# Activity- and $Ca^{2+}$-dependent Modulation of Surface Expression of Brain-derived Neurotrophic Factor Receptors in Hippocampal Neurons

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***Abstract.*** Brain-derived neurotrophic factor (BDNF) has been shown to regulate neuronal survival and synaptic plasticity in the central nervous system (CNS) in an activity-dependent manner, but the underlying mechanisms remain unclear. Here we report that the number of BDNF receptor TrkB on the surface of hippocampal neurons can be enhanced by high frequency neuronal activity and synaptic transmission, and this effect is mediated by $Ca^{2+}$ influx. Using membrane protein biotinylation as well as receptor binding assays, we show that field electric stimulation increased the number of TrkB on the surface of cultured hippocampal neurons. Immunofluorescence staining suggests that the electric stimulation facilitated the movement of TrkB from intracellular pool to the cell surface, particularly on neuronal processes. The number of surface TrkB was regulated only by high frequency tetanic stimulation, but not by low frequency stimulation. The activity dependent modulation appears to require $Ca^{2+}$ influx, since treatment of the neurons with blockers of voltage-gated $Ca^{2+}$ channels or NMDA receptors, or removal of extracellular $Ca^{2+}$, severely attenuated the effect of electric stimulation. Moreover, inhibition of $Ca^{2+}$/calmodulin-dependent kinase II (CaMKII) significantly reduced the effectiveness of the tetanic stimulation. These findings may help us to understand the role of neuronal activity in neurotrophin function and the mechanism for receptor tyrosine kinase signaling.

Key words: TrkB receptors • tetanic stimulation • calcium influx • $Ca^{2+}$/calmodulin-dependent kinase II • synaptic transmission

## *Introduction*

Brain-derived neurotrophic factor (BDNF),[1] a member of the neurotrophin family, is a potent neurotrophic protein that regulates neuronal survival and differentiation (Lewin and Barde, 1996). Signal transduction and neurotrophic functions of BDNF are mediated primarily by TrkB, a high affinity receptor tyrosine kinase (Kaplan and Stephens, 1994). BDNF is also capable of binding to the low affinity receptor p75 (p75NR) and eliciting apoptotic function in certain populations of neurons (Casaccia-Bonnefil et al., 1996, 1998; Frade et al., 1996). Evidence accumulated in the last few years suggests that BDNF is involved in synapse development and plasticity, in addition to its traditional role in neuronal survival and differentiation (Thoenen, 1995; Bonhoeffer, 1996; Berninger and Poo, 1996; Lu and Chow, 1999; McAllister et al., 1999). BDNF has been shown to exert complex modulation of dendritic and axonal growth in the brain, particularly in the visual system (Cohen-Cory and Fraser, 1996; Cohen-Cory and Lom, 1999; McAllister et al., 1995, 1996, 1997). BDNF is also involved in activity-dependent synaptic competition and formation of ocular dominance columns in the visual cortex (Maffei et al., 1992; Gu et al., 1994; Cabelli et al., 1995; Riddle et al., 1995; Galuske et al., 1996; Huang et al., 1999). BDNF is capable of rapidly regulating synaptic transmission at the neuromuscular junction and central nervous system (CNS) synapses (Lohof et al., 1993; Knipper et al., 1994; Lessmann et al., 1994; Levine et al., 1995; Takei et al., 1997). In the hippocampus, BDNF promotes tetanus-induced long-term potentiation (LTP; Korte et al., 1995, 1996; Figurov et al., 1996; Patterson et al., 1996; Kang et al., 1997). Moreover, BDNF selectively enhances high frequency but not low frequency synaptic transmission (Tanaka et al., 1997; Frerking et al., 1998; Gottschalk et al., 1998). Recent experiments using BDNF knockout

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[1]*Abbreviations used in this paper:* AIP, autocamtide-2-related inhibitory peptide; BDNF, brain-derived neurotrophic factor; CaMKII, $Ca^{2+}$/calmodulin-dependent kinase II; CNS, central nervous system; CNQX, 6-cyano-7-nitroquinozaline-2,3-dione; kyn, kynurenic acid; LTD, long-term depression; LTP, long-term potentiation; p75NR, low affinity receptor p75; TBS, theta burst stimulation; TTX, tetrodotoxin.

mice demonstrate that BDNF enhances high frequency synaptic transmission by facilitating synaptic vesicle docking in the hippocampus, possibly by increasing the levels of the vesicle protein synaptobrevin in the presynaptic terminals of CA1 synapses (Pozzo-Miller et al., 1999).

Based on the above discoveries, BDNF has been proposed to participate in several forms of activity-dependent plasticity in the CNS (Thoenen, 1995; Bonhoeffer, 1996; Lu and Chow, 1999). A critical element of such proposition is that BDNF acts preferentially on active neurons. Indeed, blockade of neuronal activity and synaptic transmission prevents the increase of dendritic arborization induced by BDNF (McAllister et al., 1996). BDNF cannot enhance the survival of retinal ganglion neurons unless they are depolarized by high $K^+$ or glutamate agonists, or their intracellular cAMP is increased (Meyer-Franke et al., 1995). Presynaptic depolarization greatly facilitates the BDNF modulation of synaptic transmission at the neuromuscular junction (Boulanger and Poo, 1999). In the hippocampus, the effect of BDNF on CA1 synapses is observed only when presynaptic neurons are stimulated at high frequency (Gottschalk et al., 1998). These results support the notion that certain levels of neuronal activity are required for neuronal responsiveness to BDNF.

As a diffusible molecule, how does BDNF distinguish active and inactive neurons or synapses, and restrict its action preferentially on active neurons/synapses? One possible mechanism is that cellular responsiveness of neurons to BDNF is enhanced by neuronal activity. Thus, whether or how well a neuron can respond to BDNF may depend on its activity levels. It is unclear, however, how activity-dependent regulation of BDNF responsiveness is achieved. Neuronal activity could increase the number of BDNF receptors on the cell surface, facilitate the internalization of BDNF–receptor complex, or facilitate the signaling mechanisms for BDNF. Depolarization or cAMP elevation has been shown to increase the levels of the BDNF receptor TrkB on the cell surface of retinal ganglion cells and spinal neurons (Meyer-Franke et al., 1998). Here we investigate whether physiologically relevant stimuli such as electric stimulation can modulate the BDNF receptors on the cell surface of neurons in the hippocampus, where activity-dependent plasticity is most commonly observed. Using three independent approaches (biotinylation, receptor binding, and immunocytochemistry), we show that high frequency tetanic stimulation, but not low frequency stimulation or simple depolarization, can rapidly enhance the insertion of TrkB into the cell surface. We also demonstrate that the activity-dependent modulation requires $Ca^{2+}$ influx through NMDA and $Ca^{2+}$ channels, and involves $Ca^{2+}$/calmodulin-dependent kinase II (CaMKII). Not only may these findings provide insights into the mechanistic link between activity-dependent and neurotrophic modulation of CNS neurons and synapses, but they may also have general implications in the cell biology of growth factor signaling.

