HHS Public Access

Author manuscript

Mol Neurobiol. Author manuscript; available in PMC 2016 August 01.

Published in final edited form as:

Mol Neurobiol. 2016 August ; 53(6): 4261–4273. doi:10.1007/s12035-015-9335-7.

BDNF Induces Striatal-Enriched Protein Tyrosine Phosphatase 61 Degradation Through the Proteasome

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Abstract

Brain-derived neurotrophic factor (BDNF) promotes synaptic strengthening through the regulation of kinase and phosphatase activity. Conversely, striatal-enriched protein tyrosine phosphatase (STEP) opposes synaptic strengthening through inactivation or internalization of signaling molecules. Here, we investigated whether BDNF regulates STEP levels/activity. BDNF induced a reduction of $STEP_{61}$ levels in primary cortical neurons, an effect that was prevented by inhibition of tyrosine kinases, phospholipase C gamma, or the ubiquitin-proteasome system (UPS). The levels of $pGluN2B^{Tyr1472}$ and $pERK1/2^{Thr202/Tyr204}$, two STEP substrates, increased in BDNF-treated cultures, and blockade of the UPS prevented $STEP_{61}$ degradation and reduced BDNF-induced GluN2B and ERK1/2 phosphorylation. Moreover, brief or sustained cell depolarization reduced $STEP_{61}$ levels in cortical neurons by different mechanisms. BDNF also promoted UPS-mediated $STEP_{61}$ degradation in cultured striatal and hippocampal neurons. In contrast, nerve growth factor and neurotrophin-3 had no effect on $STEP_{61}$ levels. Our results thus indicate that $STEP_{61}$ degradation is an important event in BDNF-mediated effects.

Keywords

PLCγ; GluN2B; ERK1/2; NGF; NT-3; Depolarization; $STEP_{33}$

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Electronic supplementary material The online version of this article (doi:10.1007/s12035-015-9335-7) contains supplementary material, which is available to authorized users.

Conflict of Interest None

Introduction

Synaptic strengthening depends, among others, on the phosphorylation of synaptic proteins controlled by a finely tuned balance between protein kinases and protein phosphatases [1]. Brain-derived neurotrophic factor (BDNF) is crucial for the regulation of synaptic transmission, plasticity, and cognitive functions [2–4]. Binding of BDNF to its receptor TrkB activates intracellular signaling cascades that depend on dynamic phosphorylation events. While kinases activated in response to BDNF are well characterized [5, 6], less is known about BDNF-induced regulation of phosphatases and their involvement in BDNF effects. Mitogen-activated protein (MAP) kinase phosphatase-1 is required for BDNF-dependent axonal branching [7], whereas BDNF-induced calpain activation promotes the degradation of the phosphatase tensin homolog deleted on chromosome 10 (PTEN) contributing to stimulate dendritic protein synthesis [8]. The serine/threonine phosphatase suprachiasmatic nucleus circadian oscillatory protein (SCOP) is also degraded by calpains in response to BDNF [9].

Striatal-enriched protein tyrosine phosphatase (STEP), encoded by the *Ptpn5* gene, is involved in the regulation of synaptic plasticity [10]. Its mRNA is alternatively spliced into several isoforms [11, 12] targeted to distinct cellular compartments [13–15]. Its major isoforms are cytosolic $STEP_{46}$ and membrane-associated $STEP_{61}$ [11]. Both are expressed in the striatum, central nucleus of the amygdala, and optic nerve, whereas neurons of the hippocampus, cortex, and lateral amygdala only express $STEP_{61}$ [16, 17]. STEP normally opposes synaptic strengthening by dephosphorylating neuronal signaling molecules, including the *N*-methyl-D-aspartate (NMDA) glutamate receptor subunit GluN2B [18, 19] and extracellular signal-regulated kinase 1/2 (ERK1/2) [20, 21]. In addition, STEP mediates internalization of GluA1/GluA2-containing AMPA receptors [22]. STEP also dephosphorylates p38, Fyn, and proline-rich tyrosine kinase 2, thereby controlling the duration of their signal [20, 23–25].

Multiple posttranslational modifications regulate STEP activity, including phosphorylation/dephosphorylation [21, 26, 27], calpain cleavage [15, 28–30], and ubiquitin-proteasome degradation [15, 18, 31]. Despite the accumulated knowledge about STEP function/substrates, particularly in pathological circumstances [18, 32–35], data about its physiological regulation is sparse.

Since STEP exerts an opposite effect to BDNF on synaptic strength, we hypothesized that BDNF could regulate STEP levels/activity. Our results indicate that BDNF induces $STEP_{61}$ degradation through the proteasome in primary cortical, striatal, and hippocampal neurons and suggest that $STEP_{61}$ degradation is an important event in BDNF-induced effects.

Materials and Methods

Cell Cultures and Treatments

Primary cortical, striatal, and hippocampal cultures were obtained from wild-type 18-day-old B6CBA mouse embryos following the National Institutes of Health guide for the care and use of laboratory animals, and the procedures approved by the local animal care

committee of Universitat de Barcelona (99/01) and Generalitat de Catalunya (99/1094), in accordance with the European (2010/63/UE) and Spanish (RD 53/2013) regulations for the care and use of laboratory animals. Primary rat cortical cultures were prepared from embryos at E18 (Charles River Laboratories, MA) according to procedures approved by Yale University Institutional Animal Care and Use Committee. Cells were plated at a density of 800,000 cells onto 60-mm culture dishes and 100,000 cells onto 24-well plates with coverslips for biochemical and immunocytochemical analysis, respectively. Culture dishes and coverslips were precoated with 0.1 mg/ml poly-D-lysine (Sigma Chemical Co., St. Louis, MO), and neurons were cultured in neurobasal medium supplemented with B27 and glutamax™ (all Gibco-BRL, Renfrewshire, Scotland, UK). Cultures were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2. The cultures were treated with BDNF (10 ng/ml; Peprotech, Inc., Rocky Hill, NJ) at day in vitro (DIV) 8. In different sets of experiments, cultures were treated for different time periods with BDNF or during 60 min with the tyrosine kinase inhibitor K252a (200 nM; Sigma-Aldrich), the MAPK inhibitor PD98059 (25 μM; Calbiochem; San Diego, CA), the PI-3 K inhibitor wortmannin (50 nM; Calbiochem), the PLC inhibitor U73122 (5 μM; Calbiochem), the proteasome inhibitor MG-132 (carbobenzoxy-l-leucyl-l-leucyl-l-leucinal; 10 μM; Calbiochem) and then incubated in the presence or absence of BDNF for additional 15 or 60 min. Cortical neurons were incubated for 60 min with or without 100 μM ANA-12 (Sigma-Aldrich) or 10 μM MG-132 and then depolarized by treatment with 50 mM KCl for additional 5 or 60 min. Nerve growth factor (NGF) and neurotrophin-3 (NT-3) (10 ng/ml; Peprotech, Inc.) were added to cultures for 15 min. After treatments, cultures were rinsed with phosphate-buffered saline (PBS) and processed for Western blot analysis or immunofluorescence as detailed below.

