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Presynaptic depolarization facilitates neurotrophin-induced synaptic potentiation

Lisa Boulanger and Mu-ming Poo

Department of Biology-0357, 9500 Gilman Drive, University of California at San Diego, La Jolla, California 92093-0357, USA
Correspondence should be addressed to M.-M.P. (mpoo@ucsd.edu)

Neurotrophins have been proposed to participate in activity-dependent modifications of neuronal connectivity and synaptic efficacy. Preferential strengthening of active inputs requires restriction of putative neurotrophin-mediated synaptic potentiation to active synapses. Here we report that potentiation of synaptic efficacy by brain-derived neurotrophic factor (BDNF) is greatly facilitated by presynaptic depolarization at developing neuromuscular synapses. Brief depolarization in the presence of low-level BDNF results in a marked potentiation of both evoked and spontaneous synaptic transmission, whereas exposure to either BDNF or depolarization alone is without effect. This potentiation depends on the relative timing of depolarization and reflects an enhancement of transmitter secretion from the presynaptic neuron. Thus synapses made by active inputs may be selectively strengthened by secreted neurotrophins as part of activity-dependent refinement of developing connections or of mature synapses.

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Activity-dependent synaptic modifications are crucial to the normal development and function of the nervous system, but the basic underlying mechanisms are not fully understood. Recently, it has been proposed that activity-dependent synaptic plasticity may involve a family of secreted proteins known as neurotrophins[1–5]. Neurotrophins were initially identified based on their ability to promote neuronal survival and morphological differentiation[6]. Several lines of evidence have suggested a role for neurotrophins in synaptic plasticity. First, the expression of many neurotrophins is upregulated by electrical activity[7–9], and the secretion of neurotrophins is triggered by depolarization or synaptic activity[10–12]. Second, acute application of neurotrophins results in potentiation of synaptic transmission at peripheral[13,14] and central synapses[15–18] as well as morphological changes in developing nerve processes[19–22]. Third, manipulations of the level of neurotrophins influence activity-dependent segregation of thalamocortical afferents in the developing visual system[23,24] and innervation of sympathetic ganglia[25]. Fourth, the neurotrophin BDNF is required for normal induction of long-term potentiation (LTP) in the CA1 region of the hippocampus[26–29]. How might neurotrophins influence activity-dependent synaptic plasticity? Activity can induce synthesis and secretion of neurotrophins, which in turn modulate synaptic structure or efficacy of synaptic transmission. To account for activity-dependent synaptic modifications, however, it would be important to restrict the synaptic action of secreted neurotrophins to those inputs that are active at the time when neurotrophins are released. Restriction may be accomplished by a localized secretion of neurotrophin at the site of active synapses and a limited spread of secreted neurotrophins[30,31]. Alternatively, it has been suggested that active presynaptic terminals may be more susceptible to the modulatory actions of secreted neurotrophins[29,32,33]. Such an effect of activity on neurons' synaptic responsiveness to neurotrophins has not been directly demonstrated. In the present study, we addressed this issue by studying the effect of activity on the action of neurotrophins at *Xenopus* neuromuscular synapses in culture. Our findings indicate that presynaptic depolarization in the presence of BDNF greatly facilitates the potentiation of transmitter release induced by BDNF. These results provide direct evidence for the activity-dependent synaptic action of neurotrophins, a mechanism that may be crucial in conferring an advantage to active inputs during refinement of developing nerve connections.

Results

The synaptic efficacy of developing neuromuscular junctions was monitored by whole-cell recording of synaptic currents from innervated myocytes in one-day-old *Xenopus* nerve-muscle cultures[34]. Evoked postsynaptic currents (EPCs) were triggered by extracellular suprathreshold stimulation of the presynaptic neuronal soma. Spontaneous miniature postsynaptic currents (MEPCs) were observed in the postsynaptic myocyte in the absence of presynaptic action potentials. The frequency of MEPCs was not affected by the presence of tetrodotoxin in the medium[35], indicating that they reflect spontaneous quantal release of transmitter. Confirming previous studies[13,14], we found that acute treatment with exogenous BDNF at a high concentration (50 ng per ml) resulted in potentiation of both spontaneous and evoked secretion of transmitter at these developing *Xenopus* neuromuscular synapses (data not shown), but had no effect on either EPCs or MEPCs at a lower concentration (10 ng per ml; Figs. 1a and 2a). However, when the presynaptic neuron was depolarized by a brief (50 s) perfusion with medium containing 20 mM K^+ while the postsynaptic myocyte was voltage-clamped at resting potential (–70 mV), a marked increase in EPC amplitude was observed during the same BDNF treatment (Fig. 1a and c). Perfusion with high-K^+ medium alone did not result in any change in EPCs

