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

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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, in contrast to BDNF, 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 $STEP_{61}$ levels. Reduced BDNF signaling leads to elevation of $STEP_{61}$ both in $BDNF^{+/-}$ mice and after acute BDNF knockdown in cortical cultures. Moreover, 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 $STEP_{61}$ activity. A better understanding of the opposing interaction between STEP and BDNF in normal cognitive functions and in neuropsychiatric disorders will hopefully lead to better therapeutic strategies.

Introduction

Brain-derived neurotrophic factor (BDNF) is widely expressed in many brain regions and is enriched in neocortex, hippocampus, striatum and amygdala, all 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 primarily restricted to the nervous system and present in several brain regions with the exception of the cerebellum (Lombroso *et al.* 1991), where a highly homologous tyrosine phosphatase (PTP-STEP-like, PTP-SL) is found (Hendriks *et al.* 1995). The single STEP gene is alternatively spliced to produce several STEP proteins that include the cytosolic $STEP_{46}$ and the membrane-associated $STEP_{61}$, the later targeted, in part, to the endoplasmic reticulum and post-synaptic compartments (Bult *et al.* 1996, Lombroso *et al.* 1993, Boulanger *et al.* 1995). STEP proteins are negative regulators of synaptic strengthening and do so through the dephosphorylation of regulatory tyrosine residues on their substrates. As a result, 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 amygdala-dependent memory consolidation (Olausson *et al.* 2012).

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, cognitive deficits are reversed by genetic reduction of STEP expression in SZ and FXS mouse models (Zhang *et al.* 2010, Goebel-Goody *et al.* 2012), as well as genetic or pharmacologic inhibition of STEP in an AD mouse model (Xu *et al.* 2014).

We have recently shown that BDNF induces $STEP_{61}$ degradation through the proteasome in cell cultures (Saavedra *et al.* 2015) and here we extend these findings by showing that reduction of BDNF leads to elevated $STEP_{61}$ expression in mice. Importantly, both a novel STEP inhibitor and a TrkB agonist reverse biochemical and motor alterations in $BDNF^{+/-}$ mice. These findings define a mechanism by which BDNF and $STEP_{61}$ interact in vivo and may contribute to the reciprocal expression patterns of BDNF and $STEP_{61}$ in several neuropsychiatric and neurodegenerative disorders.

Materials and Methods

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 the 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 (n = 6 per group) 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, 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). All procedures met the European Community guidelines for the care and use of laboratory animals (86/609//EEC), and were approved by the animal care committee of the University of Barcelona and by the regional autonomous government (Generalitat de Catalunya). 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; C57BL/6 background) were obtained from the Jackson Laboratory (Stock Number: 002266). 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 ANOVAs with Bonferroni *post hoc* analyses.

Results

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

Low BDNF expression is found in several neurological disorders, including AD (Caccamo *et al.* 2010) and SZ (Chen da *et al.* 2009, Buckley *et al.* 2007b), while elevated $STEP_{61}$ levels are found in these same disorders (Kurup *et al.* 2010, Carty *et al.* 2012). To investigate whether there is a correlation between decreased BDNF signaling and increased $STEP_{61}$ levels, we first examined whether mice with reduced BDNF expression ($BDNF^{+/-}$ mice) had altered $STEP_{61}$ expression in the synaptosomal membrane fraction (P2) where $STEP_{61}$ is enriched. We confirmed the approximately 50% decrease in BDNF expression in the frontal cortex (Fig. 1a) and hippocampus (Fig. 1b) of $BDNF^{+/-}$ mice. $STEP_{61}$ was elevated in synaptosomal membrane fractions (P2) 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 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 the Tyr phosphorylation of $STEP_{61}$ substrates. 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. TC-2153 inhibition of $STEP_{61}$ at baseline (scrambled siRNA transfected cells) resulted in a significant increase in the 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 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). The decreased Tyr phosphorylation of STEP substrates 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). We recently showed that BDNF treatment of neuronal cultures promotes the ubiquitination and degradation of $STEP_{61}$ (Saavedra *et al.* 2015). We wished to extend these studies *in vivo* and, since 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 an increase in the 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). We also performed time-course analyses of 7,8-DHF effects on $STEP_{61}$ levels. We found that 7,8-DHF treatment (500 nM) led to the rapid decrease of $STEP_{61}$ levels at 5 min followed by a delayed increase in the 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. 7,8-DHF treatment increased the ubiquitination of STEP (1.52 ± 0.19, $p < 0.05$), which was blocked by K252a (Fig. 4a, b). In addition, another TrkB agonist LM 22A4 also led to $STEP_{61}$ degradation (Fig. S1a) via the ubiquitin proteasome pathway (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.). Biochemical analyses of cortical 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 the Tyr phosphorylation of 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. 5). 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). These data indicate that activation of TrkB signaling leads to the ubiquitination and degradation of $STEP_{61}$ in mouse cortical synaptosomal fractions.

