European Journal of Neuroscience, Vol. 21, pp. 1457–1468, 2005

Translation of an integral membrane protein in distal dendrites of hippocampal neurons

Jeffrey C. Grigston, Hendrika M. A. VanDongen, James O. McNamara II and Antonius M. J. VanDongen
Department of Pharmacology and Cancer Biology, Duke University Medical Center, PO Box 3813, Durham, NC 27710, USA

Keywords: mRNA, protein synthesis, *Rattus norvegicus*, synaptic plasticity, TGN-38, zip code

Abstract

Maintenance of synaptic plasticity requires protein translation. Because changes in synaptic strength are regulated at the level of individual synapses, a mechanism is required for newly translated proteins to specifically and persistently modify only a subset of synapses. Evidence suggests this may be accomplished through local translation of proteins at or near synapses in response to plasticity-inducing patterns of activity. A number of proteins important for synaptic function are integral membrane proteins, which require a specialized group of organelles, proteins and enzymatic activities for proper synthesis. Dendrites appear to contain machinery necessary for the proper production of these proteins, and mRNAs for integral membrane proteins have been found localized to dendrites. Experiments are described that investigate the local translation of membrane proteins in the dendrites of cultured rat hippocampal neurons, using fluorescence recovery after photobleaching. Neurons were transfected with cDNAs encoding a fluorescently labeled transmembrane protein, TGN-38. Under conditions where the transport of this reporter construct was inhibited, the appearance of newly synthesized protein was observed via fluorescent microscopy. The dendritic translation of this protein required activation of glutamate receptors. The results demonstrate a functional capacity for activity-dependent synthesis of integral membrane proteins for distal dendrites in hippocampal neurons.

Introduction

Synaptic transmission between neurons is responsible for mediating information processing in the mammalian CNS. Lasting modification of synaptic strength underlies processes such as learning, memory, and synapse development and maturation. While changes in synaptic strength may initially result from the modification and redistribution of existing proteins, the persistence of synaptic plasticity requires synthesis of new proteins (Nguyen *et al*., 1994; Lisman & Fallon, 1999). Evidence for the necessity of new protein synthesis for the expression of lasting synaptic modification has come from experiments involving translational inhibitors. Application of pharmacological antagonists of protein synthesis blocks long-lasting forms of synaptic plasticity in hippocampal slices and other brain-derived preparations, while the initial stages of plasticity remain intact (Stanton & Sarvey, 1984; Montarolo *et al*., 1986; Frey *et al*., 1988).

An individual neuron can have thousands of synaptic inputs distributed throughout a complex network of dendrites. The requirement of protein synthesis for maintenance of synaptic plasticity and the independent regulation of the efficacy of individual synapses raises the issue of how newly synthesized proteins are incorporated only at the particular synapses undergoing changes in strength. A number of findings suggest local mRNA translation as a potential mechanism for delivering newly synthesized proteins specifically to activated synapses (Tang & Schuman, 2002). Dendritic shafts contain protein synthetic machinery and polyribosome complexes, which are frequently found localized at the base of synaptic spines (Steward & Levy, 1982; Steward & Reeves, 1988; Tiedge & Brosius, 1996; Pierce *et al*., 2001). Furthermore, while most mRNA species are confined to the neuronal cell body, certain mRNAs are selectively localized to the dendritic compartment (Wells *et al*., 2000; Steward & Schuman, 2003). Dendritic targeting elements have been identified for a number of dendritically localized mRNAs (Mori *et al*., 2000; Rook *et al*., 2000), and disruption of these elements impairs persistent plasticity and memory consolidation (Miller *et al*., 2002). Dendritic localization of specific mRNAs can be stimulated by synaptic activity or depolarization (Muslimov *et al*., 1998; Steward *et al*., 1998; Zhang *et al*., 1999; Mori *et al*., 2000; Havik *et al*., 2003). Additional support for the involvement of dendritic translation in the maintenance of synaptic potentiation is provided by findings that localized protein synthesis can be activated in dendrites by protocols shown to induce lasting plasticity (Kang & Schuman, 1996; Weiler *et al*., 1997; Ouyang *et al*., 1999; Scheetz *et al*., 2000). Experiments using a green fluorescent protein (GFP) construct expressed in cultured neurons have demonstrated that protein synthesis occurs preferentially at distinct foci along the dendrites coinciding with sites of presynaptic innervation (Aakalu *et al*., 2001).

While substantial data have been provided for the local synthesis of a number of soluble proteins, evidence for the dendritic translation of integral membrane proteins is more limited. The synthesis and processing of this class of proteins requires a set of organelles, including rough endoplasmic reticulum (RER) and Golgi, which allow for their proper membrane insertion and glycosylation. Membranous cisternae immunoreactive for markers of RER and Golgi can be found in the dendrites (Gardiol *et al*., 1999; Pierce *et al*., 2000). Many of the polyribosomes within the dendrites are associated with membranous organelles in a pattern that resembles RER (Steward & Reeves, 1988). Enzymatic activities associated with these organelles are seen in synaptoneurosomes and in isolated neuronal dendrites. A number of

Correspondence: Dr A. M. J. VanDongen, as above.
E-mail: vando005@mc.duke.edu

Received 7 January 2004, revised 12 January 2004, accepted 13 January 2004

doi:10.1111/j.1460-9568.2005.03999.x

mRNAs encoding integral membrane proteins are present in dendrites (Miyashiro *et al*., 1994; Benson, 1997; Eberwine *et al*., 2002). Direct evidence for local translation of integral membrane proteins has been restricted to epitope-tagged versions of the AMPA receptor subunits GluR1 and GluR2, which have been shown to be synthesized in severed dendrites of cultured hippocampal neurons (Kacharmina *et al*., 2000; Ju *et al*., 2004).

We have used a reporter construct consisting of a fusion of cyan fluorescent protein (CFP) and TGN-38, a single-span integral membrane protein that resides in the *trans*-Golgi network (Luzio *et al*., 1990; Girotti & Banting, 1996) to observe real-time synthesis of an integral membrane protein in the dendritic compartment of intact cultured hippocampal neurons. Synthesis of the CFP–TGN-38 reporter construct could be seen in a previously photobleached dendritic region of transfected neurons less than 30 min after stimulation of the neurons with glutamate and glycine. This synthesis was sensitive to the translational inhibitor anisomycin and required the presence of the αCaMKII ZIP code element in the 3′ untranslated region (3′UTR) of the CFP–TGN-38 construct. Finally, cell membrane permeabilization experiments indicate that the newly synthesized dendritic protein is incorporated into internal membranes.

Materials and methods

All experimental data sets include a minimum of eight cells taken from at least two separate cultures. Images shown are representative examples of the respective data sets.

Cell culture

Hippocampal brain tissue from E18 rats was obtained from Brainbits (Springfield, Illinois, USA) in Hibernate/B27 media (Brewer & Price, 1996). Cells were dissociated and plated at 20,000 cells/cm^2 on poly-D-lysine-coated glass-bottomed culture dishes (Mattek). Cells were cultured as described (Brewer *et al*., 1993), except that they were fed only weekly.

DNA constructs

Plasmids encoding CFP and yellow fluorescent protein (YFP) were obtained from Clontech. cDNA for rat TGN-38 was generated via polymerase chain reaction (PCR) amplification with Clontech's Marathon rat brain cDNA as template. CFP was inserted between amino acids 40 and 41 of TGN-38 (Girotti & Banting, 1996) using established techniques of molecular biology (CFP–TGN-38) (McNamara *et al*., 2004). Glycine residues were inserted at both the amino and carboxyl termini of CFP to facilitate the proper folding of both CFP and TGN-38. A 98-nucleotide ZIP code fragment of the αCaMKII 3′UTR with the sequence TGAATGACTATGCTAGGG TCCCTGCGCTCTTGCTTCGCAGAGATCCATTCTTTGTCCAT GGAATGTGGCTGCTGGCTCTCCCTTGGATGTTGCTGGAA was generated via PCR amplification with Clontech's Marathon rat brain cDNA as template. The fragment was inserted into the CFP–TGN-38 fusion construct 10 nucleotides following the TGN-38 STOP codon (CFP–TGN-38–ZIP).

Transfections

Cultures were transfected with plasmid DNA using Lipofectamine 2000 (Life Technologies). For each transfection 3 μL of Lipofectamine 2000 was added to 50 μL Neurobasal, and 2 μg plasmid DNA was added to a separate 50 μL Neurobasal. Both mixtures were incubated at room temperature for 5 min. The mixtures were then combined and incubated at room temperature for 20 min. Media on culture (∼ 2 mL) was then replaced with Neurobasal/Lipofectamine 2000/DNA mixture and cells were incubated at 35 °C for 10 min. Original culture media was then returned to the culture and the culture was returned to the incubator. For co-transfections, 1 μg of each plasmid was used. Neurons were transfected after 14–20 days *in vitro* and imaging/RNA hybridization was done 18 h post-transfection.

