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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 · $STEP_{33}$

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

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59 membrane-associated $STEP_{61}$ [11]. Both are expressed in the
60 striatum, central nucleus of the amygdala, and optic nerve,
61 whereas neurons of the hippocampus, cortex, and lateral
62 amygdala only express $STEP_{61}$ [16, 17]. STEP normally op-
63 poses synaptic strengthening by dephosphorylating neuronal
64 signaling molecules, including the *N*-methyl-D-aspartate
65 (NMDA) glutamate receptor subunit GluN2B [18, 19] and
66 extracellular signal-regulated kinase 1/2 (ERK1/2) [20, 21].
67 In addition, STEP mediates internalization of GluA1/GluA2-
68 containing AMPA receptors [22]. STEP also dephosphory-
69 lates p38, Fyn, and proline-rich tyrosine kinase 2, thereby
70 controlling the duration of their signal [20, 23–25].
71 Multiple posttranslational modifications regulate STEP ac-
72 tivity, including phosphorylation/dephosphorylation [21, 26,
73 27], calpain cleavage [15, 28–30], and ubiquitin-proteasome
74 degradation [15, 18, 31]. Despite the accumulated knowledge
75 about STEP function/substrates, particularly in pathological
76 circumstances [18, 32–35], data about its physiological regu-
77 lation is sparse.
78 Since STEP exerts an opposite effect to BDNF on synaptic
79 strength, we hypothesized that BDNF could regulate STEP
80 levels/activity. Our results indicate that BDNF induces
81 $STEP_{61}$ degradation through the proteasome in primary corti-
82 cal, striatal, and hippocampal neurons and suggest that
83 $STEP_{61}$ degradation is an important event in BDNF-induced
84 effects.

85 ## Materials and Methods

86 ### Cell Cultures and Treatments

87 Primary cortical, striatal, and hippocampal cultures were ob-
88 tained from wild-type 18-day-old B6CBA mouse embryos
89 following the National Institutes of Health guide for the care
90 and use of laboratory animals, and the procedures approved
91 by the local animal care committee of Universitat de Barcelo-
92 na (99/01) and Generalitat de Catalunya (99/1094), in accor-
93 dance with the European (2010/63/UE) and Spanish (RD 53/
94 2013) regulations for the care and use of laboratory animals.
95 Primary rat cortical cultures were prepared from embryos at
96 E18 (Charles River Laboratories, MA) according to proce-
97 dures approved by Yale University Institutional Animal Care
98 and Use Committee. Cells were plated at a density of 800,000
99 cells onto 60-mm culture dishes and 100,000 cells onto 24-
100 well plates with coverslips for biochemical and immunocyto-
101 chemical analysis, respectively. Culture dishes and coverslips
102 were precoated with 0.1 mg/ml poly-D-lysine (Sigma Chem-
103 ical Co., St. Louis, MO), and neurons were cultured in
104 neurobasal medium supplemented with B27 and glutamax^{TM}
105 (all Gibco-BRL, Renfrewshire, Scotland, UK). Cultures were
106 maintained at 37 °C in a humidified atmosphere containing
107 5 % CO_2. The cultures were treated with BDNF (10 ng/ml;
108 Peprotech, Inc., Rocky Hill, NJ) at day in vitro (DIV) 8. In
109 different sets of experiments, cultures were treated for differ-
110 ent time periods with BDNF or during 60 min with the tyro-
111 sine kinase inhibitor K252a (200 nM; Sigma-Aldrich), the
112 MAPK inhibitor PD98059 (25 μM; Calbiochem; San Diego,
113 CA), the PI-3 K inhibitor wortmannin (50 nM; Calbiochem),
114 the PLC inhibitor U73122 (5 μM; Calbiochem), the protea-
115 some inhibitor MG-132 (carbobenzoxy-l-leucyl-l-leucyl-l-
116 leucinal; 10 μM; Calbiochem) and then incubated in the pres-
117 ence or absence of BDNF for additional 15 or 60 min. Cortical
118 neurons were incubated for 60 min with or without 100 μM
119 ANA-12 (Sigma-Aldrich) or 10 μM MG-132 and then
120 depolarized by treatment with 50 mM KCl for additional
121 5 min. Nerve growth factor (NGF) and neurotrophin-3 (NT-
122 3) (10 ng/ml; Peprotech, Inc.) were added to cultures for
123 15 min. After treatments, cultures were rinsed with
124 phosphate-buffered saline (PBS) and processed for Western
125 blot analysis or immunofluorescence as detailed below.

