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Localized Synaptic Potentiation by BDNF Requires Local Protein Synthesis in the Developing Axon

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Summary

Brain-derived neurotrophic factor (BDNF) is known to promote neuronal survival, guide axonal pathfinding, and participate in activity-dependent synaptic plasticity. In *Xenopus* nerve-muscle cultures, localized contact of a single BDNF-coated bead with the presynaptic axon resulted in potentiation of transmitter secretion at the developing synapses, but only when the bead was placed within 60 μm from the synapse. The localized potentiation induced by BDNF is accompanied by a persistent local elevation of $[Ca^{2+}]_i$ in the axon and requires constitutive presynaptic protein translation, even for axons severed from the cell body. Thus, presynaptic local TrkB signaling and protein synthesis allow a localized source of BDNF to potentiate transmitter secretion from nearby synapses, a property suited for spatially restricted synaptic modification by neurotrophins.

Introduction

A unique feature of the nervous system is the complex morphological phenotype of neurons and the intricate network of specific nerve connections. Developing axons undergo long-range pathfinding to reach their appropriate target cells to form synaptic connections. After the initial synaptic connections are established, substantial remodeling of these connections may occur, depending on the pattern of synaptic activity. At the cell biological level, it remains largely unknown how the neuron carries out its task of transporting newly synthesized constituents to the tip of the growing axon or presynaptic nerve terminals that are distant from the cell body. It is also unclear how localized synaptic activity triggers a synapse-specific modification that requires supplies of newly synthesized components to be localized to selective synaptic sites. Local protein synthesis at the synaptic site may provide a potential mechanism for achieving synapse specificity in activity-dependent long-term synaptic modification (Schuman, 1997; Steward, 1997; Martin et al., 2000; Steward and Schuman, 2001). The existence of translational machinery, e.g., mRNAs and ribosomes, in dendrites has been reported (Steward and Levy, 1982; Steward and Reeves, 1988; Steward, 1997), and local protein translation has been demonstrated in dendrites of cultured hippocampal neurons (Aakalu et al., 2001). There is also evidence that local protein synthesis occurs in developing and mature axons (Davis et al., 1992; Olink-Coux and Hollenbeck, 1996; Bassell et al., 1998; Eng et al., 1999; Koening and Giuditta, 1999; Koening et al., 2000; Campbell and Holt, 2001; Zheng et al., 2001; Brittis et al., 2002). Local axonal protein synthesis appears to be required for effective chemotaxis of growth cone in a gradient of guidance molecules (Campbell and Holt, 2001; Ming et al., 2002). After synaptogenesis, local translation in the presynaptic axon may continue to be involved in activity-dependent synaptic plasticity. At *Aplysia* sensory to motor synapses in cell cultures, for example, presynaptic local protein synthesis is required for branch-specific long-term facilitation induced by serotonin (Martin et al., 1997). Even for mature crayfish neuromuscular junctions, long-term facilitation also requires protein synthesis in the presynaptic axon (Beaumont et al., 2001).

Brief periods of repetitive synaptic activity are known to induce long-term potentiation or depression of synaptic transmission (Malenka and Nicoll, 1999) as well as morphological changes of activated synapses and dendrites (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Yuste and Bonhoeffer, 2001). Neurotrophins have emerged as prime candidate molecules for linking electric activity to synaptic changes (McAllister et al., 1999; Poo, 2001). The synthesis of neurotrophins in many regions of the brain (Zafra et al., 1990; Patterson et al., 1992) and in skeletal muscles (Funakoshi et al., 1995) is regulated by electrical activity. Membrane depolarization and synaptic activation (Blöchl and Thoenen, 1995; Goodman et al., 1996; Wang and Poo, 1997; Kojima et al., 2001; Hartmann et al., 2001) can trigger secretion of neurotrophins, which may in turn modify synaptic structure or function. The latter possibility is supported by the findings that in some preparations, extracellularly applied neurotrophins can modify basal synaptic transmission (Lohof et al., 1993; Kang and Schuman, 1995), reduce the synaptic fatigue under high frequency stimulation (Figurov et al., 1996), and alter the growth and branching patterns of axonal and dendritic arbors (Diamond et al., 1992; Cohen-Cory and Fraser, 1995; McAllister et al., 1995, 1996).

An outstanding issue concerning the role of neurotrophins in synaptic plasticity is the synapse specificity of their effects. Is the action of locally secreted neurotrophins spatially restricted? Gene regulation and protein synthesis are likely to be required for long-term structural and functional modifications of synapses, but how does the action of neurotrophin remain synapse-specific if global protein synthesis machinery is involved? In hippocampal slices, BDNF and neurotrophin-3 (NT-3) were shown to potentiate synaptic transmission at Schaffer collateral-CA 1 synapses (Kang and Schuman, 1995). Interestingly, blocking protein synthesis in the slices in which the synaptic neuropil was isolated from both pre- and postsynaptic cell bodies attenuated the potentiation effects, suggesting that protein synthesis in either the dendritic or axonal compartment, or both, is required for the neurotrophin effect (Kang and Schuman, 1996). In the present study, we have measured quantitatively the spatial range of neurotrophin effect in synaptic potentiation and examined specifically whether presyn-

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aptic protein synthesis is involved in the potentiation effect at developing neuromuscular synapses in culture.

Results

Potentiation of Spontaneous Transmitter Secretion by BDNF Beads

In 1-day-old *Xenopus* nerve-muscle cocultures, functional synaptic transmission is established between spinal neurons and myocytes (Evers et al., 1989). Bath application of BDNF and NT-3 results in potentiation of spontaneous and evoked transmitter secretion from the presynaptic neuron (Lohof et al., 1993). We first examined whether local delivery of BDNF to a presynaptic site can modulate basal synaptic transmission in these cultures. Single beads covalently coated with BDNF (BDNF beads) were manipulated into contact with the presynaptic axon at sites 15–25 μm from the synapse (Figure 1A). The presence of BDNF on those beads was confirmed by immunostaining with specific polyclonal antibodies raised against BDNF (Figure 1B). Miniature excitatory postsynaptic currents (MEPCs) were recorded from innervated myocytes by a whole-cell recording method. Within 8–10 min following the contact of the BDNF bead, we observed a significant and persistent increase in the frequency of MEPCs (Figures 1D and 1E). Pretreatment of the culture with K252a (200 nM), an inhibitor of receptor tyrosine kinase, abolished the changes in MEPCs induced by the BDNF bead (Figures 1D and 1E), while K252b (200 nM), a compound which does not inhibit tyrosine kinase at the concentration used, had no effect ($n = 6$; data not shown). This is consistent with the previous result (Lohof et al., 1993) that the BDNF effect is mediated through TrkB receptors in the presynaptic axon. The specificity of the effect of BDNF beads was confirmed by the findings that preheating BDNF beads at 75°C for 45 min abolished the potentiation effect of the bead (Figure 1E) and that beads coated with bovine serum albumin (BSA) or nerve growth factor (NGF), which is known to have no effect on these developing synapses (Lohof et al., 1993), caused no significant increase in the frequency of MEPCs (Figure 1E). The change in the MEPC frequency appears to represent an enhanced probability of spontaneous quantal secretion of acetylcholine (ACh) rather than an elevated postsynaptic sensitivity to ACh. As shown in Figure 1F, the mean amplitude and the amplitude distribution of MEPCs did not show any significant change following the contact with the BDNF bead, as would be expected if postsynaptic ACh sensitivity were changed.

Potentiation of Evoked Synaptic Transmission by BDNF Beads

In addition to the potentiation of spontaneous ACh secretion, contact of single BDNF beads with the presynaptic axon also resulted in an increase in the mean amplitude of impulse-evoked postsynaptic currents (EPCs), which were recorded in innervated myocytes in response to stimulation of the presynaptic soma at a low frequency (0.03 Hz). The increase in the mean EPC amplitude correlated with a gradual increase in the MEPC frequency with time (Figure 2A). Within 20–40 min after the bead contact, the mean EPC amplitude was 221% $\pm$ 46% (SEM, $n = 8$ synapses) of the control value obtained before the bead contact. Furthermore, in the presence of K252a (200 nM), there was no significant change in the mean EPC amplitude after the bead contact (98% $\pm$ 10%, SEM, $n = 5$; see Figure 4B). The effect of bead contact was presynaptic specific: no change in the mean EPC amplitude was observed at 20–40 min after the contact of the BDNF bead with the postsynaptic muscle cell (88% $\pm$ 12%, SEM, $n = 3$). Together with the results on MEPCs, these findings indicate that localized bead-induced activation of TrkB in the presynaptic axon close to the synapse was sufficient to potentiate both spontaneous and evoked transmitter release at these developing synapses.