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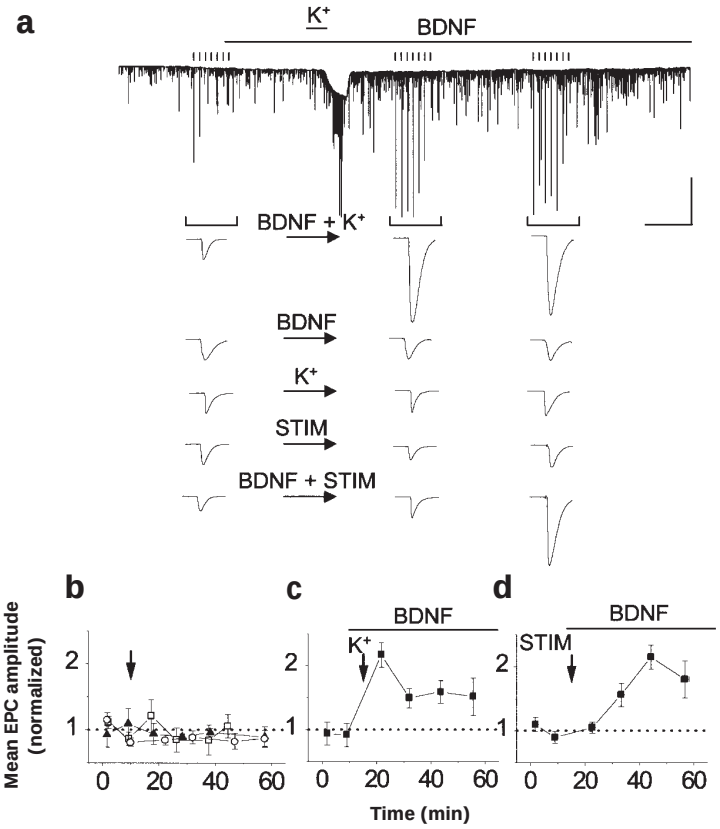


Fig. 1. Effect of depolarization on BDNF-induced changes in evoked postsynaptic currents (EPCs). **(a)** Continuous trace represents membrane currents recorded from an innervated myocyte under voltage-clamp (V_c = –70 mV, filtered at 150 Hz). EPCs were elicited at the times marked by vertical lines. BDNF (10 ng per ml) was present in the culture during the time marked by the horizontal bar. Perfusion with high-K^+ saline (20 mM, 50 s) occurred at the time marked by 'K^+'. Samples of EPCs (averages of 7–10 events) are shown below at higher time resolution. Scale, fast traces, 1 nA, 40 ms; slow trace, 1 nA, 90 s. Also shown are samples of EPCs (averages of 7–10 events) from four other experiments, elicited at similar time points as above, before (left of arrow) or after (right of arrow) the treatment with BDNF alone (10 ng per ml), high-K^+ saline alone, or repetitive electrical stimulation of the presynaptic neuron soma ('STIM') (2 Hz, 15 pulses) alone or in combination with BDNF. **(b–d)** Time course of changes in EPC amplitudes under various experimental conditions. **(b)** Synapses treated with BDNF (circles, n = 8), high-K^+ medium (triangles, n = 3) or repetitive electrical stimulation (squares, n = 6), respectively, at the time marked by the arrow. **(c)** Synapses perfused 5 min after the onset of BDNF treatment with high-K^+ medium for 50 s (n = 5). **(d)** Synapses repetitively stimulated 5 min after the onset of treatment with BDNF (n = 7). Each point in **(b–d)** represents mean EPC amplitude (± s.e.), normalized to the value during a ten-minute control period (first ten min of each recording).