126 ### Western Blot Analysis

127 Cell cultures were homogenized in lysis buffer [50 mM Tris–
128 HCl (pH 7.5), 150 mM NaCl, 10 % glycerol, 1 % Triton
129 X-100, 100 mM NaF, 5 μM $ZnCl_2$, and 10 mM EGTA] plus
130 protease inhibitors [phenylmethylsulphonyl fluoride (2 mM),
131 aprotinin (1 μg/ml), leupeptin (1 μg/ml), and sodium
132 orthovanadate (1 mM)]. The lysates were centrifuged at 16,
133 100×*g* for 20 min; supernatants were collected, and protein
134 concentration measured using the Dc protein assay kit (Bio-
135 Rad, Hercules, CA). Western blot analysis was performed as
136 previously described [34]. For the analysis of
137 $pGluN2B^{Tyr1472}$, samples were denatured in 170 mM phos-
138 phate buffer, pH 7.0, with 2.5 % (*w*/*v*) SDS, 10 % glycerol,
139 3.2 mM dithiothreitol, and 0.1 % (*w*/*v*) bromophenol blue, and
140 membranes were blocked with 5 % bovine serum albumin
141 (BSA) in Tris-buffered saline containing 0.1 % Tween 20
142 (TBS-T) for 1 h at room temperature. The primary antibodies
143 used were (1:1000, unless stated otherwise) anti-STEP (Santa
144 Cruz Biotechnology), anti-$pERK1/2^{Thr202/Tyr204}$, anti-
145 $pAkt^{Ser473}$, anti-$pPLC\gamma^{Tyr783}$, anti-$pPKAc^{Thr197}$, anti-
146 $pGluN2B^{Tyr1472}$, anti-GluN2B (Cell Signaling, Beverly,
147 MA), anti-spectrin (Chemicon International, Temecula, CA),
148 anti-TrkB (BD Transduction Laboratories, San Jose, CA), an-
149 ti-$pTrkB^{Tyr816}$, anti-TrkA (1:2000; Abcam, Cambridge, UK),
150 and anti-TrkC (1:300; Millipore, Temecula, CA). Loading
151 control was performed by reprobing the membranes with an
152 anti-α-tubulin antibody (1:50,000; Sigma-Aldrich) for 10–
153 15 min at room temperature. Then, membranes were washed
154 with TBS-T, incubated for 1 h (10–15 min for α-tubulin) at
155 room temperature with the corresponding horseradish
156 peroxidase-conjugated antibody (1:2000; Promega, Madison,
157 WI), and washed again with TBS-T. Immunoreactive bands
158 were visualized using the Western Blotting Luminol Reagent

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(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.). 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 was 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 analysis 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 $STEP_{61}$ 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 $STEP_{61}$