Spatial Range of BDNF-Induced Potentiation

Taking advantage of the precise localization of BDNF beads, we have examined quantitatively the spatial range of BDNF action by manipulating single BDNF beads into contact with presynaptic axons at various distances from the synapse. We observed a clear distance dependence in the action of BDNF beads; the potentiation of both the mean EPC amplitude and mean MEPC frequency was found only for bead contact sites within a distance of about 60 μm from the synapse (Figure 2). An example of a recording for a single bead contact at a remote location of 150 μm from the synapse is shown in Figure 2B. The restricted spatial range of BDNF action cannot be attributed simply to a limited source of BDNF on a single bead, since we observed no synaptic potentiation when the source of BDNF was increased at a site remote to the synapses by placing three or more BDNF beads into contact with the presynaptic axon (distance from synapses $\geq$ 90 μm) or by locally puffing BDNF (5 μg/ml) to the axon (distance from synapses $\geq$ 110 μm) (Figure 2D). The spatial restriction of BDNF action thus reflects not a limited extent of TrkB activation by a single bead, but a spatially restricted range of action of downstream signal cascades triggered by the activated TrkB. Finally, we found no apparent correlation between the degree of synaptic potentiation and the distance between the bead and the soma (Figure 2E), suggesting that localized BDNF action may not depend on signaling to or from the soma. Thus locally secreted BDNF at the synapse can produce highly localized modulatory effects on synaptic efficacy.

Localized Elevation of Ca^{2+}

Synaptic potentiation by BDNF is known to depend on extracellular Ca^{2+} (Stoop and Poo, 1995). Bath application of BDNF increased the cytoplasmic Ca^{2+} level ($[Ca^{2+}]_i$) persistently in the soma, axon, and synaptic terminals of cultured *Xenopus* spinal neurons (Stoop and Poo, 1996). Such elevation of $[Ca^{2+}]_i$ and subsequent activation of downstream signaling molecules may be responsible for the BDNF-induced synaptic potentiation. To examine the spatial relationship between $[Ca^{2+}]_i$ elevation and synaptic potentiation induced by BDNF beads, we have measured changes in $[Ca^{2+}]_i$ within the presynaptic axon of the spinal neuron. The neurons were microinjected with a fluorescent Ca^{2+}-sensitive dye, Oregon Green BAPTA-1 conjugated to dextran (70 kDa). As shown in Figure 3A, we observed

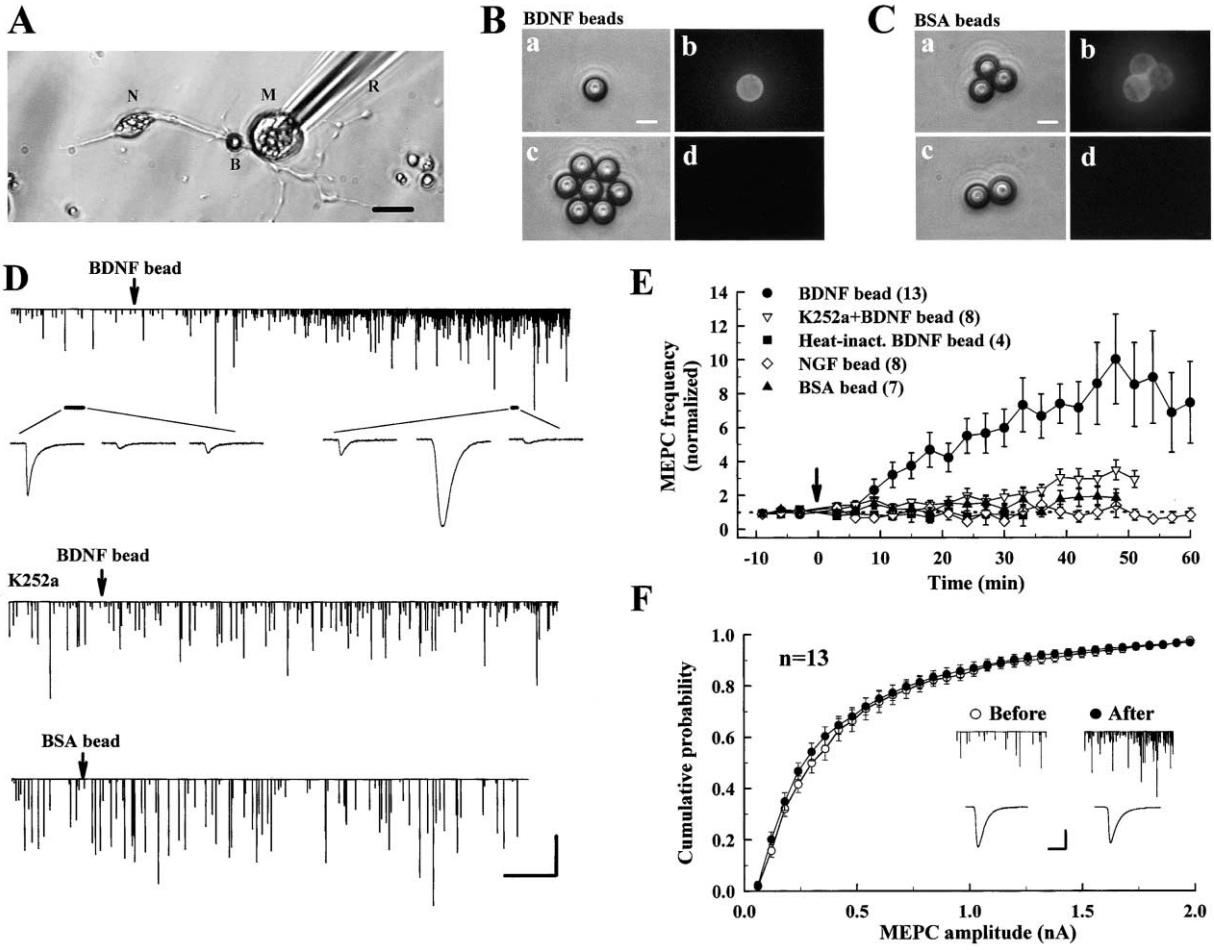

Figure 1. Potentiation of Spontaneuous ACh Secretion by BDNF-Coated Beads

(A) Phase-contrast image of a 1-day-old *Xenopus* culture in which a BDNF bead ("B") was manipulated into contact with the axon of a spinal neuron ("N") at a site ~22 μm from an innervated myocyte ("M"). R, recording pipette. Bar = 15 μm.

(B) Immunostaining of BDNF beads. (Ba and Bb) Phase-contrast and fluorescence images, respectively, of a BDNF bead immunostained with anti-BDNF polyclonal antibody and rhodamine-conjugated secondary antibody. (Bc and Bd) Same as (Ba) and (Bb), except that the primary antibodies were preincubated with the blocking peptides prior to application to BDNF beads.

(C) Immunostaining of BSA-coated beads. Similar to (B), except that anti-BSA monoclonal antibody was used as the primary antibody (Ca and Cb), and only secondary antibody was used in the control (Cc and Cd). Bar = 10 μm.

(D) Examples of recordings from innervated myocytes after contacts with a single BDNF bead or BSA bead. Continuous traces depict membrane currents of the myocyte (clamped at −70mV). Downward events are MEPCs (samples shown below at a higher time resolution). K252a: 30 min pretreatment and the drug was present throughout the recording (at 200 nM). Scales: 1.5 nA, 5 min for slow traces; 0.85 nA, 15 ms for fast traces.

(E) Changes in the MEPC frequency with time induced by the bead contact (at T = 0) at sites ≤25 μm from the synapse. Heat inactivation: 75°C for 45 min. The MEPC frequency over the 3 min bin was normalized by the mean value before the bead contact for each experiment before averaging (± SEM).

(F) Distribution of the MEPC amplitudes before and after a BDNF bead contact. Cumulative probabilities during a 5 min period before and during 35–40 min after the bead contact were analyzed. Each value represents the mean ± SEM for 13 experiments shown in (E). There was no statistically significant difference between the two data sets (Kolmogorov-Smirnov test, $p > 0.05$). Insets: sample traces of MEPCs 5 min before and 35–40 min after the BDNF bead contact. The averages of all MEPCs for the sample traces are shown below. Scales: 0.35 nA, 1 min for slow traces; 0.1 nA, 10 ms for fast traces.

a distinct and spatially restricted $[Ca^{2+}]_i$ increase ($\Delta F/Fo$ = 106%) in the presynaptic axon adjacent to the site of bead contact 30 min after the contact with a single BDNF bead at distal end of the axon, whereas only a slight change in $[Ca^{2+}]_i$ ($\Delta F/Fo$ = ~10%) was observed in the soma or other axonal regions. In another experiment (Figure 3B), the contact of a single BDNF bead at the proximal end of the axon elicited a $[Ca^{2+}]_i$ elevation at the site of contact as well as at the nearby soma, but not at the distal end of the axon. In the latter case, the rise in $[Ca^{2+}]_i$ at the soma appeared to be slightly slower than that at the bead contact site. On the average, significant $[Ca^{2+}]_i$ elevation ($\Delta F/Fo > 10\%$) was preferentially restricted within about 40 μm from the site of bead contact (Figure 3C).