(Fig. 1a and b). Because perfusion with high-K^+ medium causes only presynaptic depolarization when the postsynaptic myocyte is clamped at –70 mV, we tested whether the same effect can be induced by direct electrical stimulation of the presynaptic neuron at the soma. Repetitive electrical stimulation (2 Hz for 7.5 s) of the presynaptic neuron in the presence of 10 ng per ml BDNF also led to an increase in the amplitude of EPCs (Fig. 1a and d), whereas stimulation alone was ineffective (Fig. 1a and b). Thus the presence of presynaptic activity facilitated the action of BDNF in potentiating synaptic function.

The influence of presynaptic depolarization on BDNF's action was also reflected in the frequency of spontaneous MEPCs. Without depolarization, 10 ng per ml BDNF had no effect on the MEPC frequency (Fig. 2a). Without BDNF, MEPC frequency showed a transient elevation immediately following perfusion with high-K^+ medium, but quickly returned to control levels following reintroduction of normal recording medium (Fig. 2a), consistent with depolarization-induced Ca^{2+} elevation in the presynaptic nerve terminal[36]. However, in the presence of BDNF, brief perfusion with high-K^+ medium produced a marked and persistent elevation of MEPC frequency (Fig. 2a). Similar changes in MEPC frequency were observed after depolarization by repetitive presynaptic stimulation (Fig. 2c). In the presence of TrkB-IgG[37], a recombinant scavenger protein that selectively binds BDNF in the medium, the combined treatments of depolarization and BDNF were without effect (Fig. 2b). Thus depolarization-triggered synaptic potentiation by BDNF required binding, presumably to its specific membrane receptor, TrkB. Finally, we voltage-clamped both the presynaptic neuron and the postsynaptic myocyte at the resting membrane potential (–70 mV) during high-K^+ perfusion and in the presence of BDNF. No significant potentiation of MEPC frequency was observed (Fig. 2c), suggesting that presynaptic depolarization is necessary for BDNF potentiation. Presynaptic voltage-clamp efficacy at the nerve terminal was confirmed by lack of increase in MEPC frequency during perfusion with high K^+ (Fig. 2d, compared to Fig. 2a and b).

The potentiation of EPCs and MEPCs by BDNF following brief depolarization seems to be presynaptic in origin. First, the mean and distribution of MEPC amplitudes did not change despite a marked increase of EPC amplitude and MEPC frequency (Fig. 3a), nor was there any significant change in the time course of EPCs and MEPCs (Fig. 3a and c). If the apparent potentiation of MEPC frequency were the result of an increase in MEPC amplitude, which permitted detection of small events previously lost in baseline noise, both the mean amplitude and shape of the amplitude distribution would change. Thus our findings are inconsistent with changes in properties or density of postsynaptic transmitter receptors. Second, the fluctuation of EPC amplitudes, as reflected by the coefficient of variation (c.v.; see legend, Fig. 3b), are consistent with a presynaptic change (Fig. 3b). Assuming that EPC amplitudes can be described by a binomial distribution, c.v. is independent of quantal size and should remain constant if the change in EPC amplitude is due only to a change in postsynaptic receptor sensitivity[38,39]. We found that the ratio of squared coefficients of variation before and after potentiation showed a clear dependence on the extent of synaptic potentiation (Fig. 3b), which is consistent with a presynaptic increase in probability of vesicular exocytosis or in number of available release sites[38,39]. Third, paired-pulse facilitation (PPF) of the evoked responses (a phenomenon associated with presynaptic changes[40]) was significantly reduced in

