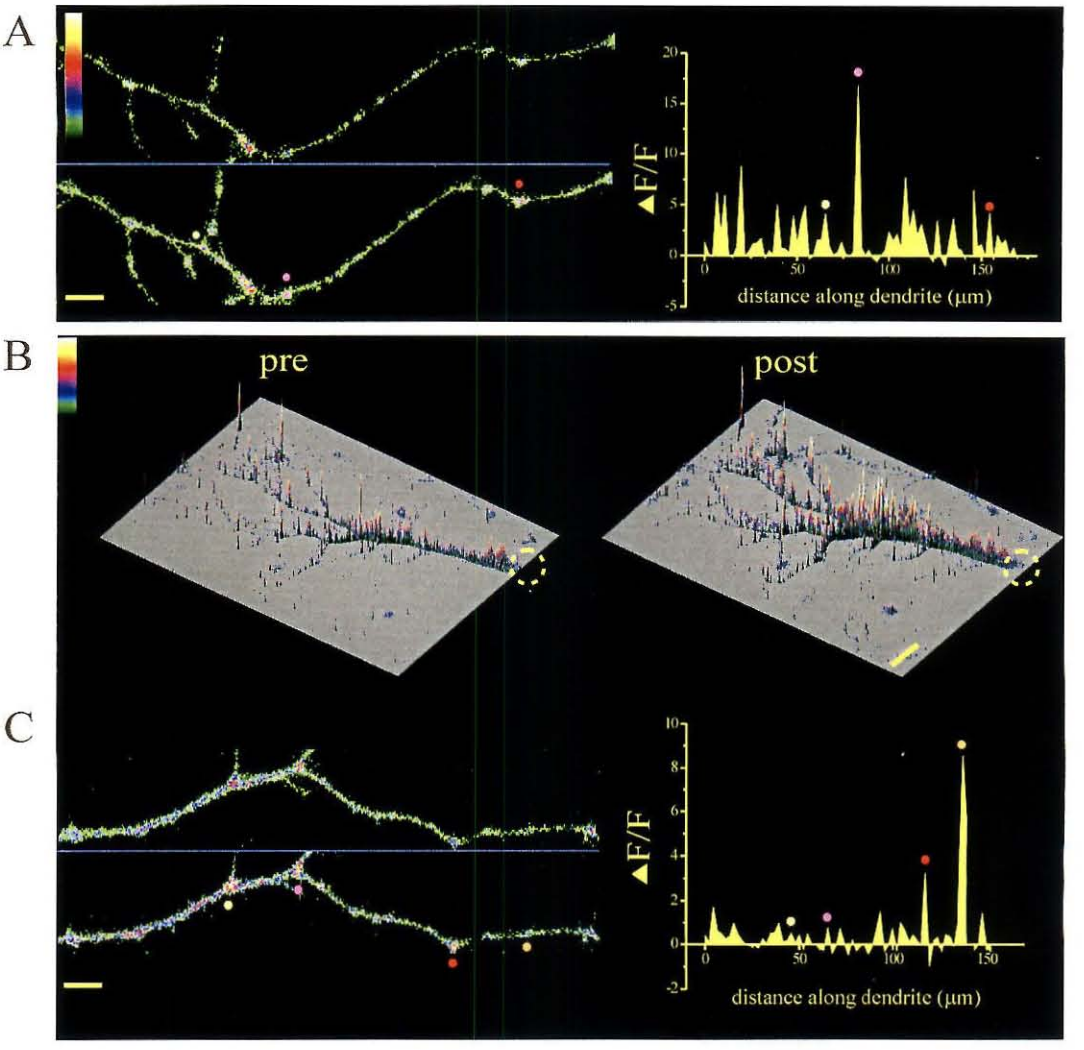

Figure 7. BDNF stimulates protein synthesis in healthy, "optically-isolated" dendrites. A. Shown is enhancement of GFP translation in an optically isolated dendrite at two consecutive time points. The profile of fluorescence change between the two time points shown is plotted on the right. Individual hot-spots are identified by colored circles on the image and the corresponding profile. B. An X–Z 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. 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.

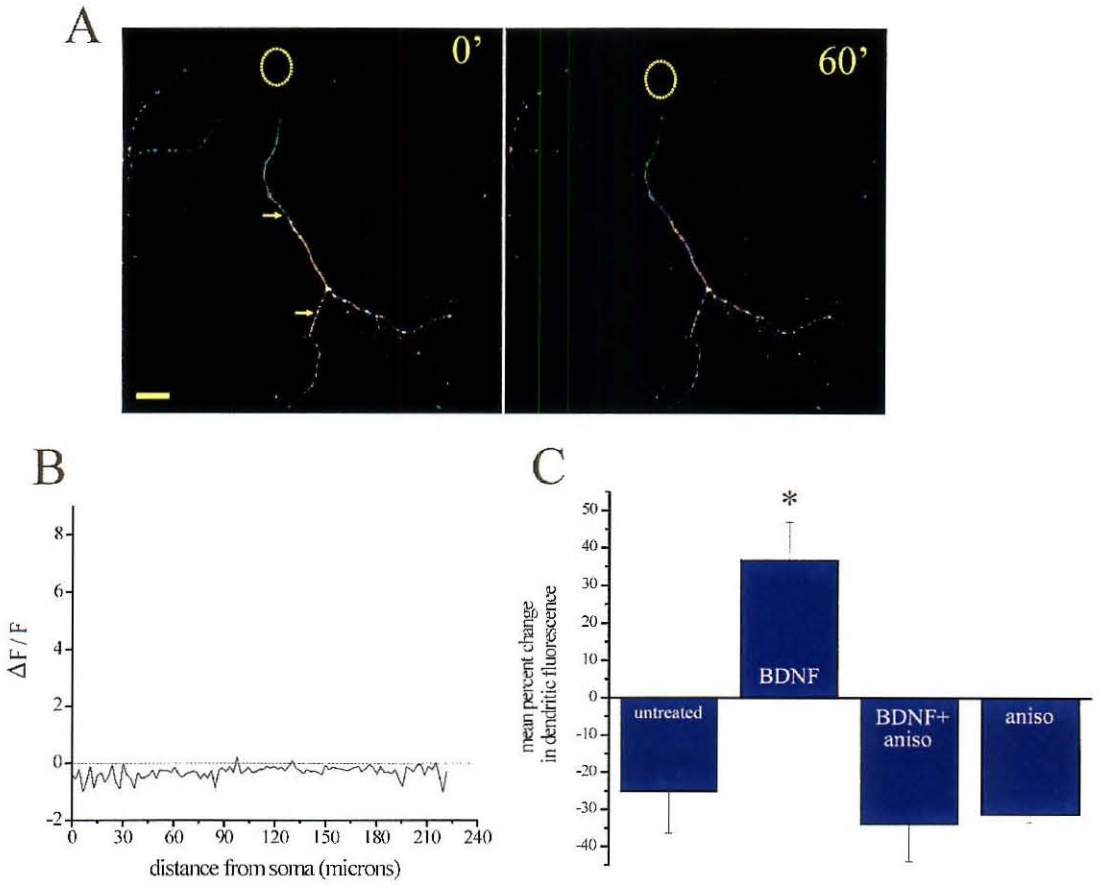

Figure 8. Anisomycin blocks BDNF-induced increases in protein synthesis in optically-isolated dendrites. A. Timelapse images of an optically isolated dendrite that was treated with BDNF + anisomycin. The optically-isolated dendrites are shown at two consecutive time points. There was an overall decline 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 minutes 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 + anisomycin-, and anisomycin-treated optically isolated dendrites. Only the BDNF-treated dendrites showed a signficant increase in fluorescence intensity over time ($p < 0.01$).

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 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 $5'_{myr}$dGFP3'-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 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 (analysis courtesy of W. B. Smith). 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 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.

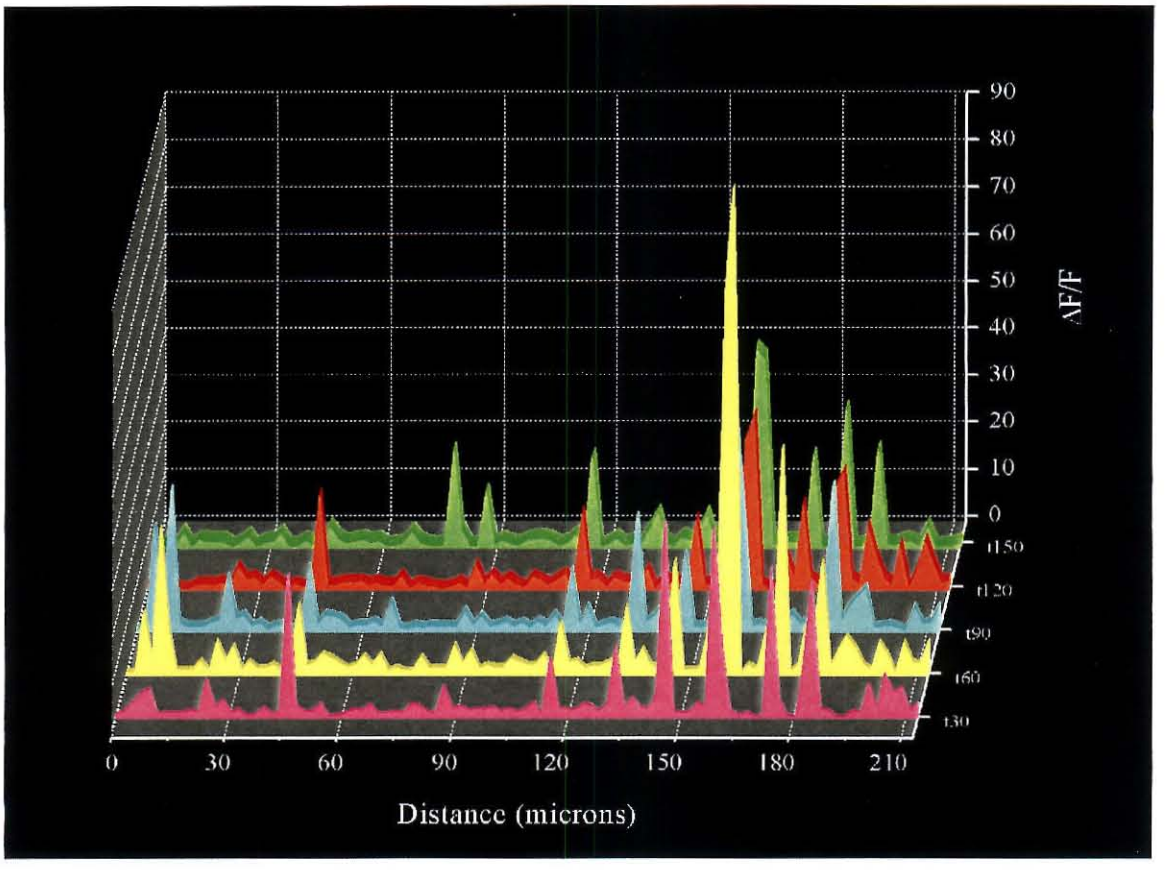

Figure 9. GFP reporter signals are spatially and temporally persistent. Shown are the ΔF/F profiles for a single optically-isolated dendrite at several time-points. The increased GFP signal is concentrated at relatively stable sites along the length of the dendrites. These sites also appear to be temporally stable over a 2 hour time period.

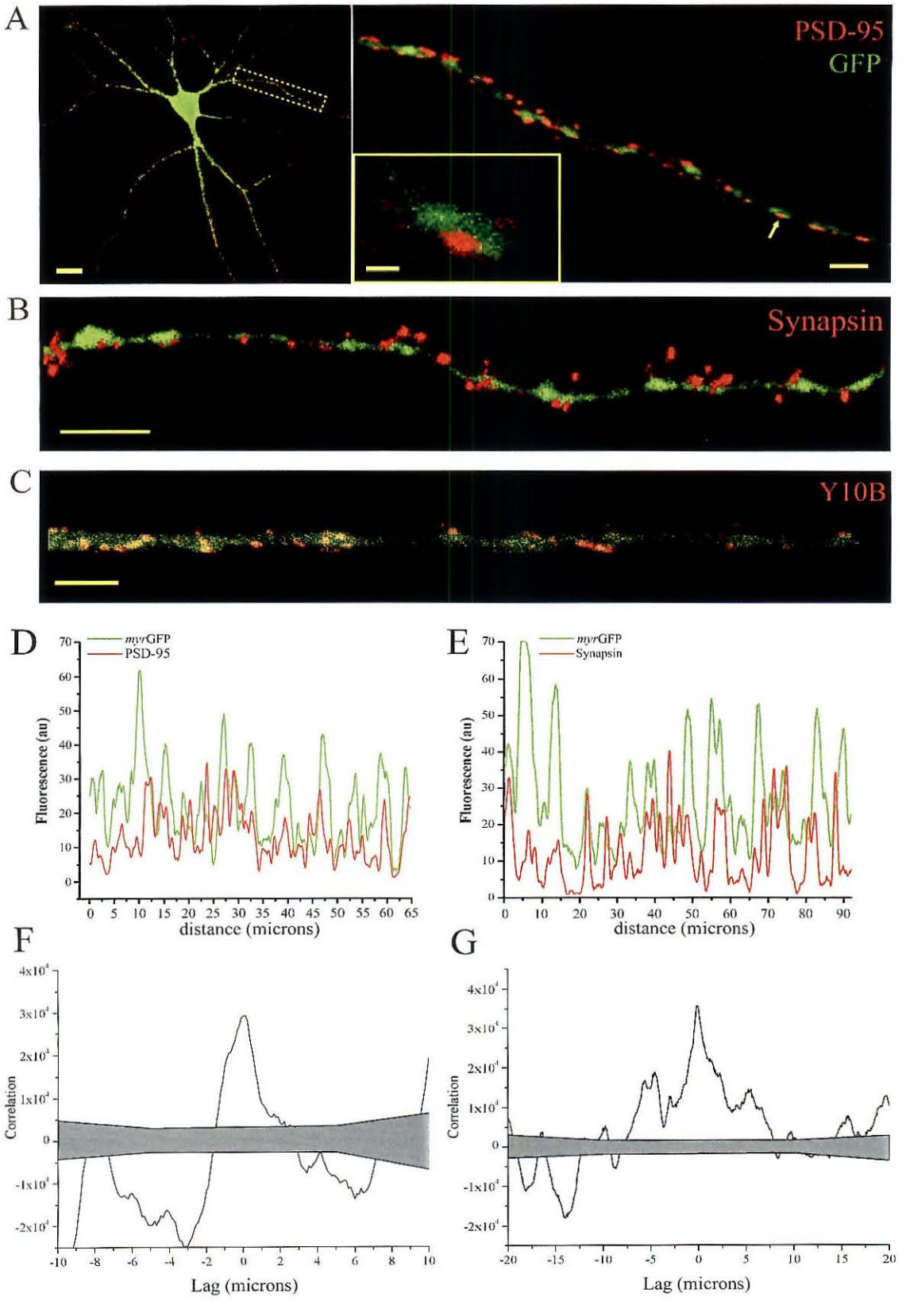
A
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Figure 10. GFP reporter signals colocalize with ribosomes and synaptic markers.
(A) Low- (left) and high-power (right) images of a GFP reporter-expressing neuron immunostained with an antibody against the synaptic marker PSD-95. The inset shows the GFP signal that forms a cloud around the punctate PSD-95 signal. Scale bars 5, 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 are 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 (Casadio et al., 1999; Huber et al., 2000; Kang and Schuman, 1996; Martin et al., 1997). 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 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). 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 CAMKII-α since our reporter contains both the 5' and 3'UTR from the CAMKII-α gene. Indeed, a stimulation of dendritic CAMKII-α 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 CAMKII-α 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 CAMKII-α 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; S.J. Tang et al., 1998, Soc. Neurosci., abstract). 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.

Materials and Methods

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/cm^2 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 seconday 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 CamKII-α 3'UTR sequence obtained from plasmid (Mayford et al., 1996) was PCR amplified (forward primer: 5'-ttatatttgcggccgcggtcgctaccattaccagtt-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 CamKII-α 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'$_{myr}$dGFP3': 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 $_{myr}$dGFP was subcloned into the BamHI-NotI sites of pcDNA3.1-5'dGFP3'. pSinRep5-5'$_{myr}$dGFP3': The 5'$_{myr}$3 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 αfamily 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 1×10^{-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, Scion Image, or Image J),

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 $_{myr}$dGFP 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.

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CHAPTER 3

Silencing of Intrinsic Synaptic Activity Results in Increased Dendritic Protein Synthesis in Cultured Hippocampal Neurons

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Summary

A number of studies have recently shown that exogenous stimuli can induce or increase dendritic protein synthesis. We wished to study the role of intrinsic, or native, synaptic activity in the regulation dendritic protein synthesis. In order to do this, we made use of a diffusion-limited GFP-based dendritic protein synthesis reporter that contains the 5' and 3' UTRs of CaMKIIα (Aakalu et al., 2001). This reporter's rate of synthesis can be stimulated by the application of brain derived neurotrophic factor (BDNF). However, neurons transfected with this construct also express significant amounts of the reporter in the absence of any exogenous stimulus. Indeed, under control (unstimulated) conditions regions of high expression of the reporter are in the vicinity of synaptic markers (Chapter 2, Figure 10). We wished to investigate the possibility that the reporter's expression may be modulated by intrinsic synaptic activity. To do so, we employed a variety of pharmacological agents that block synaptic activity and then assessed the effects on the synthesis of our reporter in dendrites. Our findings indicate that silencing synaptic activity results in increased protein synthesis in the distal dendrites. This increased synthesis may represent the synthesis required to establish "disuse hypersensitivity", a form of synaptic plasticity resulting from chronic blockade of synaptic transmission (Murthy et al., 2001).

Introduction

Compelling evidence has been provided in a number of systems demonstrating that dendrites are capable of protein synthesis (Aakalu et al., 2001; Martin et al., 1997; Ouyang Y, 1999). Indeed this synthesis has been shown to be able to support long-lasting forms of synaptic plasticity (Huber et al., 2000; Kang and Schuman, 1996; Martin et al., 1997). However, very little is known about the mechanisms that govern dendritic protein synthesis. Some studies have suggested the involvement of cytoplasmic polyadenylation and the mammalian target of rapamycin (mTOR) pathway in the regulation of dendritic protein synthesis (Tang et al., 2002; Wu et al., 1998). Little is known, however, about the upstream signals that control these regulatory pathways. Previous studies have given some insight into this area by the use of exogenous stimuli. The dendritic translation of CaMKIIα is stimulated by exogenous electrical stimulation or growth factors that modulate synaptic activity (Aakalu et al., 2001; Ouyang Y, 1999). We wished to explore whether endogenous synaptic activity present in the network of cultured hippocampal neurons could regulate dendritic protein synthesis. Thus, we visualized the dendritic expression level of a GFP-based reporter in neurons under normal, synaptically active conditions and in those in which activity was silenced by pharmacological agents.

