Journal of Neurochemistry JNC

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Journal:	*Journal of Neurochemistry*
Manuscript ID:	JNC-2015-0481
Manuscript Type:	Original Article
Date Submitted by the Author:	18-Jun-2015
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Area/Section:	Signal Transduction & Synaptic Transmission
Keywords:	BDNF, STEP, ubiquitination, TrkB agonist, STEP inhibitor, locomotor activity

Down-regulation of BDNF in cell and animal models increases striatal-enriched protein tyrosine phosphatase 61 ($STEP_{61}$) levels.

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Running title: BDNF signaling regulates $STEP_{61}$ levels

Key words: BDNF, STEP, ubiquitination, TrkB agonist, STEP inhibitor, locomotor activity

Pages: 27; Figures: 6; Table: 0

Manuscript including legends: 5862; Abstract: 224; Introduction: 451

Supplemental Figures: 1; Supplemental Table: 1

Abbreviations

7,8-DHF, 7,8-dihydroxyflavone; AKAP, A-kinase anchoring protein; AMPAR, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; BDNF, brain-derived neurotrophic factor; DIV, days in vitro; ERK, extracellular-signal regulated kinase; HRP, horseradish peroxidase; IP, immunoprecipitation; KO, knock out; MAPK, mitogen-activated protein kinase; NMDAR, N-methyl-D-aspartate receptor; PAGE, polyacrylamide electrophoresis; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLCγ, phospholipase Cγ; pyk2, proline-rich tyrosine kinase 2; RIPA buffer, radioimmunoprecipitation assay buffer; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; siRNA, short interfering RNA; $STEP_{61}$, STriatal-Enriched protein tyrosine Phosphatase, 61 kDa; TrkB, tropomyosin receptor kinase B; WT, wild type.

Abstract

Brain-derived neurotrophic factor (BDNF) regulates synaptic strengthening and memory consolidation, and altered BDNF expression is implicated in a number of neuropsychiatric and neurodegenerative disorders. BDNF potentiates NMDAR function through activation of Fyn and ERK1/2. STriatal-Enriched protein tyrosine Phosphatase (STEP) is also implicated in many of the same disorders as BDNF, but STEP opposes the development of synaptic strengthening. STEP-mediated dephosphorylation of the NMDA receptor subunit GluN2B promotes internalization of GluN2B-containing NMDA receptors, while dephosphorylation of the kinases Fyn, Pyk2 and ERK1/2 leads to their inactivation. Thus, STEP and BDNF have opposing functions. In this study, we demonstrate that manipulation of BDNF expression has a reciprocal effect on STEP levels. Reduced BDNF signaling leads to elevation of $STEP_{61}$ both in $BDNF^{+/-}$ mice and in cortical cultures after acute BDNF knockdown, and a newly identified STEP inhibitor reverses the biochemical and motor abnormalities in $BDNF^{+/-}$ mice. In contrast, increased BDNF signaling upon treatment with a TrkB agonist results in degradation of $STEP_{61}$ and a subsequent increase in the tyrosine phosphorylation of STEP substrates in cultured neurons and in mouse frontal cortex. These findings indicate that BDNF-TrkB signaling leads to degradation of $STEP_{61}$ while decreased BDNF expression results in increased STEP activity. A better understanding of the opposing interaction between STEP and BDNF in normal cognitive functions and neuropsychiatric disorders will hopefully lead to novel therapeutic strategies.

Introduction

Brain-derived neurotrophic factor (BDNF) is widely expressed in many brain regions and is enriched in neocortex, hippocampus, striatum and amygdala, regions critical for normal learning and memory (Skup 1994, Kawamoto *et al.* 1996, Dugich-Djordjevic *et al.* 1995, Bekinschtein *et al.* 2008, Liu *et al.* 2004, Lu *et al.* 2008). BDNF signaling is required for neurogenesis, axonal and dendritic growth, neuronal survival and migration, as well as the development of synaptic strengthening (Buckley *et al.* 2007a, Hu *et al.* 2005, Mamounas *et al.* 1995, Yoshii & Constantine-Paton 2010, Segal 2003). The tropomyosin receptor kinase B (TrkB) receptor mediates the biological functions of BDNF by activating the phosphoinositide 3-kinase (PI3K), phospholipase C γ (PLCγ) and mitogen-activated protein kinase (MAPK) pathways (Yoshii & Constantine-Paton 2010, Segal 2003). BDNF signaling activates ERK1/2 and Fyn and potentiates N-methyl-D-aspartate receptor (NMDAR) signaling through ERK1/2 and Fyn-dependent mechanisms (Li & Keifer 2009, Xu *et al.* 2006).

STriatal-Enriched protein tyrosine Phosphatase (STEP) is a negative regulator of synaptic strengthening and does so through the dephosphorylation of its substrates. STEP promotes the internalization of surface glutamate receptors (NMDARs and AMPARs) (Snyder *et al.* 2005, Xu *et al.* 2009, Zhang *et al.* 2008, Zhang *et al.* 2011), inactivation of Fyn (Nguyen *et al.* 2002), Pyk2 (Xu *et al.* 2012) and ERK1/2 kinases (Venkitaramani *et al.* 2009, Paul *et al.* 2003), or regulation of PTPα localization (Xu *et al.* 2015). Mice null for STEP have increased phosphorylation and activity of these kinases, increased surface expression of glutamate receptors, and enhanced cognitive function for hippocampal (Venkitaramani *et al.* 2011) and amygdalar-dependent memory consolidation (Olausson *et al.* 2012). In contrast, increased expression of STEP is found in several neuropsychiatric and neurodegenerative disorders, including schizophrenia (SZ) (Carty

et al. 2012), fragile X syndrome (FXS) (Goebel-Goody *et al.* 2012), Parkinson's disease (PD) (Kurup *et al.* 2015) and Alzheimer's disease (AD) (Kurup *et al.* 2010, Zhang *et al.* 2010). Importantly, genetic reduction of STEP expression in SZ and FXS mouse models (Zhang et al. 2010, Goebel-Goody et al. 2012) and genetic or pharmacologic inhibition of STEP in an AD model enhances cognitive functions (Xu *et al.* 2014).

