Chapter III

Dopaminergic Stimulation of Local Protein Synthesis Enhances Surface GluR1 Expression and Synaptic Transmission in Hippocampal Neurons

Introduction

The use-dependent modification of synapses is strongly influenced by the actions of the neuromodulator dopamine, a transmitter that participates in both the physiology and pathophysiology of animal behavior. In the hippocampus, dopaminergic signaling acting via the cAMP-PKA pathway is thought to play a key role in protein synthesis-dependent forms of synaptic plasticity (Frey et al., 1993; Huang and Kandel, 1995; Bach et al., 1999). The molecular mechanisms by which dopamine influences synaptic function, however, are not well understood. Using a green fluorescent protein (GFP)-based reporter of translation, as well as a novel, small-molecule reporter of endogenous protein synthesis, we show that dopamine D1/D5 receptor activation stimulates local protein synthesis in the dendrites of cultured hippocampal neurons. Furthermore, we identify the GluR1 subunit of AMPA receptors as one protein upregulated by dopamine receptor activation. In addition to enhancing GluR1 synthesis, dopamine receptor agonists increase the incorporation of surface GluR1 at synaptic sites. The insertion of new GluRs is accompanied by an increase in the frequency, but not the amplitude, of miniature synaptic events. Together, these data suggest a local protein synthesis-dependent activation of previously silent synapses as a result of dopamine receptor stimulation.

Results

In initial experiments, we examined the ability of a dopamine D1/D5 receptor agonist (SKF-38393) to stimulate protein synthesis by visualizing a GFP protein synthesis reporter molecule (Aakalu et al., 2001) in cultured hippocampal neurons. We compared the levels of GFP signal in control (untreated) neurons to neurons that had been exposed to bath application of the dopamine agonist. Relative to controls, neurons treated with SKF (100 μM for 15 min) showed significantly enhanced protein synthesis in both the soma and dendrites (Figure 3.1A,C). Similar results were obtained with a different D1/D5 receptor agonist, dihydrexidine (DHX; data not shown). The stimulation of protein synthesis by SKF was completely prevented by the co-application of a D1/D5 receptor antagonist (SCH-23390; 10 μM), confirming that the observed effects are due to dopamine receptor activation [mean percent inhibition of SKF-stimulated protein synthesis: 97.3 ± 5.1%; n = 12]. We next examined the time course of SKF-induced protein synthesis using time-lapse imaging of dendrites. Control dendrites exhibited relatively stable levels of GFP fluorescence over a 60-minute imaging period (Figure 3.1B). In contrast, a brief (15 min) exposure to SKF increased the GFP signal in dendrites within 60 minutes (Figure 3.1B,D). In both sets of experiments, the effects of SKF were completely prevented by co-application of the protein synthesis inhibitor anisomycin (Figure 3.1C,D), indicating that D1/D5 receptor activation stimulates protein synthesis in hippocampal neurons.