The amplitude and spatial range of bead-induced changes in $[Ca^{2+}]_i$ along an axon varied among the seven presynaptic neurons examined. In 3/7 cases, axonal

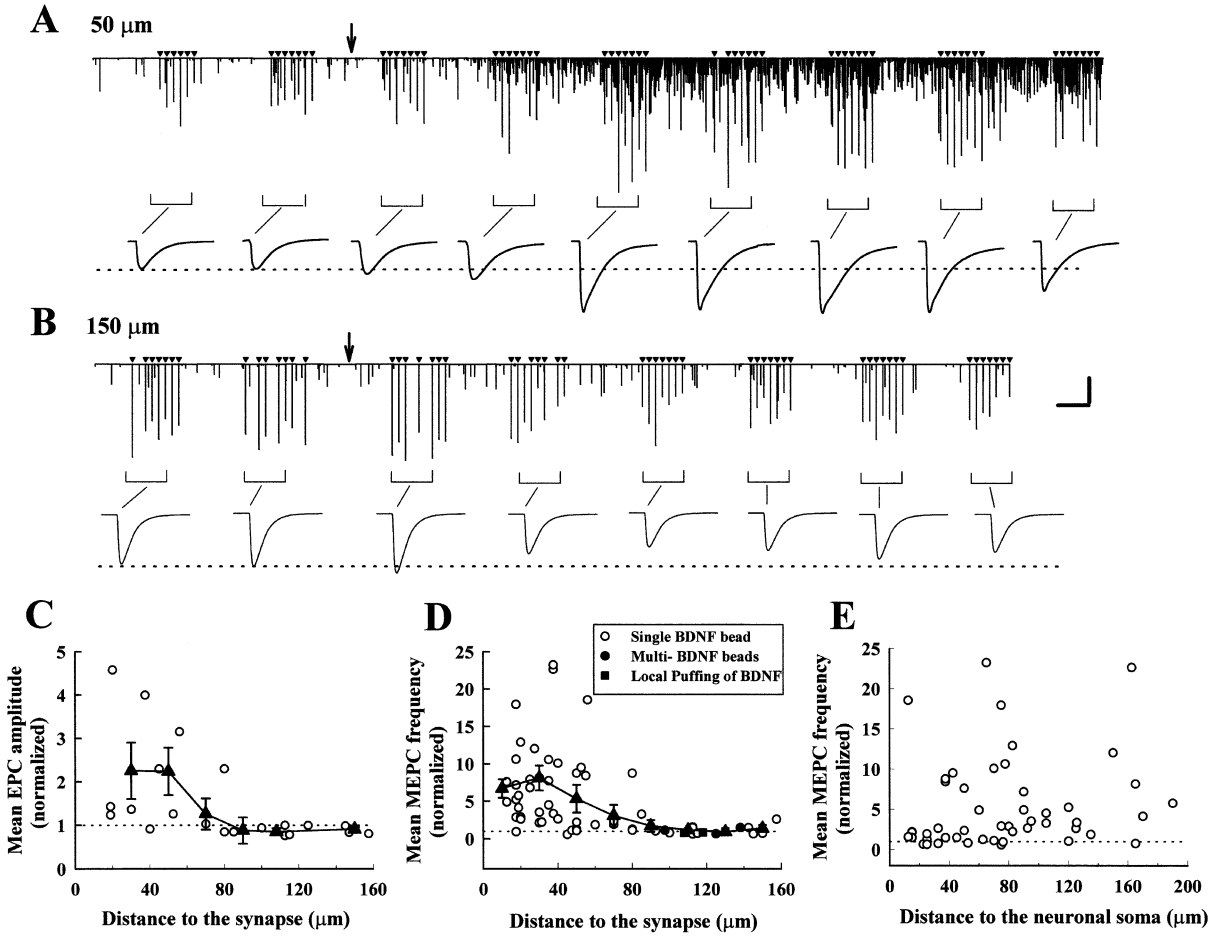

Figure 2. BDNF Bead Effects Depend on the Distance to the Synapse

(A and B) Examples of synaptic currents before and after the contact (at the time marked by the arrow) of a single BDNF bead at sites 50 (A) and 150 μm (B) from the synapse. Trains of test stimuli (7–8 pulses at 0.033 Hz) were applied at 5 min intervals to evoke EPCs at the times marked by triangles. Shown below are averaged traces of EPCs at a higher time resolution. Dotted lines represent the mean EPC amplitude before the bead contact. Scales: 2 nA, 2.5 min for slow traces; 2.5 nA, 15 ms for fast traces.

(C and D) Summary of all data for the dependence of synaptic potentiation on the distance from the BDNF bead contact site to the synapse. Each circle represents the mean EPC amplitude (C) and mean MEPC frequency (D) 20–40 min after the bead contact, normalized by the mean control values observed at the same synapse before the bead contact. Solid triangles: mean ± SEM over 20 μm bins. Multiple BDNF beads: 3 or 4 beads were manipulated into contact with axon ≥90 μm from the synapse. Local puffing of BDNF: repetitive pressure injections of BDNF from a micropipette at sites >100 μm from the synapse.

(E) Dependence of synaptic potentiation (as shown by the normalized MEPC frequency at 20–40 min) on the distance of the BDNF bead contact site from the soma (same data set as in [D]).

$[Ca^{2+}]_i$ elevation was found to be restricted to a range of less than 10 μm from the contact site. The rise in $[Ca^{2+}]_i$ was gradual over tens of minutes after the bead contact and persisted at the elevated level for as long as the recording was made (up to 1 hr in 4/7 cases). Consistent with its ineffectiveness in inducing the synaptic potentiation, contact of the BDNF bead with the muscle cell, either innervated or isolated, caused no change of $[Ca^{2+}]_i$ in the muscle cell ($\Delta F/Fo$ = 2.5% ± 2.3%, 30 min after bead contact, SEM, n = 4). In further control experiments, we found that contact with BSA beads did not change $[Ca^{2+}]_i$ in the presynaptic axon (Figure 3E). In addition, we found that local $[Ca^{2+}]_i$ elevation was induced at sites both close (<50 μm, n = 7) and distant (>90 μm, n = 3) from the synapse, suggesting that failure of synaptic potentiation by distant contact of the BDNF bead was not due to the absence of TrkB signaling.

To further determine whether soluble BDNF, when applied locally to the presynaptic axons, also induces a localized elevation of $[Ca^{2+}]_i$, we focally puffed soluble BDNF (1 μg/ml) to the presynaptic axons by repetitive pulsatile ejections of BDNF from a micropipette (2 Hz, 20 ms, 3 psi, and 1 μm tip opening) in the presence of a constant perfusion flow that washed the ejected BDNF away from the neuron. As shown in Figure 3C, localized increase of $[Ca^{2+}]_i$ was observed within 10 min after the onset of BDNF puffing, and the $[Ca^{2+}]_i$ gradually declined to the control level with a time course of about 20–25 min after the puffing was terminated. Similar local changes in $[Ca^{2+}]_i$ were observed in two other puffing experiments.

Effects of Protein Synthesis Inhibitor

Using local application of BDNF beads, which avoids direct postsynaptic actions of BDNF, we have examined