We have recently shown that BDNF induces $STEP_{61}$ degradation through the proteasome in cell cultures (Saavedra *et al.,* in press) and here we extend these findings by showing that reduction of BDNF leads to elevated $STEP_{61}$ expression both *in vitro* and *in vivo*. Importantly, both a novel STEP inhibitor and a TrkB agonist normalize BDNF signaling and reverse the biochemical deficits *in vitro* as well as reverse the biochemical and motor alterations in $BDNF^{+/-}$ mice. These findings define a mechanism by which BDNF and $STEP_{61}$ interact and whose alteration may contribute to the pathophysiology of several neuropsychiatric and neurodegenerative disorders.

Materials and reagents

Antibodies and reagents

All antibodies used in this study are listed in the Table S1. The proteasome inhibitors lactacystin and MG-132 were obtained from Calbiochem (San Diego, CA). The tyrosine kinase inhibitor K252a, the TrkB agonists 7,8-dihydroxyflavone (7,8-DHF) and LM 22A4 were purchased from Tocris Biosciences (Ellisville, MO). TC-2153 was purified as described (Xu *et al.* 2014).

Treatment of primary neuronal cultures

All experimental procedures were approved by the Yale University Institutional Animal Care and Use Committee and in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals. Primary cortical cultures were derived from rat Sprague-Dawley E18 embryos (Jackson Laboratory, Bar Harbor, Maine) as described (Xu *et al.* 2014). Both male and female embryos were used in this study. Neuronal cultures were maintained in Neurobasal with B27 supplement (Invitrogen, San Diego, CA) for 12-14 days until treatment. Cultures were treated with three doses of 7,8-DHF (100, 250 and 500 nM) for 5-30 min. In some experiments, inhibitors (K252a: 100 nM and lactacystin: 5 μM) were pre-incubated for 30-60 min, followed by 7,8-DHF (500 nM, 30 min) or LM 22A4 (500 nM, 30 min) treatment. Neurons were lysed in 1×RIPA buffer (Pierce Biotechnology, Rockford, IL) with complete phosphatase and protease inhibitors (Roche, Indianapolis, IN).

BDNF knockdown using small interfering RNA (siRNAs)

BDNF siRNAs and non-targeting negative control siRNA were purchased from Ambion (Austin, TX). Twenty nM of BDNF or control siRNAs were transfected into cortical neurons on DIV 7

using Lipofectamine RNAiMAX transfection reagent following manufacturer's protocol (Invitrogen). Neurons were harvested 3 days post transfection and lysed in 1×RIPA buffer. In some experiments, transfected neurons were treated with vehicle (0.1% DMSO) or TC-2153 (1 μM) for 1 h prior to lysis (Xu *et al.* 2014).

Measurement of ubiquitinated STEP

Cortical neurons were pretreated with MG-132 (10 μM) for 30 min, followed by 7,8-DHF (500 nM, 30 min) or LM 22A4 treatment. Neurons were lysed in 1×RIPA buffer with phosphatase and protease inhibitors and spun at 12,000×g for 10 min. Equal amount of supernatants were precleared with protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) to minimize non-specific binding. A monoclonal anti-STEP antibody (clone 23E5) was used to pull-down STEP. Ubiquitinated STEP species were visualized by probing with anti-ubiquitin antibody.

Drug administration for biochemical analyses

Male C57BL/6J mice (3-4 months old) and $BDNF^{+/-}$ mice (15-weeks old) were obtained from the Jackson Laboratory (Bar Harbor, Maine). Mice were injected with vehicle (2% DMSO in saline), TC-2153 (10 mg/kg, i.p.) or 7,8-DHF (5 mg/kg, i.p.). The effective doses of TC-2153 and 7,8-DHF were chosen based on previous publications (Xu *et al.* 2014, Carty *et al.* 2012, Jang *et al.* 2010, Andero *et al.* 2012). Frontal cortices were collected 1-2 h post injections and snap frozen in dry-ice.

Sample preparation and immunoblotting

Mouse brain tissues were homogenized in ice-cold TEVP buffer (10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 5 mM NaF, 320 mM sucrose) supplemented with complete protease inhibitor cocktail (Roche). Homogenates were centrifuged to obtain synaptosomal membrane fractions (P2) as described (Xu *et al.* 2015). Protein concentrations were determined using bicinchoninic acid (BCA) kit (Pierce) and 30 μg of each sample were separated on 8% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad, Richmond, CA). Membranes were blocked in 5% BSA in TBS + 1% Tween-20 and incubated with primary antibodies and horseradish peroxidase (HRP)-coupled secondary antibodies following standard procedures. Membranes were developed using Chemiluminescent Substrate kit (Pierce) and visualized by a G:BOX with the GeneSnap software (Syngene, Cambridge, UK). All densitometric bands were quantified using the Genetools program (Syngene).

Locomotor activity in BDNF$^{+/-}$ mice

Locomotor activity was measured by using a white melamine circular open field (40 cm diameter and 40 cm high) during the dark phase of the light cycle as described (Giralt *et al.* 2009). Male BDNF$^{+/-}$ mice and their WT littermates (15-weeks old; B6CBA background) were administrated with vehicle or TC-2153 (10 mg/kg, i.p.). One hour post injection mice were tested. At the beginning of the session, mice were left in the periphery of the apparatus and during 1 h the total distance travelled was recorded and traced with an Image tracking system (SMART, Panlab SL, Barcelona, Spain). A second cohort of BDNF$^{+/-}$ and WT mice (male, 15-weeks old) were obtained from the Jackson Laboratory. One hour prior to test, mice were administrated with vehicle or 7,8-DHF (5 mg/kg, i.p.), mice were then kept in the activity chamber for 1 h. Total

distance traveled was measured with Activity Monitor version 5 software (MED Associates) and used as an indication of general activity.

Data analyses

All experiments were repeated at least three times. Data were expressed as means ± SEM. Statistical significance ($p < 0.05$) was determined using Student's *t*-test, one-way or two-way ANOVA with Bonferroni's *post hoc* test.