Western Blot Analysis

Cell cultures were homogenized in lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 % glycerol, 1 % Triton X-100, 100 mM NaF, 5 μM $ZnCl_2$, and 10 mM EGTA] plus protease inhibitors [phenylmethylsulphonyl fluoride (2 mM), aprotinin (1 μg/ml), leupeptin (1 μg/ml), and sodium orthovanadate (1 mM)]. The lysates were centrifuged at 16, 100×*g* for 20 min; supernatants were collected, and protein concentration measured using the Dc protein assay kit (Bio-Rad, Hercules, CA). Western blot analysis was performed as previously described [34]. For the analysis of pGluN2B^{Tyr1472}, samples were denatured in 170 mM phosphate buffer, pH 7.0, with 2.5 % (*w*/*v*) SDS, 10 % glycerol, 3.2 mM dithiothreitol, and 0.1 % (*w*/*v*) bromophenol blue, and membranes were blocked with 5 % bovine serum albumin (BSA) in Tris-buffered saline containing 0.1 % Tween 20 (TBS-T) for 1 h at room temperature. The primary antibodies used were (1:1000, unless stated otherwise) anti-STEP (Santa Cruz Biotechnology), anti-pERK1/2$^{Thr202/Tyr204}$, anti-pAkt^{Ser473}, anti-pPLCγ^{Tyr783}, anti-pPKAc^{Thr197}, anti-pGluN2B^{Tyr1472}, anti-GluN2B (Cell Signaling, Beverly, MA), anti-spectrin (Chemicon International, Temecula, CA), anti-TrkB (BD Transduction Laboratories, San Jose, CA), anti-pTrkB^{Tyr816}, anti-TrkA (1:2000; Abcam, Cambridge, UK), and anti-TrkC (1:300; Millipore, Temecula, CA). Loading control was performed by reprobing the membranes with an anti-α-tubulin antibody (1:50,000; Sigma-Aldrich) for 10–15 min at room temperature. Then, membranes were washed with TBS-T, incubated for 1 h (10–15 min for α-tubulin) at room temperature with the corresponding horseradish peroxidase-conjugated antibody (1:2000; Promega, Madison, WI), and washed

again with TBS-T. Immunoreactive bands were visualized using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and quantified by a computer-assisted densitometer (Gel-Pro Analyzer, version 4, Media Cybernetics).

Immunofluorescence

Primary cortical cultures were fixed in 4 % paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 min. The cells were then washed with PBS, incubated with PBS containing 0.2 M glycine for 15 min, and washed. To permeabilize cells, coverslips were treated with 0.1 % saponin in PBS for 10 min. After washing with PBS, cells were incubated with 15 % normal horse serum in 0.1 M PBS for 30 min at room temperature. The cells were then incubated overnight at 4 °C with anti-STEP (Santa Cruz Biotechnology) and anti-MAP2 (Abcam) antibodies, prepared at 1:500 in 0.1 M PBS with 5 % normal horse serum. After washing three times with PBS, cells were incubated for 2 h at room temperature with Alexa Fluor 488-conjugated AffiniPure donkey anti-mouse and Cy3-conjugated AffiniPure donkey anti-rabbit (both 1:100; Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Coverslips were then washed three times with PBS and finally with water before mounting with Mowiol. Immunofluorescence was analyzed by confocal microscopy using a TCS SL laser scanning confocal spectral microscope (Leica Microsystems Heidelberg, Mannheim, Germany). Images were taken using 63× numerical aperture objective.

Pulldown of Ubiquitinated Proteins

Primary cortical neurons were incubated with or without BDNF (10 ng/ml, 15 min) in the presence of MG-132 (10 μM; 1 h preincubation), and ubiquitinated proteins were isolated using Agarose-TUBE2 (Tandem Ubiquitin Binding Entities, Lifesensors, Malvern, PA) affinity pulldown as described previously [32]. Briefly, cultured neurons were resuspended in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 1 % NP-40, 1 mM EDTA, 150 mM NaCl, 10 % glycerol supplemented with 10 mM *N*-ethylmaleimide and complete protease inhibitor cocktail (Roche, Indianapolis, IN), followed by centrifugation at 12,000×*g* for 10 min at 4 °C. Two hundred micrograms from the supernatants were precleared with control agarose (Lifesensors, Malvern, PA) for 1 h at 4 °C and incubated overnight with 20 μl Agarose-TUBE2 beads at 4 °C. Then, the beads were washed four times (10-min intervals each) in the wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 % Tween-20), and bound proteins were eluted with 50 μl 2× SDS sample buffer and then subjected to SDS-PAGE. To aid the transfer of higher molecular weight proteins, the gels were incubated with gel soaking buffer (63 mM Tris-HCl, pH 6.8, 2.3 % SDS, 5.0 % β-mercaptoethanol) for 30 min. After transfer, the membranes were probed with anti-STEP antibody (1:2000), to visualize high molecular weight STEP-ubiquitin conjugates and with an anti-ubiquitin antibody (1:5000; Affinity Bioreagents, Golden, CO) as control.

Statistical Analysis

All data are expressed as mean±SEM. Statistical analyses were performed by using the unpaired Student's *t* test (95 % confidence) or the one-way ANOVA with Dunnett's or Bonferroni's post hoc test, as appropriate and indicated in the figure legends. Values of $p<0.05$ were considered as statistically significant.

Results

BDNF Reduces $STEP_{61}$ Levels in Primary Cortical Neurons

To investigate whether BDNF regulates STEP levels, we used mouse primary cortical cultures at DIV 8. First, we analyzed whether TrkB, the high affinity receptor for BDNF, was expressed in cortical neurons in culture. As we found TrkB expression in 8-day-old primary cortical neurons (Fig. 1a), cultures were then treated with 10 ng/ml BDNF for 24 h and STEP levels were analyzed by Western blot. As in cortical tissue, cultured cortical neurons only express the $STEP_{61}$ isoform (Fig. S1). We found that $STEP_{61}$ levels were significantly reduced in BDNF-treated cultures compared with cultures incubated in the absence of BDNF (Fig. 1b; Ctr: 100.00±6.94 % and BDNF: 49.83±14.37 %; n=3; p= 0.0347, Student's t test). To further characterize this effect, we examined $STEP_{61}$ levels at different time points after BDNF treatment. We observed a significant reduction of $STEP_{61}$ as early as 5 min after BDNF addition, and this effect was sustained for up to 6 h (Fig. 1c).