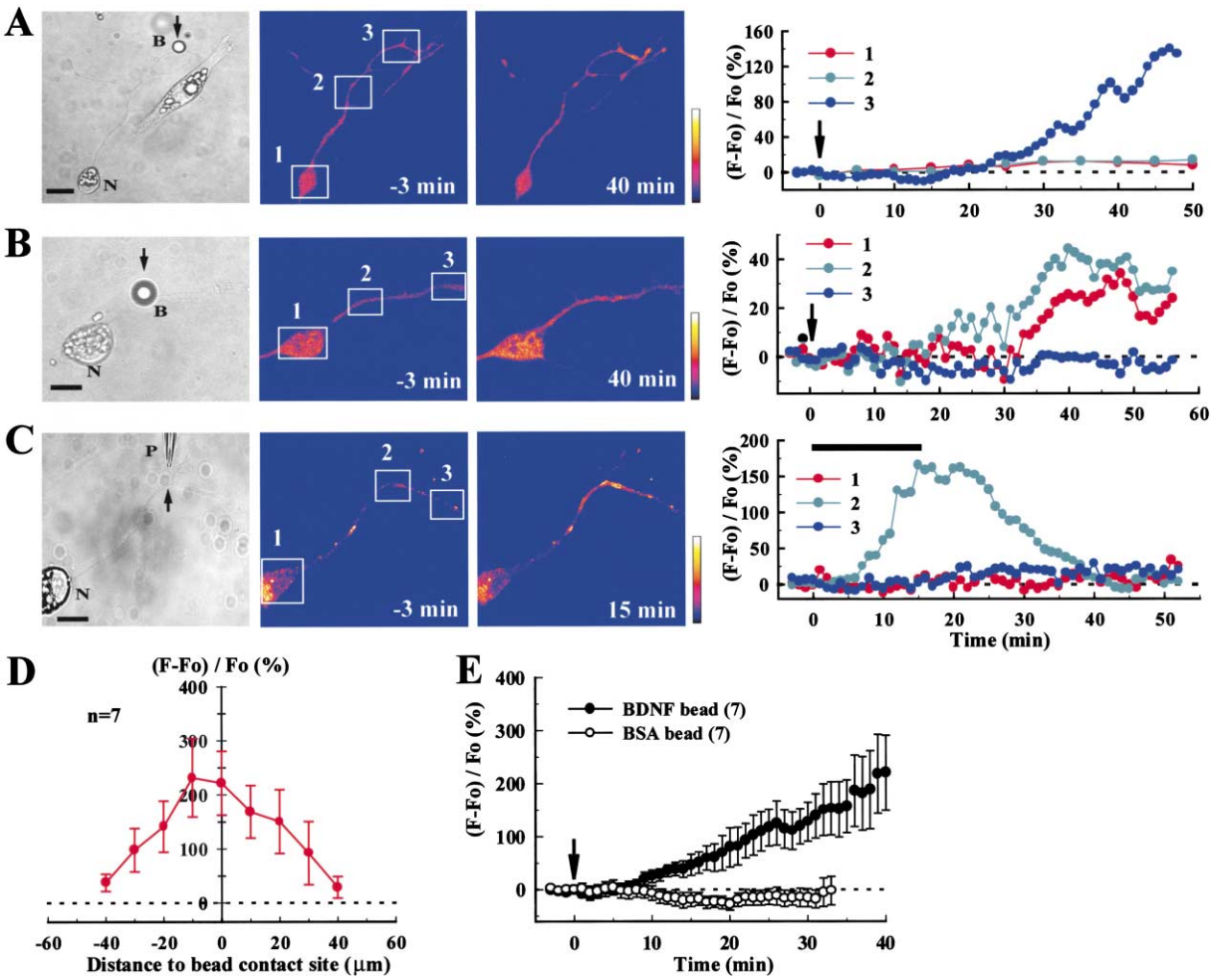

Figure 3. Localized Ca^{2+} Elevation Induced by the BDNF Bead Contact

(A–C) Bright field and Oregon Green-488 BAPTA fluorescence images of the spinal neuron ("N") 3 min before and 40 min after BDNF bead ("B", arrows in [A] and [B]) contact with the axon, or 15 min after focal puffing of soluble BDNF with a micropipette ("P" in [C]). The intensity of the fluorescence is coded by pseudocolors (brighter colors corresponding to higher values, see color bar). Scale: 20 μm (A), 10 μm (B and C). Graphs on the right show fluorescence intensity changes with time at selected regions in the neurons shown on the left (marked by numbered boxes). Black bar: duration of local puffing of BDNF.

(D) Spatial distribution of fluorescence changes along the axon induced by the BDNF bead contact (at the site defined as "0" μm). The values from each neuron were normalized to the mean value during the control period before the bead contact before grand averaging (mean ± SEM, n = 7 neurons).

(E) Changes in fluorescence intensity over time after the contact with BDNF beads or BSA beads (same data set as in [C]).

the role of protein synthesis in BDNF-induced synaptic potentiation. We found that a 45 min pretreatment of anisomycin (30 μM), a protein synthesis inhibitor, did not significantly affect synaptic potentiation induced by BDNF beads, which made contacts with the presynaptic axon at 20–40 μm from the synapse, as shown by an increased EPC amplitude and MEPC frequency (Figures 4A and 4B). However, after the 2 hr incubation with anisomycin, BDNF beads became ineffective in inducing synaptic potentiation at contact sites 20–40 μm from synapses (Figures 4A–4C). The inhibitory effect of 2 hr anisomycin incubation was not due to a deleterious effect of the drug on the transmitter secretion machinery, because after the same treatment of anisomycin, NT-3-coated beads were still effective in potentiating the synapse (Figures 4A–4C). Assuming that 45 min treatment of anisomycin had completely prevented new protein synthesis, the above results suggest that synaptic potentiation does not require BDNF-induced protein synthesis. On the other hand, continuous protein synthesis is required for synaptic potentiation, since prolonged (2 hr) absence of translation prevented synaptic potentiation. Taken together, our results indicate that the presynaptic action of localized BDNF depends on constitutive protein synthesis, whereas that of NT-3 does not.

We have also examined whether Ca^{2+} signaling induced by BDNF in these spinal neurons also requires protein synthesis. As shown in Figure 5A, we found that the $[Ca^{2+}]_i$ increase in the axon induced by bath application of BDNF (100 ng/ml) can be completely blocked by a 2 hr pretreatment of the culture with anisomycin (30 μM). Those treated axons, although unresponsive to bath-applied BDNF, still responded with $[Ca^{2+}]_i$ eleva-

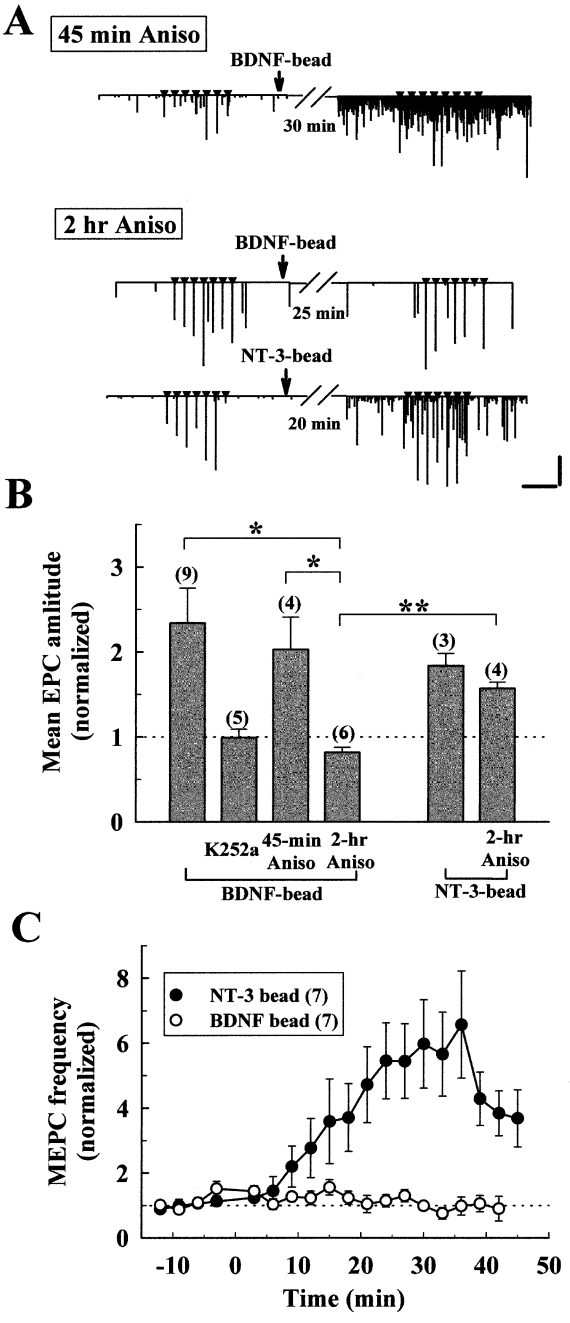

Figure 4. Synaptic Potentiation Induced by BDNF Beads Requires Protein Synthesis

(A) Experiments similar to that illustrated in Figure 2A, except that the culture was pretreated with anisomycin ("Aniso," 30 μM) for 45 min or 2 hr. Traces shown are recordings of synaptic currents made during a 10 min period before and at 20–30 min after the contact (at the time marked by the arrow) with a single BDNF bead or NT-3 bead. Scales: 1 nA, 1.5 min.

(B) Summary of data for the mean EPC amplitude (± SEM) at 20–40 min after the bead contact under various treatments, normalized by the mean control values from the same synapse before the bead contact. $0.01 < *p < 0.05$; $**p < 0.01$ (Student's t test). K252a, 200 nM.

(C) Changes in the MEPC frequency with time induced by contact of BDNF beads or NT-3 beads in cultures pretreated with anisomycin for 2 hr. Each data point represents the mean ± SEM.

tion when a high K^+ (60 mM) solution was subsequently applied. Pretreatment of the culture with anisomycin for 45 min partially reduced the BDNF-induced $[Ca^{2+}]_i$ elevation in the axon (Figures 5B and 5C). Thus, Ca^{2+} signaling mediated by BDNF/TrkB also requires continuous protein synthesis in these neurons.

Pre- versus Postsynaptic Protein Synthesis

To further determine whether pre- or postsynaptic protein synthesis is required for the synaptic potentiation induced by the BDNF bead, we selectively injected gelonin—a membrane-impermeant ribosome-inactivating protein (Stirpe et al., 1980)—into the pre- or postsynaptic cell, together with fluorescently labeled dextran as a marker. When BDNF beads were manipulated into contact with the presynaptic axon near the synapse 2 hr after presynaptic injection of gelonin and dextran, we observed no potentiation of spontaneous ACh release (Figures 6Aa and 6B). In contrast, postsynaptic injection of gelonin and dextran did not affect the potentiation of spontaneous ACh release induced by the BDNF bead (Figures 6Ab and 6B). Injection of fluorescent dextran alone into either the pre- or postsynaptic cell also had no effect on the BDNF-induced potentiation (Figure 6B). Thus, presynaptic protein synthesis appears be required for BDNF-induced synaptic potentiation.