Results

Hypofunction of BDNF signaling leads to elevated $STEP_{61}$ levels

Low BDNF expression was found in several neurological disorders, including AD (Caccamo *et al.* 2010) and SZ (Chen da *et al.* 2009, Buckley *et al.* 2007b). On the other hand, $STEP_{61}$ levels are elevated in these same disorders (Kurup *et al.* 2010, Carty *et al.* 2012). To investigate whether there is a correlation between hypomorphic BDNF signaling and increased $STEP_{61}$ levels, we first examined whether mice with reduced BDNF expression ($BDNF^{+/-}$ mice) had altered $STEP_{61}$ expression. We confirmed the approximately 50% decrease in BDNF expression in the frontal cortex (Fig. 1a) and hippocampus (Fig. 1b) of $BDNF^{+/-}$ mice. Conversely, $STEP_{61}$ was elevated in synaptosomal membrane fractions in both regions compared to WT littermates (frontal cortex: 1.44 ± 0.15; hippocampus: 1.44 ± 0.17, *p values* < 0.05, Fig. 1a, b).

We then acutely knocked down BDNF with small interfering RNA (siRNA) in primary cortical cultures. We first confirmed that siRNA transfection resulted in decreased BDNF expression (0.58 ± 0.12 of scrambled siRNA, $p < 0.05$, Fig. 2a). BDNF knock down led to elevated $STEP_{61}$ expression (1.46 ± 0.11, $p < 0.05$) and decreased Tyr phosphorylation of the $STEP_{61}$ substrates GluN2B, Pyk2 and ERK1/2, compared with control siRNA treated cultures (pGluN2B: 0.56 ± 0.11; pPyk2: 0.72 ± 0.05; pERK1/2: 0.69 ± 0.07, *p values* < 0.05, Fig. 2a).

We next used a recently identified STEP inhibitor TC-2153 (Xu *et al.* 2014) to test whether STEP inhibition might reverse the effects of BDNF knock down on $STEP_{61}$ activity and Tyr phosphorylation of $STEP_{61}$ substrates in cultures. TC-2153 is a pentathiepin that is a potent STEP inhibitor (IC_{50} = 25 nM) with a mechanism of action that involves covalent

binding to the catalytic cysteine. TC-2153 is relatively specific for STEP and does not inhibit homologous protein tyrosine phosphatases in neuronal cultures and mouse brains. We treated cortical neurons with TC-2153 (1 μM for 1 h) in the presence of normal or reduced BDNF signaling and examined the Tyr phosphorylation of $STEP_{61}$ substrates. In agreement with previous findings (Xu *et al.* 2014), inhibition of $STEP_{61}$ at baseline (scrambled siRNA transfected cells) resulted in significant increases in Tyr phosphorylation of STEP substrates (pGluN2B: 1.47 ± 0.13; pPyk2: 1.58 ± 0.10; pERK1/2: 1.51 ± 0.08, *p values* < 0.05) without changes in $STEP_{61}$ or BDNF levels (Fig. 2b). BDNF siRNA knock down led to increased $STEP_{61}$ and decreased Tyr phosphorylation of STEP substrates (BDNF siRNA Veh vs scrambled siRNA Veh, pGluN2B: 0.72 ± 0.05; pPyk2: 0.65 ± 0.09; pERK1/2: 0.67 ± 0.15, *p values* < 0.05), which was significantly reversed by TC-2153 (BDNF siRNA TC, pGluN2B: 1.42 ± 0.19; pPyk2: 1.61 ± 0.12; pERK1/2: 1.39 ± 0.09, *p values* < 0.05 compared to BDNF siRNA TC, Fig. 2b).

TrkB activation leads to the ubiquitination and degradation of $STEP_{61}$ in neuronal cultures

$STEP_{61}$ is normally ubiquitinated and degraded by the proteasome (Kurup *et al.* 2010, Xu *et al.* 2009). Moreover, we recently showed that BDNF treatment of neuronal cultures promotes the ubiquitination and degradation of $STEP_{61}$ (Saavedra *et al.* in press) indicating that activation of BDNF signaling reduces $STEP_{61}$ expression. Since we wished to carry out studies *in vivo* (see below) and BDNF is poorly transported across the blood-brain barrier, we characterized a selective TrkB agonist 7,8-dihydroxyflavone (7,8-DHF) that has better bioavailability after peripheral administration (Jiang *et al.* 2013, Zeng *et al.* 2012, Jang *et al.*

2010). We first incubated cortical cultures with increasing concentrations of 7,8-DHF and found that 250 and 500 nM 7,8-DHF resulted in a robust reduction of $STEP_{61}$ levels (250 nM: 0.64 ± 0.09; 500 nM: 0.66 ± 0.08, *p values* < 0.05), which was completely blocked by the tyrosine kinase inhibitor K252a (1.13 ± 0.08, $p > 0.05$, Fig. 3a). The loss of $STEP_{61}$ expression was accompanied by the increased Tyr phosphorylation of STEP substrates (500 nM 7,8-DHF, pGluN2B: 1.34 ± 0.16; pPyk2: 1.73 ± 0.13; pERK1/2: 1.46 ± 0.24, *p values* < 0.05, Fig. 3a). Next, we examined the time course of 7,8-DHF treatment (500 nM) and found it led to rapid decrease of $STEP_{61}$ levels at 5 min followed by a delayed increase in Tyr phosphorylation of STEP substrates (starting from 10 min). Thirty min incubation showed robust reduction $STEP_{61}$ (0.58 ± 0.06, $p < 0.05$) and increased Tyr phosphorylation of its substrates (pGluN2B: 1.44 ± 0.10; pPyk2: 1.70 ± 0.10; pERK1/2: 1.51 ± 0.14, *p values* < 0.05, Fig. 3b). This finding is consistent with previous work demonstrating that lowering STEP levels genetically (Venkitaramani *et al.* 2009) or lowering STEP activity with an inhibitor (Xu *et al.* 2014) results in increased phosphorylation of its substrates.

Next we investigated whether the loss of $STEP_{61}$ upon 7,8-DHF treatment was through the ubiquitin proteasome system. Like BDNF (Saavedra *et al.,* in press), 7,8-DHF treatment increased the ubiquitination of STEP, which was blocked by K252a (Fig. 4a, b). In addition, another TrkB agonist LM 22A4 also led to degradation of $STEP_{61}$ (Fig. S1a) via ubiquitination (Fig. S1b).