## Materials and Methods

### Culture Preparations

Cultures of hippocampal neurons were prepared according to the published procedure (Feng et al., 1999) with minor modifications. In brief, hippocampus was dissected from embryonic day 18 rats, dissociated in $Ca^{2+}$- and $Mg^{2+}$-free HBSS containing 0.125% tyrosine for 15 min, triturated in DMEM/10% FBS, and plated at $2 \times 10^5$ cells per well in 12-well plates. Cells were grown at 37°C, 5% $CO_2$ and 95% humidity, first in 10% FBS/DMEM, and 1 d later switched to serum-free medium Neurobasal plus B27 (Life Technologies). Cultures were grown in serum-free medium for 11–14 d before being used for experiments, and the medium was changed every 3 d. Fresh medium was applied 24 h before each experiment. These cultures yielded virtually pure neurons (data not shown). Drugs were applied immediately before electric stimulation. In some cases, serum-free medium was replaced with $Ca^{2+}$-free medium ($Ca^{2+}$-free DMEM; Life Technologies) for 30 min before electric stimulation.

### Electric Stimulation of Neuronal Cultures

Hippocampal neurons were stimulated using a method similar to one that has been described previously (Bito et al., 1996; Deisseroth et al., 1996; Fields et al., 1997). Field electric stimulation was applied across a 12-well dish through a homemade lid, which contained platinum wires contacting the medium in each well. Each stimulation pulse (1 msec, 2–8 V) was sufficient to elicit action potentials in these cultured neurons (see Fig. 1 A). The entire electric stimulation was performed in a 37°C, 5% $CO_2$ incubator. The following stimulation paradigms were used. (i) TBS: each episode consisted of four bursts, each with five biphasic pulses at 100 Hz (10-msec interval), separated by an interburst interval of 200 msec. One episode was given every 5 s throughout the whole incubation period. (ii) Tetanic stimulation: 1 s, 100 Hz, given every 10 min for for 30 min. (iii) Low frequency stimulation: 0.16 Hz during the entire incubation period. (iv) Long-term depression (LTD)-inducing stimulation: 4 min, 5 Hz. Whole-cell recording was performed under the current-clamped or voltage clamped conditions as previously described (Kim et al., 1994). Data were collected by an Axopatch 200B amplifier, filtered at 5 kHz, digitized at 10 kHz, and analyzed by P-clamp software (Axon Instruments).

### Surface Biotinylation and Western Blot Analysis of TrkB

Surface TrkB receptors was measured by biotinylation followed by Western blot using either a TrkB antibody or a p75NR antibody, as described elsewhere (Meyer-Franke et al., 1998). In brief, various blockers were added to the hippocampal cultures, and electric stimulation was applied immediately in a 37°C incubator. At the end of electric stimulation (60 min), ice-cold PBS, pH 7.4, with $Ca^{2+}$ and $Mg^{2+}$, pH 7.4; Life Technologies) was added to the cultures to prevent receptor internalization. After three washes with ice-cold PBS, cells were incubated in Sulfo-NHS-LC-biotin (0.25 mg/ml in cold PBS; Pierce) for 30 min. The surface biotinylation was stopped by removing the above solution and incubating the cells in 10 mM ice-cold glycine in PBS for 20 min. Cells were then washed three times with cold PBS and lysed by RIPA buffer, which contains 20 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM EGTA, 1 mM $Na_3VO_4$, 50 mM NaF, 1% NP-40, 1% deoxycholate, 0.1% SDS, 1 mM [4-(2-aminoethyl)-benzenesulfluoride hydrochloride], 10 μg/ml leupeptin, and 1 μg/ml aprotinin. Biotinylated proteins (160 μg) were precipitated with 100 μl of ImmunoPure Immobilized Streptavidin (Pierce). Western blots were performed by separating the biotinylated protein precipitates on SDS-PAGE gel and transferring the proteins to Immobilon *P* membrane. The membranes were probed with a monoclonal anti-TrkB antibody (1:250; Transduction Laboratories), or an anti-p75NR antibody (1:250; Upstate Biotechnology), followed by peroxidase-conjugated goat anti–rabbit IgG (1:10,000; Vector Laboratories). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech). The ECL signal intensities were quantified by NIH Image program. To measure the total amount of TrkB, cultured hippocampal neurons were simply harvested by RIPA buffer and processed for Western blot. Quantitation for each experimental condition was based on three to six independent experiments (samples), each was repeated at least two to three times. The results were pooled and averaged, and presented as mean ± SE.

### BDNF Receptor Binding

Binding assays were performed in hippocampal cultures in a 37°C, 5% $CO_2$ incubator in quadruplicates. In brief, cells were washed three times with warm DMEM, and then incubated in binding buffer (DMEM plus 0.5

mg/ml protamine sulfate and 10 mM Hepes, pH 7.4) containing $I^{125}$-BDNF (2,200 Ci/mmol, $5 \times 10^{-11}$ M; NEN Life Science Products) with or without excess cold BDNF ($5 \times 10^{-8}$ M; provided by Regeneron Pharmaceuticals, Inc.) for 30 min. During the entire period of incubation, the hippocampal neurons were electrically stimulated in the incubator in the presence or absence of various blockers. After incubation, the 12-well dishes were placed on ice to prevent receptor internalization. Nonspecifically bound BDNF was removed by washing three times with 1 ml of ice-cold PBS. The $I^{125}$-BDNF bound to cell surface was obtained by a 10-min acid wash on ice (0.2 M acetic acid, pH 2.2, 0.5 M NaCl, 0.5 ml), and the counts were used as the measure for BDNF surface binding. An LKB $\gamma$ counter was used to count the radioactivity. Raw data (quadruplicates) from a specific experimental condition were normalized to the mean in control condition. The results in several experiments were pooled and averaged, and presented as mean ± SE.

### *Immunofluorescence Staining of TrkB Receptors*

To visualize surface TrkB, cultured hippocampal neurons were fixed with 2% paraformaldehyde, 120 mM sucrose in PBS at room temperature for 3 min. After paraformaldehyde was quenched with 0.1 M glycine in PBS, the nonspecific binding was blocked with 50% goat serum, 1% BSA, and 100 mM lysine in PBS for 40 min. The cells were then incubated with a chicken antibody against extracellular domain of TrkB (a gift from Dr. Louis Reichardt, University of California, San Francisco, CA) in blocking solution overnight at 4°C, or in room temperature for 40 min. The secondary antibody was Cy3-conjugated goat anti–chicken Y antibody (1:100; Jackson ImmunoResearch Laboratories). After several washes, cells were mounted with mounting medium Vectashield (Vector Laboratories). To visualize both surface and intracellular TrkB, the cells were fixed with 4% paraformaldehyde, 120 mM sucrose in PBS for 20 min at room temperature, followed by quenching with 0.1 M glycine in PBS. The cells were permeabilized and nonspecific binding was blocked with 10% goat serum, 0.4% Triton X-100 in PBS for 40 min at room temperature. The cells were stained with rabbit anti-TrkB (1:50; Chemicon) overnight at 4°C. After several washes the cells were incubated with Cy3-conjugated anti–rabbit antibody (1:200; Jackson ImmunoResearch Laboratories) in 5% goat serum in PBS for 1 h at room temperature. The cells were washed three times and then mounted with Vectashield. Fluorescence images were acquired by a MicroMax 1300 cool CCD camera mounted on a Nikon Eclipse E800 microscope, and assigned to a pseudo color (green or red). In some cases, immunostained cells were examined using a confocal microscope (MRC1024; Bio-Rad). The images were processed by IPLab software. Each experimental condition was repeated at least three times.

