CLATHRIN-DEPENDENT ENDOCYTOSIS IS REQUIRED FOR TRKB-DEPENDENT AKT-MEDIATED NEURONAL PROTECTION AND DENDRITIC GROWTH

Jing Zheng, Wan-Hua Shen, Ting-Jia Lu, Yang Zhou, Qian Chen, Zi Wang, Ting Xiang, Yong-Chuan Zhu, Chi Zhang, Shumin Duan and Zhi-Qi Xiong

Institute of Neuroscience and State Key Laboratory of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China;

Running head: Clathrin-dependent TrkB endocytosis on BDNF signaling

Address correspondence to: Dr. Zhi-Qi Xiong, Institute of Neuroscience, 320 Yueyang Road, Shanghai 200031, China. Tel: 86-21-5492-1716, Fax: 86-21-5492-1735, E-mail: xiongzhiqi@ion.ac.cn

Endocytosis of tropomyosin-related kinase (Trk) receptors is critical for neurotrophin signal transduction and biological functions. However, the mechanism governing endocytosis of tropomyosin-related kinase B (TrkB) and the specific contributions of TrkB endocytosis to downstream signaling are unknown. In this study, we report that blocking clathrin, dynamin or AP2 in cultured neurons of the central nervous system inhibited brain-derived neurotrophic factor (BDNF)-induced activation of Akt but not ERK. Treating neurons with the clathrin inhibitor MDC or a peptide that blocks dynamin function specifically abrogated Akt pathway activation in response to BDNF but did not affect the response of other downstream effectors, or the upregulation of immediate early genes NPY and ARC. Similar effects were found in neurons expressing AP2 RNAi or a dominant negative form of dynamin that inhibits clathrin-mediated endocytosis. In PC12 cells, ERK but not Akt activation required TrkA endocytosis following stimulation with nerve growth factor (NGF) whereas the opposite was true when TrkA expressing neurons were stimulated with NGF in central nervous system. Thus, the specific effects of internalized Trk receptors likely depend on the presence of cell-type specific modulators of neurotrophin signaling and not on differences inherent to Trk receptors themselves. Endocytosis-dependent activation of Akt in neurons was found to be critical for BDNF-supported survival and dendrite outgrowth. Together these results demonstrate the functional requirement of clathrin- and dynamin-dependent endocytosis in generating the full intracellular response of neurons to BDNF in central nervous system.

INTRODUCTION

Members of the Neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin 3 and 4/5 (NT3, NT4/5), regulate neuronal development, survival, differentiation and synaptic plasticity (1-3). Neurotrophins initiate these cellular responses by binding to and activating specific tropomyosin-related kinases, known as Trk receptors. NGF is specific for TrkA, whereas BDNF and NT4/5 are specific for TrkB. NT3 primarily activates TrkC, but also other Trks with less efficiency. Neurotrophin binding to Trk induces Trk dimerization and autophosphorylation at specific tyrosine residues in the cytoplasmic domain, creating docking sites for adaptor proteins that trigger activation of PI3K/Akt, MEK/ERK, and PLCγ signaling pathways (4).
Formation of the neurotrophin-Trk complex also initiates its endocytosis (5) and endocytosis is required for some biological functions of neurotrophins (6). It has long been known that communication of neurotrophin signals from nerve terminals to neuronal cell bodies requires endocytosis and retrograde transport of neurotrophin-Trk complexes (7). Growing evidence suggests that internalization of neurotrophin-Trk complexes may also be a necessary step for activating some downstream signaling pathways. For example, blocking TrkA endocytosis with pharmacological inhibitors of clathrin-dependent endocytosis or dominant-negative dynamin inhibits NGF-induced MEK/ERK phosphorylation in dorsal root ganglion neurons (8), PC12 cells (9) and sympathetic neurons (10). Internalization of NGF-TrkA complexes is also required for NGF-induced differentiation (9). Stimulating cultured sympathetic neurons with NGF covalently cross-linked to beads that preclude ligand-receptor internalization promotes TrkA and Akt phosphorylation, but not MEK/ERK phosphorylation which normally occurs in the presence of NGF (10). These data suggest that endocytic trafficking of NGF-Trk complexes is necessary for the activation of some Trk downstream pathways but not others.

It is currently unknown whether other neurotrophins-Trk complexes, such as BDNF-TrkB, depend on endocytosis for the activation of selective downstream signaling cascades. In the central nervous system, TrkB is widely expressed in cortical and hippocampal pyramidal neurons, as well as cerebellar granule cells, while TrkA is restricted to small populations of neurons in other brain regions (11,12). Endocytosis of BDNF-TrkB complexes has been reported. In cultured hippocampal neurons, exogenous BDNF promotes a rapid translocation of adaptor protein 2 (AP2) and clathrin to the plasma membrane (13), and both neuronal activity and Ca2+ influx facilitate TrkB endocytosis in the presence of BDNF (14). In cultured sensory neurons, BDNF induces rapid endocytosis and retrograde movement of TrkB in axons (15). In the present study, we report that BDNF-induced TrkB endocytosis in cultured neurons of the central nervous system depends on clathrin, dynamin and AP2, and blocking endocytosis prevents BDNF-induced activation of Akt but not ERK. Endocytosis inhibitors likewise block NGF-induced Akt activation in TrkA-expressing neurons of the central nervous system whereas Erk activity is unaffected, the opposite effect as that observed in PC12 cells. Furthermore, we show that TrkB endocytosis is required for BDNF-induced neuronal protection and dendritic growth, but not the up-regulation of NPY and ARC. Together our results demonstrate activation of Akt signaling downstream of TrkB relies on ligand-receptor endocytosis for its initiation and BDNF-TrkB endocytosis is a required step in the regulation of biological functions.