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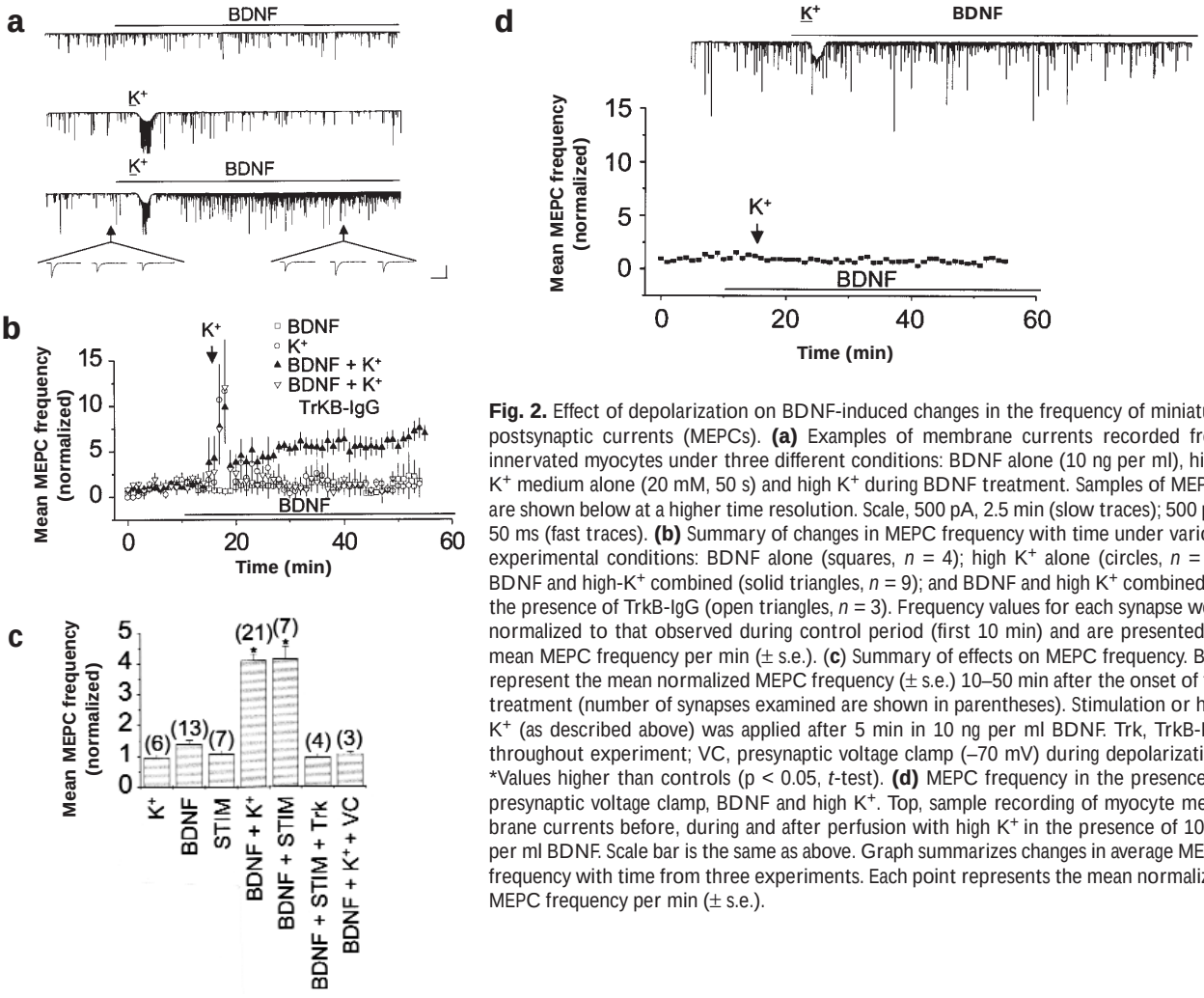