Imaging and fluorescence recovery after photobleaching (FRAP)

Cells were treated for 1–2 h prior to imaging with 50 mM 1,2-butanedione monoxime (BDM) in Dulbecco's phosphate-buffered saline (DPBS) at 35 °C. For glutamate/glycine or *N*-methyl-D-aspartate (NMDA)/glycine stimulation, cells were treated with 1 μM glycine and either 100 μM glutamate or 100 μM NMDA in DPBS for 30 s in the presence of BDM 10 min prior to imaging. After stimulation, cells were washed four times in DPBS. Cells were imaged in DPBS + BDM at 35 °C with a Nikon Diaphot inverted fluorescence microscope. A neutral density filter 16 was placed in the illumination pathway for all images taken. A Princeton Instruments cooled CCD digital camera (MicroMax) was used to record images. ImagePro version 4.0 imaging software was used to control image acquisition and for image analysis. Filters for imaging CFP and YFP were purchased from Chroma. For photobleaching, an iris was contracted in the illumination pathway to isolate a dendritic region of the cell being imaged. The neutral density filter was removed and the isolated area was exposed to illumination for 2 min. Cells were permeabilized with either prechilled methanol at −20 °C for 20 min or 0.1% saponin in DPBS for 20 min before being imaged in DPBS.

In situ *RNA hybridization*

The first 225 nucleotides of the coding region of CFP were amplified by PCR and cloned non-directionally into the pcDNA3.1 (–) vector (Invitrogen) to generate plasmids containing either sense or antisense orientations of the CFP fragment. The plasmids were linearized with Not I, and digoxigenin-labeled RNA probes were generated using the MAXIscript T7 *In vitro* Transcription Kit (Ambion). DIG RNA Labeling Mix (Roche) was substituted for the nucleotides from the MAXIscript kit. Neurons transfected with CFP–TGN-38 or CFP–TGN-38–ZIP were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) + 4% sucrose at 4 °C for 20 min and then permeabilized with methanol at −20 °C for 20 min. The cells were prehybridized in hybridization buffer [4 × standard sodium citrate (SSC), 10% dextran sulfate, 1 × Denhardt's solution, 2 mM EDTA, 50% deionized formamide, 500 μg/mL salmon testes DNA] for 1 h at 35 °C. RNA hybridization was performed in hybridization buffer containing approximately 200 ng/mL of digoxigenin-labeled RNA probe for 16 h at 35 °C. The cells were washed 1 × 5 min with 2 × SSC at 35 °C, 3 × 5 min with 60% formamide in 0.2 × SSC at 35 °C and 2 × 5 min with 2 × SSC at 25 °C. The cells were incubated in blocking buffer (PBS + 10% goat serum + 2% bovine serum albumin) at 25 °C for 1 h and incubated in blocking buffer containing a monoclonal anti-digoxigenin antibody (1 : 1000, Boehringer Mannheim) at 25 °C for 1 h. The cells were washed 3 × 10 min in blocking buffer and incubated in blocking buffer containing an Alexa Fluor

488-conjugated goat antimouse antibody (1 : 1000, Molecular Probes) at 25 °C for 1 h. The cells were washed 3 × 10 min in blocking buffer and 2 × 10 min in PBS. Neurons were imaged in DPBS as described above. Filters for YFP were used to image Alexa Fluor 488 labeling.

Data analysis

Images of fluorescent CFP–TGN-38 proteins in distal dendrites of hippocampal neurons were analysed using Image-Pro Plus version 4.5 (Media Cybernetics). Three images were analysed for each neuron: before (B) and immediately after (A) photobleaching and after 15 min of recovery (R). Using the B image, a polygonal 'area of interest' (AOI) was defined that outlined the region of a distal dendrite that was selected for photobleaching. Total fluorescence intensity was measured for this AOI in all three images and fractional recovery was calculated as $(R - A)/B$. Image analysis was performed in a 'double-blind' fashion, where the investigator doing the measurements had no knowledge of the experimental conditions associated with the images. The mean and standard error of the mean (SEM) was calculated for each experimental condition and a Student's *t*-test (two-sample, equal variances) was used to evaluate statistical significance ($a = 0.05$) between groups.

Results

A fluorescent reporter was designed that can be used to study local synthesis of an integral membrane protein in dendrites of living, intact neurons. We based the reporter on TGN-38, a type I integral membrane protein found in the *trans*-Golgi network (TGN), recycling endosomes and at the cell surface (Luzio *et al*., 1990; Girotti & Banting, 1996; McNamara *et al*., 2004). The function of this protein is not well characterized, but it is believed to be involved in the sorting and trafficking of proteins between the TGN and the cell surface. TGN-38 was chosen because it has a distinctive, highly punctate appearance in hippocampal dendrites, which is a requirement for FRAP studies. TGN-38 was fluorescently labeled by inserting the CFP

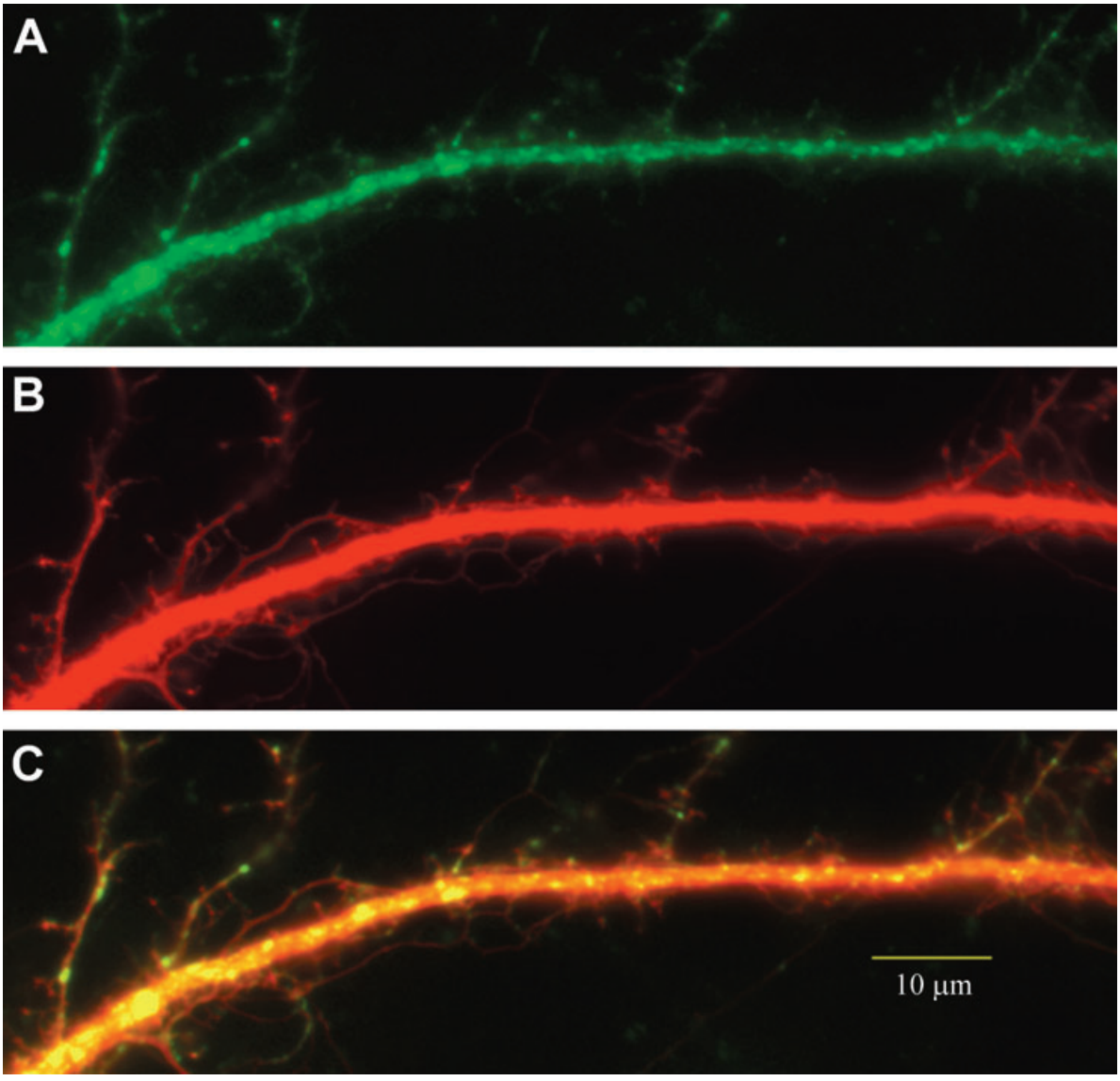

FIG. 1. Distribution of CFP–TGN-38 in hippocampal dendrites. CFP–TGN-38 and YFP were co-expressed in hippocampal neurons. A dendrite of a transfected cell is shown here. (A) The CFP–TGN-38 channel. (B) The YFP channel. (C) The images of A and B are merged.