**EXPERIMENTAL PROCEDURES**

**Primary cortical and hippocampal neuron cultures**—Cortical and hippocampal neurons were prepared from E18 rat embryos and maintained for 10–12 days in vitro as described previously (16). Primary cultures of hippocampal and cortical neurons were plated on poly-D-lysine-coated 6-well plates at 1.5×10^6 per well for biochemical experiments or on poly-D-lysine-coated coverslips at 5,000 per coverslip for microscopic analysis. All cell culture reagents were purchased from Gibco. Two hours prior to experimentation, cells were rinsed with serum-free media and then incubated in serum-free conditions. Monodansylcadaverine (MDC, 50 μM, Sigma) or dynamin proline-rich domain peptide (Dyn-pep,
50 μM, Princeton BioMolecules) was applied to the bath for 30 min prior to BDNF treatment (25 ng/ml, Sigma) to inhibit clathrin and dynamin, respectively. Cultures were treated with Trk inhibitor K252a (200 nM, Sigma), MEK1 inhibitor PD98059 (10 μM, Sigma) or PI3K/Akt inhibitor LY294002 (50 μM, Sigma) for 30 min prior to BDNF treatment. All pharmacological agents were dissolved in DMSO, control cultures were stimulated with the same amount of DMSO solution. The Animal Experiment Committee of Shanghai Institutes for Biological Sciences approved all experimental procedures.

Primary cerebellar granule neuron cultures—Cerebellar granule cells were prepared from P6 rats as described previously (17). Cerebella were dissected and then incubated for 20 min at 37°C in phosphate-buffered saline (PBS) containing 10 mM glucose, 10 mg/ml DNase, and 0.5 mg/ml papain. Cells were carefully dissociated by triturating through a plastic pipette, pelleted at 900 g for 5 min, and resuspended in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS). The medium was changed after 24 h to serum-free medium with or without BDNF (25 ng/ml) for cell survival assay. Cerebellar granule cell lysate used for Western blotting was collected after 3-4 days in vitro.

Biotinylation assay—Following treatments, cultures were quickly rinsed in ice-cold PBS-Ca-Mg (PBS, pH 7.4, containing 1 mM CaCl₂ and 0.5 mM MgCl₂). Cell surface proteins were biotinylated for 60 min with Sulfo-NHS-LC-Biotin (0.25 mg/ml, Pierce) diluted in PBS-Ca-Mg. Biotinylation was stopped by removing the biotin solution and washing cultures with 10 mM ice-cold glycine in PBS-Ca-Mg for 20 min. Cells were then lysed with RIPA buffer and biotinylated proteins were precipitated with ImmunoPure Immobilized Streptavidin (25 μl, Pierce) by constant mixing overnight at 4°C. Biotinylated protein precipitates were washed with RIPA buffer and processed for Western blotting analysis. Experiments were performed in triplicate.

Enzyme-linked immunosorbent assay (ELISA)—ELISAs were performed in 96-well plates. Following treatment, cultures were fixed and incubated with anti-TrkB (N-20; 1:50; Santa Cruz) for 3 h at 37°C. Cultures were then washed, incubated with horseradish peroxidase—conjugated goat anti-rabbit secondary antibody (1:1,000, Pierce). For end-point assays, the level of surface TrkB was measured by a ready-to-use peroxidase substrate containing 2, 3’-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid, Sigma). Absorbance was read at 405 nm.

Transfection of primary cultured neurons—Hippocampal neurons grown on glass coverslips (Corning) for 7 days were transfected with the full length TrkB-GFP, truncated TrkB-GFP constructs (kindly provided by Dr. Rosalind A Segal), wild-type dynamin and dominant negative dynamin constructs (kindly provided by Dr. Bai Lu). Constructs were mixed with 250 mM CaCl₂ and an equal volume of 2×Hepes-buffered saline (274 mM NaCl, 10 mM KCl, 1.4 mM Na₂HPO₄, 15 mM D-glucose, and 42 mM Hepes, pH 7.06). The DNA-Calcium complex was incubated for 20 min and added to cultures for 1 hour in DMEM without glutamine. After transfection, hippocampal neurons were washed three times with DMEM, and transferred to the original medium. We used the Amaxa Nucleofector device (Amaxa Biosystems) to transfect cerebellar granule neurons for the neuronal survival assay and to transfect primary hippocampal neurons for MAP2 staining. Transfection of primary neurons with GFP, Becl2-GFP, HA-tagged wild type and constitutively active Akt constructs (kindly provided by Dr. Alfonso Bellacosa, ref. (18)) was carried out...
immediately after dissociation. Plasmids (5 µg each) were introduced into dissociated neurons by electroporation using the Amaxa Nucleofector before plating onto 35 mm dishes according to the manufacturers' recommendations. Transfection efficiencies were determined in each experiment by GFP fluorescence and only cultures with more than 75% of cells transfected were considered for analysis. We also examined transfection efficiency by Western Blotting with anti-HA tag or anti-GFP antibodies.