Protein Synthesis Is Required for Synaptic Potentiation in Transected Axons

Previous studies on these *Xenopus* cultures have shown that synaptic potentiation by bath-applied BDNF does not require signaling to and from the presynaptic soma, whereas that induced by ciliary neurotrophic factor does (Stoop and Poo, 1995). This is consistent with our present finding that there is no clear correlation between the degree of synaptic potentiation and the distance of the bead from the soma (Figure 2E). These findings suggest that local signaling and protein synthesis in the axon may be sufficient for the BDNF action. To directly test the role of local protein synthesis, we examined the effect of BDNF beads on synaptic efficacy after transection of the axon near the soma with a sharp micropipette (Figure 7A). Immediately after the transection, spontaneous ACh secretion exhibited a transient increase, presumably due to a surge of Ca^{2+} influx at the injured site, and then gradually subsided to a normal level (Figure 7A). In these experiments, only synapses with long axons (≥100 μm) were used, and the "cut loose" axons were allowed to recover for 2 hr before a BDNF bead was manipulated into contact with the axon near the synapse. We found that the frequency of spontaneous ACh release gradually decreased within the 2 hr recovery period after transection. However, for bead contacts at 20–40 μm from the cut-loose synapse, we observed a significant increase in MEPC frequency similar to that found in intact neurons. Moreover, when transected axons were treated for 2 hr after axon transection with either anisomycin (30 μM) or cycloheximide (50 μg/ml), two structurally different protein synthesis inhibitors, we found no potentiation effect of the BDNF bead on the cut-loose synapse (Figure 7B). In contrast, the same treatment with the transcription inhibitor actinomycin D (10 μg/ml) did not affect the potentiation induced by the

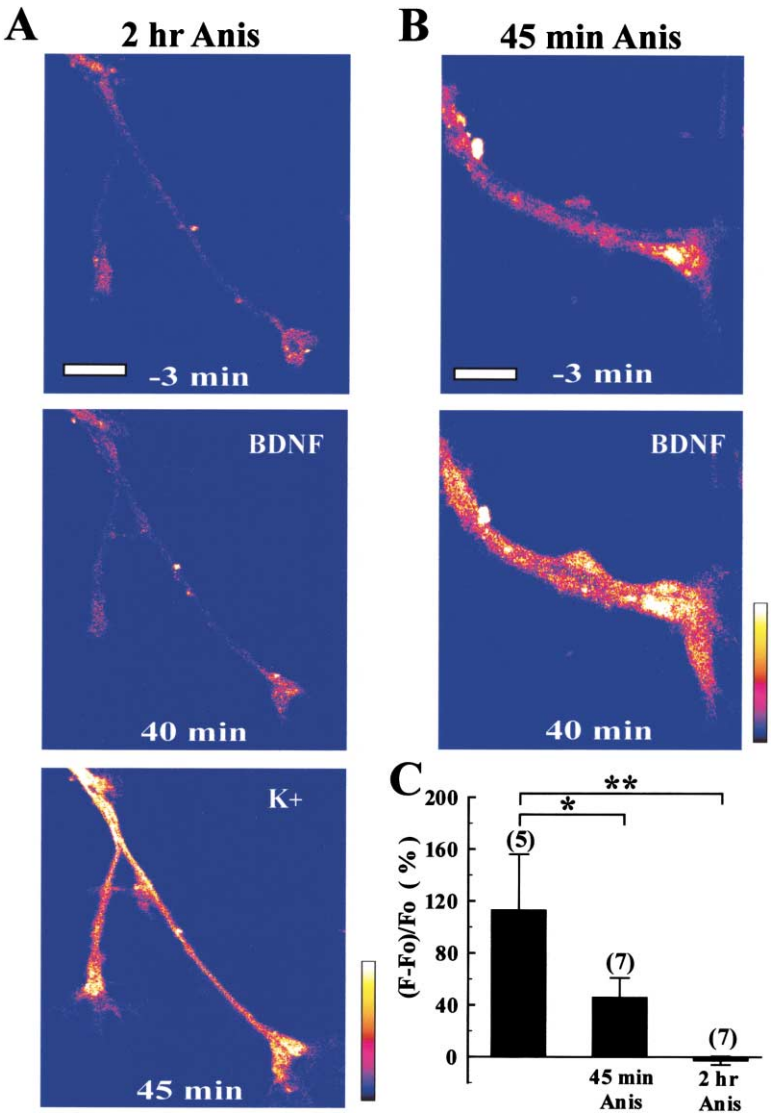

Figure 5. Inhibitory Effects of Anisomycin on BDNF-Induced Ca^{2+} Elevation

(A and B) Fluorescence images of the axon of spinal neurons 3 min before and 40 min after bath application of BDNF (100 ng/ml) in the cultures pretreated with anisomycin (30 μM) for 2 hr (A) or 45 min (B). In (A), 60 mM K^+ was added 45 min after BDNF application. Scale bars: 20 μm (A), 10 μm (B).

(C) Summary of changes in $[Ca^{2+}]_i$ induced by bath application of BDNF in normal cultures and in cultures pretreated with anisomycin for 45 min or 2 hr. $0.01 < *p < 0.05$; $**p < 0.01$.

BDNF bead (n = 4; data not shown). Taken together, these results strongly support the notion that presynaptic protein synthesis, but not RNA transcription, within the developing axon is required for synaptic potentiation induced by localized exposure to BDNF.

The Presence of Translation Machinery in the Axon

To investigate the distribution of RNAs and ribosomes in these neurons, we used a membrane-permeable dye, SYTO 64, that fluoresces on binding to nucleic acids and an anti-ribosomal P antigen antibody that recognizes three protein components (P0, P1, and P2) of the 60S subunit of the ribosome. As shown in Figure 7C, the axon of the spinal neuron showed SYTO 64 staining. That this SYTO 64 staining of the axon represents RNA rather than DNA staining was further confirmed by the staining of the nucleus by Hoechst 33342, a membrane-permeable dye that specifically binds to DNA (Figure 7C). The axons also showed strong positive staining with the anti-ribosomal P antigen antibody (Figure 7D). Labeling of F-actin with Alex568-phallotoxin helped to visualize the fixed cells in the latter experiments. Taken together, these staining results confirmed the presence of key components of the translation machinery in the developing axon of these cultured spinal neurons.

Discussion

In this study, we have examined the role of axonal protein synthesis in BDNF-induced synaptic potentiation of *Xenopus* neuromuscular synapses in culture, using beads covalently coated with BDNF. We find that (1) synaptic potentiation induced by local application of BDNF to the presynaptic axon is spatially restricted to nearby synapses located within about 60 μm from the site of contact; (2) Ca^{2+} signals elicited by local BDNF application also spread over a similar spatial range along the axon; and (3) this localized potentiation effect of BDNF requires continuous protein synthesis within the presynaptic axon. Together, our data suggest that the spatially restricted action of BDNF is achieved by local

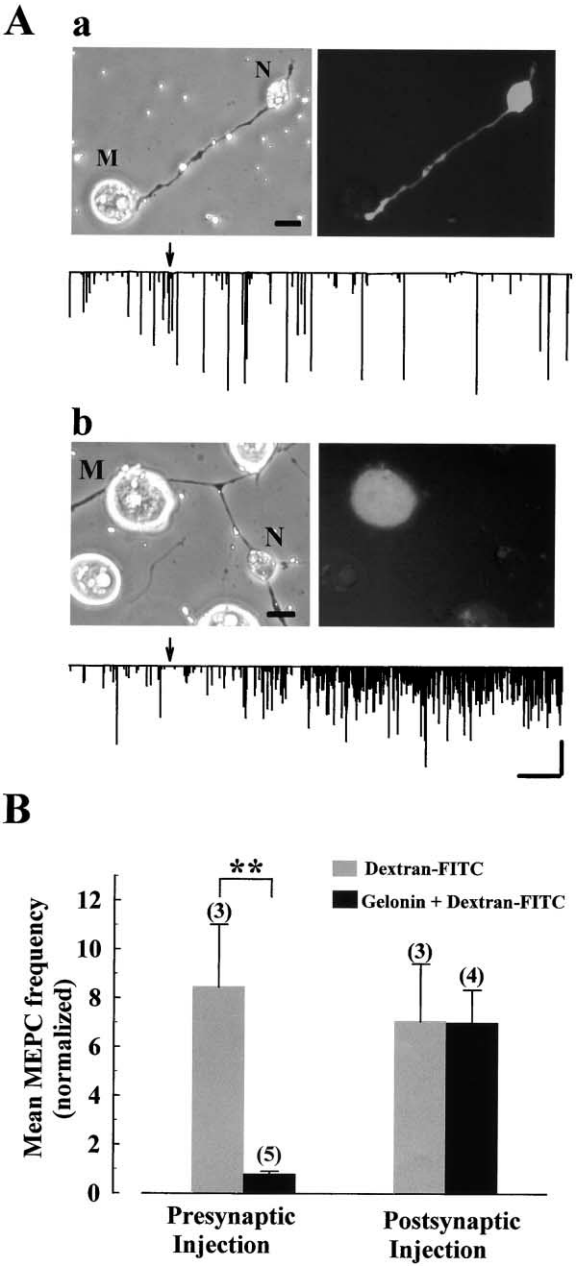

Figure 6. Synaptic Potentiation by BDNF Beads Requires Presynaptic Protein Synthesis

(A) Effects of microinjection of gelonin and FITC-dextan into the presynaptic neuron (Aa) or the postsynaptic myocyte (Ab) on BDNF bead-induced synaptic potentiation. Phase-contrast and FITC fluorescence images (bar = 10 μm) are shown together with example recordings of MEPCs before and after the bead contact (marked by the arrow, scales: 1 nA, 5 min).

