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DOWNSTREAM EFFECTS OF STRIATAL-ENRICHED PROTEIN TYROSINE PHOSPHATASE REDUCTION ON RNA EXPRESSION *IN VIVO* AND *IN VITRO*

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Abstract—Striatal-enriched protein tyrosine phosphatase (STEP) is a brain-specific tyrosine phosphatase that has been shown to de-phosphorylate several key neuronal signaling proteins, including kinases (extracellular signal-regulated kinase (ERK1/2), FYN, PYK2) and glutamate receptor subunits (N-methyl-D-aspartate receptor subtype 2B (NR2B), glutamate receptor 2 (GLUR2)). Step knock-out mice have increased phosphorylation of these substrates in the brain, with potential functional consequences in synaptic plasticity and cognitive tasks. It is therefore of interest to identify the molecular pathways and downstream transcriptional targets that are impacted by Step knockdown. In the present study, striatal RNA samples from Step wild-type, knock-out and heterozygous mice were hybridized to Affymetrix microarray chips and evaluated for transcriptional changes between genotypes. Pathway analysis highlighted Erk signaling and multiple pathways related to neurotrophin signaling, neuronal development and synaptic transmission. Potential genes of interest identified by microarray were confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) in the cortex and hippocampus, which shared several transcriptional alterations with the striatum. In order to evaluate Step knockdown in an *in vitro* system, a panel of genes were evaluated using qRT-PCR in rat cortical neurons that were transduced with lentivirus expressing short hairpin RNA against Step or a non-targeting control. Our data suggest that Step has a role in the expression of immediate early genes relevant to synaptic plasticity, in both *in vitro* and *in vivo* systems.

Key words: PTPN5, STEP, qRT-PCR, microarray, NMDA, schizophrenia.

INTRODUCTION

Over the past decade, striatal-enriched protein tyrosine phosphatase (STEP) has been proposed as a gene of interest to multiple neurologic and psychiatric disorders, including schizophrenia, Alzheimer's disease, Huntington's disease, and Fragile X (Goebel-Goody et al., 2012). STEP is encoded by the protein tyrosine phosphatase non-receptor type 5 gene (PTPN5), and is expressed exclusively in the cerebrum (Lombroso et al., 1991). STEP has two major splice variants: STEP61, which has a 172-amino acid sequence containing the transmembrane regions at the amino terminus and is localized to the post-synaptic density and endoplasmic reticulum throughout the forebrain, and STEP46, which lacks the transmembrane sequence and is localized in the cytosol primarily in the striatum (Boulanger et al., 1995; Oyama et al., 1995; Bult et al., 1997; Venkitaramani et al., 2009).

The substrates of STEP include the ionotropic glutamate receptor 2 (GLUR2) subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionicacid receptor (AMPA) (Zhang et al., 2008), the N-methyl-D-aspartate (NMDA) receptor subtype 2B (NR2B) subunit of the NMDA receptor (Braithwaite et al., 2006), as well as several kinases: FYN (Nguyen et al., 2002), PYK2 (Xu et al., 2012), and mitogen-activated protein kinases extracellular signal-regulated kinase (ERK1/2) and p38 (Munoz et al., 2003; Paul et al., 2003). Tyrosine de-phosphorylation by STEP inactivates kinase substrates, and leads to receptor internalization in the case of GLUR2 and NR2B (Braithwaite et al., 2006; Zhang et al., 2008). In this manner, STEP acts as a negative regulator of glutamatergic transmission and multiple signal transduction pathways. Step knockout (KO) mice have elevated phosphorylation levels of multiple substrates, including Erk and Nr2b (Zhang et al., 2010; Venkitaramani et al., 2011), which play key roles in synaptic plasticity, learning and memory. Consequently, Step KO mice have been shown to outperform wild-type littermates in hippocampal learning

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Abbreviations: ANOVA, analysis of variance; Arc, associated protein gene; Camk2a, calcium/calmodulin-dependent protein kinase II alpha gene; DMEM, Dulbecco's modified Eagle's medium; Dusp4, dual specificity phosphatase 4 gene; Egr2, Early growth response 2; ERK, extracellular signal-regulated kinase; Fos, FBJ murine osteosarcoma viral oncology homolog gene; GluR2, glutamate receptor, ionotropic, AMPA 2; GO, gene ontology; GOI, gene of interest; HET, heterozygous; HOM, Homozygous; Igf1, insulin-like growth factor 1 gene; Ipw, imprinted in Prader–Willi syndrome gene; KO, knock-out; LTP, long-term potentiation; NMDA, N-methyl-D-aspartic acid; NR2B, N-methyl-D-aspartate receptor subtype 2B; Pitpnm3, Pitpnm family member 3; PSD95, postsynaptic density 95; qRT-PCR, quantitative real-time polymerase chain reaction; RT, room temperature; Sept11, septin 11 gene; shRNA, short hairpin ribonucleic acid; Sik1, salt-inducible kinase 1 gene; STEP, striatal-enriched protein tyrosine phosphatase; Stxbp1, syntaxin-binding protein gene; Tmem114, transmembrane protein 114; WT, wild-type.

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tasks (Venkitaramani et al., 2011), and crossing Step KO mice with Alzheimer's disease mouse models has been reported to mitigate cognitive deficits intrinsic to those models (Kurup et al., 2010; Zhang et al., 2010).