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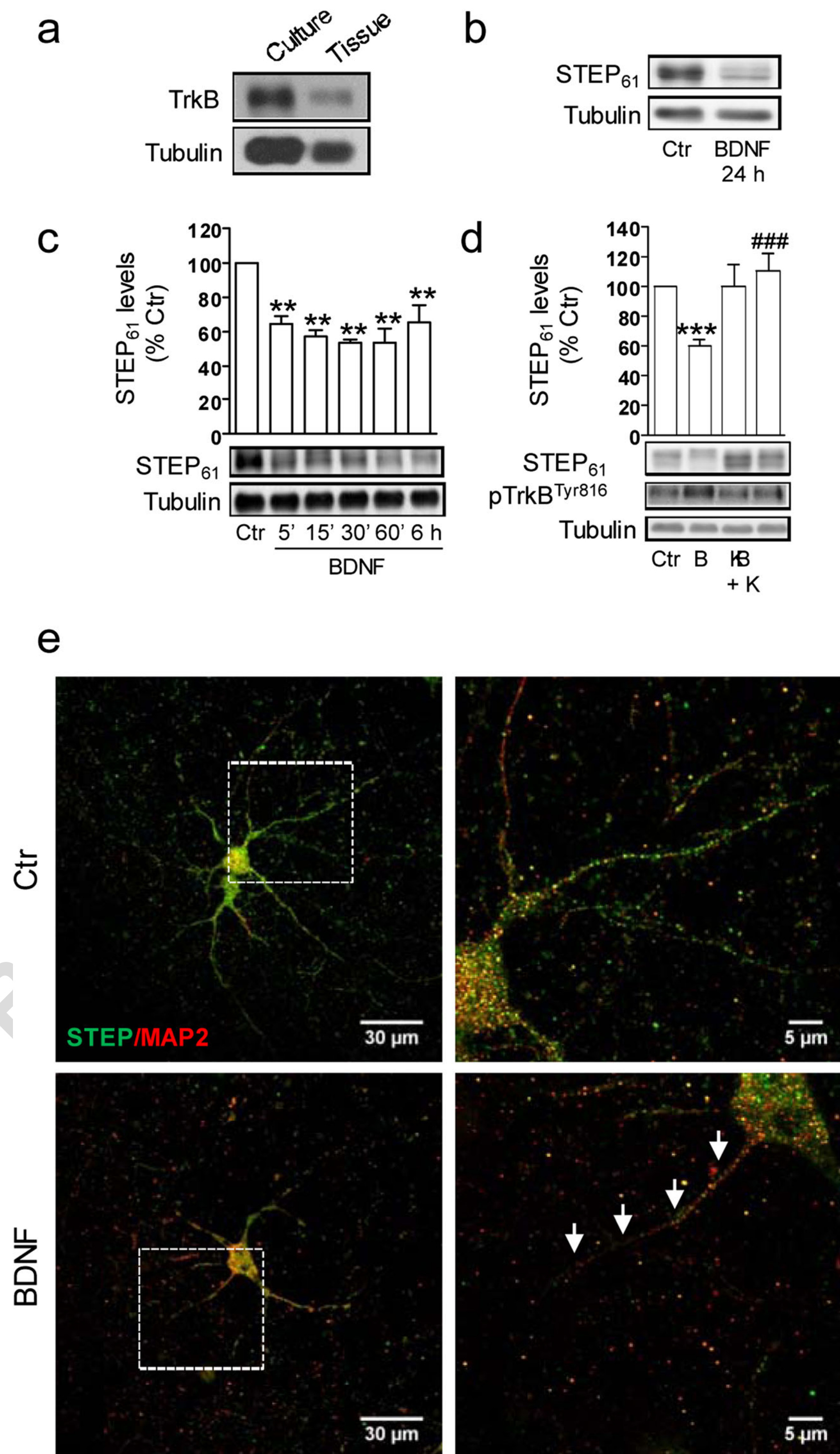

Fig. 1 Effect of BDNF on $STEP_{61}$ levels in primary cortical neurons. **a** The expression of TrkB was analyzed by Western blot of protein extracts obtained from mouse primary cortical cultures. Mouse adult tissue served as positive control. Primary cortical cultures were incubated with 10 ng/ml BDNF **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 Student's *t* test (**b**), one-way ANOVA with Dunnett's (**c**), and one-way ANOVA with Bonferroni's (**d**) post hoc test. $*p<0.05$, $**p<0.01$, 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

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255 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. 262

263 Finally, we stimulated cortical neurons with BDNF for 15 min and performed immunocytochemistry against STEP. As shown in Fig. 1e, $STEP_{61}$ expression was detected in cell body and neurites, and after BDNF treatment, STEP 266

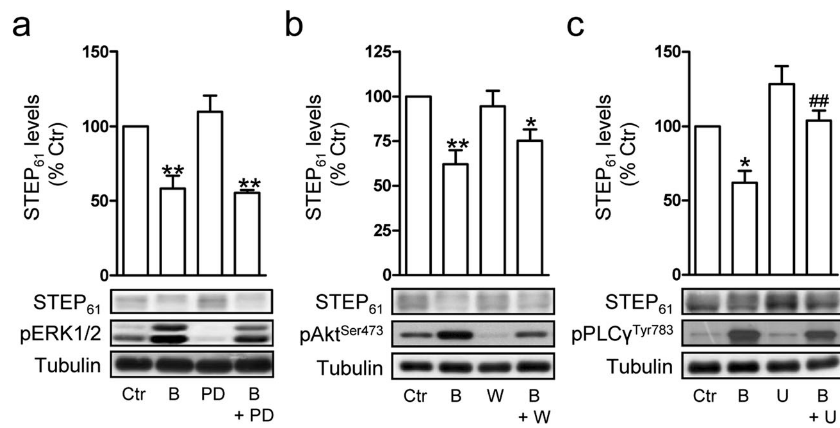

Fig. 2 PLCγ mediates the degradation of $STEP_{61}$ by BDNF in primary cortical cultures. Mouse primary cortical cultures were treated for 60 min with or without **a** the MAPK inhibitor PD98059 (25 μM; *PD*), **b** the PI-3 K inhibitor wortmannin (50 nM; *W*), or **c** the PLC inhibitor U73122 (5 μM; *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

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267 immunoreactivity was mainly decreased in neurites. Taken
268 together, the data indicate that BDNF-TrkB signaling induces
269 $STEP_{61}$ reduction in cortical neurons.