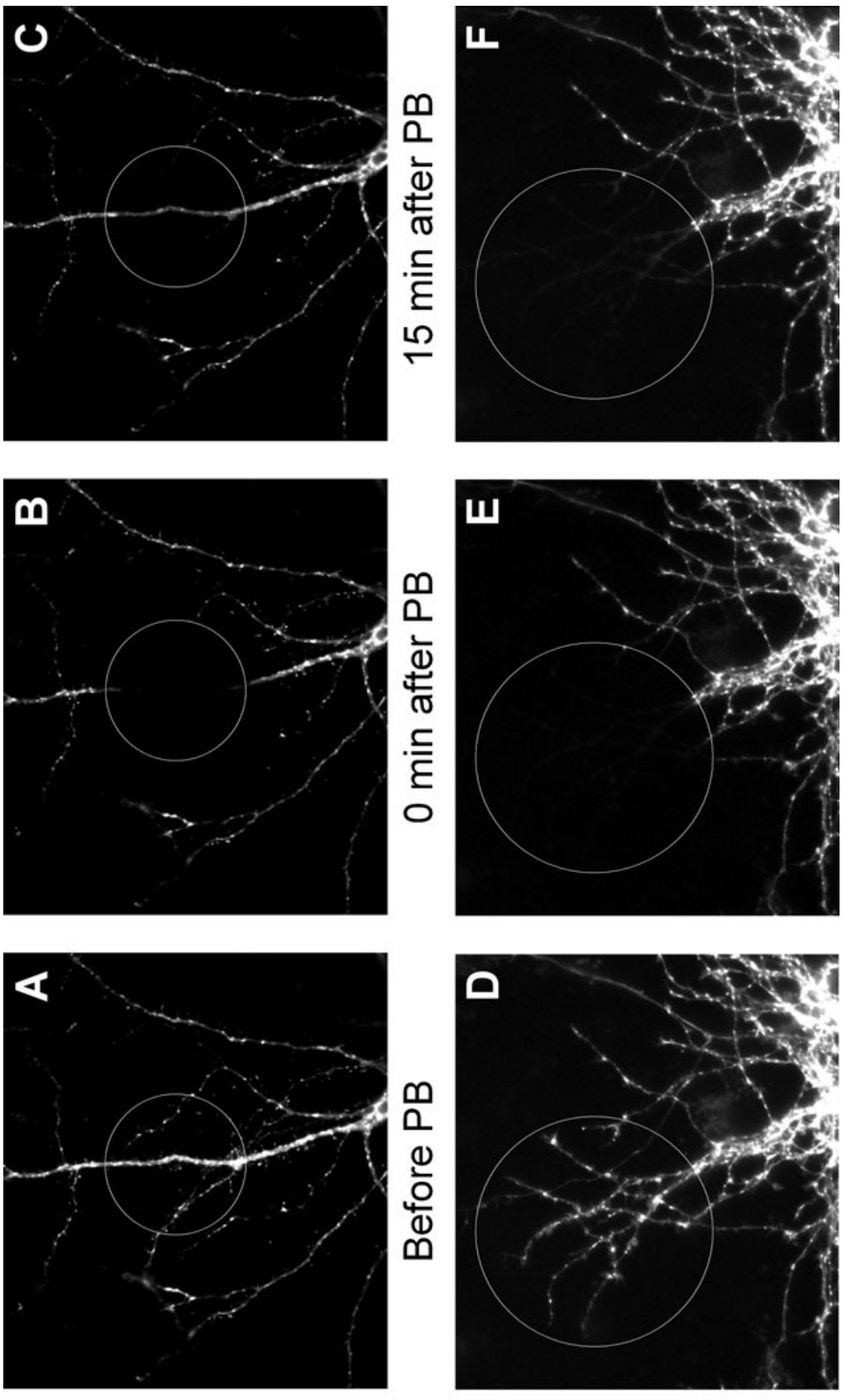

FIG. 2. BDM inhibits CFP–TGN-38 protein transport in cultured hippocampal neurons. (A–C) The CFP–TGN-38–ZIP construct was expressed in hippocampal neurons. CFP–TGN-38 fluorescence was observed before (A), 0 min after (B), and 15 min after photobleaching (C) of an isolated dendritic region. A white circle outlines the photobleached area. Recovery in the photobleached area is attributed to transport of existing CFP–TGN-38 protein into the region from non-photobleached areas of the neuron. (D–F) After pretreatment for 1–2 h with the myosin inhibitor butanedione monoxime (BDM), CFP–TGN-38 fluorescence was observed before (D), 0 min after (E), and 15 min after photobleaching (F) of an isolated dendritic region. Inhibition of CFP–TGN-38 transport results in a large decrease in fluorescence recovery within the photobleached area.

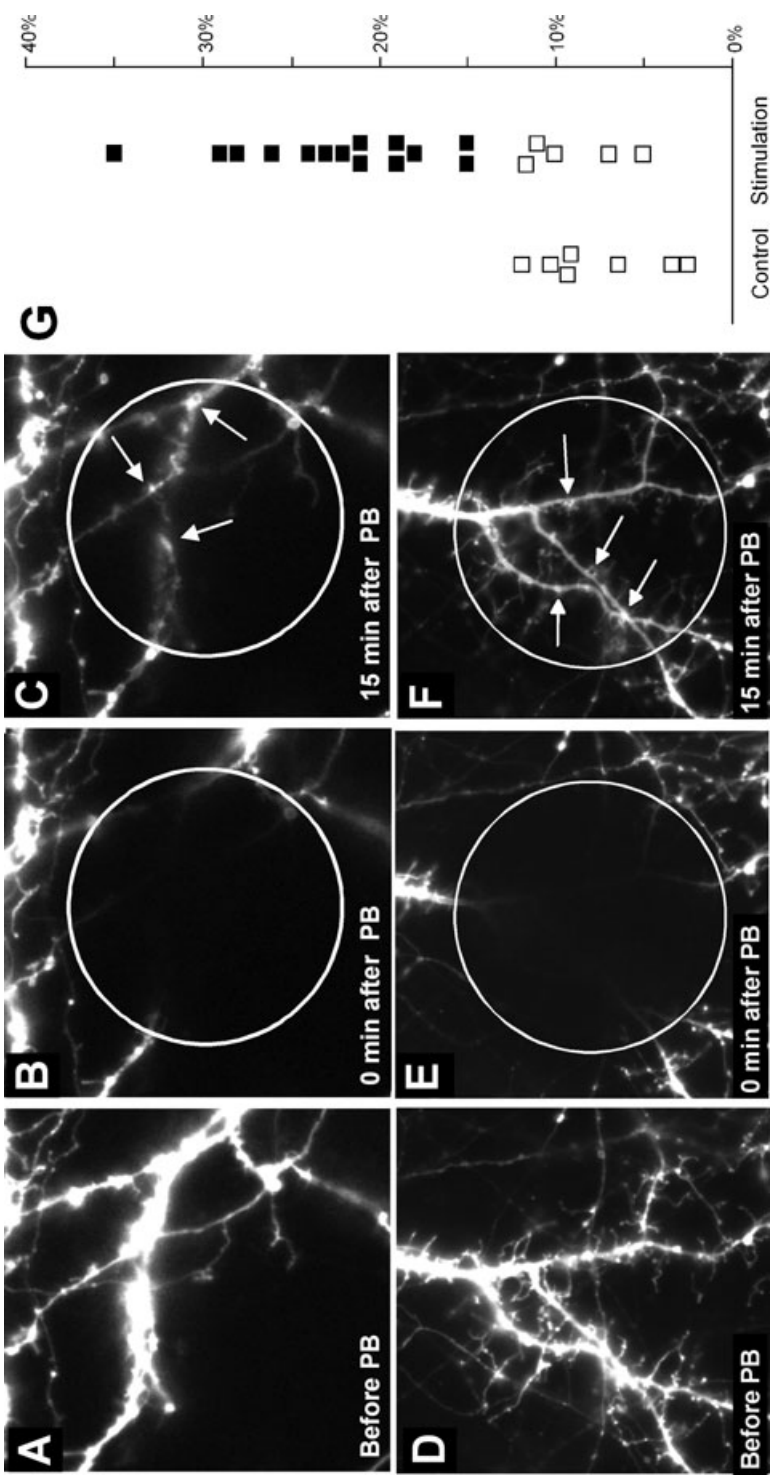

FIG. 3. Stimulation of CFP–TGN-38–ZIP translation in dendrites of hippocampal neurons. (A–F) The CFP–TGN-38–ZIP construct was expressed in cultured hippocampal neurons. Neurons were pretreated with the myosin inhibitor BDM and stimulated with 1 μM glycine, amended with either 100 μM glutamate or 100 μM NMDA, for 30 s 10 min prior to imaging. Two representative neurons are shown in (A–C) and (D–F). CFP–TGN-38 fluorescence was observed before (A and D), 0 min after (B and E), and 15 min after photobleaching (C and F) of an isolated distal dendritic region. A white circle outlines the photobleached area. Arrows indicate 'hot spots' of fluorescence recovery. The percent fluorescence recovery for control, unstimulated neurons (Fig. 2) was 7.5 ± 1.5% ($n = 7$). Stimulation significantly increased the percent recovery to 18.9 ± 1.9% ($n = 19$, $P = 0.001$). The distribution of the data points in the stimulated group suggested that there were two populations of neurons (G): in a small number of stimulated neurons the recovery was similar to the unstimulated control population (white squares), while for the majority of the neurons (14 out of 19, black squares) the recovery was much higher (22.5 ± 1.5%, $n = 14$). However, the entire set of 19 neurons was used for subsequent statistical comparisons (Fig. 4).