Although first-order signaling alterations and behavioral outcomes in Step KO mice have been described, the downstream implications of Step reduction in *in vivo* and *in vitro* models are not well understood. Identifying transcriptional changes that result from reduced Step activity may both elucidate mechanisms underlying higher order phenotypes and identify novel pathways and interactors of this phosphatase. In the present study, we evaluated transcriptional changes in samples with reduced or eliminated Step expression using two methods: first, using Affymetrix microarray analysis and follow-up quantitative real-time PCR (qRT-PCR) in homozygous (HOM) Step KO, heterozygous (HET) and wild-type (WT) mouse striatum, hippocampus and cortex; second, using qRT-PCR in rat cortical neurons treated with a lentivirus expressing Step short hairpin RNA (shRNA). Through these methods, we were able to identify pathways which are impacted by reductions in Step expression and provide additional support for Step's role in regulating glutamatergic activity.

EXPERIMENTAL PROCEDURES

Animals

Five-month-old male HET ($n = 5$) and HOM ($n = 4$) Step KO mice and WT littermates ($n = 4$) were provided by Dr. Paul Lombroso (Yale University). Procedures were performed in compliance with the National Institutes of Health guide for the Care and Use of Laboratory animals (Institute of Laboratory Animal Resources, 1996) under the approval of the Pfizer site Institutional Animal Care and Use Committee. Mice were euthanized by cervical dislocation followed by decapitation. The frontal cortex, striatum and hippocampus were dissected and snap-frozen on dry ice.

Cell culture

Rat primary hippocampal neurons were isolated at embryonic day 18 and cultured at a density of 250,000 cells/well in 12-well plates coated with poly-d-lysine and laminin in serum-free neurobasal media supplemented with B27 (Invitrogen). Neurons were transduced with virus after 7 days *in vitro*. After 3 h, virus was removed and media were replaced. Neurons were harvested for RNA or protein at 14–21 days *in vitro*.

HEK293T cells were plated in poly-D-lysine coated 12-well plates at 10% confluence on Day 0. On Day 1, cells were transfected using 100 μL serum-free Dulbecco's modified Eagle's medium (DMEM) dilution media, 1.6-μg total DNA, and 4 μL Lipofectamine 2000 (Life Technologies) per well. DNA and Lipofectamine were incubated separately in DMEM dilution media for 5 min at room temperature (RT) before being combined, after which the DNA and Lipofectamine together were incubated for 20 min at RT. The mixture was then added to the cells for 36 h and harvested on day 3.

Gene expression profiling

Total RNA was isolated using Trizol (Life Technologies, Carlsbad, CA, USA) and hybridized to Affymetrix Mouse 430_2 microarrays at Expression Analysis (Durham, NC, USA). CEL file data were normalized using Probe Logarithmic Intensity Error (PLIER), and linear models were used to calculate p-values between all groups. Benjamini and Hochberg false discovery rate correction was performed to control for multiplicity of testing (Benjamini and Hochberg, 1995). Because few changes remained following FDR correction, unadjusted *p*-values were used for subsequent pathway analysis. Only probe-sets with mean absolute normalized intensities > 60, unadjusted *p*-value < 0.05 and fold change > 1.2 were considered for pathway analysis. Pathway analysis was performed using Nextbio (Kupershmidt et al., 2010). The top 10 MSigDB pathways and gene ontology (GO) terms were identified based on significant enrichment in both HET and HOM Step KO comparisons, ranked by the highest net enrichment score across both conditions. Raw data and a supplementary spreadsheet of differentially expressed genes have been uploaded to GEO (Accession number GSE51678; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51678).

For qRT-PCR analysis, RNA samples were converted to cDNA using Vilo Master Mix (Life Technologies). qRT-PCR was run using Life Technologies TaqMan assays and Gene Expression Master Mix (Life Technologies) using the Viia7 Real-Time PCR System. Each sample was run in quadruplicate. In addition to the genes of interest (GOI), six control genes were run: hydroxymethylbilane synthase (Hmbs), hypoxanthine phosphoribosyltransferase 1 (Hprt1), peptidylprolyl isomerase A (Ppia), proteasome (prosome, macropain) subunit beta type 2 (Psmb2), ribophorin 1 (Rpn1) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (Ywhaz). Amplification curve replicates were evaluated using Viia7 1.2 software and analyzed using the ddCT method normalized to endogenous controls, which were picked using Normfinder software. KO mouse data were analyzed by a single factor analysis of variance (ANOVA) with Bonferroni post hoc analyses using GraphPad Prism v5.0. For *in vitro* experiments, which were performed on rat cortical neurons in 12-well dishes, $n = 6$ well replicates were run for each experiment, and each experiment was repeated three times. A multivariate ANOVA with 2-way factorial design was used to analyze these data. A linear mixed effect model was used with experiment # (1–3) as a random factor to eliminate variation due to individual experiments.

STEP shRNA production

Step shRNA constructs were generated in the lentiviral vector pLL3.7H1-GFP. The target sequence for Step shRNA was: 5′-GCATGACTCTTTGGCAACATG-3′ using a loop sequence of 5′-TTCAAGAGA-3′. A BamHI restriction site was added at the 5′ end of the sense