TrkB activation leads to degradation of $STEP_{61}$ and increased tyrosine phosphorylation of STEP substrates *in vivo*

Having established that 7,8-DHF was effective in neuronal cultures, we administered it to WT mice (5 mg/kg, i.p.) and collected tissue from frontal cortex. Biochemical analyses of synaptosomal membrane fractions showed that $STEP_{61}$ was degraded after 7,8-DHF administration (1 h post injection: 0.58 ± 0.11 of vehicle, $p < 0.05$) and Tyr phosphorylation of the STEP substrates was significantly increased (1 h post injection, pGluN2B: 1.79 ± 0.19; pPyk2: 1.55 ± 0.13; pERK1/2: 1.57 ± 0.16, *p values* < 0.05, Fig. 4a). GluN2B levels were also increased in synaptosomal membranes after 7,8-DHF administration (1 h post injection: 1.53 ± 0.12, $p < 0.05$, Fig. 5). Together, these data suggest that activation of TrkB signaling leads to ubiquitination and degradation of $STEP_{61}$ both in neuronal cultures and *in vivo*.

STEP inhibition rescues behavioral and biochemical alterations in $BDNF^{+/-}$ mice

$BDNF^{+/-}$ mice display behavioral alterations that include hyperactivity (Kernie *et al.* 2000, Autry & Monteggia 2012, Chan *et al.* 2006). We therefore tested whether inhibition of $STEP_{61}$ by TC-2153, which leads to decreased $STEP_{61}$ activity, or 7,8-DHF, which leads to decreased $STEP_{61}$ expression, could attenuate the increase in locomotion in $BDNF^{+/-}$ mice. We first administered vehicle or TC-2153 to WT and $BDNF^{+/-}$ mice 1 prior to behavioral assessment in an open-field chamber, and the distance traveled over the next hour was determined. A two-way ANOVA analysis revealed significant effects of TC-2153 treatment ($F(1,37) = 9.554$, $p < 0.01$), genotype ($F(1,37) = 6.440$, $p < 0.05$) and treatment × genotype interaction ($F(1,37) = 20.93$, $p < 0.001$) on locomotion. Bonferroni's *post hoc* test showed that $BDNF^{+/-}$ mice were hyperactive when compared with WT mice ($p < 0.05$), in consistence with previous findings (Kernie *et al.* 2000, Chan *et al.* 2006). TC-2153 significantly attenuated the increased locomotor behavior in these mice ($BDNF^{+/-}$ TC vs $BDNF^{+/-}$ Veh, $p < 0.01$, two-way ANOVA with Bonferroni's *post hoc* test),

but did not alter locomotion in WT mice (Fig. 6a). These data indicate that inhibition of STEP was sufficient to reverse the hyperlocomotion present in $BDNF^{+/-}$ mice.

Activation of the TrkB signaling by 7,8-DHF has been shown to be effective in reversing behavioral and cognitive deficits in several mouse models of neurological diseases (Jang *et al.* 2010, Yang *et al.* 2014, Castello *et al.* 2014, Zhang *et al.* 2014, Tsai *et al.* 2013, Jiang *et al.* 2013, Zeng *et al.* 2012, Andero *et al.* 2012). Having showed that inhibition of STEP by TC-2153 rescued hyperlocomotion in the $BDNF^{+/-}$ mice, we examined whether 7,8-DHF-induced degradation of $STEP_{61}$ might also achieve similar efficacy. A second cohort of male WT and $BDNF^{+/-}$ mice was administered vehicle or 7,8-DHF (5 mg/kg, i.p.) 1 h prior to behavioral assessment in an open-field chamber for an additional hour. A two-way ANOVA analysis revealed a significant genotype (WT or $BDNF^{+/-}$) and treatment (Veh or DHF) interaction ($F(1,34) = 5.971$, $p < 0.05$) in the locomotor activity. $BDNF^{+/-}$ mice displayed hyperactivity at baseline when compared with WT mice ($p < 0.05$, Bonferroni's *post hoc* test). Similar to TC-2153, 7,8-DHF also showed a main effect ($F(1,34) = 4.796$, $p < 0.05$) in attenuating the increased locomotor activity in $BDNF^{+/-}$ mice ($p < 0.05$, Bonferroni's *post hoc* test), without alteration of locomotion in WT mice (Fig. 6b).

Discussion

BDNF is essential for neurodevelopment and normal brain function (Poo 2001). Dysfunction of the BDNF/TrkB signaling is implicated in a number of disorders with prominent cognitive deficits, including AD, PD, Huntington's chorea, SZ, depression, and the cognitive decline that occurs with aging (reviewed in (Autry & Monteggia 2012, Nagahara & Tuszynski 2011)). An increase of $STEP_{61}$ expression is found in many of the same neurodegenerative and neuropsychiatric disorders, resulting in decreased Tyr phosphorylation of its substrates (Carty *et al.* 2012, Kurup *et al.* 2010, Goebel-Goody *et al.* 2012, Gladding *et al.* 2014, Gladding *et al.* 2012, Saavedra *et al.* 2011). We propose that insufficient BDNF signaling results in increased $STEP_{61}$ activity, with the concomitant removal of glutamate receptors from synaptic membranes and inactivation of key signaling kinases.

In agreement with this model, $STEP_{61}$ levels are elevated in $BDNF^{+/-}$ mice as well as in cortical neurons after acute knockdown of BDNF expression. We demonstrated that a novel STEP inhibitor (TC-2153) was sufficient to enhance the Tyr phosphorylation of STEP substrates when BDNF signaling was reduced in these two models. Administration of TC-2153 was also sufficient to reverse the hyperlocomotion in $BDNF^{+/-}$ mice.

Considering its role in regulating cognitive function, BDNF treatment reverses cognitive deficits in rodent models of disorders. However, due to the poor pharmacokinetic profile of recombinant BDNF, such as its short half-life, poor diffusion, and difficulty in crossing the blood-brain barrier, clinical trials have not been successful (Ochs *et al.* 2000, Beck *et al.* 2005). Small molecule TrkB agonists (BDNF mimetics) are emerging as new therapeutic agents because of their superior pharmacokinetic properties. Indeed, 7,8-DHF and its analogs confer neuroprotection and improve cognitive functions in variety of rodent models of neuropsychiatric

and neurodegenerative disorders (Jang *et al.* 2010, Yang *et al.* 2014, Castello *et al.* 2014, Zhang *et al.* 2014, Tsai *et al.* 2013, Jiang *et al.* 2013, Zeng *et al.* 2012, Andero *et al.* 2012). We demonstrated that activation of the TrkB receptor by 7,8-DHF resulted in degradation of $STEP_{61}$, increased Tyr phosphorylation of STEP substrates and attenuated hyperactivity in $BDNF^{+/-}$ mice. The data suggest that the beneficial effects of TrkB agonists involve the degradation of $STEP_{61}$ that normally opposes the development of synaptic strengthening.