Fig. 2. Effect of depolarization on BDNF-induced changes in the frequency of miniature postsynaptic currents (MEPCs). **(a)** Examples of membrane currents recorded from innervated myocytes under three different conditions: BDNF alone (10 ng per ml), high-K^+ medium alone (20 mM, 50 s) and high K^+ during BDNF treatment. Samples of MEPCs are shown below at a higher time resolution. Scale, 500 pA, 2.5 min (slow traces); 500 pA, 50 ms (fast traces). **(b)** Summary of changes in MEPC frequency with time under various experimental conditions: BDNF alone (squares, n = 4); high K^+ alone (circles, n = 4); BDNF and high-K^+ combined (solid triangles, n = 9); and BDNF and high K^+ combined, in the presence of TrkB-IgG (open triangles, n = 3). Frequency values for each synapse were normalized to that observed during control period (first 10 min) and are presented as mean MEPC frequency per min (± s.e.). (**c**) Summary of effects on MEPC frequency. Bars represent the mean normalized MEPC frequency (± s.e.) 10–50 min after the onset of the treatment (number of synapses examined are shown in parentheses). Stimulation or high K^+ (as described above) was applied after 5 min in 10 ng per ml BDNF. Trk, TrkB-IgG throughout experiment; VC, presynaptic voltage clamp (–70 mV) during depolarization. *Values higher than controls ($p < 0.05$, t-test). **(d)** MEPC frequency in the presence of presynaptic voltage clamp, BDNF and high K^+. Top, sample recording of myocyte membrane currents before, during and after perfusion with high K^+ in the presence of 10 ng per ml BDNF. Scale bar is the same as above. Graph summarizes changes in average MEPC frequency with time from three experiments. Each point represents the mean normalized MEPC frequency per min (± s.e.).

synapses treated with a combination of depolarization and BDNF, but not in synapses treated with either alone (Fig. 3c and d). Taken together, these results support the notion that BDNF paired with depolarization enhanced synaptic transmission by increasing the level of evoked and spontaneous transmitter secretion from the presynaptic nerve terminal.

Effective facilitation depended on the timing of depolarization relative to addition of BDNF to the culture. We found that when depolarization was applied immediately before BDNF was added to the culture, no synaptic potentiation was induced (Fig. 4a). In contrast, depolarization coincident with or during the first 20 min after BDNF addition led to a marked potentiation (Fig. 4a). Thus depolarization must occur in the presence of BDNF for the induction of synaptic potentiation and leaves no effect five minutes after its termination. We noted, however, that depolarization applied after the cells have been exposed to BDNF for 20 min was ineffective in facilitating synaptic potentiation (data not shown). This may be accounted for by desensitization or downregulation of TrkB receptors, mechanisms demonstrated in other systems[41,42].

An immediate and transient effect of depolarization is the opening of voltage-dependent Ca^{2+} channels in the presynaptic neuron, raising the possibility that Ca^{2+} influx may be necessary for the facilitation of BDNF-induced synaptic potentiation by depolarization. We addressed this question by using Ca^{2+}-free solution or medium containing a Ca^{2+} channel blocker (10 mM Co^{2+}) during high-K^+ depolarization. These experimental conditions were effective in preventing Ca^{2+} influx, as they completely abolished EPCs as well as any K^+-induced transient elevation of MEPC frequency (Fig. 4b). Surprisingly, neither treatment with Co^{2+} nor Ca^{2+}-free solution had any effect on the facilitation of BDNF-induced potentiation by depolarization, so these data are grouped together (Fig. 4b). Furthermore, perfusion of Ca^{2+}-free, high-K^+ solution, followed by the return of normal Ca^{2+} medium to restore synaptic transmission, induced elevation of the EPC amplitude similar to that found after treatment with BDNF and depolarization in normal medium (3.2 ± 0.24 times control values at 10–40 min after the K^+ treatment; n = 3). Thus, depolarization does not seem to act by opening Ca^{2+} channels. One intriguing possibility is that binding of BDNF and/or subsequent signal transduction via the TrkB receptor, including receptor dimerization and tyrosine phosphorylation, are directly influenced by membrane potential.

