The receptor tyrosine kinase TrkB signals without dimerization at the plasma membrane

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Tropomyosin-related tyrosine kinase B (TrkB) is the receptor for brain-derived neurotrophic factor (BDNF) and provides critical signaling that supports the development and function of the mammalian nervous system. Like other receptor tyrosine kinases (RTKs), TrkB is thought to signal as a dimer. Using cell imaging and biochemical assays, we found that TrkB acted as a monomeric receptor at the plasma membrane regardless of its binding to BDNF and initial activation. Dimerization occurred only after the internalization and accumulation of TrkB monomers within BDNF-containing endosomes. We further showed that dynamin-mediated endocytosis of TrkB-BDNF was required for the effective activation of the kinase AKT but not of the kinase ERK1/2. Thus, we report a previously uncharacterized mode of monomeric signaling for an RTK and a specific role for the endosome in TrkB homodimerization.

INTRODUCTION

Neurotrophin (NT) signaling is initiated by the binding of the nerve growth factor (NGF) family of protein ligands to their specific cell-surface receptors. NT signaling is critical for neuronal development and function in vertebrates. Brain-derived neurotrophic factor (BDNF) is a well-studied and highly abundant neuronal growth factor in the central and peripheral nervous systems whose canonical signaling is mediated through its interaction with tropomyosin-related kinase B (TrkB), a receptor tyrosine kinase (RTK) (1, 2). The TrkB-BDNF interaction activates the Ras–ERK (extracellular signal–regulated kinase), PI3K (phosphatidylinositol 3-kinase)–AKT, and PLCγ signaling pathways, which orchestrate their diverse cellular effects such as proliferation, differentiation, axon guidance, cell survival, and synaptic long-term potentiation (3, 4). Similar to other signaling receptors at the plasma membrane, TrkB undergoes ligand-induced endocytosis, a process playing a predominant role in the delivery of a NT signal emanating from a distal source via its retrograde transport to the neuronal soma, where it activates specific transcriptional programs and inhibits apoptosis (5).

The subcellular spatial organization of signaling receptors is considered to be directly linked to their activity (6). According to the currently accepted model, RTKs form homodimers at the plasma membrane upon ligand binding that enable the transphosphorylation of one receptor by the kinase of its cognate partner and thus initiates their signaling activity (7, 8). The precise involvement of ligand binding in the reorganization of RTK from monomers to dimers is not entirely clear but apparently differs between RTK subtypes and cellular contexts (9, 10). Trk receptors are considered ligand-dependent dimers based mainly on biochemical, structural, and cell-imaging studies of TrkA-NGF complexes, as well as their structural and functional similarity to other dimeric RTK family members (11–13). Nevertheless, direct evidence is still lacking for dimerization of TrkB upon binding of BDNF.

RESULTS

Monomeric organization of TrkB at the plasma membrane

Protein clustering in response to ligand stimulation is a common feature in various signaling systems (6, 14). To assess clustering in a relevant cellular context, we used mouse embryonic spinal cord neurons (SCNs) cultured for 2 to 3 days, which are highly responsive to BDNF and express TrkB as their predominant Trk receptor (figs. S1 and S7, B and C) (15). Endogenously labeled Trk receptors were localized by direct stochastic optical reconstruction microscopy (dSTORM) at the substrate-proximal region of the neuron soma. To test the localization precision of the system, we used an Alexa 647–only sample as a control to measure the spread of single secondary antibody molecule localization (fig. S10A). Trk clustering analysis revealed no substantial change in clustering in response to BDNF ligand interaction compared with media treatment alone (Fig. 1A). This result prompted us to further investigate the stoichiometry of the receptor at the plasma membrane to determine whether TrkB undergoes ligand-mediated dimerization or oligomerization at the single-molecule level. For this, we used a photobleaching step counting analysis of plasma membrane labeled acyl carrier protein (ACP)–tagged TrkB imaged by total internal reflection fluorescence microscopy (TIRFM) to determine receptor numbers (Fig. 1B and fig. S2) (16). Validation of TIRFM illumination setup was done by measuring a stable drop in fluorescent bead intensity in different illumination beam angles (fig. S10B). We examined ectopically expressed TrkB-ACP in rodent SCNs and in human embryonic kidney (HEK) 293T (hereafter, HEK) cells, the latter do not express TrkB endogenously (figs. S1B and S2 and data files S1 and S2). The majority of TrkB receptors were counted as monomers, and only 5 to 8% of the receptors in both types of cells were found in a dimeric or oligomeric state (Fig. 1B). By contrast, the same measurement of the epidermal growth factor receptor (EGFR) showed a significantly higher dimer or oligomer ratio of 18% in nonstimulated HEK cells (fig. S3), in accordance with previous reports on ligand-independent dimers and oligomers of the EGFR (17–19). Notably, stimulation with BDNF for 5 min did not induce an increase in the ratio of the TrkB dimers at the plasma membrane (Fig. 1B), although it induced a robust signaling response (figs. S1, B and C, and S2, B and C). Single-particle–based colocalization analysis of dual-color–labeled ACP-TrkB receptors revealed colocalization in 1 to 2% of the particles under both naïve and BDNF stimulation conditions, similar to the colocalization rates of randomly positioned spots (Fig. 1C).
Original evidence of Trk ligand–induced dimerization is based on molecular weight analysis after chemical cross-linking (13). Cross-linking with membrane impermeable BS3 reagent revealed monomer-sized bands of TrkB in lysates of SCN and of HEK cells expressing TrkB, whereas EGFR was detected as a dimer (Fig. 1E and figs. S3 and S4A). We observed that TrkA dimerizes at the cell surface after quick exposure to its ligand NGF, affirming the special organization of TrkB at the plasma membrane (fig. S4B). Given that receptor interactions might be disrupted by fixation and cross-linking procedures, we sought to examine TrkB-TrkB interactions at the plasma membrane in live cells using FRET in TIRFM mode. The FRET ratio as measured by sensitized emission in TIRFM between TrkB receptors was negligible, whereas the
heterodimeric γ-aminobutyric acid B receptor subunit 1a and subunit 2 (GB1a and GB2) yielded a robust donor-normalized per-cell FRET ratio (Fig. 1F) (20).

**Ligand binding and activation of monomeric TrkB in the plasma membrane**

Ligand binding and the subsequent tyrosine phosphorylation (pTyr) are the initial steps required for ligand-based RTK activation and signaling (7). After the unexpected observation that TrkB is organized as a monomer at the plasma membrane, we proceeded to determine whether monomeric TrkB receptors can bind BDNF. Live TIRFM of colabeled BDNF and cell-surface TrkB in HEK cells showed a coordinated lateral movement of TrkB and BDNF particles (Fig. 2, A and B). Accordingly, we observed colocalization of BDNF and labeled TrkB particles in fixed cells (Fig. 2C), demonstrating that BDNF and TrkB bind at the plasma membrane under our experimental conditions. Rapid induction of TrkB tyrosine phosphorylation at the cell surface by BDNF was confirmed by the observed enrichment in global pTyr and specific TrkB Tyr515 phosphorylation in cell-surface–purified fractions of TrkB-ACP (fig. S5). Furthermore, the increased density of pTyr labeling seen by TIRFM after BDNF treatment (Fig. 2, D and E) suggests that BDNF-TrkB induces a fast cell signaling activation at the cell surface. Confirming that TrkB binds to BDNF and is tyrosine-phosphorylated at the cell surface, we sought to detect the stoichiometry of activated TrkB at the cell surface by TIRFM. Discrete TrkB particles found in colocalization with BDNF- or pTyr-labeled particles were counted by photobleaching step analysis (Fig. 2F), resulting in monomer rates of 88 to 92% in both SCN and HEK cells (Fig. 2G).

