# Mechanism underlying activity-dependent insertion of TrkB into the neuronal surface

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## Summary

**Activity-dependent insertion of tyrosine kinase receptor type 2 (TrkB receptor) into the plasma membrane can explain, in part, the preferential effect of brain-derived neurotrophic factor (BDNF) on active neurons; however, the detailed cellular and molecular mechanisms underlying this process are still unclear. In our study, we developed a fluorescence ratiometric assay for surface TrkB receptors to investigate the mechanisms of recruitment of TrkB to the plasma membrane following chemical long-term potentiation (cLTP) induction. We found that, in hippocampal neurons, the effect of cLTP-induced TrkB surface-recruitment occurred predominantly on neurites with rapid kinetics ($t_{1/2}$ of ~2.3 minutes) and was dependent on an intact cytoskeleton structure. Mutagenesis studies revealed that the juxtamembrane domain of TrkB is necessary and sufficient for its activity-dependent insertion into the plasma membrane. Moreover, we found that the phosphorylation of TrkB receptor at the Ser478 site by cyclin-dependent kinase 5 (Cdk5) is essential for cLTP-induced TrkB insertion into the neuronal surface. Finally, the degree of cLTP-induced TrkB surface-recruitment is higher in postsynaptic regions, which provides a potential mechanism for rapid enhancement of postsynaptic sensitivity to incoming BDNF signaling. Our studies provide new insights regarding neuronal activity-dependent surface delivery of TrkB receptor, which will advance our understanding of the modulatory role of TrkB in synaptic plasticity.**

Supplementary material available online at
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Key words: TrkB, Activity, cLTP, Neuron, Surface insertion, Juxtamembrane domain

## Introduction

Although neurotrophins are best known for their ability to promote neuronal survival and differentiation, recent studies have established a novel function of neurotrophins in synapse development and plasticity, particularly in the central nervous system (Arancio and Chao, 2007; Huang and Reichardt, 2001; Lu, 2003). Among the neurotrophins, brain-derived neurotrophic factor (BDNF) is widely expressed and has been shown to be crucial for structural and synaptic plasticity in the developing and mature central nervous system (Bramham and Messaoudi, 2005; Cohen and Greenberg, 2008; Nagappan and Lu, 2005). BDNF possesses acute regulatory effects on neurotransmitter release, synaptic strength, and connectivity (Bonhoeffer, 1996; McAllister et al., 1999; Poo, 2001). The maintenance of long-term potentiation (LTP) in the CA1 region is impaired in hippocampal slices from mice deficient in BDNF, which can be rescued by addition of exogenous BDNF (Korte et al., 1995; Patterson et al., 1996). BDNF also plays an important role in mediating higher order activities, such as learning, memory and behavior (Lu et al., 2008; Martinowich et al., 2007). Remarkably, neuronal activity could modulate responsiveness to BDNF. For example, inhibition of spontaneous electrical activity or synaptic transmission could prevent the dramatic increase in dendritic arborizations elicited by BDNF in developing cortex (McAllister et al., 1996). Moreover, BDNF modulation of postsynaptic NMDA receptors requires concurrent neuronal activity (Crozier et al., 2008).

As a diffusible molecule, an interesting question is how BDNF preferentially acts on active neurons and synapses. First, BDNF is synthesized and released in a manner that is dependent on neuronal activity (Lessmann et al., 2003; Lu, 2003; Poo, 2001). Second, neuronal activity might enhance BDNF signaling. The actions of BDNF are dictated by two classes of receptors on the cell surface: the TrkB receptor and the p75 neurotrophin receptor. BDNF binding to TrkB receptors triggers its phosphorylation and subsequent activation of several intracellular signaling cascades, which modulate neuronal survival, neurite outgrowth and synaptic plasticity (Chao, 2003; Huang and Reichardt, 2003; Kaplan and Miller, 2000). Elevation of intracellular cAMP concentrations in hippocampal neurons can enhance TrkB phosphorylation and its translocation to spines (Ji et al., 2005). Third, neuronal activity could increase the number of TrkB receptors on cell surface, thus facilitating the enhanced responsiveness of active neurons to BDNF. Indeed, a previous study has shown that depolarization or cAMP elevation could increase the levels of the TrkB receptor on the cell surface of retinal ganglion cells and spinal neurons (Meyer-Franke et al., 1998). Electrical stimulation can also modulate the levels of cell-surface TrkB receptors in hippocampal neurons, requiring $Ca^{2+}$ influx through NMDA receptors, and involving $Ca^{2+}$/calmodulin-dependent kinase II (CaMKII) (Du et al., 2000). Activity-dependent enhancement of surface TrkB receptor levels might define an important mechanism by which the specificity of BDNF-activity-

dependant modulation is achieved. However, the detailed molecular and cellular mechanisms underlying this phenomenon remain unclear.

## Results

### Ratiometric fluorescence assay to measure recruitment of TrkB receptor to the cell surface

We made a FLAG-TrkB-GFP construct that contained a FLAG epitope just after the signal peptide (extracellular) and a GFP tag fused to the C-terminal (intracellular) of TrkB receptor (Fig. 1A). When we transfected FLAG-TrkB-GFP into hippocampal neurons; surface TrkB was measured by staining with anti-FLAG M2 antibody, under non-permeabilized conditions. The GFP fluorescence throughout the cell represented the total TrkB levels (Fig. 1B). To avoid the effect of having different TrkB expression levels in different cells, due to transient transfection, cell-surface TrkB levels were quantified by fluorescence intensity of FLAG staining normalized to GFP intensity per cell (see Materials and Methods), which was designated the 'surface TrkB ratiometric fluorescence assay'. Through this assay based on ratiometric immunocytochemical staining, the cell-surface TrkB levels could be measured in a quantitative manner.

A previous study showed that depolarization induced by a high concentration of KCl (50 mM) or elevation of cAMP by forskolin, a specific activator of adenylyl cyclase, could rapidly recruit TrkB to the plasma membrane in retinal ganglion cells (RGCs) (Meyer-Franke et al., 1998). As anticipated, with our surface TrkB ratiometric fluorescence assay, high $K^+$ or forskolin treatment for 1 hour could significantly increase cell-surface TrkB levels in hippocampal neurons (Fig. 1B). Next, we investigated whether rapid TrkB insertion into plasma membrane provides a potential source of TrkB receptor supply at synapses. To this end, we tested whether activation of synaptic NMDA receptors by glycine stimulation, a model used to induce chemical LTP (cLTP) in cultured hippocampal neurons (Lu et al., 2001; Musleh et al., 1997; Park et al., 2004), could increase TrkB levels at the cell surface. We found that brief glycine stimulation markedly increased surface TrkB levels (Fig. 1B). By normalization to the control group, we found that cLTP, induced by 200 μM glycine treatment, led to a 1.82±0.08-fold increase in surface TrkB levels ($P<0.01$). Depolarization or cAMP elevation led to a 1.5±0.18-fold ($P<0.05$) or 1.45±0.14-fold ($P<0.05$) increase of surface TrkB receptor, respectively (Fig. 1C). Thus, cLTP induced the most robust increase in surface TrkB levels and was then chosen as the stimulation condition in our subsequent studies.

There was a concern that overexpressed FLAG-TrkB-GFP might behave differently to the endogenous TrkB receptor because it might saturate the intracellular machinery for rapidly recruiting TrkB to the plasma membrane upon stimulation. To exclude this possibility, cLTP induction of insertion of endogenous TrkB to the cell surface was measured by using a surface biotinylation assay in non-transfected hippocampal neurons. Quantitative analysis of TrkB immunoblotting revealed a 1.75-fold increase in surface full-length TrkB (TrkB-FL) levels after glycine treatment; however, surface truncated TrkB (TrkB.T1) levels were unchanged (Fig. 1D,E). Given that the degree of cLTP-induced insertion of FLAG-TrkB-GFP to the cell surface was similar in magnitude to that of endogenous TrkB, this finding suggests that the transiently transfected FLAG-TrkB-GFP behaves similarly to the endogenous TrkB in cLTP-induced recruitment of TrkB to the plasma membrane.

To exclude the possibility that the C-terminal GFP might interfere with activity-dependent TrkB surface insertion, hippocampal neurons were transfected with FLAG-TrkB construct and stained with a monoclonal anti-FLAG M2 antibody before permeabilization (which represents the surface pool of TrkB receptor) followed by staining with a polyclonal rabbit anti-FLAG antibody after permeabilization (representing total TrkB) (Fig. 2A). Another set of experiments was performed by first feeding neurons a mouse anti-FLAG antibody and then, after glycine treatment, staining the same neurons with a rabbit anti-FLAG antibody under non-permeabilized conditions to show the cLTP-induced FLAG-TrkB recruitment within a single cell (Fig. 2C). Using fluorescence quantification, both experiments indicated a similar effect in activity-induced TrkB surface recruitment as the FLAG-TrkB-GFP ratiometric fluorescence assay (Fig. 2B,D).

### Measurement of plasma membrane TrkB

From our cell-surface TrkB ratiometric fluorescence assay, we found that cLTP stimuli could dramatically increase plasma membrane TrkB levels in hippocampal neurons. Next, we sought to determine the percentage of TrkB receptors on the cell surface. Neurons transfected with FLAG-TrkB-GFP were stained against the FLAG epitope under permeabilized and non-permeabilized conditions. In permeabilized cells, FLAG staining represented total TrkB, just like GFP fluorescence, which was indicated by the complete colocalization between the GFP and anti-FLAG signals (Fig. 3A). Under non-permeabilized conditions, only surface TrkB was stained by anti-FLAG antibody, and GFP represents total TrkB. To assess the proportion of TrkB on the cell surface, we plotted the anti-FLAG fluorescence signal in the whole cell region in permeabilized neurons against the GFP fluorescence signal. The FLAG versus GFP plot was fit by a line through the origin with a slope of 1.01 (Fig. 3B). We used this relationship between the anti-FLAG and the GFP signals to calculate the fraction of TrkB on the neuronal surface using the formula: surface intensity (FLAG) / [total intensity (GFP) $\times$ 1.01]. Using this formula, the surface percentage of FLAG-TrkB-GFP in hippocampal neurons at the basal state was calculated to be 0.34±0.03, indicating that about 34% of the FLAG-TrkB-GFP is on the cell surface.

To test whether FLAG-TrkB-GFP transfection changed the fraction of TrkB at the cell surface, we calculated the proportion of surface TrkB at endogenous levels using surface protein biotinylation in cultured hippocampal neurons. After biotinylation, the cell lysate was equally divided into two halves; one half was precipitated by avidin beads to pull down cell surface receptors and the other half was precipitated by C-14 anti-Trk antibody to represent the total amount of TrkB-FL. Then, western blots were performed with an antibody against the TrkB extracellular domain (Fig. 3C). Quantitative analysis with ImageJ software showed that the ratio of surface TrkB to total TrkB was 31.4% (Fig. 3D), which was in accordance with the data from the ratiometric fluorescence assay of FLAG-TrkB-GFP transfection. Therefore, we adopted 34% as the surface TrkB fraction at the basal state in the following experiments. Our data suggested that at basal state most TrkB is located intracellularly with only a small portion being detected at the surface, thus allowing for quick recruitment of intracellular TrkB pools to the cell surface upon stimuli.