### *Antennapedia Fusion Peptide Experiments*

The autocamtide-2–related inhibitory peptide (AIP) was as a specific inhibitor for CaMKII (Ishida et al., 1995). To facilitate the translocation of AIP across the cell membrane, we fused AIP to a 16-residue antennapedia homeopeptide (antp-AIP; Prochiantz, 1996; Passafaro et al., 1999). A control peptide was made using antp and scrambled AIP sequences (antp-AIPscm). This scrambled sequence was analyzed by Program Blastp, and no significant similarity was found in the current database. The sequences of the peptides are listed below:

[H]-RQIKIWFQNRRMKWKKALRRRAVEDAL—Antp-AIP

[H]-RQIKIWFQNRRMKWKKRLAAADLVERR—Antp-AIPscm

The peptides were synthesized and HPLC-purified by Princeton Biomolecules. To allow sufficient penetration of the peptides into the cytoplasm of hippocampal neurons, the cells were pretreated with Antp-AIP or Antp-AIPscm (20 μM) 3 h before the biotinylation or binding assay was performed.

## *Results*

BDNF receptors on the surface of cultured hippocampal neurons were determined by a biotinylation assay. All membrane proteins were biotinylated, followed by precipitation with ImmunoPure Immobilized Streptavidin and Western blot analysis using antibodies against the high affinity TrkB receptors or the p75NRs. Field electric stimulation was applied to the culture dishes in a 37°C incubator to induce neuronal firings (Bito et al., 1996; Deisseroth et al., 1996; Fields et al., 1997). Whole-cell current clamp recording indicated that field stimulation reliably elicited action potentials (Fig. 1 A). The firing patterns of the neurons followed well with either TBS (Fig. 1 A) or 100 Hz tetanus (data not shown). When equal amounts of biotinylated proteins were loaded into the SDS gel, significant differences in the amount of both full-length (145 kD, or p145) and truncated (95 kD, or p95) forms of TrkB receptors on the cell surface were observed between active and inactive hippocampal neurons (Fig. 1, B and C). Neurons stimulated with TBS exhibited significantly more surface TrkB as compared with those in unstimulated cultures (Fig. 1, B1 and C). To control for any nonspecific effects of electric stimulation on surface expression of TrkB, we performed most of our experiments in cultures stimulated with TBS either alone (active) or in the presence of activity blockers (inactive). The stimulation alone group is referred to as control (Ctr). Tetrodotoxin (TTX; 1 μM), which blocks $Na^+$ channels and therefore all neuronal action potentials, significantly reduced the amount of surface TrkB (Fig. 1 C). TBS had no effect on surface expression of p75NRs (Fig. 1 D), suggesting that the effect of TBS is specific for TrkB receptors. In these cultures, neurons were well connected, and electric stimulation often elicited excitatory synaptic transmission (data not shown). Inhibition of excitatory transmission, either by the general glutamate receptor antagonist kynurenic acid (kyn; 1 mM) or a combination of the non-NMDA receptor antagonist 6-cyano-7-nitroquinozaline-2,3-dione (CNQX; 100 μM) and the NMDA receptor antagonist MK801 (80 μM), significantly attenuated the TBS-induced increase in surface TrkB (Fig. 1, B1 and C). Blockade of high frequency transmission by kyn had no effect on surface p75NR (Fig. 1 D). Thus, high frequency neuronal activity modulates TrkB, but not p75NR, on the surface of hippocampal neurons, and this effect appears to require action potentials coupled to excitatory synaptic transmission.

The increase of surface TrkB could be due to an increase in TrkB insertion into the cell surface, a decrease in TrkB internalization, or an increase in TrkB synthesis. Several pieces of evidence argue against a general increase in TrkB synthesis. First, there was no difference in the total amount of TrkB between active (TBS) and inactive (TBS plus TTX or Kyn) hippocampal neurons (Fig. 1 B2). Second, inhibition of protein synthesis by anisomycin (10 μg/ml) or cycloheximide (10 μg/ml) did not decrease the levels of surface TrkB in neurons stimulated with TBS (Fig. 1 B3). Thus, the synthesis of TrkB receptors is not enhanced by high frequency neuronal activity. Finally, electric stimulation of hippocampal neurons resulted in an increase, rather than a decrease in TrkB internalization (data not shown). These results, together with the immunocytochemistry experiments (see below), suggest that TBS facilitates the insertion of TrkB onto the surface membrane, rather than its production, in hippocampal neurons.

A previous study showed that depolarization induced by high concentration of $K^+$ (50 mM) resulted in a significant increase in the surface TrkB in retinal ganglion cells (Meyer-Franke et al., 1998). In our study, we found that simple depolarization by high $K^+$ did not affect the amount of surface TrkB in hippocampal neurons. The lev-