**Single cell fluorescence imaging**—Forty eight hours after calcium phosphate transfection of full length or truncated TrkB-GFP constructs, hippocampal neurons were mounted onto a heated stage (37 °C) on the confocal microscope (Zeiss LSM 510) and incubated in 25 ng/ml BDNF for 5-30 min. GFP fluorescence was visualized under 488-nm wavelength light. Z-series stacks of confocal fluorescence images were acquired with consecutive optic slices (1 µm interval) and reconstructed with LSM Image Browser. At least 8 neurons were captured per condition in each experiment. Statistical significance (p < 0.05) was assessed using Student’s t-test.

**AP2 RNAi lentivirus generation**—AP2-targetting oligonucleotides were designed and generated from full-length rat AP2 by Shanghai GeneChem Co. Ltd. After testing knock-down efficiencies, stem-loop DNA oligonucleotides were synthesized by Shanghai GeneChem Co. Ltd. (Sense: 5’-TAA GAA AGT CCA GCA TTC AAA TTT CAA GAG AAT TTG AAT GCT GGA CTT TCT TTT TTT TC-3’; antisense: 5’-TCG AGA AAA AAA AGA AAG TCC AGC ATTCAA ATT CTC TTT AAA TTT GAA TGC TGG ACT TTC TTA-3’) and cloned into the lentivirus-based RNAi vector PsicoR (Addgene). A non-targeting stem-loop DNA PsicoR vector was also generated for use as a negative control. Lentiviral particles were prepared as described previously (19). Neurons were infected with AP2-RNAi-lentivirus or negative control virus at DIV7, and examined at DIV10.

**Western blotting and antibodies**—Cultured neurons were lysed in ice-cold RIPA lysis buffer (0.1 % SDS, 1 % NP-40, 50 mM HEPES, pH 7.4, 2 mM EDTA, 100 mM NaCl, 5 mM Sodium Orthovanadate, 40 µM PNPP, 1 % Protease Inhibitor Cocktail Set I, Calbiochem, La Jolla, CA) after treatments. Whole cell lysates or biotinylated fractions were mixed with sample buffer for SDS-PAGE. The following antibodies were used: rabbit anti-phosphor-TrkA (Tyr490) (1:1,000; Cell Signaling Technology), rabbit anti-TrkB (H-181) (1:500; Santa Cruz), rabbit anti-TRK-Akt1/2/3 (1:1,000; Santa Cruz), rabbit anti-Akt1/2/3 (1:1,000; Cell Signaling Technology), rabbit anti-phosphor-ERK1/2 (1:2,000; Cell Signaling Technology), rabbit anti-Akt1/2/3 (1:1,000; Cell Signaling Technology), anti-phosphor-PLCγ1 T783 (1:1,000; Cell Signaling Technology), anti-PLCγ1(1:1,000; Cell Signaling Technology), anti-phosphor-CREB (1:1,000; Cell Signaling Technology), rabbit anti-CREB (1:1,000; Cell Signaling Technology), rabbit anti-GFP (1:1,000; Santa Cruz), mouse anti-HA tag (1:2000; Sigma) and mouse anti-Actin (1:2000; Sigma). Horseradish peroxidase–conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (1:10,000, Pierce) were used. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Pierce). Densitometric analysis was conducted using ImageQuant 5.2 software (Amersham) and statistical evaluations were assessed by one-way ANOVA analysis of variance followed by student t test (p < 0.05 was considered significant). Each experiment was repeated at least three times.

**RT-PCR reactions**—TRIZOL reagent kit (Invitrogen) was used to isolate mRNA from cultured neurons. First-strand synthesis and PCR
were performed using M-MLV Reverse Transcriptase and Tag polymerase (Promega) according to the manufacturer’s instructions. Primers sets used were: ARC, 5’-CCG CCG GCT CTG AAT CC-3’ and 5’-AAG TTG TTC TCC TCC AGC TTG CCC-3’; NPY, 5’-TGT GGA CTG ACC CTC CTA-3’ and 5’-ATT GGG TGG GAC AGG CAG AC-3’; AP2, 5’- CGG TGG GTT ACT GGT GGA -3’ and 5’- AAC TGC GTG GAG GTC TTG -3’; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5’-ACC ACA GTC CAT GCC ATC AC-3’ and 5’- GCC ACC ACC CTG TTG CTG TA -3’.

**Cell survival assay**—Cell death was detected by enzymatic labeling of DNA strand breaks using the ApopTag peroxidase in situ Apoptosis Detection Kit (Chemicon) following the manufacturer's instructions. Briefly, neurons were grown on coverslips for 24-well chambers. After treatment, media was removed and neurons were washed three times with PBS. Cells were fixed in 1% paraformaldehyde for 10 min at room temperature and washed three times in PBS. Neurons were permeabilized in ethanol:acetic acid (2:1), washed in PBS and then incubated with TUNEL solution (TdT and digoxigenin-dUTP) in humidified atmosphere at 37°C for 60 min. The reaction was terminated by rinsing with PBS prior to incubation with peroxidase-conjugated anti-digoxigenin antibody (1:2000) for 60 min. To visualize the immuno-complexed peroxidase, substrate solution (75 µl) was added. The experiment was repeated three times in duplicate. TUNEL labeling was quantified by counting the number of positively stained neurons per hundred cells for each treatment group. Values are represented as the mean percentage of positively stained cells ± SEM. The survival of cerebellar granule neurons was also estimated using the 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay (20).