270 BDNF Reduces $STEP_{61}$ Levels in Primary Cortical 271 Cultures Through the PLCγ Pathway

272 BDNF can activate calpains [9, 39], and calpains cleave
273 $STEP_{61}$, generating a 33-kDa fragment [15, 28–30].
274 Thus, we first analyzed whether BDNF activated
275 calpains in our model by looking at spectrin breakdown
276 products (SBDPs) at 145–150 kDa, which are generated
277 specifically by calpain-dependent cleavage. We detected
278 higher levels of SBDPs in BDNF-treated cultures than
279 in controls, indicating that there was activation of
280 calpains in response to BDNF (Fig. S3a). Next, we
281 evaluated whether reduced $STEP_{61}$ levels in BDNF-
282 treated cultures correlated with an accumulation of the
283 $STEP_{33}$ fragment. As shown in Fig. S3b, $STEP_{33}$ was
284 barely detected both in control and in cultures incubated
285 for 15 min with 10 ng/ml BDNF. Thus, although BDNF
286 activates calpains, a different mechanism is responsible
287 for the reduction of $STEP_{61}$ levels in BDNF-treated cor-
288 tical neurons.

289 To identify the intracellular pathways responsible for
290 BDNF-mediated reduction of $STEP_{61}$ levels in primary
291 cortical cultures, we inhibited three downstream effec-
292 tors of BDNF [6]: the MAPK pathway (PD98059), the
293 phosphoinositide-3 kinase (PI-3 K) pathway
294 (wortmannin), and the phospholipase Cγ (PLCγ) path-
295 way (U73122). Treatment with PD98059 (25 μM) or
296 wortmannin (50 nM) reduced both basal and BDNF-
297 induced ERK1/2 and Akt phosphorylation, respectively,
298 but did not affect $STEP_{61}$ levels at baseline and did not
299 block the reduction of $STEP_{61}$ after BDNF treatment
300 (Fig. 2a, b). Similarly, incubation with U73122
301 (5 μM) alone did not modify $STEP_{61}$ levels in basal
302 conditions, but it blocked the reduction of $STEP_{61}$
303 levels in BDNF-treated cultures (Fig. 2c). Thus,
304 BDNF-TrkB signaling induces a reduction of $STEP_{61}$
305 levels in cortical neurons through the activation of
306 PLCγ.

307 $STEP_{61}$ is Ubiquitinated and Degraded by the Proteasome 308 in Response to BDNF Stimulation

309 $STEP_{61}$ can be degraded through the ubiquitin-
310 proteasome system (UPS) [18, 31]. Since BDNF pro-
311 motes the ubiquitination of several synaptic proteins
312 [40], we next investigated the effect of proteasome in-
313 hibition on $STEP_{61}$ levels in BDNF-treated cultures. The
314 proteasome inhibitor MG-132 (10 μM) alone had no
315 effect on basal $STEP_{61}$ levels in cortical neurons, but
316 it prevented the reduction of $STEP_{61}$ in response to
317 BDNF (Fig. 3a). To confirm these findings, we analyzed
318 $STEP_{61}$ ubiquitination in control and in BDNF-treated
319 cultures. To detect $STEP_{61}$ ubiquitination, BDNF stimu-
320 lations were performed in the presence of MG-132
321 (10 μM) and polyubiquitinated proteins were enriched
322 using ubiquitin affinity beads and probed with an anti-
323 STEP antibody. Consistent with the effect of proteasome
324 inhibition on $STEP_{61}$ levels (Fig. 3a), polyubiquitinated
325 $STEP_{61}$ was detected in BDNF-treated cultures, while
326 the total level of polyubiquitined proteins was not