(B) Summary of data for the mean MEPC frequency (± SEM) at 30–40 min after the BDNF bead contact, normalized by the control values prior to the contact. **$p < 0.01$.

TrkB signaling and protein synthesis within the axonal cytoplasm.

It has been proposed that during activity-dependent synapse refinement, nerve terminals compete for limiting amounts of neurotrophic factors secreted by the target cell (Purves and Lichtman, 1985). There is evidence that neurotrophins are required for the formation of ocular dominance columns and act as activity-dependent signals to modulate the pattern of synaptic connectivity (Maffei et al., 1992; Cabelli et al., 1995, 1997). Application of exogenous neurotrophins can also induce changes in the morphology of axons and dendrites (Diamond et al., 1992; Cohen-Cory and Fraser, 1995; McAllister et al., 1995, 1996) and promote transmitter secretion from the presynaptic nerve terminals (Lohof et al., 1993; Kang and Schuman, 1995). To serve for their roles in the formation and refinement of synaptic connections, the action of neurotrophins must be under precise spatial and temporal regulation. The present results demonstrate directly that the synaptic action of BDNF can be localized. There appears to be an upper limit of about 60 μm for the spread of BDNF/TrkB-activated cytoplasmic effectors within the axon. These results are also consistent with an earlier finding that showed a similar range of restricted spread of synaptic potentiation caused by NT-4 secreted from the postsynaptic myocyte (Wang et al., 1998). Thus, the restricted range of the synaptic action allows neurotrophins to serve as localized neuromodulators, specific to synapses adjacent to the secretion site. In the CA1 region of the hippocampus, activity-induced long-term potentiation (LTP) requires BDNF (Korte et al., 1995; see review by Poo, 2001). Interestingly, when LTP is locally induced at Schaffer collateral-pyramidal cell synapses, there is a spatially restricted spread of potentiation to nearby synapses within a range of about 70 μm (Engert and Bonhoeffer, 1997). Localized action of activity-induced BDNF secretion may account for the spatially restricted spread of LTP at central synapses.

Given the limited extracellular diffusion of secreted neurotrophins (Blöchl and Thoenen, 1995; Wang et al., 1998), spatial specificity in BDNF-induced synaptic potentiation may be achieved by differential localization of TrkB receptors or spatially restricted propagation of intracellular signaling molecules triggered by the activated TrkB. The TrkB receptors are found at distinct neuronal compartments in various brain regions (Cabelli et al., 1996; Tongiorgi et al., 1997; Drake et al., 1999; Aoki et al., 2000). The pattern of expression and subcellular localization of TrkB in visual cortical neurons undergo developmental changes: an early appearance of TrkB on axon fibers and later expression on cell bodies and dendrites (Cabelli et al., 1996). Following the transfection of neurons in hippocampal cultures or intact cortical slices with epitope-tagged *trkB* and *trkC* cDNAs, both receptors exhibited homogeneous distribution along the axon (Kryl et al., 1999). The distribution of endogenous TrkB (S. Andersen, personal communication) and TrkC (Chang and Popov, 1999) in developing *Xenopus* axons also appeared to be relatively uniform. This is consistent with our finding that BDNF beads can induce local Ca^{2+} elevation at regions distant from the synaptic sites. Thus, localized synaptic actions of BDNF observed in the present study were unlikely to result from a differential distribution of TrkB receptors near the synapse.

To further examine the spatial range of intracellular signals triggered by TrkB activation, we had focused our attention on Ca^{2+}, which is known to mediate cytoplasmic signaling cascades initiated by BDNF. Synaptic

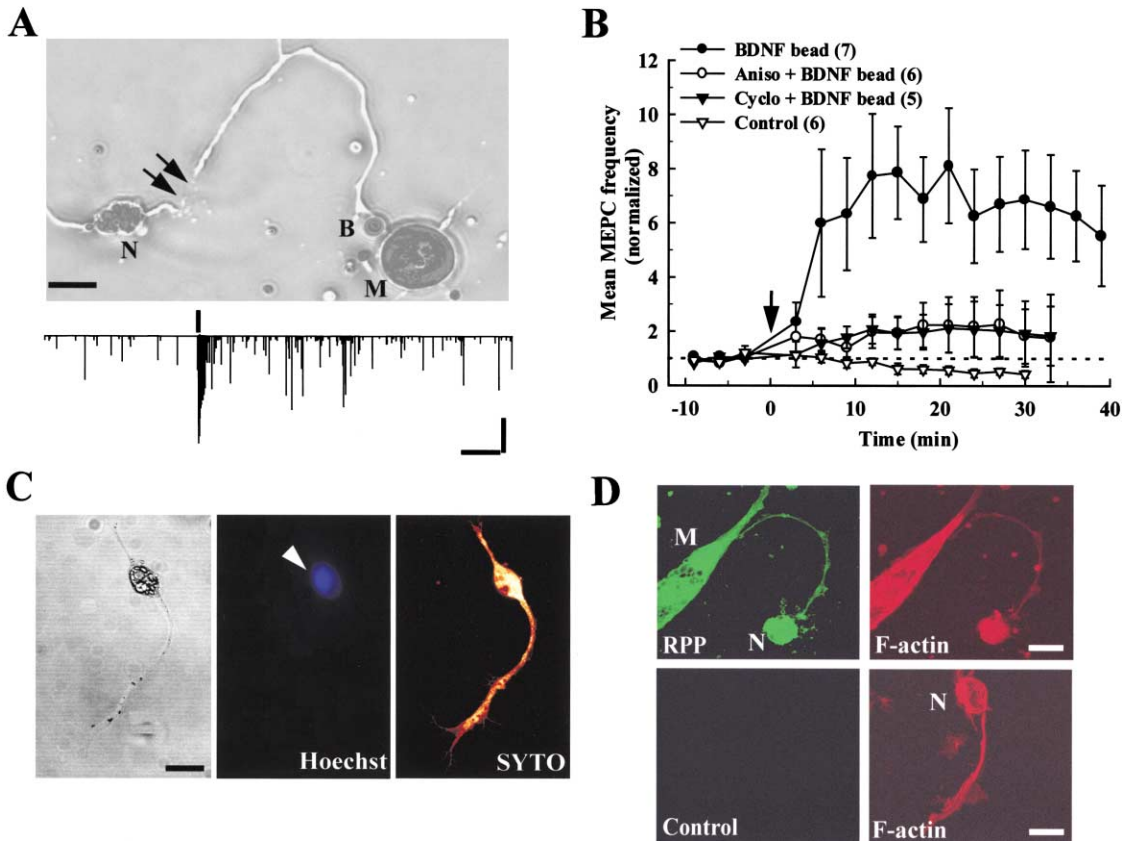

Figure 7. Synaptic Potentiation by BDNF Beads Requires Local Protein Synthesis.

(A) A phase-contrast image of a cut-loose synapse after the axon was transected (at marked arrows) from the soma of a spinal neuron ("N"). A BDNF bead ("B") was manipulated into contact with the axon at a site close to the innervated myocyte ("M"). Scale bar: 20 μm. An example recording of MEPCs from a synapse in which the presynaptic axon was transected at time marked by the vertical bar. Scale: 1 nA, 4 min.

(B) Summary of data for changes in the MEPC frequency with time after contact with the BDNF bead (T = 0) in the absence and the presence of protein synthesis inhibitors anisomycin (30 μM) or cycloheximide (50 μg/ml), which was applied for 2 hr after the transection. Control: no BDNF bead contact and no inhibitor treatment.

(C) Nucleic acid labeling of a spinal neuron with Hoecchast 33342 and SYTO 64 dyes. White arrowhead: Hoechst staining of the nucleus. Scale bar: 15 μm.