**Immunocytochemical staining and fluorescence microscopy**—Hippocampal neurons grown on glass coverslips (Corning) for 7 days were transfected with GFP, HA-tagged WT-dynamin and DN-dynamin constructs by the calcium phosphate method. Forty eight hours later, transfected neurons were stimulated with BDNF (25 ng/ml, 10 min), fixed, and then incubated overnight at 4 °C with rabbit anti-phosphor-Akt1/2/3 (1:200; Santa Cruz), or rabbit anti-phosphor-ERK (1:100;Cell Signaling Technology) and mouse anti-HA (1:1000; Sigma) in PBS containing 0.2% goat serum. Cultures were washed and incubated with Alexa Fluar 546 and 488-conjugated secondary antibodies (Molecular Probes) diluted in PBS containing 0.2% goat serum for 1 h at room temperature. Images were acquired under a Zeiss confocal microscope (40×, 488 and 546 nm laser). For the dendritic growth assay, transfection of hippocampal neurons was carried out immediately after dissociation using the Amaxa Nucleofector device. Anti-MAP2 (1:1000; Chemicon) and anti-HA (1:1000; Sigma) were used in the following immunocytochemical procedures. The number of the primary dendrites per neuron was counted from confocal images and the total dendrite length was determined by tracing all dendritic processes in Neurolucida software. At least 25 neurons were quantified per condition in each experiment, and each experiment was repeated for at least three times. Statistical significance ($p < 0.05$) was assessed using paired Student’s t-test.

**RESULTS**

**BDNF induces TrkB endocytosis in a clathrin- and dynamin-dependent manner**—We began our investigation of BDNF-TrkB endocytosis by performing a surface biotinylation assay to measure surface expression of TrkB in cultured...
cortical neurons when exposed to BDNF. Cells were stimulated with 25 ng/ml BDNF for 0-60 min and then incubated with biotin for 60 min. Biotin-conjugated surface proteins were fractionated on an immobilized streptavidin column and the amount of surface-TrkB was measured by blotting the biotin fraction with anti-TrkB and comparing it to total TrkB in whole cell lysate (Fig. 1A). Within 15 min of BDNF exposure, a reduction in surface-expressed TrkB was apparent and the biotinylated fraction was reduced by about 39% (Fig. 1B, upper). After 60 min of exposure only 38% remained at the surface (Fig. 1B, upper) but no change in total TrkB expression was observed (Fig. 1A). We further confirmed this finding by ELISA with an antibody specific for the extracellular domain of TrkB. Approximately 33% of cell surface TrkB was internalized by 15 min after BDNF application and only 45% remained at the surface after 60 min (Fig. 1B, lower). These results are reminiscent of NGF-induced TrkA endocytosis in PC12 cells (6) and suggest that TrkB receptors are endocytosed in response to exogenous BDNF stimulation.

To visualize TrkB receptor re-localization, we expressed a GFP-tagged, full-length TrkB fusion protein in cultured hippocampal neurons and monitored the response to BDNF stimulation by time-lapse imaging. TrkB-GFP was observed to undergo endocytosis within 5 min of exposure to 25 ng/ml BDNF (data not shown). Movement from the cell-surface into intracellular compartments accelerated in the following minutes and 10 min after application of BDNF cell surface localized GFP signals (within 2µm of the plasma membrane) had decreased by 42% while fluorescence had increased by 51% in the medulla (Fig. 1C a and c; 1D, upper). In contrast, we did not observe GFP internalization into hippocampal neurons transfected with a GFP-tagged, truncated TrkB receptor lacking the cytoplasmic domain (T-TrkB) in response to BDNF exposure (Fig. 1C, b and d; 1D, lower), even after 30 min (data not shown).

NGF-induced endocytosis of TrkA is both clathrin- and dynamin-dependent in PC12 cells and BDNF can rapidly increase AP2 and clathrin translocalization to the plasma membrane (13). We therefore examined whether inhibiting the assembly of the clathrin coat of endosomes with the putative inhibitor monodansylcadaverine (MDC, ref. (21)) could block TrkB internalization. We found that bath application of 50 µM MDC to cortical neuron cultures for 30 min prior to BDNF stimulation completely blocked TrkB endocytosis (Fig. 1E and F). Pre-treatment with dynamin-derived proline-rich peptide (Dyn-pep, 50 µM, ref. (22)) for 30 min also blocked BDNF-induced TrkB endocytosis (Fig. 1E and F).

**BDNF-induced PI3K/Akt activation requires clathrin-, dynamin-, and AP2-mediated endocytosis**—We next examined whether endocytosis of TrkB is required for downstream signaling. Interestingly, pre-treating primary cortical neuron cultures with MDC prevented a sustained BDNF-induced Akt activation (Fig. 2A and B, upper), as did Dyn-pep (Fig. S1), without significantly affecting the activation of TrkB, SHC, ERK, PLCγ or CREB (Fig. 2A and B, Lower). Neither MDC nor Dyn-pep affected basal phosphorylation levels of any of these proteins (data not shown). In primary hippocampal neuron cultures and cerebellar granule cells, we similarly found that inhibition of endocytosis by bath application of either MDC or Dyn-pep selectively prevented BDNF-induced Akt activation, without affecting the activation of TrkB and ERK (Fig. 3B, C and data not shown). As expected, the tyrosine kinase inhibitor K252a (23) completely blocked BDNF-induced activation of TrkB, Akt and ERK.