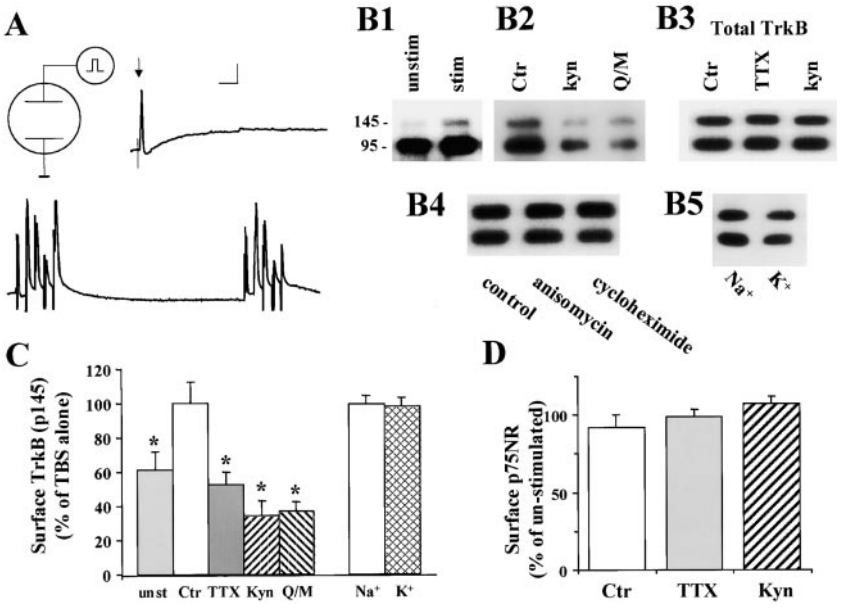


*Figure 1.* Effects of TBS on BDNF receptors on neurons. Hippocampal neurons were grown in serum free medium for 11–14 d. Two platinum electrodes were positioned on opposite sides of the culture well, and the hippocampal neurons were stimulated with TBS for 60 min in a 37°C incubator in the presence or absence of various inhibitors. After electric stimulation, proteins on the surface of hippocampal neurons were biotinylated, and then examined by Western blot using antibodies against the high affinity receptor TrkB or the low affinity receptor p75. (A) Electrophysiological recording of firing patterns in cultured hippocampal neurons elicited by field electric stimulation. Top traces show that a supra-threshold field stimulation induced a single action potential. Arrow indicates application of a single field electric pulse. Scale: 20 mV and 100 ms. Bottom trace shows an example of action potentials elicited by TBS. 2 out of the 10 bursts are shown. (B) Western blot analysis of cell surface TrkB receptors. Cultures were stimulated with TBS in the presence of indicated agents as follows: TTX (1 μM); kyn (1 mM); Q/M (0.1 mM CNQX plus 80 μM MK801); anisomycin (10 μg/ml); cycloheximide (10 μg/ml); $Na^+$ (50 mM) and $K^+$ (50 mM). (B1) High frequency electric stimulation increases surface expression of TrkB. Both full length (145 kD) and truncated (95 kD) TrkB receptors in stimulated (stim) or unstimulated (unstim) cultures are shown. (B2) Blockade of excitatory synaptic transmission prevents the TBS-induced increase in surface TrkB. The surface TrkB was measured in cultures in the presence or absence of blockers for excitatory transmission, kyn or Q/M. (B3) The total levels of TrkB are not changed by electric stimulation. Hippocampal neurons were stimulated with TBS in the presence or absence of TTX or kyn, harvested by RIPA buffer. Total amount of TrkB were measured directly by Western blot. (B4) TBS-induced increase in surface TrkB does not require protein synthesis. Cultures were stimulated by TBS in the presence or absence of the protein synthesis inhibitor anisomycin or cycloheximide. The surface TrkB were determined by biotinylation. (B5) Simple depolarization induced by high $K^+$ does not change the levels of surface TrkB. Biotinylation was used to determine the levels of surface TrkB in cultures that were treated with 50 mM of either control agent $Na^+$ or the depolarizing agent $K^+$. (C) Summary of the biotinylation experiments for surface TrkB (full length, p145). (Left) The levels of surface TrkB in TBS-stimulated cultures (set as 100%) are compared with unstimulated cultures (unst) and cultures stimulated with TBS in the presence of TTX, kyn, or Q/M; $n = 7$. (Right) Simple depolarization by high $K^+$ has no effect; $n = 4$. The surface TrkB levels were determined in cultures that were treated with $Na^+$ (50 mM, set as 100%) and $K^+$ (50 mM). Asterisk indicates statistically different results ($P < 0.05$, ANOVA followed by post hoc tests). (D) Summary of the biotinylation experiments for surface p75NR. TTX and kyn have no effect on surface p75NR; $n = 3$. In this and all other bar graph figures, data from a specific experimental condition (e.g., TBS plus TTX) were normalized to the mean in control (TBS stimulation alone) groups. The results in several independent experiments ($n$) were pooled and averaged, and presented as mean ± SE.

els of surface TrkB were the same in neurons treated with 50 mM of $K^+$, 50 mM of $Na^+$, or nothing at all (Fig. 1 B4 and data not shown). In retinal ganglion cells, the full-length (p145) but not the truncated (p95) form of TrkB was detected (Meyer-Franke et al., 1998). Both the full-length and truncated TrkB receptors were found on the cell surface of hippocampal neurons and both were increased by TBS (Fig. 1 B1). Moreover, very low levels of p75NR were detected in the hippocampal neurons (data not shown), whereas those in the retinal cells are known to be high (Frade et al., 1996; von Bartheld et al., 1996). Thus, modulation of surface TrkB receptors in hippocampal neurons may be different from that in retinal ganglion neurons.

Application of BDNF significantly reduced the amount of surface TrkB, presumably due to ligand-induced receptor internalization (data not shown). It was difficult to determine by the biotinylation assay whether electric activity could still modulate TrkB in the presence of BDNF, a situation more likely to occur in the physiological conditions in vivo. We thus turned to a more sensitive assay using radiolabeled BDNF ($I^{125}$-BDNF). The neuronal cultures were incubated at 37°C with $I^{125}$-BDNF ($5 \times 10^{-11}$ M) with or without cold BDNF ($5 \times 10^{-8}$ M) for 30 min. Surface BDNF receptors were determined by the amount of $I^{125}$-BDNF that can be washed off from the cell surface by mild acid (0.2 M acetic acid, 0.5 M NaCl, pH 2.2). Surface binding of $I^{125}$-BDNF was reduced by 80–90% when an excess amount of cold BDNF was added to the cultures (data not shown). Thus, the binding was specifically mediated by BDNF receptors. Stimulation of the hippocampal neurons with TBS reliably elicited an increase in surface binding of $I^{125}$-BDNF (Fig. 2). Compared with neurons stimulated with TBS alone, $I^{125}$-BDNF surface binding was significantly reduced in neurons stimulated with TBS plus TTX, which completely blocked action potentials in these neurons (Fig. 2 A). The glutamate receptor antagonists kyn or CNQX/MK801 also blocked the effect of TBS (Fig. 2 A), suggesting that the excitatory synaptic transmission is required for TBS-induced increase in surface TrkB.

As with the biotinylation experiments, the enhancement of BDNF receptor binding was dependent on high fre-

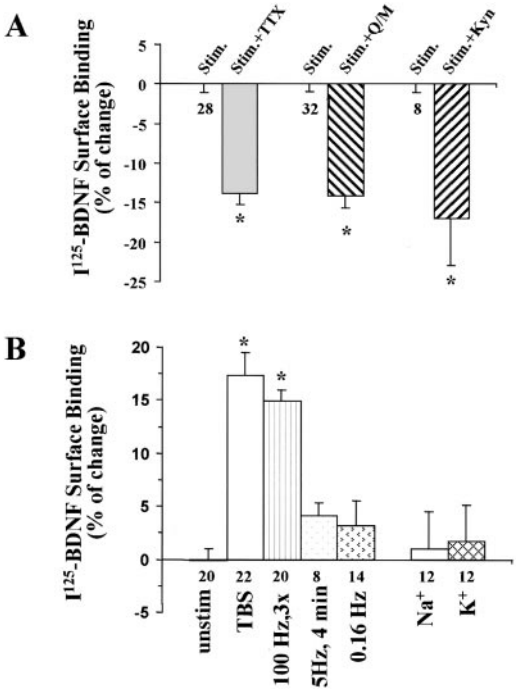


*Figure 2.* Activity-dependent modulation of the surface binding of BDNF receptors. Cultured hippocampal neurons were incubated with $I^{125}$-labeled BDNF ($5 \times 10^{-11}$ M) with or without cold BDNF ($5 \times 10^{-8}$ M) for 30 min while stimulated with TBS. Surface binding is defined as acid washable radioactivity at the end of $I^{125}$-BDNF incubation. The numbers associated with each column represent the total number of experiments. (A) Blockade of neuronal activity or excitatory synaptic transmission prevents the TBS-induced increase in BDNF surface binding. Hippocampal cultures were stimulated with TBS in the presence or absence of TTX, CNQX plus MK801, or kyn. All drug-treated groups were significantly lower than their paired stimulation alone (control) groups, which were set as 0% ($P < 0.01$, Student's $t$ test). (B) Modulation of BDNF receptor binding by patterned electric stimulation. Percentage of changes is presented. The data in control (no stimulation at all) are set as 0%. TBS and tetanic stimulation (three times, 100 Hz, 1 s every 10 min), but not continuous low frequency stimulation (0.16 Hz) or high $K^+$ (50 mM) stimulation, elicited much higher BDNF receptor binding as compared with control. LTD-inducing stimulus (5 Hz, 4 min) also had no effect. Asterisk indicates statistically different results ($P < 0.05$, ANOVA followed by post hoc tests).

quency tetanic stimulation. TBS reliably elicited an increase in $I^{125}$-BDNF surface binding as compared with nonstimulated controls (Fig. 2 B). Another tetanic stimulation (three times, 100 Hz, 1 s every 10 min), which elicited a train of high frequency action potentials, resulted in an increase in BDNF binding similar to the result from TBS (Fig. 2 B). In contrast, low frequency stimulation, such as the LTD-inducing stimuli (5 Hz, 4 min) or a constant low frequency train (0.16 Hz), had no effect on $I^{125}$-BDNF surface binding (Fig. 2 B). It is worth pointing out that the same number of pulses was delivered during 30 min of stimulation in both the 100 Hz tetanic stimulation and the 0.16 Hz stimulation. Thus, the modulation of surface BDNF receptors appears to depend on the stimulation frequency, rather than the number of pulses. Again, simple depolarization induced by high $K^+$ had little effect on surface binding (Fig. 2 B).