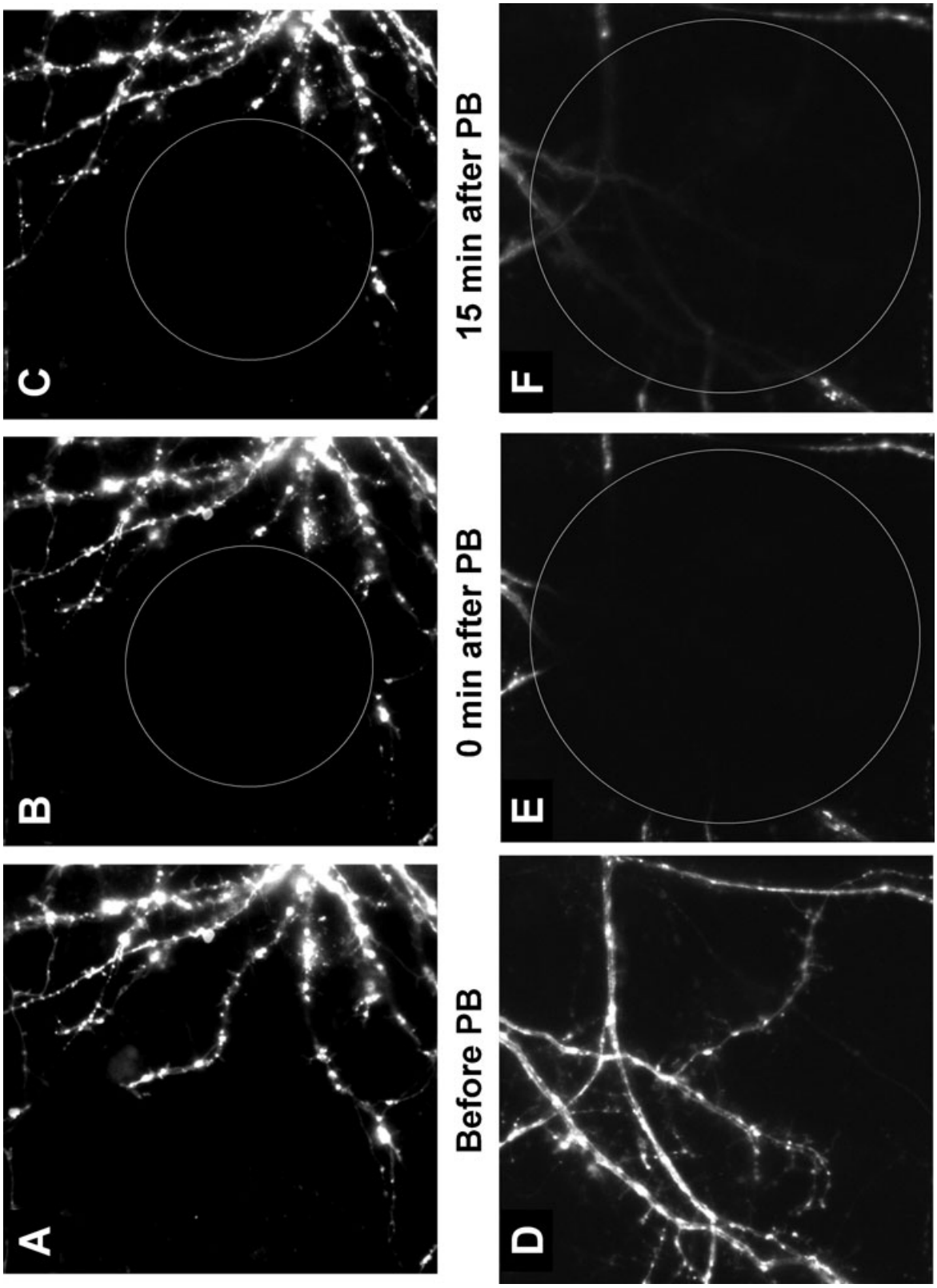

FIG. 4. Stimulation-induced FRAP depends on mRNA translation and dendritic targeting. (A–C) The CFP–TGN-38–ZIP construct was expressed in cultured hippocampal neurons. Cells were pretreated with BDM and with the translational inhibitor anisomycin. Cells were stimulated with 1 μM glycine, amended with 100 μM glutamate or 100 μM NMDA, for 30 s 10 min prior to imaging. CFP–TGN-38 fluorescence was observed before (A), 0 min after (B), and 15 min after photobleaching (C) of an isolated, distal dendritic region. A white circle indicates the photobleached area. Fluorescence recovery within the photobleached area in anisomycin-treated neurons (10.0 ± 1.6%, $n = 13$) was not significantly different from that observed in unstimulated neurons ($P = 0.28$). The anisomycin-treated neurons were significantly different from the entire stimulated group ($P = 0.001$). (D–F) A CFP–TGN-38 construct lacking the mRNA dendritic targeting element was expressed in cultured hippocampal neurons. Cells were pretreated with BDM and were stimulated with 100 μM glutamate or 100 μM NMDA +1 μM glycine for 30 s 10 min prior to imaging. CFP–TGN-38 fluorescence was observed before (D), 0 min after (E), and 15 min after photobleaching (F) of an isolated, distal dendritic region. A white circle indicates the photobleached area. Fluorescence recovery within the photobleached area in CFP–TGN-38-transfected neurons (12.0 ± 2.1%, $n = 12$) was not significantly different from that observed in unstimulated neurons ($P = 0.11$). The neurons expressing the CFP–TGN-38 construct were significantly different from the entire stimulated group ($P = 0.03$).