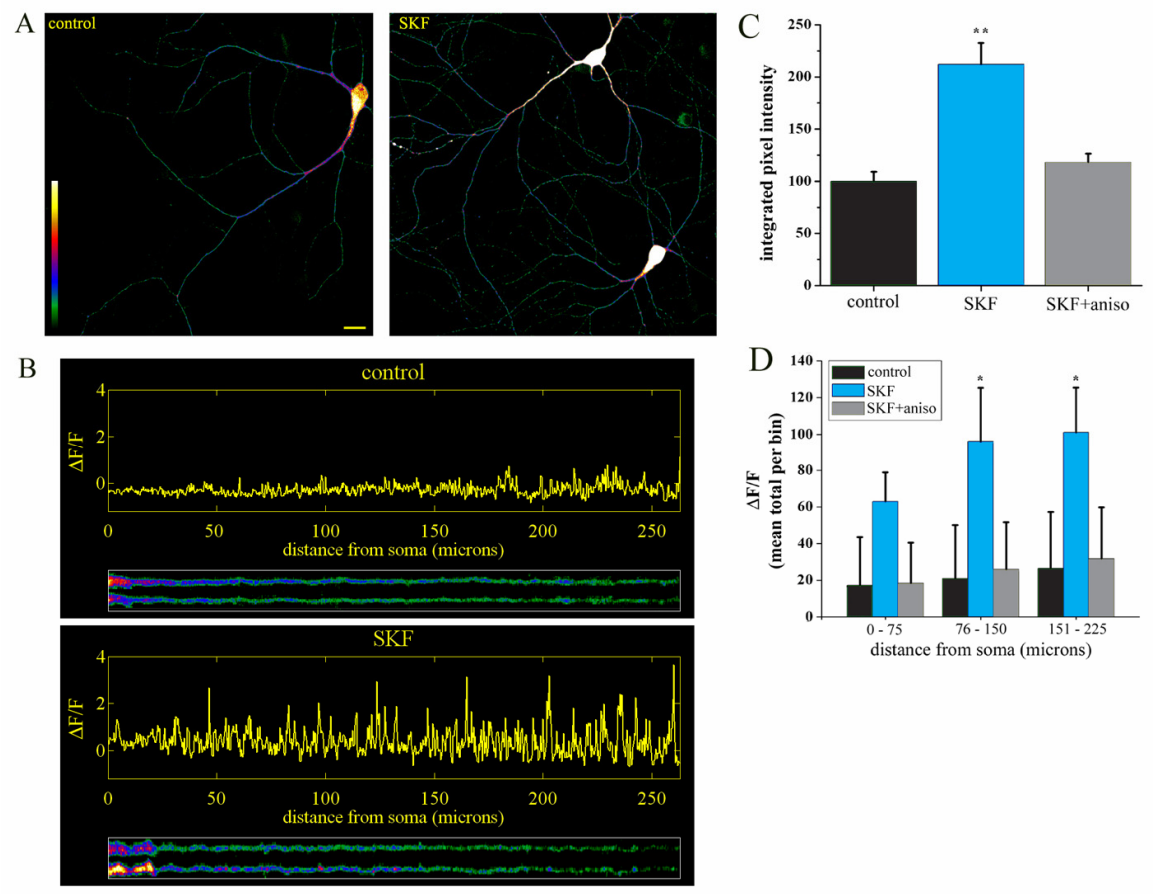

Figure 3.1 Dopamine D1/D5 Receptor Activation Stimulates Protein Synthesis in Hippocampal Neurons

(A) P2 cultured hippocampal neurons infected with a sindbis virus encoding a GFP reporter. Shown are a control neuron (left) and neurons treated for 15 minutes with the D1/D5-selective agonist SKF-38393 (right). The pseudocolor scale at left in the control image indicates GFP fluorescence levels. Scale bar = 15 μm.

(B) Time-lapse imaging of a control neuron (top panel) shows a small decrease in GFP signal as seen in the ΔF/F plot for images before and 60 minutes after vehicle treatment. In contrast, a neuron treated with SKF for 15 minutes (bottom panel) shows an overall increase in GFP signal, with small hotspots of high-intensity fluorescence throughout the dendrite. Images of the dendrites before (top) and 60 minutes after vehicle or SKF treatment (bottom) are shown in the white box beneath each ΔF/F plot, which is aligned to the dendrite shown.

(C) Between-dish (A) summary data showing a significant increase in GFP fluorescence in the dendrites of SKF-treated neurons relative to control neurons (n = 28 dendrites per condition, $p < 0.01$).

(D) Time-lapse (B) summary data 60 minutes after agonist application showing a significant increase in GFP signal at distances greater than 75 microns from the cell soma (n = 12 dendrites per condition, asterisk indicates $p < 0.05$).

The above data show that dopamine agonists can stimulate the synthesis of a fluorescent protein synthesis reporter that contains the 5' and 3' untranslated regions from CaMKIIα (Aakalu et al., 2001). To determine whether D1/D5 receptors activate the translation of endogenous mRNAs in living neurons, we used fluorescein-dC-puromycin (F2P), a novel protein synthesis reporter based on the peptidyl transferase inhibitor puromycin (Starck and Roberts, 2002). Because puromycin is a structural analog of an amino-acyl tRNA molecule, it enters ribosomes actively engaged in translation where it becomes covalently attached to the carboxy-terminus of nascent proteins through a peptide linkage (Nathans, 1964). Initially, we examined whether F2P can serve as a protein synthesis reporter in cultured hippocampal neurons (Figure 3.2A,B). A brief (~15 min) bath application of F2P resulted in fluorescence detected in both the cell body and the dendrites (Figure 3.2A). The majority of the fluorescence observed in the dendrites reflects basal protein synthesis as it was significantly attenuated by co-application of anisomycin or unlabeled puromycin (data not shown). When neurons were treated with the dopamine agonist SKF in the presence of F2P, a dramatic stimulation of protein synthesis in the cell body, dendrites and spines was observed [mean percent increase in F2P signal relative to control: 91.3 ± 11.2 %; n = 14] (Figure 3.2B). These data indicate that dopamine agonists can stimulate the synthesis of endogenous protein(s) in hippocampal neurons.

