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Regulation of TrkB receptor tyrosine kinase and its internalization by neuronal activity and Ca^{2+} influx

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Internalization of the neurotrophin–Trk receptor complex is critical for many aspects of neurotrophin functions. The mechanisms governing the internalization process are unknown. Here, we report that neuronal activity facilitates the internalization of the receptor for brain-derived neurotrophic factor, TrkB, by potentiating its tyrosine kinase activity. Using three independent approaches, we show that electric stimulation of hippocampal neurons markedly enhances TrkB internalization. Electric stimulation also potentiates TrkB tyrosine kinase activity. The activity-dependent enhancement of TrkB internalization and its tyrosine kinase requires Ca^{2+} influx through *N*-methyl-D-aspartate receptors and Ca^{2+} channels. Inhibition of internalization had no effect on TrkB kinase, but inhibition of TrkB kinase prevents the modulation of TrkB internalization, suggesting a critical role of the tyrosine kinase in the activity-dependent receptor endocytosis. These results demonstrate an activity- and Ca^{2+}-dependent modulation of TrkB tyrosine kinase and its internalization, and they provide new insights into the cell biology of tyrosine kinase receptors.

Introduction

Although neurotrophins are best known for their ability to promote neuronal survival and differentiation, more recent studies have established a novel function of neurotrophins in synapse development and plasticity, particularly in the central nervous system (Huang and Reichardt, 2001; Lu, 2003). Neurotrophins initiate their signal transduction by interacting with the Trk receptors: NGF with TrkA, brain-derived neurotrophic factor (BDNF) and NT4 with TrkB, and NT3 with TrkC. Consequently, Trk receptor tyrosine kinases are activated, triggering multiple signaling pathways (Kaplan and Miller, 2000). Formation of the ligand–receptor complex also initiates internalization of the activated receptors (Ehlers et al., 1995; Grimes et al., 1996). Internalization of the neurotrophin–Trk complex seems to be critical in signal transduction that initiates cell body responses to target-derived neurotrophins (Bhattacharyya et al., 1997; Riccio et al., 1997; Senger and Campenot, 1997; Zhang et al., 2000). This is quite unusual because the internalization for most other growth factor receptors is to inactivate the signaling process (Sorkin and Waters, 1993). The neurotrophin–Trk complex is internalized through clathrin-mediated endocytosis, leading to the formation of signaling endosomes (Grimes et al., 1996, 1997; Beattie et al., 2000). The internalized Trk receptor remains tyrosine phosphorylated and activated, with its extracellular domain bound to the ligand neurotrophin inside the signaling endosomes, and the intracellular domain tightly associated with a number of signaling molecules such as PLC-γ, PI3 kinase, and proteins of the Ras–MAP kinase pathway in the cytoplasm of the responsive neurons (Grimes et al., 1996; Howe et al., 2001). The molecular mechanisms that govern the internalization of Trk receptors are poorly understood.

Neuroelectric activity, like neurotrophins, is known to regulate the structure and function of synapses during development and refinement of neuronal connectivity in the

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Key words: BDNF; endocytosis; calcium influx; hippocampus; activity dependent

Abbreviations used in this paper: BDNF, brain-derived neurotrophic factor; CNQX, 6-cyano-7-nitroquinozaline-2,3-dione; Kyn, kynurenic acid; MDC, monodansylcadavenrine; NMDA, *N*-methyl-D-aspartate; p75NR, p75 NGF receptors; TBS, theta burst stimulation; TTX, tetrodotoxin.

adult (Katz and Shatz, 1996). The relationship between activity and neurotrophins in neuronal modulation remains largely unknown. Interestingly, neuronal activity often influences the effectiveness of neurotrophins, particularly BDNF. For example, regulation of dendritic arborization by BDNF requires neuronal activity and Ca^{2+} influx through *N*-methyl-D-aspartate (NMDA) receptors (McAllister et al., 1996). BDNF regulation of the survival of retinal ganglion neurons is also dependent on neuronal depolarization (Meyer-Franke et al., 1995). Presynaptic depolarization greatly facilitates BDNF modulation of synaptic transmission at the neuromuscular junction (Boulanger and Poo, 1999). In the hippocampus, the effect of BDNF on CA1 synapses appears to be restricted to highly active presynaptic neurons (Gottschalk et al., 1998). Thus, whether or how well a neuron can respond to BDNF may depend on its intrinsic neuronal activity. One strategy is to increase the number of BDNF receptors on the cell surface. Indeed, treatment with depolarizing agents results in an increase in the amount of the BDNF receptor TrkB on the plasma membranes of retinal ganglion cells and spinal neurons (Meyer-Franke et al., 1998). In the hippocampus, tetanic stimulation, but not simple depolarization or low frequency stimulation, has been shown to facilitate the insertion of TrkB into the cell surface (Du et al., 2000). This effect requires Ca^{2+} influx through NMDA receptors or voltage-gated Ca^{2+} channels, but appears to be independent of ligand binding (Du et al., 2000). Binding of ligands to Trk receptor induces their tyrosine kinase activity and internalization, both of which are important for neurotrophin signaling (Kaplan and Stephens, 1994; Bothwell, 1995; Riccio et al., 1997; Zhang et al., 2000). Thus, an alternative and physiologically relevant way to control BDNF responsiveness is to regulate the tyrosine kinase activity and/or the internalization of TrkB receptor.

Here, we aimed to investigate whether and how neuroelectric activity and consequent Ca^{2+} influx regulates the internalization of the TrkB receptor and its relationship with TrkB tyrosine kinase function. Field electric stimulation was applied to hippocampal neurons to elicit action potentials. Three independent approaches were used to measure receptor internalization induced by BDNF. We show that neuroelectric activity facilitates the internalization of TrkB, as well as its tyrosine kinase activity. We also demonstrate that the tyrosine kinase activity of TrkB is critical for the activity-dependent modulation of TrkB internalization. These results identified a novel mechanism by which biological responses to BDNF might be regulated, and they provided new insights into the cell biology of tyrosine kinase receptors.

Results

We have used three independent approaches to investigate BDNF receptor internalization. Our first approach was to visualize the internalization of cell surface TrkB receptors after binding to BDNF. Conventional immunocytochemistry using TrkB antibodies cannot distinguish the newly synthesized receptors to be inserted onto the cell surface from the internalized receptors. Therefore, we used biotinylated BDNF (BDNF-biotin) to detect ligand-induced TrkB internalization. Application of this BDNF-biotin to cultured hippocampal neurons induced the same level of TrkB tyrosine phosphorylation as the recombinant BDNF, suggesting that BDNF-biotin is bioactive (Fig. 1 G). Field electric stimulation was applied to the cultured hippocampal neurons dur-