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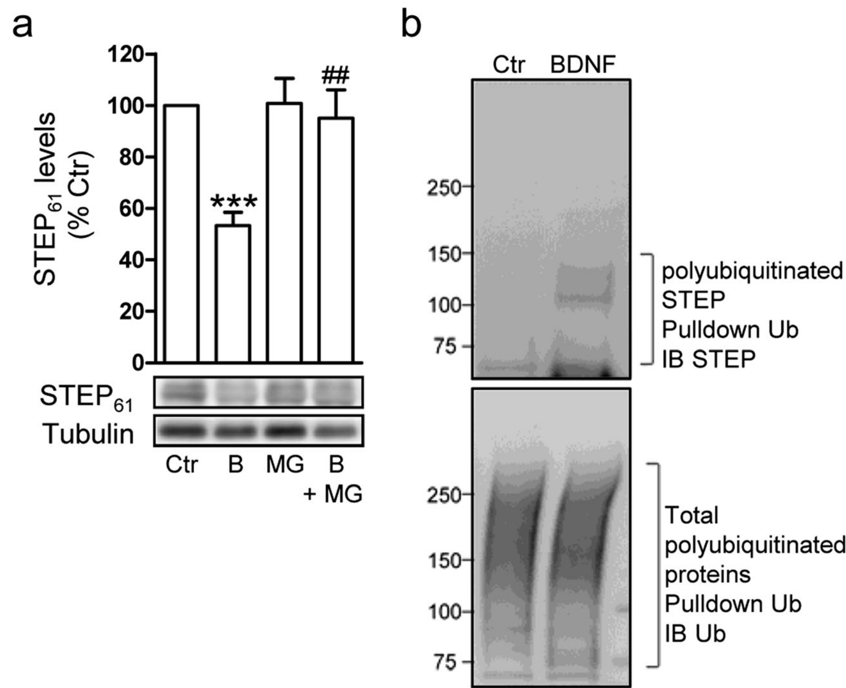

Fig. 3 BDNF promotes $STEP_{61}$ ubiquitination and degradation through the proteasome. **a** $STEP_{61}$ levels were analyzed by Western blot of protein extracts obtained from mouse primary cortical cultures treated for 60 min with or without the proteasome inhibitor MG-132 (10 μM; *MG*) and then incubated 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. $^{**}p<0.01$ and $^{***}p<0.001$ compared with Ctr cultures; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ compared with cultures incubated with BDNF alone

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327 altered by BDNF treatment (Fig. 3b). Altogether, these
328 results indicate that $STEP_{61}$ is ubiquitinated in response
329 to BDNF and degraded by the UPS through a PLCγ-
330 mediated pathway.

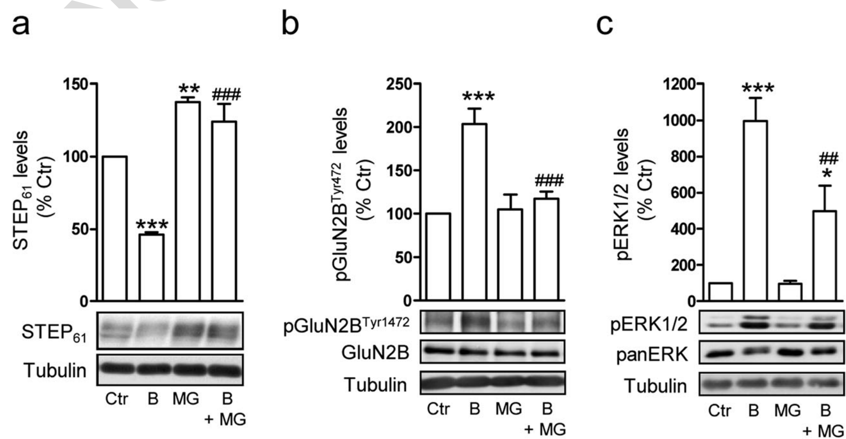

Fig. 4 Effect of BDNF-induced $STEP_{61}$ degradation on $GluN2B^{Tyr1472}$ and $ERK1/2^{Thr202/Tyr204}$ phosphorylation levels in primary cortical cultures. **a** $STEP_{61}$, **b** $pGluN2B^{Tyr1472}$, and **c** $pERK1/2^{Thr202/Tyr204}$ levels were analyzed by Western blot of protein extracts obtained 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

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

333 BDNF induces GluN2B [41, 42]and ERK1/2 [43–45] phos-
334 phorylation in cortical neurons, and both phospho-proteins are
335 STEP substrates [19, 21, 46]. Thus, to address the functional
336 relevance of BDNF-induced $STEP_{61}$ degradation, we ana-
337 lyzed the phosphorylation level of GluN2B and ERK1/2 in
338 cultures exposed to BDNF in the presence or absence of the
339 proteasome inhibitor MG-132. In an attempt to avoid the ini-
340 tial BDNF-induced kinase-dependent effect on the phosphor-
341 ylation status of GluN2B and ERK1/2, we analyzed their
342 phosphorylation levels at 1 h. BDNF-induced $STEP_{61}$ degra-
343 dation was prevented by MG-132, and in agreement with
344 $STEP_{61}$ being degraded by the proteasome, incubation for
345 120 min with MG-132 alone (but not for 75 min, Fig. 3a)
346 significantly increased $STEP_{61}$ levels compared to control cul-
347 tures (Fig. 4a). Both pGluN2B^{Tyr1472} (Fig. 4b) and pERK1/
348 2$^{Thr202/Tyr204}$ (Fig. 4c) levels were significantly increased in
349 cultures exposed to BDNF for 1 h. In contrast, in cultures
350 incubated with BDNF plus MG-132, the phosphorylation
351 levels of GluN2B (Fig. 4b) and ERK1/2 (Fig. 4c) were signif-
352 icantly reduced compared to cultures incubated with BDNF
353 alone. Taken together, these results indicate that $STEP_{61}$ deg-
354 radation through the proteasome is necessary for full phos-
355 phorylation of GluN2B and ERK1/2 in response to BDNF.