Ultrastructural analysis has revealed that TrkB receptors are localized in diverse subcellular compartments, including dendritic spines, axon initial segments, axon terminals, dendritic shafts and cell bodies (Drake et al., 1999). Using ratiometric fluorescence assay, we measured the cLTP-induced increase in surface TrkB levels in neuronal processes, which are the physiologically more

relevant area in the context of activity-dependent modulation (Fig. 3E,F). Compared with the cell body, the cLTP-induced receptor surface insertions were greater in neurites (1.35-fold in cell body versus 1.98-fold in processes, $P<0.01$) (Fig. 3E,F), which might provide a functional pool of TrkB receptor in neuronal dendrites or axons and mediate the biological function of BDNFs in modulation of synaptic plasticity. In the following studies, we focused our quantification on neurites.

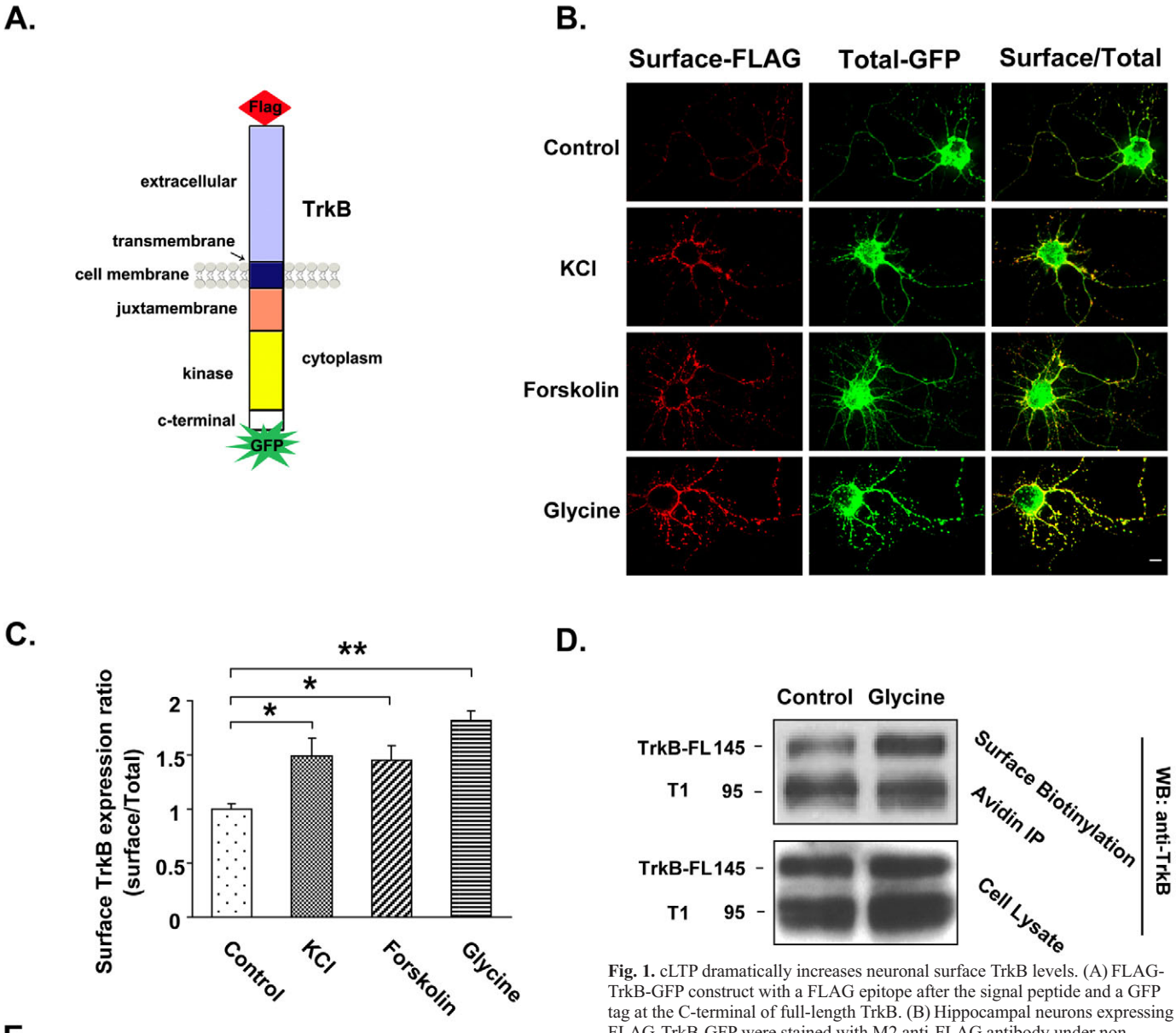


**Fig. 1.** cLTP dramatically increases neuronal surface TrkB levels. (A) FLAG-TrkB-GFP construct with a FLAG epitope after the signal peptide and a GFP tag at the C-terminal of full-length TrkB. (B) Hippocampal neurons expressing FLAG-TrkB-GFP were stained with M2 anti-FLAG antibody under non-permeabilized conditions to label surface TrkB. Total TrkB was represented by GFP fluorescence. The neurons were treated with 50 µM KCl, 5 µM forskolin or 200 µM glycine for 1 hour before fixation. Immunofluorescence images are from a representative cell for each condition. Scale bar: 10 µm. (C) Quantification of surface-to-total TrkB ratio by ratiometric fluorescence assay of immunofluorescence images shown in B. The data were normalized to the control. (D) Increased endogenous TrkB surface levels following cLTP stimuli, as measured by surface biotinylation. Hippocampal neurons were surface biotinylated before or after 200 µM glycine treatment. Surface-labeled receptors were detected by streptavidin pull-down followed by anti-TrkB antibody (against extracellular domain) immunoblotting. Total TrkB in the cell lysate was also immunoblotted as an internal control. (E) Quantitative analysis (using ImageJ software) of endogenous surface TrkB-FL and T1 levels from the biotinylation experiments shown in D. All of the data are presented as a means ± s.e.m. determined from analysis of more than three independent experiments ($n\geq20$ cells for each condition per experiment, $^{**}P<0.01$, $^{*}P<0.05$, ANOVA followed by post hoc tests or Student's *t*-test).

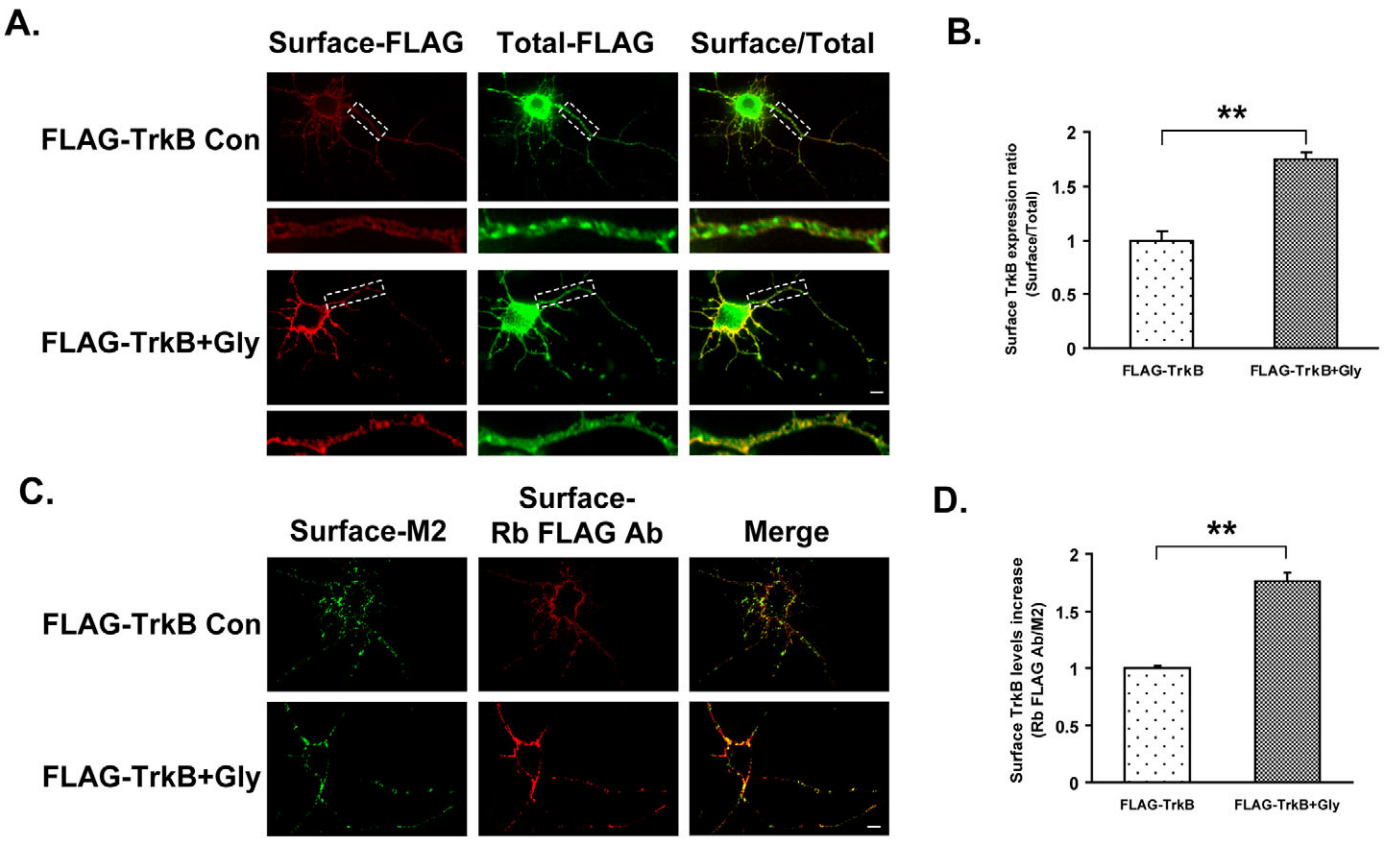


**Fig. 2.** C-terminal GFP tag does not interfere with activity-dependent insertion of TrkB to the cell surface. (A) Hippocampal neurons transfected with FLAG-TrkB were stained with M2 antibody before permeabilization and then stained with a polyclonal rabbit anti-FLAG antibody after permeabilization. Lower panels show enlarged images of framed regions. (B) Histogram of the FLAG-TrkB surface-to-total ratio in A. (C) cLTP-induced FLAG-TrkB cell-surface recruitment within a single neuron. Neurons were fed with M2 antibody first and then stained with a rabbit anti-FLAG antibody after vehicle or glycine treatment. (D) Fluorescence quantitation of surface TrkB levels change by the ratio of red to green (after versus before cLTP stimuli) in C. All of the data are presented as a means ± s.e.m. determined from analysis of more than three independent experiments ($n \geq 20$ cells for each condition per experiment, $^{**}P<0.01$, $^{*}P<0.05$, Student's $t$-test). Scale bars: 10 μm.