Results

To visualize dendritic protein synthesis, we utilized a GFP based reporter that we have previously described (Aakalu et al., 2001). This reporter construct consists of the coding sequence for a destabilized variant of EGFP fused to a myristoylation signal. The coding sequence is flanked by the 5' and 3' untranslated regions (UTRs) of calcium/calmodulin-dependent kinase II α (CaMKIIα), thereby endowing the mRNA for the reporter with dendritic localization and translational regulation similar to CaMKIIα (Mayford et al., 1996; Mori et al., 2000; Blichenburg et al., 2001; Aakalu et al., 2001). As Ouyang et al. have demonstrated the dendritic synthesis of CaMKIIα can be elicited by exogenous synaptic stimulation (Ouyang Y, 1999), the translation of this reporter construct may also be regulated by synaptic activity. Indeed the dendritic translation of this reporter is stimulated by brain derived neurotrophic factor (BDNF) – a growth factor that can increase both the frequency and the amplitude of mEPSCs in culture (Aakalu et al., 2001; Li et al., 1998).

The reporter was introduced into cultured P2 rat hippocampal neurons via infection with a sindbis viral vector. The cultures were simultaneously treated with either a cocktail of pharmacological agents to silence synaptic activity (Chu and Hablitz, 2000) or a vehicle control. The neurons were then incubated at 37°C for 12 hours and subsequently transferred to a HEPES buffered saline solution for imaging. Fluorescence images were taken of neurons expressing the reporter using a confocal microscope. Analysis was carried out by an individual blind to the experimental condition. The

neurons in both the silenced and control dishes displayed robust expression of the reporter (Figure 1).

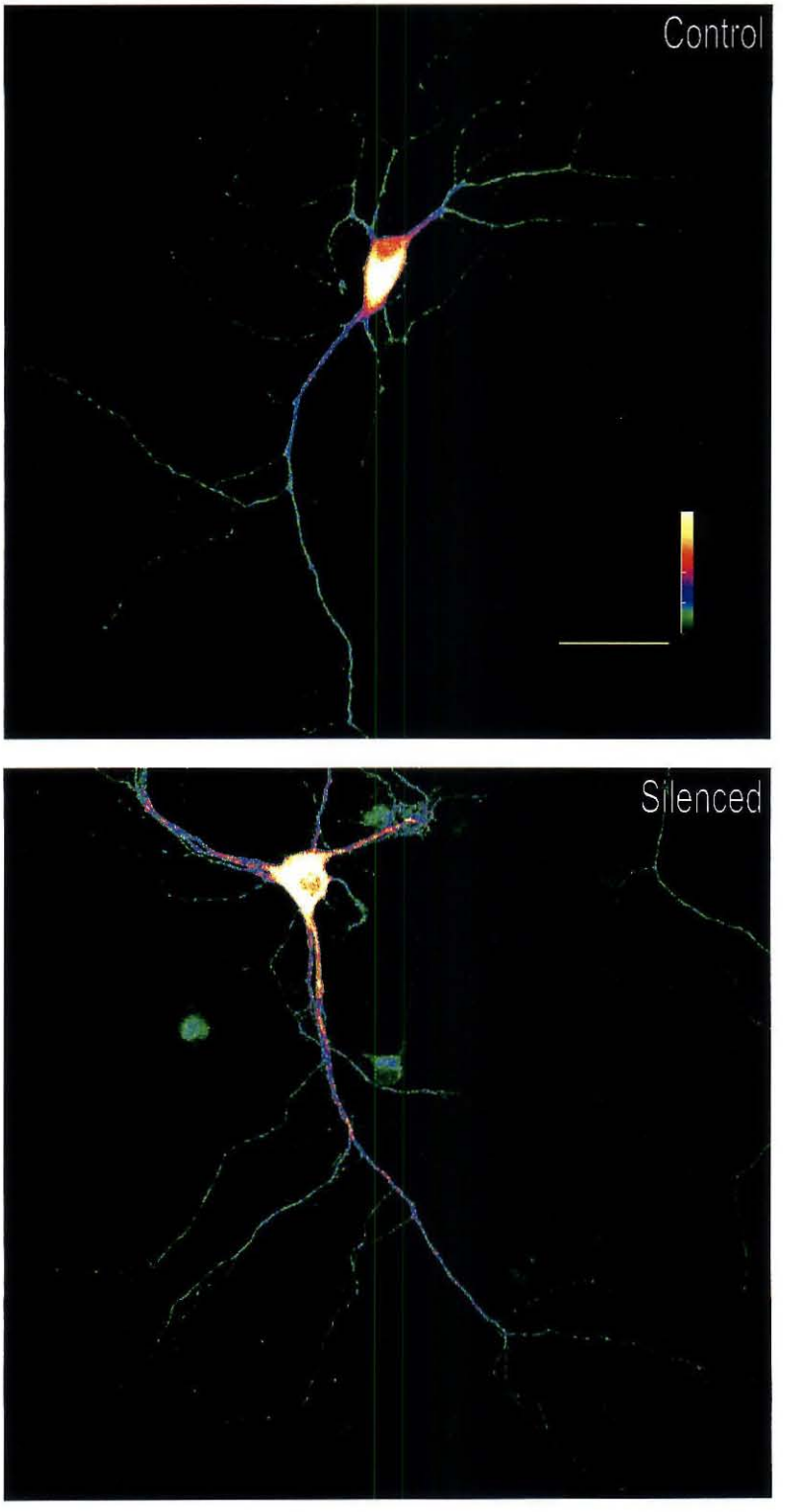

Figure 1. Representative neurons from live-cell silencing experiments. Shown here are two neurons expressing the dendritic synthesis reporter. The cell in the top panel is a vehicle treated control and the cell in the bottom panel is a cell that had been silenced.

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 = 50 μm.

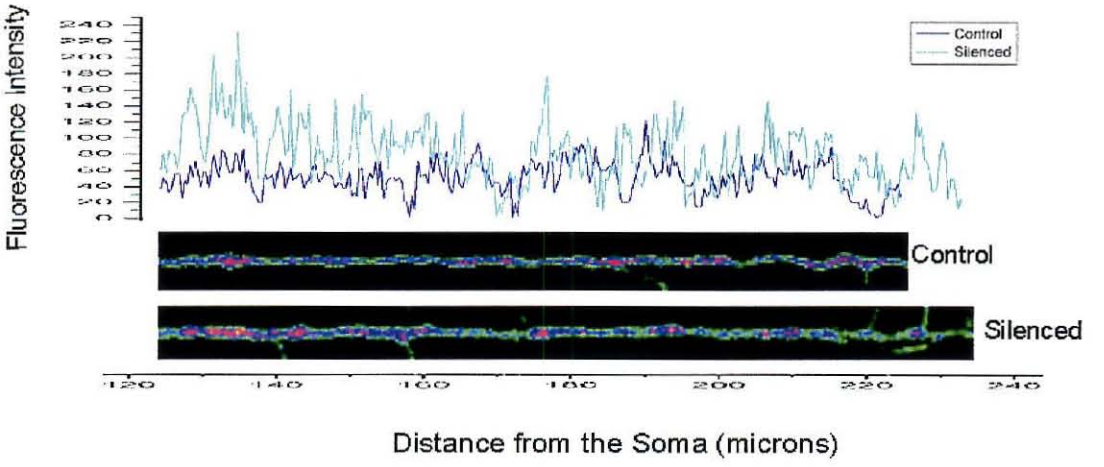

Figure 2. Fluorescence levels in distal dendrites of representative neurons from live-cell silencing experiments. Fluorescence levels are plotted against distance from the soma. The images of the analyzed dendrites are given below the graph. In this example, the distal dendrite of the silenced neuron shows higher levels of dendritic synthesis than the vehicle treated control.

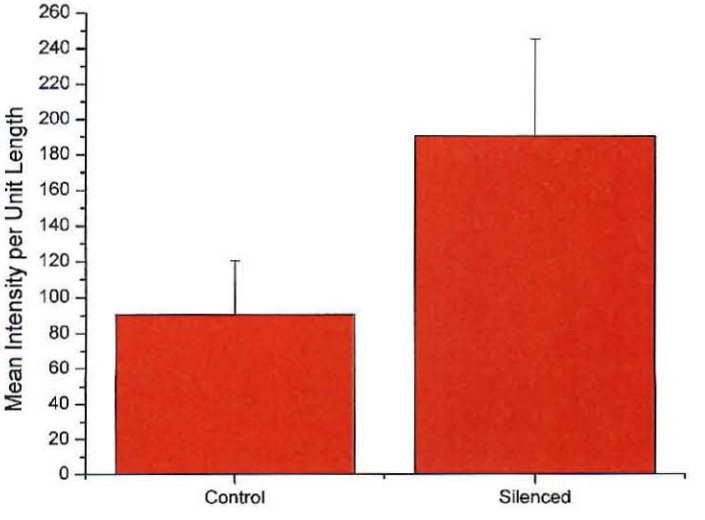

Figure 3. Summary data for live-cell silencing experiments. The mean fluorescence intensity per unit length in the distal dendrites of silenced cells is not statistically different from vehicle treated controls ($P \geq 0.01$).

Although the reporter is diffusion limited, the period of time the neurons had expressed the reporter may have been long enough to allow some somatically synthesized reporter molecules to enter the dendrites. In order to minimize this somatic contribution we measured reporter expression levels solely in the distal dendrites (greater than 125 μm from the soma). When distal dendrites were examined, the synaptically silenced group showed increased reporter synthesis when compared with controls (Figure 3), but this difference was not statistically significant. This lack of statistical significance was likely due to the large neuron-to-neuron variation in reporter expression within experimental groups. Thus we carried out a larger scale study to determine if silencing synaptic activity did have a significant effect on dendritic protein synthesis.

A second, larger, study was conducted in which neurons were fixed prior to imaging and following treatment with control solution or the activity blocker cocktail (Figure 4). Imaging and analysis for this study were both carried by individuals that were blind to the experimental condition. There was a significant difference when expression was analyzed in the distal dendrites (Figure 5). The dendrites of silenced neurons exhibited a greater level of reporter expression in their distal dendrites than dendrites of vehicle treated controls ($p = 0.006$).

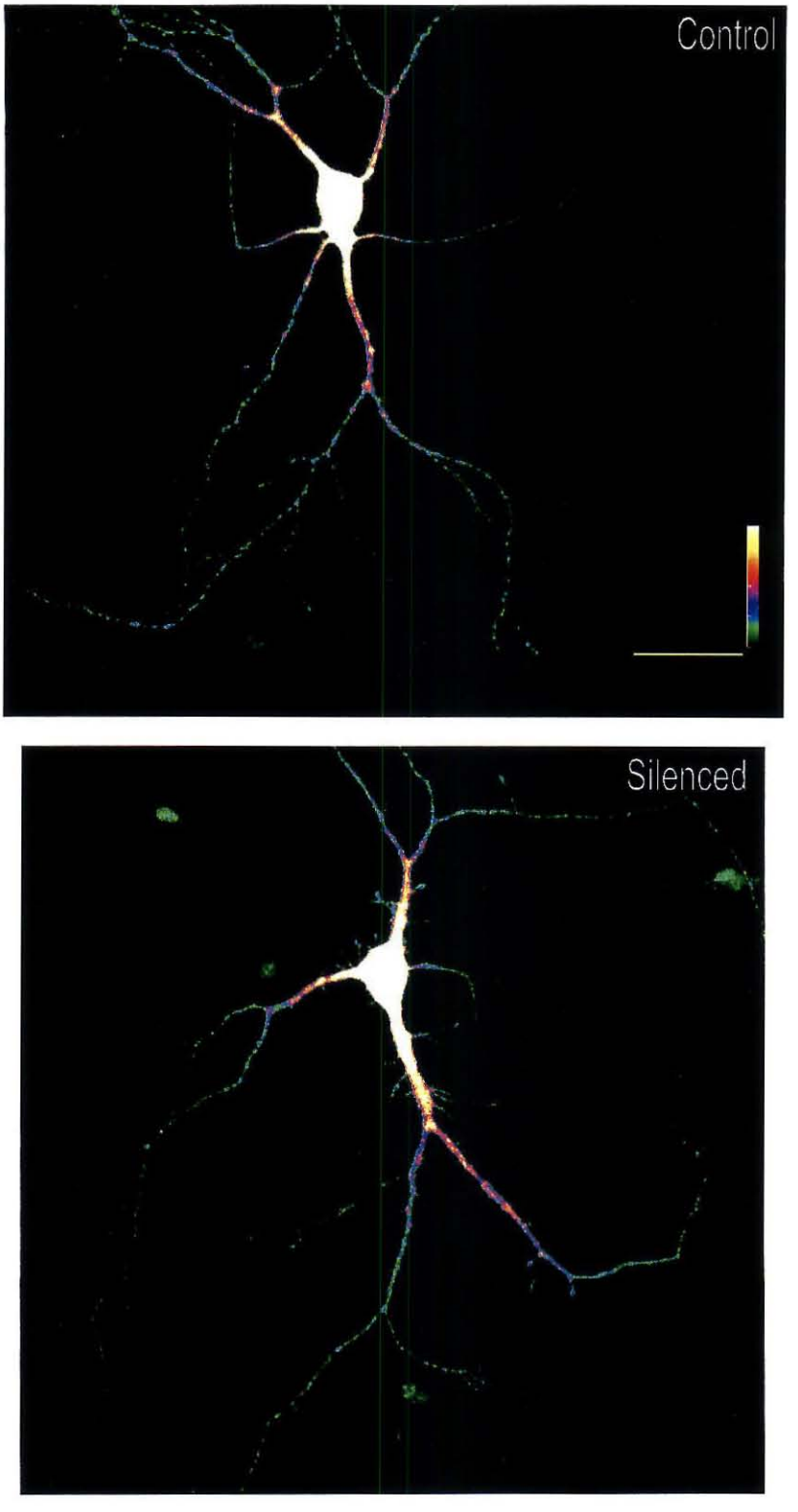

Figure 4. Representative images from fixed-cell silencing experiments. Shown here are two neurons expressing the dendritic synthesis reporter. The cell in the top panel is a

vehicle treated control and the cell in the bottom panel is a cell that had been silenced. Scale bar = 50 μm

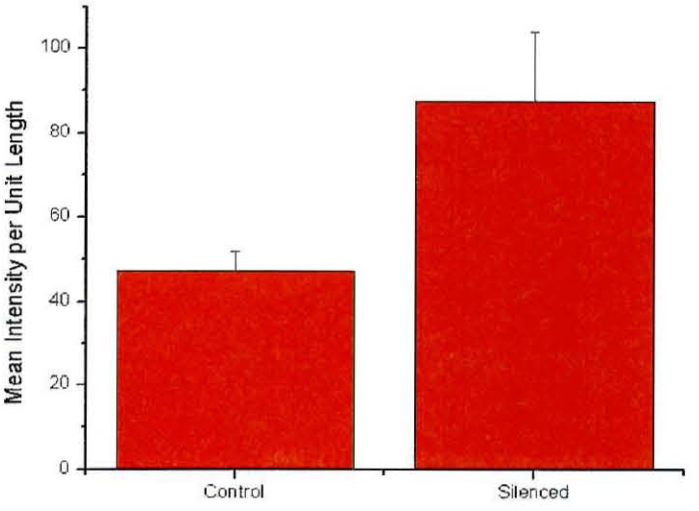

Figure 5. Summary data for fixed-cell silencing experiments. The distal dendrites of silenced cells show higher levels of protein synthesis (as measured by mean fluorescence intensity per unit length) than vehicle treated controls ($P \leq 0.01$).

Discussion

The finding that silenced neurons exhibited increased dendritic synthesis was surprising, but not entirely unexpected. When a synapse has been silenced or otherwise inactive for an extended period of time, it is strengthened (Murthy et al., 2001). This phenomenon is termed disuse hypersensitivity. It is thought to be the result of a homeostatic mechanism typically used for the establishment of synaptic efficacy (Murthy et al., 2001). Disuse hypersensitivity is associated with a number of disorders and diseases including tardive dyskinesia, nicotine addiction, and glaucoma (Casey, 2000; Clark, 1989; Dani and Heinemann, 1996).

Disuse hypersensitivity requires new protein synthesis (Fambrough, 1970; Grampp et al., 1972). The proteins synthesized to generate this hypersensitivity are known to include neurotransmitter receptors (Fambrough, 1970; Grampp et al., 1972) (Follesa and Ticku, 1996; Rao and Craig, 1997). The synthesis of other molecules required for other forms of synaptic plasticity may be needed as well. The translational regulation of the reporter molecule used in this study has been designed to mimic just such a molecule – CaMKIIα. Therefore it is plausible to propose that the increase in dendritic reporter expression that we observe is the result of the protein synthesis required for disuse hypersensitivity initiated by synaptic activity blockade. However, this apparent increase in dendritic reporter expression was not strictly shown to be protein synthesis-dependent. This was because in order to demonstrate the increase in reporter concentration was dependent on new synthesis, the neurons would have to be incubated in protein synthesis inhibitors for the period of synaptic silencing to show that this would abolish the increase. However, incubation in protein synthesis inhibitors for longer than 5 hours has been shown to be detrimental to neuronal viability (G. Aakalu, unpublished results) and the length of synaptic silencing studied in this series of experiments was 12-13 hours. Therefore, there remain other possible explanations for the greater fluorescence levels observed in the dendrites of silenced neurons. The first and most reasonable alternative explanation for our results is that silencing induces a decrease in the degradation rate of the reporter rather than increasing its synthesis. In theory this explanation could be ruled out by showing that inhibitors of protein degradation do not abolish the increase in reporter signal. Unfortunately, much like incubation in the synthesis inhibitors, incubation in degradation inhibitors negatively impacts neuronal

health after 8 hours (G. Patrick, unpublished results). The technical difficulties in working with the synthesis and degradation inhibitors could be overcome if the increase in dendritic reporter expression occurred with a shorter period of synaptic silencing. Preliminary data indicate that this does occur (G. Aakalu, unpublished results) and experiments are underway to confirm this finding. Although unlikely and never previously described, synaptic silencing may also increase membrane fluidity allowing increased diffusion of reporter generated in the soma into the dendrites. As there is a high concentration of reporter in the soma this could then result in increased fluorescent signal in the dendrites of silenced neurons. The possibility of increased membrane fluidity due to silencing could be studied by staining the somas of synaptically silenced and control neurons with a lipophilic dye (e.g., dil) and observing the rates of diffusion of the dye into the dendrites in the two different groups of cells. If there was no significant difference in the rates of diffusion, it would rule out the contribution of this hypothetical effect to dendritic reporter expression.