**Localized receptor dimerization in BDNF-TrkB endosomes**

BDNF stimulation of TrkB induces endocytosis of the receptor-ligand complex and its sorting into signaling endosomes that propagate the neurotrophic signal spatially and temporally (21). Ligand-induced redistribution of EGFR receptors into endosomes has been shown to be affected in terms of the quantity and spatial organization of the receptor within the endosomes (22). In agreement with a previous work, we noticed the appearance of intracellular TrkB puncta in media-only–treated neurons and after BDNF treatment (fig. S6A) (23, 24). Labeled BDNF was robustly internalized into HEK cells that were infected with TrkB (fig. S6, B and C). Dual-color surface labeling of TrkB, followed by stimulation with BDNF in SCN, resulted in the colocalization of the two colors inside the cell together with the late endosome–associated protein Rab7 after 30 min, as well as with labeled BDNF after 60 min (Fig. 3, A and B). By contrast, we observed a distinct lack of dual-labeled TrkB colocalization at the cell surface (Fig. 1C), further demonstrating the accumulation and aggregation of multiple TrkB molecules within endosomes induced by BDNF stimulus.

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**Fig. 2.** TrkB binds to BDNF and is activated at the plasma membrane as a monomer. (A) TIRF images of TrkB-ACP and Qdot-605 (QD-605)–labeled BDNF puncta comovement in HEK cells. TrkB-ACP was labeled with CoA-488, then BDNF-QD was added, and cells were subsequently imaged up to 60 min after addition. Arrowheads in green and magenta mark individual puncta. White arrowheads mark colocalized TrkB and BDNF. Scale bar, 1 μm. (B) Traces of tracked TrkB and BDNF spots shown in (A) were plotted in a three-dimensional trace. Image at the lower base of the plot depict first frame of analysis. Images and tracking in (A) and (B) are representative of two experiments. (C) TIRF image of colocalized TrkB-ACP-488 and streptavidin-647–labeled BDNF in fixed HEK cells. Arrowheads mark colocalized spots. Quantification of colocalization is shown in (G). Scale bar, 2 μm. Image is representative of three experiments. (D) Representative TIRF images of phospho-tyrosine immunofluorescence and TrkB-ACP in HEK cells treated with media or BDNF for 5 min. Scale bar, 5 μm. (E) TIRF imaging analysis of the density of phospho-tyrosine (pTyr) spots in HEK cells and SCNs treated as in (D). Data are means ± SEM density per cell from n = 50 (HEK + media), 56 (HEK + BDNF), 27 (SCN + media), and 26 (SCN + BDNF) cells pooled from three experiments; ***P < 0.001, Student’s two-tailed t test. (F) Depiction of the analysis presented in (D) and (F). TrkB and BDNF or phospho-tyrosine spots were detected, and the ratio of TrkB-ACP monomers and dimers was assessed by photobleaching step analysis. (G) The ratio of monomers (Mono.) and dimers or oligomers (Di/oligo.) from TrkB-ACP spots detected in colocalization with streptavidin-647–labeled biotin-BDNF and immunolabeled pTyr in HEK and SCN cultures. Data are from n = 28 (HEK and BDNF), 16 (SCN and BDNF), 37 (HEK and pTyr), and 25 (SCN and pTyr) spots are pooled from three experiments.
To examine the aggregation of TrkB together with BDNF in intracellular compartments, we used a dSTORM technique, focusing here on intracellular rather than surface-proximal regions of the neuron. We observed distinct dense clusters of Trk receptors in proximity to BDNF concentrations inside the cell after a 30-min exposure to BDNF (Fig. 3C), suggesting that the accumulated receptors cluster in specific domains within the endosome. Clustering of TrkB receptors in ligand-induced endosomes may serve as a platform for receptor homointeractions. To probe these interactions, we used FRET analysis of intracellular TrkB after stimulation with streptavidin-647-labeled BDNF or with the label alone (Fig. 3, D and E). Despite their lower abundance compared to BDNF-treated cells, enough intracellular TrkB puncta were detected in BDNF-untreated cells for FRET analysis. We measured a significantly higher FRET value in endosomes containing BDNF versus those in unstimulated cells (Fig. 3F), suggesting that a ligand-dependent dimerization occurs within signaling endosomes.

**Fig. 3. TrkB clusters and dimerizes in BDNF-induced endosomes.** (A and B) Confocal images of SCN expressing TrkB-ACP that was dual-labeled with 647- and 488-conjugated CoA. After labeling, SCNs were incubated with BDNF for 30 min, then fixed, and immunolabeled with Rab7 (A) or treated with streptavidin-568–tagged biotin-BDNF for 60 min (B). Arrowheads mark intracellular colocalization between dual-labeled TrkB-ACP and Rab7 or BDNF puncta. Zoomed insets show internalized particles. Images are representative of two experiments. Scale bars, 10 μm and 2 μm (inset). (C) dSTORM localization images of SCNs treated with biotin-BDNF for 30 min, then fixed, and stained with Trk-647 and Qdot-525–streptavidin to label TrkB (red) and BDNF (green). Zoomed insets show intracellular TrkB-BDNF clusters. Images are representative of two experiments. Scale bars, 2 μm, 200 nm, and 100 nm (left to right). (D and E) Representative oblique illumination images of HEK cells coexpressing TrkB-Citrine and TrkB-Cerulean for FRET measurement, treated with BDNF–streptavidin-647 (D), or with streptavidin-647 alone (E) for 30 min. Arrowheads denote intracellular endosomal puncta containing TrkB donor and acceptor signals (E) and TrkB together with labeled BDNF (D). Zoomed insets show areas with internalized TrkB and TrkB with BDNF. Scale bars, 5 μm. (F) FRET ratio analysis of intracellular TrkB-Citrine and TrkB-Cerulean spots. Endosomes that were positive (BDNF-streptavidin-647) or negative for BDNF (in streptavidin-647–treated cells) were delineated, and the FRET ratio was calculated per spot. Data are means ± SEM FRET ratio in endosomes of cells 30 min after treatment with media with streptavidin-647 only or BDNF-647. **P < 0.001, Student’s two-tailed t test; n = 160 and 77 endosomes for streptavidin-only and BDNF treatments, respectively. The left image shows exemplary FRET measurement of the BDNF-TrkB endosome. Scale bar, 2 μm. Results and images in (D) and (F) are representative of three experiments.