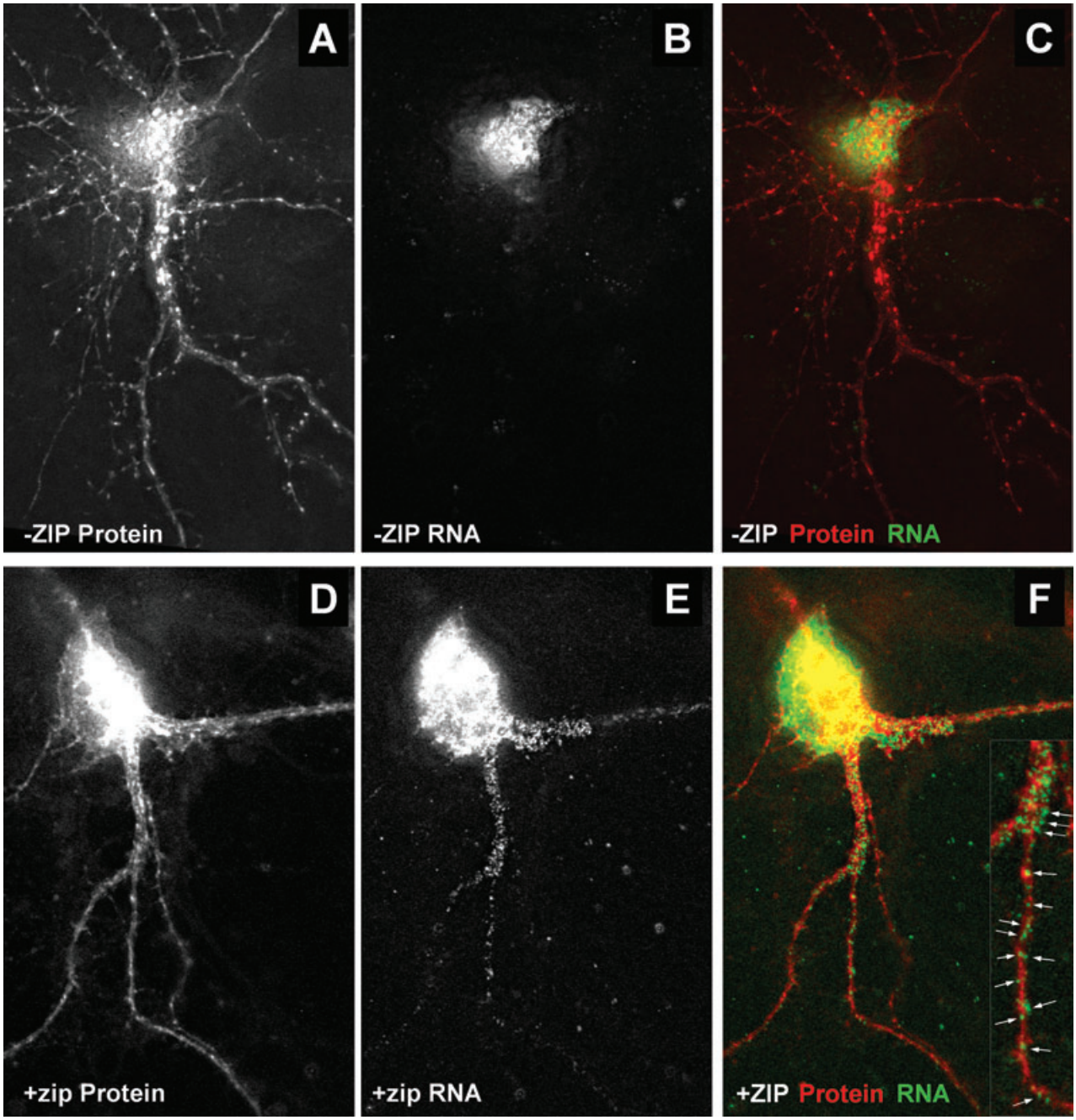

FIG. 5. Localization of CFP–TGN-38 and CFP–TGN-38–ZIP mRNAs in hippocampal neurons. Cultured hippocampal neurons were transfected with plasmids encoding either CFP–TGN-38 (A–C) or CFP–TGN-38–ZIP (D–F). CFP–TGN-38 protein was visualized by fluorescence microscopy (A and D). CFP-TGN38 and CFP-TGN38-ZIP mRNA were localized using *in situ* RNA hybridization with a digoxigenin-labeled antisense RNA probe directed against the first 225 nucleotides of CFP (B and E). CFP–TGN-38 mRNA was found to be restricted to the cell body in cultured hippocampal neurons (C). *In situ* RNA hybridization of CFP–TGN-38–ZIP mRNA demonstrates that this mRNA species was present throughout the dendrites in cultured hippocampal neurons (F), indicating that the αCaMKII mRNA ZIP sequence was sufficient to mediate mRNA transport in this system. The *inset* in (F) shows the distinct punctate nature of the CFP–TGN-38–ZIP mRNA in a dendrite at higher magnification. *In situ* hybridization experiments with antisense probe failed to label cells not expressing CFP–TGN-38 or CFP–TGN-38–ZIP, while no labeling was observed in expressing or non-expressing cells probed with a digoxigenin-labeled sense CFP RNA probe (data not shown.).

sequence in frame into the N-terminal domain of TGN-38. The CFP sequence was inserted after the TGN-38 signal peptide such that, when expressed, the C-terminus of the resulting protein is located in the cytosol while the N-terminus, including the fluorescent protein, is located in the lumen of the Golgi. Additionally, a 98-nucleotide segment of the αCaMKII 3′UTR shown to be sufficient for dendritic transport of mRNA was inserted immediately following the TGN-38 stop codon (Mori *et al*., 2000). This modification is predicted to result in an enrichment of CFP–TGN-38–ZIP mRNA in the dendrites of transfected neurons.

Treatment with BDM blocks TGN-38 transport

Cultured hippocampal neurons were transfected with the CFP–TGN-38–ZIP cDNA and localization of fluorescently labeled

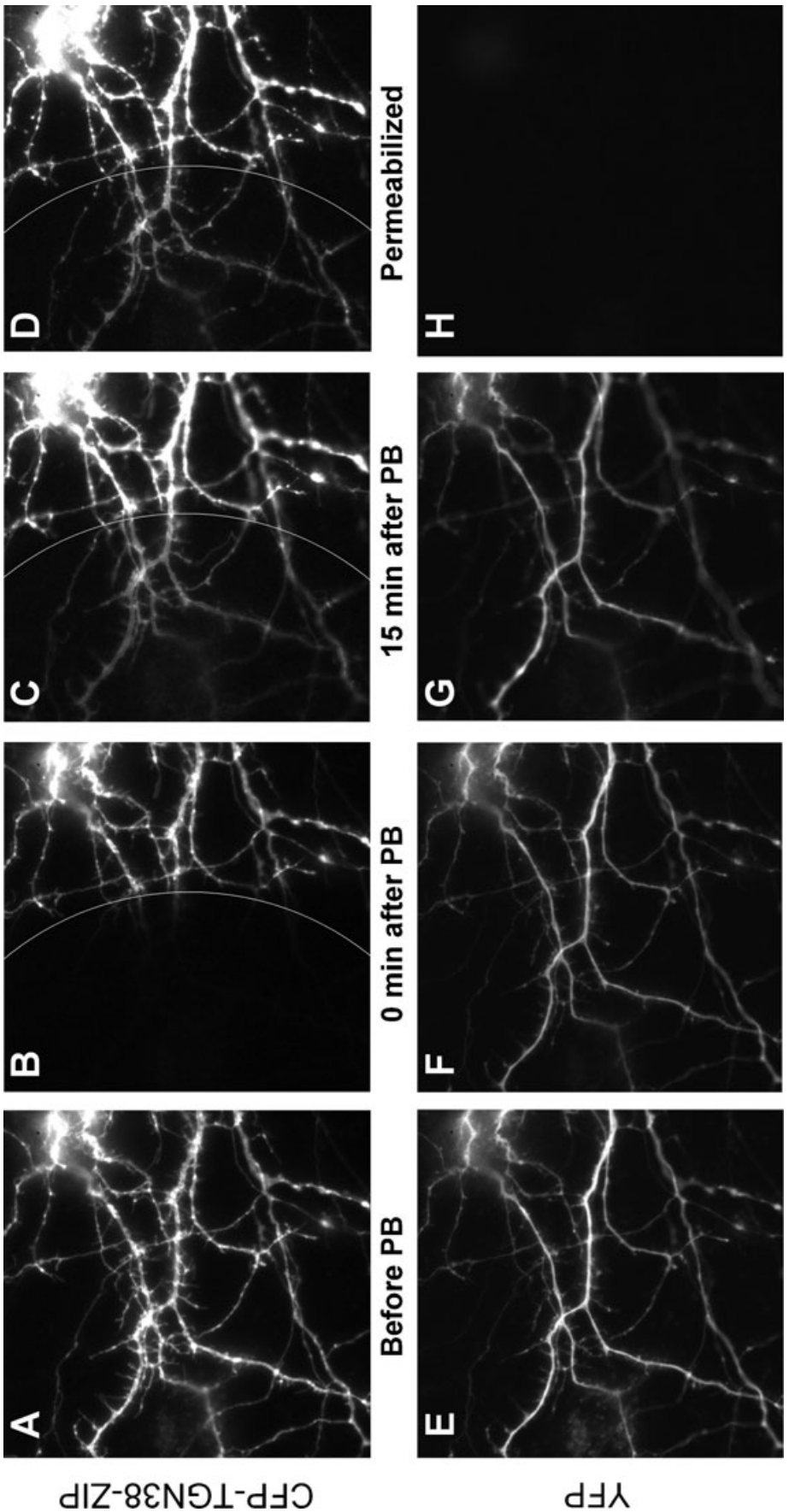

FIG. 6. CFP–TGN-38 protein translated in dendrites is inserted into internal membranes. (A–C) The CFP–TGN-38–ZIP construct was co-expressed with a plasmid encoding the spectrally distinct, yellow fluorescent protein, YFP. Cells were pretreated with BDM and stimulated with 1 μM glycine, amended with 100 μM glutamate or 100 μM NMDA, for 30 s 10 min prior to imaging. CFP–TGN-38 fluorescence was observed before (A), 0 min after (B), and 15 min after photobleaching (C) of an isolated dendritic region. A white arc borders the photobleached area. Arrows indicate 'hot spots' of fluorescence recovery. (D) After permeabilization of the neuron shown in (A–C) with methanol, the CFP–TGN-38 fluorescence signal remains intact. Similar results were obtained using saponin for permeabilization (data not shown). (E–G) YFP fluorescence in the same neuron (A–D) is illustrated before (E), 0 min after (F), and 15 min after photobleaching (G). (H) After permeabilization of the cells, the fluorescence signal of the soluble YFP signal was eliminated.

protein was observed with fluorescence microscopy. The CFP–TGN-38 protein was found to be distributed throughout the cell body and dendrites of transfected cells (Fig. 1). In neuronal dendrites, TGN-38-fusion proteins were found to localize to organelles that were rapidly transported bi-directionally. This transport appeared to be mediated by actin filaments because it was effectively blocked with the actin-depolymerizing agent cytochalasin B and the myosin inhibitor BDM, but not by nocodazole, a microtubule de-polymerizing agent (McNamara *et al*., 2004). The implication that dendritic transport of TGN-38 may be mediated by actin and myosin was unexpected, given that previous work has suggested a role for microtubules in long-range trafficking (Prekeris *et al*., 1999; Burack *et al*., 2000). We are currently investigating this matter further; here we have exploited the effect of BDM on the dynamics of TGN-38 transport to assess local translation of our reporter construct.