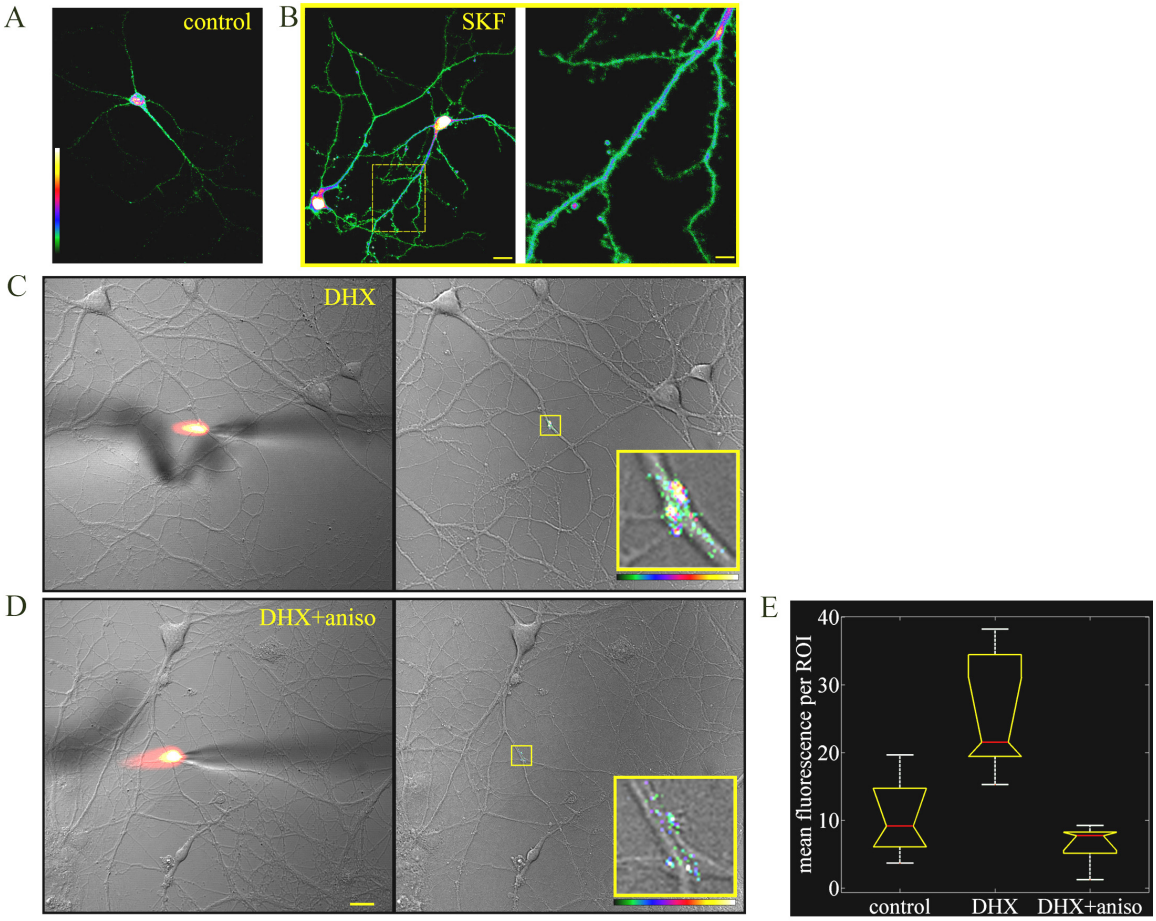

Figure 3.2 A Dopamine Agonist Stimulates the Local Translation of Endogenous Proteins as Indicated by Novel Puromycin-Based Reporter of Protein Synthesis

(A) A control neuron incubated for 15 minutes in F2P (left) exhibits moderate levels of fluorescence primarily due to basal rates of protein synthesis in the unstimulated cell.

(B) Neurons treated with the dopamine agonist SKF for 15 minutes in F2P show markedly higher fluorescence, with signal apparent throughout the dendritic arbor. The region boxed in yellow is shown at high power (right), where signal in the spines is clearly evident. Scale bars = 20 (left), and 5 μm (right).

(C) A solution containing dihydrexidine (DHX), F2P, and the dye Alexa 568 (to mark solution flow) was perfused for 15 minutes onto a small dendritic segment of a cultured hippocampal pyramidal cell (left; shown is dye spot) resulting in a strong dendritic F2P signal (right). The high-power image (right, inset) shows high levels of F2P incorporation indicating local protein synthesis in the stimulated dendrite.

(D) Pretreatment and perfusion with a solution containing anisomycin abolished most of the DHX-induced F2P incorporation in the dendrite (compare high-power insets at right).

(E) The average F2P pixel intensity in each perfused region of interest (ROI, defined by the area of dendrite beneath the Alexa 568 dye) is shown as a series of box plots (see methods for a description of box plots). Perfusion of dendrites with DHX resulted in significantly greater F2P incorporation when compared to control dendrites ($p < 0.05$). The enhancement produced by DHX was completely blocked by preincubation and perfusion with anisomycin ($p < 0.01$, $n = 8$ dendrites for each condition).

Local, dendritic protein synthesis (Steward and Levy, 1982; Torre and Steward, 1992) is required for some forms of synaptic plasticity (Huber et al., 2000; Kang and Schuman, 1996; Martin et al., 1997). We directly examined whether dopamine receptor activation stimulates local protein synthesis by restricting the F2P application to a small (3-10 μm) region of the dendrite (Figure 3.2C). Using a red fluorescent dye to monitor the size and location of the perfusion spot, we locally delivered a continuous stream of both F2P and the D1/D5 agonist DHX (Figure 3.2C) to dendritic regions at least 100 μm from the cell body. A 15-minute exposure to DHX caused a dramatic stimulation of local protein synthesis (Figure 3.2C,E) that was completely prevented when anisomycin was included in the perfusate (Figure 3.2D,E). Because the perfusate was delivered only to a small region of dendrite, remote from the cell body, the F2P signal detected is the result of local protein synthesis. These data firmly establish that D1/D5 agonists stimulate protein synthesis in dendrites.