An immediate consequence of tetanus-induced neuronal activity is $Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels or NMDA receptors. To determine the mechanisms underlying TBS-induced increase in the surface expression of TrkB, we studied effects of a number of manipulations known to interfere with $Ca^{2+}$ influx. Using the biotinylation assay, we found that blockade of $Ca^{2+}$ influx by the NMDA receptor blocker MK801 (80 μM) markedly reduced the amount of both full-length and truncated TrkB receptors on the surface of hippocampal neurons stimulated with TBS (Fig. 3 A). Inhibition of $Ca^{2+}$ influx by the general $Ca^{2+}$ channel blockers $Cd^{2+}$ (0.2 mM) had similar effects (Fig. 3 A). These results were further confirmed by the $I^{125}$-BDNF surface binding assays. The surface binding was reduced when TBS was applied together with the general $Ca^{2+}$ channel blockers $Cd^{2+}$ (Fig. 3 B) or

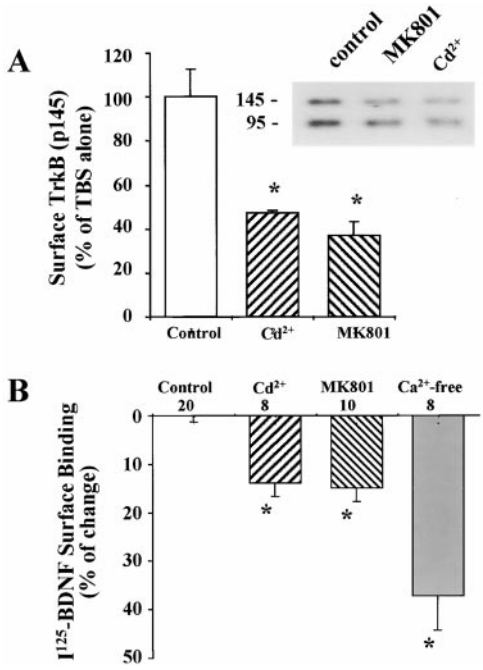


*Figure 3.* Role of $Ca^{2+}$ influx in activity-dependent modulation of cell surface TrkB receptors. Western blot analysis of cell surface TrkB receptors (A) and surface binding of $I^{125}$-BDNF (B) were performed under conditions that affect $Ca^{2+}$ influx. TBS was applied in all conditions. Asterisk indicates statistically different results ($P < 0.05$, ANOVA followed by post hoc tests). (A) Summary of the effect of $Ca^{2+}$ influx on surface TrkB. Inset shows an example of biotinylation analysis of surface TrkB receptors showing that MK801 and $Cd^{2+}$ reduce both p145 and p95 TrkB proteins in TBS-stimulated cultures. The amount of full length TrkB (p145) in control conditions (TBS in regular medium) was set as 100%. $Ca^{2+}$ channels blocked by $Cd^{2+}$ (0.2 mM) and NMDA receptors blocked by MK801 (80 μM) all inhibited BDNF receptor on the cell surface; $n = 7$. (B) Summary of the effect of $Ca^{2+}$ influx on BDNF surface binding. Controls (TBS in regular medium) were set as 0%. In $Ca^{2+}$-free condition, culture medium was replace and pretreated for 30 min with $Ca^{2+}$-free DMEM before experiments were performed. The numbers associated with each column represent the total number of experiments.

$Co^{2+}$ (3 mM, not shown), or NMDA antagonists MK801 (80 μM; Fig. 3 B) or 2-amino-5-phosphonovalerate (50 μM; data not shown). Moreover, surface BDNF receptors were significantly reduced in neurons stimulated by TBS in $Ca^{2+}$-free medium, as compared with that in regular medium (Fig. 3 B). Thus, the TBS modulation of surface expression of BDNF receptors appears to depend on $Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels and/or NMDA receptors.

To determine the changes in the distribution of TrkB receptors on the cell surface, we performed immunocytochemistry under nonpermeable (no detergent) conditions. Cultured hippocampal neurons were fixed in 2% paraformaldehyde for 3 min, and TrkB antibodies were incubated with the fixed cells in buffers containing no detergent. Under these conditions, an antibody against intracellular domains of the TrkB did not stain hippocampal neurons, suggesting that antibodies or proteins can not penetrate the cells (Fig. 4, A and B). The same antibody was able to detect a substantial amount of TrkB if the staining was performed in the presence of detergent (Fig. 4 C). Under the nonpermeable (no detergent) conditions, surface TrkB were detected using an antibody against extracellular domains of the TrkB. In cultures stimulated with TBS, many TrkB receptors were found on the surface of the cell body. More importantly, a large number of TrkB receptors were distributed along the neuronal processes (Fig. 4 D). In contrast, TrkB receptors were mainly clustered on the cell body and there were very few receptors on the neuronal processes in cultures stimulated with TBS in the presence of kyn and $Cd^{2+}$ (Fig. 4 E). The reduction in surface TrkB in the cultures treated with kyn and $Cd^{2+}$ was not due to a decrease in the number of neuronal processes, which were obviously observed under phase contrast microscopy (data not shown). To better visualize the surface TrkB, we used confocal microscopy. Thin section (2 μm) confocal images revealed a substantial increase in the amount of surface TrkB receptors in active (TBS alone) neurons as compared with the inactive (TBS plus kyn and $Cd^{2+}$) neurons (Fig. 4, F and G). These experiments were repeated many times and striking differences in surface TrkB were always observed between active and inactive neurons. Unstimulated neurons also exhibited less surface TrkB receptors than stimulated neurons (data not shown).

To further investigate the changes in the TrkB receptors inside the cells, we stained the hippocampal neurons under permeable (with detergent) conditions using the antibody against the intracellular domain of TrkB. Both surface and intracellular TrkB were detected. In the stimulated cultures, it appeared that majority of TrkB receptors were on the cell membrane (arrows) and only small amounts of the receptors were inside the cells (Fig. 5 A). Significantly fewer TrkB receptors were found on the cell surface but a lot more receptors were detected inside cells (arrowheads) in cultures stimulated with TBS in the presence of kyn and $Cd^{2+}$ (Fig. 5 B). Confocal microscopy was again used to better separate surface and cytoplasmic TrkB. Indeed, active neurons exhibited a "ring" pattern of staining in sections across the middle of the cell body region, with a lot of TrkB receptors on the cell surface but very little in the cytoplasm (Fig. 5 C). In contrast, a great deal of cytoplasmic TrkB was observed in inactive neurons in similar sections (Fig. 5 D). These results further support the notion that high frequency neuronal activity facilitates the insertion of TrkB receptor into the cell surface.