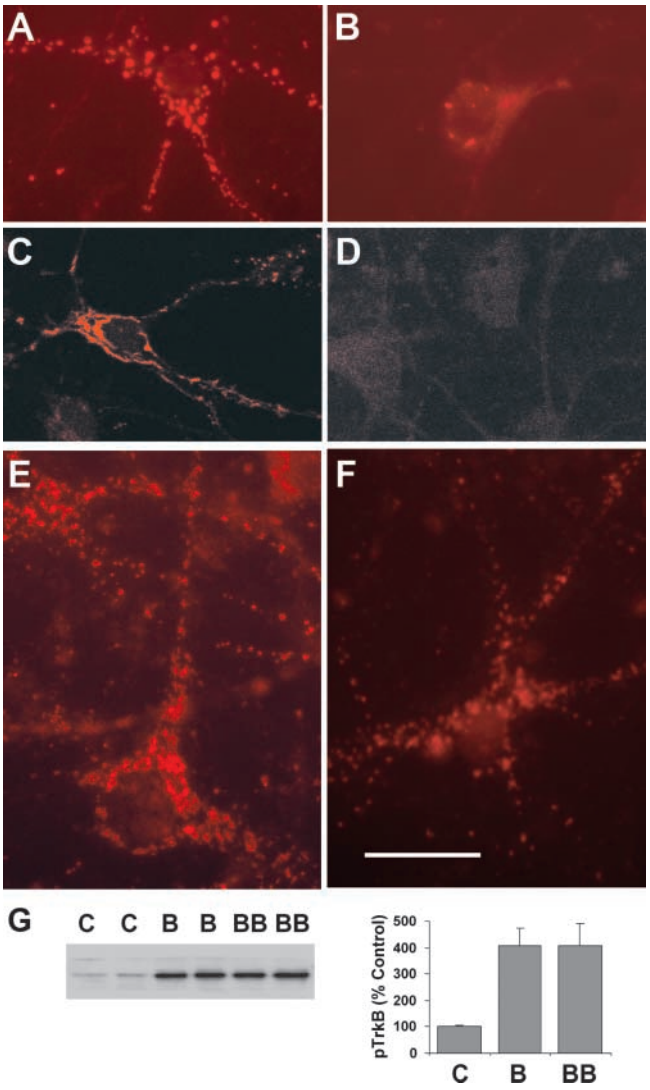

Figure 1. **Effect of TBS on BDNF-receptor internalization, detected by BDNF-biotin imaging.** (A and B) Detection of the internalized receptor–BDNF–biotin complex by Cy3-conjugated avidin. Hippocampal cultures were first incubated with BDNF-biotin on ice to achieve saturated binding, and then switched to 37°C for 30 min to allow receptor internalization. Many fluorescent puncta are seen in cultures incubated with BDNF-biotin (A), but not in those incubated with BDNF-biotin plus excess cold BDNF (B). (C and D) Blockade of BDNF-receptor internalization at low temperature. Confocal microscopy showing exclusive surface staining of BDNF-biotin when cultures were kept on ice (C). The staining was completely eliminated after acid wash (D). (E and F) Effect of TBS on the internalization of BDNF-biotin. Hippocampal neurons were incubated with BDNF-biotin, and stimulated with TBS in the presence (F) or absence (E) of activity blockers Cd^{2+} and Kyn. Bar, 10 μm. (G) Effect of BDNF-biotin on TrkB phosphorylation. Cultured hippocampal neurons were treated with or without recombinant BDNF (B) or BDNF-biotin (BB). C, no treatment. TrkB phosphorylation was detected by Western blot using an anti-pTrkB antibody specific for phosphor-Tyr490. (left) An example of Western blot; (right) summary of results ($n = 4$).

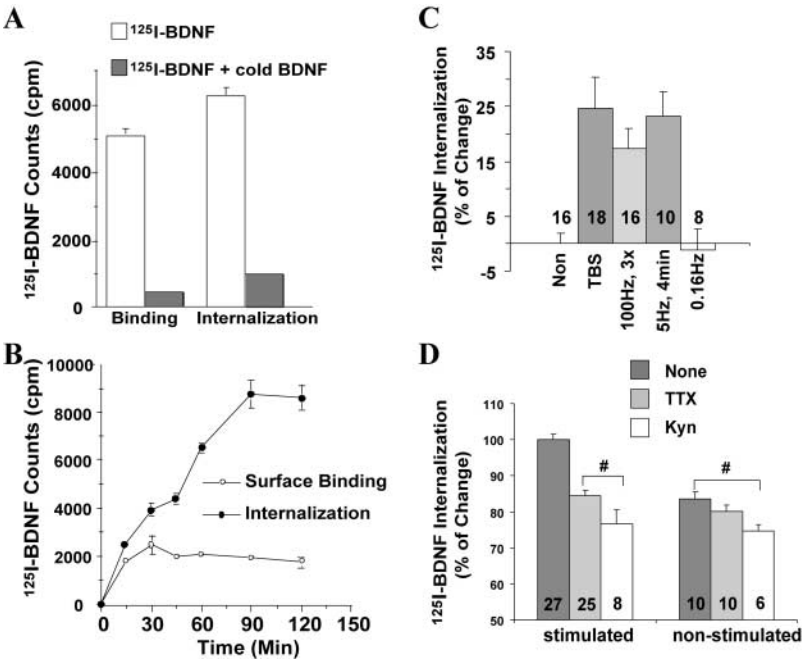

Figure 2. **Activity-dependent modulation of BDNF receptor internalization, measured by ^{125}I-BDNF internalization.** (A) ^{125}I-BDNF binding assay. Surface binding is defined as acid-washable radioactivity, and internalization is the remaining radioactivity in the cells after the acid wash. Total and nonspecific (blockable by cold BDNF) binding and internalization of ^{125}I-BDNF are shown. (B) Time courses of BDNF binding and internalization. Specific ^{125}I-BDNF counts, which were obtained by subtracting the nonspecific counts from their respective total counts, are shown. (A and B) Error bars represent standard errors. (C) Effect of electric stimulation on ^{125}I-BDNF internalization. The cultures were incubated with ^{125}I-BDNF while various indicated stimuli were applied for 30 min, followed by acid wash. The data in control (no stimulation) are set as 0%, and the percentage of changes is presented. In this and all other experiments, data in a specific experimental condition were normalized to the mean in control. Results from several experiments were averaged and presented as mean ± SEM. The number associated with each column represents the number of experiments performed. (D) Role of neuronal activity or excitatory transmission. The stimulated and nonstimulated cultures were pretreated with or without TTX, or kynurenic acid (Kyn) for 30 min. All data are normalized to TBS-stimulated groups, which are set as 100%. #, Significantly different; $P < 0.05$, *t* test.

ing a 30-min incubation with BDNF-biotin. We attempted to distinguish whether neuronal activity directly facilitates TrkB internalization, or indirectly as a consequence of enhancement of membrane insertion of TrkB as described previously (Du et al., 2000). Hippocampal cultures were first incubated with BDNF-biotin on ice to achieve saturated surface binding without any internalization (Fig. 1 C), followed by extensive washes to remove unbound BDNF-biotin. The cultures were then switched to a 37°C incubator, and stimulated for 30 min while BDNF receptors were being internalized. Thus, this method measured only the internalization of the receptors bound to BDNF-biotin, and the newly inserted receptors were not detected.

Numerous fluorescent puncta, typical of endocytotic particles, were observed inside the cells treated with BDNF-biotin (Fig. 1 A). BDNF-biotin staining was markedly decreased when the cultures were coincubated with 200-fold excess of cold BDNF (Fig. 1 B). If the cultures were kept on ice without switching to 37°C, internalization did not occur and BDNF-biotin staining was only observed on the cell surface (Fig.1 C). Acid wash after BDNF-biotin binding on ice completely eliminated surface BDNF-biotin staining (Fig. 1 D). Careful examination of the cultures by phase-contrast or differential interference contrast microscopy indicated that this mild acid treatment did not damage or lyse the cells (unpublished data). Thus, the fluorescent puncta observed in this assay reflected true receptor internalization induced by BDNF. When electric stimulation was applied during the period of receptor internalization in 37°C, BDNF receptor internalization was significantly increased. In cultures stimulated with theta burst stimulation (TBS; every 5 s for 30 min), many intracellular BDNF-biotin puncta were observed, especially in the dendritic regions (Fig. 1 E). In contrast, much less BDNF-biotin puncta were observed in cultures stimulated with TBS, whereas neuronal and synaptic activities were completely blocked by the general glutamate receptor antagonist kynurenic acid (Kyn; 1 mM) and the general Ca^{2+} channel blocker Cd^{2+} (0.2 mM; Fig. 1 F). The TBS-stimulated cultures also exhibited more BDNF-biotin puncta than the nonstimulated cultures (Fig. 1, compare E with A).