Next, we analyzed whether BDNF-induced reduction of $STEP_{61}$ levels was dependent on activation of the BDNF receptor TrkB. Treatment with the tyrosine kinase inhibitor K252a (200 nM) blocked BDNF-induced TrkB phosphorylation and reduction of $STEP_{61}$ levels, whereas it had no effect on $STEP_{61}$ basal levels (Fig. 1d). cAMP-dependent protein kinase (PKA) phosphorylates STEP $_{6\,1}$ at Ser^{221} within the kinase interacting domain thereby inactivating it [26]. Since BDNF transiently activates PKA in neurons [36, 37], we investigated whether BDNF also leads to $STEP_{61}$ inactivation by PKA-mediated phosphorylation in cortical neurons. To analyze whether PKA was activated by BDNF, we examined the phosphorylation of Thr197 in the activation loop of the catalytic subunit of PKA (PKAc), an essential step for its proper function [38]. $pPKAc^{Thr197}$ levels were not altered after 15-min incubation with 10 ng/ml BDNF (Fig. S2), indicating that PKA was not activated.

Finally, we stimulated cortical neurons with BDNF for 15 min and performed immunocytochemistry against STEP and MAP2. As shown in Fig. 1e, $STEP_{61}$ expression was detected in cell body and neurites, and after BDNF treatment, STEP immunoreactivity was mainly decreased in neurites. Taken together, the data indicate that BDNF-TrkB signaling induces $STEP_{61}$ reduction in cortical neurons.

BDNF Reduces $STEP_{61}$ Levels in Primary Cortical Cultures Through the PLCγ Pathway

BDNF can activate calpains [9, 39], and calpains cleave $STEP_{61}$, generating a 33-kDa fragment [15, 28–30]. Thus, we first analyzed whether BDNF activated calpains in our model by looking at spectrin breakdown products (SBDPs) at 145–150 kDa, which are generated specifically by calpain-dependent cleavage. We detected higher levels of SBDPs in BDNF-treated cultures than in controls, indicating that there was activation of calpains in response to BDNF (Fig. S3a). Next, we evaluated whether reduced $STEP_{61}$ levels in BDNF-treated cultures correlated with an accumulation of the $STEP_{33}$ fragment. As shown in Fig. S3b, $STEP_{33}$ was barely detected both in control and in cultures incubated for 15 min with 10 ng/ml BDNF. Thus, although BDNF activates calpains, a different mechanism is responsible for the reduction of $STEP_{61}$ levels in BDNF-treated cortical neurons.

To identify the intracellular pathways responsible for BDNF-mediated reduction of $STEP_{61}$ levels in primary cortical cultures, we inhibited three downstream effectors of BDNF [6]: the MAPK pathway (PD98059), the phosphoinositide-3 kinase (PI-3 K) pathway (wortmannin), and the phospholipase Cγ (PLCγ) pathway (U73122). Treatment with PD98059 (25 μM) or wortmannin (50 nM) reduced both basal and BDNF-induced ERK1/2 and Akt phosphorylation, respectively, but did not affect $STEP_{61}$ levels at baseline and did not block the reduction of $STEP_{61}$ after BDNF treatment (Fig. 2a, b). Similarly, incubation with U73122 (5 μM) alone did not modify $STEP_{61}$ levels in basal conditions, but it blocked the reduction of $STEP_{61}$ levels in BDNF-treated cultures (Fig. 2c). Thus, BDNF-TrkB signaling induces a reduction of $STEP_{61}$ levels in cortical neurons through the activation of PLCγ.

$STEP_{61}$ is Ubiquitinated and Degraded by the Proteasome in Response to BDNF Stimulation

$STEP_{61}$ can be degraded through the ubiquitin-proteasome system (UPS) [18, 31]. Since BDNF promotes the ubiquitination of several synaptic proteins [40], we next investigated the effect of proteasome inhibition on $STEP_{61}$ levels in BDNF-treated cultures. The proteasome inhibitor MG-132 (10 μM) alone had no effect on basal $STEP_{61}$ levels in cortical neurons, but it prevented the reduction of $STEP_{61}$ in response to BDNF (Fig. 3a). To confirm these findings, we analyzed $STEP_{61}$ ubiquitination in control and in BDNF-treated cultures. To detect $STEP_{61}$ ubiquitination, BDNF stimulations were performed in the presence of MG-132 (10 μM) and polyubiquitinated proteins were enriched using ubiquitin affinity beads and probed with an anti-STEP antibody. Consistent with the effect of proteasome inhibition on $STEP_{61}$ levels (Fig. 3a), we detected polyubiquitinated $STEP_{61}$ in BDNF-treated cultures, while the total level of polyubiquitined proteins was not altered by BDNF treatment (Fig. 3b). Altogether, these results indicate that $STEP_{61}$ is ubiquitinated in response to BDNF and degraded by the UPS through a PLCγ-mediated pathway.

BDNF-Induced $STEP_{61}$ Degradation Contributes to Sustain High Levels of pGluN2B and pERK1/2

BDNF induces GluN2B [41, 42] and ERK1/2 [43–45] phosphorylation in cortical neurons, and both phospho-proteins are STEP substrates [19, 21, 46]. Thus, to address the functional relevance of BDNF-induced $STEP_{61}$ degradation, we analyzed the phosphorylation level of GluN2B and ERK1/2 in cultures exposed to BDNF in the presence or absence of the proteasome inhibitor MG-132. In an attempt to avoid the initial BDNF-induced kinase-dependent effect on the phosphorylation status of GluN2B and ERK1/2, we analyzed their phosphorylation levels at 1 h. BDNF-induced $STEP_{61}$ degradation was prevented by MG-132, and in agreement with $STEP_{61}$ being degraded by the proteasome, incubation for 120 min with MG-132 alone (but not for 75 min, Fig. 3a) significantly increased $STEP_{61}$ levels compared to control cultures (Fig. 4a). Both $pGluN2B^{Tyr1472}$ (Fig. 4b) and $pERK1/2^{Thr202/Tyr204}$ (Fig. 4c) levels were significantly increased in cultures exposed to BDNF for 1 h. In contrast, in cultures incubated with BDNF plus MG-132, the phosphorylation levels of GluN2B (Fig. 4b) and ERK1/2 (Fig. 4c) were significantly reduced compared to cultures incubated with BDNF alone. Taken together, these results indicate that $STEP_{61}$ degradation through the proteasome is necessary for full phosphorylation of GluN2B and ERK1/2 in response to BDNF.

