Zn
sup2+ -dependent Activation of the Trk Signaling Pathway Induces Phosphorylation of the Brain-enriched Tyrosine Phosphatase STEP

MOLECULAR BASIS FOR ZNsup2+ -INDUCED ERK MAPK ACTIVATION*

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Excessive release of Zn
sup2+ in the brain is implicated in the progression of acute brain injuries. Although several signaling cascades have been reported to be involved in Zn
sup2+ -induced neurotoxicity, a potential contribution of tyrosine phosphatases in this process has not been well explored. Here we show that exposure to high concentrations of Zn
sup2+ led to a progressive increase in phosphorylation of the striatal-enriched phosphatase (STEP), a component of the excitotoxic-signaling pathway that plays a role in neuroprotection. Zn
sup2+ -mediated phosphorylation of STEP at multiple sites (hyperphosphorylation) was induced by the up-regulation of brain-derived neurotrophic factor (BDNF), tropomyosin receptor kinase (Trk) signaling, and activation of cAMP-dependent PKA (protein kinase A). Mutational studies further show that differential phosphorylation of STEP at the PKA sites, Ser-160 and Ser-221 regulates the affinity of STEP toward its substrates. Consistent with these findings we also show that BDNF/Trk/PKA mediated signaling is required for Zn
sup2+ -induced phosphorylation of extracellular regulated kinase 2 (ERK2), a substrate of STEP that is involved in Zn
sup2+ -dependent neurotoxicity. The strong correlation between the temporal profile of STEP hyperphosphorylation and ERK2 phosphorylation indicates that loss of function of STEP through phosphorylation is necessary for maintaining sustained ERK2 phosphorylation. This interpretation is further supported by the findings that deletion of the STEP gene led to a rapid and sustained increase in ERK2 phosphorylation within minutes of exposure to Zn
sup2+. The study provides further insight into the mechanisms of regulation of STEP and also offers a molecular basis for the Zn
sup2+ -induced sustained activation of ERK2.

A growing body of evidence indicates that Zn
sup2+, the second most abundant transition metal in the brain, is released at high concentrations during excitotoxic neurological conditions and can contribute to neuronal injury (1–7). The proposed mechanisms associated with Zn
sup2+ -dependent neurotoxicity include alteration of activity of a diverse group of post-synaptic receptors like α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA), N-methyl-L-aspartic acid (NMDA), voltage-gated calcium channels and neurotrophic receptors (7–12). Receptor activation in turn modulates the function of several intracellular kinases including protein kinase C, mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 (ERK MAPK), and the Src family of tyrosine kinases resulting in impaired energy production, excitability and oxidative stress (8,9,13–15). However, the role of phosphatases in Zn
sup2+ -induced neurotoxicity is currently not well understood.

The intracellular tyrosine phosphatase, STEP (striatal-enriched tyrosine phosphatase) also known as PTPN5) is expressed exclusively in the central nervous system (16,17) and is emerging as a key regulator of neuronal survival and death. The STEP-family of PTPs includes both membrane-associated (STEP) and cytosolic (STEP) variants that are formed by alternative splicing of a single gene (18). STEP is expressed in neurons of the cortex, hippocampus, and striatum and its substrate affinity is regulated through phosphorylation/dephosphorylation of a serine residue in a conserved regulatory domain termed the KIM (kinase interacting motif) domain (18–22). Dopamine/D1 receptor/cAMP/PKA pathway-mediated phosphorylation of this residue hinders the ability of STEP to bind to its substrates (22). In contrast, dephosphorylation of this residue following glutamate-NMDA receptor mediated activation of calcineurin allows STEP to bind to its substrates and inhibit their activities (21). This dephosphorylated form of STEP also referred to as the active form has been shown to contribute to neuroprotection in cell culture models of excitotoxicity and oxygen glucose deprivation (23,24). Evidence for a neuroprotective role of STEP also comes from in vivo studies demonstrating that degradation of active STEP following an ischemic insult allows activation of detrimental cascades involved in neuronal injury and brain damage. In contrast, restoration of STEP function, using a brain-permeable STEP-derived peptide, is effective in limiting ischemic brain injury (24).

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3 The abbreviations used are: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid; STEP, striatal-enriched phosphatase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; NMDA, N-methyl-D-aspartic acid; MMP, matrix metalloproteinases; BDNF, brain-derived neurotrophic factor.
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These findings indicate that loss of function of endogenous STEP increases the vulnerability of neurons to excitotoxic insult. Since Zn\(^{2+}\) has been associated with excitotoxic brain injury, the present study sought to examine the role of excessive Zn\(^{2+}\) exposure in regulating the function of STEP\(_{61}\), the predominant isoform expressed in cultured neurons, cortex, and hippocampus. The results show that Zn\(^{2+}\)–mediated Trk receptor activation leads to phosphorylation of STEP\(_{61}\) at multiple PKA sites with a concomitant increase in the phosphorylation of ERK MAPK. The findings suggest that loss of affinity of phosphorylated STEP\(_{61}\) toward its substrates facilitates the sustained phosphorylation of ERK MAPK that is known to be involved in Zn\(^{2+}\)-induced neurotoxicity (13).