The above data indicate that dopamine agonists can stimulate the dendritic synthesis of endogenous proteins, but do not establish which particular protein(s) are the targets of dopamine receptor activation. Since dopamine has been shown to enhance synaptic transmission at hippocampal synapses (Bouron and Reuter, 1999; Frey et al., 1993; Huang and Kandel, 1995; Otmakhova and Lisman, 1996), we considered the possibility that a glutamate receptor subunit might be a target for local, dopamine-stimulated translation. We first examined the effect of dopamine agonist treatment on total GluR1 protein detected by Western blot analysis of lysates prepared from hippocampal neurons. We found that a brief (20 min) exposure to DHX resulted in a significant increase

in GluR1 levels relative to untreated controls (Figure 3.3A). In individual experiments, we observed a 9–20% increase in GluR1 levels following D1/D5 receptor activation, which was blocked by anisomycin (Figure 3.3A). A DHX-stimulated increase in dendritic GluR1 was also observed in a parallel set of experiments in which quantitative immunocytochemical labeling was conducted on cultured hippocampal neurons (Figure 3.3B). We next examined whether the new synthesis of GluR1 alters the population of receptors on the cell surface at synaptic sites. Using an antibody that recognizes the GluR1 extracellular domain, we conducted live immunolabeling for surface GluR1 (Figure 3.3C). To determine the location of surface-expressed GluR1 relative to synaptic sites, presynaptic compartments were labeled with an anti-synaptophysin antibody (Figure 3.3C). In control conditions we observed 24 synaptophysin-positive puncta per 10 μm; on average, 21.6% of these puncta colocalized with the surface GluR1. Neurons briefly exposed to DHX (15 min) showed a large, anisomycin-sensitive increase in surface GluR1 (Figure 3.3C-E; Table 1). This increase was evident in both the number and average size of GluR1 puncta detected on the surface; the average intensity of GluR1 puncta was not changed (Table 1). The DHX-stimulated increase in GluR1 was accompanied by an increase in the overlap of GluR1-positive puncta with synaptophysin (Figure 3.3C-E). To analyze the overlap, the number of colocalized (GluR1 and synaptophysin) particles was divided by the number of synaptophysin particles; the result estimates the fraction of synaptic sites that are associated with GluR1 puncta. This analysis revealed that DHX treatment also increased the colocalization of the surface pool of GluR1 and synaptophysin. Importantly, the observed increase in colocalization cannot be attributed to a decrease in the number of synaptophysin particles (Figure 3.3E). Both the increased surface GluR1 expression and the increased

synaptic localization were prevented by anisomycin (Figure 3.3C-E), indicating that protein synthesis is required for these changes.