The mechanism by which the dendritic synthesis could be stimulated (or protein degradation could be suppressed) by synaptic silencing is unclear. One possibility is that after a period of time, lack of synaptic activity triggers protein synthesis in a manner similar to other exogenous stimuli that elicit dendritic synthesis (e.g., 5HT, BDNF, etc.) (Aakalu et al., 2001; Martin et al., 1997; Ouyang Y, 1999). Synaptic silencing may be detected by an intracellular sensor that may be in one state in the presence of synaptic activity (e.g., phosphorylated or not, etc.) and in another when there is a lack of activity. Alternatively, the absence of synaptic activity may be detected by the neuron through the buildup of a molecule that is regularly degraded by activity. One such molecule is IκB.

It is normally complexed to NF-κB, but upon NMDA activation NF-κB is activated and IκB is rapidly degraded (Whiteside and Israel, 1997; Lopez-Salon et al., 2001; Wellmann et al., 2001). IκB concentration increases in cultured hippocampal neurons when there is a blockade of basal synaptic transmission, presumably due to the concomitant block of NF-κB activation and thus IκB degradation (M. Meffert, unpublished results). There is another mechanism that could account for the stimulation of dendritic synthesis by synaptic silencing: It is possible that dendritic protein synthesis is normally inhibited by of synaptic activity; a lack of activity would then relieve this inhibition. This activity-dependent translational repression may be mediated by factors such as eukaryotic initiation factor 2 alpha (eIF-2α). In cultured neurons, eIF-2α is phosphorylated in response to elevated levels of calcium, and in this phosphorylated state it inhibits protein synthesis (Alcazar et al., 1996). During periods of normal synaptic activity there may be sufficient calcium influx, due to NMDAR and L-type calcium channel activation, to maintain a certain level of phospho-eIF-2α. When synaptic activity is silenced, the calcium levels in the dendrites may drop, thereby decreasing the phosphorylated form of eIF-2α and increasing protein synthesis. In addition there may be other activity-dependent repressors of protein synthesis similar to those used in regulating protein synthesis in developmental systems (Mendez and Richter, 2001; Richter and Theurkauf, 2001; Smith et al., 2001).

As disuse hypersensitivity develops over the time frame of days (Murthy et al., 2001), observing the effect of synaptic silencing over shorter periods of time than in this study may give a more clear insight into the interplay of endogenous activity and dendritic synthesis.

Materials and Methods

Neuronal culture preparation. Dissociated hippocampal neuron cultures are prepared from post-natal 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 days (fixed cell experiments) or 15-21 days (live cell experiments) before use.

Sindbis virus. Construction and production of sindbius virus containing the 5'$_{myr}$dGFP3' reporter has been previously described (Aakalu, et al., 2001).

Live cell experiments. Dissociated P2 hippocampal neurons were incubated for 12 hr in growth medium containing the Sindbis virus as well as a cocktail of pharmacological agents (20 μM APV, 40 μM CNQX, 0.5 μM TTX, and 500 μM MCPG) or a vehicle control. After the incubation, growth medium was removed and replaced with a HEPES-buffered solution (HBS) (Malgaroli and Tsien, 1992) (without glycine or picrotoxin) for imaging. N = 10 for silenced neurons, 8 for vehicle treated neurons. N = 8 for silenced neurons with dendrites over 125 μm, 7 for control neurons with dendrites over 125 μm.

Fixed cell experiments. Dissociated P2 hippocampal neurons were incubated for 13 hr in growth medium containing the Sindbis virus as well as a cocktail of pharmacological agents (20 μM APV, 40 μM CNQX, 0.5 μM TTX, and 500 μM MCPG) or a vehicle control. After the incubation, neurons were fixed at room temperature with

4% paraformaldehyde for 20 min. These specimens were imaged in PBS. N = 38 for silenced neurons, 46 for vehicle treated neurons. N = 25 for silenced neurons with dendrites over 125 μm, 40 for control neurons with dendrites over 125 μm.

Confocal microscopy. Confocal images were acquired in 0.3 μm sections; image analysis was conducted on z-compressed image stacks, which 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 silenced neurons in a given experimental set. All neurons used in our experiments had a pyramidal neuron-like morphology with one or two major dendrites emanating from the cell body.

Analysis. To analyze the GFP of individual dendrites, we calculated the mean pixel intensity for each dendrite along its length or its length past 125 μm (Image J).

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CHAPTER 4

A Method to Locally Control Protein Synthesis

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Summary

Protein synthesis is required for persistent forms of synaptic enhancement, such as LTP (Krug et al., 1984; Montarolo et al., 1986; Frey et al., 1988; Otani et al., 1989). Whether the required proteins are synthesized in the soma or the dendrites has not yet been determined. To explore the necessity of dendritic protein synthesis, one would have to inhibit protein synthesis in the dendrites of neurons while maintaining normal somatic protein synthesis and an intact route for protein transport from the soma to the dendrites. If late phase LTP were abolished under these conditions, it would demonstrate the insufficiency of somatic protein synthesis and the necessity of dendritic protein synthesis in sustaining late-phase LTP. In order to inhibit protein synthesis exclusively in the dendrites, we have developed a photo-releasable anisomycin compound. The cage consists of a 6-bromo-7-hydroxycoumarin-4-ylmethyl group, which can be removed through exposure to UV light. Therefore, the area of protein synthesis inhibition can be restricted to a small region defined by the limits of UV light exposure, or, potentially, multiphoton excitation if a pulsed IR laser is the light source. We have tested the compound's effectiveness using *in vitro* protein translation systems and cultured CHO cells. These tests indicate that the uncaged compound can effectively inhibit protein synthesis in a spatially restricted manner and does not affect protein synthesis in its caged form. This ability to "locally" inhibit protein synthesis will enable experiments in hippocampal slices where one can specifically inhibit protein synthesis in the cell body or synaptic layers. These experiments will elucidate the roles of somatic and/or dendritic protein synthesis during LTP with both spatial and temporal precision.

Introduction

A large body of work has implicated the dendrites as the likely site of the protein synthesis required for the maintenance of long-lasting forms of synaptic plasticity (Smith et al., 2001; Steward and Schuman, 2001; Aakalu et al., 2001; Casadio et al., 1999; Huber et al., 2000; Kang and Schuman, 1996; Martin et al., 1997; Rao and Steward, 1991; Torre and Steward, 1992; Torre and Steward, 1996; Weiler and Greenough, 1991; Weiler and Greenough, 1993)). The strongest evidence for this model has been provided by Kang and Schuman and Huber et al. (Huber et al., 2000; Kang and Schuman, 1996). Both groups demonstrated that synaptic neuropil isolated from their cell bodies can maintain persistent forms of synaptic enhancement in a protein synthesis-dependent fashion. They additionally showed that it would likely take too long for somatically synthesized proteins to travel to the dendrites to efficiently support such enhancement. Kang and Schuman and Huber et al.'s temporal demonstration of dendritic synthesis necessity pertains only to forms of synaptic enhancement that have sensitivity to protein synthesis inhibition in the time scale of a few minutes, as at physiological temperatures somatically synthesized proteins may reach the dendrites in 5 to 10 minutes (Feig and Lipton, 1993; Kang and Schuman, 1996). Temporal arguments, however, are less than definitive, given that transport rates in dendrites have not been perfectly defined. A more convincing case for the necessity of dendritic protein synthesis would be made if long-lasting plasticity were abolished under conditions in which dendritic synthesis was inhibited while somatic

synthesis was permitted. However, in order to conduct an experiment of this nature, one would need to be able to inhibit protein synthesis in a spatially restricted manner.

One method for controlling and manipulating cell physiology in a spatially-restricted manner utilizes photolabile protecting groups to render physiologically active messengers inactive ("caged") until they are pulsed with a flash of light. This cleaves away the protecting group ("uncaging"), effecting a rapid concentration increase of the messenger. This technique is an excellent way to examine the kinetics or spatial heterogeneity of biochemical responses in cell or tissue culture and achieve temporal control over messenger release. A number of photolabile protecting or "caging" groups have been developed for this purpose (Adams and Tsien, 1993; Marriott, 1998; Nerbonne, 1996), including *p*-nitrobenzyl (*p*-NB), 4,5-dimethoxy-2-nitrobenzyl (DMNB), and 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc) (Furuta et al., 1999), the latter of which is especially useful for controlling the *spatial* release biological effectors because of its sensitivity to multiphoton excitation, a process whereby two or more coincident infrared photons promote a chromophore to the excited state (Denk et al., 1995; Denk et al., 1990; Williams et al., 1994; Xu and Webb, 1997; Xu et al., 1996). Messenger release is confined to the focus of an infrared laser beam, ~1 $(\mu m)^3$; in addition, photodamage to the tissue, light absorption, and scattering are minimized, allowing much deeper and more accurate penetration into complex tissue samples than can be achieved with UV light. This paper describes a method for the spatially restricted, or local, inhibition of protein synthesis using a photoactivated anisomycin, which blocks the peptide bond formation reaction in eukaryotic (80S) ribosomes: K_i = 0.43 (yeast), 1.6 (human tonsil), and 0.65 (rabbit reticulocyte) μM (Barbacid and Vazquez, 1974; Ioannou et al., 1998).

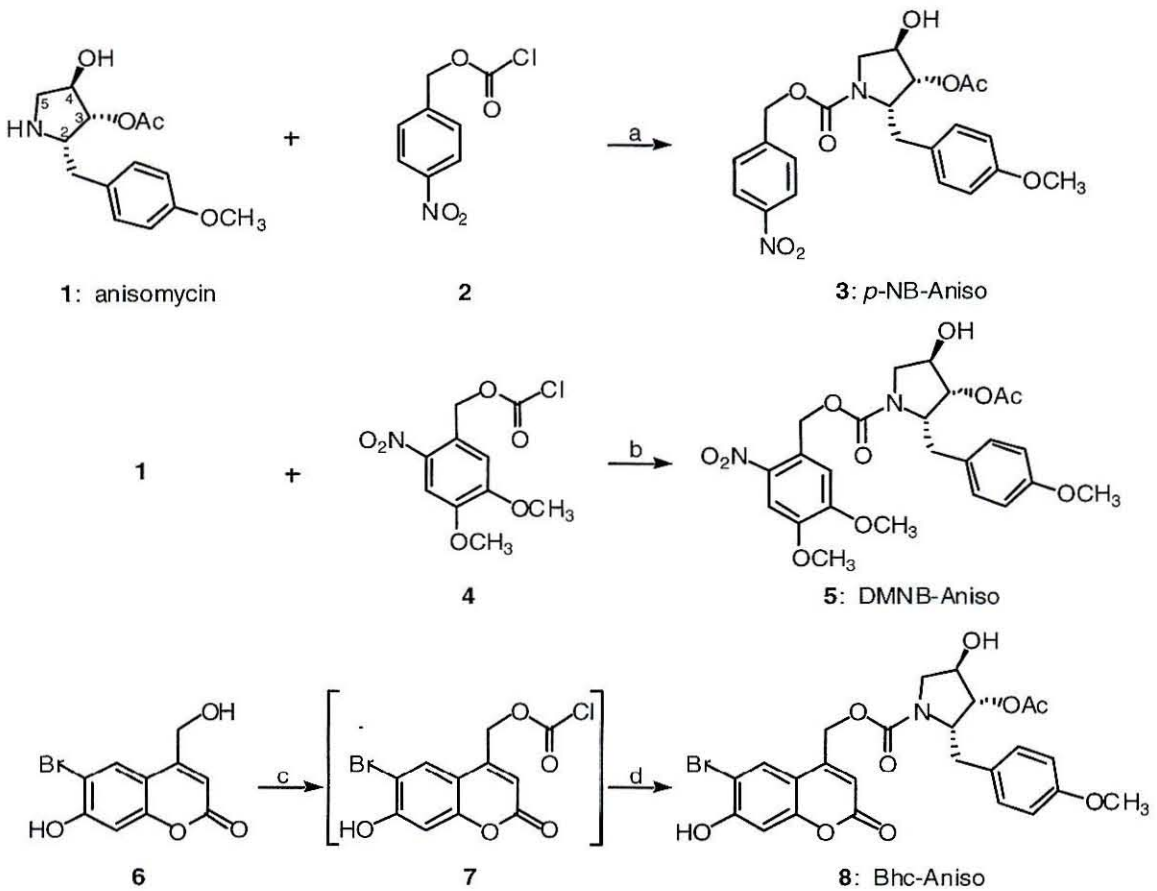
Results

Synthesis and Chemical Characterization

The "caged" anisomycins, *p*-NB-Aniso (**3**) and DMNB-Aniso (**5**), were synthesized by treating anisomycin (**1**) with the commercially available chloroformates **2** and **4**, respectively (Scheme 1). Treatment of bromohydroxycoumarin **6** (prepared from 4-bromoresorcinol and ethyl 4-chloroacetoacetate by published methods (Furuta et al., 1999)) with phosgene produced the chloroformate **7**, which upon exposure to anisomycin yielded Bhc-Aniso (**8**) (Iino et al., 1995).

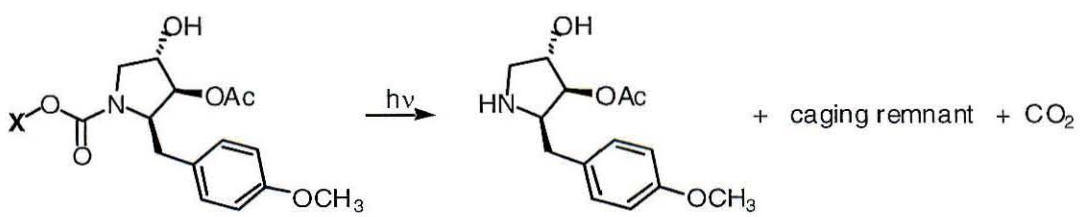
In order to determine which of the three caged anisomycins would be most suitable for regulating protein synthesis in neurons, the 1- and 2-photon photochemical properties of each compound were evaluated for their photolytic properties (Table 1). Upon irradiation with 365-nm light (Scheme 2), the rate of 1-photon photolysis of compounds **3**, **5**, and **8** fits to a simple decaying exponential (Figure 1) with time constants (τ) of 22173, 187, and 20.9 s, respectively, which correspond to uncaging quantum efficiencies (Q_{u1}) of 0.00007, 0.012, and 0.040 mol/ein, respectively. The maximum measured concentration of anisomycin was reached after 100 s of irradiation of Bhc-Aniso (**8**). Clearly, Bhc-Aniso possesses superior 1-photon uncaging kinetics to the nitrobenzyl ester-based compounds. The 2-photon uncaging cross-section (δ_u), a measure of the sensitivity of a molecule to 2-photon photolysis, at 740 nm of DMNB-Aniso (**5**) and Bhc-Aniso (**8**) were found to be 0.048 GM and 0.59 GM, respectively (Figure 2, 1 GM = 10^{-50} cm^4 s/photon). Thus, DMNB-Aniso lacks a sufficiently high uncaging cross-section to be useful in vivo, while Bhc-Aniso is well suited for experiments requiring the

highly localized regulation of anisomycin release combined with minimal phototoxicity obtainable by multiphoton excitation.



(a) Na_2CO_3, THF, 58%; (b) Na_2CO_3, THF, 67%; (c) phosgene, THF, rt, 1 h; (d) anisomycin (**1**), THF, Na_2CO_3, water, rt, 1 h, 35%

Scheme 1. Synthesis of caged anisomycins.

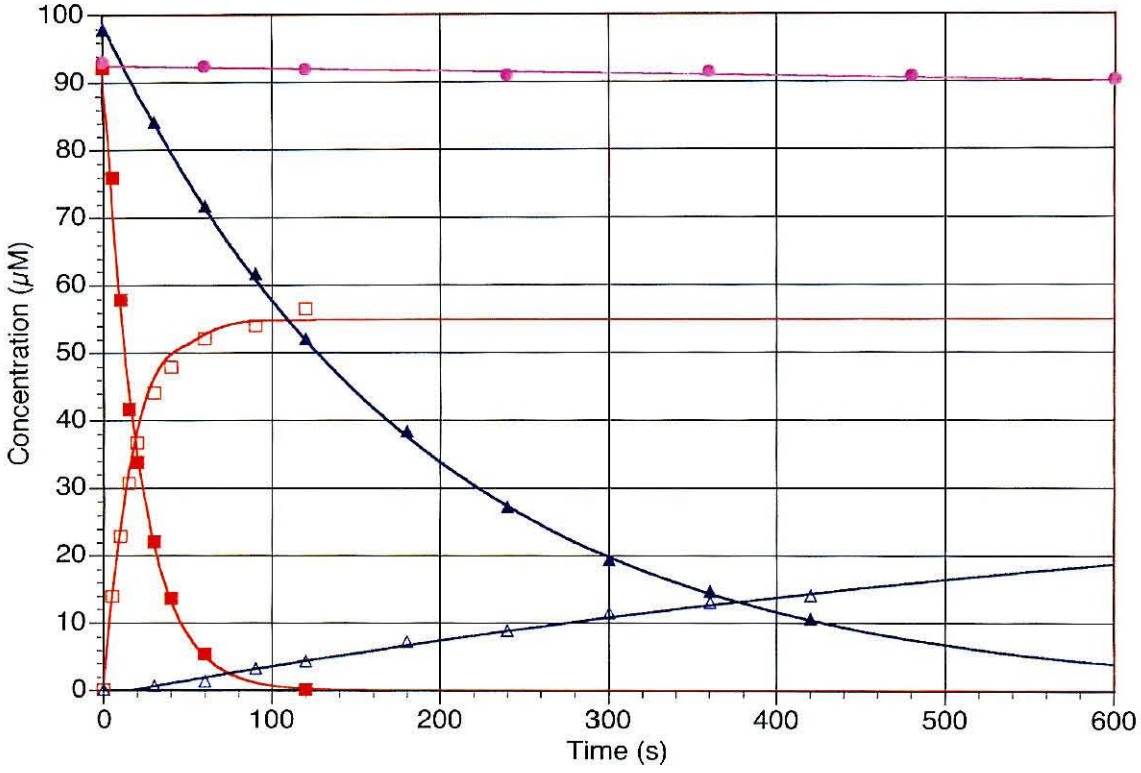


caged anisomycin

3: **X** = *p*-NB
5: **X** = DMNB
8: **X** = Bhc

anisomycin (**1**)

Scheme 2. Photolytic release of anisomycin.