**Dynamin-mediated endocytosis in BDNF-induced AKT signaling**

Next, we sought to examine the dynamics of TrkB-BDNF signaling in our cellular models. The kinetics of BDNF-induced ERK1/2 and AKT phosphorylation in SCN were probed by immunoblotting for phosphorylated ERK1/2 and AKT in SCN after 5, 30, and 60 min of incubation with BDNF and the staurosporine analog and nonselective protein kinase inhibitor K252a (fig. S7, A and B). Incubation with K252a at 1 μM abolished BDNF-induced AKT and ERK1/2 signaling in HEK and SCN cells (fig. S7, B and C), suggesting that Trk receptors mediate this response, albeit at a higher concentration than has been previously reported to effectively inhibit Trk signaling in other cell types (25). Dynamin- and clathrin-mediated endocytosis has been previously shown to control downstream signaling of the ERK and AKT pathways induced by NT ligands (24, 26–28). Considering that our results indicate a specific endosomal organization for TrkB, we proceeded to investigate the role of endocytosis in BDNF-TrkB signaling in SCN. To efficiently and acutely inhibit dynamin-mediated endocytosis, we used Dynasore, a specific inhibitor of dynamin guanosine triphosphatase (GTPase), which effectively abolished TrkB-dependent BDNF internalization (fig. S6C) (29). The SCNs used in this study endogenously express dynamin-1 and dynamin-2 (data file S2). Dynasore markedly reduced the 5-min BDNF-induced phosphorylation of AKT and abolished the 30- and 60-min BDNF-induced phosphorylation of AKT in SCNs (Fig. 4A) but had no apparent effect on BDNF-induced ERK1/2 phosphorylation (Fig. 4B). To validate this, we transfected SCNs with a green fluorescent protein (GFP)–tagged, GTase-deficient K44A mutant of dynamin, which acts as a dominant-negative (DN) isoform (30). Because the transfection efficiency in SCNs was insufficient for Western blot analysis, we analyzed ERK1/2 activation by immunofluorescence, focusing our analysis...
on DN-dynamin-GFP–positive cells compared to nontransfected cells. DN-dynamin expression induced a slight increase in ERK1/2 activation after 5-min incubation with BDNF but no effect after 30-min incubation (fig. S9A).

AKT induction by NT signaling is mediated by the activation of PI3K, which catalyzes the formation of phosphatidylinositol(3,4,5)-trisphosphate (PIP$_3$,4,5) at the membrane, thereby recruiting and activating AKT (31). To follow our results on the selective effect of endocytosis on AKT signaling, we sought to investigate these dynamics of AKT recruitment and activation at the plasma membrane. To do so, we used live TIRFM to measure the signal of PH-AKT-GFP, a probe for PIP$_3$,4,5, to monitor its recruitment and activation at the plasma membrane. To follow our results on the fast responsiveness to changes in PIP$_3$,4,5, we monitored the initial signaling response after BDNF stimulation, observing a remarkable reduction in the amplitude and duration of BDNF stimulation after Dynasore addition during the first 2 to 3 min of treatment (Fig. 4, E and F). Thus, dynamin-dependent endocytosis of TrkB by BDNF is critical to the early and sustained phases of AKT signaling at the plasma membrane. Notably, expression of DN-dynamin effectively inhibited BDNF endocytosis but did not induce an increase in TrkB-TrkB interactions at the cell surface, as measured by TIRF-FRET (fig. S9B and C).

**DISCUSSION**

Our findings suggest that TrkB is activated as a monomeric receptor for BDNF on the plasma membrane and as a dimer in endosomes. Furthermore, we suggest that these subcellular spatial alterations in receptor organization can regulate specific signaling activity of BDNF, controlling its robustness by tuning the amplitude and duration of TrkB signaling. We propose a model in which BDNF binds monomeric TrkB on the plasma membrane to activate ERK1/2 signaling, whereas BDNF-dependent dimerization of TrkB at the endosomes activates robust PI3K-AKT signaling (Fig. 4G). We postulate that the TrkB-BDNF endosome functions as a cellular platform that spatially and temporally tunes the efficiency of receptor autointeractions and signaling. These findings propose a previously unknown mechanism by which spatial organization of TrkB receptor may regulate diverse BDNF signaling and functions.

The findings we report here suggest that ligand binding and specific signaling activation of a Trk family receptor can occur independent of its homodimerization. Notably, the earliest report describing TrkA as a dimer shows that most of NGF-TrkA–cross-linked complexes are

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**Fig. 4. Dynamin-mediated endocytosis facilitates BDNF-induced AKT but not ERK1/2 signaling in SCNs.** (A and B) Western blot analysis of phospho-AKT (pAKT) (A) and phospho-ERK1/2 (pERK1/2) (B) in SCNs treated with BDNF or BDNF and Dynasore (Dyn) for the duration specified. Whole-cell lysates were gel electrophoresed and blotted to assess the phosphorylation of AKT and pERK1/2. Blots of pERK1/2 were reprobed for total ERK1/2; because both pAKT and AKT antibodies are rabbit-derived, comparison between conditions is not possible. *P* < 0.05, **P* < 0.01 by Student’s two-tailed *t* test; *n* = 43 and 32 cells for BDNF and BDNF + Dynasore conditions, respectively, representative of four experiments. **(C)** Representative time-series images of SCNs expressing a PH-AKT-GFP probe by live TIRF imaging. Signal represents AKT recruitment to the plasma membrane in SCNs treated as described in (A) and measured before BDNF exposure or 5, 30, and 60 min after. Scale bar, 5 μm. **(D)** Graph shows means ± SEM of normalized PH-AKT-GFP TIRF signals per cell in each time point after treatments as in (A) and (C). *P* < 0.05, **P* < 0.01, Student’s two-tailed *t* test; *n* = 26 [dimethylsulfoxide (DMSO)], 51 (BDNF), and 44 (BDNF + Dynasore) cells. Data and images in (C) and (D) are from 4 (DMSO), 7 (BDNF), and 6 (BDNF + Dynasore) experiments, respectively. **(E)** Representative trace of a PH-AKT-GFP signal after addition of BDNF or BDNF with Dynasore. **(F)** Means ± SEM of duration (left) and peak amplitude over baseline (right) of PH-AKT-GFP-TIRF signal rise events after BDNF or BDNF + Dynasore treatments. *P* < 0.01, Student’s two-tailed *t* test; *n* = 43 and 32 cells for BDNF and BDNF + Dynasore conditions, respectively, representative of four experiments. **(G)** Schematic model of TrkB endosome-specific dimerization and signaling role in SCN: TrkB receptor monomer binds to BDNF at the plasma membrane to activate ERK1/2 signaling. Dimerization of TrkB and robust downstream activation of the PI3K-AKT pathway occur in BDNF-induced endosomes.
detected as TrkA monomers (13). Using similar techniques, we confirmed that TrkA dimersize at the plasma membrane in response to NGF, whereas TrkB is monomeric. However, it should be noted that, because the cross-linking reagents used here react only with free amine residues, the lack of observable TrkB dimers could be a result of insufficient proximity between lysine residues or the N termini of TrkB proteins in a putative dimer conformation. Nevertheless, the monomeric organization of TrkB at the plasma membrane, even in the presence of BDNF, we report here is validated by the combined results of single-molecule analysis, super-resolution imaging, and FRET-TIRF measurements. Therefore, although we cannot ultimately rule out the possibility of highly transient TrkB dimer assembly at the plasma membrane, our results show that TrkB is quantitatively different in its propensity to form and maintain a homodimeric organization at the plasma membrane, compared with the homodimeric EGFR and TrkA receptors and the heterodimeric GABA receptors. We thus demonstrate that stabilization of TrkB dimers does not occur in the initial phase of BDNF binding and activation at the plasma membrane but that its dimerization is endosomal and ligand-dependent. The increase in interaction between TrkB receptors in BDNF-containing endosomes is supported by FRET analysis based on C-terminal proximity, where TrkB was fluorescently tagged. Because the tyrosine kinase domain of TrkB is close to its C terminus, the proximity between them in the homodimer may support enhanced transactivation and signaling from the endosome, which may underlie the role of endosomes in AKT signaling. The precise conformation of the TrkB-BDNF complex in the endosome versus that in the plasma membrane merits further examination, especially in light of recent evidence on EGF-EGFR, which reveals a unique conformation in which EGF allosterically supports EGFR dimerization but is not part of the direct dimerization interface (9). Available structural data of TrkA/NGF demonstrate a dimer/dimer stoichiometry (12), which may reflect the dimerization of TrkB we found in endosomes. However, our findings regarding its plasma membrane organization invite a re-examination of the structural mechanisms by which ligand initially activates TrkB. Possibly, interactions between the intracellular domain of TrkB and cytosolic Src family kinases initiate the phosphorylation and activation of TrkB, such as the reciprocal kinase activity between TrkB and the kinase Fyn (33), or heterodimeric interaction with a co-receptor at the plasma membrane. In addition, according to our results, the DN effect of truncated TrkB isoform (TrkB.T1) on full-length TrkB (TrkB-FL) signaling is not through the formation of inactive TrkB-FL/TrkB.T1 dimers at the plasma membrane but rather through downstream signaling by BDNF-TrkB.T1 interactions (34).