**Experimental Procedures**

**Materials**—Pregnant female Sprague-Dawley rats (16-day gestation) were obtained from Harlan Laboratories. STEP knock-out mice (STEP KO or STEP\(^{-/-}\)) were developed on a C57BL6 background (25) and were bred at the University of New Mexico Animal Care Facility. ZnCl\(_2\), glutamate, kainic acid, APV, CNQX, pyrithione sodium salt, Ca-EDTA, and BDNF were from Sigma-Aldrich; BDNF were from R&D Systems. GM6001 was bisindolylmaleimide I (Bis), thapsigargin, and H89 were from Millipore; polyclonal anti-

**DNA Constructs**—Full-length STEP\(_{61}\) cDNA was constructed in mammalian expression vector pcDNA3.1 encoding C-terminal V5 and His tags. Mutations of serine residues in STEP\(_{61}\) were obtained by polymerase chain reaction (PCR)-based site-directed mutagenesis using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. All mutations were verified by nucleotide sequencing.

**Cell Culture and Stimulation**—Primary neuronal cultures were obtained from 16–17-day-old rat or wild type (WT) and STEP knock-out (KO) mice embryos as described previously (21). Briefly, the cortex was dissected under a microscope, the tissue dissociated mechanically and resuspended in DMEM/F-12 (1:1) containing 5% fetal calf serum. Cells were plated on 60 mm poly-d-lysine-coated tissue culture dishes and grown for 12–14 days at 37 °C in a humidified atmosphere (95:5% air:CO\(_2\) mixture). To inhibit proliferation of non-neuronal cells, 10 \(\mu\)M of cytosine d-arabinofuranoside was added to the cultures 72 h after plating. For neuronal stimulation, cells were washed twice with minimum essential medium (MEM) followed by treatment with ZnCl\(_2\), BDNF, or NGF for the indicated times at 37 °C. For some experiments, cells were returned back to its original medium following treatment with Zn\(^{2+}\). APV, CNQX, MK801, nifedipine, K252a, thapsigargin, Ca-EDTA, PP2, bisindolylmaleimide, H89, or GM6001 were added 15 min before stimulation with Zn\(^{2+}\) or BDNF. The selective Zn\(^{2+}\) chelator, Ca-EDTA or the Zn\(^{2+}\) ionophore, pyrithione were added along with Zn\(^{2+}\) without any pre-incubation. For some experiments, function blocking anti-BDNF antibody was added 15 min prior to treatment with BDNF. Some cultures were treated with thapsigargin for the specified times without stimulation with Zn\(^{2+}\), BDNF, or NGF. Cells were harvested at the specified times after stimulation and processed for immunoblot analysis.

HeLa cell lines were routinely maintained in DMEM supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere (95:5% air:CO\(_2\) mixture). Cells were transiently transfected with 1 \(\mu\)g of each DNA using Lipofectamine 2000 (Invitrogen). After 24–48 h, the cells were treated with forskolin (40 \(\mu\)M) or phorbol ester (10 ng/ml) at 37 °C. Both treated and untreated cells were harvested at the specified times after stimulation and either processed for immunoprecipitation with anti-ERK2 antibody or immunoblot analysis.

**Immunoprecipitation**—Cells were lysed in a buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 10 mM MgCl\(_2\), 0.1 mM DTT, 0.5% Nonidet P40, and a mixture of protease inhibitors for 30 min on ice. Lysates were centrifuged for 10 min at 14,000 rpm and the supernatant was used for the immunoprecipitation assay. Equal amount of protein from each sample was incubated with 0.5 \(\mu\)l of alkaline phosphatase from calf intestine (257 units/\(\mu\)l) at 37 °C for 30 min. The reactions were terminated by the addition of Laemmli sample buffer (1X, final concentration) and the samples were then boiled at 100 °C for 10 min followed by immunoblot analysis.

**Immunoblotting**—Equal amount of protein lysates, as estimated using bicinchoninic acid protein assay kit (Pierce Biotechnology), were resolved by 8% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membranes were blocked for 1 h at room temperature with 5% nonfat dry milk or 5% bovine serum albumin and then incubated for 1 h at room temperature or at 4 °C overnight with the appropriate primary antibody. Horseradish peroxidase coupled to anti-rabbit or anti-mouse IgG that were raised in goat were used as secondary antibodies. Immune complexes were detected on x-ray film...
Zn²⁺-mediated Phosphorylation of STEP₆₁—In initial studies, neuronal cultures (12–14 days in vitro) were treated with increasing concentrations of Zn²⁺ (0, 25, 50, 100, 200, and 300 µM) for 30 min and analyzed by immunoblotting with an anti-STEP antibody (Fig. 1A). As previously reported STEP₆₁, the predominant isofrom expressed in cultured neurons was present as a doublet (21), where the lower band (band 1) represents the non-phosphorylated form and the upper band (band 2) represents the phosphorylated form of STEP₆₁ (Fig. 1A, upper row, lane 1). Treatment with Zn²⁺ progressively decreased the intensity of band 1 with a concomitant increase in the intensity of band 2 and the appearance of a third band at an even higher molecular weight (band 3) suggesting concentration-dependent hyperphosphorylation of STEP₆₁. To examine the temporal profile of STEP₆₁ phosphorylation, we next treated neuronal cultures with Zn²⁺ (300 µM) for various time periods (0, 5, 15, 30, 45, and 60 min). A rapid increase in the phosphorylation of band 2 of STEP₆₁ was observed within 5 min of stimulation with Zn²⁺ that remained unchanged over time (Fig. 1B). The level of the hyperphosphorylated band 3 of STEP₆₁ increased progressively, peaked at 30 min and then remained elevated throughout the rest of the time studied (Fig. 1B, upper row). This increase in phosphorylation and hyperphosphorylation of STEP₆₁ (bands 2 and 3) was completely blocked following addition of a cell-impermeable selective Zn²⁺ chelator (CaEDTA, 1 mM) either at the onset of Zn²⁺ exposure (Fig. 2A) or 15 min after the onset of Zn²⁺ exposure (Fig. 2B).