Mechanistically, the findings suggest TrkB agonists, like BDNF (Saavedra *et al.* in press), induce $STEP_{61}$ degradation through the ubiquitin proteasome system. Dysfunction of this pathway in AD patients and animal models results in the accumulation of $STEP_{61}$ (Kurup *et al.* 2010, Zhang *et al.* 2010). Recent studies have shown that disruptions of the ubiquitin proteasome pathway likely contribute to the accumulation of $STEP_{61}$ in SZ (Carty *et al.* 2012) and PD patients (Kurup et al. 2015). Consistent with our findings, BDNF/TrkB signaling promotes the ubiquitination and degradation of several synaptic proteins, including the catalytic subunit of PKA, A-kinase anchoring protein (AKAP) 79/150, and spinophilin (Jia *et al.* 2008).

In summary, our data support a mechanism by which a disruption in BDNF signaling leads to high levels of $STEP_{61}$ that likely contributes to the pathophysiology of a number of disorders through reduced tyrosine phosphorylation and inactivation of key signaling kinases and/or endocytosis of glutamate receptors from the synaptic membrane.

Acknowledgements

This work was supported by the NIH grants MH091037 and MH52711 (PJL), MH090963 (ACN), and GM054051 (JAE); and project PI13/01250 to [EP-N], integrated in "Plan Nacional de I+D+I y cofinanciado por el ISCIII-Subdirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER), Spain. The authors declare no conflict of interest.

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Figure legends

Figure 1. $STEP_{61}$ is elevated in $BDNF^{+/-}$ mice. (a, b) Frontal cortices (a) or hippocampi (b) from male WT and $BDNF^{+/-}$ mice (15-weeks old) were collected for biochemical analyses. Tissues were processed to obtain synaptosomal membrane fractions (P2). Samples were subjected to western blotting and blots were probed with anti-BDNF or anti-STEP antibodies. β-actin was used as a loading control. All data were expressed as mean ± SEM and statistical significance determined using Student's *t*-test (*$p < 0.05$, **$p < 0.01$, n = 6 per group).

Figure 2. Knock down of BDNF increases $STEP_{61}$ in neuronal cultures. (a) Rat cortical neurons (DIV 7) were transfected with scrambled siRNA (Scr siRNA) or BDNF siRNA and lysed 3 days post transfection. Samples were subjected to western blotting and probed with phospho-specific and pan-antibodies. Data were expressed as mean ± SEM (*$p < 0.05$, Student's *t*-test, n = 6). (b) Primary cortical neurons (DIV 7) were transfected with scrambled siRNA (Scr siRNA) or BDNF siRNA. Three days post transfection cultures were treated with control (0.1% DMSO) or TC-2153 (1 μM) for 1 h and lysed in RIPA buffer. Quantification of phospho-protein levels was normalized to total protein levels and then to β-actin as a loading control. All data were expressed as mean ± SEM and statistical significance determined using two-way ANOVA with Bonferroni's *post hoc* test (*$p < 0.05$, n = 4 per group).

Figure 3. Concentration-response and time-course analyses of 7,8-DHF effect on $STEP_{61}$ levels. (a) Primary rat cortical neurons (DIV12-14) were treated with various doses (100, 250 or 500 nM) of a TrkB agonist (7,8-dihydroxyflavone, DHF) in the absence or presence of the tyrosine kinase inhibitor, K252a (100 nM) for 30 min. (b) Cultures were treated with DHF (500 nM) for 5-30

min in the absence or presence of K252a (100 nM). Equal amounts of lysates were used for western blotting with phospho-specific and pan-antibodies as indicated in the figure. Quantification of phospho-protein levels was normalized to total protein levels and then to β-actin as a loading control. Data were expressed as mean ± SEM (*$p < 0.05$, **$p < 0.01$, one-way ANOVA with Bonferroni's *post hoc* test, n = 4 separate cultures).

Figure 4. The TrkB agonist 7,8-DHF induces ubiquitination and degradation of $STEP_{61}$ in cortical neurons. (a) Primary rat cortical neurons (DIV12-14) were treated with 7,8-DHF (500 nM) in the absence or presence of the tyrosine kinase inhibitor (K252a, 100 nM) or a proteasome inhibitor (lactacystin, 5 μM) for 30 min. $STEP_{61}$ and tyrosine phosphorylation levels of STEP substrates were analyzed by western blotting. (b) Cultures were pretreated with another structurally different proteasome inhibitor (MG-132, 10 μM), followed by 7,8-DHF treatment (500 nM, 30 min). All STEP species were immunoprecipitated with anti-STEP (23E5) antibody and probed with anti-ubiquitin or anti-STEP antibodies. Quantification of phospho-protein levels was normalized to total protein levels and then to β-actin as a loading control. All data were expressed as mean ± SEM (*$p < 0.05$, one-way ANOVA with Bonferroni's *post hoc* test, n = 4).