BDNF Also Induces $STEP_{61}$ Degradation Through the Proteasome in Striatal and Hippocampal Neurons

Next, we investigated whether BDNF-induced $STEP_{61}$ degradation is a general mechanism that occurs in distinct neuronal types. We first analyzed TrkB expression by Western blot of mouse primary striatal and hippocampal neuronal extracts. Both striatal (Fig. 5a) and hippocampal (Fig. 5b) neurons expressed TrkB at DIV 8. In agreement with the postnatal ontogeny of $STEP_{46}$ [47], we did not detect expression of this STEP isoform in 8-day-old primary striatal neurons (Fig. 5b). Moreover, like hippocampal tissue, cultured hippocampal neurons only express the $STEP_{61}$ isoform (Fig. 5b). Addition of 10 ng/ml BDNF for 15 min activated PLCγ and reduced $STEP_{61}$ protein levels in striatal (Fig. 5c) and hippocampal (Fig. 5d) cultured neurons, an effect that was prevented by the addition of the proteasome inhibitor MG-132. Thus, BDNF-induced $STEP_{61}$ degradation through the proteasome is a mechanism common to cortical, striatal, and hippocampal neurons.

NGF and NT-3 do not Induce $STEP_{61}$ Degradation

We next examined whether $STEP_{61}$ degradation was exclusively induced by BDNF or could also be promoted by other neurotrophin family members. To this end, we first checked in mouse primary neurons the expression of TrkA, the high affinity receptor for NGF, and TrkC, the high affinity receptor for NT-3. As shown in Fig. 6a, TrkA and TrkC were expressed in mouse primary cortical, striatal, and hippocampal neurons at DIV 8. In contrast to BDNF, addition of 10 ng/ml NGF or NT-3 for 15 min did not alter $STEP_{61}$ levels in cortical, striatal, or hippocampal primary neurons (Fig. 6b). Of note, neither NGF nor NT-3 induced PLCγ, Akt, or ERK1/2 phosphorylation in these conditions (Fig. 6b). NT-3 [48–50], but not NGF [49, 51], stimulates Trk phosphorylation and downstream signaling in rat primary cortical neurons. As Trk receptors were detected in 8-day-old rat primary cortical neurons (Fig. 6c), we analyzed the effect of the distinct neurotrophins on $STEP_{61}$ levels and intracellular signaling. We found that a 15-min exposure to 10 ng/ml NT-3 or BDNF, but not NGF, increased the phosphorylation level of PLCγ, Akt, and ERK1/2 (Fig. 6d). Importantly, despite PLCγ activation in both NT-3- and BDNF-treated cultures, only BDNF induced $STEP_{61}$ degradation in rat cortical neurons (Fig. 6d). Therefore, the effect of BDNF on $STEP_{61}$ levels is specific as other neurotrophin family members are unable to induce $STEP_{61}$ degradation.

$STEP_{61}$ Levels are Reduced Upon Cell Depolarization

Neuronal depolarization induced by high extracellular KCl levels is commonly used as an in vitro model to study activity-dependent processes, and it induces BDNF release [52–55]. We therefore determined whether depolarization could promote $STEP_{61}$ degradation. To address this, we analyzed $STEP_{61}$ levels after depolarization of primary cortical neurons with 50 mM KCl for 5 min, in the presence and absence of ANA-12, a TrkB antagonist [56], and the proteasome inhibitor MG-132. We found that, like BDNF (Fig. 2c), depolarization significantly increased the levels of $pPLC\gamma^{Tyr783}$ (Ctr: 100.01±0.04 % and KCl: 141.84± 19.07 %; n=3–4; p=0.0470, Student's t test) with a concomitant reduction of $STEP_{61}$ levels, which was prevented in cultures where TrkB signaling or the proteasome were inhibited (Fig. 7a). After a prolonged incubation (60 min) with 50 mM KCl to mimic

neuronal stimulation in pathological conditions, $STEP_{61}$ levels were also reduced, but in this case there was an increase in $STEP_{33}$ levels (Fig. 7b), indicating cleavage of $STEP_{61}$ by calpains [15]. Consistent with this, we detected calpain activation in cultures incubated with KCl for 60 min as assessed by the increase in SBDPs at 145–150 kDa (Fig. 7b). Hence, neuronal depolarization promotes a reduction in $STEP_{61}$ levels by a mechanism that is dependent on whether it is physiological or pathological.

Discussion

In the present study, we show that BDNF induces $STEP_{61}$ degradation in primary cortical neurons through a PLCγ-UPS pathway (Fig. S4). BDNF-induced $STEP_{61}$ degradation contributes to the sustained high levels of pGluN2B and pERK1/2. This effect is reproduced in cultured striatal and hippocampal neurons and is specific for BDNF since the neurotrophins NGF and NT-3 do not induce degradation of $STEP_{61}$. In addition, a brief depolarization of cortical neurons with KCl also promotes TrkB-mediated and UPS-dependent $STEP_{61}$ degradation, whereas a sustained depolarization induces proteolytic cleavage of $STEP_{61}$ by calpains. Taken together, our results indicate that BDNF promotes the rapid degradation of $STEP_{61}$ and elucidate a novel mechanism that likely participates in regulating neuronal function and synaptic strengthening.

Our results show a reduction of $STEP_{61}$ levels in BDNF-treated cortical cultures, an effect that was abrogated when the proteasome was inhibited, indicating that BDNF-TrkB signaling modulates $STEP_{61}$ protein levels through the UPS. In accordance with these results, previous studies have shown that $STEP_{61}$ is degraded by the proteasome [15, 18, 31] and that BDNF promotes the ubiquitination of synaptic proteins [40] and induces UPS-dependent degradation of proteins that inhibit neurite outgrowth [57–59]. Importantly, active STEP is more prone to degradation through the proteasome than inactive STEP [31] suggesting that BDNF-induced $STEP_{61}$ degradation constitutes a rapid way to attenuate its phosphatase activity.

In addition to its effect on UPS-dependent protein degradation, BDNF can also promote calpain activation in cultured neurons and hippocampal slices [8, 9, 39]. BDNF-induced activation of calpains stimulates the proteolysis of phosphatases such as PTEN [8] and SCOP [9]. Calpains are known to cleave $STEP_{61}$ to generate a $STEP_{33}$ fragment [15, 28, 30]. However, the levels of this fragment were not increased in BDNF-treated cultures, strongly suggesting that proteolysis by calpains was not implicated in the effect of BDNF on $STEP_{61}$. In this sense, both BDNF and neuronal activity induced by a brief incubation with KCl promoted PLCγ activation and $STEP_{61}$ degradation through the proteasome. In agreement with this, depolarization induces BDNF release from primary cortical neurons [60], and KCl-induced $STEP_{61}$ degradation was prevented by antagonizing TrkB signaling with ANA-12. In contrast, prolonged KCl-induced depolarization promoted calpain cleavage of $STEP_{61}$. Importantly, the 33 kDa fragment neither interacts with nor dephosphorylates STEP substrates [15, 28]. Therefore, $STEP_{61}$ levels/activity are dynamically regulated in very specific manners. Reinforcing our observation of a signal-dependent mechanism of $STEP_{61}$ degradation, synaptic stimulation of NMDA receptors promotes $STEP_{61}$

degradation through the UPS, while extrasynaptic stimulation induces calpain-mediated $STEP_{61}$ proteolysis, leading to a differential regulation of ERK and p38 [15].