Although the imaging assay allowed visualization of BDNF receptor internalization, it was not quantitative. We used a BDNF binding assay that simultaneously quantified both cell surface receptors and internalized receptors. Cultured hippocampal neurons were incubated at 37°C with radiolabeled BDNF (^{125}I-BDNF, 50 pM) with or without cold BDNF (50 nM). At the end of the 30-min incubation, ^{125}I-BDNF bound to the receptors on neuronal surfaces was washed off by mild acid, and the amount of acid-washable radioactivity was used to quantify the cell surface BDNF receptors. The radioactivity inside the cells after acid wash was used to quantify receptor internalization. Both surface binding and internalization were markedly reduced when excess amount of cold BDNF was included in the incubation, suggesting that the assay is specific for BDNF receptors (Fig. 2 A). Time course studies indicated that binding was saturable within 30 min, whereas internalization continued to increase over ~2 h (Fig. 2 B). Incubation of hippocampal neurons in ^{125}I-BDNF at 4°C for 4 h still yielded high levels of surface binding, but there was virtually no radioactivity inside cells after acid wash (unpublished data), suggesting that the assay measures the true BDNF receptor-mediated internalization.

Electric stimulation significantly increased the amount of ^{125}I-BDNF inside the hippocampal neurons (Fig. 2 C). Several LTP-inducing protocols, such as TBS or tetanus (100 Hz, 1 s, three times in 30 min), elicited a significant increase

in ^{125}I-BDNF internalization, compared with those in nonstimulated cultures (Fig. 2 C). A 30-min continuous 0.16-Hz stimulation that delivers the same number of pulses as the tetanus had no significant effect (Fig. 2 C). Given that TBS also facilitates the insertion of TrkB into the cell surface of these hippocampal neurons (Du et al., 2000), the TBS-induced increase in ^{125}I-BDNF internalization may reflect endocytosis of the existing, as well as the newly inserted receptors. However, a long-term depression-inducing protocol (5 Hz, continuous for 4 min) that does not affect the insertion of BDNF receptors (Du et al., 2000) increased ^{125}I-BDNF internalization, as well as TBS (Fig. 2 C). Thus, neuronal activity can facilitate the receptor endocytosis independent of its effect on receptor insertion. Next, we examined whether the effect of TBS is mediated by action potential by stimulating the cultures with TBS either alone (active) or in the presence of 1 μM tetrodotoxin (TTX), a Na^+ channel blocker that completely blocked action potentials in these neurons (unpublished data). TTX significantly attenuated the TBS effect (Fig. 2 D, left). The activity-dependent modulation appears to involve action potentials coupled to excitatory synaptic transmission. Inhibition of excitatory transmission by 1 mM Kyn further reduced the BDNF receptor internalization compared with that in cultures treated with TTX (Fig. 2 D, left). Similar results were obtained by using a combination of the AMPA receptor antagonist 6-cyano-7-nitroquinozaline-2,3-dione (CNQX, 100 μM) and the NMDA receptor antagonist MK801 (80 μM; unpublished data). Thus, spontaneous synaptic transmission (TTX should block all evoked transmission) may also contribute to TrkB internalization. The role of spontaneous synaptic activity was further investigated by using nonstimulated cultures, which should fire relatively fewer action potentials. TTX was relatively ineffective in inhibiting receptor internalization in these cultures (Fig. 2 D, right). However, application of Kyn resulted in a small, but statistically significant, decrease in receptor internalization over control (Fig. 2 D, right). These results suggest that two distinct mechanisms are at play: one through action potential and subsequent evoked transmission and the other through spontaneous synaptic activity.

The BDNF-biotin and ^{125}I-BDNF assays cannot distinguish whether the internalized receptors are TrkB receptors or p75 NGF receptors (p75NR). Our third approach to detect receptor internalization was a biotinylation assay using a TrkB antibody. Cultured hippocampal neurons were incubated with BDNF on ice for 30 min to achieve saturated BDNF binding. All proteins on the cell surface were labeled with NHS-SS-biotin. By placing the cultures in a 37°C incubator for 30 min, we initiated BDNF-induced receptor internalization (because BDNF was already bound to the receptors on the surface of these cells). The internalization was terminated by placing the cultures on ice, and remaining biotinylated surface proteins were debiotinylated by cleavage of the NHS-SS-biotin disulfide bond with glutathione. The cells were lysed, and all internalized biotinylated proteins were precipitated by streptavidin, and separated by gel electrophoresis. Immunoblotting was then performed using an antibody against the extracellular domain of TrkB. The total surface biotinylated TrkB was determined in cells held on

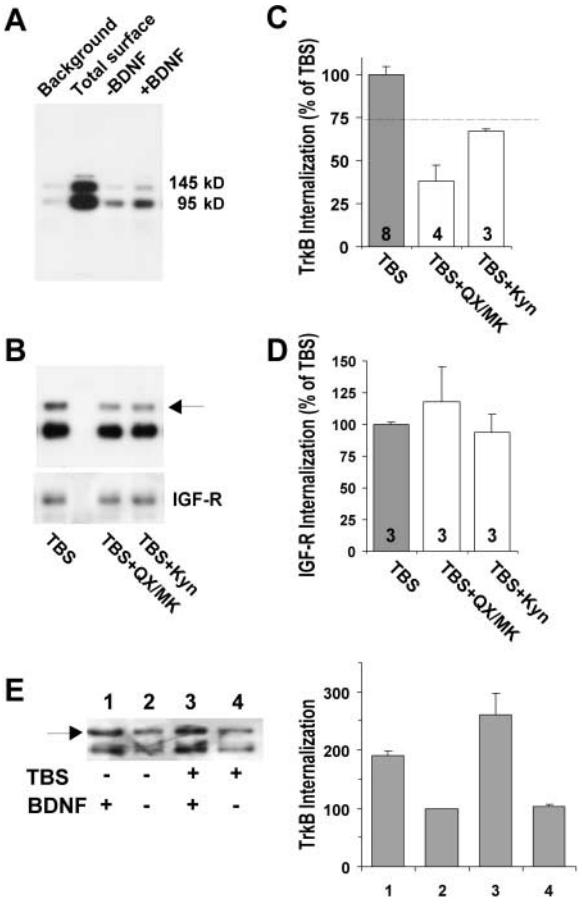

Figure 3. **Effect of TBS on the internalization of TrkB, determined by surface biotinylation.** Cell surface proteins were labeled by NHS-SS-biotin before initiation of TrkB internalization by BDNF at 37°C. The remaining biotin on surface proteins was removed by glutathione. The internalized TrkB was precipitated by streptavidin followed by Western blot using a TrkB antibody. (A) Detection of TrkB internalization by surface biotinylation. Both full-length (145 kD) and truncated (95 kD) TrkB receptors are observed. (Background) Internalization measured in cells debiotinylated without initiation of internalization; (Total surface) surface biotinylated TrkB measured in cells held on ice; (−BDNF) spontaneous TrkB internalization measured in the absence of BDNF; and (+BDNF) TrkB internalization induced by BDNF. (B) Enhancement of BDNF-induced TrkB internalization by TBS. Cultured hippocampal neurons were incubated with BDNF on ice to reach saturated receptor binding. Unbound BDNF was washed off, and surface proteins were labeled by NHS-SS-biotin. The cultures were moved to 37°C to initiate internalization while neurons were stimulated with TBS, with or without QX/MK or Kyn. The internalized membrane proteins were precipitated by immobilized avidin, blotted, and probed first with anti-TrkB antibodies, and then reprobed with anti–IGF1-R antibodies. (C) Quantification on full-length TrkB. The data were normalized to those of TBS stimulation alone (100%). (dashed line) Levels of TrkB internalization in cells not stimulated by TBS. The TBS group is significantly higher than the TBS + QX/MK and TBS + Kyn groups. $P < 0.01$, ANOVA followed by post-hoc test. (D) Quantification on IGF1-R using the same method as for TrkB. (E) Effect of TBS on ligand-independent internalization of TrkB. (left) An example of Western blot; (right) summary of results ($n = 4$). In the absence of the ligand (BDNF), TBS had no effect on the spontaneous internalization of TrkB (no significant difference between lanes 2 and 4). Results from several experiments were averaged and presented as mean ± SEM. The number associated with each column represents the number of experiments performed. (D and E) Data in a specific experimental condition were normalized to the mean in control. (B and E, arrow) Full-length TrkB.