In support of these findings, cultured
Hippocampal neurons stained with an antibody specific to phosphorylated Akt were more robustly labeled following 10 min of BDNF stimulation than at rest. However, staining intensity of phosphorylated Akt (pAkt) was not increased in response to BDNF in cells transfected with a construct expressing HA-tagged, dominant negative dynamin (K44E-dynamin, Fig. S2A and B), which is known to inhibit clathrin-mediated endocytosis (24). In contrast, staining of phosphorylated ERK (pERK) was not affected by K44E-dynamin expression (Fig. S2A and B). Transfecting with wild-type dynamin did not alter the levels of pAkt or pERK after BDNF application (Fig. S2A and B). To further confirm our results, we used a lentivirus-based RNAi delivery system to infect cultured hippocampal neurons with short interfering RNAs (siRNAs) targeting clathrin adaptor protein 2 (AP2), a critical regulator of endocytosis. AP2 siRNA markedly reduced the expression of AP2 protein and mRNA compared to non-target control RNAi (Fig. 3A) and blocked BDNF-induced activation of Akt but not ERK (Fig. 3B and D). Taken together, these results demonstrate that BDNF-induced PI3K/Akt activation in the central nervous system requires clathrin-dependent endocytosis of TrkB.

Given the divergent effects of endocytosis on the activation of ERK and Akt pathways by BDNF/TrkB signaling in cultured neurons and by NGF/TrkA in PC12, DRG cells and sympathetic neurons (9), we set out to determine whether cell type plays a determinant role in the mechanism of activation of neurotrophin signaling. We first confirmed that 50 µM MDC and 50 µM Dyn-pep indeed blocks NGF-induced activation of ERK, but not Akt, in PC12 cells (Fig. S2C). We next transfected cortical neurons with wildtype TrkA and found that NGF-induced activation of Akt, but not ERK, was inhibited by MDC and Dyn-pep (Fig. S2D). These results demonstrate that the selective effect of Trk endocytosis on downstream signaling pathways is dependent on cell type rather than differences in the intrinsic properties of Trks.

**BDNF-induced up-regulation of NPY and ARC does not require clathrin-mediated endocytosis**—BDNF activity regulates the expression of a multitude of genes (25). To examine whether endocytosis of TrkB is required for BDNF-induced gene expression, we investigated the effect of BDNF on the expression of two immediate early genes, Activity-Regulated Cytoskeleton-associated protein (ARC) and neuropeptide Y (NPY) under conditions of endocytic blockade. RT-PCR analysis revealed that 3h stimulation with BDNF (25 ng/ml) led to expression of ARC and NPY mRNA. This upregulation was blocked by K252a (200 nM) and MEK1 inhibitor PD98059 (10 µM) but not, however, by MDC and Dyn-pep (Fig. 4A and B). The PI3K inhibitor LY294002 (LY, 50 µM) also had no effect on BDNF-induced ARC and NPY expression. Thus, ARC and NPY upregulation are not dependent on clathrin-mediated TrkB endocytosis or PI3K/Akt signaling but instead are likely to be driven by the activity of MEK/ERK signaling and possibly other pathways regulated by BDNF.

**BDNF-dependent neuronal protection requires clathrin-mediated endocytosis**—Akt is a downstream target of PI3K and a critical mediator of growth factor-induced neuronal survival. Previous studies have demonstrated that BDNF/TrkB-dependent survival of cultured neurons under serum deprivation depends on PI3K/Akt activity and, to a lesser extent, on MEK activity (26,27). We hypothesized that TrkB endocytosis is thus necessary for
BDNF-dependent neuronal survival. To test this, we deprived cultured cerebellar granule cells of serum and measured the neuroprotective effect of BDNF in the presence of endocytosis inhibitors. TUNEL staining revealed a significant increase in apoptosis 24 h after deprivation, and application of BDNF dramatically attenuated apoptosis of serum-starved granule neurons (Fig. 5A). Treatment with MDC (50 µM) or Dyn-pep (50 µM) significantly reduced the protective effect of BDNF (Fig. 5A and B), whereas the inhibitors themselves did not have a significant effect on neuronal survival (data not shown). Expression of HA-tagged, constitutively active Akt (CA-Akt) also significantly alleviated apoptosis in serum-free medium even in the absence of BDNF and expression of CA-Akt in the presence of MDC rescued the protective effect of BDNF (Fig. 5C and D). Furthermore, inhibition of PI3K/Akt with the inhibitor LY294002 (LY, 50 µM) abolished the protective effect of BDNF (Fig. 5B) and the MEK1 inhibitor PD98059 (10 µM) partially blocked the effect of BDNF (Fig. 5B). These data indicate that clathrin-mediated endocytosis of TrkB and subsequent PI3K/Akt activation is required for the neuroprotective effect of BDNF.