The endosomal clustering and dimerization of TrkB upon ligand stimulation, along with the AKT-specific signaling output, support a model by which the neurotrophic signaling endosome serves as a signaling organization platform with roles beyond the spatial propagation of a neurotrophic signal or its termination by endolysosomal degradation. These signaling endosomes specify not only the organization of the TrkB receptor but also its capacity to induce and sustain PI3K-AKT activation at the plasma membrane, in agreement with a study by Zheng et al., reporting a neuron-specific AKT, but not ERK1/2, activation dependence on clathrin- and dynamin-mediated endocytosis (24). In addition, we observed a fast PI3K-AKT signaling response to BDNF at the plasma membrane that occurs as early as 30 to 60 s after its addition and is dependent on dynamin-mediated endocytosis. A similar dependence of early signaling events on clathrin-mediated endocytosis was also reported previously (24). A possible explanation for the endosome dependence of early PI3K-AKT signaling was presented in a recent study that followed PIP dynamics during and after clathrin-mediated endocytosis, suggesting that the budding vesicle is a phosphatase-protected membrane substrate, allowing the localized increase in the concentration of specific PIP species (35). Such a mechanism could explain why dynamin-mediated endocytosis is required for the fast onset of membrane-proximal PIP3 accumulation after BDNF stimulation. Further examination is required to pinpoint and dissect the possible mechanism by which the endosome regulates specific downstream signaling events by sequestering and concentrating signaling receptors, substrates, and positive and negative effectors. In the case of growth factor–induced receptor endocytosis, the high local concentration of the ligand within the endosome could be a potential mechanism to drive receptor dimerization by direct ligand-induced coupling. An alternative or additional mechanism could be the selective formation of membrane domains in these BDNF-TrkB endosomes, which locally concentrate TrkB receptors, with its low affinity toward its dimeric state, and thereby increase the likelihood of homointeractions, stabilizing TrkB dimers and clusters. A similar concept was previously brought by Villasenor et al., who showed EGFR-localized clustering in EGF-induced endosomes, which, in that case, was related to endosome-specific down-regulation of EGF signaling (22).

Endosome-specific heterodimerization of monomeric plasma membrane receptors, similar to our findings, has been reported for interleukin (IL) receptors IL-4Rα and IL-2Rγ after ligand stimulation, whereas other IL receptor pairs readily dimerize at the plasma membrane (36). Our findings on TrkB highlight a possible general role for subcellular compartmentalization in controlling RTK homodimerization and signaling. Further work is needed to elucidate the mechanisms of how the endosome may locally facilitate receptor dimerization and how receptor dimerization directly regulates specific downstream signaling.

**MATERIALS AND METHODS**

**Animals**

Animals were maintained in Tel Aviv University Specific Pathogen Free animal facility. Either ICR mice from Harlan or B6.Cg-Tg(Hlx9-GFP)1Tmj/j males crossed with ICR females were used. In case of Hlx9-GFP crossings, timed pregnancy embryos at embryonic days 11 to 12 (E11 to E12) were screened under a fluorescence microscope, and only negative embryos were used. Embryos of both sexes were used. Before dissection, pregnant mice were sacrificed by placing them in a chamber with a slow flow of CO₂ until breathing ceased and mice were unconscious, followed by cervical dislocation. All animal experiments were supervised by the Tel Aviv University animal ethics committee.

**Culturing of SCN cultures**

SCNs derived from mouse embryos were cultured for 2 to 3 days in vitro (DIV 2 to 3) in all experiments, unless specifically noted otherwise. Preparation of motoneuron-enriched dissociated neuron cultures was based on a modified protocol of the Henderson Laboratory (37). ICR embryos were used for all experiments. Culture plates were prepared for plating by coating overnight with poly-l-ornithine solution (1.5 μg/ml) in PBS in a cell culture incubator at 37°C and then replaced with 1:333 laminin (L2020, Sigma-Aldrich) in double-distilled water (DDW) for 2 to 3 hours before plating the SCN. Complete Neurobasal medium (CNB) was used in culture preparation and maintenance. CNB was freshly prepared for each culture by mixing 2% B27 (17504044, Thermo Fisher Scientific),
oxyribonuclease (DNase) (0.1 mg/ml; DN-25, Sigma-Aldrich), 0.4% salt solution, and trypsin (T1426, Sigma-Aldrich) was added for a final concentration 0.025%, incubated for 5 min at 37°C water bath, mixed by tapping, and incubated for another 5 min, after which the treated spinal cord pieces should have formed a clump. The clump was transferred to a tube containing freshly prepared 1-ml mixture of deoxyribonuclease (DNase) (0.1 mg/ml; DN-25, Sigma-Aldrich), 0.4% bovine serum albumin (BSA) (A9418, Sigma-Aldrich) in Leibovitz's L-15 (11415064, Thermo Fisher Scientific). Clumps were triturated by pipetting up-down twice and left to settle for 2 min, and then, the supernatant was transferred to the collection tube without disturbing the precipitate. One milliliter of freshly prepared CNB supplemented with 0.4% BSA and DNase (0.02 mg/ml) was added to the remaining clumps, followed by eight rounds of trituration. The collection and trituration steps were repeated for a total of three rounds of trituration, and any remaining tissue clumps were discarded. Cell homogenate was added to a centrifuge tube with 1 ml of 4% BSA cushion and spun at 400 gg for 5 min. Pellet was collected and resuspended in 6 ml of CNB and divided into two tubes. For each tube, 1 ml of 10% OptiPrep solution [10.4% OptiPrep (D1556, Sigma-Aldrich), 10 mM tricine, and 4% glucose in DDW] was slowly added to the bottom of the tube to create a fine interface. Tubes were centrifuged at 775g for 20 min with low acceleration and deceleration, and cells at the OptiPrep media interface were collected, mixed with 3 ml of CNB, and loaded on 1 ml of 4% BSA cushion in a centrifuge tube. Cells were spun at 400g for 5 min, then the pellet was resuspended with 1 to 2 ml of CNB, and cells were counted, diluted in CNB, and plated.

HEK cell culturing and transfection
HEK cells were received from the laboratory of E. Bachrach (Tel Aviv University), maintained in Dulbecco’s minimum essential medium (DMEM) (01-053, Biological Industries) [supplemented with 1% GlutaMAX (35050061, Thermo Fisher Scientific), 10% fetal bovine serum, and 1% penicillin-streptomycin (Biological Industries)], and kept in a humidified incubator at 37°C and 5% CO₂. Cells were passaged by 1:10 dilution every 3 to 4 days or when nearing full confluence. Passage numbers 1 to 15 were used for experiments. For lentiviral preparation, cells were transfected with calcium phosphate method as described below. For live- and fixed-cell imaging experiments, cells were transfected 1 day after plating with Fugene-6 reagent (E2691, Promega) in DMEM at a ratio of 1:3 μg DNA/μl of reagent. For cells plated on six-well plate, 4 μg of DNA was used at a transfection mixture volume of 200 μl per well. For cells in 8-mm polydimethylsiloxane (PDMS) wells, 0.1 to 0.2 μg of DNA per well was used at a total volume of 25 μl per well.