(D) Immunostaining of cultured spinal neurons ("N") and muscle cells ("M") with an anti-ribosomal P (RPP) antigen antibody. The F-actin labeling by Alex-568-phallotoxin helped to visualize the fixed cells. Control: no primary antibody was applied. Scale bar: 15 (upper) or 10 μm (lower).

potentiation induced by bath-applied BDNF is accompanied by a gradual increase of $[Ca^{2+}]_i$ in the soma, along the axon, and in the axonal terminals of cultured *Xenopus* spinal neurons (Stoop and Poo, 1996). A similar $[Ca^{2+}]_i$ increase in the soma has also been observed in cultured hippocampal neurons (Berninger et al., 1993; Li et al., 1998). In the present study, we found that the spread of axonal Ca^{2+} signals elicited by the contact of the BDNF bead is restricted to a similar range as that found for bead-induced synaptic potentiation along the axon (Figure 3D), suggesting that Ca^{2+} elevation in axon terminals may be responsible for triggering synaptic potentiation. As shown in Figure 3C, spatially restricted $[Ca^{2+}]_i$ elevation can also be elicited by focal application of soluble BDNF. Thus the spatial restriction of $[Ca^{2+}]_i$ elevation induced by BDNF beads was not due to the fact that BDNF covalently bound to the bead cannot be internalized. It reflects a spatially confined cytoplasmic signaling triggered by localized TrkB activation. The nature of this local signal remains to be determined.

It has been shown that the release of Ca^{2+} from internal endoplasmic reticulum via inositol 1,4,5-trisphosphate ($InsP_3$) receptors and store-operated capacitative Ca^{2+} entry are critical for BDNF-induced $[Ca^{2+}]_i$ elevation in cultured neurons (Li et al., 1998; Kleiman et al., 2000). Blocking either of these two Ca^{2+} sources abolishes BDNF-induced synaptic potentiation. The BDNF-elevated Ca^{2+} can promote spontaneous transmitter release directly by elevating the basal rate of synaptic vesicle exocytosis (Miledi, 1973) or indirectly by modulating the efficacy of vesicular fusion machinery via activation of the downstream effector(s) of Ca^{2+}. Indeed, synaptic vesicle-associated proteins—synapsin, synaptophysin, and synaptobrevin—have been implicated as downstream targets of the BDNF/TrkB signaling pathway (Pozzo-Miller et al., 1999). Application of BDNF to cortical synaptosomes also elicits a mitogen-activated protein (MAP) kinase-dependent phosphorylation of synapsin-I, leading to an increase in the availability of synaptic vesicles for release (Jovanovic et al., 2000).

Previous studies have shown that the neurotrophin-Trk complex can be rapidly internalized and transported retrogradely or anterogradely over long distances within the neuron and across the synapse, providing a mechanism for long-range signaling in neural circuits (von Bartheld et al., 1996a, 1996b, 2001; Bhattacharyya et al., 1997; Grimes et al., 1997). This long-range signaling is critical for promoting neuronal survival and maintaining global neuronal properties of the presynaptic neuron (Lewin and Barde, 1996; Huang and Reichardt, 2001; Sofroniew et al., 2001). Using beads covalently coated with NGF, MacInnis and Campenot (2002) found that the survival signal initiated by NGF binding to TrkA at the axon terminals was transported retrogradely to the soma without the internalization of NGF, suggesting that long-range TrkA signaling is mediated by internalized TrkA itself or downstream effectors, such as activated phosphatidylinositol 3-kinase (PI-3 kinase). In contrast, we have shown that BDNF, in the absence of internalization, can exert a localized action on synaptic transmission, presumably by a spatially restricted spread of downstream effector(s) of TrkB. Moreover, BDNF-induced synaptic potentiation does not require signaling to or from the neuronal soma (Stoop and Poo, 1995). In agreement with the latter finding, we found that the contact of single BDNF beads remained effective in inducing the synaptic potentiation even after the axon was severed from the soma (Figure 6). These findings underscore the notion of two distinct modes of neurotrophin action: a local action resulting from spatially restricted spread of cytoplamic downstream effectors triggered by Trk activation, and a global action that involves long-range trafficking of internalized neurotrophin-Trk complexes, Trk alone, or downstream effectors of Trk signaling pathway.

Although BDNF and NT-3 were found to exert similar potentiating effects at several synapses (Lohof et al., 1993; Kang and Schuman, 1995), signal transduction mechanisms mediated by their receptors, TrkB and TrkC, respectively, differ significantly (Segal and Greenberg, 1996; Patapoutian and Reichardt, 2001). For example, BDNF/TrkB-induced synaptic potentiation depends on extracellular Ca^{2+} and is regulated by the level of cytosolic cAMP, whereas synaptic potentiation by NT-3/TrkC depends on Ca^{2+} release from internal stores and is independent of Ca^{2+} influx and the cytosolic cAMP level (Boulanger and Poo, 1999, He et al., 2000; Yang et al., 2001). A similar difference in signal transduction was also found for the growth cone turning induced by gradients of BDNF and NT-3 (Song et al., 1998; Song and Poo, 1999). Furthermore, the NT-3-induced synaptic potentiation effect is independent of MAP kinase activation, but requires activation of both PI-3 kinase and phospholipase C-γ (Yang et al., 2001), whereas activation of MAP kinase is required for synaptic modulation by BDNF (Gottschalk et al., 1999; Jovanovic et al., 2000; Ying et al., 2002). Interestingly, unlike the localized synaptic effect of BDNF reported here, synaptic potentiation of NT-3 was found to spread over long distances (300–400 μm) along the presynaptic axon from the site of local perfusion of NT-3 (Chang and Popov, 1999). Taken together, the differences in signal transduction between BDNF and NT-3 are likely to account for their differential dependence of synaptic potentiation on axonal protein synthesis observed in the present study and for the different spatial ranges of synaptic action of these two neurotrophins.

Our results on the effects of translation inhibition on BDNF bead actions clearly suggest that local constitutive protein synthesis within the developing axon is required for the synaptic potentiation induced by BDNF beads (Figures 6 and 7). A previous study has shown that treatment of hippocampus slices with protein translation inhibitors markedly attenuated the BDNF-induced potentiation on synaptic transmission at the Schaffer collateral synapses on CA1 pyramidal cells, even for slices in which the synaptic neuropil was isolated from both pre- and postsynaptic cell bodies, suggesting that this synaptic action of BDNF requires local protein synthesis in either axonal or dendritic compartments, or both (Kang and Schuman, 1996). Recent evidence has further indicated that BDNF can initiate local protein synthesis in the dendrites of cultured hippocampal neurons (Aakalu et al., 2001). In the present study, we found that BDNF beads were effective in potentiating the synapse in the presence of translation inhibitor anisomycin, unless anisomycin treatment starts 2 hr before the bead contact. Assuming 45 min incubation with anisomycin is effective in blocking protein synthesis, BDNF-initiated protein synthesis is not required for synaptic potentiation. Instead, continuous synthesis of a critical presynaptic component seems to be required for the potentiation effect. Since prolonged inhibition of protein synthesis also abolished BDNF-induced Ca^{2+} signaling, this critical component appears to act at a step upstream from Ca^{2+} signaling. It has been shown recently that the protein synthesis inhibitor anisomycin can reversibly block synapse formation and functional maturation of *Alysia* sensorimotor synapses in the absence of cell bodies (Schacher and Wu, 2002), suggesting that synaptic proteins may be rapidly synthesized and degraded at or near synaptic sites. Our present finding on the effect of 2 hr treatment of anisomycin is consistent with the requirement of constitutive protein synthesis in BDNF-induced synaptic potentiation. Constitutive local protein synthesis in the developing axon was also found to be critical for the adaptation of growth cone turning responses (Ming et al., 2002), a process essential for long-range growth cone chemotaxis in a gradient of diffusible guidance cues. Local protein synthesis thus plays a role not only in axonal guidance, but also in neurotrophin-dependent modulation of developing synapses.

Experimental Procedures

Cell Culture Preparation

Xenopus nerve-muscle cultures were prepared as described previously (Tabti et al., 1998). Briefly, the neural tube and associated myotomal tissue of 1-day-old *Xenopus* embryos (stage 20–22) were dissociated in Ca^{2+}/Mg^{2+}-free saline containing 115 mM NaCl, 2.6 mM KCl, 0.4 mM EDTA, and 10 mM HEPES (pH 7.6) for 20–25 min. The cells were plated on clean glass coverslips and were cultured for 22–24 hr at room temperature (20°C–22°C) before use. The culture medium consisted of (v/v) 50% Leibovitz's medium (L-15, GIBCO Life Technologies), 1% fetal bovine serum (Hyclone, Longan, UT), and 49% Ringer's solution containing 115 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, and 10 mM HEPES (pH 7.6).