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, Zhu *et al.* 2009). We therefore tested whether inhibition of $STEP_{61}$ by TC-2153, which decreases $STEP_{61}$ activity, or 7,8-DHF, which decreases $STEP_{61}$ 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 (Fig. 6a). 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 *post hoc* test showed that $BDNF^{+/-}$ mice were hyperactive when compared with WT mice ($p < 0.05$) (Fig. 6a), consistent with previous findings (Kernie *et al.* 2000, Chan *et al.* 2006, Zhu *et al.* 2009). TC-2153 significantly attenuated the increased locomotor behavior in these mice ($BDNF^{+/-}$ TC vs $BDNF^{+/-}$ Veh, $p < 0.01$, two-way ANOVA with Bonferroni *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 hyperlocomotor activity present in $BDNF^{+/-}$ mice.

Activation of the TrkB signaling by 7,8-DHF is 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 shown 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 reduce hyperlocomotion in these mice (Fig. 6b). A second cohort of male WT and $BDNF^{+/-}$ mice was administered vehicle or 7,8-DHF (5 mg/kg, i.p.) prior to behavioral assessment in an open-field chamber. 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 locomotor activity. $BDNF^{+/-}$ mice displayed hyperactivity at baseline compared with WT mice ($p < 0.05$, Bonferroni *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 *post hoc* test), without altering locomotion in WT mice (Fig. 6b).

Next we investigated whether changes in $STEP_{61}$ and the phosphorylation of its substrates correlated with the reversal of locomotor activity in $BDNF^{+/-}$ mice by TC-2153 or 7,8-DHF. We first treated $BDNF^{+/-}$ mice with vehicle (Veh) or TC-2153 (TC, 10 mg/kg, i.p.) for 1 h and analyzed synaptosomal membrane fractions (P2) from frontal cortices. We found increased Tyr phosphorylation of STEP substrates (TC vs Veh, pGluN2B: 1.67 ± 0.17, $p < 0.05$; pPyk2: 1.80 ± 0.14; pERK1/2: 1.69 ± 0.14, *p values* < 0.01) upon TC-2153 administration without changes in $STEP_{61}$ levels (Fig. 6c). Moreover, $STEP_{61}$ inhibition by TC-2153 also increased total GluN2B level (1.53 ± 0.17, $p < 0.05$) in synaptosomal membranes (Fig. 6c).

We also examined whether degradation of $STEP_{61}$ by 7,8-DHF had similar effects in $BDNF^{+/-}$ mice. As expected, 7,8-DHF resulted in loss of $STEP_{61}$ (0.60 ± 0.04, $p < 0.01$) and increased the Tyr phosphorylation of STEP substrates (DHF vs Veh, pGluN2B: 1.81 ± 0.14, $p < 0.01$; pPyk2: 1.61 ± 0.16; pERK1/2: 1.59 ± 0.15, *p values* < 0.05) and increased total GluN2B (1.54 ± 0.18, $p < 0.05$) in synaptosomal membrane fractions (P2) from $BDNF^{+/-}$ mice (Fig. 6d), presumably through the degradation of $STEP_{61}$. Together, these findings suggest that inhibition of $STEP_{61}$ activity by TC-2153 or degradation of $STEP_{61}$ induced by 7,8-DHF is sufficient to increase Tyr phosphorylation of STEP substrates and reverse hyperactivity in $BDNF^{+/-}$ mice.