Preparation of glass-bottom culture plate samples
For TIRF and dSTORM imaging, cells were plated on round coverslip-bottom 35-mm dishes with #1.5 thickness (FD35-100, World Precision Instruments). Before use, the plates were cleaned as follows: Plates were treated with 20% NaOH for 45 min, washed in DDW, then treated with 30% sulfuric acid for 30 min, rinsed in DDW, cleaned with 70% ethanol, then washed with sterile DDW, and left to dry in a sterile hood. Before plating cells, PDMS (Sylgard 184, Dow Corning) was mixed, cast, cured in round plates, and then cut to fit on the glass bottom dishes. For plating, the cells were punched using 6-mm wells (four wells per plate) or 8-mm wells (two wells per plate). PDMS well cast was cleaned with tape and 70% ethanol and left to dry inside a biological hood. The cast was firmly attached to the glass surface, and the plate was incubated for 5 to 10 min in 60°C. PDMS well device was pressed against the glass to ensure tight bondage. For plating SCNs, wells were treated overnight with poly-l-ornithine and 2 hours of laminin before, as described in the description of dissociated neuron culture preparation. For plating HEK cells, wells were coated with 0.1% poly-l-lysine (P4707, Sigma-Aldrich) for 45 min before plating. Unless specified otherwise, SCNs were plated at a density of 10,000 cells per 6-mm PDMS well. HEK cells were plated on 8-mm wells at a density of 15,000 cells per well.

Expression plasmids and vector constructs
TrkB-GFP plasmid encoding rat TrkB-FL fused to enhanced GFP (EGFP) at the C terminus under a cytomegalovirus (CMV) promoter was gifted by R. Segal (Harvard University). The pLL3.7-CMV-EGFP lentiviral vector backbone (Addgene #11795) was gifted by U. Ashery (Tel Aviv University). LV-TrkB-GFP was cloned by inserting TrkB and p75 from TrkB-GFP into pLL3.7-CMV-EGFP multiple cloning site downstream of the CMV promoter, in frame with the EGFP. LV-TrkB-ACP was subcloned by inserting the ACP sequence from pACP-tag(m)-2 (New England Biolabs) immediately after the TrkB signal sequence and cloning into pLL3.7-CMV whose EGFP reporter was excised. Similarly, LV-EGFR-AEC was cloned by inserting human EGFR (ErbB1) from EGFRTGFP expression plasmid (gifted by R. Pinkas-Kramarski, Tel Aviv University) together with ACP inserted after its signal sequence into pLV lentiviral backbone (VectorBuilder, Cyagen) under CMV promoter. LV-TrkB-ACP-GFP was created by inserting TrkB-ACP into pLL3.7-CMV. TrkB-Citrine and TrkB-Cerulean FRET was created by subcloning TrkB from TrkB-GFP into Cerulean and Citrine expression vectors under the CMV promoter. TrkB-Citrine, TrkB-Cerulean, GabaB1a-YFP, and GabaB2-Cerulean were gifts from I. Slutsky (Tel Aviv University). Cyan fluorescent protein (CFP)–YFP linker plasmid used to calibrate the FRET analysis parameters was a gift from K. Hirschberg (Tel Aviv University). Lentiviral expression vector for Rab5a-mCherry was cloned by inserting human Rab5a into pLL3.7-CMV-mCherry, which was created by replacing the EGFP of pL3.7-CMV-EGFP with mCherry open reading frame (ORF). The LV-PH-AKT-GFP was subcloned by inserting the ORF of the probe from PH-AKT-GFP plasmid (gifted by B. Aroeti, Hebrew University of Jerusalem) into pL3.7-CMV. Hemagglutinin (HA)-tagged TrkB (TrkB-HA) was obtained from Addgene (#39978). TrkA-HA cloned in pCDNA3 vector under CMV promoter was gifted by M. Fainzilber (Weizmann Institute). Expression plasmids for DN-dynamin (K44A) fused to EGFP and to monomeric red fluorescent protein were obtained from Addgene (#55795 and # 34681, respectively).

Lentivirus production and transduction of SCNs
Lentiviral particles were produced in HEK cells using a second generation packaging system based on Gag-Pol helper and vesicular stomatitis virus glycoprotein (VSVG) coat constructs with selected lentiviral vector construct. The helper pVSVG and pGag-Pol were gifts from E. Bachrach (Tel-Aviv University). For lentiviral production, HEK cells
grown on a 60-mm dish at 70 to 80% confluence were transfected with 10 μg of lentiviral vector, 7.5 μg of pCMV-Pol, and 2.5 μg of pSVSVG. Plasmids were placed in a calcium-phosphate transfection mix (25 mM Hepes, 5 mM KCl, 140 mM NaCl, and 0.75 mM Na₂PO₄ with 125 mM CaCl₂) immediately before their addition to cells, in a volume of 0.5 ml per plate. Culture supernatants were harvested 2 days after transfection and concentrated x10 using the PEG Virus Precipitation kit (ab102538, Abcam). Final pellets were each resuspended in Neurobasal media, aliquoted, and kept in –80°C until use. For transduction of SCNs, 2 to 10 μl of concentrated lentiviral suspension was used per well containing 10,000 SCNs. Lentiviral vectors were added 1 to 2 hours after plating SCNs and were washed out three times in CNB 24 hours later.

**Lipofectamine transfection in SCNs**

For immunofluorescence-based analysis of ERK1/2 signaling (fig S9A), SCNs plated in 6-mm wells were transfected with DN-dynamin-GFP at DIV 2. Briefly, in each well, 0.5 μl of Lipofectamine 2000 (11668-019, Thermo Fisher Scientific) was mixed with 0.2 μg of plasmid in 50 μl of Opti-MEM and added to the SCNs for 25 min. Cells were washed two times with Neurobasal media and were then incubated with 1:1 (v/v) fresh and conditioned Neurobasal growth media (conditioned media are the media aspirated from the wells before transfection and kept for this step). BDNF stimulation experiment was carried out at DIV 4.

**BDNF stimulation experiments procedure**

Recombinant human BDNF was used at a concentration of 50 ng/ml for the times indicated. Cells were deprived of serum and neurotrophic supplements for 16 to 24 hours before the experiments were initiated. SCNs were incubated with Neurobasal medium supplemented with 1% GlutaMAX and 2% B27 (poor Neurobasal media), and HEK cells were kept in DMEM and 1% GlutaMAX (poor DMEM). For ACP-CoA labeling experiments (further detailed below), poor media were supplemented with 0.5% BSA for blocking. For inhibition of dynamin and Trk activity, cells were preincubated with 100 μM Dynasore (D7693, Sigma-Aldrich) or 1 μM K252a (K-150, Alomone Labs) in poor DMEM/Neurobasal for 1 hour to allow complete blockage before stimulation with BDNF. When Dynasore was applied to SCNs, B27 was not added to the media to avoid nonspecific binding of Dynasore to proteins in the supplement.

**Streptavidin labeling of biotin-BDNF**

Biotin-BDNF (B-250-B, Alomone Labs) at a concentration of 10 μg/ml was mixed with streptavidin-647 (Jackson ImmunoResearch 016-600-084), streptavidin-568 (S11226, Thermo Fisher Scientific), streptavidin–Qdot-525, or streptavidin–Qdot-605 (Q10143MP and Q10101MP, Thermo Fisher Scientific) in DMEM or Neurobasal at a molar ratio of 1:1 streptavidin to biotin (assuming an average of 1.5 biotin per BDNF molecule, according to the product details by the supplier) for 20 min at room temperature. Mixture was added to the cells so that the final concentration of biotin-BDNF on the cells was 50 ng/ml.