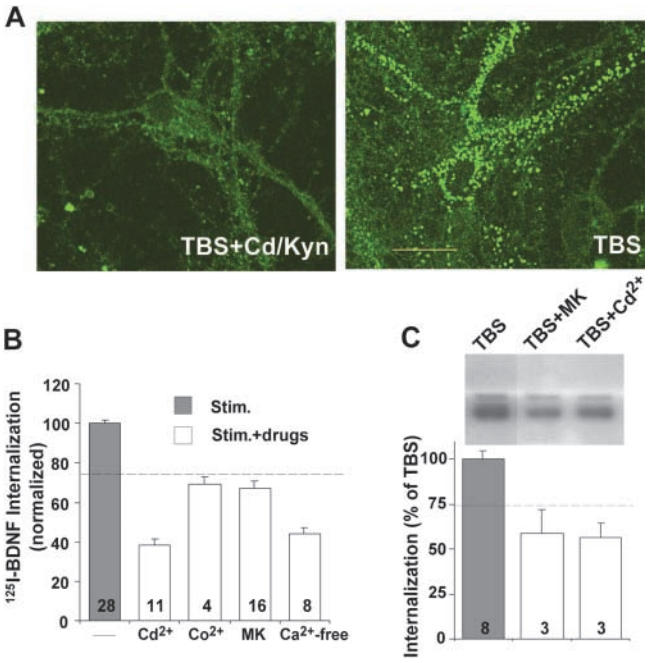

Figure 4. **TrkB internalization in the absence of BDNF or Ca^{2+} influx.** (A) Effect of TBS on BDNF receptor internalization in BDNF−/− neurons derived from BDNF knockout mice. BDNF−/− hippocampal neurons (cultured for 12 d) were stimulated with TBS in the presence (left) or absence (right) of activity blocker Cd^{2+} and Kyn. BDNF-biotin imaging assay similar to that shown in Fig. 1 was performed. Green color was assigned the fluorescent puncta representing internalized BDNF-biotin. Bar, 10 μm. (B) Role of Ca^{2+} influx in TrkB internalization measured by ^{125}I-BDNF binding assay. Controls (TBS in regular medium) are set as 100%. Ca^{2+}-free medium, Ca^{2+} channel blockers Cd^{2+} or Co^{2+}, and NMDA receptor blocker MK801 (MK) all inhibited internalization ($P < 0.001$, ANOVA). (C) Role of Ca^{2+} influx in TrkB internalization measured by the biotinylation assay. Significantly less internalized TrkB receptors were detected in cultures stimulated with TBS in the presence of MK801 or Cd^{2+} ($P < 0.05$, ANOVA). (B and C, dashed line) Levels of TrkB internalization in cells not stimulated by TBS. Results from several experiments were averaged and presented as mean ± SEM. The number associated with each column represents the number of experiments performed.

ice without glutathione cleavage, and background internalization was measured in cells that were debiotinylated without initiation of internalization (Fig. 3 A). A substantial amount of TrkB was internalized when the cultures were warmed to 37°C in the presence of BDNF (Fig. 3 A, +BDNF). Spontaneous internalization of TrkB was also detected in cells not treated with BDNF, although the levels were lower (Fig. 3 A, −BDNF). The difference in these two signals was used to quantify ligand-induced TrkB internalization. Using this assay, we found that BDNF induced significantly more TrkB internalization in active neurons (stimulated with TBS) than in inactive neurons (Fig. 3 B; TBS + CNQX/MK801 or Kyn). Quantitative analysis of the blots showed that the activity blockers reduced TrkB internalization to levels below those by application of BDNF alone (nonstimulated cultures, as indicated by the dashed line in Fig. 3 C). This result again suggests that spontaneous synaptic activity in nonstimulated cultures may also influence BDNF-induced TrkB endocytosis. However, TBS stimulation did not affect spontaneous internalization of insulin-like growth factor-1 receptors (IGF1-R) because reprobing of the same blots with anti–IGF1-R antibody did not detect any significant decrease in cells treated with the activity blockers (Fig. 3, B and D). Thus, neuronal activity does not have a general effect on membrane protein endocytosis. Finally, we found that in the absence of exogenous BDNF, TBS has no effect on TrkB internalization (Fig. 3 E, compare lane 2 with lane 4), suggesting that activity selectively enhances ligand-induced TrkB internalization.

Activity-dependent secretion of BDNF has been observed in hippocampal neurons (Goodman et al., 1996; Mowla et al., 1999). To exclude the possibility that the increase in BDNF receptor internalization was due to an elevated secretion of endogenous BDNF induced by electric stimulation, we measured the internalization in hippocampal neurons derived from BDNF knockout mice using the imaging assay. Again, neurons were treated with BDNF-biotin while stimulated with TBS in the presence or absence of the activity blockers Cd^{2+}/Kyn. As shown in Fig. 4 A, neuronal activity still enhanced BDNF-biotin internalization in cultured BDNF−/− neurons that cannot secrete BDNF at all, suggesting an activity-dependent TrkB internalization in the absence of endogenous BDNF secretion.

A direct consequence of electric stimulation is the influx of Ca^{2+} into the hippocampal neurons. Therefore, we studied the effects of a number of manipulations known to interfere with Ca^{2+} influx through voltage-gated Ca^{2+} channels or NMDA receptors. The ^{125}I-BDNF internalization assay showed that blockade of Ca^{2+} influx prevented the effect of electric stimulation (Fig. 4 B). BDNF internalization was reduced by 52.7% in neurons stimulated by TBS in Ca^{2+}-free medium, as compared with normal medium (Fig. 4 B). BDNF internalization was also reduced when TBS was applied in the presence of the general Ca^{2+} channel blockers Cd^{2+} (0.2 mM) or Co^{2+} (3 mM; Fig. 4 B). Furthermore, treatment of the cultures with the NMDA receptor antagonist MK801 (80 μM) reduced the TBS effect (Fig. 4 B). These results were further confirmed by the biotinylation assay (Fig. 4 C). Thus, activity-dependent modulation of TrkB receptor internalization appears to depend on Ca^{2+} influx through voltage-gated Ca^{2+} channels and/or NMDA receptors.