$Ca^{2+}$ influx is known to activate CaMKII, which has been implicated in the activity-dependent insertion of AMPA-type glutamate receptors into the postsynaptic membrane during LTP (Hayashi et al., 2000). To determine whether CaMKII is also involved in the insertion of the tyrosine kinase receptor TrkB, we measured the amount of surface TrkB in cultures stimulated with TBS in the presence or absence of CaMKII inhibitors. Biotinylation experiments demonstrated that inhibition of CaMKII by either KN62 or KN93 significantly reduced the amount of TrkB receptors in cultures stimulated with TBS, whereas inactive compound KN92 had no effect (Fig. 6, A and B). KN62 and KN93 also significantly inhibited BDNF surface binding on hippocampal neurons (Fig. 6 C). Since KN62 and KN93 have been shown to inhibit other CaM kinases and may cause some nonspecific effects in certain conditions, we used AIP, a peptide known to selectively inhibit CaMKII (Ishida et al., 1995). The $NH_2$ terminus of AIP or a control peptide with scrambled sequence (AIP-scm) was fused to the antennapedia homeopeptide (antp, 16 residues) derived from antennapedia gene to facilitate the translocation of the peptide across the cell membrane of hippocampal neurons (Prochiantz, 1996; Passafaro et al., 1999). To determine whether the peptides can penetrate into the hippocampal neurons, we labeled the peptides with biotin, and treated the cells with the biotinylated peptides for a few hours. Cy3-conjugated streptavidin detected the biotinylated peptides inside the hippocampal neurons 3 h after peptide incubation (data not shown). In cultures stimulated with TBS in the presence of antp-AIP, significantly lower levels of surface TrkB were detected as compared with those in cultures stimulated with TBS alone (Fig. 6, A and B). The control peptide antp-AIPscm had no effect (Fig. 6, A and B). Similar results were obtained using the $I^{125}$-BDNF surface binding assay (Fig. 6 C). These results strongly suggest the involvement of CaMKII in the activity-modulation of surface TrkB.

## *Discussion*

A critical but unresolved question in the neurotrophin research is how a diffusible molecule such as BDNF achieves preferential regulation of active neurons or synapses. In our study, we have investigated whether neuronal responsiveness to BDNF is dependent on, or modified by, neuronal activity. Using three independent approaches, we demonstrate that several forms of tetanic stimulation, but not low frequency stimulation or simple depolarization, promotes the insertion of the BDNF receptor TrkB into the cell surface of hippocampal neurons. We also show that excitatory synaptic transmission, $Ca^{2+}$ influx, and activation of CaMKII are important for the cell membrane insertion of TrkB. Thus, activity-dependent increase in the number of surface TrkB receptors may explain why BDNF acts preferentially on active neurons. This study reveals a novel mechanism by which neurotrophin signaling and function may be regulated, and provides a potential link between activity-dependent and BDNF-induced mod-

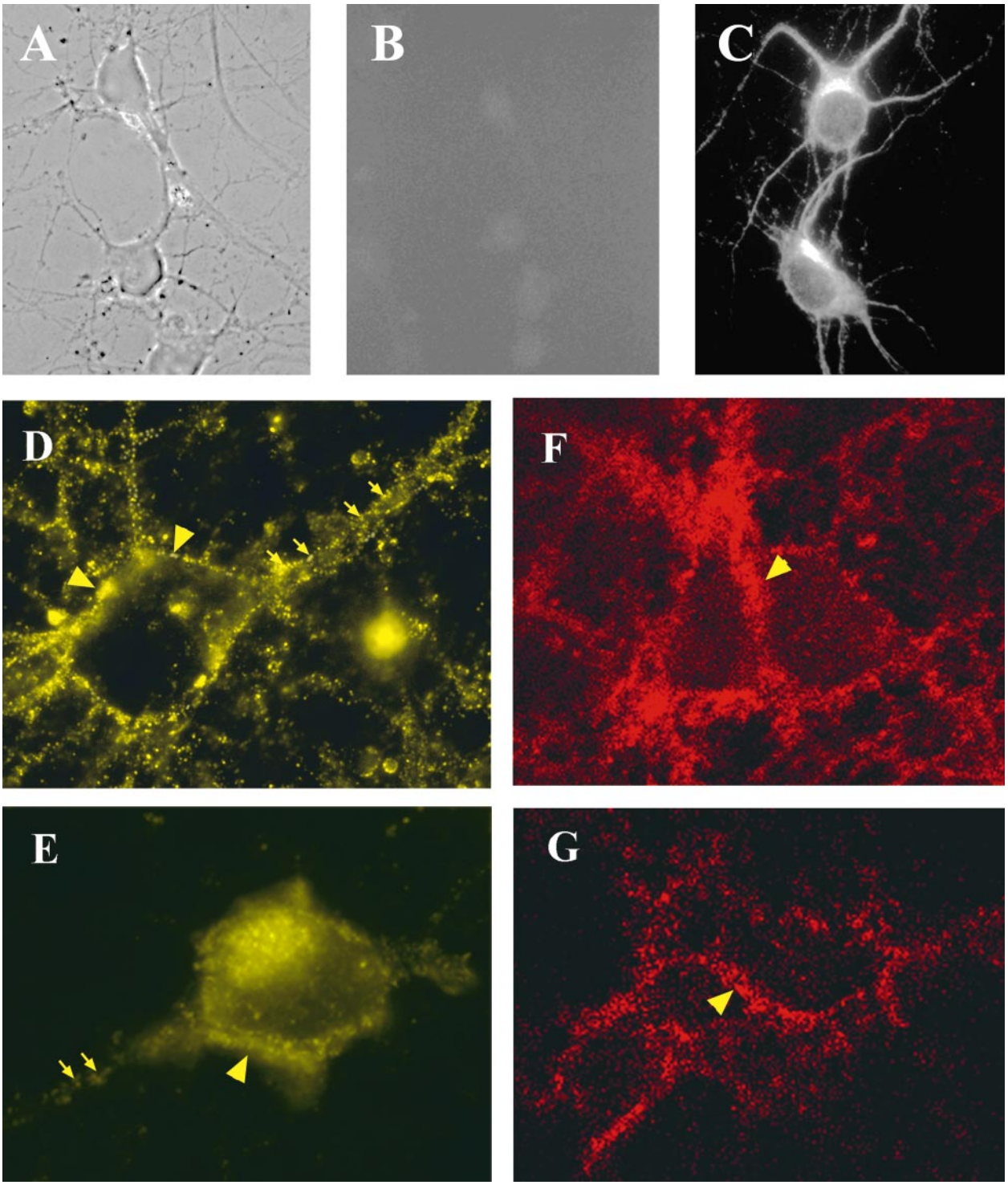


*Figure 4.* Immunocytochemistry of TrkB on the surface of hippocampal neurons. (A and B) Phase and fluorescence images of neurons stained with an antibody against intracellular domain of TrkB under nonpermeabilizing conditions. (C) Immunofluorescence images of neurons stained with the same antibody permeabilizing conditions. There is no staining in B but good staining in C, indicating that the antibody cannot penetrate inside cells under nonpermeabilizing conditions. (D–G) Immunocytochemistry staining using an antibody against the extracellular domain of TrkB under non-permeabilizing conditions. Hippocampal neurons were stimulated with TBS in the presence (E and G) or absence (D and F) of $Cd^{2+}$ (0.2 mM) and kyn (1 mM) for 30 min. Cells were fixed with 2% paraformaldehyde, 120 mM sucrose in PBS at room temperature for 3 min, followed by conventional immunofluorescence procedure without any detergent. D and E are conventional immunofluorescence images, and F and G are confocal immunofluorescence images. Arrows indicate surface TrkB stainings on neuronal processes and arrowheads indicate those on cell body. Note that far more surface TrkB receptors are seen in cultures stimulated with TBS alone, especially in neuronal processes. Bar, 5 μm.