### cLTP stimuli rapidly recruit TrkB receptor to the plasma membrane

Although previous work showed that neuronal activity could rapidly increase TrkB cell surface levels (Du et al., 2000; Meyer-Franke et al., 1998), the time course of this effect remains unknown. In our studies we performed time-lapse images of immunofluorescence and quantitative ratiometric analysis of surface TrkB levels after glycine treatment to study the dynamics of activity-dependent insertion of TrkB to the plasma membrane. cLTP-induced TrkB membrane insertion was plotted against time, which fit a single exponential curve. We observed that a plateau was reached at 10 minutes after glycine treatment and the $t_{1/2}$ time was 2.3 minutes (Fig. 4A). This result further demonstrated that cLTP stimuli could rapidly recruit TrkB receptor to the plasma membrane and we chose the 10-minute time point for our following experiments. Inhibition of protein synthesis by 20 μg/ml of cycloheximide had no effect on surface TrkB recruitment after glycine stimuli (Fig. 4B), which is consistent with previous reports (Du et al., 2000; Meyer-Franke et al., 1998). This result suggests that cLTP could induce rapid TrkB insertion from an intracellular reserve pool rather than promote newly TrkB synthesis. To test whether cLTP-induced recruitment of TrkB to the cell surface requires NMDA receptor activity and $Ca^{2+}$, MK-801 (80 μM), BAPTA/AM (an intracellular calcium chelator, 20 μM) or EGTA (an extracellular calcium chelator, 2 mM) were applied 30 minutes before glycine stimulation. All the three inhibitors completely abolished glycine-induced surface TrkB recruitment compared with vehicle treatment controls (Fig. 4B). Our results indicate that cLTP-induced TrkB surface insertion requires NMDA receptor and $Ca^{2+}$ influx, which is consistent with previous studies done with theta burst stimulation (Du et al., 2000).

It is still unknown whether rapid TrkB surface insertion triggered by cLTP relies on an intact cytoskeleton system. Nocodazole, a microtubule-depolymerizing agent (Lu et al., 2001), or cytochalasin D, which inhibits actin polymerization (Cooper et al., 1987), was applied to cultured hippocampal neurons (10 μM for 30 minutes) followed by glycine stimulation. Compared with vehicle (DMSO) treatment, both nocodazole and cytochalasin D significantly inhibited cLTP-induced TrkB plasma membrane insertion (Fig. 4C,D). It suggests that rapid TrkB recruitment to the plasma membrane requires both an intact microtubule and microfilament.

### The juxtamembrane domain of TrkB is necessary and sufficient for cLTP-induced TrkB surface recruitment

To define the potential region in the TrkB receptor that is required for its activity-dependent plasma membrane insertion, we generated a series of constructs in which various TrkB domains

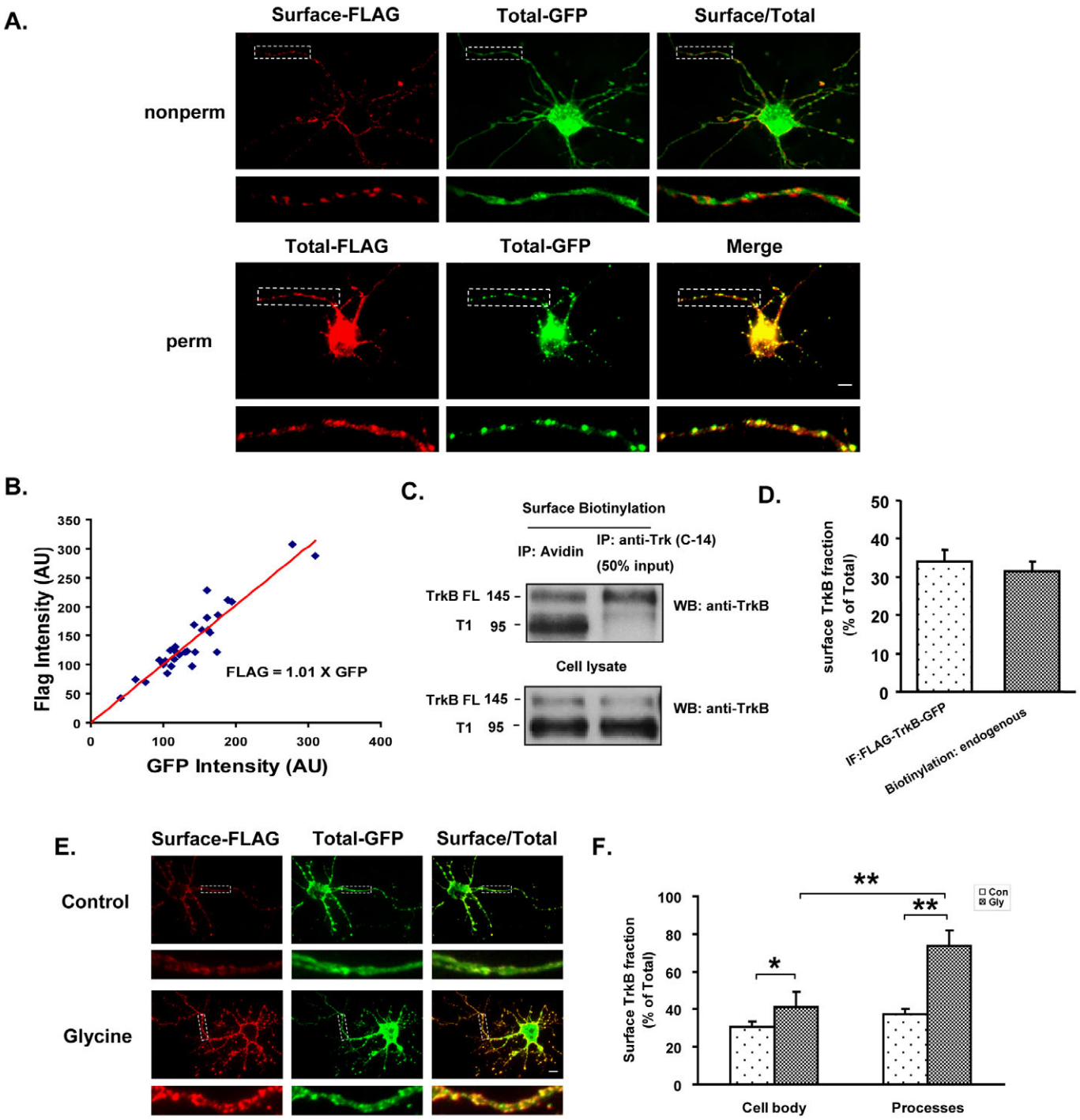


**Fig. 3.** Measurement of percentage TrkB at the neuronal surface. (A) Hippocampal neurons expressing FLAG-TrkB-GFP were stained with M2 anti-FLAG antibody under non-permeabilization (nonperm) or permeabilization (perm) conditions. Lower panels show enlarged images of framed regions. (B) Plot of anti-FLAG fluorescence signals against the GFP fluorescence signals in permeabilized neurons transfected with FLAG-TrkB-GFP. The relationship between the total FLAG and GFP intensities was fit by a linear equation. The slope gives the ratio of FLAG intensity to GFP intensity (1.01), which is the constant used in further calculations. (C) Endogenous TrkB neuronal surface fraction was measured by surface biotinylation. Non-transfected hippocampal neurons were surface biotinylated, then half of the cell lysate was immunoprecipitated (IP) by C-14 to give the total amount of TrkB; the other half was precipitated by avidin beads followed by anti-TrkB antibody (against extracellular domain) immunoblotting to give the amount of surface TrkB. Total TrkB in cell lysate was also immunoblotted as an internal control. (D) Histogram of the surface TrkB fraction in total TrkB in hippocampal neurons measured in A and C. The surface TrkB fraction measurement by surface biotinylation was quantified with the ImageJ software. (E) cLTP can significantly induce more TrkB insertion into the plasma membrane in cell processes than in the cell body. Hippocampal neurons transfected with FLAG-TrkB-GFP were treated by glycine. Lower panels are magnified images of framed regions and show the distribution of surface TrkB in neuronal processes. (F) Quantitative analysis of surface levels of FLAG-TrkB-GFP receptor in E using ratiometric fluorescence assay in neuronal processes and in the cell body. All immunoflurescence images are from representative cells for each condition and the fluorescence intensity was quantified by MetaMorph software. All of the data are presented as a means ± s.e.m. determined from analysis of more than three independent experiments ($n \geq 20$ cells for each condition per experiment, $^{**}P<0.01$, $^{*}P<0.05$, Student's $t$-test). Scale bars: 10 µm.