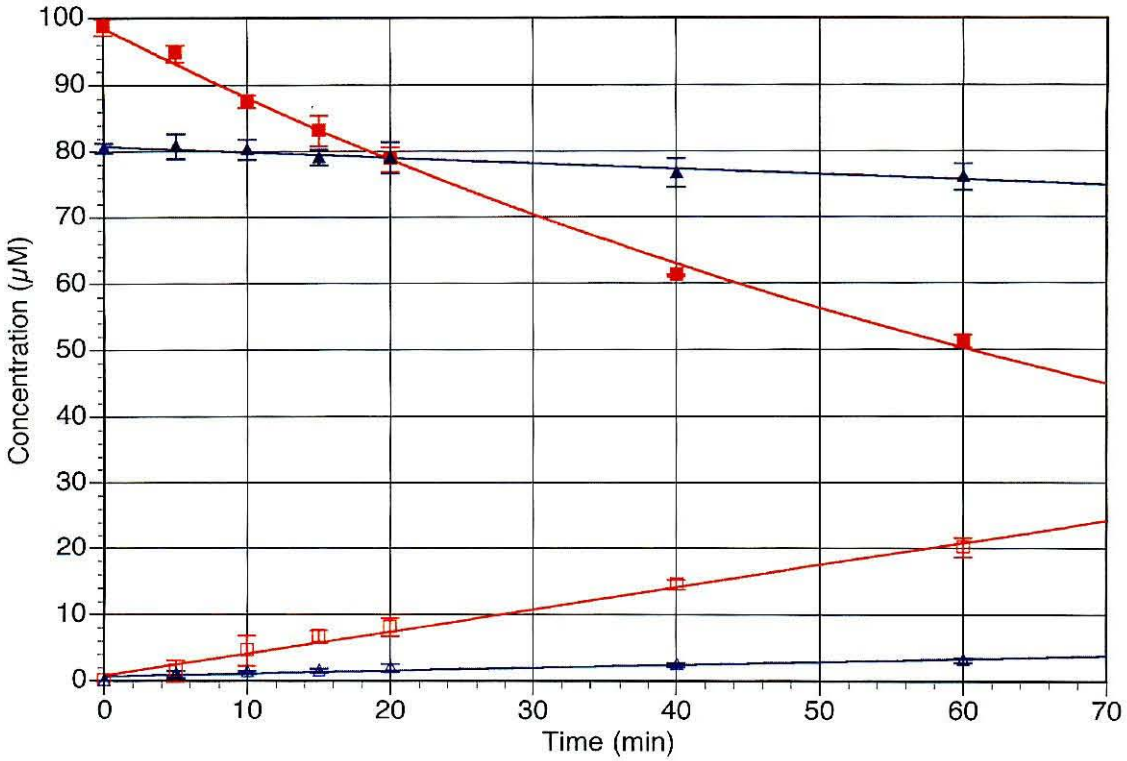
■ Bhc-Anisomycin (τ = 20.9 s, R^2 = 0.998)*
□ Anisomycin from photolysis of Bhc-Anisomycin

▲ DMNB-Anisomycin (τ = 187 s, R^2 = 1.000)*
△ Anisomycin from photolysis of DMNB-Anisomycin

● *p*-NB-Anisomycin (τ = 22,173 s, R^2 = 0.890)*
No anisomycin was detected in the time course of the photolysis of *p*-NB-Anisomycin

*Time constants (τ) were computed from a least squares fit to a simple decaying exponential with the coefficient of determination (R^2) indicated.

Figure 1. Typical 1-photon photolysis kinetics of caged anisomycins at 365 nm.



■ Bhc-Anisomycin (τ = 89.3 min, R^2 = 0.996)*
□ Anisomycin from photolysis of Bhc-Anisomycin

▲ DMNB-Anisomycin (τ = 917 min s, R^2 = 0.943)*
△ Anisomycin from photolysis of DMNB-Anisomycin

Ti:Sapphire laser average power 350 mW, 131 fs pulse width.
Error bars represent the standard deviation from three measurements.
*Time constants (τ) were computed from a least squares fit to a simple decaying exponential with the coefficient of determination (R^2) indicated.

Figure 2. Typical 2-photon photolysis kinetics of caged anisomycins at 740 nm.

Table 1: Spectroscopic and Photolytic Characteristics of Caged Anisomycins

Compound	λ_{max} (nm)	ε (M^{-1} cm^{-1})	Q_{u1} (mol/ein)*	δ_u (GM)†
p-NB-Aniso (**3**)	274	9,400	$0.007 \pm 0.003 \times 10^{-2}$	—
DMNB-Aniso (**5**)	350	6,200	$1.2 \pm 0.1 \times 10^{-2}$	0.048 ± 0.003
Bhc-Aniso (**8**)	373	14,500	$4.0 \pm 0.4 \times 10^{-2}$	0.59 ± 0.05

*1-photon quantum efficiency for uncaging at 365 nm. Uncertainty is the standard deviation from at least 3 experiments.
†2-photon uncaging action cross section at 740 nm (GM = 10^{-50} cm^4 s/photon). Uncertainty is the standard deviation from at least 3 experiments.

All compounds tested were reasonably stable in the dark and behaved similarly under simulated physiological conditions: 98.1% of **3**, 97.8% of **5**, and 96.5% of **8** remained after 19 h at 24 °C and pH 7.2 (data not shown). Liquid Chromatography / Mass Spectrometry (LC/MS) monitoring of the dark hydrolysis of Bhc-Aniso revealed that the disappearance of substrate is not a result of hydrolysis of the carbamate linkage, but rather the loss of the acetate at C3. No products resulting from carbamate hydrolysis were observed during the time course of the experiment (190 h).

Assessment of Uncaging Efficiency by *In Vitro* Translation

The ability of the caged anisomycin compounds to inhibit protein synthesis following exposure to UV light was initially assessed using *in vitro* translation assays. In the first set of experiments, Bhc and DMNB caged anisomycins were activated by exposure to 280 to 390 nm light for varying periods of time. The UV exposure was delivered through a 20X objective (N.A. 0.46) on an Olympus Provis AX 70 microscope using a 100 W mercury arc lamp as the light source. After UV exposure, of different durations, the compounds were added to *in vitro* translation reactions at a final concentration of 40 μM (Figure 3A).

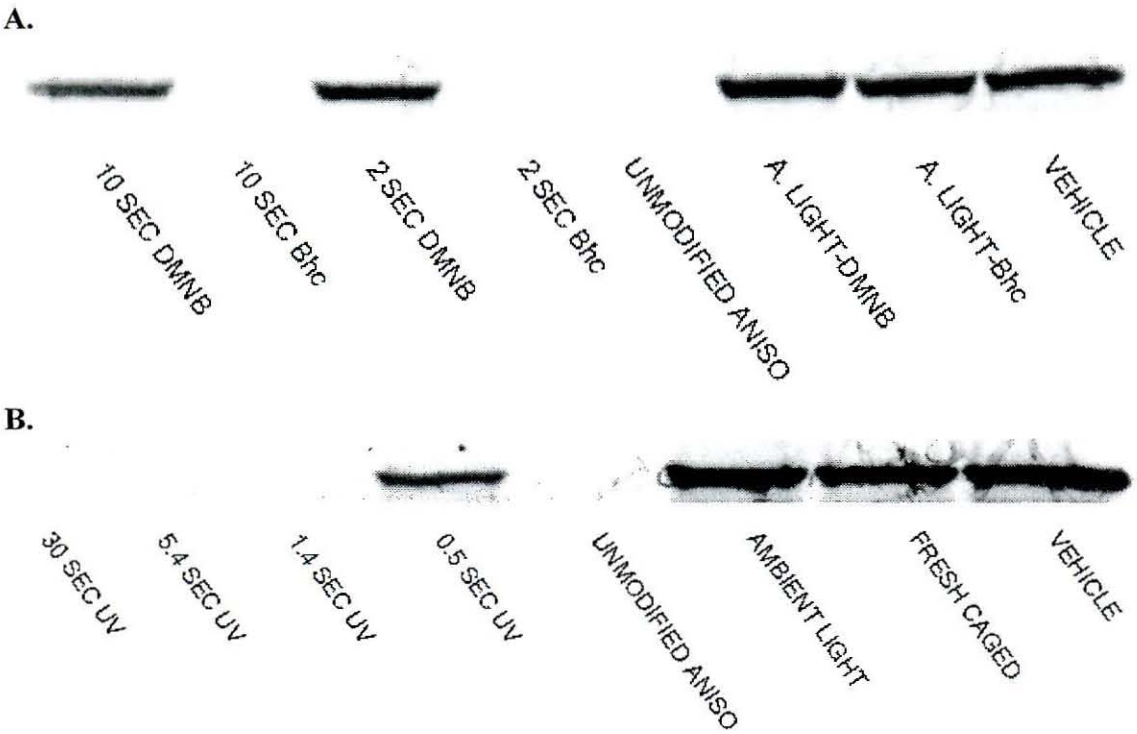


Figure 3. Bhc-Aniso can be efficiently uncaged to inhibit protein synthesis. Caged anisomycin compounds, DMNB-Aniso (DMNB) or Bhc-Aniso (Bhc), that were exposed to UV light for different durations, compounds exposed to ambient room light for 45 minutes (A. Light), unexposed Bhc-Aniso (Fresh Caged), regular anisomycin (Unmodified Aniso), or a vehicle control (Vehicle) was added to an *in vitro* translation reaction. Following a one-hour incubation, the [^{35}S]-Methionine incorporation in these reactions was analyzed using SDS-PAGE. **A.** Comparison of the relative uncaging efficiencies DMNB-Aniso and Bhc-Aniso. **B.** Characterization of Bhc-Aniso uncaging with 365 nm light exposure.

The DMNB-Aniso demonstrated a significant inhibition of protein synthesis after 10 seconds of UV exposure, but little inhibition after 2 seconds of exposure. The Bhc-Aniso was able to completely inhibit protein synthesis after as little as 2 seconds of exposure. Both compounds were very stable and showed no inhibition of synthesis after 45 minutes of exposure to ambient light when compared with the vehicle control.

Since Bhc-Aniso had a greater uncaging efficiency than the DMNB-Aniso, while being equivalently stable for our purposes, it was chosen as the compound to use for the

local inhibition experiments. Further characterization of the Bhc-Aniso, carried out with 365 nm (+/- 10 nm) wavelength light (the wavelength that was to be utilized in the local inhibition experiments), indicated that complete inhibition of protein synthesis could be achieved through 1.4 seconds of exposure or less (figure 3B).

The *p*-NB-Aniso was also able to inhibit protein synthesis following UV exposure but the required time of exposure, 5 minutes, and compound concentration, 400 μM, were both very high (data not shown).

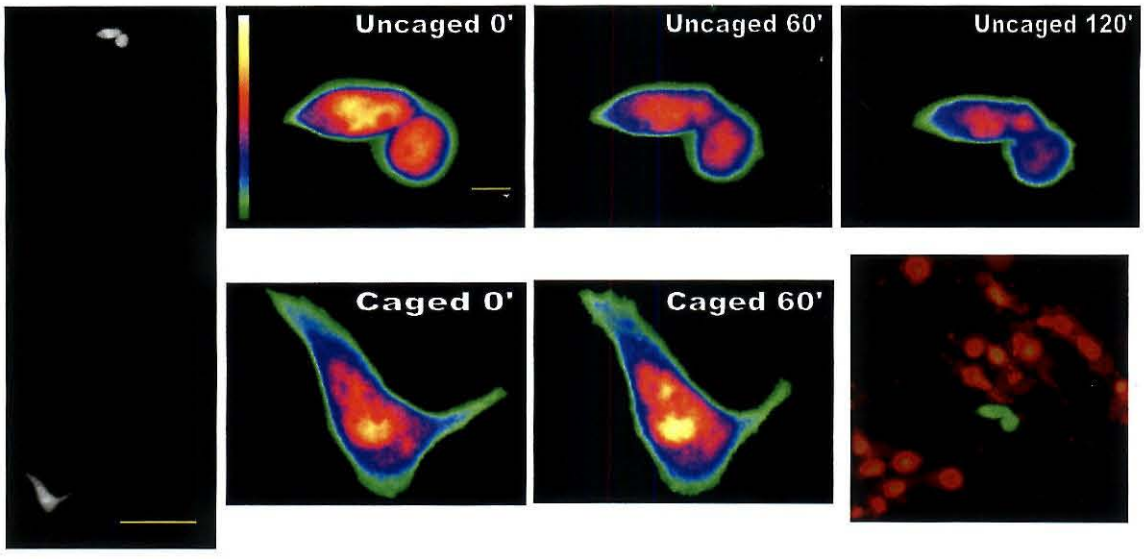
Bhc-Anisomycin can be used to Locally Inhibit Protein Synthesis

Visualizing GFP expression serves as simple and accurate way to monitor of protein synthesis in cells (e.g., Aakalu et al., 2001). Thus we monitored fluorescence levels in CHO cells transfected with GFP in the presence of bath applied Bhc-Aniso in order to ascertain if we could locally inhibit protein synthesis. In regions of a culture dish where the Bhc-Aniso was uncaged by two 500 msec pulses (with a 500 msec interpulse interval) of ~365 nm light every ten minutes, there was a significant reduction in cellular fluorescence over the course of two hours (Figure 4 and 6). The UV light was delivered through a 40X water immersion objective (N.A. 0.80) using a 100 W mercury arc lamp as the light source (through a neutral density 25 filter). We attribute the observed reduction in fluorescence to the inhibition of GFP synthesis by the uncaged anisomycin. In regions of the same dish where there was no UV light exposure, and therefore no uncaged Bhc-Aniso, the fluorescence levels of cells increased due to new GFP synthesis (Figure 4 and 6). Thus we were able to locally inhibit synthesis in cells in

one region of the culture dish while maintaining uninterrupted protein synthesis in other cells in the same dish.

There is a concern that UV exposure itself may be capable of bleaching GFP fluorescence and thereby simulate the predicted effects of protein synthesis inhibition in the cells. We found that this was not the case with our UV exposure protocol. When not in the presence of Bhc-Aniso, cells expressing GFP and treated with our UV exposure protocol showed a steady increase in GFP fluorescence that is statistically indistinguishable from the increases in fluorescence demonstrated by unexposed controls (Figure 5 and 6). Compromised cell health was another concern, since the UV exposure or the free radicals generated in the uncaging process could damage the cells. We found, however, that cell health, as assessed by morphology and propidium iodide staining, was not compromised by our local inhibition protocol (Figure 4).

A.



B.

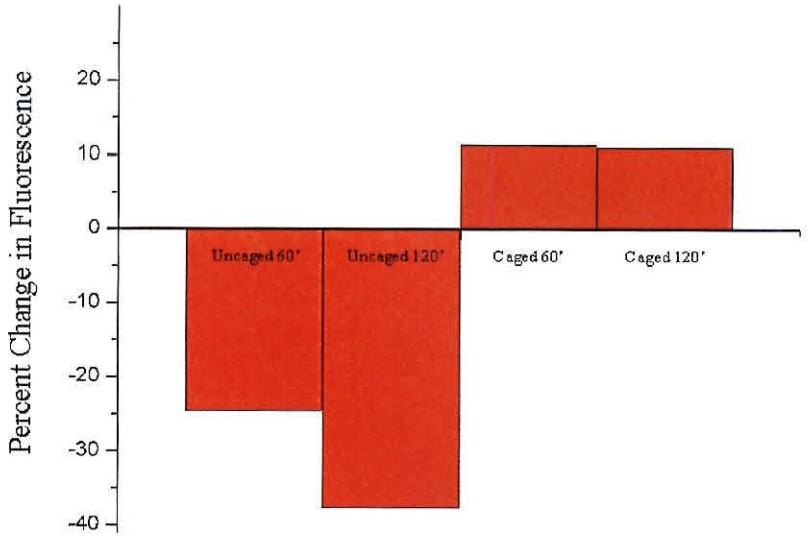
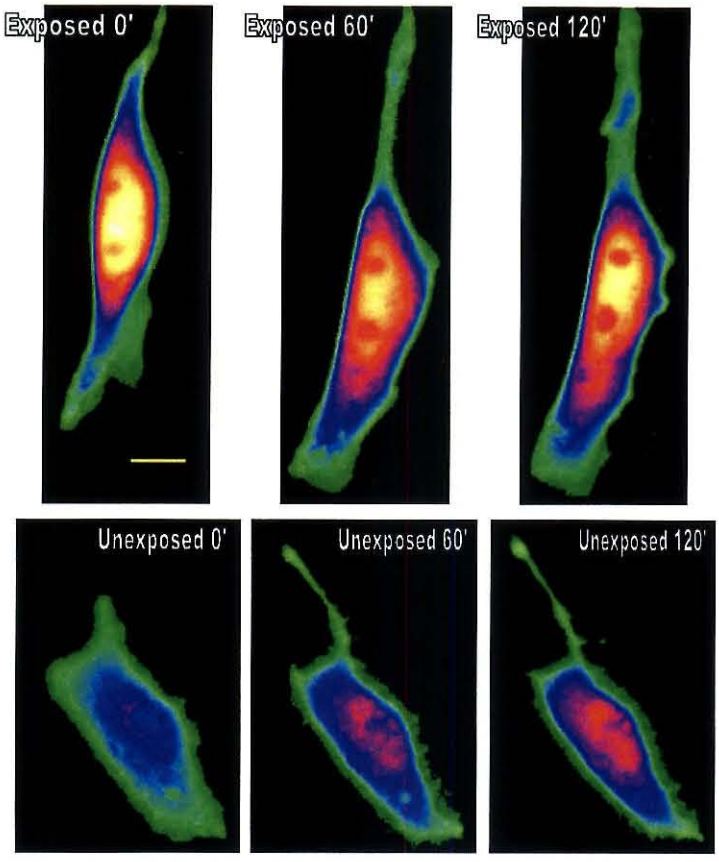


Figure 4. Local inhibition of protein synthesis in living cells. A. Protein synthesis can be locally inhibited by spatially restricted uncaging of Bhc-Aniso. Leftmost panel shows the relative positions of the UV exposed cells (top) and unexposed cell (bottom). Scale bar = 100 μm. Fluorescence decreases in the cell where Bhc-Aniso is uncaged and increases in the cell where Bhc-Aniso remains caged (scale bar = 10 μm). 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. The bottom right panel shows propidium iodide exclusion by the UV exposed cell at the conclusion of the experiment, indicating that it remains healthy (Green = GFP, Red = PI). **B.** Percent change in fluorescence from t=0 for both a UV exposed cell and the unexposed cell.

A.



B.