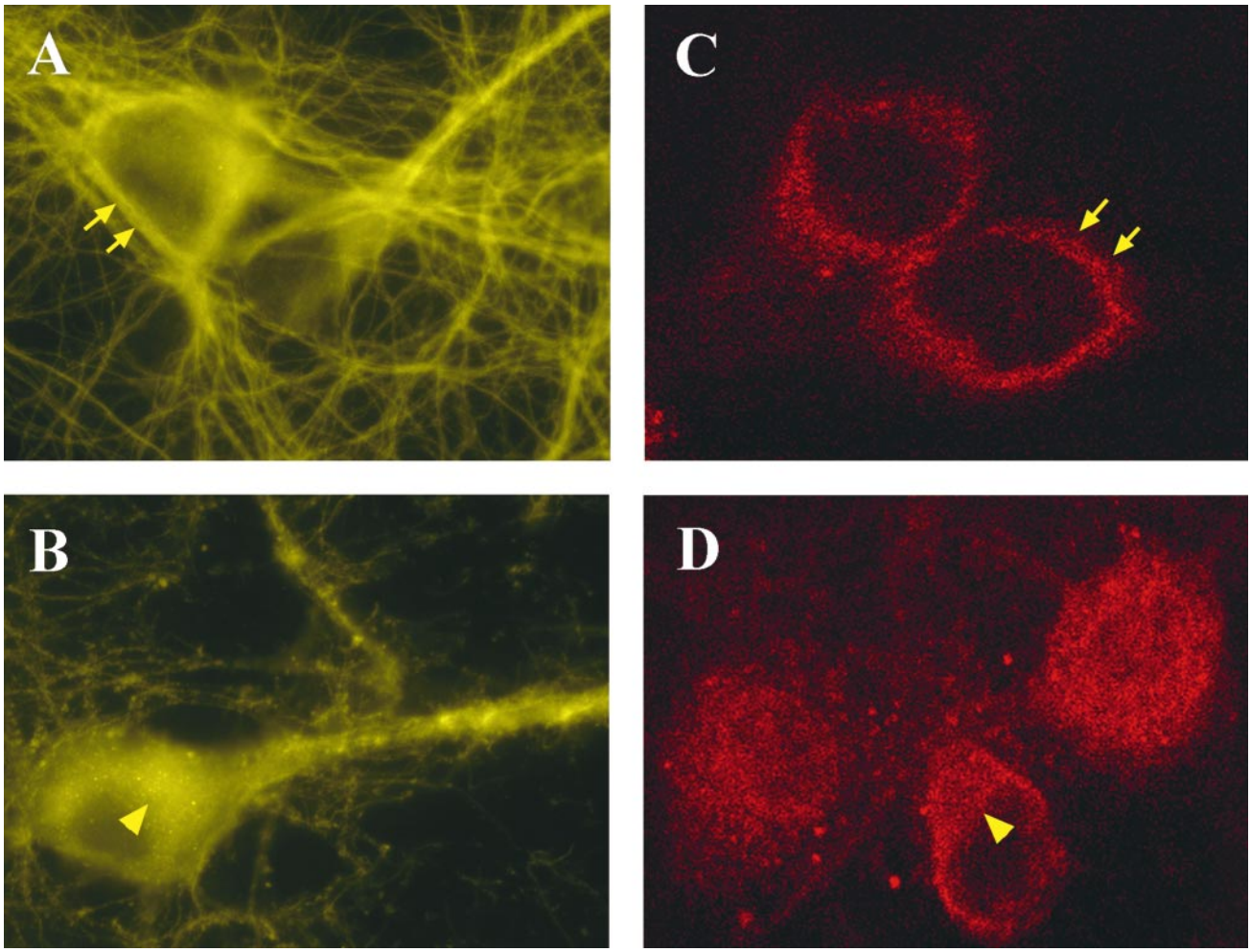


*Figure 5.* Immunocytochemistry of hippocampal neurons stained by an antibody against the intracellular domain of TrkB under permeabilizing conditions. TBS was applied to the hippocampal neurons in the presence (B and D) or absence (A and C) of $Cd^{2+}$ and kyn for 30 min. The cultures were then fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.4% Triton X-100 for 60 min, and processed for immunofluorescence staining of TrkB. A and B are conventional immunofluorescence images, and C and D are confocal immunofluorescence images. Arrows indicate cell surface and arrowheads indicate cytoplasmic stainings, respectively. Note that active cells (stimulated by TBS) exhibit TrkB receptors mostly on the cell surface, whereas inactive cells (TBS plus $Cd^{2+}$ and kyn) show more cytoplasmic staining of TrkB.

ulation of neuronal and synaptic function in the hippocampus.

In this study, hippocampal neurons were grown for 11–14 d. Electrophysiological recording indicated that these neurons were well connected synaptically (data not shown). We show that excitatory synaptic activity plays an important role in regulating surface expression of the TrkB receptor tyrosine kinase. Electric stimulation is a more physiological form of stimulation that has been successfully used to study activity-dependent regulation of signal transduction and gene transcription in cultured hippocampal neurons (Bito et al., 1996; Deisseroth et al., 1996; Fields et al., 1997). LTP-inducing tetanic stimuli enhanced surface expression of TrkB, whereas blockade of excitatory synaptic transmission inhibited the tetanus-induced insertion. Immunocytochemical studies demonstrate that the increase in surface TrkB induced by TBS occurred mostly on neuronal processes rather than cell bodies. Remarkably, low frequency stimulation such as those used to induce LTD, or continuous 0.16 Hz (which delivers the same number of pulses as the 100 Hz tetanus), had no effect. These results suggest that temporal pattern of neuronal activity and the kinetics of changes in intracellular $Ca^{2+}$ concentrations, rather than the number of action potentials or the total amount of $Ca^{2+}$ influx, are the critical factors for the activity-dependent insertion of TrkB receptors. Consistent with this idea, simple depolarization by high $K^+$ (Figs. 1 and 2) or veratridine (data not shown) had no effect on the number of surface TrkB. Using freshly dissociated retinal ganglion cells as a model, Barres and colleagues demonstrated that depolarization by high $K^+$ or glutamate agonists increases the number of cells expressing surface TrkB (Meyer-Franke et al., 1998). The mechanisms underlying the apparent discrepancy re-