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

359 Next, we investigated whether BDNF-induced $STEP_{61}$ degra-
360 dation was a general mechanism that occurs in distinct neuro-
361 nal types. We first analyzed TrkB expression by Western blot
362 of mouse primary striatal and hippocampal neuronal extracts.
363 Both striatal (Fig. 5a) and hippocampal (Fig. 5b) neurons
364 expressed TrkB at DIV 8. In agreement with the postnatal
365 ontogeny of $STEP_{46}$ [47], we did not detect expression of this
366 STEP isoform in 8-day-old primary striatal neurons (Fig. 5b).
367 Moreover, like hippocampal tissue, cultured hippocampal
368 neurons only express the $STEP_{61}$ isoform (Fig. 5b). Addition
369 of 10 ng/ml BDNF for 15 min activated PLCγ and reduced
370 $STEP_{61}$ protein levels in striatal (Fig. 5c) and hippocampal
371 (Fig. 5d) cultured neurons, an effect that was prevented by
372 the addition of the proteasome inhibitor MG-132. Thus,
373 BDNF-induced $STEP_{61}$ degradation through the proteasome
374 is a mechanism common to cortical, striatal, and hippocampal
375 neurons.

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

377 We next examined whether $STEP_{61}$ degradation was exclu-
378 sively induced by BDNF or could also be promoted by other

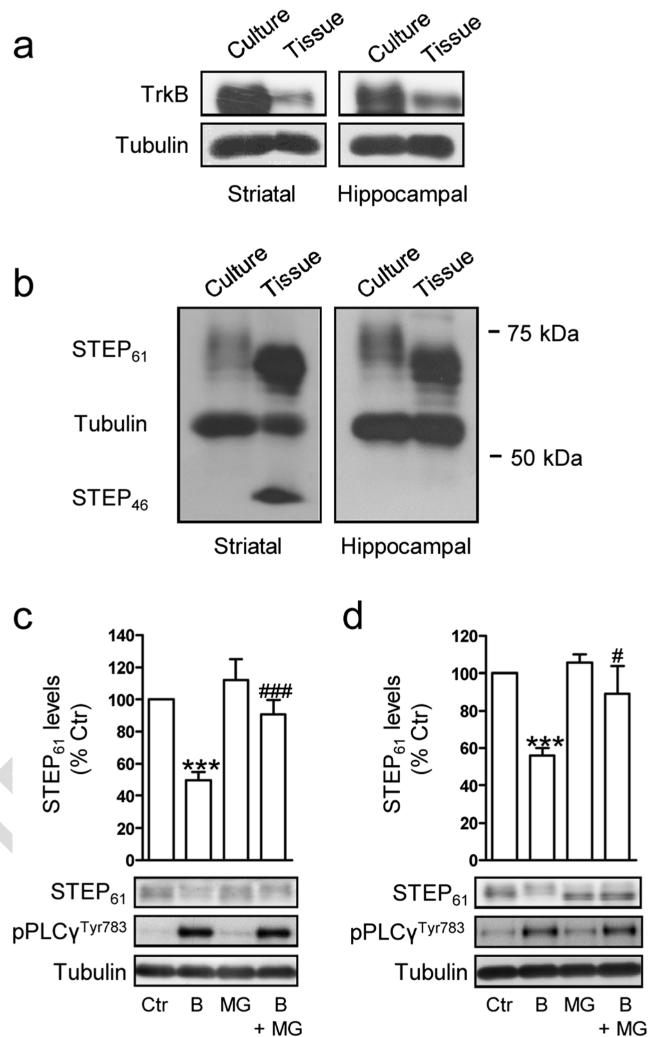

Fig. 5 BDNF induces $STEP_{61}$ degradation in primary striatal and hippocampal cultures through the proteasome. **a** The expression of TrkB was analyzed by Western blot of protein extracts obtained from mouse primary striatal and hippocampal cultures at DIV 8. Mouse adult striatal and hippocampal tissue 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

neurotrophin family members. To this end, we first checked in 379
mouse primary neurons the expression of TrkA, the high af- 380
finity receptor for nerve growth factor (NGF), and TrkC, the 381
high affinity receptor for NT-3. As shown in Fig. 6a, TrkA and 382
TrkC were expressed in mouse primary cortical, striatal, and 383