Here, we dissect the intracellular pathway leading to UPS-dependent $STEP_{61}$ degradation in response to BDNF, and our findings demonstrate that BDNF promotes $STEP_{61}$ degradation through PLCγ. Importantly, the PLCγ pathway is necessary for BDNF-induced hippocampal plasticity [5]. As BDNF-induced PLCγ activation leads to $STEP_{61}$ degradation by the proteasome, we propose that the effects of BDNF on synaptic plasticity might be mediated, at least in part, by a reduction of $STEP_{61}$ levels.

Interestingly, BDNF-induced $STEP_{61}$ degradation through the proteasome was not neuronal type-dependent, as it occurred in cortical, striatal, and hippocampal neurons. BDNF plays an important role in the cellular mechanisms underlying neuronal plasticity in these neuronal types [4, 61]. Thus, the present results improve our understanding of the mechanisms underlying BDNF regulation of neuronal function and suggest that $STEP_{61}$ degradation could play an important role in this process. In agreement, STEP levels/activity are altered in neurodegenerative diseases in which cortical, striatal, and/or hippocampal function is impaired, including Alzheimer's disease, Huntington's chorea, Parkinson's disease, schizophrenia, and fragile X syndrome [18, 32–35, 62]. Since BDNF also regulates survival, maturation, and differentiation of these neurons [63–68], future studies are needed to address the contribution of BDNF-induced $STEP_{61}$ degradation to these phenomena.

Additionally, here, we show that $STEP_{61}$ degradation is specifically induced by BDNF since we did not observe decreased levels of $STEP_{61}$ after treatment with NGF or NT-3, two related neurotrophin family members. Remarkably, although we detected TrkA protein in mouse primary cortical, striatal, and hippocampal, as well as rat primary cortical neurons, addition of NGF did not activate PLCγ, PI-3 K, or MAPK pathways in any condition analyzed. Actually, data regarding the expression of TrkA and its activation by NGF in primary neuronal cultures are inconsistent [48, 51, 69–72]. These discrepancies could be due to different species, culture conditions, concentration of NGF used, or duration of the treatment. Here, we did not detect NGF-induced intracellular signaling in culture conditions in which BDNF promoted a strong activation of PLCγ, PI-3 K, and MAPK, suggesting that these neurotrophins could elicit different biological responses depending on neuronal type and/or maturation. On the other hand, we did not detect NT-3-induced signaling in mouse primary neurons but, consistent with other reports [48–50], NT-3 induced intracellular signaling in rat primary cortical neurons. Nevertheless, despite activation of PLCγ, $STEP_{61}$ levels were not altered after NT-3 exposure supporting a specific effect of BDNF on $STEP_{61}$ levels.

Changes in STEP activity modulate the phosphorylation levels of several proteins like GluN2B and ERK1/2 [19–21]. Consistent with this, we observed decreased levels of $pGluN2B^{Tyr1472}$ and $pERK1/2^{Thr202/Tyr204}$ when BDNF-induced $STEP_{61}$ degradation was prevented by treatment with a proteasome inhibitor. These results indicate that in normal conditions, sustained high levels of $pGluN2B^{Tyr1472}$ and $pERK1/2^{Thr202/Tyr204}$ after BDNF treatment are due, in part, to a reduction in $STEP_{61}$ levels. Notably, $GluN2B^{Tyr1472}$ phosphorylation is critical for memory formation and modulates NMDA receptor function

[73, 74], while its blockade prevents BDNF-induced enhancement of synaptic transmission [75, 76]. Moreover, ERK1/2 is an essential component of the signal transduction mechanisms underlying learning and memory [77–79]. In view of the functional importance of pGluN2B and pERK in the regulation of synaptic plasticity and memory, alterations in BDNF-induced $STEP_{61}$ degradation may have deleterious effects in these processes. In line with our proposal, in conditions where $STEP_{61}$ activity is increased, like in Alzheimer's disease and schizophrenia, there is a dysregulation of NMDA receptors and reduced cognitive function [18, 32, 35]. In contrast, STEP knockout mice have higher pGluN2B and pERK1/2 levels, enhanced hippocampal long-term potentiation, and improved performance in hippocampal-dependent learning and memory tasks [35, 80].

In conclusion, we demonstrate for the first time that BDNF induces $STEP_{61}$ degradation in primary cortical, striatal, and hippocampal neurons through a PLCγ-UPS pathway. Since BDNF-induced $STEP_{61}$ degradation leads to higher phosphorylation levels of GluN2B and ERK1/2, our results unravel a novel mechanism that likely contributes to BDNF-induced effects.

Supplementary Material

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Acknowledgments

We are very grateful to Ana López and Maria Teresa Muñoz for their technical support and Dr. Cristina Malagelada and to laboratory members for helpful discussions and critical reading of the manuscript. This work was supported by projects PI10/01072, PI13/01250, and RD12/0019/0002, integrated in the "Plan Nacional de I+D+I y cofinanciado por el ISCIII-Subdirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER)," Ministerio de Ciencia e Innovación, Spain (grants SAF2012-39142 to S.G. and SAF2011-29507 to J.A.), Generalitat de Catalunya, Spain (grant 2009SGR-00326 to J.A.), and the National Institutes of Health (grants MH091037 and MH52711 to P.J.L.). A.S. was supported by Ministerio de Economia y Competitividad, Spain (Juan de la Cierva subprogram, grant JCI-2010-08207) and S.T. by Generalitat de Catalunya, Spain (grant AGAUR ST067914).