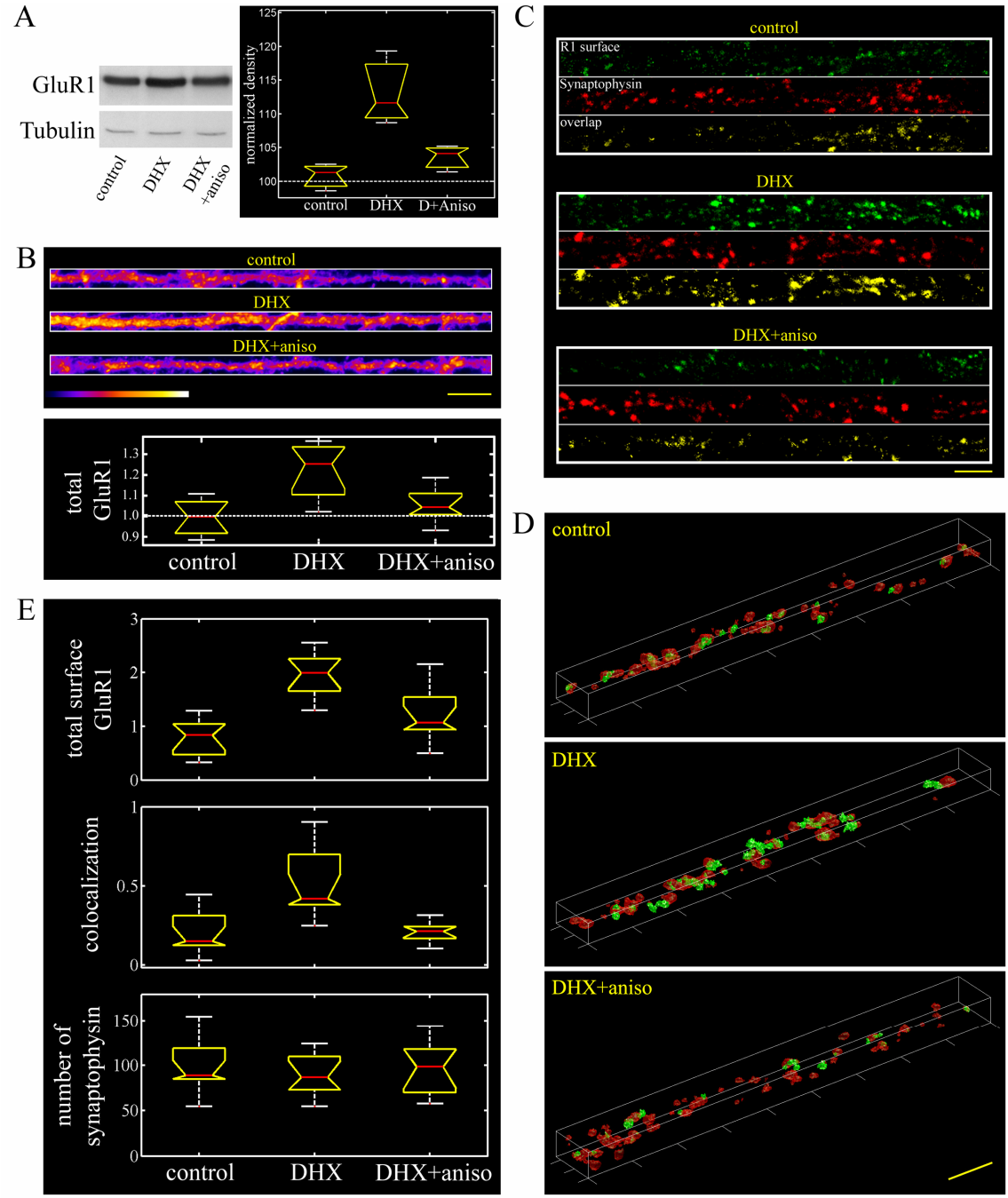

Figure 3.3 Treatment With a Dopamine Agonist Produces a Protein Synthesis-Dependent Increase in Total and Surface GluR1 Expression at Synaptic Sites

(A) Western blot of whole-cell lysates prepared from cultured hippocampal neurons treated for 20 minutes with DMSO (control), dihydrexidine (DHX), or DHX plus anisomycin (DHX+aniso). Blots were probed with antibodies against GluR1 (top), and β-Tubulin as a loading control (bottom). A box plot summary of GluR1 band densities normalized to the Tubulin band in each lane (control mean = 100%) shows significantly higher levels of GluR1 protein in neurons treated with DHX, which is blocked by anisomycin ($n = 3$, $p < 0.05$).

(B) Neurons treated with DMSO (control), dihydrexidine (DHX), or dihydrexidine in the presence of anisomycin (DHX+aniso) show significantly increased levels of total GluR1 protein detected in the dendrites by immunofluorescence labeling ($n = 12$ dendrites per condition, $p < 0.05$). As shown in the box plot summary, the effect is blocked by anisomycin, with no significant difference detected between control and DHX+aniso groups. The pseudocolor scale ranging from dark blue to white is shown at bottom left. Scale bar = 15 μm.

(C) Neurons treated as in (B) were immunostained under nonpermeabilizing conditions with antibodies recognizing extracellular epitopes of GluR1 (green), and synaptophysin (red), and are shown as 2-D projections. The yellow image (overlap) of each 2-D set represents the colocalization between GluR1 and synaptophysin. Increased surface GluR1 as well as increased colocalization of GluR1 with synaptophysin is evident in dendrites treated with DHX. Scale bar = 5 μm.

(D) Representative examples of immunostained dendritic segments are shown as 3-D surface renderings. In these images, only those GluR particles that overlap with synaptophysin are shown whereas all synaptophysin particles are shown. Scale bar = 5μm.

(E) Analysis of 3-D immunostaining data shows significantly increased surface GluR1 and GluR1/synaptophysin colocalization in dendrites treated with DHX ($n = 15$ dendrites per condition, $p < 0.01$). DHX-induced increases were blocked by anisomycin ($p < 0.01$), with no significant difference in synaptophysin labeling between groups.

Given the increase in the total and synaptic GluR1 population, we next examined the effects of dopamine agonists on synaptic transmission. To monitor synaptic strength before and after exposure to a dopamine agonist, we recorded miniature excitatory postsynaptic currents (mEPSCs) in cultured hippocampal neurons. After a baseline recording period (Figure 3.4A_i,B_i) neurons were treated with DHX (Figure 3.4A) or DHX in the presence of anisomycin (Figure 3.4B). We observed that DHX induced a rapid increase in mEPSC frequency that was completely prevented when protein synthesis was inhibited. On average, DHX induced a 2-fold increase in mEPSC frequency (Figure 3.4C). There was, however, no change in mEPSC amplitude elicited by the dopamine agonist

(Figure 3.4D). To determine whether the mEPSC frequency increase was due to a pre- or postsynaptic mechanism, we included the membrane impermeant PKA inhibitor peptide PKI_{6-22} in the recording pipette. Blocking the activity of PKA postsynaptically completely prevented the DHX-induced increase in mEPSC frequency (Figure 3.4C). These data indicate that activation of D1/D5 receptors induces a postsynaptically-driven increase in the frequency, but not amplitude, of mEPSCs.