In the next series of experiments, we determined whether neuronal activity also regulates TrkB tyrosine kinase activity. This was achieved by measuring tyrosine phosphorylation of the TrkB receptor itself, using Western blots with an antibody that specifically recognizes TrkB phosphorylated on the tyrosine residue 490 (pTrkB; Segal et al., 1996). In nonstimulated cultures, there was virtually no detectable pTrkB. Application of 2 nM BDNF to the cultures elicited a rapid phosphorylation of TrkB (Fig. 5 A, group I). Stimulation of the hippocampal neurons with TBS significantly enhanced the auto-phosphorylation of TrkB (Fig. 5 A, compare groups I and III; #, $P < 0.05$, *t* test). Moreover, blockade of synaptic transmission by a cocktail of CNQX/MK801 reduced pTrkB in stimulated cultures (Fig. 5 A, compare groups III and IV; *, $P < 0.01$, *t* test). Thus, the activity-dependent modulation TrkB tyrosine phosphorylation also involves action potentials coupled to excitatory synaptic transmission. In nonstimulated cultures, inhibition of excitatory

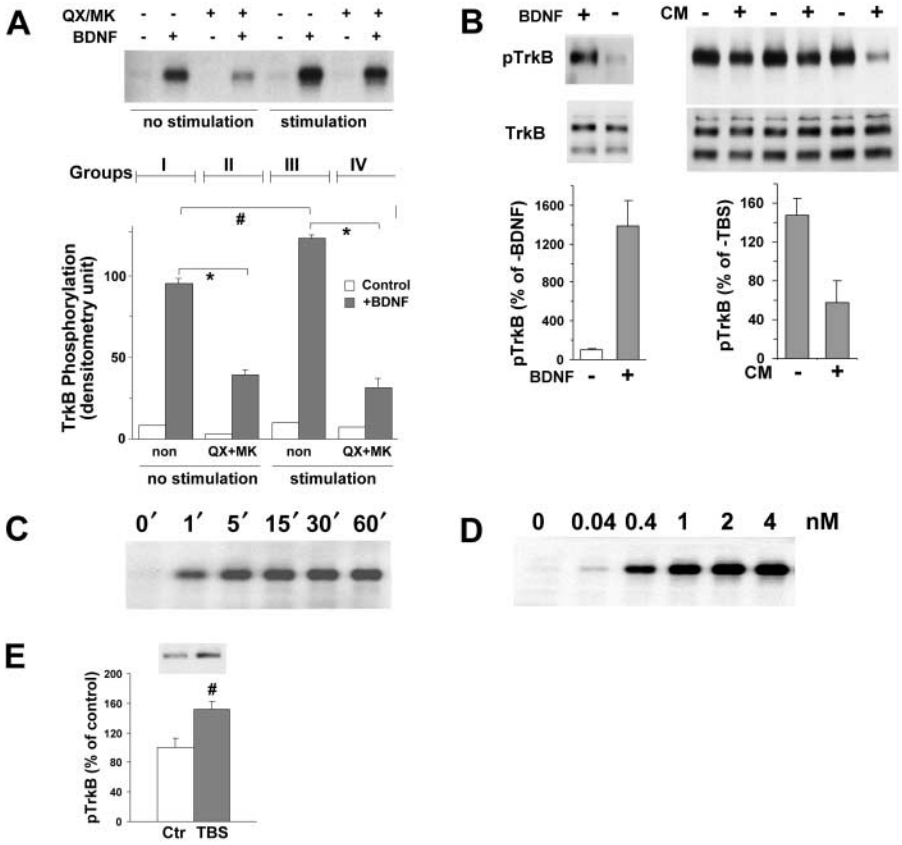

Figure 5. **Regulation of TrkB tyrosine phosphorylation.** TrkB phosphorylation was detected by an anti-pTrkB antibody specific for phosphor-Tyr490. (A) Enhancement of TrkB phosphorylation by TBS. 2 nM BDNF was applied to the cultures under various conditions indicated for 15 min, and cells were harvested for pTrkB assay. (top) A sample Western blot; (bottom) quantification of pTrkB based on six to seven experiments. # and *, significantly lower and higher than control, respectively. $P < 0.01$, ANOVA plus post-hoc. (B) Loading control of the TrkB phosphorylation assay. Blots were reprobed with an anti-TrkB antibody, and signals for pTrkB were normalized to those for total TrkB signal on a lane-by-lane basis. Quantifications are shown below the blots ($n = 4$ for both experiments). (CM) CNQX + MK801. (C) Time course of TrkB phosphorylation induced by 1 nM BDNF application. (D) Dose–response relationship. TrkB phosphorylation was measured 15 min after the application of various concentrations of BDNF. (E) TBS-induced increase in TrkB phosphorylation is not due to an enhanced TrkB surface expression. Hippocampal neurons were pretreated with BDNF on ice, extensively washed, and then switched to 37°C to allow kinase activation. Cells were harvested 15 min later for TrkB phosphorylation assay. #, Significantly different; $P < 0.05$, *t* test.

transmission inhibited TrkB phosphorylation, suggesting that spontaneous firing and/or synaptic transmission in the hippocampal cultures potentiate the TrkB tyrosine kinase activity (Fig. 5 A, compare groups I and II; *, $P < 0.01$). Total TrkB served as an internal loading control, and did not show any significant change in any of these conditions (Fig. 5 B).

Electric stimulation has been shown to induce the secretion of a small amount of BDNF ($\sim 10^{-12}$ M) in cultured hippocampal neurons (Balkowiec and Katz, 2002; Gartner and Staiger, 2002). To examine whether the stimulation-induced BDNF secretion could explain the activity-dependent increase in TrkB tyrosine phosphorylation, we performed the following experiments. First, we tested the possibility that an additional amount of endogenous BDNF secreted from hippocampal neurons could cause more TrkB tyrosine phosphorylation under our experimental conditions. In control cultures, TrkB tyrosine phosphorylation reached its maximal level 15–30 min after BDNF application (Fig. 5 C). Therefore, we performed dose–response experiments at the 15-min time point, and found that maximal TrkB tyrosine phosphorylation was achieved when the concentrations of BDNF approached 1 nM (Fig. 5 D). However, even at 2 nM of BDNF, which induced maximal TrkB phosphorylation in control cultures, electric stimulation still increased TrkB tyrosine phosphorylation (Fig. 5 A, compare groups I and III). Second, electric stimulation alone did not cause any increase in TrkB phosphorylation in the absence of BDNF (Fig. 5 A, top, compare the first lanes of groups I and III). Thus, even if electric stimulation induced BDNF secretion into the culture medium, this amount of BDNF was not sufficient to cause a further increase in TrkB phosphorylation. Our previous work demonstrated that electric stimulation enhances the surface expression of TrkB in these hippocampal neurons (Du et al., 2000). To test whether this phenomenon contributed to the enhancement of TrkB phosphorylation by TBS, we pretreated the hippocampal neurons with BDNF on ice for 30 min to achieve saturated binding, and extensively washed the cells to remove any unbound BDNF. The cultures were switched to 37°C to allow kinase activation for 15 min, with or without TBS stimulation, followed by Western blot analysis with anti-pTrkB antibody. Therefore, the TrkB receptors newly inserted on the cell surface would not have the opportunity to see free BDNF and should not be phosphorylated. Under these conditions, TrkB phosphorylation was still enhanced by TBS (Fig. 5 E). Thus, neuronal activity may have a direct effect on TrkB tyrosine kinase. Together, these results suggest that the activity-induced increase in TrkB tyrosine phosphorylation is due to an increased TrkB tyrosine kinase activity, rather than an increase in BDNF secretion and/or surface expression of TrkB.