Discussion

BDNF is essential for neurodevelopment and normal brain function (Poo 2001). A decrease in BDNF/TrkB signaling is implicated in a number of disorders with prominent cognitive deficits, including AD, PD, Huntington's disease (HD), 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). 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 and STEP levels increased in these two models. Administration of TC-2153 was also sufficient to reverse the hyperlocomotion in $BDNF^{+/-}$ mice.

BDNF treatment reverses cognitive deficits in rodent models of disorders including AD, PD, SZ and HD (Canals *et al.* 2004, Levivier *et al.* 1995, Nagahara *et al.* 2009, Nagahara & Tuszynski 2011). 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 compared to recombinant BDNF (Ochs *et al.* 2000, Beck *et al.* 2005).

Indeed, 7,8-DHF and its analogs confer neuroprotection and improve cognitive functions in a 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 7,8-DHF activation of the TrkB receptor 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 of the current study and our previous results suggest TrkB agonists 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). BDNF/TrkB-induced degradation of $STEP_{61}$ resulted in increased Tyr phosphorylation of STEP substrates, including GluN2B, Pyk2 and ERK1/2. Blockade of $STEP_{61}$ degradation by lactacystin reversed the effects of 7,8-DHF on phosphorylation of GluN2B and Pyk2, whereas phosphorylation of ERK1/2 was not abolished. The differential regulation of these substrates may be due to the distinct localization STEP substrates, i.e. both $STEP_{61}$ and GluN2B enriched in postsynaptic densities, while ERK1/2 is not. The close proximity of $STEP_{61}$ and GluN2B may facilitate their interactions in a more rapid manner. In addition, ERK1/2 activation by BDNF is

expected to be a $STEP_{61}$-independent process, as $STEP_{61}$ normally determines the duration of ERK1/2 activation by its dephosphorylation. Consistently, it has been shown that immediate BDNF-induced phosphorylation of ERK1/2 was not blocked by a structurally different proteasome inhibitor, MG-132 (Jia *et al.* 2008).

In summary, our data support a mechanism by which 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.

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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 (DIV7) 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 (DIV7) 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 *post hoc* test (*$p < 0.05$, n = 4 per group).

Figure 3. Concentration-response and time-course analyses of 7,8-DHF effects 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 *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. Quantification of phospho-protein levels was normalized to total protein levels and then to β-actin as a loading control. (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 Ub-STEP species was performed between 75 and 250 kDa. All data were expressed as mean ± SEM (*$p < 0.05$, one-way ANOVA with Bonferroni *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. 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 were expressed as mean ± SEM (*$p < 0.05$, one-way ANOVA with Bonferroni *post hoc* test, n = 4 per group).

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 *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 *post hoc* test (*$p < 0.05$, n = 9-10 per group). (c) TC-2153 and (d) 7,8-DHF administration in $BDNF^{+/-}$ mice results in $STEP_{61}$ inhibition and increased Tyr phosphorylation of STEP substrates. Fifteen-week old male $BDNF^{+/-}$ mice were administrated vehicle (Veh), TC-2153 (TC, 10 mg/kg, i.p.) or 7,8-DHF (5 mg/kg, i.p.). Frontal cortices were collected 1 h later for biochemical analysis. Tissues from $BDNF^{+/-}$ mice were processed to obtain synaptic membrane fractions (P2). Samples were subjected to western blotting and blots were probed with phospho-specific and pan-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 and statistical significance determined using Student's *t*-test (*$p < 0.05$, **$p < 0.01$, n = 6 per group).