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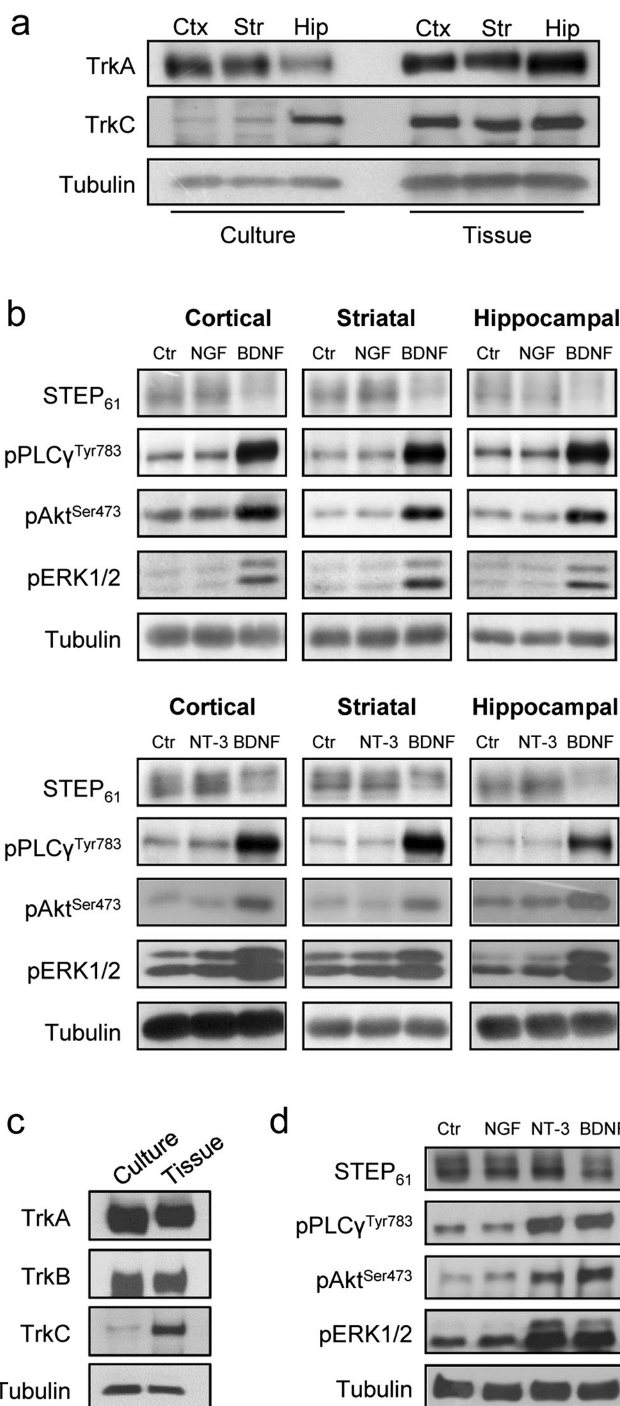

Fig. 6 NGF and NT-3 have no effect on $STEP_{61}$ levels in primary neurons. **a** The expression of TrkA and TrkC was analyzed by Western blot of protein extracts obtained from mouse primary cortical, striatal, and hippocampal cultures. Mouse adult tissue served as positive control. **b** Mouse primary 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

384 hippocampal neurons at DIV 8. In contrast to BDNF, addition 385 of 10 ng/ml NGF or NT-3 for 15 min did not alter $STEP_{61}$ 386 levels in cortical, striatal, or hippocampal primary neurons 387 (Fig. 6b). Of note, neither NGF nor NT-3 induced PLCγ, 388 Akt, or ERK1/2 phosphorylation in these conditions 389 (Fig. 6b). NT-3 [48–50], but not NGF [49, 51], stimulates 390 Trk phosphorylation and downstream signaling in rat primary 391 cortical neurons. As Trk receptors were detected in 8-day-old 392 rat primary cortical neurons (Fig. 6c), we analyzed the effect 393 of the distinct neurotrophins on $STEP_{61}$ levels and intracellu- 394 lar signaling. We found that a 15-min exposure to 10 ng/ml 395 NT-3 or BDNF, but not NGF, increased the phosphorylation 396 level of PLCγ, Akt, and ERK1/2 (Fig. 6d). Importantly, de- 397 spite PLCγ activation in both NT-3- and BDNF-treated cul- 398 tures, only BDNF induced $STEP_{61}$ degradation in rat cortical 399 neurons (Fig. 6d). Therefore, the effect of BDNF on $STEP_{61}$ 400 levels is specific as other neurotrophin family members are 401 unable to induce $STEP_{61}$ degradation.