Next, we determined whether the activity-dependent enhancement of TrkB tyrosine phosphorylation also requires Ca^{2+} influx. The effect of electric stimulation was significantly attenuated when normal culture medium was re-

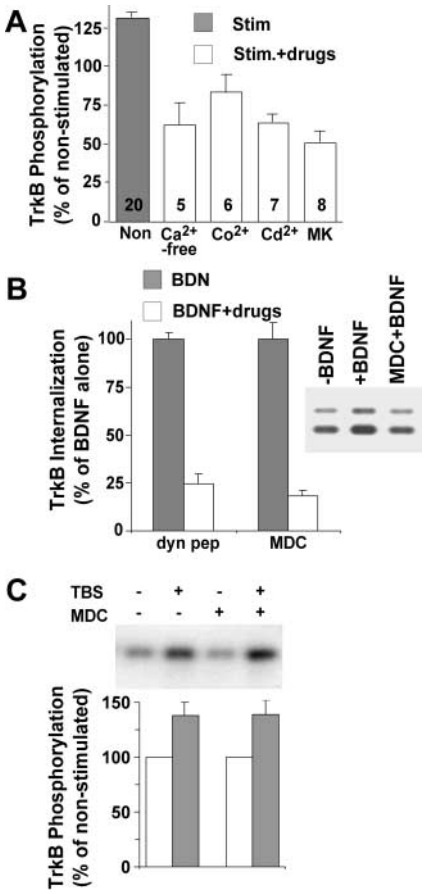

Figure 6. **TrkB phosphorylation in the absence of Ca^{2+} influx or TrkB internalization.** (A) Regulation of TrkB phosphorylation by Ca^{2+} influx. pTrkB in nonstimulated condition in regular medium were set as 100%. Ca^{2+}-free medium, Ca^{2+} blockers Cd^{2+} or Co^{2+}, and NMDA receptor blocker MK801 all significantly inhibit TrkB phosphorylation compared with TBS alone. $P < 0.001$, ANOVA plus post-hoc. Results from several experiments were averaged and presented as mean ± SEM. The number associated with each column represents the number of experiments performed. (B) Effect of MDC on TrkB internalization. Cultures were treated with or without MDC for 15 min, and TrkB internalization was measured by biotinylation assay. (right) Example showing MDC blocks the BDNF-induced TrkB internalization; (histogram) relative effects of dynamin proline-rich domain peptide (dyn pep) and MDC. MDC appears to be just as effective in blocking TrkB internalization as dyn pep, which is known to block clathrin-mediated endocytosis. (C) Effect of MDC on TrkB tyrosine kinase. Cultures were treated with or without MDC for 15 min. BDNF was applied and cells were harvested 15 min later for pTrkB assay ($n = 7$ in all conditions).

placed by Ca^{2+}-free medium (Fig. 6 A). TrkB phosphorylation was also severely reduced when hippocampal neurons were stimulated with TBS in the presence of the general Ca^{2+} channel blockers Cd^{2+} (0.2 mM) or Co^{2+} (3 mM), or the NMDA receptor blocker MK801 (80 μM; Fig. 6 A). Thus, Ca^{2+} influx through voltage-gated Ca^{2+} channels and/or NMDA receptors play an important role in activity-induced potentiation of TrkB tyrosine kinase activity in hippocampal neurons. Specific types of Ca^{2+} channels involved in modulating TrkB tyrosine phosphorylation remain unknown. To determine whether neuronal activity/Ca^{2+} influx enhances the TrkB tyrosine kinase by facilitating TrkB internalization, we measured the effects of electric stimulation on TrkB phosphorylation in the presence of monodansylcadavenrine (MDC), a widely used agent known to block clathrin-mediated receptor internalization for a variety of cell types (Schutze et al., 1999). Application of BDNF to cultured hippocampal neurons induced a significant TrkB internalization over the spontaneous or ligand-independent internalization of TrkB (Fig. 6 B, right, lanes 1 and 2; and Fig. 3 A, lanes 3 and 4). Pretreatment with 10 μM MDC for 15 min reversed BDNF-induced TrkB internalization (Fig. 6 B). The residual bands are likely to represent spontaneous TrkB internalization. To determine the specificity of MDC in hippocampal neurons, we compared its effect with that of a dynamin proline-rich domain peptide, which is known to block clathrin-mediated endocytosis (Wang and Linden, 2000). Treatment of the hippocampal cultures with this peptide, but not the control, scrambled peptide, inhibited BDNF-induced endocytosis to the same extent as MDC (Fig. 6 B). These results suggest that MDC could effectively block BDNF-induced TrkB endocytosis in hippocampal neurons. In MDC-treated cultures, there was no significant change in the TBS-induced increase in TrkB tyrosine phosphorylation (Fig. 6 C), suggesting that the enhancement of TrkB tyrosine kinase by electric stimulation does not require TrkB internalization. Thus, activity-dependent modulation of TrkB tyrosine phosphorylation occurs upstream of TrkB internalization.

Does the activity/Ca^{2+} modulation of TrkB tyrosine kinase contribute to activity-dependent TrkB internalization? A number of approaches were used to answer this question. First, we applied a low concentration (0.2 μM) of k252a, which is known to specifically inhibit the tyrosine kinase activity of Trk receptors (Berg et al., 1992). k252a (pretreated for 30 min) reliably prevented TrkB tyrosine phosphorylation induced by BDNF (Fig. 7 A). Next, we performed the ^{125}I-BDNF binding and internalization assays in the presence or absence k252a. Treatment with k252a significantly inhibited the potentiation effect of TBS on BDNF-induced receptor internalization, without affecting the surface binding in these neurons (Fig. 7 C). Thus, BDNF surface binding and internalization in the hippocampal neurons may be regulated through different mechanisms. The effect of k252a on BDNF receptor internalization was further confirmed by the TrkB biotinylation assay. Treatment with k252a markedly reduced the amount of TrkB receptors internalized by hippocampal neurons in cultures stimulated with TBS (Fig. 7 B). Neurons in the nonstimulated cultures also have ongoing spontaneous neuronal/synaptic activities, which could contribute to the ligand-induced TrkB internalization. To test this possibility, we pretreated the nonstimulated cultures with k252a for 30 min, and then performed the ^{125}I-BDNF binding assay. We found that k252a also inhibited BDNF-induced TrkB internalization in the absence of TBS (Fig. 7 C), suggesting that the tyrosine kinase activity is involved in TrkB endocytosis in nonstimulated hippocampal neurons.

k252a inhibits the tyrosine kinase activity of all Trks. As an alternative and more specific way to inhibit TrkB tyrosine kinase, we transfected a dominant-negative TrkB mutant (kinase dead TrkB; $TrkB^{kd}$; Atwal et al., 2000) with cDNA for