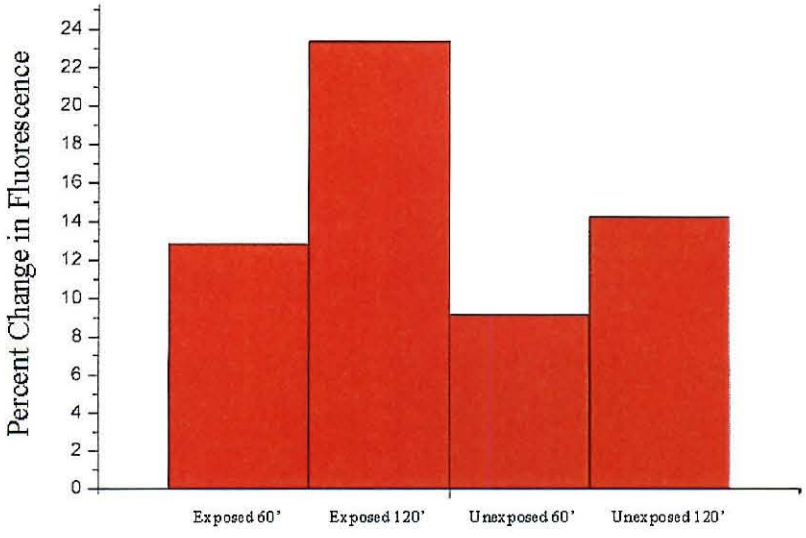


Figure 5. UV exposure alone does not bleach GFP or inhibit synthesis. A. The top set of panels show a cell treated with the same UV exposure protocol as locally uncaged cells exhibiting a steady increase in fluorescence over time. The bottom set of panels

show another transfected cell that is not exposed to UV light in the same dish as the exposed cell exhibiting a similar increase in fluorescence (scale bar = 10 μm). **B.** Percent change in fluorescence from t=0 for both the UV exposed and unexposed cell.

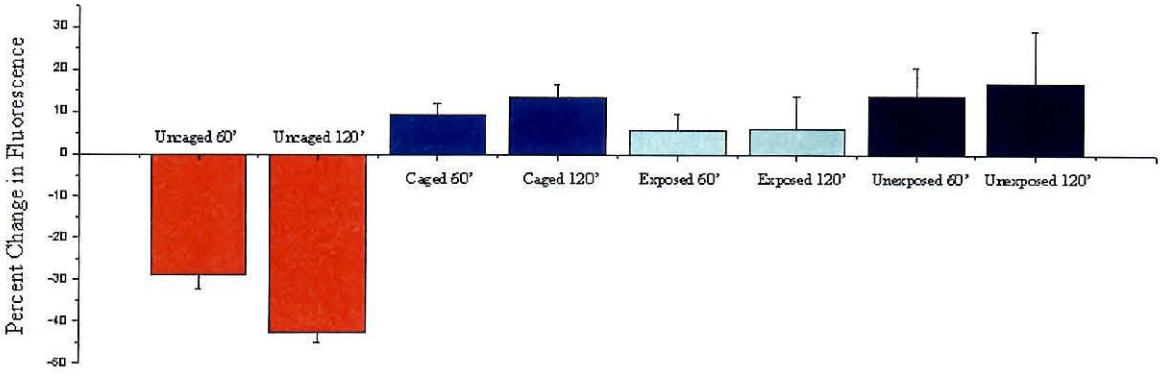


Figure 6. Local inhibition summary data. Cells exposed to uncaged Bhc-Aniso displayed a significant decrease in fluorescence over time when compared to cells exposed to caged Bhc-Aniso, UV exposed cells and untreated controls ($P \leq 0.01$ for all controls at both 60' and 120'). All controls (cells exposed to caged Bhc-Aniso, UV exposed cells and untreated controls) exhibited increases in fluorescence that were statistically indistinguishable from one another. N (cells) for each group are as follows: uncaged (6); caged (10); UV exposed (6); unexposed (6).

Discussion

Here we have described a method for the local inhibition of protein synthesis, by using a photocaged protein synthesis inhibitor, Bhc-Aniso. We have shown that we can significantly inhibit protein synthesis in cells in one region of a culture dish while allowing protein production to go unhindered in other cells (~ 500 μm away) in the same dish. Additionally, this local inhibition of protein synthesis has no significant impact in cell health over the time frame that we have observed (2 hours). Bhc-Aniso is highly stable in the presence of ambient light as well as in the presence of the type and intensity of light used in fluorescence microscopy (data not shown). Bhc-Aniso is also efficiently

uncaged. This allows significant amounts of anisomycin to be liberated from its cage with limited UV exposure, thereby minimizing any toxic effects to cells. Its rapid rate of uncaging also allows for high temporal precision of protein synthesis inhibition, as a high concentration of anisomycin can be rapidly generated.

We have demonstrated local inhibition with a simple system available to most biological investigators: a microscope objective lens with a 100 W mercury arc lamp light source. This has allowed us to demonstrate the control protein synthesis within a 500 μm radius in a monolayer of cultured cells. However, the use of more sophisticated light delivery systems offers the possibility of greater spatial control in more complex systems. Fiber optics can be used to deliver light with a higher spatial specificity than can typically be achieved with a standard microscope objective (Parpura and Haydon, 1999; Kandler et al., 1998). Spot sizes of UV illumination as small as 1 μm can be generated by controlling the fiber tip size or using mirrored micropipettes with small apertures. By using these small spot sizes to activate the caged synthesis inhibitor, one can precisely restrict synthesis in structures as small as a dendritic spine. This would allow one to not only determine if dendritic protein synthesis is necessary for long-lasting plasticity, but if it is necessary, where within the dendrite it needs to take place.

The local inhibition of protein synthesis could be a valuable tool for investigators in developmental biology as well. It would be possible to inhibit protein synthesis in a particular cell or tissue within an embryo with both spatial and temporal precision. Such studies may be particularly suited to take advantage of the robust 2-photon uncaging cross section of Bhc-Aniso. Two-photon uncaging, although requiring a sophisticated and expensive apparatus, would allow one to avoid possible toxicity from long term UV

exposure to cells as well as allow spatial specificity without the insertion of a fiber or micropipette into tissue (Denk et al., 1995; Denk et al., 1990).

This technique has a broad range of applications in the biological sciences. Local inhibition of protein synthesis using Bhc-Aniso can be conducted by employing a variety of light delivery systems, many of which are readily available to most investigators. Hopefully, this technique will facilitate studies that require precise spatial and/or temporal control over protein synthesis.

Experimental Procedures

General. Anisomycin was purchased from AG Scientific and Sigma. Phosgene was purchased from Fluka as a 20% solution in toluene (1.93 M). 6-Bromo-7-hydroxy-4-hydroxymethyl-coumarin (**6**) was prepared according to published procedure (Furuta et al., 1999). Other reagents and solvents were purchased from Aldrich or Fisher and used without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AMX 400 MHz or OTHER. FTIR spectra were recorded on a Bruker Vector 22 spectrophotometer. UV spectra were recorded on a Cary 300 Bio UV-Visible spectrophotometer (Varian). ESI MS and LC/MS were determined on a Sciex API-1 quadrupole mass spectrometer with an electrospray ionization source. HPLC analysis (analytical and preparative) was performed on a Varian ProStar HPLC system with an autosampler and diode array detector using Microsorb C-18 reverse phase columns. KMOPS buffer consisted of 100 mM KCl and 10 mM MOPS titrated to pH 7.2 with

KOH. Thin layer and column chromatography were performed on precoated silica gel 60 F_{254} plates (EM Science) and 230-400 mesh silica gel 60 (EM Science), respectively.

N-(4-nitrobenzyloxycarbonyl)anisomycin (3). Anisomycin (**1**, 90.2 mg, 0.34 mmol), 4-nitrobenzylchloroformate (**2**, 75.3 mg, 0.34 mmol), and sodium carbonate (40.7 mg, 0.38 mmol) were stirred in anhydrous THF (4 ml) for 2 hours. The reaction was filtered, and the solvent was removed by rotary evaporation, leaving a yellow oil, which was purified over silica gel using ethyl acetate/toluene (1:1) to yield was 87.0 mg (0.197 mmol, 58%) of product **3**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.71 (1H, s), 7.2-7.0 (3H, m), 6.77 (2H, d), 5.52 (2H, m), 4.88 (1H, m), 4.46 (1H, m), 4.02 (1H, m), 3.95 (6H, s), 3.76 (3H, s), 3.6-3.5 (2H, m), 3.22 (1H, d), 2.9-2.8 (1H, m), 2.60 (1H, m), 2.11 (3H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.8, 158.4, 154.9, 153.6, 148.3, 140.2, 130.4, 129.8, 128.2, 127.6, 114.0, 110.3, 79.1, 78.5, 72.4, 71.8, 64.3, 60.2, 56.6, 55.4, 51.7, 32.9, 21.1; ESI MS, *m/z* = -437, -479 (Cl^- adduct).

N-(4,5-dimethoxy-2-nitrobenzyloxycarbonyl)anisomycin (5). Prepared in 67% yield as described for **3**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.19 (2H, s), 7.46 (2H, d), 7.00 (2H, d), 6.75 (2H, s), 5.20 (2H, m), 4.88 (1H, m), 4.44 (1H, broad s), 4.12-4.04 (1H, m), 3.74 (3H, s), 3.58-3.49 (2H, m), 3.19 (2H, m), 2.81 (1H, m), 2.09 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.8, 158.4, 154.8, 147.7, 144.2, 130.3, 129.8, 128.4, 123.9, 114.0, 79.1, 72.2, 66.0, 60.0, 55.3, 51.7, 29.8, 21.0; ESI MS, *m/z* = +527.2 (Na^+ adduct).

N-[(6-Bromo-7-hydroxycoumarin-4-yl)methyloxycarbonyl]anisomycin (8). Chloroformate **7** was prepared by adding a 20% phosgene solution (1.26 ml, 2.22 mmol) via syringe to a solution of 6-bromo-7-hydroxy-4-hydroxymethylcoumarin (**6**, 100 mg, 0.37 mmol) in dry THF (4 ml) under nitrogen. The solution was stirred for 1 h and then

purged with nitrogen to concentrate it to approximately half the original volume. In a separate reaction vessel, an aqueous solution of 1.0 M sodium carbonate (0.34 ml) was added to a solution of anisomycin (**1**, 42 mg, 0.16 mmol) in THF (1 ml). The solution of the chloroformate was added dropwise over 10 min to this mixture while stirring and cooling on ice. The reaction mixture was stirred at room temperature for 1 h. After addition of another solution of chloroformate **7**, prepared as described above, the mixture was stirred for 2 h. The reaction was quenched with 15% citric acid, diluted with chloroform, and extracted with water. The organic layer was dried ($MgSO_4$) and evaporated to a residue on the flask wall. The crude product was purified by flash column chromatography (ethyl acetate/hexane 6:4) to yield 31 mg (0.055 mmol, 35%) of product **8**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67 (1H, s), 7.1 (2H, d), 7.02 (1H, s), 6.83 (2H, d), 6.5 (1H, broad s), 6.37 (1H, s), 5.38-5.2 (2H, dd), 5.0-4.93 (2H, m), 4.5 (1H, d), 4.16-4.08 (1H, dd), 3.78 (3H, s), 3.64-3.53 (2H, m), 2.9-2.85 (1H, m), 2.17 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.3, 155.4, 154.5, 153.8, 149.1, 130.3, 129.4, 126.7, 113.9, 112.1, 110.6, 106.7, 104.4, 91.7, 78.4, 72.2, 61.7, 60.1, 59.4, 55.2, 51.5, 32.6, 21.0; FTIR (neat) 3430, 3188, 3016, 2930, 1750, 1719, 1703, 1688, 1602, 1422, 1219, 656 cm^{-1}; ESI MS *m/z* 562, 564. Samples of **8** for photochemical analysis and biological studies were further purified by preparative HPLC (60% CH_3CN/40% H_2O containing 0.1% TFA).

Determination of Quantum Efficiency for One-Photon Excitation of Caged Anisomycin Derivatives. A KMOPS-buffered solution (3 ml) of the substrates (100 μM) was irradiated with 365-nm UV light from a mercury lamp (Spectroline SB-100P; Spectronics Corporation, Westbury, NY). The duration of each irradiation period ranged from 5 to 360 s. After each period of irradiation, a 20 μl aliquot of the solution was

removed for analysis by HPLC using an external standard method to determine concentrations. The compound was eluted with an isocratic mixture of 50% acetonitrile and 50% water containing 0.1% trifluoroacetic acid (flow rate of 1 ml/min). Absorbance was detected at 325 nm (Bhc-Aniso), 345 nm (DMNB-Aniso), 270 nm (*p*-NB-Aniso), and 225 nm (Anisomycin). The progress curves were plotted as simple decaying exponentials. Quantum efficiencies (Q_{u1}) were calculated according to published methods (Adams et al., 1988; Furuta et al., 1999; Livingston, 1971) using $Q_{u1} = (I\sigma t_{90\%})^{-1}$, where I is the irradiation intensity in ein·cm^{-2}·s^{-1}, σ is the decadic extinction coefficient (10^3 times ε, molar extinction coefficient) in cm^2·mol^{-1}, and $t_{90\%}$ is the irradiation time in seconds for 90% conversion to product. The UV intensity of the lamp I was measured by using potassium ferrioxalate actinometry (Hatchard and Parker, 1956) in the same setup.

Determination of Dark Hydrolysis Rates of Caged Anisomycin Derivatives. Substrates were placed into a KMOPS-buffered solution and stored in the dark at room temperature. HPLC analysis was carried out periodically as described for 1-photon photolysis. The identity of the hydrolysis products was determined by LC/MS.

Measurements of 2-Photon Uncaging Cross Sections. Measurements were carried out in microcuvettes (10 × 1 × 1 mm illuminated dimensions) with an effective filling volume of 20 µl (26.10F-Q-10, Starna, Atascadero, CA) using a femtosecond IR pulses from a mode-locked Ti:Sapphire laser (Mira 900 pumped by a Verdi, Coherent, Santa Clara, CA) focused on the center of the cuvette chamber with a 25 mm focal length lens optimized for IR lasers (06LXP003/076, Melles-Griot, Irvine, CA). The 2-photon uncaging cross-section (δ_{u2}) could be estimated by referencing to fluorescein, a compound with a known 2-photon fluorescence quantum yield (Q_{f2} = 0.9 mol/ein) and

absorbance cross-section (δ_{aF} = 30 GM at 740 nm) (Xu and Webb, 1996), by the following equation:

$$\delta_{i2} = \frac{N_p \phi Q_{f2} \delta_{aF} C_F}{< F(t) > C_S},$$

where N_p is the number of product molecules formed per unit time (molecules/s, determined by HPLC analysis as in the 1-photon analysis); ϕ is the collection efficiency of the detector (SED033 on an IL-1700, International Light, Newburyport, MA) used to measure the fluorescence of fluorescein emitted at a right angle to the beam and passed though a 535/45 nm bandpass filter (Chroma Technologies, Brattleboro, VT); C_F is the concentration of fluorescein (mol/L); $<F(t)>$ is the time averaged fluorescent photon flux (photons/s) collected by the detector; and C_S is the initial concentration of caged substrate (mol/L) (Furuta et al., 1999).

In vitro translation experiments. 4 mM of a given caged anisomycin compound (in DMSO) was exposed to 280-390 nm or 365 (+/- 10 nm) UV light (filters from Chroma Technology Corp., Brattleboro, VT) for the given amount of time. The UV exposure was delivered through a 20X objective (N.A. 0.46) on an Olympus Provis AX 70 microscope using a 100 W mercury arc lamp as the light source. After exposure, the caged compound (or a anisomycin or a vehicle control) was added at a final concentration of 40 μm to an *in vitro* translation reaction (Promega, Madison, WI) using [^{35}S]-Methionine incorporation to detect protein synthesis. The level of incorporation after incubation with the inhibitor or control was assessed by SDS-PAGE.

GFP construct. The GFP reporter plasmid used was pEGFP-C1 (GenBank Accession # U55763, Clontech, Palo Alto, CA).

CHO cell culture and transfection. CHO cells were maintained and propagated under standard conditions (5% CO2 in Ham's F12 medium containing 10% fetal bovine serum). Prior to transfection, cells were grown in 35 mm culture dishes or 6 well plates at 95% confluence. Cells were transfected utilizing Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described by the protocol provided by Invitrogen.

Local inhibition experiments. After 5 hours of transfection, CHO cell growth media was replaced with a HEPES-buffered solution (HBS) (Malgaroli and Tsien, 1992) (without glycine or picrotoxin) with 2 mM ascorbic acid and 40 μM Bhc-Aniso (or vehicle control). Cells were incubated in this media for 1 hour at room temperature. Following incubation transfected cells were imaged and then one cell or set of cells was exposed to 365 (+/- 10 nm) UV light (filters from Chroma Technology Corp., Brattleboro, VT) twice for 0.5 seconds (with a 0.5 second interpulse interval) every ten minutes. The UV exposure was delivered through a 40X objective (N.A. 0.80) on an Olympus Provis AX 70 microscope using a 100W mercury arc lamp as the light source. Subsequent fluorescent images were taken every hour.

Cell death assay. Cell health was assessed after each local inhibition experiment by propidium iodide (PI) exclusion. Cells incubated in PI solution (50 μg/ml) for five minutes and then washed. They were subsequently imaged to assess cell health.

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CHAPTER 5

Conclusion: Future Directions and Clinical Relevance

Girish Aakalu

Summary

The study of local protein synthesis (LPS) in neurons has now moved past its long infancy. In the past six years, the phenomenon of neuronal LPS has been convincingly demonstrated (Aakalu et al., 2001) and its functions in mature neuronal physiology have begun to be described (Huber et al., 2000; Kang and Schuman, 1996; Martin et al., 1997; Ouyang Y, 1999). Even the means by which LPS is regulated has started to come to light (Kiebler et al., 1999; Kohrmann et al., 1999; Tang et al., 2001; Tang et al., 2002; Wu et al., 1998). However, there is still a great deal of work that remains to be done in this field. In addition to the questions of specificity and regulation of LPS, there is the question of its necessity. Is LPS necessary for neuronal function or does it play a permissive or redundant role? Finally, there is much to learn about LPS dysfunction and its possible clinical outcomes.

Specificity and Regulation of Neuronal LPS

Does protein synthesis take place over broad regions of the dendrites with subsequent targeting of proteins or does synthesis occur right at the site where the proteins are needed? And what are the regulatory events and molecules that initiate and suppress neuronal LPS? Although exactly where and when translation occurs in the dendrites is still unclear, answers to these questions are emerging.

Specificity. The idea that synthesis does occur in a site-specific manner is supported by a number of observations. Steward et al. (Steward et al., 1998) provide a

striking demonstration of Arc mRNA localization to synaptically activated sites and its subsequent translation at those sites. Ouyang et al. (Ouyang Y, 1999) show that CaMKIIα can also be locally synthesized in regions of the dendrites that have been synaptically activated. Finally, Aakalu et al. (Aakalu et al., 2001) have observed that regions of high dendritic synthesis correspond with the synaptic markers Synapsin (De Camilli et al., 1983) and PSD-95 (Walsh and Kuruc, 1992). In fact, they found that these regions of high dendritic synthesis occur within 2 microns of the Synapsin immunofluorescence signal. One interpretation of this data is that synaptic activity is driving translation in a *very* localized manner.