To monitor new synthesis of CFP–TGN-38 protein, a method has been developed for selectively photobleaching an isolated region containing distal dendrites of a cultured neuron expressing the fluorescently labeled construct. A selected dendritic region of cells expressing CFP–TGN-38–ZIP was isolated by contracting an iris located in the fluorescence optical pathway. The isolated regions were exposed to illumination for 2 min, eliminating a majority of the fluorescence of pre-existing proteins in the photobleached area and allowing for the observation of the appearance of new fluorescent proteins. Images of distal dendrites of neurons transfected with ECFP–TGN-38–ZIP were taken using fluorescence microscopy before and after photobleaching, as well as after a 15-min recovery interval. Images after the recovery from photobleaching were then compared with those collected before and immediately after photobleaching to determine the percentage of fluorescence recovery.

In photobleached dendritic regions of CFP–TGN-38–ZIP-expressing cells, transport of pre-existing CFP–TGN-38 protein from other areas of the cell resulted in a rapid recovery of fluorescence (Fig. 2A–C). However, CFP–TGN-38–ZIP-expressing neurons treated with the myosin inhibitor BDM to prevent transport of preexisting ECFP–TGN-38 protein showed minimal fluorescence recovery in photobleached regions (Fig. 2D–F). This fluorescence was very diffuse and may have been the result of passive diffusion of ECFP–TGN-38 into the photobleached area. This finding indicated that BDM-mediated inhibition of CFP–TGN-38 protein transport was sufficient to eliminate transport of pre-existing protein into photobleached areas, allowing the reappearance of fluorescence after photobleaching to be used as a measure of local translation of new protein. Importantly, the lack of significant fluorescence recovery under these basal conditions indicates that little or no local translation of the CFP–TGN-38–ZIP message is occurring in unstimulated cultures.

Glutamate receptor agonists stimulate CFP–TGN-38 protein synthesis

We next investigated the effects of glutamate receptor stimulation on the recovery of fluorescence in photobleached dendritic regions of ECFP–TGN-38–ZIP-expressing cells. Neuronal cultures transfected with CFP–TGN-38–ZIP were treated with 50 mM BDM for 1–2 h. These cultures were then treated with 1 μM glycine and either 100 μM glutamate or 100 μM NMDA for 30 s 10 min prior to photobleaching. Following glutamate receptor stimulation, isolated distal dendritic regions were photobleached and recovery was observed at 15-min intervals, as described above. In stimulated neurons, a significantly increased fluorescence recovery was observed in the photobleached region. Figure 3A–F illustrates this for two representative neurons. The distribution of the percent fluorescence recovery suggested that a small subset of neurons (four out of 19) did not respond to the stimulation (Fig. 3G). The stimulation-induced fluorescence was typically concentrated at foci along the photobleached dendrites. The majority of the fluorescence recovery appeared to occur in the first 15 min after photobleaching (the first 25 min after stimulation). All subsequent experiments were therefore performed at this time point. Similar results were obtained with either NMDA or L-glutamate as the glutamate site agonist.

In order to test whether the stimulation-induced increase in fluorescence recovery resulted from *de novo* protein synthesis, the experiments were repeated in the presence of the translational inhibitor anisomycin. Under these conditions, we failed to observe a significant recovery of fluorescence in the photobleached region (Fig. 4A–C). These data suggest that the increase in fluorescence seen in stimulated neurons in the absence of anisomycin resulted from newly synthesized CFP–TGN-38 proteins. Next, it was investigated whether local synthesis of CFP–TGN-38 was dependent on the presence of a dendritic targeting element (ZIP) in its mRNA. A stimulation-induced increase in fluorescence recovery was not seen in neurons expressing the ECFP–TGN-38 construct lacking the αCaMKII mRNA ZIP sequence (Fig. 4D–F). *In situ* RNA hybridization experiments were performed to verify that the αCaMKII mRNA ZIP sequence resulted in dendritic targeting of the mRNA as expected. In neurons transfected with CFP–TGN-38 (lacking the ZIP sequence), the expressed mRNA was isolated within the neuronal cell body (Fig. 5A and B). However, in neurons transfected with CFP–TGN-38–ZIP, the expressed mRNA extended throughout the dendrites (Fig. 5C and D). The dendritically targeted mRNAs were localized to distinct punctae (Fig. 5D, insert), suggesting they are present in heterogeneous nuclear ribonucleoprotein complexes (hnRNP particles). This finding suggests that the stimulation-dependent recovery of fluorescence required the dendritic localization of ECFP–TGN-38 mRNA, providing further support that the observed fluorescence recovery represents local protein synthesis.

Newly synthesized CFP–TGN-38 protein is membrane-bound

To ensure that the newly expressed fluorescent TGN-38 protein was properly inserted into the membrane, neurons were co-transfected with ECFP–TGN-38–ZIP and enhanced yellow fluorescent protein (EYFP). Neurons were stimulated with glutamate receptor agonist and distal dendritic regions were photobleached and allowed to recover, as described above. Subsequently, neurons were permeabilized with methanol or saponin. The ECFP–TGN-38 fluorescence signal, including the fluorescence recovered in the photobleached region, was not affected by permeabilization (Fig. 6A–D). In contrast, the EYFP fluorescence signal was eliminated following permeabilization, suggesting that the soluble EYFP protein had diffused into the medium (Fig. 6E–H). The retention of the newly synthesized ECFP–TGN-38 protein within the cell after permeabilization provides support for its membrane integration.

Discussion

The ability of synapses to alter their strength and responsiveness to synaptic input is believed to be the molecular basis for learning and memory. The requirement of lasting forms of synaptic plasticity for the

production of new protein makes the idea of local protein synthesis within dendrites an attractive and promising one. The exceedingly complex layout of the dendritic arborization and the timing and precision required to deliver any newly made proteins specifically to synapses undergoing modification seems to raise a number of regulatory problems for the neuron. Recent research from a number of groups has demonstrated that dendritic translation is one mechanism by which neurons are able to circumvent these problems (Steward & Schuman, 2003).

Here we find that a fluorescently tagged reporter construct encoding an integral membrane protein (CFP–TGN-38) was translated in distal dendrites of hippocampal neurons in culture. The translation requires the presence of a dendritic targeting element within the CFP–TGN-38 mRNA. In addition, dendritic translation required stimulation of glutamate receptors. The dendritic targeting element used here was contained within the first 98 nucleotides of the 3′UTR of the αCaMKII mRNA, a region previously shown to be necessary and sufficient for trafficking of this mRNA into distal dendrites (Mori *et al*., 2000). In two additional studies, regions containing this short sequence failed to support dendritic targeting (Blichenberg *et al*., 2001; Miller *et al*., 2002). In both cases, the element was present in the context of a larger segment of the 3′UTR, which has been suggested to contain a *cis*-acting element that inhibits extrasomatic sorting (Blichenberg *et al*., 2001). Also, these 3′UTRs were preceded by the coding region of αCaMKII, whereas our experiments and those of Mori *et al*. (2000) used TGN-38 and GFP coding regions. Our *in situ* data (Fig. 5) indicate that the 98-nucleotide region described by Mori *et al*. (2000) is sufficient to target the CFP–TGN-38 reporter mRNA to the dendritic compartment.

Because dendritic translation of αCaMKII mRNA is regulated by NMDA receptor activity (Scheetz *et al*., 2000; Huang *et al*., 2002), it is conceivable that the dendritic targeting element in the reporter construct was responsible for the activity-dependence of its dendritic translation. RNA transcripts destined for distal dendrites are typically packaged into specific hnRNP particles, which mediate their transport and regulate their translation. The highly distinctive punctate appearance of the dendritic CFP–TGN-38 mRNAs (Fig. 5D, insert) is consistent with their localization to hnRNP complexes. During their transport, mRNAs in hnRNP particles are translationally inactive and this inhibition can be relieved by neuronal activity (Krichevsky & Kosik, 2001). Consequently, dendritically targeted mRNAs may display a low basal level of translation in the absence of synaptic stimulation. This could explain the low level of CFP–GFP-38 protein synthesis in the absence of stimulation seen in our experiments (Fig. 2) and the subsequent increase in translation rate observed after treatment with glutamate and glycine (Fig. 3). The activity-dependence of CFP–TGN-38 mRNA translation is consistent with the localization of this mRNA species to hnRNP particles.

A small subset of neurons failed to show enhanced translation of the reporter construct following glutamate receptor stimulation (Fig. 3G). The reasons for this failure are currently not clear. A possible explanation may be the heterogeneous nature of the hippocampal cultures. Neurons may display differences in a number of properties, including but not limited to the ability to transport mRNAs to dendrites, the density of functional synapses, the ratio of synaptic vs. extra-synaptic glutamate receptors and the local availability of translational machinery. Despite the variable nature of neuronal phenotypes in these cultures, it was possible to demonstrate a significant increase in translatability for a large majority of the stimulated neurons.