Discussion

Here we have shown that presynaptic depolarization facilitates the synaptic potentiation induced by BDNF. This effect was revealed by using a level of exogenous BDNF that was by itself ineffective in

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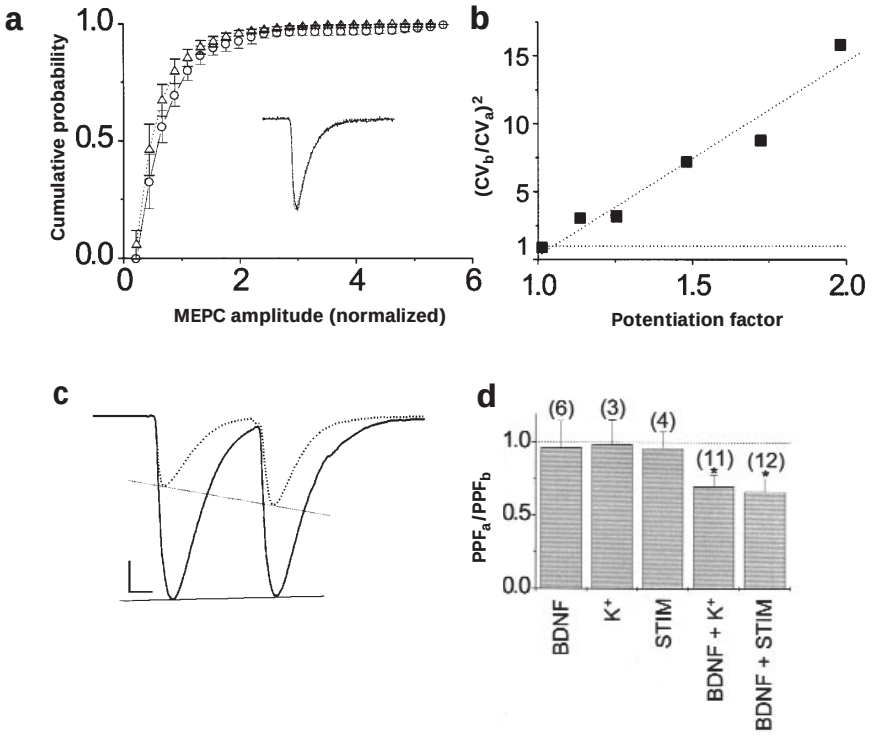


Fig. 3. Analyses of MEPCs and EPCs. **(a)** Amplitude distributions of MEPCs before and after treatment with low-level BDNF (10 ng per ml) and high K^+ (20 mM, 50 s). Cumulative probability refers to the probability of observing events with amplitudes smaller than or equal to a given value. Data were obtained from 10 synapses before (circles) and 10–50 min after (triangles) exposure to the combined treatment of high-K^+ and BDNF. Inset, superimposed average traces of MEPCs before and after treatment. Dashed line depicts MEPCs (average of 178 events) for a typical synapse, 10–50 min after the treatment. The superimposed solid line depicts MEPCs (average of 38 events) during a control period (first 10 min of recording). **(b)** Analysis of amplitude fluctuation of EPCs. The ratio of coefficients of variation squared [$c.v.^2$ = variance/$(mean)^2$] before and after synaptic potentiation ($c.v._b^2/c.v._a^2$) was plotted against the extent of synaptic potentiation induced by combined high-K^+ and BDNF treatment from six experiments. Potentiation factor is defined as the mean EPC amplitude 10–50 min after treatment divided by mean EPC amplitude before the treatment. Only synapses with more than 35 EPC events recorded were used for this analysis. The horizontal dotted line represents prediction from binomial statistics for postsynaptic changes, and the diagonal line represents the best linear fit of the data. **(c, d)** Paired-pulse facilitation of evoked responses. Shown in **(c)** are samples of EPCs (averages of 40 events) in response to presynaptic paired-pulse stimuli (25 ms interval) before (dotted trace) and 10–50 min after (solid trace) exposure to a combined treatment of high K^+ and BDNF. Lines connecting peaks of each pair are for comparison of the degree of facilitation. Scale, 0.33 nA, 6 ms. In **(d)**, ratio of average paired-pulse facilitation (after treatment/before treatment) is shown for synapses treated with BDNF, high K^+ or stimulation alone or in combination. For each cell, PPF was normalized to initial PPF during a ten-minute control period; a value of less than one represents a decrease in PPF. *Treatments for which PPF changed significantly after application ($p < 0.05$, t-test). All error bars represent s.e.