**Immunofluorescence**

 Cultures were fixed in 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and then blocked for 15 min at 37°C with blocking solution [5% normal serum from donkey or goat (017-000-121 and 005-000-121, Jackson ImmunoResearch) and BSA (1 mg/ml; A2058, Sigma-Aldrich)] in PBS. Primary antibodies [Trk c-14 (1:400; sc11, Santa Cruz Biotechnology), phospho-tyrosine (1:1000; 05-1050, Millipore), Rab7 (1:200; ab50533, Abcam), phospho-ERK1/2 (1:2000; M8159, Sigma-Aldrich), and total-ERK1/2 (1:2000; M5670, Sigma-Aldrich)] were diluted in blocking solution and incubated for 45 min or overnight at 37°C, followed by three washes with PBS. Unless otherwise specified, secondary antibodies against mouse or rabbit immunoglobulin G conjugated to appropriate Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 647 dyes were obtained from Thermo Fisher Scientific, diluted 1:1000 in blocking buffer, incubated with blots for 45 min or 2 hours in blocking buffer, and then washed three times with PBS before imaging.

**Cell lysis and SDS-PAGE**

HEK cells were grown on six-well cell culture plates, plated at a density of 300,000 cells per well, and harvested 1 day after transfection at a confluence of 70 to 90%. SCNs were plated at a density of 100,000 per well on a 24-well plate and harvested at DIV 2 to 3. After the experimental procedure, culture plates were washed once with cold PBS and lysed on ice using cold radioimmunoprecipitation assay (RIPA) lysis buffer containing 1% TX-100, 0.1% SDS, 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA supplemented with phosSTOP phosphatase inhibitor (Sigma-Aldrich), and complete protease inhibitor (Roche). Volumes of 100 and 50 μl per well were used for 24- and 6-well samples, respectively. After addition of lysis buffer, cells were scraped off plates and homogenized by pipetting. Homogenates were centrifuged at 10,000 g for 5 min, and the supernatant was collected, mixed in 1:4 (v/v) 5× Laemli sample buffer, and heated to 95°C for 5 min before loading on gel for SDS–polyacrylamide gel electrophoresis (PAGE). Equal sample volumes were loaded and run on 7 to 10% polyacrylamide gels. Molecular weight markers for most runs were in the range of 17 to 230 kDa, with the exception of representative samples of cross-linking experiments that were run with HiMark protein marker (Thermo Fisher Scientific) in the range of 30 to 460 kDa. Samples were blotted on nitrocellulose membranes for subsequent immunodetection by Western blotting (see below).

**Surface ACP-CoA labeling**

Reagents of ACP-CoA labeling mixture were purchased from New England Biolabs and were used according to the supplier’s instruction. For single-dye ACP labeling, either 2 μM fluorescent-conjugated CoA (488, 547, or 647; for imaging-based experiments) or biotin-CoA (for surface TrkB pulldown) was mixed with 1 mM MgCl₂ and 0.8 μM M₄-phosphopantetheinyl transferase synthase enzyme in labeling media. Labeling media were composed of DMEM with 1% GlutaMAX (for HEK cells) or Neurobasal medium with 2% B27 and 1% GlutaMAX (for SCNs), either of which supplemented with 0.5% BSA to block nonspecific CoA attachment. For dual-color labeling, CoA-488 was mixed with Coa-547 or Coa-647 at 1:4 ratio (0.5:2 μM of 488 and 547/647, respectively), which resulted in similar labeling density in the two channels. Then, a volume of 25 and 50 μl was added to 6- and 8-mm PDMS wells, respectively, and placed in the cell culture incubator for 30 min. Cells were then washed three times with labeling media, and subsequent treatment was applied as detailed for each assay.

**Avidin pulldown of surface-labeled TrkB**

HEK cells were plated in six-well plates at a density of 500,000 cells per well. One day later, cells were transfected with 4 μg of DNA of TrkB-ACP-GFP construct using Fugene-6 reagent. One or two wells were used for either BDNF or media-only conditions. Two days after plating and one day after transfection, cells were surface-labeled using biotin-CoA in a total labeling volume of 500 μl, incubated for 20 min, and then
washed three times. Cells were then stimulated with either BDNF or media alone for 5 min, then washed with cold PBS, and lysed with RIPA buffer supplemented with protease and phosphatase inhibitors as mentioned above. Samples were preclarified by 10,000 g centrifugation in a cooled microcentrifuge for 5 min and then rotated at room temperature with 25 μl NeutraAvidin beads (#1859388, Thermo Fisher Scientific) for 1 h. Beads were washed three times with PBS supplemented with protease and phosphatase inhibitors by 1000 g centrifugation steps. Conjugated proteins were eluted from the beads by boiling in 35 μl of dithiothreitol (DTT)–SDS buffer (50 mM DTT) for 5 min, and the supernatant was collected. Trace amount of bromophenol was added, and samples were run on SDS-PAGE gel for Western blot analysis.

Western blotting procedure and image analysis
Membranes were blocked in 5% milk in tris-buffered saline–Tween buffer and then incubated overnight at 4°C with primary antibody [ERK1/2 (1:15,000 to 40,000; M5670, Sigma-Aldrich), phospho-ERK1/2 (1:10,000 to 20,000; M8159, Sigma-Aldrich), α-tubulin (loading control; 1:1000; ab7291, Abcam), AKT1 (1:1000; #4691, Cell Signaling Technology), phospho(S473) AKT1 (1:250 to 1000; ab66138, Abcam), TrkB (mouse; 1:250 to 1000; 610101, BD Biosciences), TrkB (rabbit; 1:300; ANT-019-AG, Alomone Labs), EGFR (1:1000; sc-03, Santa Cruz Biotechnology), pTrk (Tyrosine 490/494; 1:500; #4619, Cell Signaling Technology), phospho-tyrosine (1:500; 05-1050, Millipore), and TrkA (1:200; ANT-018, Alomone Labs)]. Unless otherwise specified, the mouse-derived antibody from BD Biosciences was used to detect TrkB. Secondary horseradish peroxidase (HRP)–conjugated antibodies were obtained from Jackson ImmunoResearch and used at 1:10,000 for 1 hour at room temperature. Membranes were then incubated with enhanced chemiluminescence (ECL) reagent WestPico or WestFemto for enhanced sensitivity, specifically for cross-linking experiments (Thermo Fisher Scientific). ECL images were obtained with the Thermo Fisher Scientific myECL imager and quantified with the gel analysis plugin in Fiji software. In phospho-AKT and phospho-ERK1/2 assays, intensity of antibody activity, membranes were incubated with 1 mM of sodium azide for 1 hour between probeds with phospho- and total-ERK1/2 antibodies to eliminate HRP activity of bound antibodies.

Chemical cross-linking of TrkB and EGFR
SCN or HEK cells expressing TrkB or EGFR constructs were washed with PBS before cross-linking. Cells were then incubated with 3 mM BS3 cross-linker (Thermo Fisher Scientific) and dissolved in 0.45 M sucrose in PBS. Cells were incubated for 30 min at 37°C or on ice. After incubation, cross-linker was washed out, and cells were treated with 40 mM tris-HCl (pH 8.0) containing 0.45 M sucrose for 15 min at room temperature to quench remaining cross-linker. Cells were then washed with cold PBS and lysed for SDS-PAGE and Western blot analysis (see above).