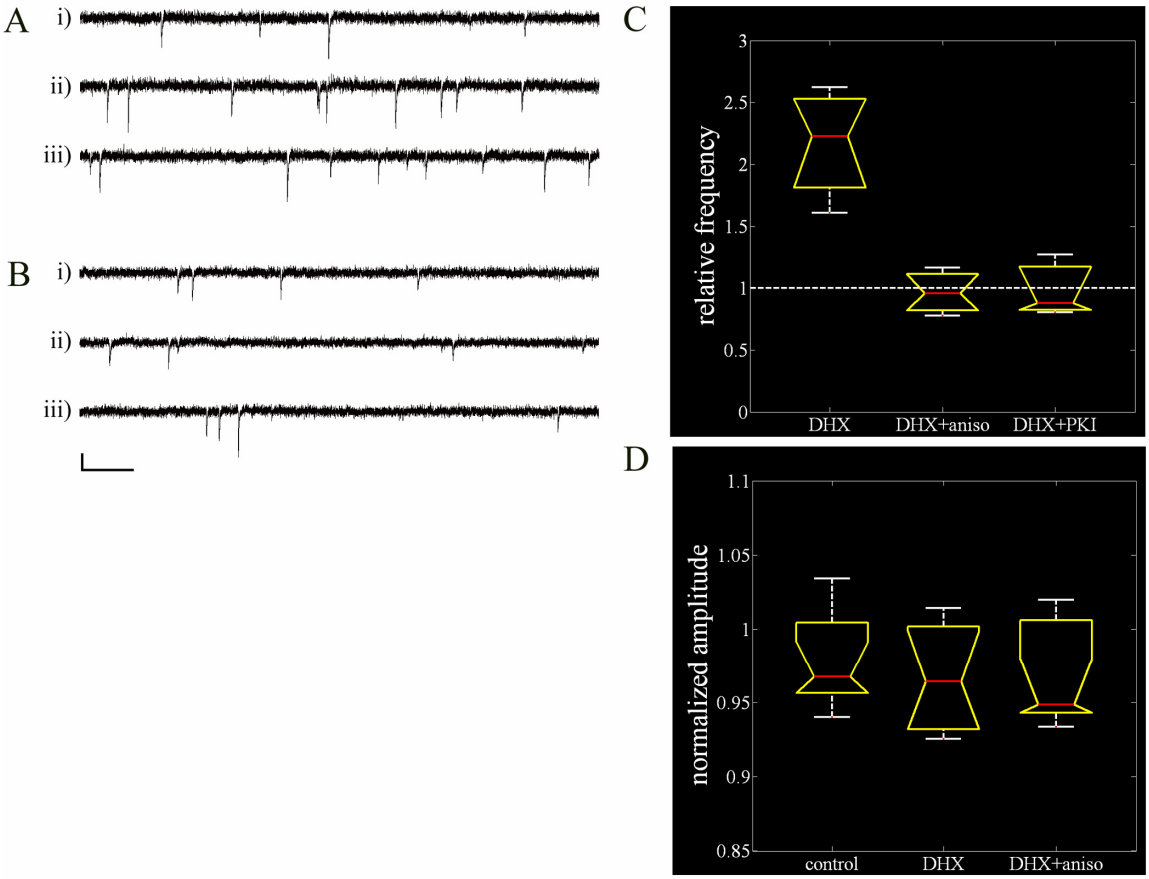

Figure 3.4 A Dopamine Agonist Increases the Frequency, but Not the Amplitude, of Miniature Excitatory Postsynaptic Currents in a Protein Synthesis-Dependent Manner

(A) Whole-cell voltage clamp recording from a cultured hippocampal neuron during a baseline period (i), and 10-12 minutes after bath application of dihydrexidine (ii and iii) shows a rapid increase in mini frequency after agonist application.

(B) Whole-cell voltage clamp recording from a neuron pretreated with anisomycin for 30 minutes shows no difference in mini frequency between the baseline recording period (i) and 10-12 minutes after bath application of dihydrexidine (ii and iii). Scale bar = 10 pA, 333 ms.

(C) Summary analysis illustrating a 2-fold increase in mini frequency in neurons treated with dihydrexidine (DHX), which is completely blocked by preincubation with anisomycin (DHX+aniso, n = 10 cells per condition, $p < 0.01$). Relative frequency is plotted as the mean frequency 10-12 minutes after agonist application, normalized to the mean frequency during the final 2 minutes of the baseline recording period for each experiment.

(D) Analysis of mini amplitudes shows no significant difference between groups. Normalized amplitudes are plotted as the mean amplitude 10-12 minutes after agonist application, normalized to the mean amplitude during the final 2 minutes of the baseline recording period for each experiment.

Using both a GFP-based reporter of local translation and a novel, small molecule reporter, we observed the stimulation of local protein synthesis in the dendrites of cultured hippocampal neurons by dopamine receptor agonists. We identify GluR1 as one synaptic protein whose synthesis is stimulated by dopamine receptor activation; dopamine agonists also induced an increase in surface GluR1, as has been observed in the nucleus accumbens (Chao et al., 2002; Mangiavacchi S, 2004). The agonist-stimulated increase in surface GluR1 required new protein synthesis and increased the fraction of synapses that possess a surface GluR1 cluster. The stimulated synthesis and surface expression of GluR1 was accompanied by a dopamine agonist-stimulated increase in the frequency, but not amplitude, of mEPSCs. Because these changes occur rapidly (10-15 minutes), our data are most consistent with the idea that GluR1 is locally synthesized. Indeed, two recent studies have demonstrated that glutamate receptors can be locally synthesized in dendrites (Ju et al., 2004; Kacharmina et al., 2000). Taken together, these data suggest that D1/D5 receptor activation stimulates a local protein synthesis-dependent increase in surface GluR1 at synaptic sites that did not previously possess functional postsynaptic GluRs, consistent with

the activation of postsynaptically silent synapses (Isaac et al., 1995; Liao et al., 1999; Liao et al., 1995; Petralia et al., 1999).