**BDNF-induced dendritic growth requires clathrin-mediated endocytosis—**PI3K/Akt signaling also regulates dendrite growth and arborization downstream of BDNF (28). DIV2 hippocampal cultures exposed to BDNF for 48 h significantly increased total dendrite length and branch points, as revealed by MAP2 staining (Fig. 6A). Blocking clathrin-dependent endocytosis with MDC or Dyn-pep abolished BDNF-induced dendritic growth (Fig. 6A and B) whereas expression of CA-Akt greatly promoted dendritic growth in the absence of BDNF and rescued the effect of BDNF in the presence of MDC (Fig. 6C). In contrast, overexpressing wild-type Akt did not rescue BDNF-mediated dendritic growth in the presence of MDC (Fig. 6D). BDNF-induced dendrite growth was also inhibited by MDC in hippocampal neurons expressing GFP-tagged Bcl2 (Fig. S3A-D). Thus, endocytosis-dependent activation of PI3K/Akt signaling directly affects dendrite growth and is not simply an indirect effect of enhanced cell survival. These results suggest that TrkB endocytosis is required for BDNF-induced dendritic growth.

**DISCUSSION**

This study investigated the mechanism and functional consequences of BDNF-induced TrkB endocytosis in neurons of central nervous system. Four principle findings emerged: 1) stimulation with BDNF induced TrkB endocytosis in cultured primary cortical, hippocampal, and cerebellar granule neurons; 2) clathrin, dynamin and AP2 were all required for TrkB endocytosis; 3) endocytosis was required for BDNF-induced activation of PI3K/Akt but not MEK/ERK nor PLCγ in all central nervous system neuronal cell types investigated; 4) endocytosis was required for BDNF-induced neuron survival and dendrite growth but not TrkB-mediated up-regulation of immediate-early genes.

Several studies have reported the importance of Trk endocytosis in regulating NGF signaling. Endocytosis of TrkA is required for downstream activation of ERK signaling in PC12 cells, dorsal root ganglion cells and sympathetic neurons (8,9). Binding of NGF to TrkA triggers the recruitment of receptors to clathrin-coated pits, leading to the formation of clathrin-coated vesicles (CCVs) and entry into the endocytic pathway (13,29). It is now known that CCVs also contain signaling molecules, including proteins involved in the Ras-MAP kinase pathway, that serve as a platform for ERK activation (29). Accordingly, blocking
TrkA endocytosis selectively inhibits NGF-induced activation of ERK in PC12 cells but does not, however, affect Akt activation (9). Surprisingly, we found that blocking endocytosis selectively inhibited NGF-induced Akt activation, but not ERK activation, in TrkA expressing neurons of central nervous system, indicating that NGF-TrkA signaling differs fundamentally in its dependence on endocytosis in different cell types. Thus, the selective effects of Trk endocytosis on downstream signaling cascades are not likely dependent on inherent differences in the physiological properties of Trk receptors but rather depend on the partnership with unique adaptor proteins.

Isoforms of Trk receptors lacking the intracellular tyrosine kinase domain are expressed in numerous cell types and have been found to be biologically active. Truncated TrkB and TrkC do not support the activation of canonical downstream signal cascades but can bind to both neurotrophins and full-length Trks, thereby inhibiting neurotrophin signaling. In addition to dominant-negative effects, truncated Trks can also elicit non-canonical intracellular responses (30-32). Previous studies have shown that truncated TrkB can bind to and internalize with BDNF (33), possibly by dimerizing with full-length TrkB, and internalization of truncated TrkB can be regulated by neuronal activity (14,34,35). As shown in Fig. 1, BDNF induced rapid and robust endocytosis of full-length TrkB, but failed to induce obvious endocytosis of truncated TrkB, even after 30 min of BDNF stimulation. However, it is possible that the kinetics of truncated TrkB endocytosis in hippocampal neurons are much slower than full-length Trks and were thus not detected during our observation period. Indeed, it has been shown that in cultured 3T3 NIH fibroblasts cells truncated TrkB-dependent BDNF internalization occurs slowly and peaks at 90 min after BDNF stimulation (36). In cultured astrocytes, internalization of BDNF is undetectable by 30 min of stimulation but uptake slowly takes effect thereafter, reaching a steady-state level by 120–240 min (33).

BDNF-induced activation of the PI3K/Akt pathway plays an important role in a diversity of cellular functions, such as promoting cell survival and dendrite morphogenesis. BDNF-TrkB signaling is capable of rescuing cultured neurons from apoptosis when grown in conditions of low potassium or in serum-free medium. Our findings confirm the essential role of PI3K/Akt signaling in protecting serum-deprived cerebellar granule cells and demonstrate that TrkB endocytosis is also necessary for the protective effect of BDNF. Pharmacological blockers of clathrin and dynamin were all able to reverse the protective effect of BDNF on serum-deprived granule cells. However, constitutive activation of the PI3K/Akt pathway with CA-Akt precluded the inhibitory effect of endocytic blockade.