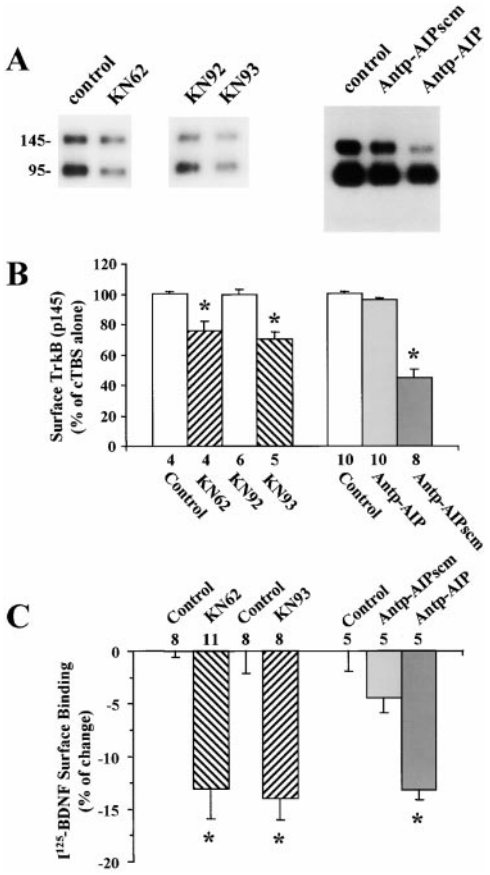


*Figure 6.* Role of CaMKII in activity-dependent modulation of cell surface TrkB receptors. Hippocampal neurons were stimulated with TBS in the presence or absence of KN compounds, or pre-treated with peptides for 3 h before TBS stimulation. Cells were processed either for biotinylation assay for cell surface TrkB receptors (A and B) or for surface binding of $I^{125}$-BDNF (C). The numbers associated with each column represent the number of experiments. Asterisk indicates statistically different results ($P < 0.05$, ANOVA followed by post hoc tests). (A) Examples of biotinylation experiments showing that KN62 (10 μM), KN93 (20 μM), and antp-AIP (20 μM), but not KN92 (20 μM) and antp-AIPscm (20 μM), reduce both p145 and p95 TrkB proteins in TBS-stimulated cultures. (B) Summary of the effects of CaMKII inhibitors on surface TrkB (p145). Controls (TBS in regular medium) were set as 100%. (C) Summary of the effect of CaMKII inhibitors on BDNF surface binding. In both B and C, KN62, KN93, and antp-AIP were all effective, whereas KN92 and antp-AIPscm were not.

main unclear. A simple explanation is that different types of neurons may use different mechanisms to regulate surface expression of TrkB. In the retinal ganglion cells, simple depolarization by high $K^+$ may increase intracellular cAMP concentrations, which in turn facilitate the incorporation of TrkB into the surface through a number of unknown steps (Meyer-Franke et al., 1998). In the hippocampal neurons, high frequency stimulation may induce $Ca^{2+}$ influx that is qualitatively different from that induced by high $K^+$, leading to the activation of CaMKII. Alternatively, freshly dissociated retinal ganglion neurons and synaptically connected hippocampal neurons (grown for 11–14 d) may respond differently to high $K^+$. In retinal ganglion cells, high $K^+$ may be sufficient to generate the intracellular signals needed to facilitate surface expression of TrkB (Meyer-Franke et al., 1998). In the hippocampal neurons, however, high $K^+$ could not produce the specific temporal pattern of neuronal activity and $Ca^{2+}$ influx and subsequent CaMKII activation required for the modulation.

Our results suggest that the modulation of TrkB receptors by tetanic stimulation is mediated, at least in part, by high frequency excitatory synaptic transmission. We have also demonstrated that the activity-dependent modulation of TrkB requires $Ca^{2+}$ influx. Since $Ca^{2+}$-free medium and $Cd^{2+}$ not only prevent $Ca^{2+}$ influx into the postsynaptic neurons through $Ca^{2+}$ channels but also inhibit transmitter release from presynaptic terminals, it is unclear whether blockade of $Ca^{2+}$ influx directly affects the insertion of TrkB receptor into the cell surface, or indirectly by blocking excitatory synaptic transmission. However, blockade of NMDA receptors, which are primarily localized in the postsynaptic cells rather than presynaptic nerve terminals, also attenuates surface expression of TrkB (Fig. 3). Moreover, CaMKII appears to be involved in the activity-dependent insertion of TrkB (Fig. 6). Given that CaMKII has been shown to be important for the insertion of AMPA-type receptors postsynaptically during hippocampal LTP (Hayashi et al., 2000), it is conceivable that similar postsynaptic mechanisms may be used for the insertion of the tyrosine kinase receptor TrkB. It is important to point out, however, that tetanic stimulation does not increase all surface molecules. The surface p75NR is not increased in neurons stimulated with TBS (Fig. 1 D).

One of the remarkable features of the nervous system is that neuronal activity can modulate synaptic efficacy and connectivity in a local and synapse-specific manner (Stent, 1973; Goodman and Shatz, 1993; Katz and Shatz, 1996; Constantine-Paton et al., 1990). Recent studies strongly implicate a role of BDNF in activity-dependent synaptic modulation, such as the formation of ocular dominance columns in the visual cortex and hippocampal LTP (Thoenen, 1995; Lu and Chow, 1999; McAllister et al., 1999). It is important to understand how diffusible factors such as BDNF achieve local and synapse-specific modulation, and how BDNF strengthens active synapses without affecting their neighbors. One such mechanism would be a localized secretion of BDNF at the site of active synapses. Although there is some evidence for an activity-dependent secretion of BDNF (Wang and Poo, 1997; Goodman et al., 1996; Heymach et al., 1996), so far local or synapse-specific secretion of any neurotrophins has not been demonstrated. It is difficult to imagine that locally secreted factors would not spread to their neighboring, less active synapses. Our results demonstrate an alternative and more practical strategy. Active neurons may respond better to BDNF, and this is achieved by an activity-dependent control of the number of TrkB receptors on the cell surface. These results provide a molecular basis for the facilitation of BDNF-induced synaptic potentiation when coupled to presynaptic depolarization at the neuromuscular junction (Boulanger and Poo, 1999), and the restricted action of BDNF on highly active synapses in the hippocampus (Gottschalk et al., 1998). In this context, it is important to

note that the tetanic stimuli such as TBS or tetanic stimulation were capable of modulating TrkB receptors, whereas low frequency stimulation was not. Since all of our experiments were done using cultured neurons, their relevance to the BDNF modulation of hippocampal synaptic plasticity in vivo has yet to be established. Nevertheless, activity-dependent enhancement of the number of surface TrkB receptor may define an important mechanism by which the specificity of BDNF modulation is achieved.

The results in our study may have general implications in the cell biology of tyrosine kinase receptors. First, we have demonstrated an activity-dependent increase in the number of surface TrkB receptors in the hippocampal neurons. This is due to an increase in the insertion of TrkB receptors into the neuronal cell surface, rather than an increase in TrkB synthesis or decrease of TrkB internalization. The mechanisms underlying membrane insertion of TrkB receptors, and tyrosine kinase receptors in general, are largely unexplored. This study may trigger further interests in investigating the mechanisms for membrane insertion of tyrosine kinase receptors. It will be interesting to examine whether molecules important for vesicle fusion, such as NSF and SNAP, are involved in the delivery of tyrosine kinases onto cell membrane. Second, we show that the membrane insertion of the TrkB receptors is enhanced by $Ca^{2+}$ influx. To our knowledge, this is the first report for $Ca^{2+}$-dependent modulation of the number of surface tyrosine kinase receptors. Thus, our results suggest a novel mechanism for cross-talk between $Ca^{2+}$ and tyrosine kinase signaling pathways. Whether the tyrosine kinase activity of the TrkB receptors can be regulated by intracellular $Ca^{2+}$ is an interesting topic for future study. Finally, CaMKII has recently been implicated in the insertion of AMPA-type glutamate receptors onto the postsynaptic membrane of hippocampal neurons (Hayashi et al., 2000). Our study demonstrates that similar mechanisms are used for the tetanus-induced increase in the tyrosine kinase receptor TrkB on the surface of hippocampal neurons. It remains to be established whether CaMKII also regulates the membrane insertion of other tyrosine kinases in neurons and in other cell types.

We thank Dr. Phillip Nelson for his valuable advice throughout this work, Drs. Doug Fields, Serena Dudek, Lin Mei, and Hao-Chia Chen, as well as the members of the Lu laboratory for critical comments on the manuscript. We also thank Regeneron Pharmaceuticals for providing recombinant BDNF.

This work is supported in part by a grant from Shanghai Science and Technology Commission and National Natural Science Foundation of China.

Submitted: 5 May 2000
Revised: 22 June 2000
Accepted: 17 July 2000

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