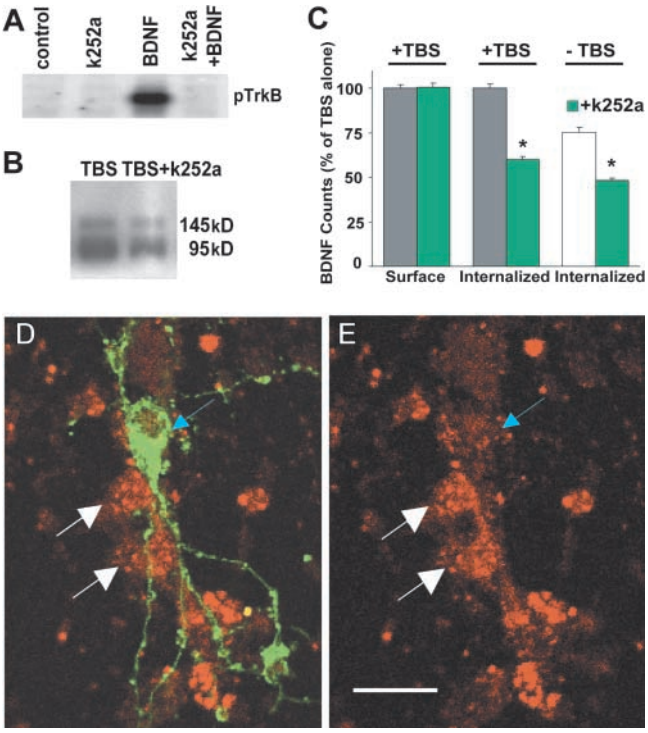

Figure 7. **Role of tyrosine kinase in activity-dependent modulation of TrkB internalization.** (A) Inhibition of TrkB phosphorylation by k252a. k252a was applied 1 h before BDNF application, and the hippocampal cultures were harvested 15 min later to measure TrkB phosphorylation. (B) Inhibition of TrkB internalization by k252a. Hippocampal cultures were pretreated with or without 0.2 μM k252a, and internalized TrkB was measured by biotinylation assay with or without TBS (n = 5–7). (C) Attenuation of BDNF receptor internalization, but not on surface binding, by k252a. Binding and internalization assays were performed in cultures stimulated with or without TBS and averaged counts for binding and internalization in TBS-stimulated cultures were set as 100% (gray bars). The data from nonstimulated cultures were normailzed to the stimulated cultures (white bars); n = 12 for TBS-stimulated cultures, and n = 4 for nonstimulated cultures. *, $P < 0.001$, t test. (D and E) Inhibition of TrkB internalization by dominant-negative TrkB ($TrkB^{kd}$). Hippocampal neurons were transfected with $TrkB^{kd}$ tagged with a myc indicator. Internalization was visualized by BDNF-biotin. (D) The superimposed image of a $TrkB^{kd}$-transfected cell (green, detected by an anti-myc antibody) and cells that internalized BDNF-biotin (red). (E) The same image as D with BDNF-biotin staining only. White arrows indicate $TrkB^{kd}$ negative neurons; blue arrows indicate a $TrkB^{kd}$-positive cell. Note the difference in BDNF-biotin fluorescence spots between these two types of cells. Bar, 20 μm.

GFP directly into cultured hippocampal neurons. The $TrkB^{kd}$ has been mutated on its ATP binding domain, and contains a myc-tag at its NH_2 terminus (Atwal et al., 2000). Staining with anti-myc antibody confirmed that all GFP positive cells expressed myc-containing TrkB mutant (unpublished data). Approximately 20% of neurons were transfected, as indicated by myc-tag immunofluorescence (Fig. 7 D, green). BDNF-biotin staining was used to measure BDNF receptor internalization (Fig. 7 D, red). Incubation of BDNF-biotin at 37°C for 20 min resulted in a substantial BDNF receptor internalization in untransfected cells (Fig. 7 E, white arrows). In marked contrast, neurons transfected with $TrkB^{kd}$ in the same culture exhibited fewer fluorescent puncta (Fig. 7 E, blue arrow). Of 19 $TrkB^{kd}$ transfected neurons, only one showed very limited internalization, whereas in 113 out of 124 nontransfected neurons, a substantial number of fluorescent spots was observed in their cell bodies and dendrites. Transfection of wild-type TrkB did not affect BDNF receptor internalization (unpublished data). These results strongly suggest that inhibition of TrkB tyrosine kinase prevents the activity-dependent modulation of TrkB internalization.

Discussion

In many cases, BDNF acts preferentially on active central nervous system neurons/synapses. An important but unresolved question in neurotrophin research is how diffusible molecules such as BDNF modulate active neurons/synapses without affecting their less active neighbors. Such modulation requires that neurotrophic actions be restricted to active neurons/synapses (Lu, 2003). One way to achieve this is to have local secretion and restricted diffusion of BDNF at or near the active synapses (Biffo et al., 1995; Wang et al., 1998). Alternatively, active neurons may be more susceptible to BDNF modulation. This could be manifested by activity-dependent enhancement of TrkB expression on the cell surface, facilitation of TrkB internalization, and/or potentiation of TrkB signaling. It has been previously shown that neuronal activity and Ca^{2+} influx facilitate the insertion of TrkB onto neuronal surface membranes (Meyer-Franke et al., 1998; Du et al., 2000). Here, we show that electric stimulation potentiates both the internalization and the tyrosine kinase signaling of the TrkB receptor in hippocampal neurons, and that both effects require Ca^{2+} influx. Surprisingly, neuronal activity facilitates TrkB internalization by enhancing TrkB tyrosine kinase. Thus, this work reveals a novel mechanism by which BDNF signaling and function may be restricted to active neurons/synapses.

Unlike other tyrosine kinase receptors, internalization of Trk receptors often serves as an important step that mediates some biological functions of neurotrophins, rather than as a process that inactivates neurotrophin signaling. For example, substantial evidence suggests that TrkA internalization is required to initiate cell body responses to target-derived NGF (Grimes et al., 1996; Bhattacharyya et al., 1997; Riccio et al., 1997). Blockade of TrkA internalization by dominant-negative dynamin prevents NGF-induced neurite outgrowth in PC12 cells (Zhang et al., 2000). At the neuromuscular synapses, acute application of NT3 rapidly potentiates transmitter release (Lohof et al., 1993), whereas long-term treatment with NT3 induces both structural and functional changes at the neuromuscular synapses (Wang et al., 1995). Using dominant-negative dynamin and bead-conjugated NT3, we recently found that the long-term, but not acute, synaptic modulation by NT3 requires dynamin-dependent internalization of the NT3 receptor TrkC (unpublished data). Thus, the internalization of Trk receptors is critical for many neurotrophin actions, and modulation of this process by neuronal activity has a profound physiological relevance.

The internalization process could be measured semi-quantitatively by ^{125}I-BDNF binding or the TrkB biotinylation. However, the latter seemed to detect bigger effects of neu-

ronal activity (Fig. 3 C; TBS-stimulated neurons exhibited twice as much internalization compared with neurons stimulated by TBS in the presence of activity blockers) than the former (Fig. 2 D; internalization was 33–55% more in active neurons than in inactive neurons). A possible explanation is that ^{125}I-BDNF assay may reflect the internalization of both TrkB and p75NR. The presence of p75NR, which is expressed at a quite low level in these hippocampal neurons (Frade et al., 1996), could interfere with the measurement of the TrkB signals that are specifically regulated by neuroelectric activity. Thus, although less efficient, the TrkB-biotinylation assay was more reliable in quantifying TrkB internalization. Using this assay, we often observed that manipulation of neuroelectric activity affected the internalization of both full-length and truncated TrkB receptors, which lack the kinase domain. In contrast, several lines of experiments clearly indicated that TrkB tyrosine kinase activity is important for the activity-dependent modulation of internalization. Therefore, it is puzzling why the internalization of the kinase-deficient truncated TrkB was still regulated by neuronal activity. One possibility is that the full-length and the truncated TrkB receptors are located very close to each other on the cell surface. When exposed to BDNF, the full-length receptor is internalized, carrying the truncated TrkB in the same endocytotic vesicles into the hippocampal neuron.

Neuronal activity has recently been shown to rapidly activate TrkB, but this effect has been interpreted as a consequence of activity-dependent secretion of BDNF (Aloyz et al., 1999; Patterson et al., 2001). Two pieces of evidence suggest that the activity-dependent enhancement of TrkB tyrosine kinase in our cultured hippocampal neurons is not due to an elevated BDNF secretion: (1) in the absence of BDNF, electric stimulation did not activate TrkB receptor; and (2) electric stimulation still increases TrkB tyrosine phosphorylation induced by BDNF at a saturated concentration. Thus, we have observed a direct effect of neuroelectric activity on TrkB tyrosine kinase. The fact that electric stimulation potentiates TrkB tyrosine phosphorylation when TrkB internalization is blocked by MDC, and that inhibition of TrkB tyrosine kinase attenuates TrkB internalization, suggests that activation of TrkB tyrosine kinase is upstream of its internalization. Neuroelectric activity and Ca^{2+} influx potentiate both the insertion and the internalization of TrkB, but these two effects differ in several ways. The internalization of TrkB is triggered by the ligand BDNF, and is regulated by its tyrosine kinase. In contrast, TrkB insertion is ligand independent, and is not influenced by TrkB tyrosine kinase activity. Moreover, high frequency stimulation is required for TrkB insertion, whereas low frequency neuronal firing seems to be sufficient to enhance Trk internalization. Thus, the molecular mechanisms underlying the insertion and internalization of TrkB receptor may be quite different.