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inducing potentiation. The increase in synaptic strength depends on the timing of depolarization and seems to occur through enhancement of transmitter secretion from the presynaptic nerve terminal. The effect of depolarization does not require Ca^{2+} influx, suggesting that depolarization may act directly on BDNF receptor binding or signal transduction. These results indicate that neuronal activity can confer rapid synaptic responsiveness to BDNF.

Studies of activity-dependent refinement of the developing nervous system have led to the hypothesis that competition for limited amounts of target-derived factors underlies the selective

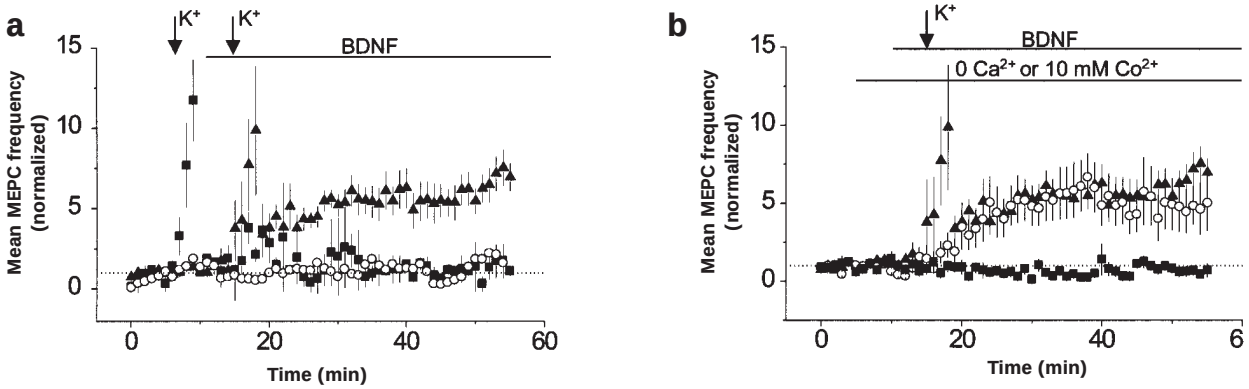


Fig. 4. The importance of timing of depolarization and the role of Ca^{2+} influx. **(a)** Changes in MEPC frequency in three sets of experiments. Squares, high K^+ (20 mM, 50 s) was applied 5 min before the onset of BDNF treatment (10 ng per ml, $n = 9$). Triangles, high K^+ was applied 5 min after the onset of BDNF treatment ($n = 12$). Circles, only BDNF was applied to the culture ($n = 4$). **(b)** The role of Ca^{2+} influx: changes in MEPC frequency in three sets of experiments. Triangles, same as in **(a)** Circles, high K^+ was applied 5 min after the onset of BDNF treatment in the presence of Ca^{2+}-free saline with ($n = 6$) and without ($n = 4$) 10 mM Co^{2+}. Squares, Ca^{2+}-free treatment alone, with ($n = 3$) and without ($n = 3$) 10 mM Co^{2+}. The triangles in **(a)** and **(b)** represent the same data presented in Fig. 2b, shown here for comparison with other treatments. Each point represents mean normalized MEPC frequency (± s.e.).

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stabilization and elimination of nerve connections[43]. Neurotrophins are candidates for such target-derived factors[3]. The expression of NGF and BDNF in central neurons is regulated by neuronal activity[7,8], and the secretion of both of these factors can be triggered by membrane depolarization[10,11]. In rat skeletal muscle, activity elevates the production of NT-4 (ref. 9). In nerve-muscle co-cultures, repetitive activity triggers the release of NT-4 from postsynaptic muscle cells, resulting in potentiation of synaptic responses[12]. All of these results implicate activity-dependent availability of neurotrophins in mediating synaptic modifications. However, they do not suggest any obvious mechanism for selective stabilization or elimination of specific synaptic inputs. In principle, activity may confer an advantage to a synaptic input if the action of the target-derived neurotrophin is regulated by activity. Depolarization promotes cortical dendritic growth[33] and retinal ganglion cell survival[44] induced by neurotrophin as well as enhancing membrane insertion of neuronal TrkB receptors[45]. Activity could also promote the response of the presynaptic neuron to neurotrophins by regulating neurotrophin binding or signal transduction. Voltage-dependent ligand binding has been demonstrated for membrane receptors associated with ion channels such as dihydropyridine receptors[46]. It is possible that, being a transmembrane protein, TrkB also exhibits voltage-dependent ligand binding. Voltage-dependent changes in the conformation of neurotrophin-receptor complexes might affect the efficiency of receptor dimerization and autophosphorylation, although all these possibilities have yet to be examined. In addition, depolarization triggers neuronal membrane recycling through increased endocytosis. Endocytotic uptake of neurotrophin–receptor complexes, which may be an integral part of intracellular signaling[47], could also be enhanced by depolarization.