PCR analysis for TrkB (NTRK2) mRNA expression
To collect RNA, cells (150,000 HEK; 500,000 SCN) cultured on 24 wells were lysed 3 days after plating with 233 μl of TRI Reagent (T9424, Sigma-Aldrich) per well. Lysates from three wells were combined. Chloroform (0.5 ml) was added to the lysate, mixed, and incubated for 15 min. Samples were centrifuged at 12,000 g for 15 min, and the supernatant was collected. Chloroform addition and centrifugation were repeated once. Isopropanol (0.5 ml) was added, incubated for 15 min, and then centrifuged at 12,000 g for 30 min. Pellets were washed with 0.75 ml of ethanol 75% and then centrifuged for 5 min. Ethanol was discarded and allowed to evaporate, and the pellet was resuspended in 50 μl of ultrapure water. To produce complementary DNA (cDNA), 50 ng of RNA was taken for reverse polymerase chain reaction (PCR) with SuperScript II First-Strand system (#11904018, Thermo Fisher Scientific) according to the supplier’s instructions. PCR analysis was performed by combining 1 μl of cDNA with primers [seq., 5′-CMGACACTCACGATTGTACG-3′ (forward) and 5′-TGTTTCTGTCATCCITCCATAC-3′ (reverse)], which amplify a 615-bp (base pair) segment in the full-length NTRK2 mRNA of either human or mouse origin in the HEK and SCN cells, respectively.

Mass spectrometry analysis of TrkB
Cell lysate (30 μg) from HEK and SCN samples were run on 10% SDS-PAGE, and the area around and between 100 and 130 kDa was excised from the gel. In this region, we occasionally observed in HEK samples faint bands after Western blot against TrkB and therefore proceeded to rule out endogenous TrkB expression in those cells. Briefly, the excised gel was processed by trypsin and chymotrypsin, analyzed by liquid chromatography–tandem mass spectrometry on Q Exactive (Thermo Fisher Scientific), and identified by Discoverer software against specific mouse and human databases. All the identified peptides were filtered with high confidence, top rank, mass accuracy, and minimum of two peptides. High-confidence peptides passed the 1% false discovery rate threshold. Protein hits are in data files S1 and S2.

dSTORM setup and image acquisition
For optimal localization microscopy imaging, SCN samples were immunolabeled with primary TrkB antibody and Alexa 647 secondary antibody as described above. An additional step of quenching after paraformaldehyde (PFA) fixation was done using 200 mM tris-HCl (pH 8.0) buffer with 50 mM NH4Cl to remove PFA autofluorescence. During imaging, samples were kept in freshly prepared imaging buffer [50 mM tris-HCl (pH 8.0), 10 mM NaCl, and 10% glucose with glucose oxidase [170 active units (AU)/ml] and catalase (1400 AU/ml)] to scaveng oxygen and enable switching of fluorophores under intense illumination conditions. Imaging and localization analysis was carried out on a Vutara 350 dSTORM system (Bruker) equipped with a water-immersion 60×/1.3–numerical aperture (NA) objective and a 4-megapixel scientific complementary metal-oxide semiconductor camera for localization imaging. Before each imaging session, the system was calibrated using fluorescent beads. Illumination used for Alexa 647 switching was a high-intensity 640-nm laser (10 to 30% of 1-W output power) together with a low-intensity 405-nm laser (3% of 100 mW). For Qdot-525, illumination was a 20% 488-nm laser. Intensity was tuned for each experiment based on observation of efficient Alexa 647 blinking and was kept identical between treatment conditions of the same experiment. For membrane clustering analysis of Trk (Fig. 1), focus was set to the lower part of the cell to specifically image the membranal region of the cell. Using the two-focal plane imaging capacity of the system, localizations could be accurately assigned in the z plane at a depth range of 1 μm around a single acquisition plane. When imaging samples labeled with Trk-647, only 10,000 frames were acquired. For Trk-647 with BDNF–Qdot-525, 5000 frames were acquired in each fluorophore channel at a rate of 10 ms per frame.

dSTORM localization parameters and Trk clustering analysis
Localization signal-over-background parameter for fluorophore detection was set to 12 to 15. Localizations that were consistent (meaning that
they did not blink) for three consecutive frames were discarded from localization analysis and used instead as fiducial markers. Localizations were further filtered on the basis of their location and sparsity. Localizations of the first 1000 frames of imaging were discarded from analysis. Accuracy of localization threshold was 0.8, with a denoise threshold at 0.2 to filter out spatially sparse localization, which could represent localization of autofluorescence of unlabeled particles. For membranal clustering analysis, localizations at a maximum z depth of 200 nm into the cell were considered and measured from the lowest z-positioned localization. Localizations were rendered as point splats, with size based on accuracy. For clustering analysis, localization images were analyzed for cluster size using Fiji software by manual examination and drawing the diameter of Trk localization clusters with at least three localizations grouped together. Radii of representative cells were plotted as histograms using MATLAB software (MathWorks).

**TIRFM and oblique microscopy**

Live- and fixed-cell TIRFM imaging was carried out on a FEI-Munich (formerly TiLL Photonics) custom-made iMic-42 digital microscope. A through-the-objective TIR via an Olympus 100× 1.49-NA TIRF objective was used for TIRF illumination. As the illumination source, four solid-state laser lines at 405, 488, 561, and 640 nm were used with a maximum output power of 50 mW each. To produce homogeneous TIR between the sample glass-media interface, the illuminating laser beam was transmitted through two scanning galvo mirrors, which enable fast deflection and scanning of the beam in the circumference of the objective back-focal plane. This application of azimuthal scanning creates a conical illumination geometry in TIR mode, which effectively eliminates the typical interference pattern of the evanescent wave created by unidirectional illumination (38). Control of stage, excitation, and acquisition parameters was via Live Acquisition 2 software. Images were captured using a Lxlon897 electron-multiplying charged-coupled device camera (Andor). In all live imaging experiments, 37°C, 5% CO₂, and humidity conditions were maintained using a custom environmental control system (Live Imaging Services), and gain was set to 300 to maximize signal capture and minimize exposure of the sample. To illuminate the sample in TIR mode, the illumination beam was shifted to spin the periphery of the TIRF objective back-focal plane by a pair of galvo mirrors. The beam position range for epifluorescence and TIRF was automatically calibrated by a built-in reflection detector system that measured reflection from the sample coverslip-medium interface (see also fig. S10). Oblique illumination mode was used to image the depth of the cell for TrkB endocytosis, Rab5a colocalization, and FRET in endosomes experiments. For oblique illumination, laser was directed at the sample at a critical angle below that of total reflection so that the excitation beam illuminates the sample at a sharp angle, creating a confocal effect. A similar method termed pseudo-TIRF was used by Cui et al. to visualize NGF–quantum dot axonal transport (39).