Figure 5. 7,8-DHF induces degradation of $STEP_{61}$ and elevation of phosphorylation of STEP substrates *in vivo*. Three-months old male C57BL/6 mice were given vehicle (Veh) or 7,8-DHF (DHF, 5 mg/kg, i.p.) and sacrificed 1 h and 2 h post injections. Crude synaptic membranes fractions (P2) of frontal cortices were used for biochemical analyses. Quantification of phospho-protein levels was normalized to total protein levels and then to β-actin as a loading control. Data

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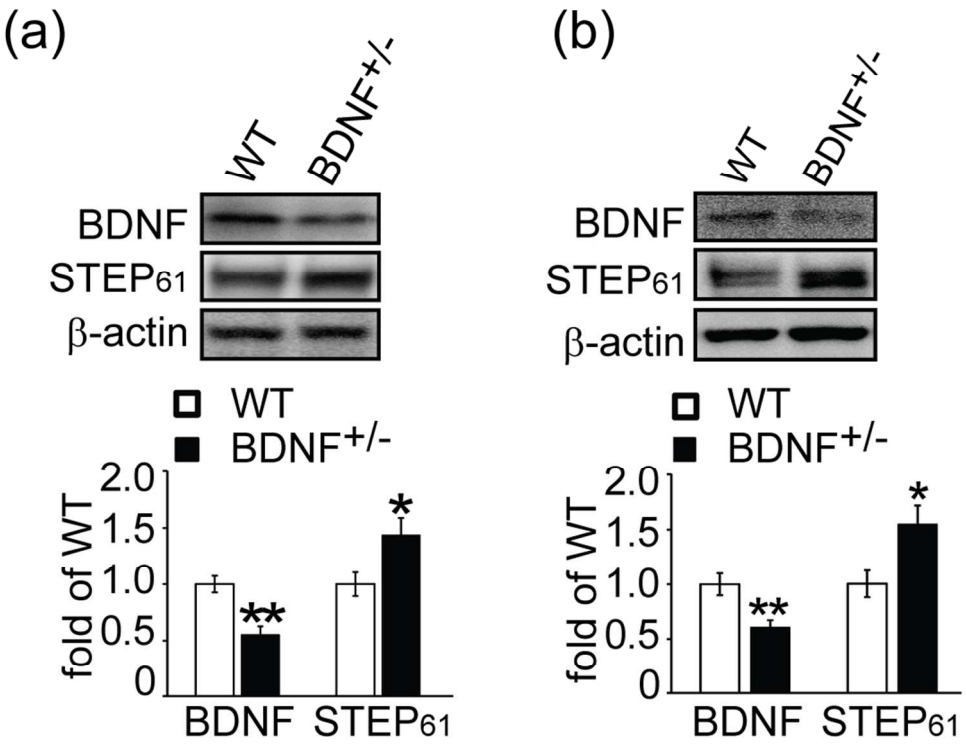
Figure 6. TC-2153 or 7,8-DHF inhibition of $STEP_{61}$ reverses hyperlocomotor activity in $BDNF^{+/-}$ mice. (a) Fifteen-weeks old male WT and $BDNF^{+/-}$ mice were administrated with vehicle or TC-2153 (10 mg/kg, i.p.). One hour post injection mice were tested in activity chambers for 1 h. Differences in total distance traveled were analyzed using two-way ANOVA with Bonferroni's *post hoc* test (*$p < 0.05$, **$p < 0.01$, n = 10-11 per group) (b) A second cohort of WT and $BDNF^{+/-}$ (15-weeks old) male mice were administrated with vehicle or 7,8-DHF (5 mg/kg, i.p.). One hour post injection mice were tested in activity chambers for 1 h. Differences in total distance traveled was analyzed using two-way ANOVA with Bonferroni's *post hoc* test (*$p < 0.05$, n = 9-10 per group).



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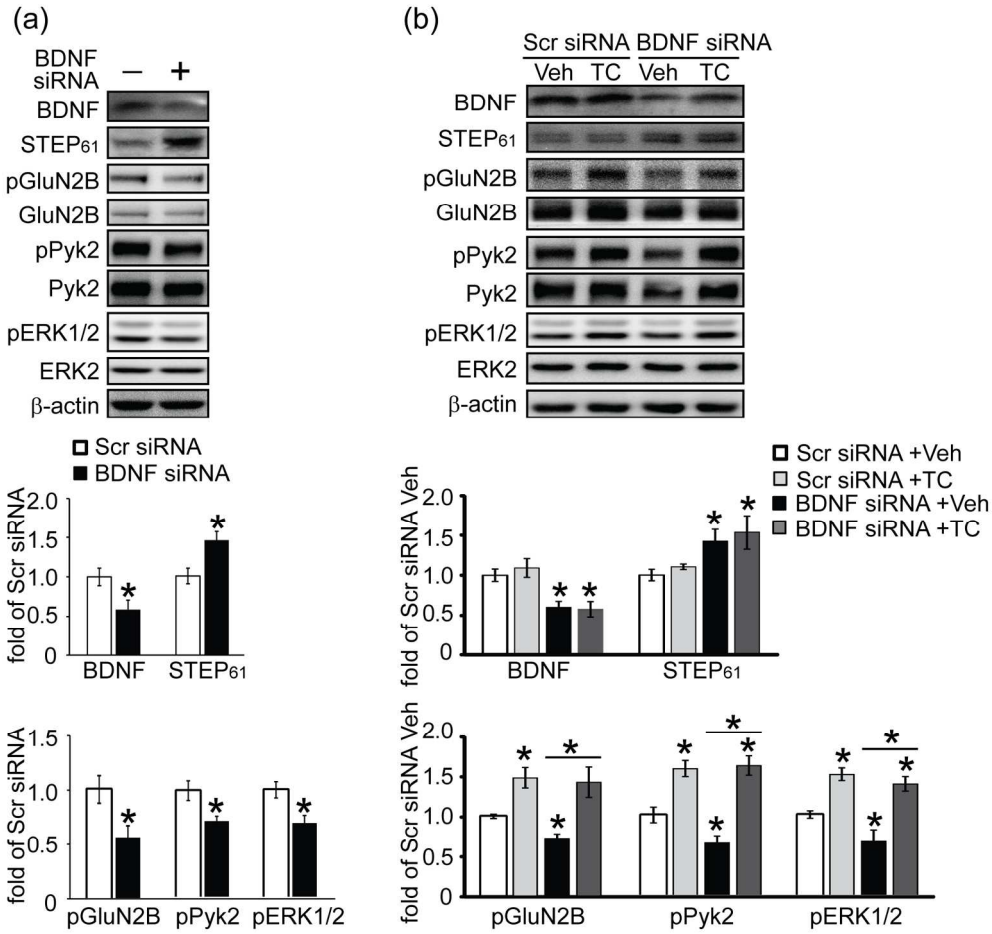