Previous studies have uncovered the importance of neurotrophins for dendrite morphogenesis (28,37). We confirmed the effect of BDNF on dendritic outgrowth and demonstrated that this effect is dependent on endocytosis. Blockade of clathrin-dependent endocytosis repressed the effects of BDNF in cultured hippocampal neurons, thus revealing the importance of PI3K/Akt pathway in mediating the growth response of dendrites to BDNF. Recently, it has been demonstrated that activation of both PI3K/Akt and MEK/ERK pathways contributes to the formation of primary dendrites in response to BDNF (37). Our present data would suggest that PI3K/Akt signaling alone is sufficient for BDNF-dependent growth since blockade of endocytosis with MDC completely abrogated the response to BDNF. Furthermore, expression of
CA-Akt had the same growth enhancing effect as BDNF, and applying BDNF to CA-Akt expressing cells did not promote further dendrite outgrowth. These data together suggest that endocytosis-dependent activation of PI3K/Akt signaling is both necessary and sufficient for BDNF-induced neuronal survival and dendrite outgrowth.

ACKNOWLEDGEMENTS

We thank Dr. Muming Poo for engaging in helpful discussions throughout the course of this project, Dr. Rosalind A Segal for providing TrkB constructs, Dr. Bai Lu for providing the dynamin constructs, and Dr. Alfonso Bellacosa for providing Akt constructs. This work was supported by grants of the National Basic Research Program of China (2006CB806600), the Key State Research Program of China (2006CB943900), the National “863” high-tech research and development program (2006AA02Z166), the Innovative Research Group of The National Natural Science Foundation of China (30721004) and the Chinese Academy of Sciences (KSCX2-YW-R-099) to Z.-Q.X.

REFERENCES


FOOTNOTES

Tropomyosin-related kinase, Trk; nerve growth factor, NGF; brain-derived neurotrophic factor, BDNF; neurotrophin 3, NT3; neurotrophin 4/5, NT4/5; phospholipase C-γ, PLC-γ; monodansylcadaverine, MDC; membrane permeable Dynamin I proline-rich domain peptide, Dyn-pep; fetal bovine serum, FBS; days in vitro, DIV; RNA interference, RNAi; green fluorescent protein, GFP; adaptor protein 2, AP2; reverse transcription polymerase chain reaction, RT-PCR; neuropeptide Y, NPY; the activity-regulated cytoskeleton-associated protein, ARC; glyceraldehyde-3-phosphate dehydrogenase, GAPDH.

FIGURE LEGENDS

FIGURE 1. BDNF induces TrkB endocytosis. A, Western blots showing the time-course of BDNF-induced TrkB endocytosis. Cultured cortical neurons were stimulated with 25 ng/ml BDNF for 0, 3, 5, 15, 30 or 60 min. Surface TrkB was detected by Western blotting with anti-TrkB after surface protein biotinylation and fractionation of biotinylated proteins. Total TrkB was determined by Western blot of whole cell lysates. B, Quantification of surface TrkB levels assessed by biotinylation assay (n = 3; *, p < 0.05, upper) and ELISA (n = 9 per group; *, p < 0.05, lower). Surface-TrkB levels were measured relative to total TrkB in whole cell lysate and values shown are normalized to levels detected at the 0 min time
point. Error bars in this and all other figures represent S.E.M. C, Representative Z-stack images of a hippocampal neuron transfected with GFP-tagged TrkB (a, c) or truncated TrkB lacking the cytoplasmic domain (b, d) before and 10 min after application of 25 ng/ml BDNF. Optical slices are spaced by 1 μm increments; scale bar = 10 μm. D, Quantification of GFP signal intensity within 2μm of the plasma membrane (Periphery) and in the cell body cytoplasm (Medulla) in transfected cells normalized to unstimulated levels (n = 8 neurons per group; *, p < 0.05). E, Pre-treating cultures with MDC (50 μM) or Dyn-pep (50 μM) for 30 min prior to 15 min of exposure to 25 ng/ml BDNF influences surface expression of TrkB. Surface and total TrkB were detected as in A. F, Quantification of surface expression of BDNF-stimulated TrkB in cortical neurons pre-treated with MDC or Dyn-pep determined by biotinylation assay (n = 3; *, p < 0.05, upper) and ELISA (n = 9 per group; *, p < 0.05, lower).

FIGURE 2. Blocking endocytosis with MDC selectively inhibits sustained BDNF-induced Akt activation. A, Representative Western blots showing the time course of BDNF-induced phosphorylation of TrkB and downstream signal transduction proteins in cortical neurons pre-treated for 30 min with 50 μM MDC (Right) or control solution (Left). Whole cell lysates were blotted with antibodies against phosphorylated TrkB (pTrkB), SHC (pSHC), Akt (pAkt), ERK (pERK), CREB (pCREB) as well as total TrkB, SHC, Akt, ERK and CREB after stimulating with 25 ng/ml BDNF for the indicated times. B, The average level of pAkt (Upper) and pERK (Lower) normalized to and represented as a fold induction from the level of pAkt and pERK, respectively, in control treated cultures at 0 min time point (n = 3; *, p < 0.05).