Preparation of Coated Beads

The carboxyl-EDAC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride) method was used to covalently couple the protein—recombinant human BDNF (MW 22 kDa, Promega, Madison, WI), purified murine NGF (MW 26 kDa, Promega), or recombinant human NT-3 (MW 27.2 kDa, PeproTech, Rocky Hill, NJ) or BSA (Sigma)—to polystyrene carboxylated beads (9.8 $\pm$ 0.8 μm in diameter, Ploysciences, Marrington, PA). Carboxylated beads in 0.1 ml solution (2.5% w/v, $\sim$4.55 $\times$ 10^6 beads) were rinsed three times with 0.1 M carbonate buffer (pH 9.6) and 0.02 M phosphate buffer (pH 4.5) sequentially. The beads were then treated with EDAC (1% w/v) freshly prepared in the phosphate buffer for 3 hr using an end-to-end rotary shaker. After being washed three times with 0.2 M borate buffer (pH 8.5), the beads were resuspended in the borate buffer containing the protein to be coupled (at the final concentration of 16.7 μg/ml) and allowed to mix on a rotary shaker overnight ($\sim$12–14 hr) at 4°C. The beads were pelleted, while the supernatant was kept for determining the amount of proteins that had not bound to the beads, by spectrophotometric measurement (Bio-Rad, Hercules, CA). Methanolamine (0.1 M) and BSA (1% w/v) were used sequentially to treat the beads for 30 min, serving to block unreacted sites. Finally, the beads coated with the protein were stored at 4°C in 0.02 M phosphate buffer containing (in w/v) 0.88% NaCl, 1% BSA, 0.1% Na_2N_3, and 5% glycerol (pH 7.4). Based on the number of beads per ml and the amount of proteins bound, BDNF bound to each bead was estimated to be 0.85 $\pm$ 0.05 pg (SD, n = 5). Assuming BDNF binds uniformly onto the bead surface, the surface density of BDNF was $\sim$2.7 fg/μm^2. The BDNF- or NT-3-coated beads remain biologically active in the storage buffer for up to one month. The reaction used to couple the protein to the carboxylated beads is expected to have linked -NH_2 groups of the proteins with -COOH groups on the bead surface so that the BDNF molecule may have adopted various orientations on the beads. A previous study has shown that neurotrophin molecules coupled to the beads by the present method do not detach within 3 hr after exposure to the saline solution (Gallo et al., 1997). BDNF on the coated bead was confirmed by immunostaining with a rabbit polyclonal IgG antibody against BDNF (Santa Cruz Biotechnology, Santa Cruz, CA) and a rhodamine-labeled secondary antibody. A monoclonal IgG antibody against BSA (Sigma) was used to detect BSA on the BSA-coated beads.

Electrophysiology

Synaptic currents were recorded from innervated myocytes by the whole-cell patch recording method, using a patch clamp amplifier (Axonpatch 200B, Axon Instruments, Foster City, CA). Myocytes were voltage clamped at $-$70mV. Recordings were performed at room temperature in a bath solution containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). The solution inside the recording pipette (1–2 MΩ) contained 145 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$, 1 mM adenosine 5′-triphosphate, and 10 mM HEPES (pH 7.2). The perforated whole-cell recording was used to stimulate the spinal neuron by a step depolarization of membrane potential from the clamped voltage of $-$60mV to 0mV (duration $\sim$1.5–2 ms). The intrapipette solution contained 140 mM potassium gluconate, 1 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.2), and amphotericin B (200 μg/ml, CalBiochem, San Diego, CA). The test stimulus at a low frequency (7–8 pulses at 0.033 Hz, spaced by 5 min intervals) was used to minimize test-induced synaptic depression at these developing neuromuscular synapses. The membrane currents recorded were filtered at 5 kHz, digitized, and acquired with a microcomputer for further analysis using the WCP program (kindly provided by J. Dempster, University of Starthclyde, UK). In a typical experiment, 10–20 min of stable control recording was obtained before the bead contact. The bead was manipulated into the contact with the axon with a heat-polished suction pipette (tip opening of 3 μm). The distance from the bead contact site to the synapse was defined by the axon trajectory between the centers of the bead and the innervated myocyte, assuming synapses are located at the center of the myocyte. In experiments studying protein synthesis, actinomycin D, anisomycin, or cycloheximide (all from Calbiochem) was added in the culture medium for 45 min or 2 hr before the bead contact or bath application of BDNF and was continuously present in the recording solution. All three drugs were dissolved in dimethyl sulfoxide (DMSO) and diluted by the bath solution to the desired concentration, with the final DMSO concentration of 0.1% (v/v). In experiments involving axon transection, the drugs were applied to the culture medium 30 min after transection, allowing resealing of the injury site.

Local puffing of BDNF was delivered through a micropipette (tip opening $\sim$1–2 μm) placed at a distance of 15–20 μm from the axon. The micropipette was filled with the bath solution containing BDNF (5 μg/ml). Repetitive pressure injection (3 psi in amplitude, 20 ms in duration, 2 Hz) was applied to the micropipette with an electrically gated valve (Picospritzer, General Valves, Fairfield, NJ). The concentration of BDNF at the exposed axon was estimated to have reached a steady state of $\sim$1.3 μg/ml within 15 s (Lohof et al., 1992), whereas it was less than 5 ng/ml at the distant synaptic sites, a concentration insufficient to induce synaptic potentiation in the present system (Boulanger and Poo, 1999). In experiments studying Ca^{2+} elevation elicited by focal application of BDNF, the micropipette filled with 1 μg/ml BDNF was placed at a distance of 5 μm from the axon, and the bath perfusion flow ($\sim$2 ml/min) was present continuously.

Injections of Drugs and Fluorescent Dye

Gelonin (30 μM, Sigma), together with 10 kDa dextran-fluorescein (350 μM, Molecular Probes, Eugene, OR), was injected into presynatic spinal neurons or postsynaptic myocytes at the cell body with sharp microelectrodes (12–16 MΩ), using an Eppendorf Pressure Injection system (a Transjector 5246 in conjunction with a Micromanipulator 5171, Hamburg, Germany). A constant backpressure of 0.4–0.6 psi was applied to the electrode. A brief pressure pulse (0.2–0.3 s per cell and 0.1–0.3 psi above the backpressure) was used for injection. Coinjection of dextran-fluorescein was used to mark the injected cells. Calcium-sensitive dye Oregon Green-488 BAPTA-1 (conjugated to 70 kDa dextran, 330 μM, Molecular Probes) was injected into the presynaptic spinal neuron using the same technique. The drug and dye were diluted to the desired concentrations in the injection buffer containing 140 mM KCl, 1 mM NaCl, 0.1 mM EGTA, and 10 mM HEPES (pH 7.2).

Ca^{2+} Imaging

Xenopus spinal neurons were microinjected with Ca^{2+}-sensitive dye, Oregon Green-488 BAPTA-1 conjugated to 70 kDa dextran. Calcium imaging was done using a Leica confocal imaging system (TCS SP) equipped with an argon gas ion laser and a Leica inverted microscope (DM IRBE) fitted with a Leica 63$\times$ objective (HCX PL Apo; NA, 1.32). The Oregon Green BAPTA-1-dextran was excited at 488 nm, and its fluorescence signal was collected at 500–540 nm through the emission filter. Images were acquired every 15 s at 512 $\times$ 512 pixels. To monitor laser power output, transmission images also were collected simultaneously. Digital images were analyzed using ImageJ (http://rsb.info.nih.gov/ij.). To quantify changes in the Ca^{2+} level, the mean fluorescence intensity within the axon at a length of 60 μm across the bead contact sites was measured over the fixed polygon area that covered the axons throughout the measurement period. To measure the spatial profile of Ca^{2+} signals along the axon, the polygons at a length of 10 μm were used. Ratios of fluorescence intensity change ($\Delta F = F - Fo$) and basal fluorescence intensity (Fo) were used to represent the Ca^{2+} signals.

Staining for Nucleic Acids and Ribosomal Proteins

The SYTO 64 dyes and Hoechst 33342 (Molecular Probes) were used to stain nucleic acids. The culture was incubated with 0.5 μM SYTO 64 for 20 min at room temperature. After an extensive wash, the culture was incubated with the solution containing 1 μg/ml Hoechst 3342 for another 10 min. The SYTO 64 fluorescence images were acquired by the confocal microscopy (Leica TCS SP), and the Hoechst 33342 fluorescence images were captured by a CCD camera (PhotoMetrics, Huntington Beach, CA). Cultures for immunocytochemistry were plated on the poly-L-lysine-coated coverslips. The cultures were fixed with 4% paraformaldehyde (EM Science, Gibbstown, NJ) for 15 min at room temperature and washed three times with phosphate buffer saline (PBS). Fixed cells were incubated with a human autoantibody against ribosomal P protein 1:150 (ImmunoVision, Springdale, AR) at 4°C overnight after being

treated with PBS containing 0.25% Triton X-100, 5% normal goat serum, and 1% BSA for 20 min. The Alex488 goat anti-human IgG secondary antibody (Molecular Probes) was used to visualize the specific immunostaining, and Alex568-phallotoxin (Molecular Probes) was also used to label F-actin for visualizing the fixed cells. The fixed cells then were incubated with Alex568 phallotoxin (13.2 μM, Molecular Probes) for 20 min to label the F-actin. The fluorescent images were captured by confocal microscopy (Leica TCS SP).

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