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402 $STEP_{61}$ Levels are Reduced Upon Cell Depolarization

403 Neuronal depolarization induced by high extracellular KCl 404 levels is commonly used as an in vitro model to study 405 activity-dependent processes, and it induces BDNF release 406 [52–55]. We therefore determined whether depolarization 407 could promote $STEP_{61}$ degradation. To address this, we ana- 408 lyzed $STEP_{61}$ levels after depolarization of primary cortical 409 neurons with 50 mM KCl for 5 min, in the presence and 410 absence of ANA-12, a TrkB antagonist [56], and the protea- 411 some inhibitor MG-132. We found that, like BDNF (Fig. 2c), 412 depolarization significantly increased the levels of 413 $pPLC\gamma^{Tyr783}$ (Ctr: 100.01±0.04 % and KCl: 141.84± 414 19.07 %; n=3–4; p=0.0470, Student's t test) with a concom- 415 itant reduction of $STEP_{61}$ levels, which was prevented in cul- 416 tures where TrkB signaling or the proteasome were inhibited 417 (Fig. 7a). After a prolonged incubation (60 min) with 50 mM 418 KCl to mimic neuronal stimulation in pathological conditions, 419 $STEP_{61}$ levels were also reduced, but in this case, there was an 420 increase in $STEP_{33}$ levels (Fig. 7b), indicating cleavage of

$STEP_{61}$ by calpains [15]. Consistent with this, we de- 421 tected calpain activation in cultures incubated with KCl 422 for 60 min as assessed by the increase in SBDPs at 423 145–150 kDa (Fig. 7b). Hence, neuronal depolarization 424 promotes a reduction in $STEP_{61}$ levels by a mechanism 425 that is dependent on whether it is physiological or 426 pathological. 427

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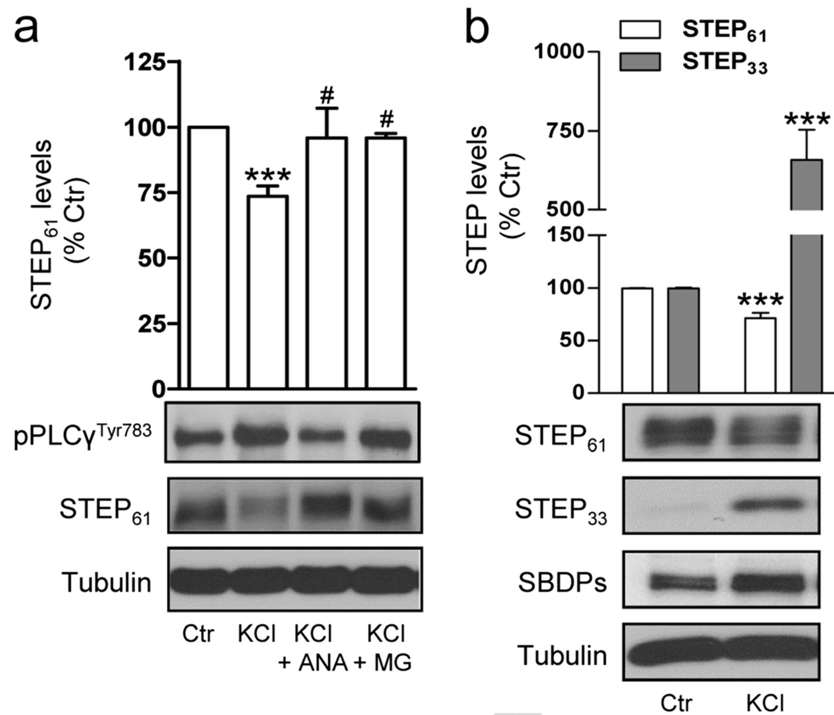

Fig. 7 $STEP_{61}$ levels after cell depolarization. **a** $STEP_{61}$ and $pPLC\gamma^{Ser783}$ levels were analyzed by Western blot of protein extracts obtained from mouse primary cortical cultures incubated with or without ANA-12 (100 μM; *ANA*) or MG-132 (10 μM; *MG*) for 60 min and then stimulated 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

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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

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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.

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Conflict of Interest None

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