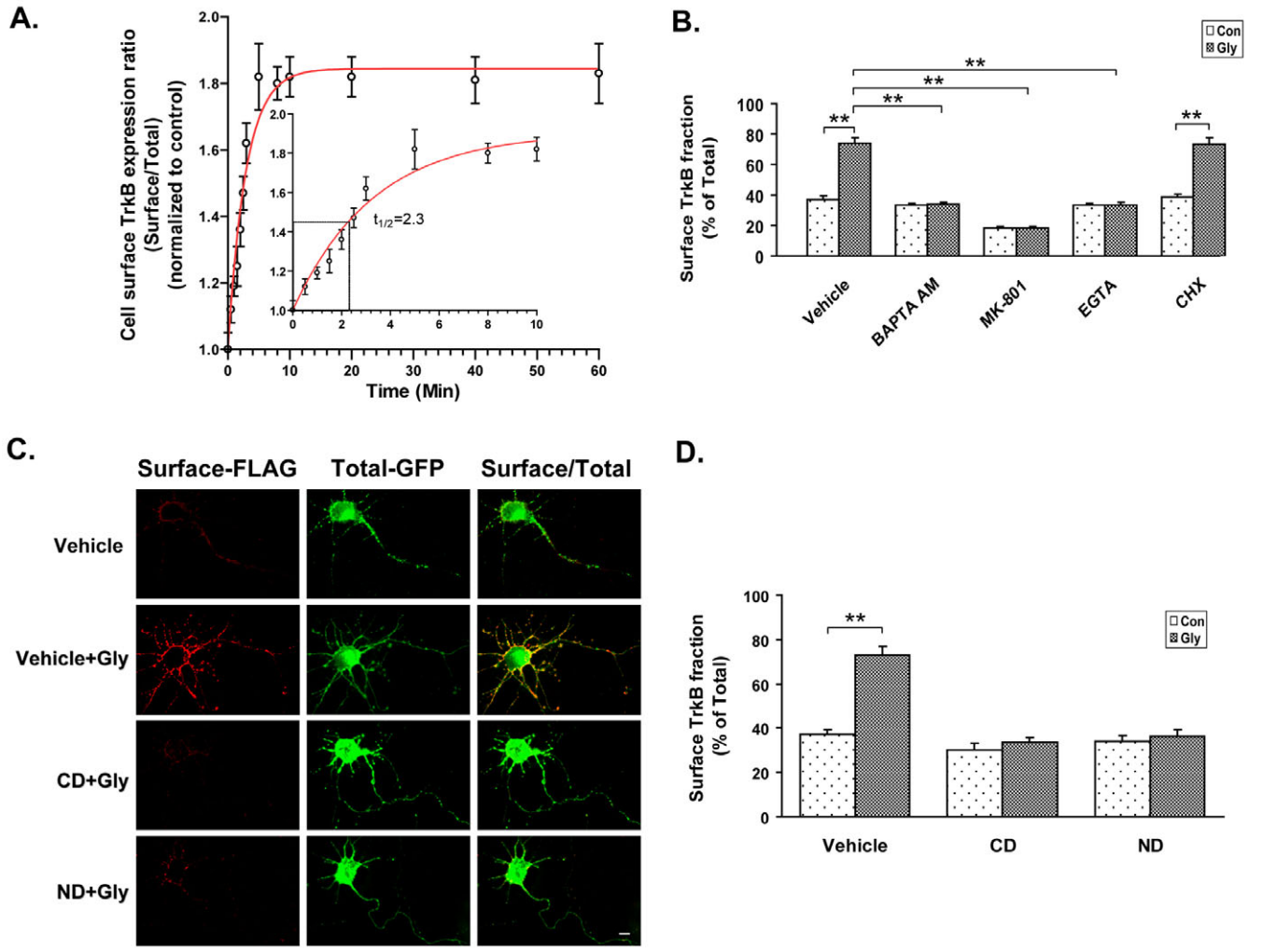


**Fig. 4.** cLTP stimuli rapidly recruit TrkB receptor to the plasma membrane. (A) Rapid recruitment of TrkB receptors to the plasma membrane with glycine treatment. Neurons expressing FLAG-TrkB-GFP were treated with 200 μM glycine at 37°C for different times up to 60 minutes. Surface TrkB levels were statistically analyzed by ratiometric fluorescence assay and plotted. The curves are based on the formula (FLAG/GFP)$t$=1–exp(–$ke$×$t$), where the (FLAG/GFP)$t$ ratio is the fraction of FLAG-to-GFP signal intensity at time $t$, and $ke$ is the receptor insertion rate constant. The inset panel is the data of an expanded time scale of 0-10 minutes. The data are the average values of more than 25 cells per time point ± s.e.m. and have been normalized to let the curve cross the zero point. (B) cLTP-enhanced TrkB surface insertion is regulated by $Ca^{2+}$ influx and does not dependent on new protein synthesis. Hippocampal neurons transfected with FLAG-TrkB-GFP were treated with BAPTA AM (20 μM), MK-801 (80 μM), EGTA (2 mM) or cycloheximide (CHX) (20 μg/ml) before cLTP stimuli. Neurons untreated with glycine were taken as control (Con). (C) cLTP-induced TrkB surface insertion relies on intact microfilaments and microtubules. Hippocampal neurons transfected with FLAG-TrkB-GFP were treated with cytochalasin D (CD) (5 μM) or nocodazole (ND) (5 μM) for 30 minutes before applying glycine (200 μM) for 10 minutes. (D) Quantification of TrkB percentages on the cell surface in hippocampal neurons from C. All immunofluorescence images are from representative cells for each condition and the ratio of surface-to-total TrkB was quantified by MetaMorph software. All of the data are presented as a means ± s.e.m. determined from analysis of more than three independent experiments ($n$≥20 cells for each condition per experiment, **$P$<0.01, ANOVA followed by post hoc tests). Scale bar: 10 μm.

(juxtamembrane, kinase, and C-terminal) were deleted from TrkB-FL. All the mutant constructs (TrkBΔJM, TrkBΔTK and TrkBΔCT) contained the same FLAG and GFP epitope tag as the wild-type FLAG-TrkB-GFP plasmid, which allowed us to measure their surface recruitment by ratiometric fluorescence assay (Fig. 5A). The basal levels of TrkB at the plasma membrane were different for the various mutants. On the one hand, this might reflect differences in constitutive versus regulated surface insertion. On the other hand, the difference might be due to different surface-receptor clearance efficiencies in TrkB mutants. Both possibilities might also be true. We found that only the TrkB mutant lacking the juxtamembrane domain (TrkBΔJM) but not the other deletion mutants abolished glycine-induced receptor surface insertion (Fig. 5B,C). TrkBΔTK mutant is still inserted in a cLTP-dependent manner, which was further confirmed by additional experiments performed with FLAG-TrkBΔTK transfection (supplementary material Fig. S1). T1 receptor is a truncated TrkB-FL isoform that lacks almost all the TrkB-FL intracellular domain but contains a very short isoform-specific cytoplasmic tail in its place (Middlemas et al., 1991). We found that, at the basal state, the surface level of T1 receptor was higher than that of TrkB-FL, which was further confirmed by quantitation of our confocal images (supplementary material Fig. S2) and was consistent with a previous report (Haapasalo et al., 2002). However, T1 did not show activity-dependent surface recruitment with cLTP

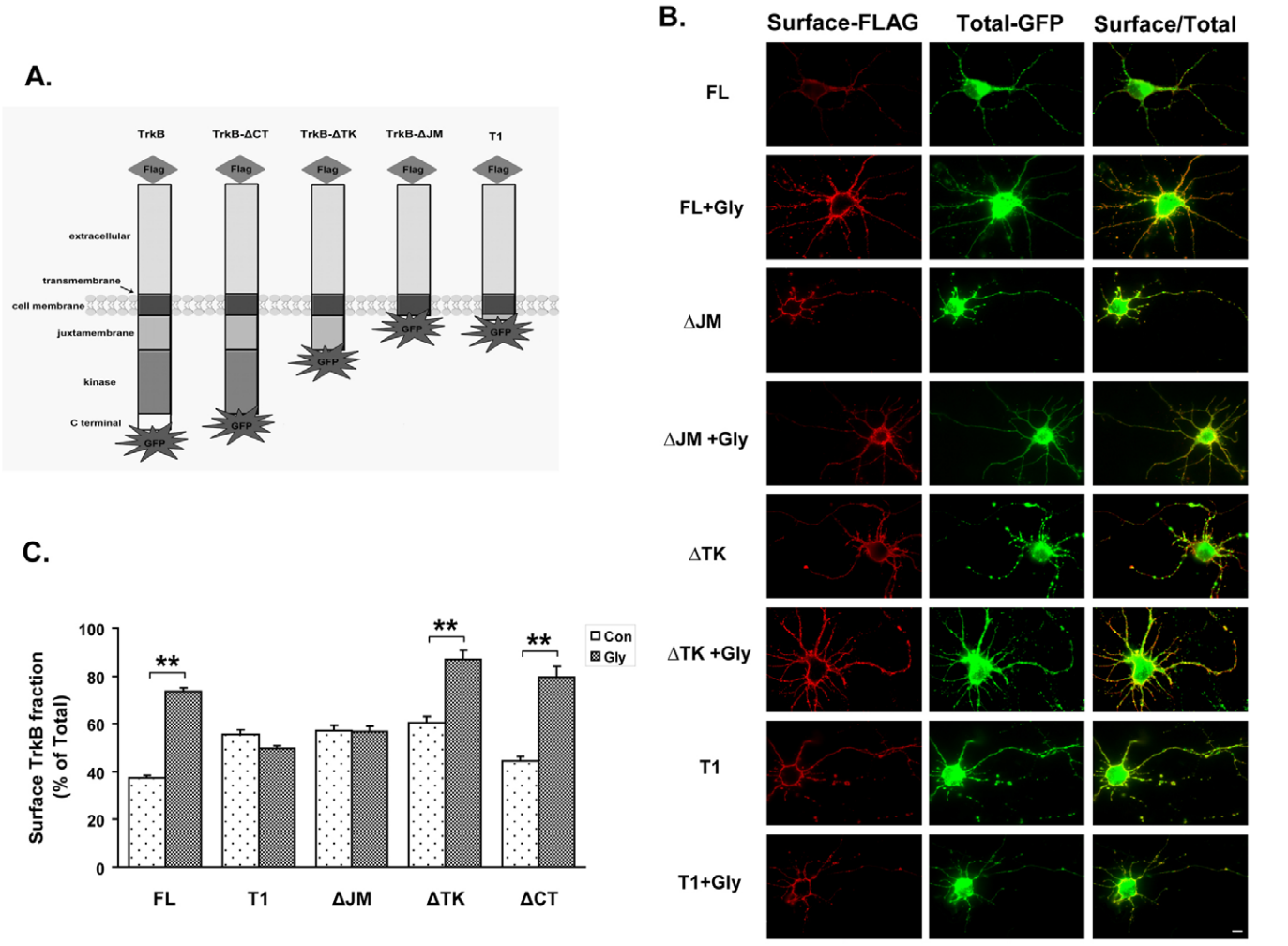


**Fig. 5.** Juxtamembrane domain of TrkB is necessary for its cLTP-induced plasma membrane insertion. (A) Schematic presentation of the TrkB deletion mutants. (B) Representative immunofluorescence images of TrkB mutants surface levels in transfected hippocampal neurons before and after glycine treatment. Scale bar: 10 µm. (C) Quantitative analysis of images in B. All of the data are presented as a means ± s.e.m. determined from analysis of more than three independent experiments ($n \geq 20$ cells for each condition per experiment, $^{**}P<0.01$, ANOVA followed by post hoc tests).

stimuli, just like TrkBΔJM (Fig. 5B,C), which was consistent with the results of our surface-receptor biotinylation studies (Fig. 1D,E). The above data suggested that the juxtamembrane domain is necessary for optimal activity-dependent insertion of TrkB receptor into the plasma membrane.

Next, we sought to determine whether the juxtamembrane domain was sufficient for activity-dependent TrkB insertion induced by glycine. Because neuronal activity could not increase levels of surface T1 receptor, we generated several chimeras in which different TrkB domains (juxtamembrane, kinase and C-terminal) were fused to the C-terminal of T1 receptor, named T1JM, T1TK and T1CT, respectively (Fig. 6A). After transfecting these chimeric constructs into hippocampal neurons, we found that only the T1 with TrkB juxtamembrane region transplantation led to a significant increase in plasma membrane receptor levels after glycine treatment (Fig. 6B,C). Together, the results suggested that the TrkB juxtamembrane region contains structural elements that are not only necessary but also sufficient for the rapid activity-dependent recruitment of TrkB to the neuronal surface.