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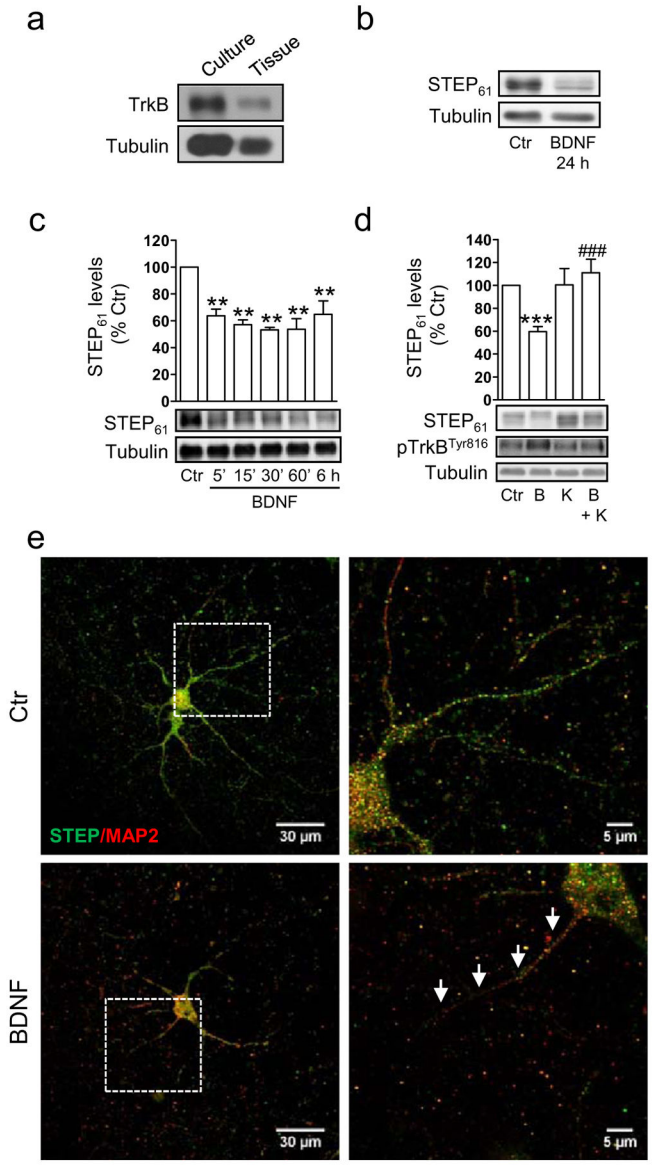

Fig. 1.

Effect of BDNF on $STEP_{61}$ levels in primary cortical neurons. **a** The expression of TrkB was analyzed by Western blot of protein extracts obtained from mouse primary cortical cultures. Mouse adult tissue served as positive control. Primary cortical cultures were incubated with 10 ng/ml BDNF **b** for 24 h or **c** during different time periods up to 6 h, and $STEP_{61}$ levels were examined by Western blot. **d** $STEP_{61}$ and $pTrkB^{Tyr816}$ levels were analyzed in cortical cultures treated for 60 min with or without the tyrosine kinase inhibitor K252a (200 nM; *K*) and then incubated in the presence or absence of BDNF (10 ng/ml; *B*) for additional 15 min. Representative immunoblots are shown. Values obtained by densitometric analysis of Western blot data are expressed as percentage of control (*Ctr*) cultures incubated in the absence of BDNF and are shown as mean ±SEM of three to seven experiments performed in duplicate in independent cultures. Data were analyzed by one-way ANOVA with Dunnett's (**c**), or Bonferroni's (**d**) post hoc test. $^{**}p<0.01$, and $^{***}p<0.001$

compared with Ctr cultures; ###$p<0.001$ compared with cultures incubated with BDNF alone. **e** STEP and MAP2 were analyzed by immunocytochemistry in untreated (*Ctr*) and BDNF-treated (10 ng/ml, 15 min) cortical cultures. High magnification insets are shown. *Arrows* denote loss of STEP immunoreactivity in a dendrite

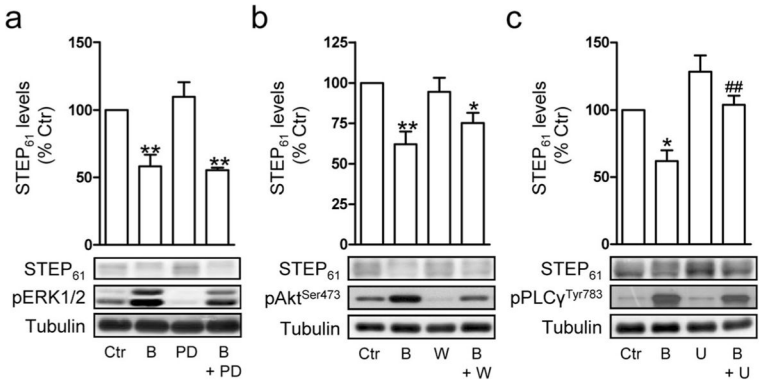

Fig. 2.

PLCγ mediates the degradation of $STEP_{61}$ by BDNF in primary cortical cultures. Mouse primary cortical cultures were treated for 60 min with or without **a** the MAPK inhibitor PD98059 (25 μM; *PD*), **b** the PI-3 K inhibitor wortmannin (50 nM; *W*), or **c** the PLC inhibitor U73122 (5 μM; *U*) and then incubated in the presence or absence of BDNF (10 ng/ml; *B*) for additional 15 min. $STEP_{61}$ and **a** $pERK1/2^{Thr202/Tyr204}$, **b** $pAkt^{Ser473}$, or **c** $pPLC\gamma^{Tyr783}$ were examined by Western blot. Representative immunoblots are shown. Values obtained by densitometric analysis of Western blot data are expressed as percentage of control (*Ctr*) cultures and are shown as mean±SEM of three experiments performed in duplicate in independent cultures. Data were analyzed by one-way ANOVA with Bonferroni's post hoc test. *p<0.05 and **p<0.01 compared with Ctr cultures; ##p<0.01 compared with cultures incubated with BDNF alone

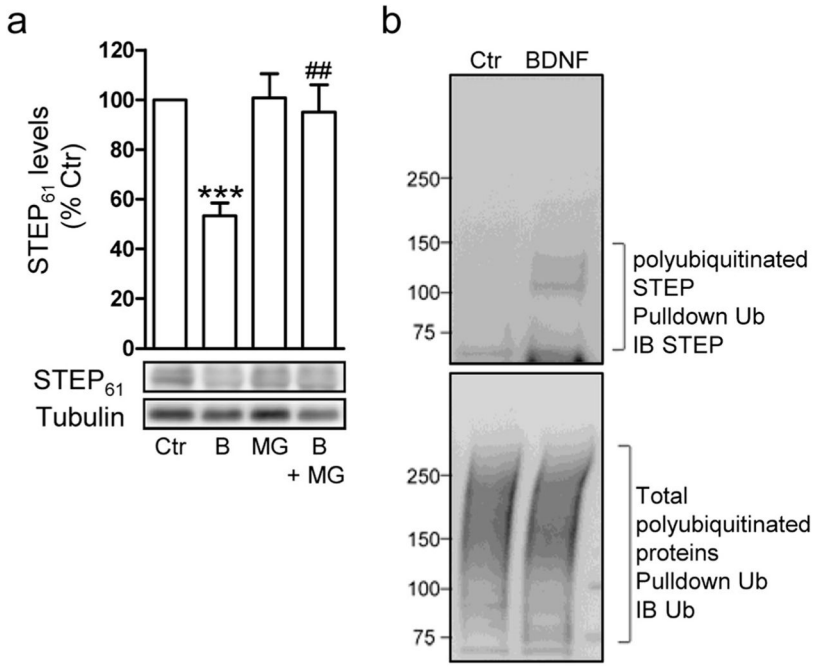

Fig. 3.