However, there are reasons to doubt the idea that highly site-specific LPS exists in neurons. Presumably LPS exists to support long-lasting changes in synaptic efficacy. If this is indeed the case, then the spatial resolution of LPS should match that of the changes in synaptic efficacy. Electrophysiological data has shown the limits of input specificity to be approximately 70 microns, as synaptic inputs that were 30-70 μm apart show a "spreading" of enhancement from one site to another (Engert and Bonhoeffer, 1997). What purpose then would the higher resolution LPS suggested by Aakalu et al. serve? When considering this question one needs to keep several things in mind. First, the electrophysiological data provided by Engert and Bonhoeffer is the most current on the topic but it is still fairly crude. They claim to be able to measure differences in input specificity with a spatial resolution of approximately 30 microns - only about half the size of the limit of input specificity that they report. Thus if there were even small errors in evaluating the limits of their technique, it could bring their observations into question. If one was to accept the findings of Engert and Bonhoeffer, one would have to still consider the physiological relevance of LTP. The intrinsic electrophysiological activity of neurons

may occur in a much more subtle manner and thus capable of generating synaptic plasticity with much greater spatial specificity. Alternatively, if LTP were similar to the molecular basis of learning and memory, the regions of potentiation defined by the input specificity may be large but precise. Then, in order to define the boundaries of long-lasting potentiation precisely, the neuron would require high-resolution LPS. Finally, there could be ways for neurons to encode information that do not alter electrophysiological readouts and the encoding of this information may generate the real need for high-resolution LPS.

Regulation. The study of neuronal LPS regulation is even more underdeveloped than the study of its specificity. Direct evidence has only been provided for one regulatory system's involvement in LPS – cytoplasmic polyadenylation (Wu et al., 1998). Nevertheless, indirect evidence has been mounting for the involvement of components of the rapamycin-dependent signaling pathway (Tang et al., 2002), as well as the translational repressors Pumilio (Dasgupta, 2002) and Bruno (Jiang, 2002). Although this indirect evidence has been limited to immunostaining that demonstrates the presence of these molecules in the dendrites, functional studies involving these molecules are underway.

Not surprisingly, all of the currently known regulators of neuronal LPS have homologs in developmental LPS systems (Albig and Decker, 2001; Hentges et al., 2001; Johnstone and Lasko, 2001; Mendez and Richter, 2001; Palacios and St. Johnston, 2001; Raught et al., 2001; Richter, 2001). There appears to be a conservation of the systems used to regulate LPS that spans from the simple yeast to complex vertebrates, such as rats (Palacios and St. Johnston, 2001). This in fact goes beyond just translational regulation

and is even true of molecules involved in mRNA localization, such as Staufen (Ferrandon et al., 1994; Kiebler and DesGroseillers, 2000; Kiebler et al., 1999; Kohrmann et al., 1999; Tang et al., 2001). Thus there is reason to believe that molecules and regulatory systems identified in diverse developmental systems will provide good leads to the molecules and systems involved in the regulation of vertebrate neuronal LPS.

Necessity of LPS for Maintenance of Synaptic Plasticity

One of the fundamental questions that remains to be answered about neuronal LPS is whether it is necessity for the maintenance of long-lasting synaptic plasticity. Is neuronal LPS required or is it simply a supplementary or fail-safe system used to augment somatic synthesis? Or is it in fact primarily used for purposes other than the maintenance of long-lasting plasticity? Currently there are no definitive answers to these questions, but several studies suggest a significant role for LPS in long-lasting forms of synaptic plasticity. Aakalu et al. and Ouyang et al. have shown that various forms of plasticity inducing stimuli are capable of eliciting dendritic LPS (Aakalu et al., 2001; Ouyang Y, 1999). Furthermore, Kang and Schuman and Huber et al. have shown that LPS alone is sufficient to maintain long-lasting forms of plasticity (Huber et al., 2000; Kang and Schuman, 1996). These two groups also provided strong temporal arguments to rule out somatic synthesis as the source of the proteins needed to maintain the forms of plasticity that they studied. Taken together these studies demonstrate a strong relationship between LPS and long-lasting synaptic plasticity.

However, in order to definitively prove the necessity of LPS in synaptic plasticity, one needs to move beyond temporal arguments (at least until transport rates are perfectly

defined) for its requirement. One way in which to do this is to conduct the experiments outlined by Aakalu et al. (Chapter 4 of this thesis). They state that a stronger argument for the necessity of LPS could be made if long-lasting plasticity could be abolished when inhibiting protein synthesis in the dendrites while maintaining normal somatic protein synthesis and an intact route for protein transport from the soma to the dendrites. Experiments of this nature can now be carried out by utilizing the local protein synthesis inhibition technique devised by Aakalu et al. (Chapter 4 of this thesis).

Clinical Relevance of Neuronal LPS

Given the broad scope of physiological processes that LPS is involved in, it is easy to imagine the pathologies resulting from its dysfunction. As many of these processes occur early in development, they would likely result in embryonic lethality (Lieberfarb et al., 1996). Yet, defects in LPS function in mature neurons may lead to more subtle pathology. There are already reports of a few neurological disorders, such as fragile X syndrome and Alzheimer disease, that may be linked to LPS dysfunction. A deeper understanding of neuronal LPS may shed light on the underlying processes that cause these disorders.

Fragile X Syndrome. Fragile X syndrome (FXS) is the single largest cause of inherited mental retardation (Pimentel, 1999). In addition to having a lower than normal IQ, individuals with FXS display autistic behaviors, macroorchidism, facial abnormalities, and morphological defects in the brain (Mazzocco, 2000; Mostofsky et al., 1998). Dendritic spine structure was found to be thinner and longer, and spine density

was found to be higher in adult fragile X mental retardation 1 (*FMR1*) gene knockout mice and affected humans when compared to controls (Comery et al., 1997; Irwin et al., 2000). This is similar to the phenotype of immature neurons. Additionally, dissociated hippocampal cultures made from *FMR1* knockout mice exhibit shorter dendrites and develop fewer functional synapses that require a longer time to develop (Braun and Segal, 2000). These synapses also produce smaller EPSCs than normal (Braun and Segal, 2000). Interestingly, these cultured neurons from *FMR1* knockout mice display a lower spine density than controls. This discrepancy between the Braun study and the Comery and Irwin studies may be due to an artifact of cell culture or it may be because of differences in the cell types examined in the different studies (Irwin et al. and Comery et al. studied cortical pyramidal neurons; Braun et al. studied hippocampal pyramidal neurons).

FXS is caused by a trinucleotide repeat (CGG) expansion in the region of *FMR1* that corresponds to the 5' UTR of the *FMR1* protein (FMRP) transcript (Pimentel, 1999). In the most severe forms of the disease (where there is a large expansion of the CGG repeat) this results in excessive methylation, and subsequent transcriptional inactivation, of the *FMR1* gene (Hagerman and Hagerman, 2001). However, in less affected individuals, the reduction of FMRP results not from reduced transcription of the gene but rather from poor translation of its mRNA (Tassone et al., 2000). This may be the result of the trinucleotide repeat hindering translation initiation, as it is maintained even in the mature mRNA (Feng et al., 1995; Tassone et al., 2000).

Several lines of evidence suggest that FMRP plays a role in neuronal LPS (Bardoni et al., 2000; Greenough et al., 2001; Hagerman and Hagerman, 2001). FMRP is

highly expressed in neurons and has been identified in dendrites and spines through immunocytochemistry (Feng et al., 1997). It has been found to associate with actively translating polyribosomes *in vivo* and bind several species of mRNA *in vitro* (Brown et al., 1998; Corbin et al., 1997; Feng et al., 1997). *FMR1* knockout mice and humans with FXS exhibit altered polyribosomal profiles (causing increases in polyribosomal association for some transcripts and decreases for others) for approximately 2% of mRNAs found in neurons (Brown et al., 2001). Many of the mRNAs that were affected contained a G quartet structure that has been proposed as the target for FMRP binding (Brown et al., 2001; Darnell et al., 2001). Additionally, Greenough et al. (Greenough et al., 2001) have shown that synaptoneurosomes prepared from *FMR1* knockout mice are capable of synthesizing significantly less proteins upon stimulation than synaptoneurosomes from control animals. One study has suggested that FMRP itself may be produced through neuronal LPS (Weiler et al., 1997), although this result has recently come into question (Steward and Schuman, 2001).

Alzheimer Disease. Alzheimer disease (AD) is a genetically complex neurodegenerative disorder that affects approximately 20 million people worldwide (Shastry, 2001; Tanzi and Bertram, 2001). The major clinical symptom of AD is the progressive loss of cognitive ability and memory. On a cellular level, AD is characterized by the presence of neurofibrillary tangles (NFT) and extracellular amyloid plaques. It is thought that the NFTs and plaques are, at least in part, responsible for the clinically observed impairments (Bierer et al., 1990; Haroutunian et al., 1998; Haroutunian et al., 1999; Haroutunian et al., 2000).

The NFTs, which are primarily composed of hyperphosphorylated tau, are suspected to be involved with the disruption of the neuronal cytoskeleton (Mandelkow, 1999; Mandelkow and Mandelkow, 1998). One of the effects of this cytoskeletal disruption is the loss of dendritic localization of normally dendritically localized mRNAs (Chang et al., 1997). Chang et al. observed a significant decrease in RC3/neurogranin mRNA dendritic localization in tissue from humans with AD when compared with controls. RC3 is a substrate for protein kinase C and is capable of binding calmodulin. It is primarily localized to the somatodendritic compartment of neurons (Houben et al., 2000).

Interestingly, RC3 may be involved in learning and memory. Miyakawa et al. have found that RC3 knockout mice perform poorly on spatial learning tasks that have anxiety related components (Miyakawa et al., 2001). Mons et al. reported a marked decrease in RC3 in the dorsal hippocampal subfields, retrosplenial and primary motor cortices in aged mice (Mons et al., 2001). This decrease occurs primarily in the dendrites (Mons et al., 2001). Taken together, these studies suggest a possible link between the decrease of dendritic RC3 and age-related declines in cognitive function. Thus the loss of RC3 mRNA localization to the dendrites in AD may cause an earlier and/or greater reduction in RC3 protein in dendrites resulting in earlier and/or greater cognitive impairment than age matched controls. Although this putative relationship between RC3 loss and AD is intriguing, it is likely to be one of many components of AD neuropathology.

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APPENDIX I

Alu sequences from GC-rich DNA are likely to be harmful and prevented from spreading in the population by natural selection. This implies no functional importance for an Alu sequence itself, but merely that, as the deletions of Alus are very unlikely to be precise, a deletion event removing an Alu is also likely to remove valuable sequences around it, and the chromosome bearing the deletion will be lost by selection.

The explanation favoured by the authors for Alu enrichment in gene-rich regions is that of positive selection in favour of Alus in GC-rich DNA. This theory, however, cannot explain the observations. The data show that Alu sequences up to five million years old are not enriched in GC-rich regions. But in human population genetics, estimated times to common ancestry of typical genomic regions show that Alu sequences which are five million years old have already been fixed (found in all individuals) in the population. This observation is also what would be expected from neutrality and genetic drift, given the human effective population size. (Alu sequences which are truly advantageous will spread to fixation much more quickly.) Earlier human ancestors would also be expected to have had similar fixation times for Alu insertions. Yet it is only during the spread to fixation of Alu sequences that positive natural selection has any opportunity to act. Thus, an increasing abundance of Alu sequences in GC-rich DNA as they age beyond five million years cannot be the result of natural selection for positive functions of Alu insertions.

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Primer

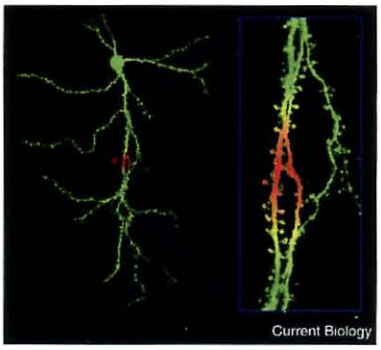
Local protein synthesis in neurons

W. Bryan Smith, Girish Aakalu and Erin M. Schuman

The processing power of the mammalian brain is derived from the tremendous interconnectivity of its neurons. An individual neuron can have several thousand synaptic connections. While these associations yield computational power, it is the modification of these synapses that gives rise to the brain's capacity to learn, remember and even recover function after injury. Inter-connectivity and plasticity come at the price of increased complexity as small groups of synapses are strengthened and weakened independently of one another (Figure 1). When one considers that new protein synthesis is required for the long-term maintenance of these changes, the delivery of new proteins to the synapses where they are needed poses an interesting problem (Figure 1). Traditionally, it has been thought that the new proteins are synthesized in the cell body of the neuron and then shipped to where they are needed. Delivering proteins from the cell body to the modified synapses, but not the unmodified ones, is a difficult task. Recent studies suggest a simpler solution: dendrites themselves are capable of synthesizing proteins. Thus, proteins could be produced locally, at or near the synapses where they are needed. This is an elegant way to achieve the synapse specific delivery of newly synthesized proteins.

Local protein synthesis is not unique to neurons. It is one of the primary mechanisms that organisms use to determine cell fate and generate differences among cells.

Figure 1

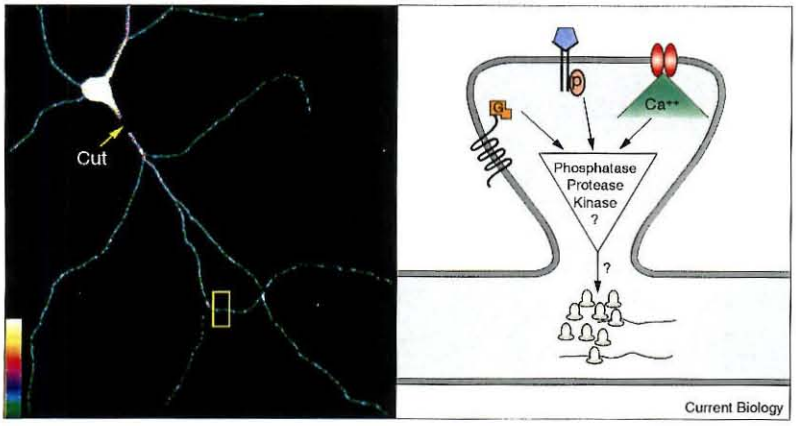


Specificity of synaptic enhancement in neurons. Shown is a single pyramidal neuron with its cell body and dendrites filled with GFP. The small protrusions that occur along the dendrites are the spines, the postsynaptic compartment onto which synapses are made. Highlighted in red is a putative area of synaptic enhancement with the adjacent yellow area depicting regions that might show a lesser amount of enhancement. During long-lasting synaptic plasticity, the spines in the enhanced area of the dendritic tree, but not the adjacent green areas, need to receive newly synthesized proteins.

For example, to achieve cellular differentiation, the *Drosophila* oocyte creates a polarized distribution of mRNA species. The mRNAs are localized and anchored to different poles of the cell *via* motifs in their 3′ untranslated regions (UTRs). The mRNAs are then locally translated to create different regions of the cell and later different daughter cells. Local protein synthesis is even used by unicellular organisms such as *Saccharomyces cerevisiae* to determine cell fate. mRNA for a factor that regulates mating type is shipped from the mother cell to the budding daughter cell, once again based on *cis*-acting elements in its 3′ UTR. The protein product restricts only the daughter cell's mating type.

Local protein synthesis is also used to create functional micro-domains within cells. Myelinating cells such as oligodendrocytes produce processes that function as

Figure 2



The physical isolation of dendrites from the cell body provides a definitive demonstration of local protein synthesis. Left panel: Shown is a hippocampal neuron expressing a dendritic protein synthesis reporter. A small cut isolates the cell body from the dendrites. In this cell, the application of a growth factor to the isolated dendrite resulted in dendritic protein synthesis. Right panel: Biochemical cascades that may result in protein synthesis activation. A single dendritic spine is illustrated with ribosomes at the base of the spine. Synaptic activation of metabotropic (black), growth factor (blue) and ionotropic (red) receptors may lead to dendritic protein synthesis activation through unknown intermediates.

lipid sheaths to electrically insulate axons. They do this by producing a protein called myelin basic protein that collapses the oligodendrocyte cell membrane, thus squeezing the cytoplasm out of the region where it is expressed. If myelin basic protein were produced in a cell-wide manner it would be toxic. Therefore, restricted translation of myelin basic mRNA is carried out in the oligodendrocyte processes.

Microdomains are useful not only for avoiding toxicity, but also for allowing distributed control of cellular function. Axonal growth illustrates this principle quite well. The growth cone of a developing axon must sort through a dizzying array of attractive and repulsive cues to determine how to reach the appropriate target tissue. To accomplish this task, the growth cone requires specific proteins based on environmental cues it encounters. However, the growth cone is frequently millimeters away from the soma. To solve this problem, the growth cone might locally synthesize the required proteins when it needs them. Several recent studies suggest that this is the case.

What is the evidence that distributed protein synthesis might occur during long-lasting plasticity? An early hint that local protein synthesis might occur within the dendritic compartment of neurons was the anatomical observation of synapse-associated polyribosome clusters at the base of the dendritic spine apparatus (Figure 2, right). Since those ultrastructural studies, an entire complement of translational machinery has been detected in dendrites. Additionally, several mRNA molecules are localized to the dendritic domain of neurons. Although these studies show that the components for local protein synthesis are present, they do not show that this synaptic translational machinery is actually used in response to synaptic activity.

Direct evidence supporting the local synthesis hypothesis was reported in a study by Feig and Lipton. The authors used [^{3}H]-leucine incorporation to show that new proteins were synthesized in the dendritic regions of hippocampal slice tissue following electrical stimulation. The stimulation protocol used in these studies, however, did not alter the strength of synaptic connections. In experiments using growth factor application to hippocampal slices, Kang and Schuman showed the first causal link between local protein synthesis and synaptic enhancement. In this study, brain-derived neurotrophic factor (BDNF) or neurotrophin-3 (NT-3) application to hippocampal slices was shown to elicit a long-lasting synaptic enhancement that was blocked when the tissue was pre-incubated with protein synthesis inhibitors.

In all local protein synthesis experiments, the burden of proof is to show that new proteins are made in the dendrites, not the cell body. Due to the laminar structure of the hippocampal slice, the authors were able to make microlesions that physically separated the cell bodies from the dendrites. Interestingly, even when the dendritic region was isolated, the growth factor-induced enhancement was still sensitive to protein synthesis inhibitors. Although consistent with a dendritic source of protein synthesis, this study could not distinguish between protein synthesis in dendrites, axons, glial cells or local interneurons — all of which are found within the isolated 'dendritic' laminae of hippocampal tissue.