### Cdk5 is involved in cLTP-induced insertion of TrkB to the plasma membrane

Protein kinases play important roles in biological activity, in neuronal activity and in mediating BDNF signaling. We pretreated neurons for 30 minutes with 100 nM K252a (a well-established Trk inhibitor), before glycine stimulation. We found that inhibiting tyrosine phosphorylation had no effect on cLTP-dependent TrkB surface insertion (Fig. 7A). To confirm this result we made a kinase-dead (KD) TrkB construct. TrkB-KD retained the ability of the wild type to insert into the neuronal surface in response to glycine stimuli ($P<0.01$) (Fig. 7A,B). This result, together with the results using the TrkBΔTK deletion mutant and K252a, further confirms that TrkB tyrosine kinase activity is not essential to this process. Because the neuronal surface level of TrkB receptors depends both on receptor insertion and on internalization, cleavable surface biotinylation experiments were performed to investigate the effect of TrkB kinase activity on its internalization. We found that there was no difference in BDNF-induced internalization between TrkB-FL and TrkB-KD. In addition, neuronal activity could enhance BDNF-induced TrkB-FL, but not TrkB-KD internalization (Fig. 7C,D), which is consistent

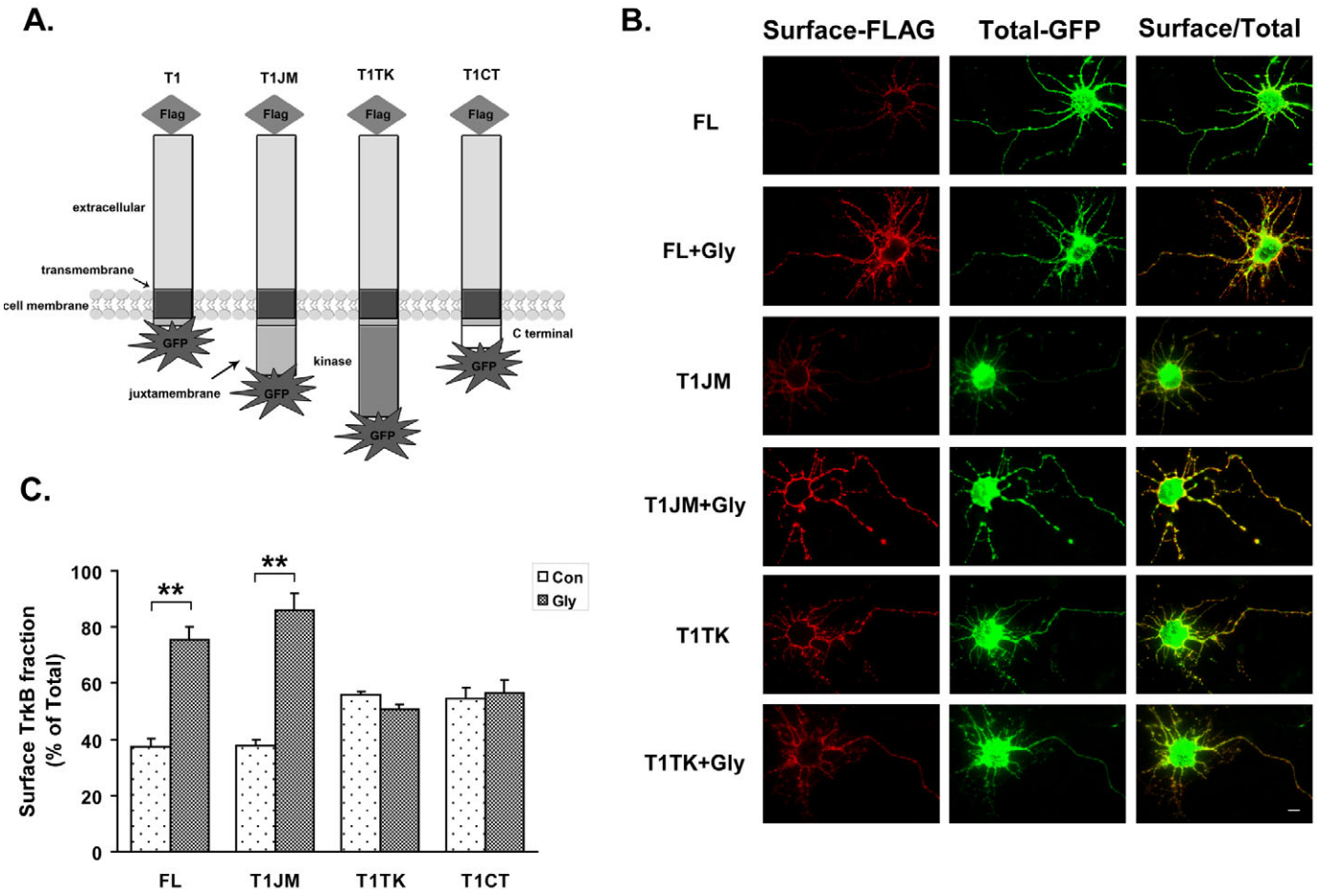


**Fig. 6.** Juxtamembrane domain of TrkB is sufficient for its cLTP-induced surface recruitment. (A) Schematic presentation of the T1 chimeras containing corresponding regions of TrkB (T1JM, TrkB juxtamembrane; T1TK, TrkB tyrosine kinase; and T1CT, TrkB C-terminal). (B) Representative immunofluorescence images of T1 chimeras surface levels in transfected hippocampal neurons before and after glycine treatment. Scale bar: 10 µm. (C) Quantitative analysis of images in B. All of the data are presented as a means ± s.e.m. determined from analysis of more than three independent experiments ($n \geq 20$ cells for each condition per experiment, $^{**}P<0.01$, ANOVA followed by post hoc tests).

with a previous report that neuronal activity facilitates BDNF-TrkB internalization (Du et al., 2003). However, in the absence of BDNF, which was the case for our measurements of TrkB recruitment to the neuronal surface, no detectable TrkB-FL or TrkB-KD internalization was found (Fig. 7C), which excluded the effect of TrkB internalization in our study. Together, our results suggest that the tyrosine kinase activity of TrkB is not essential for its activity-dependent plasma membrane insertion.

Cyclin-dependent kinase 5 (Cdk5) is a serine/threonine kinase and is implicated in neuronal development, survival, migration and synapse functions (Cheung et al., 2006). It has been shown that Cdk5 can phosphorylate TrkB at Ser478 which is pivotal in BDNF–triggered hippocampal neuron dendritic growth (Cheung et al., 2007). We found that, independently of BDNF, cLTP stimuli could lead to an acute Cdk5 tyrosine and serine phosphorylation, as detected with a phospho-Cdk5 antibody specific for Tyr15 or Ser159 sites (Fig. 8A,B). Roscovitin (ROS), a selective inhibitor of Cdk5, was used to investigate its role in cLTP-induced TrkB surface insertion. Treatment of neurons with 25 µM ROS abolished the increase in surface TrkB levels induced by glycine stimulation (Fig. 8C).

We then developed short interfering RNA (siRNA) to knock down Cdk5 expression (Fig. 8D). In neurons transfected with Cdk5 siRNA, the cLTP-induced TrkB surface recruitment was significantly abrogated (Fig. 8E,F; supplementary material Fig. S3). Given that Cdk5 can phosphorylate TrkB at the Ser478 site in the juxtamembrane region, we then examined the significance of this phosphorylation on the cLTP-induced TrkB surface insertion, by making a single point mutation at Ser478. We found that TrkBS478A mutant surface levels could not be increased in response to cLTP stimuli (Fig. 8C). Moreover, T1JM mutant with a Ser478 mutation (T1JMS478A) could not translocate to the membrane after glycine treatment (Fig. 8G,H). These results suggest that the TrkB Ser478 site, which could be phosphorylated by Cdk5, is crucial for TrkB activity-dependent surface recruitment. Protein kinase C (PKC) is another important protein kinase involved in the induction and early-phase maintenance of spinal LTP (Yang et al., 2004). The PKC inhibitor chelerythrine was also tested in our trafficking assay; however, we found that it had no effect on the cLTP-induced increase in surface TrkB levels (Fig. 8C).

### cLTP-induced TrkB surface recruitment occurs more efficiently at the postsynaptic region

cLTP-induced TrkB surface recruitment was measured in synaptic regions to test whether the TrkB insertion had any specificity to

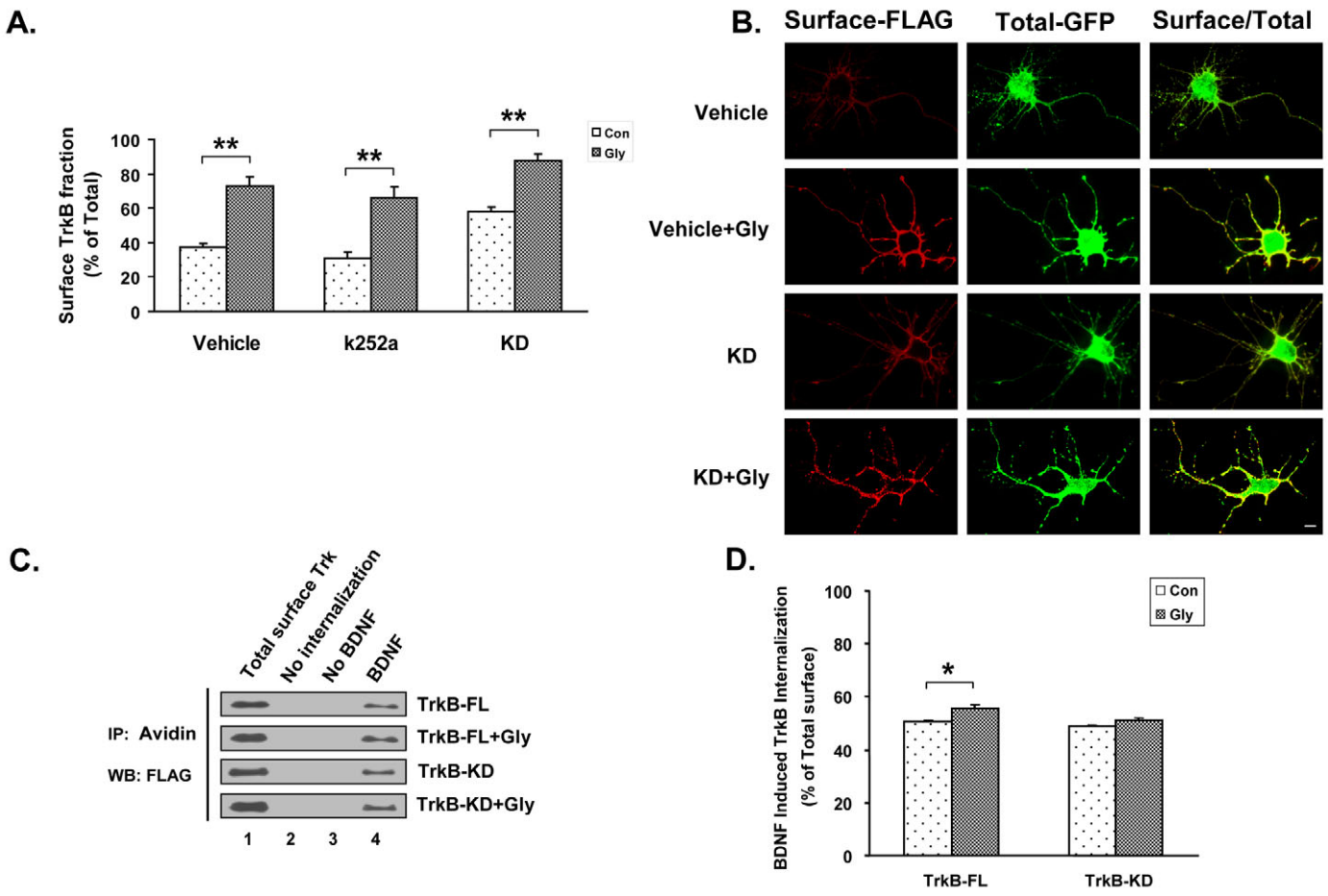


**Fig. 7.** TrkB kinase activity is not involved in cLTP-induced TrkB plasma membrane insertion. (A) Quantitative analysis of cLTP-induced surface TrkB recruitment in hippocampal neurons treated with K252a (100 nM for 1 hour before glycine treatment) or transfected with FLAG-TrkBKD-GFP (KD). (B) Representative immunofluorescence images of A. Scale bar: 10 µm. (C) Internalization of biotinylated Trk receptors in hippocampal neurons. Hippocampal neurons expressing FLAG-TrkB-GFP or FLAG-TrkBKD-GFP receptors were biotinylated with NHS-*S*-*S*-biotin and then incubated at 37°C for 10 minutes in media alone (lanes 1 and 3) or in media containing BDNF (50 ng/ml, with or without 200 µM glycine; lane 4) to allow for internalization of cell-surface proteins. The remaining cell-surface biotin was cleaved by reducing its disulfide linkage (for all samples except lane 1, which represents total biotinylated Trk receptors). Lane 2 shows a control for the efficiency of the stripping procedure, in which cells were kept at 4°C after treatment with biotin and then subjected to biotin cleavage. Neurons were subsequently lysed, biotinylated proteins precipitated with streptavidin beads, and complexes immunoblotted with anti-FLAG antibodies. (D) Quantitative analysis of the BDNF-induced internalization of TrkB-FL and TrkB-KD in C using ImageJ software. The data were presented as internalization percentage of total surface receptors. All of the data are presented as a means ± s.e.m. determined from analysis of more than three independent experiments ($n \geq 20$ cells for each condition per experiment, $^{**}P<0.01$, $^{*}P<0.05$, ANOVA followed by post hoc tests).