Our data provide a potential cellular mechanism for the dopaminergic modulation of long-lasting plasticity at hippocampal synapses. Others have reported that dopamine or activators of the cAMP/PKA pathway can induce a long-lasting protein synthesis-dependent form of potentiation in hippocampal slices (Frey et al., 1993; Huang and Kandel, 1995). It has also been shown that late-phase long-term potentiation (LTP) is diminished in hippocampal slices treated with dopamine receptor antagonists (Frey et al., 1991; Frey et al., 1990; Swanson-Park et al., 1999) or prepared from D1 receptor knock-outs (Matthies et al., 1997). In addition, a PKA-dependent increase in GluR1 synthesis has been observed during the late (3hr post-induction) phase of LTP (Nayak et al., 1998). The data presented here indicate that dopamine may exert its effects on plasticity, at least in part, by local regulation of protein synthesis.

3D particles analysis summary data										
	R1 num	R1 vol	R1 intensity	R1 total	Syn num	Syn vol	Syn intensity	Coloc num	Coloc /R1	Coloc /Syn
control	91.70	20.77	35.60	0.7e5	101.1	65.24	4.0e5	26.70	0.254	0.216
DHX	183.5	33.10	31.93	1.9e5	98.60	61.18	2.8e5	53.30	0.334	0.492
DHX+aniso	111.9	24.34	38.83	1.1e5	97.90	61.34	3.0e5	32.10	0.294	0.227
Statistical comparison between groups (ANOVA)										
ctrl/DHX	*	*		*				*		*
DHX/aniso	*	*		*				*		*

Table 3.1: 3D particle analysis values

Shown are summary data for the 3D particles analysis on surface GluR1 staining (R1) and synaptophysin (Syn) colocalization (Coloc). The variables are: number of particles (num), mean particle volume (vol), mean pixel intensity per particle (intensity), and integrated pixel intensity (total). An asterisk in the bottom portion of the table indicates statistical significance at $p < 0.05$. No significant differences were observed between the control and DHX+aniso groups.

Methods

Cultured hippocampal neurons

Dissociated hippocampal neurons were prepared and maintained as previously described (4). Briefly, hippocampi from postnatal day 2 Sprague-Dawley rat pups were enzymatically and mechanically dissociated and plated into poly-lysine coated glass-bottom petri dishes (Mattek). Neurons were maintained for 14-21 days at 37º C in growth medium (Neurobasal A supplemented with B27 and Glutamax-1, Invitrogen).

Microscopy and image analysis

All images were acquired with an Olympus IX-70 confocal laser scanning microscope running Fluoview software (Olympus America, Inc). GFP, Alexa 488, and F2P were excited with the 488 nm line of an argon ion laser, and emitted light was collected between 510 and 550 nm. Alexa 568 was excited with the 568 nm line of a krypton ion laser, and emitted light was collected above 600 nm. In experiments where two channels were acquired simultaneously, settings were chosen to ensure no signal bleed-through between channels. For between-dish comparisons on a given day, all images were acquired at the same settings, without knowledge of the experimental condition during image acquisition. All post-acquisition processing and analysis was carried out with ImageJ (NIH) and MATLAB (The MathWorks, Inc.). To facilitate the analysis of fluorescence signal as a function of distance from the soma, dendrites were linearized and extracted from the full-frame image using a modified version of the Straighten plugin for ImageJ.

Dendrites were analyzed for time lapse as follows: fluorescence was averaged across the width of linearized dendrites, generating a vector of mean pixel intensities equal to the length of the dendrite. $\Delta F/F$ (F_{tn}-F_{t0}/F_{t0}) was then computed at each pixel along the dendritic length. A value of one was added to every pixel in the linearized dendrite image, to a maximum of 255, which sets the minimum mean pixel intensity across the width of the dendrite equal to one. This prevents artificially large $\Delta F/F$ values that result from fractional mean pixel values due to zeros in the initial image. For time-lapse summary data, the sum of $\Delta F/F$ values in 75 μm bins was computed for each dendrite, and the mean ± standard error for all dendrites in a given experimental condition was plotted. 3-D colocalization and particle analysis was performed using custom-written functions in Matlab. Of particular concern in such measurements is the issue of selecting appropriate threshold values to isolate the punctate data of interest from background noise in the raw images. In order to avoid potential biases in selecting thresholds, we have relied on the *graythresh* command in Matlab. This function generates an optimal threshold based on Ostu's method, which sets a threshold that minimizes the intraclass variance of the black and white pixels. To further ensure that the experimental effects we observed were robust to threshold settings, the colocalization and particle analysis was performed with a series of 7 to 11 thresholds, using the output of graythresh as the median threshold value. All reported results were unaffected by such a range of threshold settings.

Box plots

Box plots were used because they capture a number of properties about the distribution of the data in a single graph. In each box plot, the red line indicates the median of the data, the diagonal lines leading away from the median (notches) are robust estimates of the uncertainty about the median, the horizontal white lines show the interquartile range, and the vertical whiskers represent the entire extent of the data. In general, a visual comparison of data shown in a box plot can be used to determine statistical significance by comparing the notches between groups; if the notches do not overlap, the two data sets are significantly different from one another.