BDNF promotes $STEP_{61}$ ubiquitination and degradation through the proteasome. **a** $STEP_{61}$ levels were analyzed by Western blot of protein extracts obtained from mouse primary cortical cultures treated for 60 min with or without the proteasome inhibitor MG-132 (10 μM; *MG*) and then incubated in the presence or absence of BDNF (10 ng/ml; *B*) for additional 15 min. **b** The levels of $STEP_{61}$-ubiquitin conjugates were determined in protein extracts from control cultures and cultures exposed for 15 min to BDNF (10 ng/ml) in the presence of MG-132 (10 μM) and subjected to ubiquitin (*Ub*) pulldown using Agarose-TUBE2 and immunoblotted (*IB*) with anti-STEP and anti-ubiquitin antibodies. Representative immunoblots are shown. Values obtained by densitometric analysis of Western blot data are expressed as percentage of control (*Ctr*) cultures and are shown as mean±SEM of four to seven experiments performed in duplicate in independent cultures. Data were analyzed by one-way ANOVA with Bonferroni's post hoc test. ***$p<0.001$ compared with Ctr cultures; ##$p<0.01$ compared with cultures incubated with BDNF alone

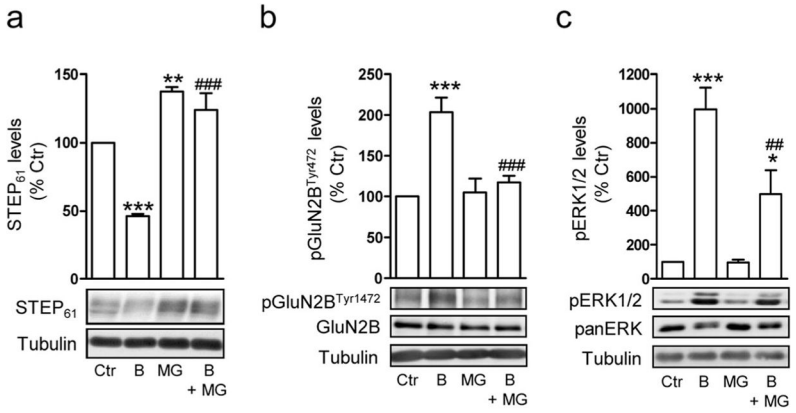

Fig. 4.

Effect of BDNF-induced $STEP_{61}$ degradation on $GluN2B^{Tyr1472}$ and $ERK1/2^{Thr202/Tyr204}$ phosphorylation levels in primary cortical cultures. **a** $STEP_{61}$, **b** $pGluN2B^{Tyr1472}$, and **c** $pERK1/2^{Thr202/Tyr204}$ levels were analyzed by Western blot of protein extracts obtained from mouse primary cortical cultures treated for 60 min in the presence or absence of the proteasome inhibitor MG-132 (10 μM; *MG*) and then incubated with or without 10 ng/ml BDNF (*B*) for additional 60 min. Representative immunoblots are shown. Values obtained by densitometric analysis of Western blot data are expressed as percentage of control (*Ctr*) cultures and shown as mean±SEM of four to eight experiments performed in duplicate in independent cultures. Data were analyzed by one-way ANOVA with Bonferroni's post hoc test. *p<0.05, **p<0.01, and ***p<0.001 compared with Ctr cultures; ##p<0.01 and ###p<0.001 compared with cultures incubated with BDNF alone

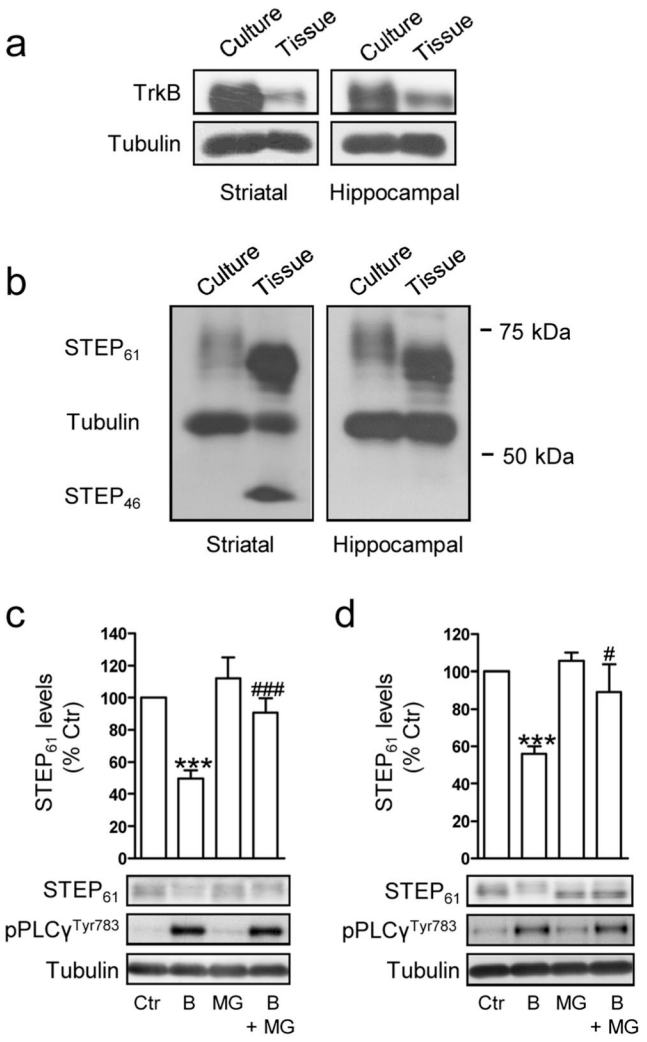

Fig. 5.