FIGURE 3. BDNF-induced activation of Akt signaling requires clathrin, dynamin and AP2. A, Western blot showing the expression of AP2 protein in cultured hippocampal neurons infected with non-target control RNAi (NC-RNAi) and AP2-RNAi lentivirus. Actin was used as a loading control in this and other figures. Histograms representing the average level of AP2 mRNA in uninfected control neurons and neurons infected with NC-RNAi or AP2-RNAi 4-5 days prior to RNA harvesting and RT-PCR (n = 3 per group; *, p < 0.05). Values were normalized to GAPDH mRNA and percentages shown are relative to the level of AP2 mRNA in uninfected neurons. B, (Left) Representative immunoblots of hippocampal neurons treated with K252a (200 nM), MDC (50 μM), Dyn-pep (50 μM) or control solution for 30 min prior to 10 min stimulation with 25 ng/ml BDNF. (Right) Representative immunoblots of hippocampal neurons infected with NC-RNAi or AP2-RNAi prior to 10 min stimulation with 25 ng/ml BDNF. Protein extracts were immunoblotted for TrkB, phosphor-TrkB (pTrkB), Akt, phosphor-Akt (pAkt), ERK and phosphor-ERK (pERK). C, Quantification of pAkt and pERK normalized to total Akt and ERK and expressed as a fold change from the level detected in control solution treated, unstimulated cultures (n = 5; *, p < 0.05).

FIGURE 4. Clathrin-dependent endocytosis does not affect BDNF-induced gene expression. Hippocampal neurons were incubated with K252a (200 nM), PD98059 (10 μM), MDC (50 μM), Dyn-pep (50 μM), LY294002 (LY, 50 μM) or control solution for 30 min prior to 3 h stimulation with 25 ng/ml BDNF. A, mRNA expression of NPY, ARC and GAPDH determined by RT-PCR. B, Histograms representing the average expression of NPY and ARC normalized to GAPDH and represented as a fold
induction of the level detected in vehicle treated, unstimulated controls (*, p < 0.05).

FIGURE 5. **Clathrin-dependent endocytosis is required for BDNF-mediated neuronal survival.** A, TUNEL staining of serum-deprived cerebellar granule cells stimulated with 25 ng/ml BDNF for 24 h. Cultures were pre-treated with control solution (b, c), 50 µM MDC (d) or 50 µM Dyn-pep (e) for 30 min prior to BDNF application (b-e); scale bar = 400 µm. B, Percentage of TUNEL-positive cells in cultures represented in A (n = 37 coverslips per group, at least 100 cells on each were counted; *, p < 0.05). Cultures were treated with MDC (50 µM), Dyn-pep (50 µM), PI3K/Akt inhibitor LY294002 (LY, 50 µM), MEK/ERK inhibitor PD98059 (10 µM) or control solution for 30 min prior to 24 h incubation in serum-free media either containing or not containing 25 ng/ml BDNF. C, Percentage of TUNEL-positive cells in CA-Akt expressing cultures challenged as in B (n = 13 coverslips per group, at least 100 cells on each were counted; *, p < 0.05). D, anti-HA immunoblot of lysate from cerebellar granule cells transfected with HA-tagged CA-Akt.

FIGURE 6. **Clathrin-dependent endocytosis is required for BDNF-mediated dendritic growth.** A, Anti-MAP2 staining highlighting dendrites of cultured hippocampal neurons treated with BDNF under control conditions or in the presence of 50 µM MDC or Dyn-pep; scale bar = 40 µm. B, Quantification of total dendrite length (Top) and branch points (Middle) in response to different treatments in GFP transfected cells (n = 30 per group; *, p < 0.05). Anti-GFP immunoblot (Bottom) of lysate from hippocampal neurons transfected with GFP. C, Total dendrite length (Top) and number of branch points (Middle) of HA-tagged CA-Akt transfected hippocampal neurons in response to different treatments (n = 30 per group). (Bottom) Anti-HA immunoblot of lysate from cerebellar granule cells transfected with HA-tagged CA-Akt. D, Total dendrite length (Top) and number of branch points (Middle) in response to different treatments in HA-tagged WT-Akt transfected cells (n = 30 per group; *, p < 0.05). (Bottom) Anti-HA immunoblot of lysate from cerebellar granule cells transfected with HA-tagged WT-Akt.
**Fig. 4**

### A

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>K252a</th>
<th>MDC</th>
<th>Dyn-pep</th>
<th>LY</th>
<th>PD98059</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>NPY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### B

![Graph showing fold induction of NPY and ARC with and without BDNF](image)

- **NPY**
  - Basal
  - BDNF

- **ARC**
  - Basal
  - BDNF

* denotes significance compared to Basal conditions.
Fig. 6

A

BDNF - Control - MDC - Dyn-pep

B

GFP

Total dendrite length (μm)

Control MDC Dyn-pep

Branch points

Control MDC Dyn-pep

C

CA-Akt

Total dendrite length (μm)

Control MDC

Branch points

Control MDC

D

WT-Akt

Total dendrite length (μm)

Control MDC

Branch points

Control MDC

anti-GFP

anti-HA

anti-Actin

anti-Actin

anti-Actin

anti-Actin
Clathrin-dependent endocytosis is required for TrkB-dependent Akt-mediated neuronal protection and dendritic growth
Jing Zheng, Wan-Hua Shen, Ting-Jia Lu, Yang Zhou, Qian Chen, Zi Wang, Ting Xiang, Yong-Chuan Zhu, Chi Zhang, Shumin Duan and Zhi-Qi Xiong

J. Biol. Chem. published online March 19, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M709930200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2008/03/21/M709930200.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2008/03/19/jbc.M709930200.citation.full.html#ref-list-1