this region. PSD-95 (postsynaptic density protein 95), a major protein constituent of postsynaptic density, was used to identify the synaptic region in our study. Forty-eight hours after transfection of hippocampal neurons with PSD-95-mCherry and FLAG-TrkBFL-GFP or FLAG-TrkBΔJM-GFP, the colocalization percentages of total TrkB (FL or ΔJM) with PSD-95 were analyzed (see Materials and Methods). We found that the total TrkB-FL (represented by GFP fluorescence) level in postsynaptic region in neuronal processes was increased about twofold after glycine treatment (Fig. 9A,B). However, TrkBΔJM could not mobilize to the postsynaptic region after glycine treatment (Fig. 9A,B). The data suggest that neuronal activity could facilitate more TrkB-FL receptors trafficking to the postsynaptic region and that the juxtamembrane domain of TrkB receptor is essential for this process. Furthermore, the percentage of surface TrkB in PSD-95 and non-PSD-95 regions was measured by quantitative ratiometric assay. After being normalized by the total TrkB amount in the corresponding region, the surface percentage of TrkB receptor increased more dramatically in PSD-95 positive regions than in PSD-95 negative regions (2.13-fold increase in PSD-95 positive region vs. 1.75-fold increase in non-PSD-95 region, $P<0.001$) (Fig. 9C,D). Thus, cLTP-triggered neuronal activity could utilize two ways to quickly provide more functional TrkB receptor to the postsynaptic membrane: facilitate more TrkB receptors trafficking to the postsynaptic region, and recruit more TrkB receptors in postsynaptic regions to the plasma membrane.

## Discussion

Activity-dependent TrkB membrane insertion can explain, in part, the preferred effect of BDNF on active neurons. Previous studies have shown that depolarization by high $K^+$, cAMP elevation or tetanic stimuli could enhance TrkB insertion to the neuronal cell surface (Du et al., 2000; Meyer-Franke et al., 1998). However, the detailed cellular and molecular mechanisms underlying this effect

are unclear. In our study, we employed a FLAG-TrkB-GFP construct to monitor TrkB recruitment to the plasma membrane. We found that, in hippocampal neurons, cLTP is more effective in TrkB surface recruitment than $K^+$ depolarization or cAMP elevation. Although previous studies have shown that insertion of a GFP tag at the C-terminal of Trk did not affect its intracellular localization, trafficking or retrograde transport (Haapasalo et al., 2002; Heerssen et al., 2004; Jullien et al., 2003), we performed additional control experiments to exclude the possibility that overexpression or the GFP tag might disturb TrkB surface insertion. cLTP-induced TrkB surface

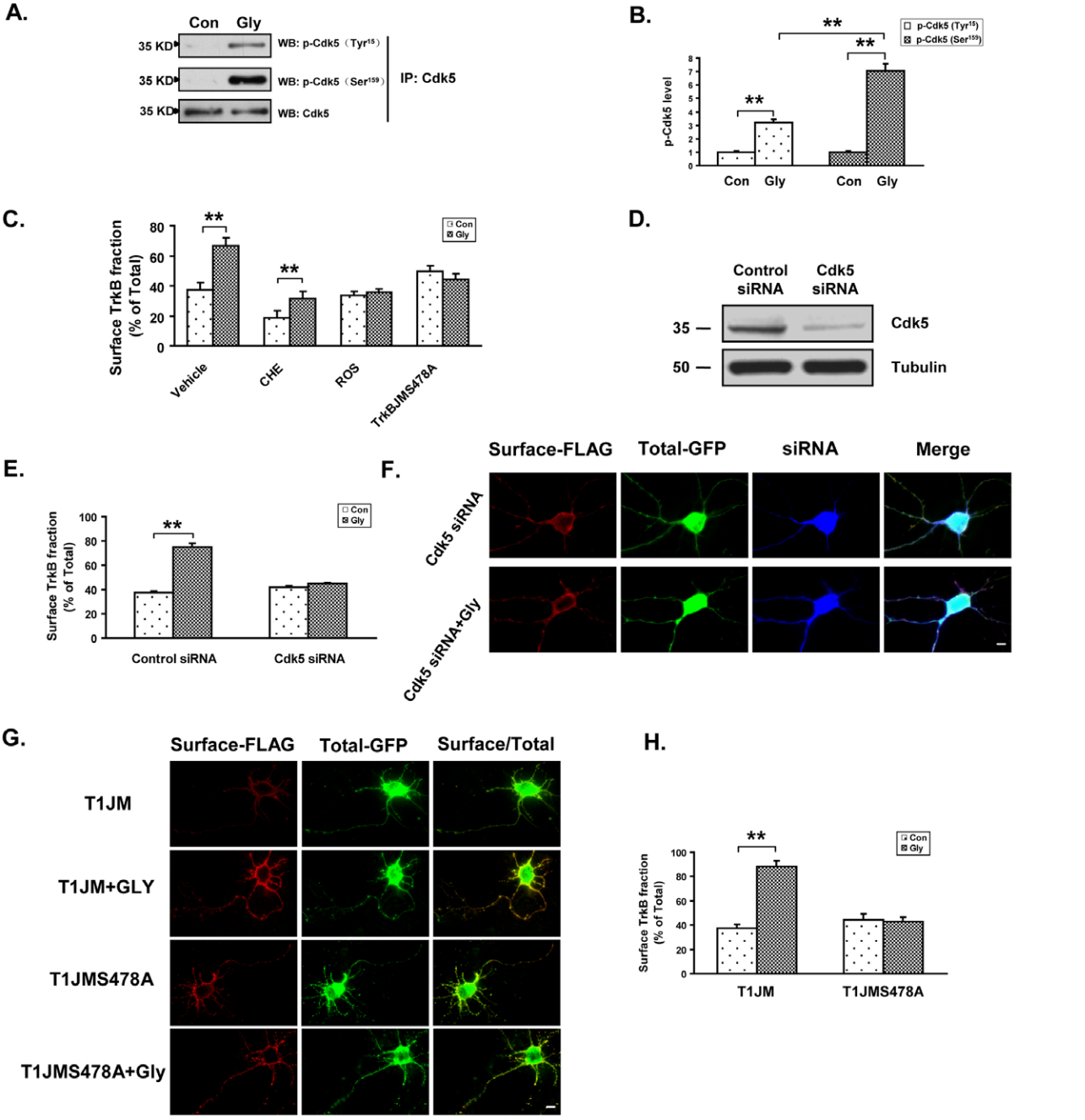


**Fig. 8.** Cdk5 activity is essential in cLTP-induced TrkB surface recruitment. (A) cLTP stimuli (Gly; 200 µM glycine treatment for 5 minutes) results in Cdk5 tyrosine and serine phosphorylation, which was detected by western blot (WB) with phospho-Cdk5 antibodies specific for Tyr15 or Ser159 sites. (B) Quantitative analysis of the Cdk5 phosphorylation level after cLTP stimulation in A using the ImageJ software. (C) Quantitative analysis of surface TrkB levels in roscovitin-treated (ROS; 25 µM for 30 minutes before glycine treatment), chelerythrine treated (CHE; 10 µM for 1 hour before glycine treatment) or neurons transfected with FLAG-TrkBS478A-GFP (S478A) using ratiometric fluorescence assay; surface TrkB is presented as the percentage of total TrkB. (D) Cdk5 level after hippocampal neurons were transfected with Cdk5 siRNA. Tubulin was used as control. (E) Quantitative analysis of cLTP-induced surface TrkB recruitment in neurons transfected with Cdk5 siRNA or control siRNA. (F) Representative immunofluorescence images of E. (G) Representative immunofluorescence images of cLTP-induced surface TrkB recruitment in neurons transfected with FLAG-T1JMS478A-GFP. (H) Quantitative analysis of G. All of the data are presented as a means ± s.e.m. determined from analysis of more than three independent experiments ($n \geq 20$ cells for each condition per experiment, $^{**}P<0.01$, ANOVA followed by post hoc tests or Student's *t*-test). Scale bars: 10 µm.