BDNF induces $STEP_{61}$ degradation in primary striatal and hippocampal cultures through the proteasome. **a** The expression of TrkB was analyzed by Western blot of protein extracts obtained from mouse primary striatal and hippocampal cultures at DIV 8. Mouse adult striatal and hippocampal tissue served as positive control. **b** The expression of STEP was analyzed by Western blot of protein extracts obtained from mouse striatal and hippocampal adult tissue and cultured neurons at DIV 8. Representative immunoblots are shown. $STEP_{61}$ levels were analyzed by Western blot of protein extracts obtained from primary **c** striatal and **d** hippocampal cultures treated for 60 min with or without the proteasome inhibitor MG-132 (10 μM; *MG*) and then incubated in the presence or absence of BDNF (10 ng/ml; *B*) for additional 15 min. Representative immunoblots are shown. Values obtained by densitometric analysis of Western blot data are expressed as percentage of control (*Ctr*) cultures and are shown as mean±SEM of three to six experiments performed in duplicate in independent cultures. Data were analyzed by one-way ANOVA with Bonferroni's post hoc test. ***$p<0.001$ compared with Ctr cultures; #$p<0.05$ and ###$p<0.001$ compared with cultures incubated with BDNF alone

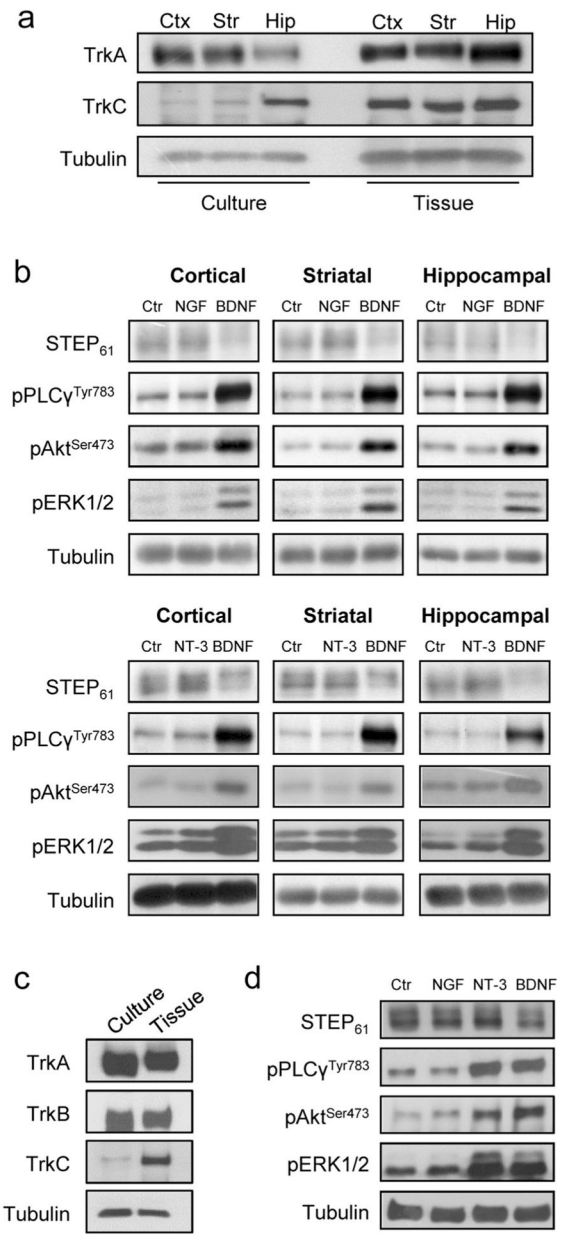

Fig. 6.

NGF and NT-3 have no effect on $STEP_{61}$ levels in primary neurons. **a** The expression of TrkA and TrkC was analyzed by Western blot of protein extracts obtained from mouse primary cortical, striatal, and hippocampal cultures. Mouse adult tissue served as positive control. **b** Mouse primary cortical, striatal, and hippocampal cultures were incubated in the presence or absence of NGF, NT-3, or BDNF (10 ng/ml) for 15 min and the levels of $STEP_{61}$, $pPLC\gamma^{Ser783}$, $pAkt^{Ser473}$, and $pERK1/2^{Thr202/Tyr204}$ were examined by Western blot. **c** The expression of TrkA, TrkB, and TrkC was analyzed by Western blot of protein extracts obtained from rat primary cortical cultures. Mouse adult cortical tissue served as positive control. **d** Rat primary cortical neurons were incubated in the presence or absence of NGF, NT-3, or BDNF (10 ng/ml) for 15 min and the levels of $STEP_{61}$, $pPLC\gamma^{Ser783}$,

pAkt^{Ser473}, and pERK1/2$^{Thr202/Tyr204}$ were examined by Western blot. Representative immunoblots are shown

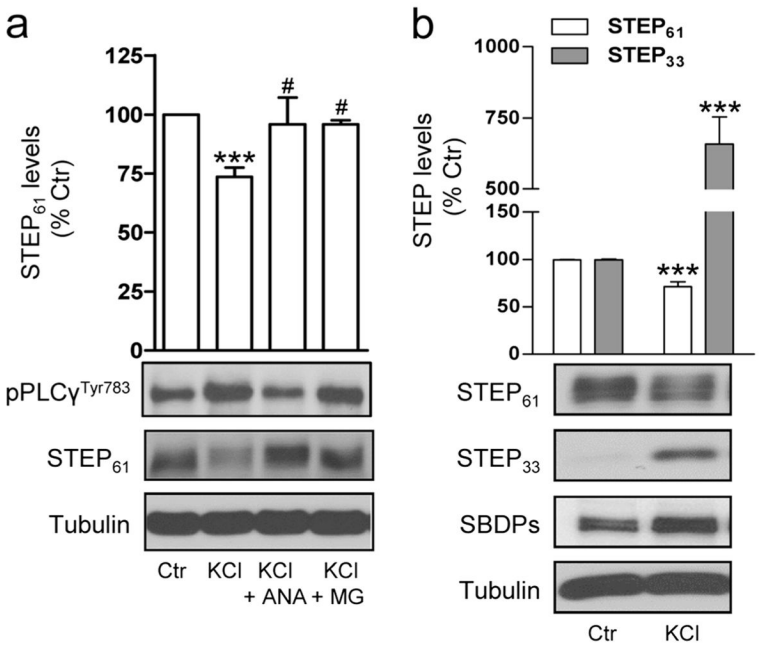

Fig. 7.

$STEP_{61}$ levels after cell depolarization. **a** $STEP_{61}$ and $pPLC\gamma^{Ser783}$ levels were analyzed by Western blot of protein extracts obtained from mouse primary cortical cultures incubated with or without ANA-12 (100 μM; *ANA*) or MG-132 (10 μM; *MG*) for 60 min and then stimulated with 50 mM KCl for 5 min. **b** The levels of STEP and spectrin breakdown products (SBDPs) at 145–150 kDa were analyzed by Western blot of protein extracts obtained from mouse primary cortical cultures incubated with 50 mM KCl for 60 min. Representative immunoblots are shown. Values obtained by densitometric analysis of Western blot data are expressed as percentage of control (*Ctr*) cultures and shown as mean ±SEM of three to five experiments performed in duplicate in independent cultures. Data were analyzed by one-way ANOVA with Bonferroni's post hoc test (**a**) and Student's *t* test (**b**). ***$p<0.001$ compared with Ctr cultures; #$p<0.05$ compared with cultures incubated with KCl alone