The results in this work may have a number of implications in the cell biology of tyrosine kinase receptors. First, we report the potentiation of TrkB tyrosine kinase by neuronal activity and Ca^{2+} influx. To our knowledge, this is the first demonstration for Ca^{2+}-dependent modulation of Trk kinases, and perhaps receptor tyrosine kinases in general. Thus, our results suggest a cross-talk between Ca^{2+} and tyrosine kinase signaling pathways. The molecular mechanisms underlying such a cross-talk remain to be investigated. Second, this work reveals an important regulatory effect of neuronal activity and Ca^{2+} influx on the internalization/endocytosis of the TrkB, a tyrosine kinase receptor. This process resembles in many ways the endocytosis of AMPA-type glutamate receptors, which is implicated in the mechanism for long-term depression in the hippocampus (Carroll et al., 2001). It will be interesting to determine whether the trafficking of AMPA receptors and TrkB receptors share similar underlying mechanisms. Finally, we show that inhibition of TrkB tyrosine kinase dramatically attenuates the activity and Ca^{2+} regulation of TrkB internalization, suggesting a key role of tyrosine phosphorylation in TrkB endocytosis. Ligand-induced endocytosis of EGF receptor has been shown to require the tyrosine kinase activity of the receptor (Lamaze and Schmid, 1995). This is achieved by EGF receptor-dependent activation of Src tyrosine kinase, leading to phosphorylation and redistribution of clathrin, a major player in ligand-induced endocytosis (Wilde et al., 1999). It is tempting to speculate that TrkB tyrosine kinase also phosphorylates and modulates some common molecules involved in endocytosis. Together, these results suggest a general role of tyrosine kinase in the endocytosis of growth factor receptors.

Materials and methods

Culture preparations and electric stimulation

Hippocampal cultures (11–14 d) and electric stimulation were performed as described previously (Du et al., 2000). Fresh medium was applied 24 h before each experiment. Drugs were applied immediately before electric stimulation. The entire electric stimulation was performed in a 37°C, 5% CO_2 incubator. Some experiments were done using hippocampal neurons derived from E16 BDNF knockout mice. Hippocampi from E16 embryos derived from crosses between BDNF heterozygous mice (+/− × +/−) were dissected, dissociated, and plated. Each embryo was handled individually to avoid cross-contamination. A piece of tail tissue was taken for genotyping. Only neurons (12 d in culture) from homozygous BDNF embryos (−/−) were used for internalization experiments.

Biotinylation of BDNF and imaging assay

100 μg BDNF (provided by Regeneron Pharmaceuticals, Inc.) was incubated with 2 mg NHS-LC-Biotin (Pierce Chemical Co.) in 100 μl PBS with Ca^{2+} and Mg^{2+} for 2 h at 4°C. Biotinylated BDNF and unbound biotin were separated with a desalting gel column (D-Salt™ polyacrylamide 1800; Pierce Chemical Co.). 20 nM biotinylated BDNF (BDNF-biotin; determined by Bio-Rad Laboratories protein assay) with or without cold BDNF (200-fold excess) was applied to cultured neurons in DME containing 0.5% protamine and 10 mM Hepes for 30 min on ice. Unbound BDNF-biotin was washed out with culture medium. Internalization was initiated by applying warm media (37°C) to the cultures with or without various inhibitors. After 30 min of incubation, the cultures were washed with ice-cold acid for 20 min to remove the surface-bound BDNF. The cultures were fixed in 4% PFA in PBS and permeabilized; and the internalized BDNF-biotin was visualized by Cy3-conjugated avidin (1:500; Jackson ImmunoResearch Laboratories) in 0.4% Triton X-100, 5% goat serum in PBS. The cells were mounted by mounting media and visualized by a 510-meta confocal microscope. Three-dimensional images were reconstructed using the Z stack function (20 sections from top to bottom, 1 μm/section). The images in the middle range of the Z stack were used to examine the internalization particles.

^{125}I-BDNF receptor binding and internalization assays

The ^{125}I-BDNF binding and internalization assays were performed as described previously (Du et al., 2000). BDNF surface binding was obtained by acid wash to remove the ^{125}I-BDNF bound to cell surface (0.2 M acetic acid and 0.5 M NaCl). BDNF receptor internalization was obtained by

measuring the remaining radioactivity inside the cells. To determine the total BDNF surface binding without internalization, the binding assay was performed at 4°C for 4 h.

Biotinylation assay of TrkB internalization

Hippocampal neurons were treated with 8 nM BDNF for 30 min on ice. Unbound BDNF was washed off three times with cold PBS. The cell surface proteins were labeled with 0.5 mg/ml NHS-SS-biotin (Pierce Chemical Co.) in PBS with Ca^{2+} and Mg^{2+} for 2.5 min at 37°C, and then washed extensively with ice-cold PBS. Internalization was initiated by switching to warm media (37°C) for 30 min. The remaining, biotinylated surface proteins were debiotinylated by washing with glutathione buffer (50 mM reduced glutathione, 100 mM NaCl, 1 mg/ml BSA, 1 mg/ml glucose, and 50 mM Tris, pH 8.6) for 3× 30 min at 4°C. The cells were washed an additional two times with PBS and harvested with the lysis buffer (the same as that for Western blot). The internalized, biotinylated proteins were precipitated by immobilized streptavidin, separated by SDS-PAGE, and subjected to Western blot using a monoclonal anti-TrkB antibody (1:200; Transduction Labs), or a polyclonal anti-IGF1 receptor antibody (1:100; Sigma-Aldrich).

BDNF-induced TrkB internalization was effectively inhibited by either MDC or dynamin proline-rich peptide (dyn-pep; Wang and Linden, 2000). We made the dyn-pep with a leading Tat sequence from HIV known to facilitate penetration of the peptide into the cells (RKKRRQRRRQVPSRPNRAP). A scrambled peptide, RKKRRQRRRQPPASNPRVR, was used as a control. Hippocampal neurons (12 d in culture) were incubated with the peptides (80 μM) for 1 h before the TrkB biotinylation experiments.

Western blot

Western blots were performed as described previously (Du et al., 2000). TrkB phosphorylation was detected by an antibody specifically recognizing the phosphorylated tyrosine residue 490 (1:500; New England Biolabs, Inc.) in 0.5% BSA in TBST (1:500), followed by ECL detection (Pierce Chemical Co.). Because these neurons do not express TrkA, and TrkC runs at a lower molecular mass, the 145-kD band seen on the gel represents phospho-TrkB.

Data analysis

The blots after ECL reactions were exposed to film (Kodak) at different exposure times. The films were scanned, and the intensities of the bands, as well as that of the background near the bands, were measured by Kodak 3.0 software. The specific signals were obtained by subtracting the background values from the total intensities. To ensure that the densitometric values faithfully reflected the relative levels of TrkB phosphorylation or internalization, the following measures were taken. First, only the films in which immunoreactive bands fell within the linear range (not saturated) were used for quantification. Second, multiple lanes of the same samples were often included in the same blot to obtain average values of a specific condition. Finally, the same experiments were repeated at least three to five times, using independent samples. For ^{125}I-BDNF assay, raw data (quadruplicates) from a specific experimental condition were normalized to the mean in TBS-stimulated condition. The results in 8–32 experiments were pooled and averaged, and presented as mean ± SEM.

To facilitate cross-experiment comparison, we generally set the average value for internalization in TBS-stimulated cells as 100%, and normalize data on all other conditions to that in "stimulation alone" condition in both ^{125}I-BDNF binding assay and TrkB biotinylation assay. To distinguish the "phosphorylation" data from the "internalization" data, we normalized all the TrkB phosphorylation data to "nonstimulated" condition.

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