Our demonstration that the dendritic compartment of intact hippocampal neurons in culture is competent for the rapid production of integral membrane proteins extends upon recent findings that showed synthesis of GluR1 and GluR2 in hippocampal dendrites (Kacharmina *et al*., 2000; Ju *et al*., 2004). Earlier studies have demonstrated the local synthesis of soluble proteins. Despite a growing body of evidence in support of dendritic protein synthesis of cytoplasmic proteins such as FMRP, Arc and αCaMKII, direct evidence for translation of integral membrane proteins outside of the neuronal cell body has been limited to AMPA receptor subunits. However, mRNAs encoding a number of integral membrane and secretory proteins have been known to be present in neuronal dendrites *in vivo* or in culture for some time (Steward & Schuman, 2003). mRNA localization in neurons is a tightly regulated process, with most mRNAs being confined to the cell body. Only a select subset of messages, generally containing *cis*-acting targeting elements, are actively transported into the dendrites. The process of RNA localization within dendrites is itself regulated in an activity-dependent manner for a number of messages, including those encoding Arc and αCaMKII. In light of the tight regulation of mRNA transport within neurons, findings that mRNAs for a diverse group of integral membrane proteins localize to dendrites strongly hint at the possibility that these proteins might be locally translated. Among the mRNAs whose localization within dendrites is best established are those encoding the NMDA receptor subunit NR1 and the InsP3 receptor (Furuichi *et al*., 1993; Benson, 1997). mRNAs for the secretory protein brain-derived neurotrophic factor (BDNF), as well as its transmembrane receptor TrkB are also dendritically localized in hippocampal neurons developing in culture (Tongiorgi *et al*., 1997). BDNF treatment has been shown to induce long-term potentiation and to stimulate dendritic protein synthesis in a number of neuronal systems (Aakalu *et al*., 2001; Job & Eberwine, 2001). *In situ* hybridization and mRNA amplification techniques have been used to identify numerous additional dendritically localized messages encoding membrane proteins, including ion channels, secreted growth factors, receptor tyrosine kinases and heterotrimeric G-protein-coupled receptors (Miyashiro *et al*., 1994).

Experiments using a GFP construct flanked by the 3′ and 5′UTRs of αCaMKII and expressed in cultured neurons demonstrated dendritic protein translation of this reporter in response to BDNF treatment (Aakalu *et al*., 2001). This protein synthesis appeared to occur preferentially at distinct foci along the dendrites, a pattern similar to that observed during stimulation-induced synthesis of CFP–TGN-38 in our experiments. Aakalu *et al*. (2001) noted that the sites of dendritic GFP synthesis co-localized with ribosomes and coincided with sites of presynaptic innervation. The results presented here do not reveal whether the sites of CFP–TGN-38 synthesis are similarly co-localized with synapses or translation machinery. However, the similar pattern of fluorescence observed and the requirement for glutamate receptor stimulation does suggest that the sites we observed may also be near synaptic contacts.

In addition to the basal machinery required for the translation of cytoplasmic proteins, dendritic synthesis of integral membrane proteins requires a unique subset of proteins and organelles for proper membrane insertion and post-translational modification. Our cell permeabilization experiments suggest that the newly synthesized CFP–TGN-38 protein is inserted into the membrane, in contrast to a cytoplasmically expressed protein such as YFP. This finding provides further support for the competence of the dendritic compartment to thread translating peptides through internal membranes. Alternatively, the retention of newly synthesized CFP–TGN-38 in the dendritic compartment following membrane permeabilization may be a consequence of TGN-38's known association with the cytoskeleton (McNamara *et al*., 2004).

A second argument for proper membrane insertion of newly synthesized CFP–TGN-38 protein extends from the co-translational mechanism used for translation of mRNAs encoding membrane and secretory proteins. The N-terminal region of CFP–TGN-38 encodes a hydrophobic signal peptide that marks the nascent protein for membrane insertion. Dendrites have been shown to contain the signal recognition particle (SRP), the ribonucleoprotein complex responsible for recognizing and binding to the signal sequence of integral membrane or secretory proteins undergoing translation (Tiedge & Brosius, 1996). After binding of the SRP to the signal sequence, translation is temporarily halted until the SRP/mRNA/ribosome complex is translocated to the ER membrane. Binding to the SRP receptor in the ER membrane dissociates the SRP from the complex and allows membrane insertion of the nascent peptide via interaction with the Sec61-complex and other components of the translocation machinery. Electron microscopic immunogold studies have identified Sec61α, a component of the Sec61-complex, in the dendritic compartment near spines (Pierce *et al*., 2000). CFP–TGN-38 has been shown to have a functional signal sequence (McNamara *et al*., 2004). Therefore, the fact that we saw newly synthesized CFP–TGN-38 protein appear within 25 min after agonist stimulation suggests a close proximity of the SRP/ZIP-tagged mRNA/ribosome complex and SRP-receptor containing RER membranes. This is in agreement with the observation of organelles resembling RER and associated polyribosomes in dendritic compartments using immunogold electron microscopy experiments (Steward & Reeves, 1988). Additionally, because the sequence encoding CFP is downstream of the signal peptide, the appearance of new CFP fluorescence indicates a resumption of protein synthesis after translocation to the ER lumen, suggesting proper membrane insertion of CFP–TGN-38.

Endogenous TGN-38 protein is heavily glycosylated to a variable extent, which increases its molecular weight from 38 kDa predicted by its amino acid sequence to a range of 85–95 kDa. Glycosylation is initiated in the ER, while further modifications to the sugar moieties occur in the Golgi apparatus. Our experiments do not allow us to draw conclusions regarding the glycosylation status of the newly synthesized CFP–TGN-38 proteins. Whereas the ER is consistently found throughout the dendritic compartment, the subcellular distribution of the Golgi apparatus is less well defined. Isolated dendrites have been found to demonstrate incorporation of sugars into newly synthesized proteins and immunostaining characteristic of the Golgi apparatus, extending only into the proximal dendrites (Torre & Steward, 1996). However, further immunogold studies have identified a full complement of secretory pathway compartments in distal dendrites, including organelles resembling Golgi cisternae in spines (Pierce *et al*., 2000, 2001). Immunocytochemistry and imaging of fluorescent chimeric protein markers in hippocampal neurons in culture found functional ER and Golgi elements in the dendritic compartment (Horton & Ehlers, 2003). While the Golgi structures identified were localized in dendritic shafts rather than in spines, they were distributed into the distal dendrites.

Synaptic plasticity depends critically on the regulated trafficking of membrane receptors and channels to and from synapses. In the case of ionotropic glutamate receptors, both stimulation-dependent and constitutive pathways have been described (Malinow & Malenka, 2002). Local synthesis of these and other membrane proteins adds another useful component to the repertoire of processes that regulate the dynamic composition of synapses. The evidence presented here provides more evidence that distal dendrites have a functional capacity for synthesizing integral membrane proteins. In the case of our reporter, translation depended critically on activation of glutamate receptors. This suggests that local translation of membrane proteins can be controlled by synaptic activity. The molecular details of this regulation need to be further investigated.

Acknowledgements

This work was supported by grants NS31557, MH61506 (A.M.J.VD.) and MH65089 (J.C.G.) from the National Institutes of Health.

Abbreviations

3′UTR, 3′-untranslated region; AOI, area of interest; BDM, butanedione monoxime; BDNF, brain-derived neurotrophic factor; CFP, cyan fluorescent protein; DPBS, Dulbecco's phosphate-buffered saline; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescence protein; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RER, rough endoplasmic reticulum; SSC, standard sodium citrate; SRP, signal recognition particle; TGN, *trans*-Golgi network; YFP, yellow fluorescent protein.

References

Aakalu, G., Smith, W.B., Nguyen, N., Jiang, C. & Schuman, E.M. (2001) Dynamic visualization of local protein synthesis in hippocampal neurons. *Neuron*, **30**, 489–502.

Benson, D.L. (1997) Dendritic compartmentation of NMDA receptor mRNA in cultured hippocampal neurons. *Neuroreport*, **8**, 823–828.

Blichenberg, A., Rehbein, M., Muller, R., Garner, C.C., Richter, D. & Kindler, S. (2001) Identification of a cis-acting dendritic targeting element in the mRNA encoding the alpha subunit of Ca2+/calmodulin-dependent protein kinase II. *Eur. J. Neurosci.*, **13**, 1881–1888.

Brewer, G.J. & Price, P.J. (1996) Viable cultured neurons in ambient carbon dioxide and hibernation storage for a month. *Neuroreport*, **7**, 1509–1512.

Brewer, G.J., Torricelli, J.R., Evege, E.K. & Price, P.J. (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J. Neurosci. Res.*, **35**, 567–576.

Burack, M.A., Silverman, M.A. & Banker, G. (2000) The role of selective transport in neuronal protein sorting. *Neuron*, **26**, 465–472.

Eberwine, J., Belt, B., Kacharmina, J.E. & Miyashiro, K. (2002) Analysis of subcellularly localized mRNAs using in situ hybridization, mRNA amplification, and expression profiling. *Neurochem. Res.*, **27**, 1065–1077.