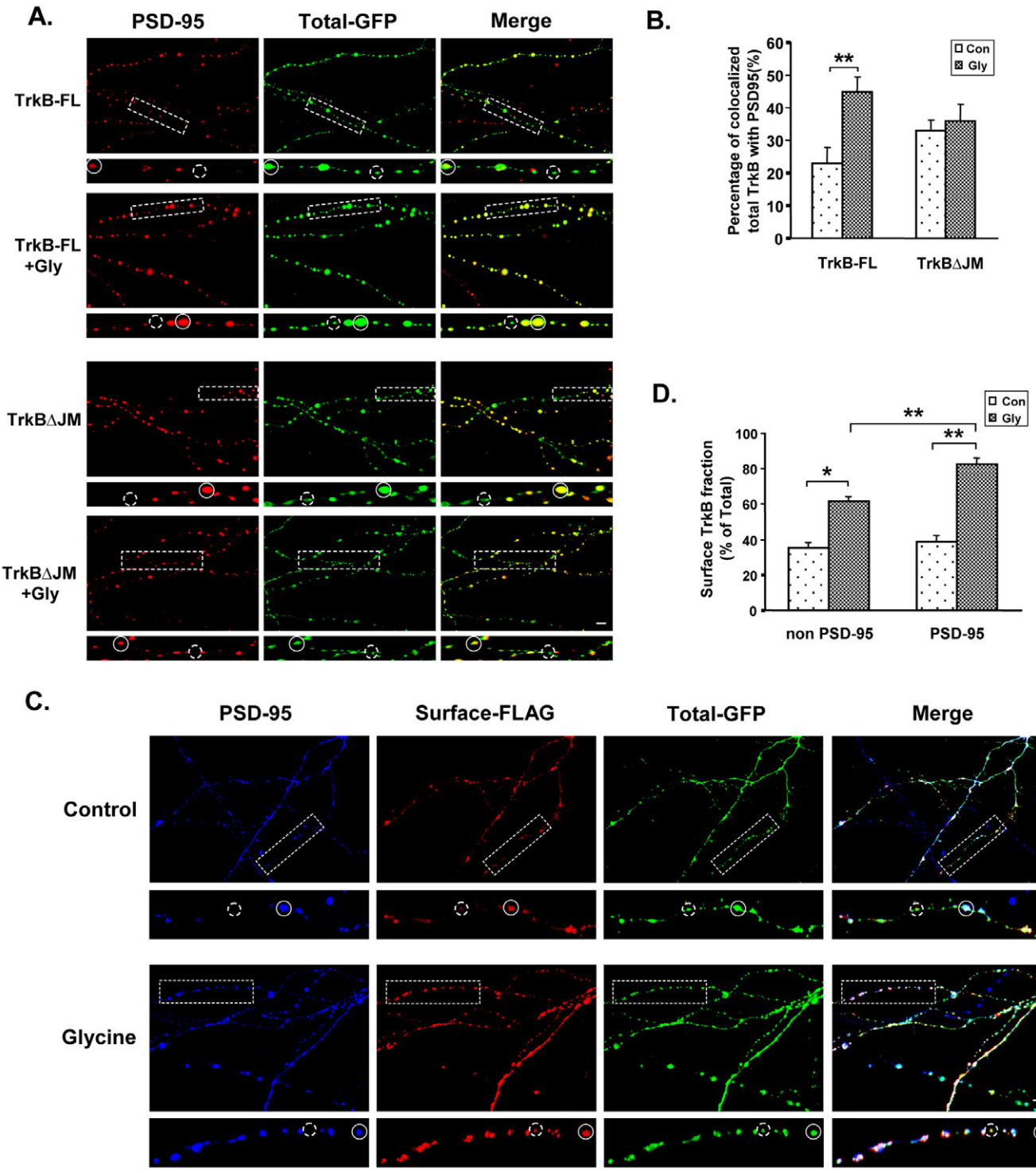


**Fig. 9.** TrkB mobilizes to the postsynaptic membrane surface following cLTP stimuli. (A) cLTP facilitate more TrkB-FL but not TrkBΔJM receptors trafficking to the postsynaptic region. PSD-95-mCherry (red) and FLAG-TrkB-GFP or FLAG-TrkBΔJM-GFP (green) constructs were cotransfected into hippocampal neurons. The dashed frames are magnified at the bottom. The colocalized region of total TrkB-FL or TrkBΔJM with PSD-95 is marked with a solid circle, and the dashed circle shows the non-colocalized region. (B) Quantification histogram of the colocalization of total TrkB-FL or TrkBΔJM with PSD-95 before (Con) and after (Gly) glycine treatment. (C) TrkB mobilization to postsynaptic membrane after glycine treatment. Hippocampal neurons were cotransfected with PSD-95-mCherry and FLAG-TrkB-GFP constructs and stained with anti-FLAG (M2) antibody under non-permeabilized conditions. The blue and red pseudo-colors represent PSD-95 and surface TrkB, respectively. GFP fluorescence indicates the total TrkB. The dashed frames are magnified at the bottom. The colocalized region of surface TrkB and PSD-95 is marked with a solid circle and the dashed circle shows the non-colocalized region of surface TrkB and PSD-95. (D) Quantification of the increase of cell surface TrkB levels upon cLTP stimuli in postsynaptic (PSD-95) and non-postsynaptic regions (non PSD-95). All of the immunofluorescence images are from representative cells for each condition. All of the data are presented as a means ± s.e.m. determined from analysis of more than three independent experiments ($n \geq 20$ cells for each condition per experiment, $^{**}P<0.001$, $^{*}P<0.01$, Student's *t*-test or ANOVA followed by post hoc tests). Scale bars: 10 µm.

recruitment was measured by surface biotinylation at endogenous levels or by immunocytochemical staining after transfection with FLAG-TrkB (without the GFP tag). The results obtained were consistent with our surface FLAG-TrkB-GFP fluorescence ratiometric assay, which suggested that FLAG-TrkB-GFP behaves similarly to endogenous TrkB and could be used to dissect the molecular and cellular mechanisms underlying this phenomenon.

Our present studies on the activity-dependent insertion of TrkB to the plasma membrane provide several new insights. Firstly, we found that the cLTP-induced enhancement of TrkB surface levels is a rapid insertion process from an intracellular TrkB pool to the cell surface with a $t_{1/2}$ of ~2.3 minutes. In addition, the inability of cycloheximide to block the effect suggests that it is not due to a transcriptional or translational increase but that it is a quick trafficking event (Du et al., 2000; Meyer-Franke et al., 1998). In this context, there are few reports on the precise time course of this recruitment kinetics. All the previous studies of activity-induced TrkB surface recruitment used 30-minute to 1-hour time frames. Here, the time-lapse experiments have shown for the first time that this effect has a short $t_{1/2}$ of ~2.3 minutes and reaches the plateau at ~10 minutes. The kinetics of this recruitment is remarkably similar to cLTP-induced surface delivery of AMPA receptor (Yudowski et al., 2007). Moreover, we found that pretreatment with nocodazole or cytochalasin D could abolish the activity-dependent recruitment of TrkB to the cell surface, which suggests that this process is microtubule- and microfilament-dependent. Interestingly, cLTP preferentially induced greater TrkB surface delivery in neuronal processes than in the soma. It has been shown that brief glycine stimulation (3 minutes) markedly increased the number of spines and the size of preexisting spines (Lu et al., 2001; Park et al., 2004; Park et al., 2006), which is within the same time course as activity-dependent TrkB surface insertion. Therefore, we speculate that the cLTP-induced rapid TrkB surface insertion might increase responsiveness to BDNF and contribute to synaptic plasticity, dendritic growth and spine formation.

Secondly, we found that cLTP-induced TrkB surface recruitment requires the cytoplasmic juxtamembrane region, which is not present in the TrkB.T1 intracellular sequence. It is important to point out, however, that cLTP stimulation does not increase surface insertion of all TrkB isoforms: surface TrkB-T1 is not increased in neurons stimulated with glycine. Using a chimeric receptor strategy, we found that the juxtamembrane domain of TrkB is not only necessary for efficient TrkB-FL surface recruitment but also sufficient, given that grafting this domain into the C-terminal of TrkB.T1 provided the ability of TrkB.T1 to be inserted in a cLTP-dependent manner. Furthermore, the juxtamembrane domain is also crucial for cLTP-induced TrkB translocation to the postsynaptic region. Our findings advance our understanding of the structural motifs responsible for activity-regulated rapid TrkB surface delivery.

Thirdly, we found a crucial protein kinase involved in cLTP-induced TrkB surface recruitment. Lu and colleagues found that $Ca^{2+}$/calmodulin-dependent kinase II (CaMKII) is involved in TrkB surface insertion induced by electric stimulation (Du et al., 2000). Whether other kinases are involved in this process is unknown. We used two loss-of-function approaches to show that Cdk5 activity is essential for cLTP-induced TrkB surface insertion: pharmacological inhibition of Cdk5 and siRNA knock-down of Cdk5 levels. Cdk5 has been shown to play an important role in synaptic plasticity (Angelo et al., 2006; Cheung et al., 2006; Hawasli and Bibb, 2007). Recently, it has been reported that Cdk5 could directly phosphorylate TrkB and is involved in BDNF-stimulated dendritic growth in hippocampal neurons (Cheung et al., 2007). Our work suggested that phosphorylation of the Ser478 site in the TrkB juxtamembrane region by Cdk5 is crucial in activity-dependent TrkB surface insertion, which further confirms that the juxtamembrane region is essential in this process. However, whether other TrkB juxtamembrane domain-interacting proteins are involved in this process, still needs to be further investigated.

Lastly, the degree of TrkB surface recruitment is greater in postsynaptic regions after cLTP stimuli. Previous studies have indicated that TrkB seems to be distributed throughout the neuron including soma, dendrite and axon (Kryl et al., 1999), and that synaptic TrkB receptors play an important role in synaptic transmission and LTP (Poo, 2001). To elicit synapse-specific modulation, it is crucial that functional TrkB is localized at the synapses, and preferably modulated by synaptic activity. It has already been shown that BDNF could trigger TrkB subcellular distribution to dendritic spines and facilitate the colocalization of TrkB with PSD-95 (Ji et al., 2005; Yoshii and Constantine-Paton, 2007). Our findings reinforce the previous conclusions, as we found that neuronal activity could both increase the distribution of total TrkB to synapses and potently drive synapse-localized TrkB to postsynaptic membranes. The efficient translocation of TrkB in the postsynaptic region to the membrane surface might present a mechanism for rapid enhancement of postsynaptic sensitivity to incoming BDNF signaling.

The rapid TrkB surface recruitment involves several intracellular trafficking steps: (1) sorting biosynthesized TrkB receptors to the intracellular reserved pool; (2) transporting the TrkB-containing vesicles to the plasma membrane region during neuronal activity; (3) docking and fusion of the TrkB-containing vesicles with the plasma membrane. There are questions remaining that need to be investigated in the future: Which step in the insertion of TrkB to the cell surface is regulated by Cdk5 activity? Are other juxtamembrane-interacting proteins involved in this process?

We found that the effect of activity-dependent TrkB surface delivery is greater in the synaptic region. However, it is unclear whether there are higher percentages of TrkB in the reserve pool in synaptic regions or whether the trafficking, docking and fusion of the vesicle containing TrkB are more efficient in the synaptic region. Previous studies have shown that BDNF produces a very rapid and transient increase in TrkB membrane localization (Haapasalo et al., 2002; Ji et al., 2005), which further emphasizes the possibility of multiple, mutually non-exclusive mechanisms underlying activity-dependent TrkB surface recruitment.

In summary, our studies provide temporal, structural and spatial information regarding activity-dependent surface delivery of TrkB receptors that will advance our understanding of how TrkB modulates synaptic plasticity. Not only might these findings provide insights into the mechanistic link between activity-dependent and neurotrophic modulation of CNS neurons and synapses, but they might also have general implications in the cell biology of growth factor signaling.

## Materials and Methods

### Reagents and antibodies

Glycine was obtained from Amresco (Solon, OH). BAPTA/AM, Roscovitine and K-252a were obtained from Calbiochem (La Jolla, CA). EGTA, forskolin, cycloheximide, MK-801, cytochalasin D, nocodazole, anti-FLAG M2 monoclonal antibody were purchased from Sigma-Aldrich (St Louis, MO). The other antibodies were purchased as follows: rabbit anti-FLAG antibody from ABR-Affinity Bioreagents (Golden, CO); mouse anti-TrkB antibody from BD Biosciences Pharmingen (San Jose, CA); rabbit anti-PSD-95 antibody from Cell Signaling Technology (Danvers, MA); rabbit Trk C-14 polyclonal antibody and Cdk5 (DC-17), p-Cdk5 (Ser159), p-Cdk5 (Tyr15)

antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); Alexa Fluor 594 goat anti-mouse IgG (H+L), and Alexa Fluor 488 goat anti-rabbit IgG (H+L) from Invitrogen (Carlsbad, CA); Cy5-conjugated goat anti-rabbit IgG (H+L) from Jackson ImmunoResarch Laboratories (West Grove, PA). The restriction enzymes were purchased from MBI Fermentas (Hanover, MD). Sulfo-NHS-biotin, sulfo-NHS-*S*-*S*-biotin and chemiluminescence were from Pierce Biotechnology (Rockford, IL). Vectashield mounting medium was obtained from Vector Laboratory (Burlingame, CA). The other reagents were from Sigma-Aldrich.