Further evidence of activity-dependent local protein synthesis within neuronal processes has since been reported. In hippocampal slices, one form of long-term depression requires local synthesis. In the marine mollusk *Aplysia*, serotonin application results in a long-term synaptic facilitation that requires protein synthesis in the

neurites. Other studies have detected local synthesis of proteins such as calcium/calmodulin-dependent protein kinase (CamKII) following synaptic plasticity. While the data from these experiments are compelling, they lack the temporal resolution to actually watch the protein synthesis occur over time. In our recent work, we used green fluorescent protein (GFP) in combination with time-lapse microscopy to investigate local protein synthesis in dendrites of cultured hippocampal neurons. In order to rule out the cell body as a potential source of the GFP signal measured in the dendrites, we used two distinct methods of isolating the dendrites from the cell bodies. The first set of experiments employed dendritic transections, in which the dendrites were physically cut away from the cell bodies (Figure 2, left). In a less-invasive approach, the GFP in the cell body was continuously photobleached, and fluorescence was monitored in the distal dendrites. We showed that BDNF application in both experimental preparations resulted in increased GFP production in the isolated dendrites. These experiments provided the first dynamic visual proof of local protein synthesis in dendrites.

An important aspect of local protein synthesis within a micro-domain of any cell type is the transport of mRNA to the appropriate location within the cell. In the case of CamKII, *cis*-acting elements have been identified within the 3′ UTR of the mRNA molecule that are necessary and sufficient to target the message to the dendritic region of hippocampal neurons. A consensus targeting sequence has yet to be identified, but it does appear as though targeting sequences reside in the 3′ UTRs of other messages as well. Several studies show that the mRNA-binding protein Staufen, which is critically involved in mRNA localization in the *Drosophila* embryo, may also participate in mRNA trafficking to neuronal dendrites.

In addition to specific transport requirements, if a message is to have a local effect only at its destination — often a distance of several hundred microns from the nucleus — there must be control over translation of the mRNA. Without translational regulation, the protein could be produced *en route* to its destination, thereby negating any specificity that arises as a result of local synthesis. It has been recently shown that a host of mRNA binding proteins work in concert to restrict translation of certain mRNA species until the appropriate time when the protein is to be produced. Central to this process is the cytoplasmic poly-adenylation element (CPE) found in the 3′ UTR of many messages. The CPE is bound by CPE-binding protein, which forms a complex with other RNA binding proteins thus regulating translation of these messages. As the 3′ UTR sequences of more mRNAs become available, it will be interesting to discover the role the CPE and other mRNA elements play in controlling local mRNA translation.

If dendritic protein synthesis is a requirement for long-term synaptic enhancement, to what extent is activity-induced protein synthesis restricted to activated synaptic sites? In order to answer this question of synapse specificity, experiments that address the precise spatial limits of protein synthesis induction during synaptic activation must be carried out. With such information, we will begin to understand the contribution of locally synthesized synaptic proteins to the subcellular specificity of neuronal communication.

Ultimately, the upstream biochemical signaling events that lead to protein synthesis activation need to be elucidated (Figure 2, right). By understanding the transduction mechanisms that various extracellular signals use to regulate protein synthesis machinery and how differences in these signaling cascades result in translation of distinct subsets of mRNA species, a defined role for activity-dependent local translation in mature dendrites will begin to unfold.

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APPENDIX II

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Dynamic Visualization of Local Protein Synthesis in Hippocampal Neurons

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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 CAMKII-α, 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, 1999a). 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, 1999a; 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 Ca^{2+}/calmodulin-dependent kinase II-α subunit (CAMKII-α) (5′GFP3′). Previous work has shown that the 3′UTR of the CAMKII-α 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 1A). In most untreated neurons, expression of the reporter was robust in the cell bodies and relatively weak in the associated dendritic processes (Figure 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 1B). The analysis of total fluorescence in the dendrites revealed that BDNF-treated neurons had significantly greater quantities of

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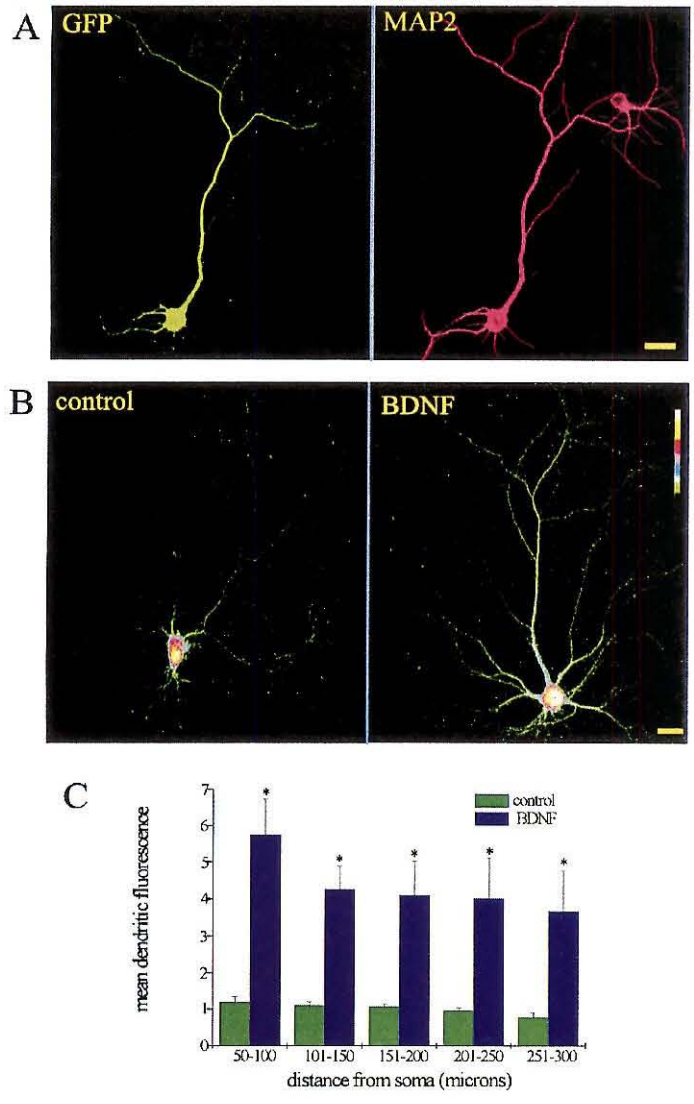


Figure 1. BDNF Stimulates Protein Synthesis of the GFP Reporter in Hippocampal Neurons

(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).

GFP throughout the length of the dendritic process (Figure 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.

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 2A); these increases were detected as early as

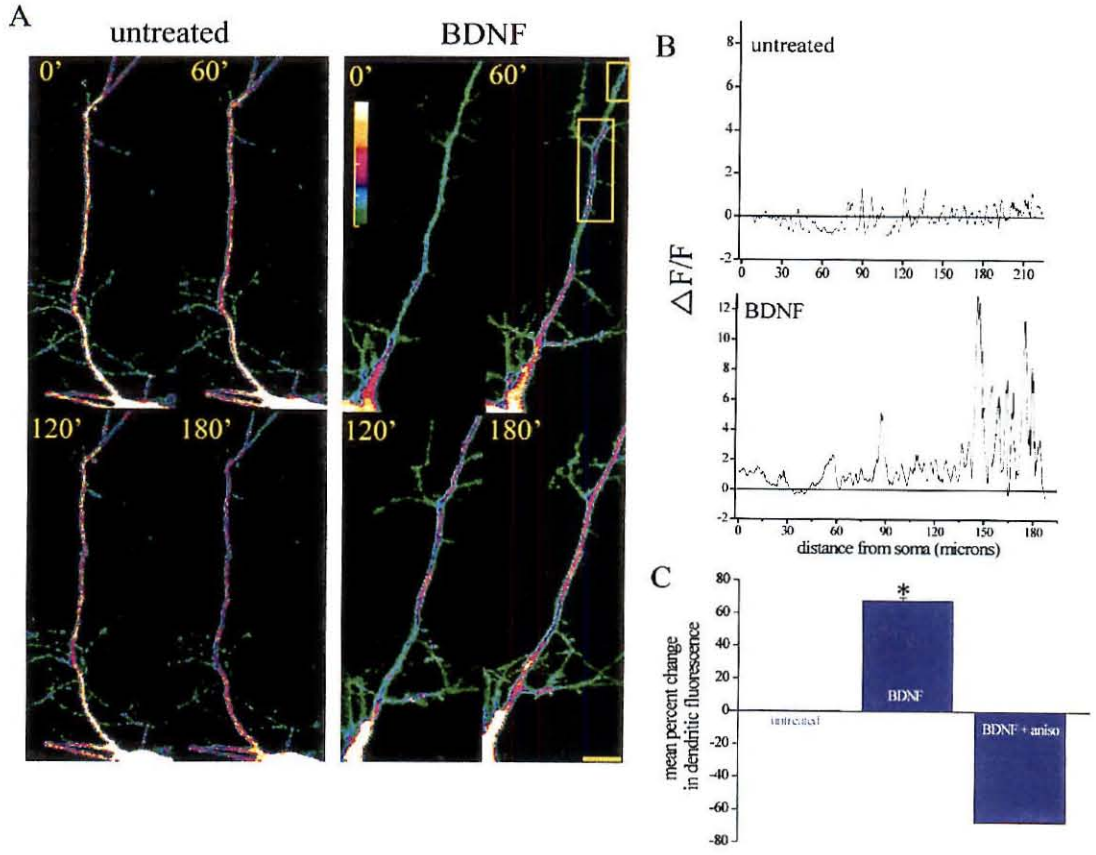


Figure 2. Time-Lapse Imaging of BDNF-Stimulated Translation

(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.

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 2C). In addition, the BDNF-induced increases were prevented by coapplication of the protein synthesis inhibitor anisomycin (Figure 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.

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

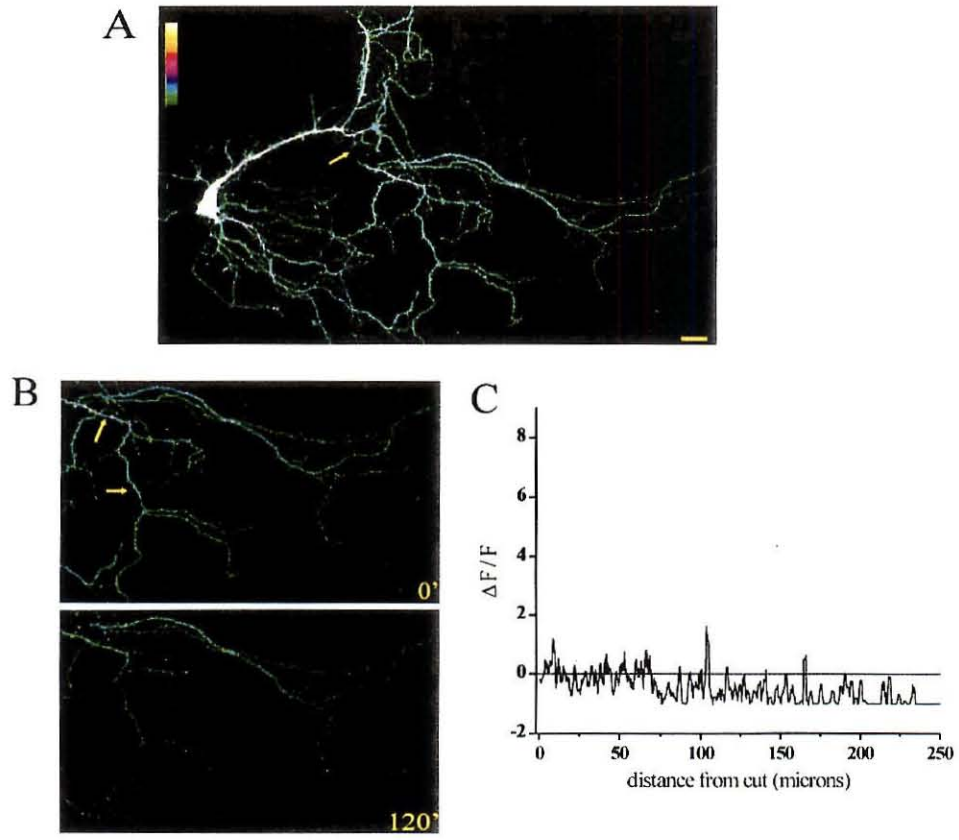


Figure 3. Untreated, Transected Dendrites Do Not Show Increases in Protein Synthesis

(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 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).

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 3). In contrast, transected dendrites treated with BDNF exhibited increases in fluorescence in the isolated dendrites (Figure 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 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 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.

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

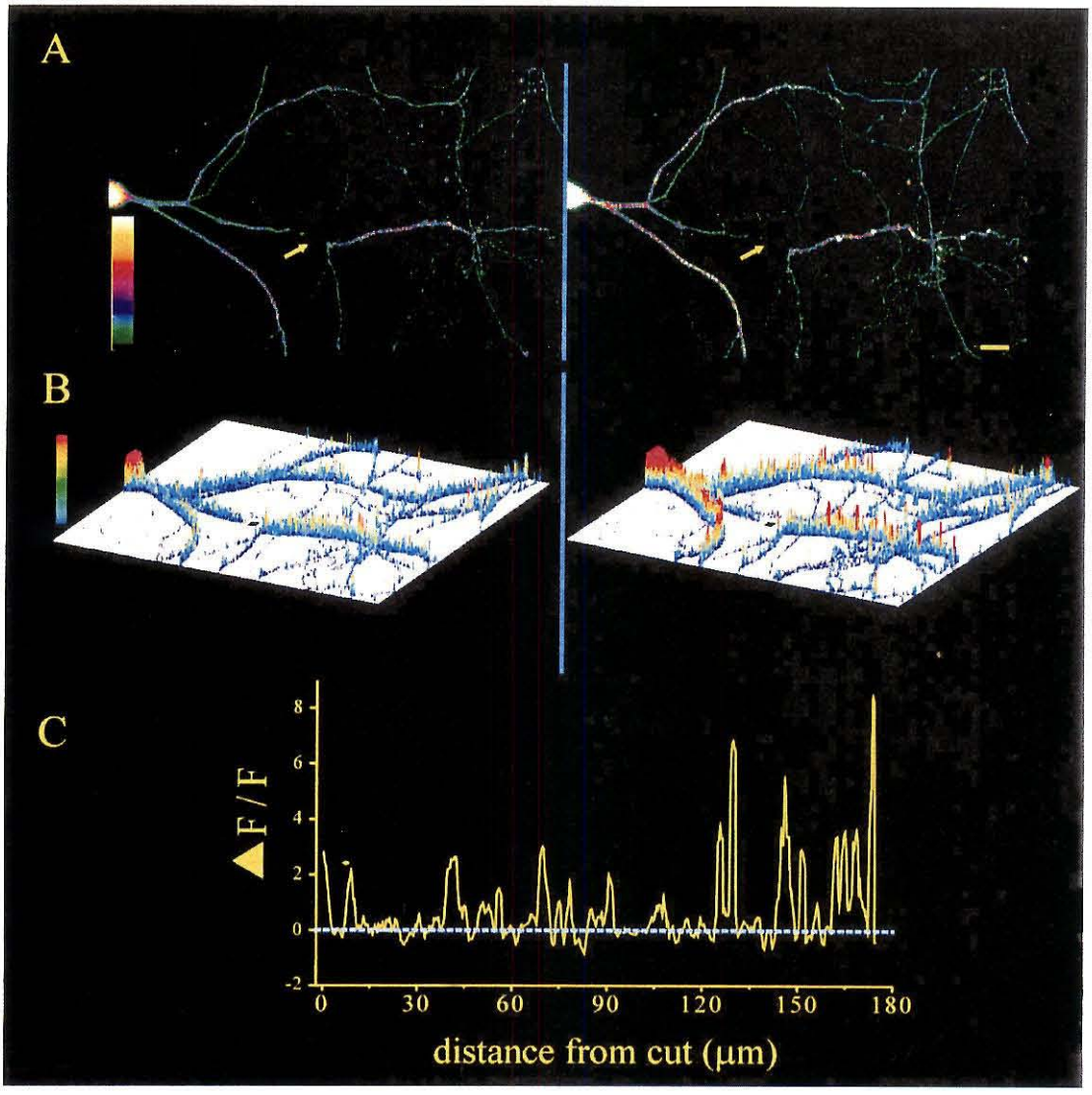


Figure 4. BDNF Stimulates Protein Synthesis in Healthy, Mechanically Isolated Dendrites

(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) X-Y-Z 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).

the N terminus of the GFP molecule and expressed this construct, Sin-5′$_{myr}$dGFP3′, 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 6A). We found that the addition of the myr sequence, however, severely retarded the diffusion of the modified (Sin-5′$_{myr}$dGFP3′)

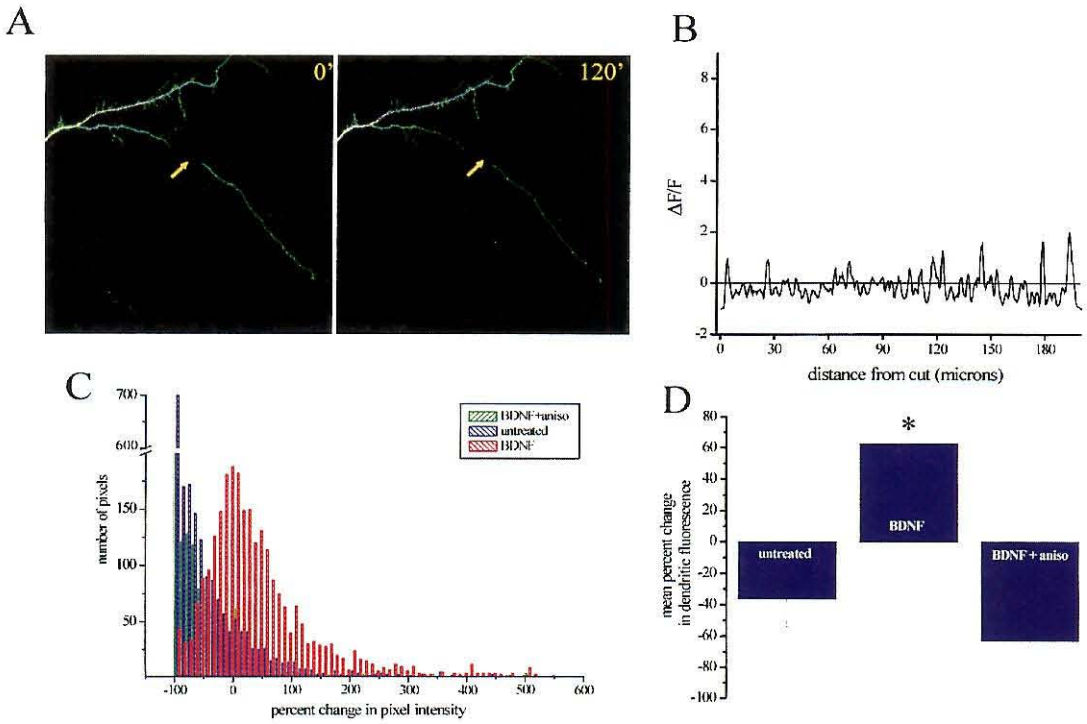


Figure 5. Anisomycin Prevents BDNF-Induced Increases in Protein Synthesis in Transected Dendrites

(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 signficant 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).