GFP experiments

The GFP construct and Sindbis virus used were described previously (Aakalu et al., 2001). Neurons 14-21 days in vitro were infected for 15-20 minutes with the virus, and allowed to incubate for 12-14 hours at 37º C before imaging commenced. After the incubation period, cells were transferred from growth medium into a HEPES-based imaging medium (HBS), containing (in mM): 119 NaCl, 5 KCl, 2 $CaCl_2 \cdot 2H_20$, 2 $MgCl_2 \cdot 6H_20$, 30 glucose, 20 HEPES. Dishes were maintained at 37º C in HBS for a minimum of 1 hour prior to imaging. For between-dish experiments (Fig 1A), neurons were treated for 15 minutes with 100 μM SKF-38393 (Sigma), rinsed with HBS, incubated at 37º C for an additional 45 minutes, and then fixed in 4% formaldehyde / 4% sucrose for 20 minutes at 4º C. For time-lapse experiments (Fig 1B), 3 baseline images were acquired at 30-minute intervals before drug treatment in order to ensure relatively stable

fluorescence in un-manipulated neurons. At time t=0, neurons were treated for 15 minutes with HBS (control) or 100 μM SKF. The next image (t=30 minutes) was acquired 15 minutes after drug washout, and images were acquired every 30 minutes thereafter. Neurons were maintained at 37º C between imaging periods, with image acquisition periods lasting no longer than three minutes at room temperature. In an effort to control for possible non-specific mechanical effects of adding or removing solution from the dishes, the number of solution exchanges was held constant across all experimental conditions.

F2P experiments

Fluorescein-dC-puromycin was synthesized using standard phosphoramidite chemistry at the California Institute of Technology oligonucleotide synthesis facility and desalted via OPC cartridge chromatography (Glen Research) and on Sephadex G-10 (Sigma). For bath application experiments, F2P was added to HBS for a final working concentration of 40-60 μM. Cells were pre-loaded with F2P for 5 minutes and then incubated in SKF (100 μM) or HBS (control) for 15 minutes in the presence of F2P at 37° C. F2P was washed out with 4x 2 ml of HBS before neurons were fixed for fluorescence microscopy. Local perfusion experiments were done for 15 minutes without pre-loading the neurons with F2P. Flow rates were manually regulated using syringes attached to patch pipettes. The was no significant difference in perfusion spot size between groups (data not shown). In experiments using anisomycin to inhibit F2P incorporation, equimolar concentrations of anisomycin were added to the neurons 30 minutes before and for the entire duration of the experiment.

Immunocytochemistry and Western blot analysis

For surface GluR1/Synaptophysin colocalization experiments, cultured neurons were pre-treated for 20 minutes at 37 º C with DMSO or 40 μM anisomycin and then treated for 15 minutes at 37º C with DMSO (control), 10 μM dihydrexidine (DHX), or DHX+anisomycin. Immunolabeling of cultured hippocampal neurons to detect total as well as surface GluR1 was done as previously described (Patrick et al., 2003; Richmond et al., 1996). For surface labeling experiments, neurons were live-labeled for 10 minutes at 37º C with an antibody against the extracellular domain of GluR1 immediately following drug treatment, and then fixed and processed for immunocytochemistry using conventional techniques. For Western blot experiments, three dishes of high-density neurons were used for each experimental condition, and samples for each condition were loaded in triplicate on each gel. Anisomycin-treated dishes were pretreated for 30 minutes with 40 μM anisomycin; control and DHX-treated dishes were pretreated with DMSO. After the preincubation period, dishes were then treated with DMSO (control), 10 μM DHX, or DHX+40μM anisomycin for 20 minutes. The analysis represents the mean GluR1 band density normalized to the β-Tubulin band for all three lanes in a given experiment. A total of three experiments (9 lanes per condition) were analyzed. The following antibodies were used: rabbit anti-β-Tubulin (1:200, Santa Cruz Biotechnology), rabbit anti-GluR1 N-terminal domain (1:10, Oncogene Research Products), rabbit anti-GluR1 for Western blot (1:1,000, Upstate), mouse anti-synaptophysin (1:1000, Sigma), goat anti-mouse Alexa 568 (1:300), goat anti-rabbit Alexa 488 (1:300, Molecular Probes).

Whole-cell recordings

Whole-cell recordings in voltage clamp mode were performed on cultured neurons 14-21 days in vitro using a whole-cell solution containing (in mM): 100 gluconic acid, 0.2 EGTA, 5 $MgCl_2 \cdot 6H20$, 40 HEPES, 2 Mg ATP, 0.3 Li GTP, pH adjusted to 7.2 with CsOH. Cells were held at a membrane potential of -60 to -65 mV with holding currents that did not exceed -250 pA. Baseline recordings from cells were acquired for 5-8 minutes for each cell, and for at least 10 minutes after drug application. For anisomycin experiments, cells were pretreated with 40 μM aniso for 30-45 minutes, and anisomycin was added to the recording pipette. The PKI_{6-22} peptide was used at 20 μM in the patch pipette. Neither anisomycin, nor PKI_{6-22} significantly affected baseline frequency or amplitude (data not shown). Cells with a change in series resistance that exceeded 15% were excluded from analysis.