Frey, U., Krug, M., Reymann, K.G. & Matthies, H. (1988) Anisomycin, an inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA1 region in vitro. *Brain Res.*, **452**, 57–65.

Furuichi, T., Simon-Chazottes, D., Fujino, I., Yamada, N., Hasegawa, M., Miyawaki, A., Yoshikawa, S., Guenet, J.L. & Mikoshiba, K. (1993) Widespread expression of inositol 1,4,5-trisphosphate receptor type 1 gene (Insp3r1) in the mouse central nervous system. *Receptors Channels*, **1**, 11–24.

Gardiol, A., Racca, C. & Triller, A. (1999) Dendritic and postsynaptic protein synthetic machinery. *J. Neurosci.*, **19**, 168–179.

Girotti, M. & Banting, G. (1996) TGN38-green fluorescent protein hybrid proteins expressed in stably transfected eukaryotic cells provide a tool for the real-time, in vivo study of membrane traffic pathways and suggest a possible role for rat TGN38. *J. Cell Sci.*, **109**, 2915–2926.

Havik, B., Rokke, H., Bardsen, K., Davanger, S. & Bramham, C.R. (2003) Bursts of high-frequency stimulation trigger rapid delivery of pre-existing alpha-CaMKII mRNA to synapses: a mechanism in dendritic synthesis during long-term potentiation in adult awake rats. *Eur. J. Neurosci.*, **17**, 2679–2689.

Horton, A.C. & Ehlers, M.D. (2003) Dual modes of endoplasmic reticulum-to-Golgi transport in dendrites revealed by live-cell imaging. *J. Neurosci.*, **23**, 6188–6199.

Huang, Y.S., Jung, M.Y., Sarkissian, M. & Richter, J.D. (2002) N-methyl-D-aspartate receptor signaling results in Aurora kinase-catalyzed CPEB phosphorylation and alpha CaMKII mRNA polyadenylation at synapses. *EMBO J.*, **21**, 2139–2148.

Job, C. & Eberwine, J. (2001) Identification of sites for exponential translation in living dendrites. *Proc. Natl. Acad. Sci. USA*, **98**, 13037–13042.

Ju, W., Morishita, W., Tsui, J., Gaietta, G., Deerinck, T.J., Adams, S.R., Garner, C.C., Tsien, R.Y., Ellisman, M.H. & Malenka, R.C. (2004) Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. *Nat. Neurosci.*, **7**, 244–253.

Kacharmina, J.E., Job, C., Crino, P. & Eberwine, J. (2000) Stimulation of glutamate receptor protein synthesis and membrane insertion within isolated neuronal dendrites. *Proc. Natl. Acad. Sci. USA*, **97**, 11545–11550.

Kang, H. & Schuman, E.M. (1996) A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. *Science*, **273**, 1402–1406.

Krichevsky, A.M. & Kosik, K.S. (2001) Neuronal RNA granules: a link between RNA localization and stimulation-dependent translation. *Neuron*, **32**, 683–696.

Lisman, J.E. & Fallon, J.R. (1999) What maintains memories? *Science*, **283**, 339–340.

Luzio, J.P., Brake, B., Banting, G., Howell, K.E., Braghetta, P. & Stanley, K.K. (1990) Identification, sequencing and expression of an integral membrane protein of the trans-Golgi network (TGN38). *Biochem. J.*, **270**, 97–102.

Malinow, R. & Malenka, R.C. (2002) AMPA receptor trafficking and synaptic plasticity. *Ann. Rev. Neurosci.*, **25**, 103–126.

McNamara, J.O., Grigston, J.C., VanDongen, H.M. & VanDongen, A.M. (2004) Rapid dendritic transport of TGN38, a putative cargo receptor. *Mol. Brain Res.*, **127**, 68–78.

Miller, S., Yasuda, M., Coats, J.K., Jones, Y., Martone, M.E. & Mayford, M. (2002) Disruption of dendritic translation of CaMKIIalpha impairs stabilization of synaptic plasticity and memory consolidation. *Neuron*, **36**, 507–519.

Miyashiro, K., Dichter, M. & Eberwine, J. (1994) On the nature and differential distribution of mRNAs in hippocampal neurites: Implications for neuronal functioning. *Proc. Natl. Acad. Sci. USA*, **91**, 10800–10804.

Montarolo, P.G., Goelet, P., Castellucci, V.F., Morgan, J., Kandel, E.R. & Schacher, S. (1986) A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in Aplysia. *Science*, **234**, 1249–1254.

Mori, Y., Imaizumi, K., Katayama, T., Yoneda, T. & Tohyama, M. (2000) Two cis-acting elements in the 3′ untranslated region of alpha-CaMKII regulate its dendritic targeting. *Nat. Neurosci.*, **3**, 1079–1084.

Muslimov, I.A., Banker, G., Brosius, J. & Tiedge, H. (1998) Activity-dependent regulation of dendritic BC1 RNA in hippocampal neurons in culture. *J. Cell Biol.*, **141**, 1601–1611.

Nguyen, P.V., Abel, T. & Kandel, E.R. (1994) Requirement of a critical period of transcription for induction of a late phase of LTP. *Science*, **265**, 1104–1107.

Ouyang, Y., Rosenstein, A., Kreiman, G., Schuman, E.M. & Kennedy, M.B. (1999) Tetanic stimulation leads to increased accumulation of Ca (2+)/ calmodulin-dependent protein kinase II via dendritic protein synthesis in hippocampal neurons. *J. Neurosci.*, **19**, 7823–7833.

Pierce, J.P., Mayer, T. & McCarthy, J.B. (2001) Evidence for a satellite secretory pathway in neuronal dendritic spines. *Current Biol.*, **11**, 351–355.

Pierce, J.P., van Leyen, K. & McCarthy, J.B. (2000) Translocation machinery for synthesis of integral membrane and secretory proteins in dendritic spines. *Nat. Neurosci.*, **3**, 311–313.

Prekeris, R., Foletti, D.L. & Scheller, R.H. (1999) Dynamics of tubulovesicular recycling endosomes in hippocampal neurons. *J. Neurosci.*, **19**, 10324–10337.

Rook, M.S., Lu, M. & Kosik, K.S. (2000) CaMKIIalpha-3′ untranslated region-directed mRNA translocation in living neurons: visualization by GFP linkage. *J. Neurosci.*, **20**, 6385–6393.

Scheetz, A.J., Nairn, A.C. & Constantine-Paton, M. (2000) NMDA receptor-mediated control of protein synthesis at developing synapses. *Nat. Neurosci.*, **3**, 211–216.

Stanton, P.K. & Sarvey, J.M. (1984) Blockade of long-term potentiation in rat hippocampal CA1 region by inhibitors of protein synthesis. *J. Neurosci.*, **4**, 3080–3088.

Steward, O. & Levy, W.B. (1982) Preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus. *J. Neurosci.*, **2**, 284–291.

Steward, O. & Reeves, T.M. (1988) Protein-synthetic machinery beneath postsynaptic sites on CNS neurons: association between polyribosomes and other organelles at the synaptic site. *J. Neurosci.*, **8**, 176–184.

Steward, O. & Schuman, E.M. (2003) Compartmentalized synthesis and degradation of proteins in neurons. *Neuron*, **40**, 347–359.

Steward, O., Wallace, C.S., Lyford, G.L. & Worley, P.F. (1998) Synaptic activation causes the mRNA for the IEG Arc to localize selectively near activated postsynaptic sites on dendrites. *Neuron*, **21**, 741–751.

Tang, S.J. & Schuman, E.M. (2002) Protein synthesis in the dendrite. *Phil. Trans. Royal Soc. Lond. – Series B: Bio. Sci.*, **357**, 521–529.

Tiedge, H. & Brosius, J. (1996) Translational machinery in dendrites of hippocampal neurons in culture. *J. Neurosci.*, **16**, 7171–7181.

Tongiorgi, E., Righi, M. & Cattaneo, A. (1997) Activity-dependent dendritic targeting of BDNF and TrkB mRNAs in hippocampal neurons. *J. Neurosci.*, **17**, 9492–9505.

Torre, E.R. & Steward, O. (1996) Protein synthesis within dendrites: glycosylation of newly synthesized proteins in dendrites of hippocampal neurons in culture. *J. Neurosci.*, **16**, 5967–5978.

Weiler, I.J., Irwin, S.A., Klintsova, A.Y., Spencer, C.M., Brazelton, A.D., Miyashiro, K., Comery, T.A., Patel, B., Eberwine, J. & Greenough, W.T. (1997) Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. *Proc. Natl. Acad. Sci. USA*, **94**, 5395–5400.

Wells, D.G., Richter, J.D. & Fallon, J.R. (2000) Molecular mechanisms for activity-regulated protein synthesis in the synapto-dendritic compartment. *Cur. Opin. Neurobiol.*, **10**, 132–137.

Zhang, H.L., Singer, R.H. & Bassell, G.J. (1999) Neurotrophin regulation of beta-actin mRNA and protein localization within growth cones. *J. Cell Biol.*, **147**, 59–70.