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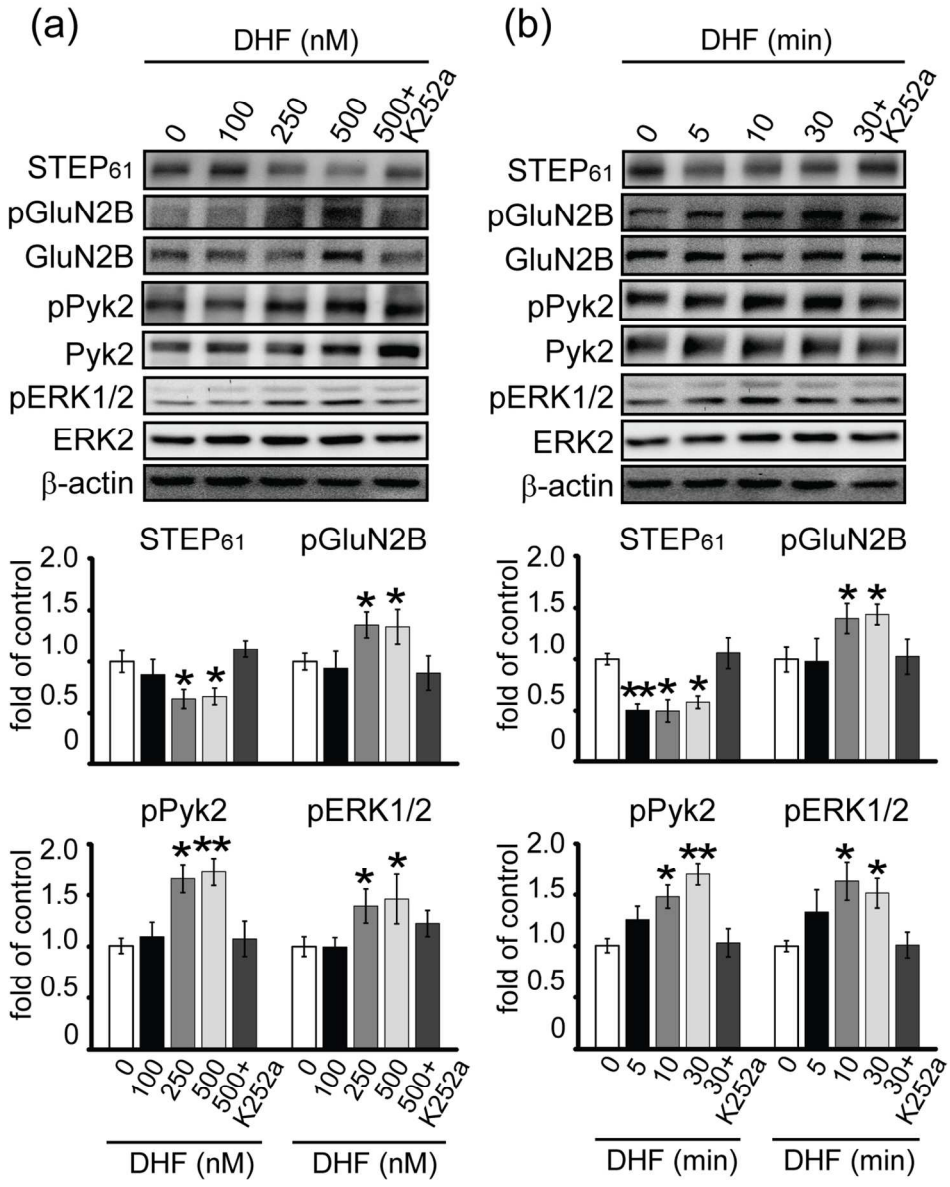

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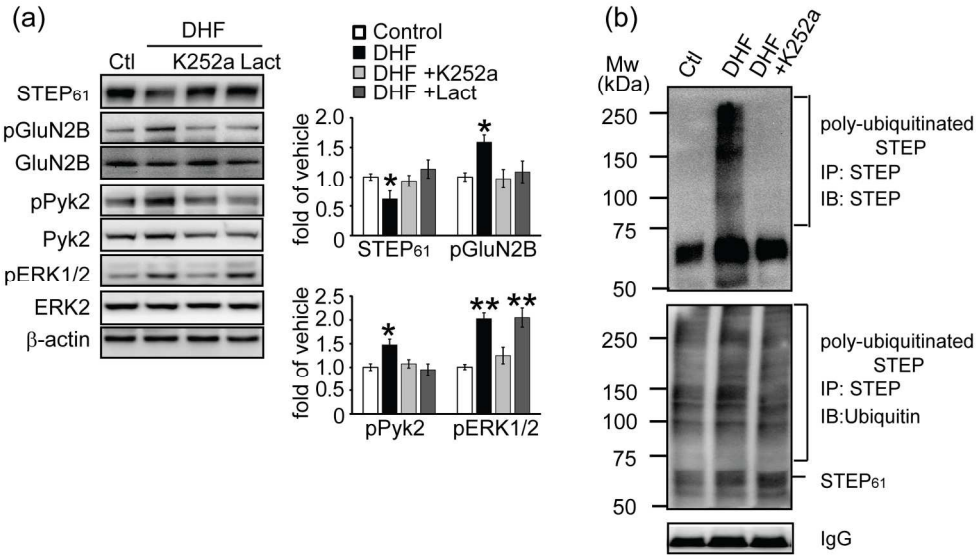

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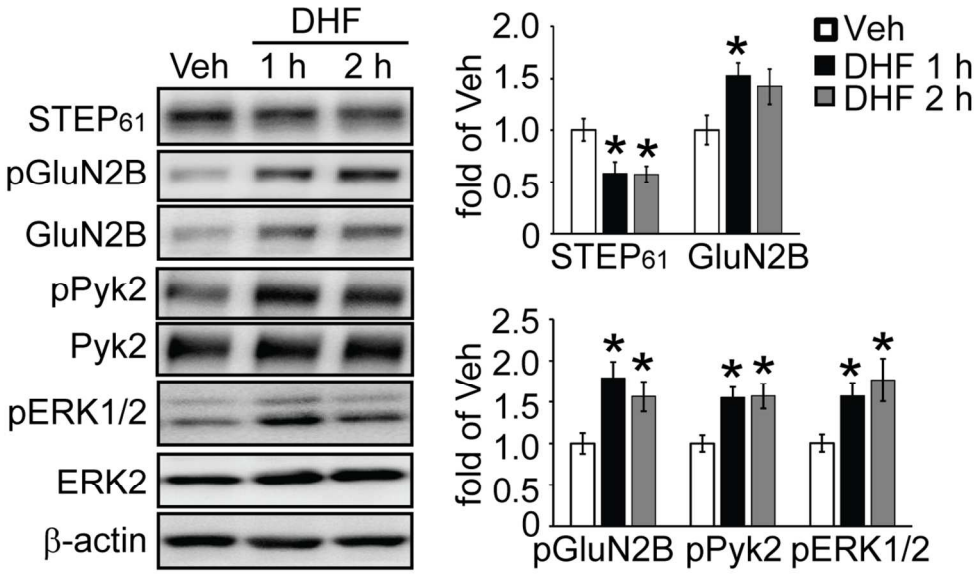