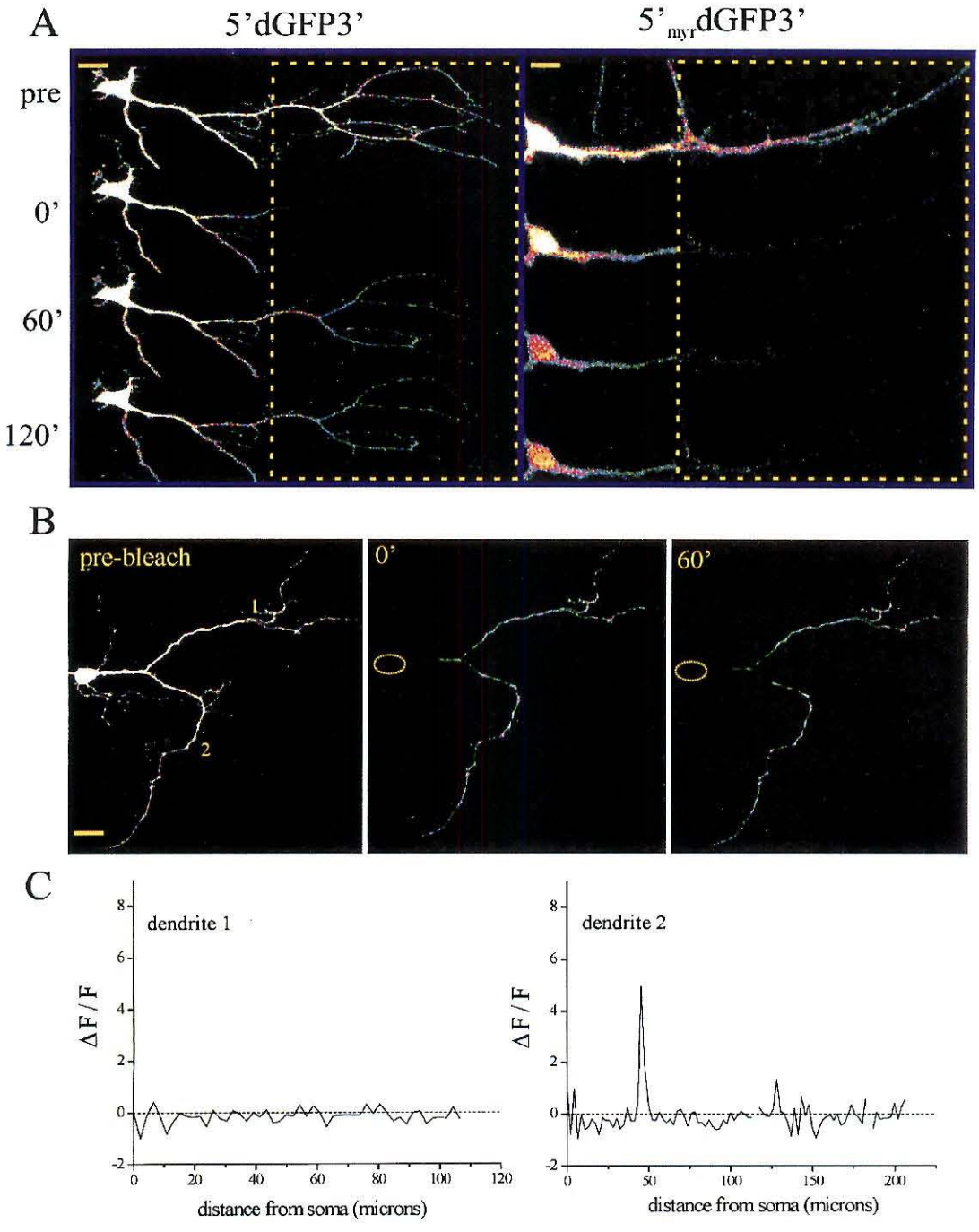
reporter (Figure 6A). Negligible recovery from the photobleached state was observed in the 120 min following the photobleaching episode. These data indicate that the myristoylated reporter exhibits limited diffusion (see Experimental Procedures), suggesting that it can be used to faithfully report local protein synthesis in intact dendrites.

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

We next used the diffusion-restricted reporter (Sin-5′$_{myr}$dGFP3′) 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

Figure 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.

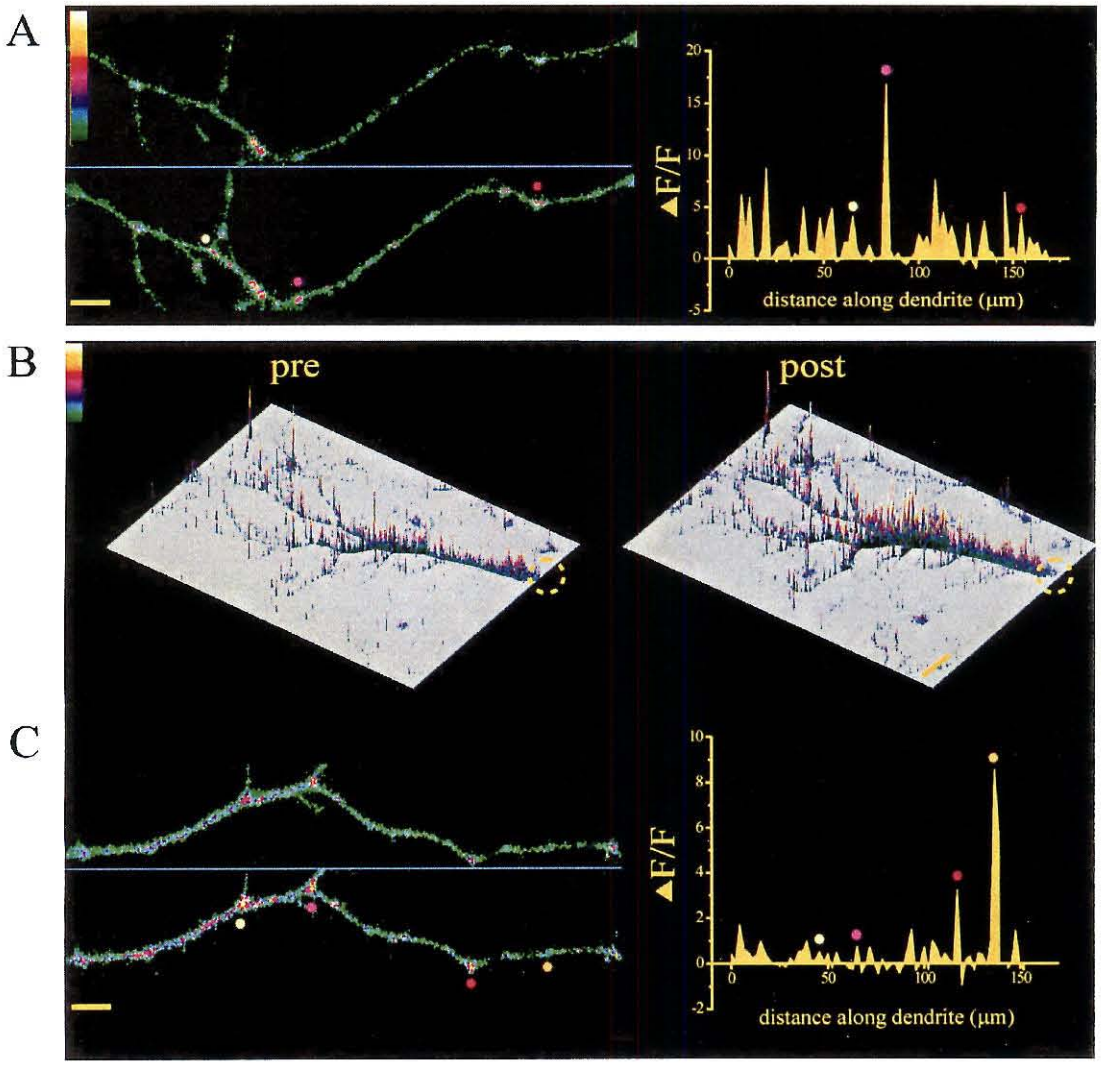


Figure 7. BDNF Stimulates Protein Synthesis in Healthy, "Optically Isolated" Dendrites

(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) An X-Y-Z 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.

fact that both untreated and anisomycin-treated dendrites showed similar average fluorescence change profiles (Figure 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 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 8). Dendrites that were treated with aniso-

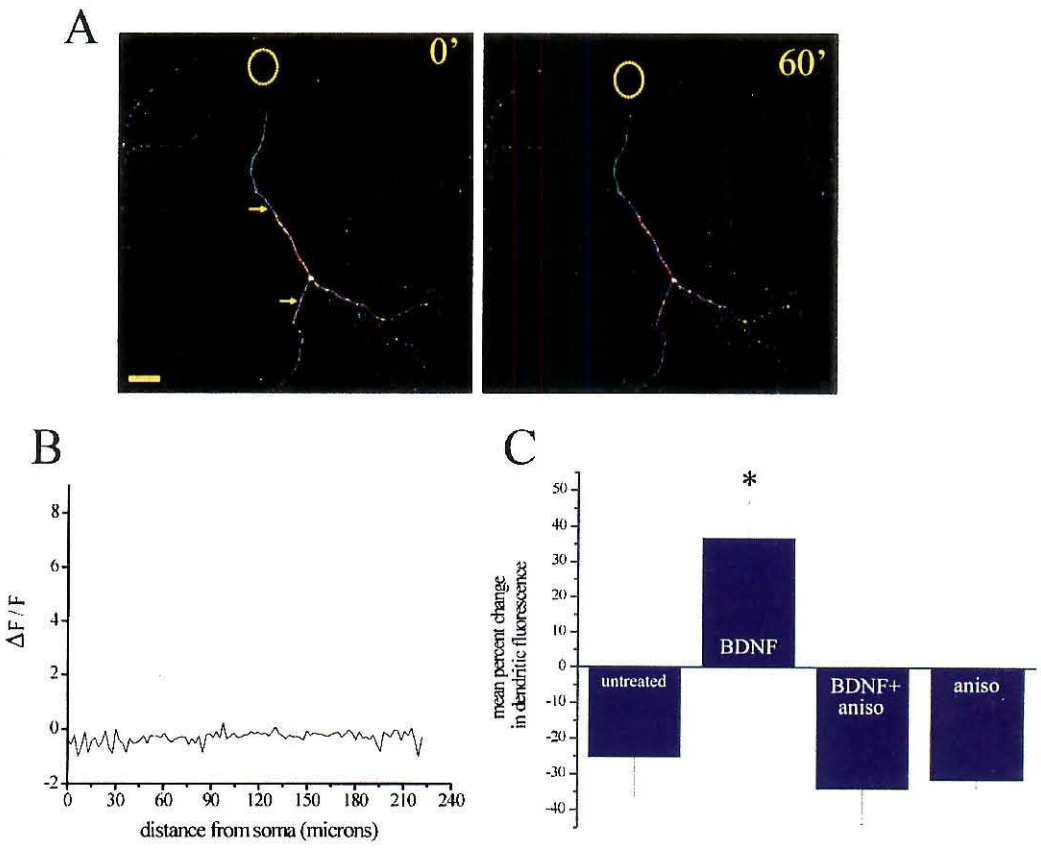


Figure 8. Anisomycin Blocks BDNF-Induced Increases in Protein Synthesis in Optically Isolated Dendrites

(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 signficant 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).

mycin 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 8).

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 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 $5'_{myr}$dGFP3′-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 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 quan-

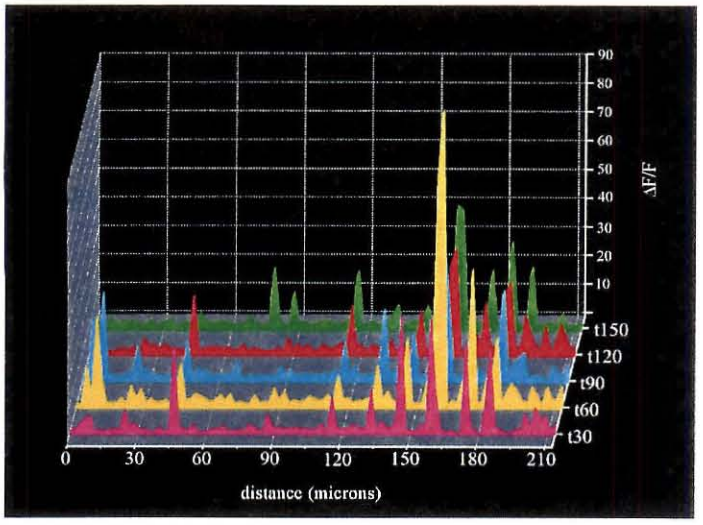


Figure 9. GFP Reporter Signals Are Spatially and Temporally Persistent

Shown are the ΔF/F profiles for a single optically isolated dendrite at several time points. 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.

tify 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 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.

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 (Casadio et al., 1999; Huber et al., 2000; Kang and Schuman, 1996; Martin et al., 1997). 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 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). 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 CAMKII-α since our reporter contains both the 5′ and 3′UTR from the CAMKII-α gene. Indeed, a stimulation of dendritic CAMKII-α 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 CAMKII-α 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 CAMKII-α 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; S.J. Tang et al., 1998, Soc. Neurosci., abstract). 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/cm^2 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 seconday 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

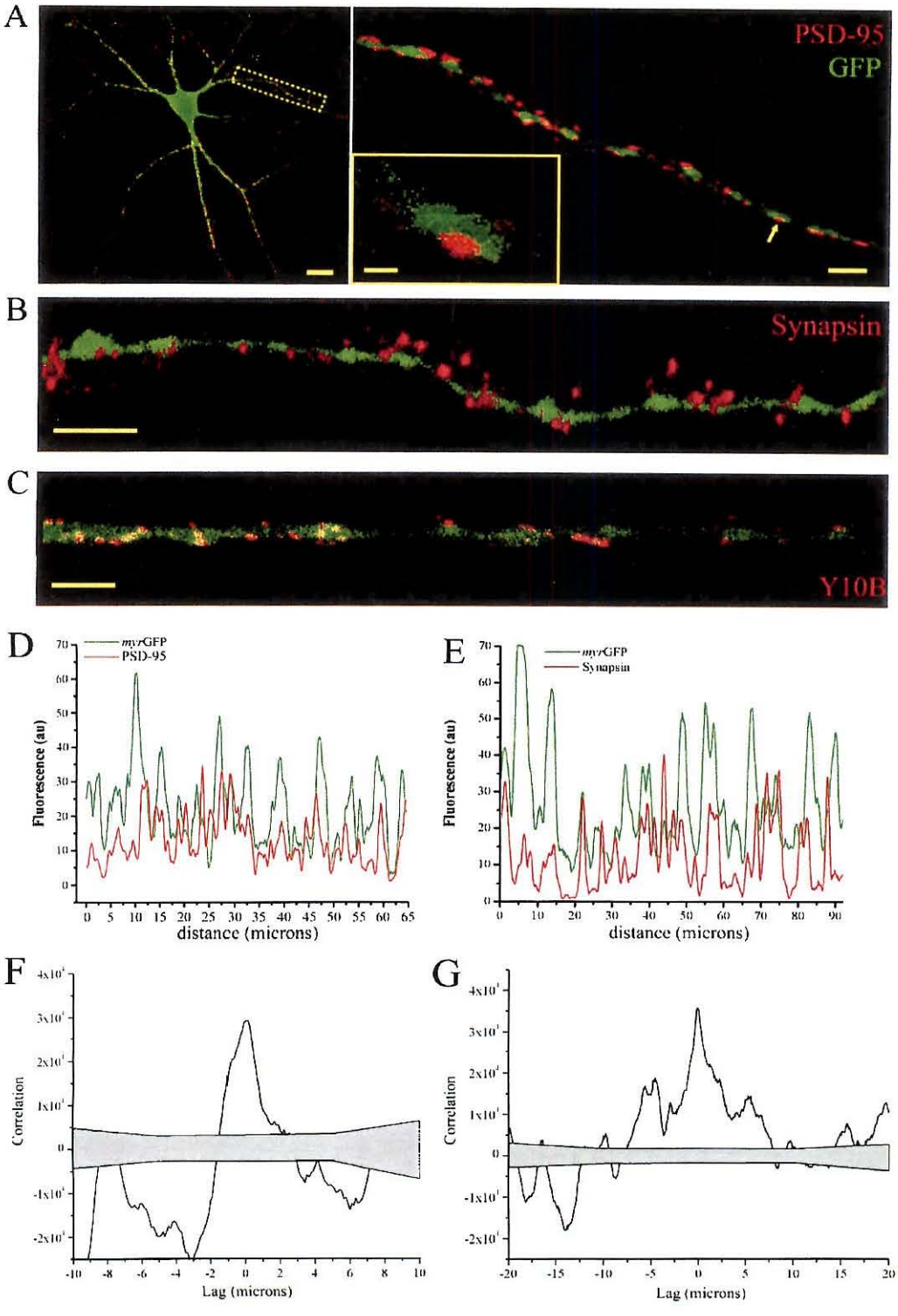
pcDNA3.1-5′dGFP3′: The CamKII-α 3′UTR sequence obtained from plasmid (Mayford et al., 1996) was PCR amplified (forward primer: 5′-ttatatttgcggccgcggtcgctaccattaccagtt-3′; reverse primer: 5′-ggcgctctctcgagtttaaatttgtagct-3′) and cloned into the NotI and XhoI sites of the pcDNA3.1 vector (Invitrogen). The resulting vector was then cleaved with BamHI and NotI for insertion of the destabilized EGFP ORF (from pd2EGFP, Clontech). The CamKII-α 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′$_{myr}$dGFP3′: 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 $_{myr}$dGFP was subcloned into the BamHI-NotI sites of pcDNA3.1-5′dGFP3′. pSinRep5-5′$_{myr}$dGFP3′: The 5′$_{myr}$3 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-


A
PSD-95
GFP
B
Synapsin
C
Y10B
D
myrGFP
PSD-95
Fluorescence (au)
distance (microns)
E
myrGFP
Synapsin
Fluorescence (au)
distance (microns)
F
Correlation
Lag (microns)
G
Correlation
Lag (microns)

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 1×10^{-8} cm²/s, which is only slightly greater than the diffusion coefficient of rhodopsin (~5×10^{-9} cm²/s) (Wey et al., 1981) and glycine receptors (~1×10^{-9} cm²/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, Scion Image, or Image J), 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 $_{myr}$dGFP 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.

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Figure 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.

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