**Receptor photobleaching step counting analysis**

After ACP-CoA surface labeling experiments, cells were fixed by 4% PFA for 30 min and imaged by TIRFM. Cells were selected for recording and analysis based on presentation of sparse surface labeling signal. A series of 1000 images were taken with 50-ms exposure time, and an electron-multiplying gain of 300 was taken with a laser intensity of 80%. For each series, the third image was then processed for spot detection using Icy image analysis software spot detection plugin (40, 41), using manually adjustable sensitivity setting of 90 to 120, which was kept the same under the different conditions of the same experiment and a spot size of 3 pixels. The list of detected spots subpixel center coordinates was exported to a dedicated MATLAB program, which filtered spots within 7 pixels or less of any neighboring spot. Manual analysis of intensity plots was performed with MATLAB. The average intensity of a 5 × 5 box around the center of each spot was traced along the 1000-frame image series. Traces in which complete bleaching to background signal occurred within the first seven frames or that had periods of increase in intensity longer than two consecutive frames were discarded from analysis. Stepwise decreases in intensity were classified as bleaching only if they met all criteria: (i) preceded by a period of a minimum of two frames in which the intensity did not distinctively increase or decrease; (ii) intensity decrease of at least 30 intensity units but not more than 100 units; (iii) after the intensity drop, intensity remained at a stable level, lasting a minimum of two frames; and (iv) duration of the intensity decrease of one or two frames (representing a complete bleach between the two acquisitions or a partial bleach during the frame with an intermediate intensity). In the instance where one or more intensity drops in a given trace did not meet the criteria, the trace was discarded. The spot image in the image series was then manually examined using Fiji to rule out movement or the occurrence of two or more adjacent spots within the 5 × 5 pixel area examined—in these cases, the analysis of the trace was discarded. The number of bleaching steps for the trace was recorded as monomer in the case of a single step or a dimer or oligomer if two or more steps were observed. The average ratio of monomers and dimers or oligomers per cell was calculated only for cells with a minimum of 10 traces whose bleaching steps were validly counted.

**Single-spot–based colocalization analysis**

TIRFM images in two acquired channels were processed separately by Icy spot detection plugin using parameters described above for step-bleaching fluorophore counting, and x and y positions were extracted for further analysis. Before colocalization analysis, spot positions were corrected for spatial mismatch between the two channels by addition or subtraction of x and y values according to the bichannel mismatch in a reference Tetraspeck 100-nm bead sample. The colocalization between the two channels was analyzed by a custom MATLAB plugin, which calculated the Euclidean distance between spot and all the other spots in both channels. First, spots with a close neighbor of 7 pixels (0.5642 μm) or less from in the same channel were discarded to avoid false colocalization due to crowding effect. The distance threshold of the nearest neighbor in the opposing channel was 2 pixels (0.1612 μm), which counted as a colocalized spot. As an internal control, positions in both channels were scrambled by randomly adding or subtracting 0 to 5 pixels from each position’s x and y value in both channels and calculating the colocalization ratio over 100 scrambled data sets to create a distribution of randomized colocalization rates. The average random colocalization ratio was taken for comparison with the measured actual ratio.

**FRET measurement and analysis**

FRET measurement was based on sensitized emission, which means the direct measurement of emission by the FRET acceptor fluorophore after excitation of the donor, and used the measurement and analysis protocol by Kaminski et al. (42). Briefly, the FRET fluorophore pair was Cerulean (donor) and yellow fluorescent protein (YFP) or Citrine (acceptor). A 405-nm laser and a 460/36-nm bandpass filter were used for donor excitation and emission, respectively; a 488-nm laser and a 535/30-nm bandpass filter were used for the acceptor. First, to obtain the α and β coefficients for FRET ratio calculation, which depend on the imaging system’s excitation, emission, and detection parameters, a standard CFP-YFP construct was expressed in cells, considering a FRET efficiency
of 38% for the linker construct, as previously reported (43). In every experiment, a set of cells expressing donor alone and the acceptor alone were measured to correct for the cross-talk between donor and acceptor emission in donor-only sample and acceptor-only excitation by donor laser. Samples were measured in all three modes: acceptor emission with acceptor excitation (AA), donor emission with donor excitation (DD), and FRET channel-acceptor emission with donor excitation (DA). The out-of-cell background intensity was subtracted from the measurement of the mean intensity in each channel across the entire cell area, and donor emission ratio (DER) and acceptor emission ratio (AER) were calculated per each cell according to the following:

\[
\text{DER} = \frac{I_{DA}}{I_{DD}}, \quad \text{AER} = \frac{I_{DA}}{I_{AA}}
\]

Averages of DER and AER across at least five cells in each experiment were used for FRET signal calculation. The samples for the FRET experiment were measured in all three channels, and the corrected FRET signal intensity was obtained according to the following formula for each cell area (in TIRF-FRET) or region of interest (for endosome FRET measurement):

\[
cFRET = I_{DA} - AER \times I_{AA} - \text{DER} \times I_{DD}
\]

To obtain the FRET normalized per donor signal (dFRET) that was used for statistical analysis and presented in the figures, the following formula was applied:

\[
dFRET = \frac{cFRET - \alpha \text{DER}}{I_{DD} + cFRET}\]

Live imaging and analysis of AKT recruitment
SCNs expressing PH-AKT-GFP by lentiviral transduction were imaged by TIRFM. Cells presenting low basal signal of GFP signal in TIRF mode were selected for measurement because they showed the most robust change in TIRF signal after BDNF, probably due to their lower cytosolic signal that is partially detected by TIRF and that could mask the increase in TIRFM PH-AKT-GFP signal resulting from plasma membrane recruitment. The GFP signal in TIRFM mode was measured to follow the recruitment of the probe to the plasma membrane, which reflects AKT recruitment and activation. For fast time-lapse imaging (5 s per frame), 10 frames were captured before addition of BDNF, and the average of the mean GFP signal across the cell area was used as reference. All measurements after the addition of BDNF were normalized to this reference and present fold change over the starting point for each cell. Both TIRF and epifluorescence images were captured in each time point, and TIRF signal was normalized to the epifluorescence signal to reduce artifacts due to changes in overall probe signal. The resulting TIRF/epifluorescence ratio in each time point after treatment was normalized to pretreatment ratio to measure the fold change in the probe’s recruitment to the plasma membrane.

Statistical analysis
Statistical parameters and test used are noted in Results and in respective figure legends. Threshold for determining statistical significance was P < 0.05. No power analysis was performed to determine sample sizes.

SUPPLEMENTARY MATERIALS
www.sciencesignaling.org/cgi/content/full/11/529/eaao4006/DC1
Fig S1. Cultured SCNs specifically express TrkB.
Fig S2. ACP tagging of TrkB receptor enables plasma membrane–specific labeling and maintains signaling capacity in response to BDNF.
Fig S3. Comparison of TrkB and EGFR dimer or oligomer ratio by step-bleaching analysis.
Fig S4. Cell-surface cross-linking of TrkA and TrkB.
Fig S5. Short BDNF treatment induces cell-surface TrkB-ACP pTyr.
Fig S6. Dynamin-dependent endocytosis of TrkB.
Fig S7. ERK1/2 and AKT signaling in response to BDNF is mediated by Trk.
Fig S8. Dynasore alone does not reduce PH-AKT-GFP signal at the plasma membrane.
Fig S9. Effects of DN dynamin on ERK1/2 signaling, TrkB TIRF-FRET, and BDNF internalization.
Fig S10. STORM and TIRF microscopy systems resolution calibration.
Data file S1. Mass spectrometry analysis of the 100 to 130 kDa proteome in rodent SCNs.
Data file S2. Mass spectrometry analysis of the 100 to 130 kDa proteome in rodent SCNs.

REFERENCES AND NOTES
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The receptor tyrosine kinase TrkB signals without dimerization at the plasma membrane
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RTKs on a new Trk
The conventional mechanism of receptor tyrosine kinase (RTK) activation is ligand-induced dimerization in the plasma membrane, upon which one monomer phosphorylates the other to initiate downstream, intracellular signaling and receptor internalization. However, using live-cell fluorescence imaging, Zahavi et al. found that the RTK TrkB in rodent neurons is internalized as a monomer. Only then in endosomes does TrkB dimerize, both independently of and (markedly more so) in response to the presence of its ligand BDNF in the endosome. The same behavior was observed when TrkB was expressed in a human cell line. These findings and future technological advances may challenge the generalized dogma of dimer-initiated RTK signaling.