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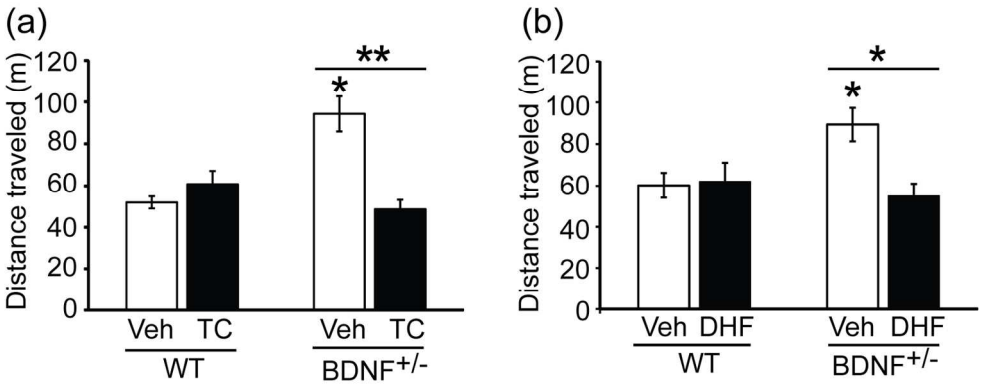

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Down-regulation of BDNF in cell and animal models increases striatal-enriched protein tyrosine phosphatase 61 ($STEP_{61}$) levels

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Supplemental Information

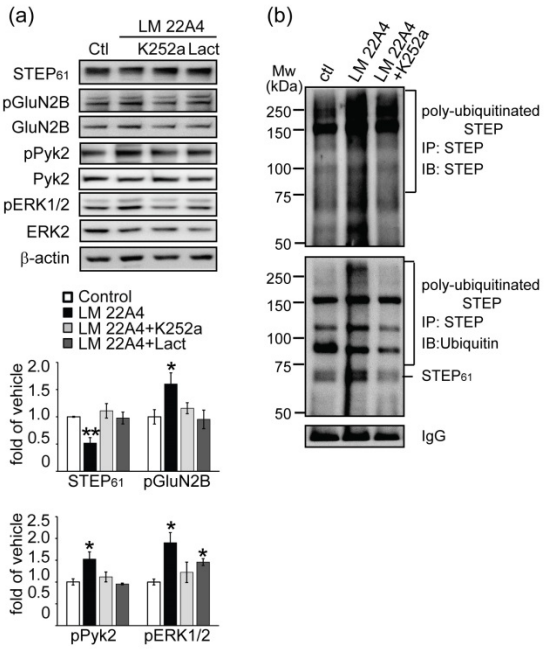

Figure S1. The TrkB agonist LM 22A4 induces ubiquitination and degradation of $STEP_{61}$. (a) Primary rat cortical neurons (DIV12-14) were treated with LM 22A4 (500 nM) in the absence or presence of the tyrosine kinase inhibitor (K252a, 100 nM) or a proteasome inhibitor (lactacystin, 5 μM) for 30 min. $STEP_{61}$ and tyrosine phosphorylation levels of STEP substrates were analyzed by western blotting. Quantification of phospho-protein levels was normalized to total protein levels and then to β-actin as a loading control. All data were expressed as mean ± SEM (*$p < 0.05$, one-way ANOVA with Bonferroni's *post hoc* test, n = 4). (b) Cultures were pretreated with another structurally different proteasome inhibitor (MG-132, 10 μM), followed by LM 22A4 treatment (500 nM, 30 min) in the absence or presence of the tyrosine kinase inhibitor (K252a, 100 nM). All STEP species were immunoprecipitated with anti-STEP (23E5) antibody and probed with anti-ubiquitin or anti-STEP antibodies.

Table S1. Antibodies used in this study.

Antibody	Immunogen	Host	Dilution	Source
anti-STEP (23E5)	N-terminal of rat $STEP_{46}$	Mouse	1:1000	Santa Cruz Biotechnology, Santa Cruz, CA
anti-BDNF	An internal region of human BDNF	rabbit	1:500	Santa Cruz Biotechnology
anti-phopsho-GluN2B	Synthetic phosphopeptide around Tyr^{1472} of rat GluN2B	rabbit	1:1000	Millipore, Billerica, MA
anti-GluN2B	C-terminus (aa 1463-1482) of mouse GluN2B	rabbit	1:1000	Millipore
anti-phospho-Pyk2	Synthetic phosphopeptide around Tyr^{402} of human Pyk2	rabbit	1:1000	Cell Signaling Technologies, Danvers, MA
anti-Pyk2	C-terminus of human Pyk2	mouse	1:1000	Cell Signaling Technologies
anti-pERK1/2	Synthetic phosphopeptide around Tyr^{204} of human ERK	mouse	1:1000	Santa Cruz Biotechnology
anti-ERK2	C-terminus of rat ERK2	rabbit	1:5000	Santa Cruz Biotechnology
anti-ubiquitin	Ubiquitin purified from bovine red blood cells	rabbit	1:5000	Thermo Scientific, Fremont, CA
anti-β-actin	Gizzard actin of avian origin	mouse	1:5000	Santa Cruz Biotechnology
anti-rabbit IgG	Rabbit IgG (H+L), Peroxidase Conjugated	goat	1:5000	Thermo Scientific
anti-mouse IgG	Mouse IgG (H+L), Peroxidase Conjugated	goat	1:5000	Thermo Scientific