Low levels of two neurotrophic factors, BDNF and ciliary neurotrophic factor (CNTF), can act in a synergistic manner to enhance synaptic efficacy at developing neuromuscular synapses[14]. It is possible that presynaptic depolarization triggers secretion of endogenous neurotrophin that acts synergistically with applied BDNF to potentiate the synapse. This secreted neurotrophin could then act in an autocrine or paracrine fashion to supplement low local levels of neurotrophin past the threshold for synaptic potentiation. Endogenous secreted neurotrophin levels are likely to be lower than those used *in vitro*. Our findings demonstrate that a previously ineffective level of neurotrophin may enhance synaptic transmission in the presence of brief presynaptic depolarization. This result underscores the notion that biological actions of neurotrophins must be considered within the context of other coincident signals received by the neuron. Consideration of coincident signals is also relevant for the actions of BDNF on neuronal growth and survival, as shown by the modulatory effect of cytosolic cAMP[44,48]. Interestingly, cAMP levels can be regulated by the presence of neuronal depolarization through voltage-sensitive (but Ca^{2+}-independent) adenylyl cyclase activity, found in several neuronal systems[49].

In conclusion, we have demonstrated that presynaptic depolarization is a critical factor in determining the synaptic action of neurotrophins. This dependence on presynaptic activity provides a natural mechanism for selective stabilization of active nerve terminals during synaptic refinement in the developing nervous system and adds a new dimension to the role of neurotrophins in synaptic plasticity.

Methods

Cell culture. Preparation of *Xenopus* nerve/muscle cultures followed described methods [34]. The cells were plated on clean glass coverslips and were used for experiments after 24 h incubation at room temperature (20–22°C). The culture medium consisted of (v/v) 50% Leibovitz's medium (L-15, Gibco), 1% fetal bovine serum (Gibco) and 49% Ringer's solution (115 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, 10 mM HEPES, pH 7.3). In zero-Ca^{2+} medium, 2 mM $MgCl_2$ or 2 mM $CoCl_2$ was substituted for $CaCl_2$; in the first instance, 10 mM NaCl was omitted from the Ringer's solution and replaced with 10 mM EGTA.

Electrophysiology. Synaptic currents were recorded from singly innervated individual spherical muscle cells by the whole-cell recording method[50] using a patch-clamp amplifier (Axopatch 1D, Axon Instruments). The solution inside the recording pipette contained 150 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$ and 10 mM HEPES (pH 7.2). Recordings were made at room temperature in culture medium. A patch electrode at the cell body under loose-seal conditions was used for extracellular stimulation of the presynaptic neuron. Recordings in which there was apparent cell damage or substantial changes (>20%) in the series or input resistance were discarded. All other recordings were included for analysis in this work. The typical series resistance was 10 MΩ, and the input resistance of the postsynaptic myocyte was in the range of 50–200 MΩ. The estimated error in voltage clamp of the myocyte was therefore about 3–12 mV when $V_c = -70$ mV. The recorded membrane currents were filtered at 10 kHz and stored by a videotape recorder for later playback onto a storage oscilloscope (2201, Tektronix) or an oscillographic recorder (RS3200, Gould) and for analysis by computer. Evoked currents were distinguished from spontaneous MEPCs by the presence of a clear stimulation artifact immediately preceding EPCs. Synaptic currents were analyzed using the SCAN program (provided by J. Dempster, Univ. of Strathclyde, Glasgow).

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