### Plasmid constructs and siRNAs

The coding region of rat full-length (FL) TrkB cDNA was subcloned into pEGFPN1 and pCDNA3.1 expression vector (Clonetech, Carlsbad, CA) to produce pEGFP-FLAGTrkB and pCDNA3.1-FLAGTrkB constructs, respectively. Truncated TrkB mutants including juxtamembrane (JM, from Lys454 to Gly821), tyrosine kinase (TK, from Ile537 to Gly821) or C-terminal (CT, from Gln807 to Gly821) deletion mutants, S478A mutant (Ser478 of TrkB was mutated to alanine), and the kinase-dead mutant (K571A) were also subcloned into pEGFPN1, named pEGFP-FLAGTrkBΔJM, pEGFP-FLAGTrkBΔTK, pEGFP-FLAGTrkBΔCT, pEGFP-FLAGTrkBS478A and pEGFP-FLAGTrkBKD, respectively. T1 receptor was also subcloned into pEGFPN1 with an N-terminal FLAG tag after the signal peptide. Different domains of TrkB (JM, TK, CT, JMS478A) were transplanted into the T1 C-terminal, named pEGFP-FLAGT1JM, pEGFP-FLAGT1TK, pEGFP-FLAGT1CT, pEGFP-FLAGT1JMS478A, respectively. All of the constructs were confirmed by DNA sequencing. The rPSD-95-mCherry in pCAGGS/ES expression vector was kindly provided by Susan M. Voglmaier (UCSF School of Medicine, San Francisco, CA). To knock down Cdk5 expression, previously reported sequences of Cdk5 (Cheung et al., 2007) were targeted with siRNA using pSuper mammalian RNA expression vector (OligoEngine, Seattle, WA) fused with RFP according to the manufacturer's instructions. The resulting Cdk5 siRNA and the control siRNA constructs were transfected into hippocampal neurons using Amaxa (Cologne, Germany), and cells lysates collected for western blot 2 days after transfection.

### Neuronal cultures and transfection

Cultures of hippocampal neurons from timed-pregnant Sprague-Dawley rats were prepared as previously described (Chen et al., 2004). In brief, hippocampus was dissected from embryonic day-18 rats, dissociated with 0.25% trypsin in Hank's balanced salt solution without $Ca^{2+}$ and $Mg^{2+}$ at 37°C for 15 minutes, triturated in DMEM/F12 medium containing 10% fetal bovine serum. For transfection, the neurons were seeded onto coverslips coated with 0.1 mg/ml poly-D-lysine in six-well plates at a cell density of $5\times10^5$ cells/ml. For biotinylation experiments, the neurons were plated at a density of $1\times10^6$ cells/ml. Neurons were grown in Neurobasal media (Invitrogen) containing 2% B27 supplement (Invitrogen), 0.5 mM L-glutamine (Invitrogen) and 100 U/ml penicillin-streptomycin (Invitrogen) at 37°C, 5% $CO_2$ and 95% humidity. Cultures were grown for 7-10 days before being used for experiments and the medium was changed every 3 days. Neurons plated on coverslips in six-well plates were transfected with Lipofectamine 2000 following the manufacturer's instructions (Invitrogen). Cells were used for experiments 48 hours after transfection.

### Surface biotinylation and western blot analysis

Insertion of endogenous TrkB receptors to the cell surface induced by cLTP was measured by biotinylation followed by western blotting using anti-TrkB extracellular-domain antibody as described (Du et al., 2000). To induce cLTP, 200 μM glycine was applied to the hippocampal cultures in an incubator at 37°C, 5% $CO_2$ and 95% humidity. At the end of glycine stimulation, neurons were washed three times with ice-cold PBS, pH 7.4, with 0.1 mM $Ca^{2+}$ and 1 mM $Mg^{2+}$ to prevent receptor internalization. Cells were then incubated in sulfo-NHS-biotin (0.3 mg/ml in cold PBS; Pierce) for 45 minutes at 4°C to biotinylate surface proteins. The surface biotinylation was stopped by removing the above solution; unreacted biotin was quenched and removed by washing twice for 10 minutes with ice-cold Tris-buffered saline (TBS) containing 0.1 mM $Ca^{2+}$, pH 7.4. Cells were then lysed in TNE lysis buffer, which contains 150 mM NaCl, 10 mM Tris, 1 mM EDTA and 1% NP-40 with protease and phosphatase inhibitors. Extracts were clarified by centrifugation (12,000 ***g*** for 20 minutes). Biotinylated proteins were precipitated with 30 μl of ImmunoPure Immobilized Streptavidin (Pierce) at 4°C overnight. Washed beads were eluted with SDS sample buffer, and eluted proteins were resolved by SDS-PAGE.

For surface TrkB fraction assay in hippocampal neurons, biotinylation was performed first and then one half of the clarified lysates were immunoprecipitated with anti-Trk polyclonal antibody (Trk C-14, Santa Cruz) overnight at 4°C followed by incubation with protein A-Sepharose beads to capture total TrkB. The other half of the lysates were immunoprecipitated with avidin beads to capture cell surface receptors. The beads were washed three times with lysis buffer; the precipitated proteins were eluted in SDS-sample buffer and analyzed using western blot. The polyvinylidene difluoride (PVDF) membranes were probed with a monoclonal anti-TrkB antibody (1:1000; BD) and immunoreactive bands visualized by enhanced chemiluminescence (ECL; Pierce) and quantified by ImageJ software.

For examining the internalization of TrkB-FL or TkB kinase-dead mutant, a cell-surface-cleavable biotinylation assay was used (Arevalo et al., 2006). Cultured hippocampal neurons were transfected with FLAG-TrkB-GFP or FLAG-TrkBKD-GFP by electroporation (Amaxa), grown in cultures for 2 days and then serum-starved for 8 hours. Neurons were washed with ice-cold PBS and subjected to biotinylation on ice with the reversible membrane-impermeable derivative of biotin (sulfo-NHS-*S*-*S*-biotin from Pierce; 1.5 mg/ml in PBS) for 45 minutes at 4°C. Internalization was allowed to occur by incubation at 37°C for 10 minutes with or without BDNF (50 ng/ml). The internalization was terminated by placing the cultures on ice; cells were washed with ice-cold TBS and the remaining biotinylated surface proteins were debiotinylated by cleavage of the NHS-*S*-*S*-biotin disulfide bond with glutathione. Then, neurons were lysed and precipitated using streptavidin-conjugated Sepharose beads, and the eluted proteins immunoblotted with an anti-FLAG antibody.

Cdk5 phosphorylation was analyzed by immunoprecipitation with an anti-Cdk5 (DC-17) antibody and followed by western blot with phospho-Cdk5 antibodies specific for Tyr15 or Ser159. Quantitative analysis for each experimental condition was based on more than three independent experiments and the results were pooled, averaged and presented as mean ± s.e.m.

### Immunofluorescence staining

To visualize cell-surface and total amount of FLAG-TrkB-GFP in transfected hippocampal neurons, cells were fixed and stained with M2 anti-FLAG monoclonal antibody followed by Alexa-Fluor-594-conjugated goat anti-mouse secondary antibody (1:500; Invitrogen); the GFP fluorescence represented total TrkB amount. To measure the surface TrkB recruitment in the postsynaptic region following cLTP stimuli, PSD-95-mCherry and FLAG-TrkB-GFP constructs were cotransfected into hippocampal neurons and the surface TrkB stained with M2 antibody followed by Cy5-conjugated goat anti-mouse antibody. To confirm that the C-terminal GFP does not interfere with activity-dependent TrkB surface insertion, hippocampal neurons were transfected with FLAG-TrkB construct and stained with a monoclonal anti-FLAG M2 antibody before permeabilization (represent surface TrkB) and then with a polyclonal rabbit anti-FLAG antibody after permeabilization (represent total TrkB). To show the cLTP-induced FLAG-TrkB surface recruitment within a single cell, neurons transfected with FLAG-TrkB construct were incubated with M2 anti-FLAG antibody at 4°C for 30 minutes to selectively label FLAG-tagged receptors present in the plasma membrane. Cells were then quickly washed three times in PBS followed by 200 μM glycine stimulation for 10 minutes. Neurons were then fixed and incubated with rabbit anti-FLAG antibody (1:500) followed by staining with Alexa-Fluor-488-conjugated goat anti-mouse and Alexa-Fluor-594-conjugated goat anti-rabbit secondary antibodies.

### Microscopic quantitative analysis

Fluorescence images were acquired by a HQ2 cool CCD camera mounted on a Nikon Eclipse TE 2000-U microscope equipped with a motorized Z drive, and assigned to a pseudo color (green, red or blue). All of the images were collected using a 60× objective lens and processed by MetaMorph software. To quantitatively analyze the amount of surface to total TrkB (FL and all other mutants) in hippocampal neurons, more than 25 cells were examined at random. A region was selected in each image and the intensity of anti-FLAG staining or GFP for each of these regions of interest (ROIs) (Delcroix et al., 2003) was measured by the MetaMorph software (Universal Imaging Corporation, West Chester, PA). In every experiment, including TrkB-FL and all the mutants, a consistent set of acquisition parameters was used for each set of images.

To calculate the ratio of TrkB surface fluorescence to total fluorescence, we selected ROIs in the Alexa Fluor 594 staining images in which surface TrkB in neurons was stained with red fluorescence and 'ring-like'. The total TrkB fluorescence of each ROI was the signal measured in the GFP (exogenous FLAG-TrkB-GFP) or Alexa Fluor 488 (exogenous FLAG-TrkB) images. Then, the ratio of red channel to green channel signal was calculated and used to represent the surface-to-total ratio. In detection of surface TrkB fraction in total TrkB at the basal state, a standard curve was constructed from the red and green signal intensity values in ~30 neurons in permeabilized condition. From this, we obtained the relationship between the red and green signals (the slope, which is a constant representing red:green signal intensity). This ratio (the surface-to-total ratio) could be converted into the surface fraction by dividing the red signal by this constant to obtain green fluorescence units and then dividing this value by the green fluorescence to obtain the surface fraction of TrkB-FL or different mutants.

For comparable analysis of the surface TrkB (FL or ΔJM) levels in PSD-95 positive and negative regions, the surface-to-total TrkB signal ratios were compared on the basis of triple-stained images in which red pseudocolor displays surface TrkB (stained with Cy5-conjugated antibody), GFP fluorescence represents total TrkB, and blue pseudocolor (from mCherry) shows PSD-95. We defined the white dots in merged images (indicated by solid circles in Fig. 9C) as the surface TrkB in the PSD-95-positive region. To perform the colocalization analysis of total TrkB (FL or ΔJM) and PSD-95, the same threshold was set to optimize the representation of puncta in GFP and mCherry channels. Fields of dendrites were randomly selected and circled as ROI. The puncta in the GFP channel were labeled with '1' to represent the total TrkB puncta, then the images were combined with the mCherry channel to determine overlaps between green and red puncta. These overlaps were labeled with '2' to show the colocalization of total TrkB with PSD-95. The percentage of TrkB puncta colocalized with PSD-95 puncta relative to total number of TrkB puncta in the same image was calculated.

Each experimental condition was repeated at least three times. All statistical analysis was performed using SPSS Software. In each experiment, more than 25 cells were examined at random, the results of more than three independent experiments were compiled, and the means ± s.e.m. calculated.

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