The increase in phosphorylation and hyperphosphorylation of STEP₆₁ returned to basal levels within 30 min of Zn²⁺ removal from the medium, implying that extracellular Zn²⁺-mediated phosphorylation of STEP₆₁ is a reversible phenomenon (Fig. 2C). To confirm that the upward shift in the mobility of the STEP₆₁ band was due to phosphorylation, lysates obtained from neuronal cultures treated with Zn²⁺ (300 µM, 30 min) were incubated with alkaline phosphatase, an enzyme that catalyzes the hydrolysis of phosphates from phosphorylated substrates (26, 27). The complete loss of band 2 and 3 under these conditions (Fig. 2D, lane 3) confirms that Zn²⁺ exposure led to increase in phosphorylation of STEP₆₁ at multiple sites resulting in a partial hypershift in the mobility of the STEP₆₁ band. Our result also reveal an additional STEP₆₁ band below the non-phosphorylated band 1 that did not change with alkaline phosphatase treatment (Fig. 2D, lane 1 versus lane 3), indicating that this band also represents a non-phosphorylated form of STEP₆₁. The difference in mobility between this band and band 1 could be attributed to other post-translational modifications and is an important topic for future study. To our knowledge this is the first study that demonstrates such a hyperphosphorylation of STEP₆₁ in response to any stimuli.

Quantification of phosphorylated STEP₆₁ and ERK MAPK levels was done by computer-assisted densitometry and Image J analysis. For quantification of the increased basal phosphorylation of STEP₆₁ (band 2) and the hyper-phosphorylation of STEP₆₁ (band 3) the intensities of all the three STEP bands (band 1, band 2, and band 3) were measured, and the intensity of band 2 or band 3 is presented as a percentage of the total intensity of band 1, band 2, and band 3. Band intensities of phosphorylated ERK were normalized to total ERK levels from the same blot and presented as the number of fold increase as compared with the untreated control.

Statistical Analysis—Data in the text and figures are expressed as means ± S.E. Statistical differences between multiple groups were assessed using one-way ANOVA followed by Bonferroni’s post-hoc comparisons test. Differences were considered statistically significant when p < 0.05.

Results

Zn²⁺ regulates phosphorylation of STEP₆₁ in neurons. Neuronal cultures were treated with (A) different concentrations of Zn²⁺ for 30 min or (B) 300 µM Zn²⁺ for the specified times. Equal amounts of protein from each sample were separated by SDS-PAGE and immunoblotted with a monoclonal antibody against STEP. β-Tubulin levels were also analyzed to indicate equal protein loading. Percentage phosphorylation of STEP₆₁, band 2 and band 3 is represented as mean ± S.E. (n = 4). A, * p < 0.01 for phosphorylated STEP₆₁ band 2 from untreated control; **, p < 0.001 for phosphorylated STEP₆₁ band 2 from 25 µM Zn²⁺ treatment; Δ, p < 0.0001 for phosphorylated STEP₆₁ band 3 from untreated control; †, p < 0.001 for phosphorylated STEP₆₁ band 3 from 25 µM Zn²⁺ treatment; ‡, p < 0.01 for phosphorylated STEP₆₁ band 3 from 50 µM Zn²⁺ treatment; ††, p < 0.001 for phosphorylated STEP₆₁ band 2 from untreated control; Δ, p < 0.001 for phosphorylated STEP₆₁ band 3 from 5 min Zn²⁺ treatment.

FIGURE 1. Zn²⁺ regulates phosphorylation of STEP in neurons. Neuronal cultures were treated with (A) different concentrations of Zn²⁺ for 30 min or (B) 300 µM Zn²⁺ either at the onset of Zn²⁺ exposure (Fig. 2A) or 15 min after the onset of Zn²⁺ exposure (Fig. 2B).
**STEP Phosphorylation by Zn²⁺ and TrkB Signaling**

![Graphs showing relative density of phosphorylated STEP61](image)

**FIGURE 2. Role of extracellular Zn²⁺ in the phosphorylation of STEP.** A–D, neuronal cultures were treated with 100 μM or 300 μM Zn²⁺ for 30 min. A, CaEDTA (1 mM) was added along with Zn²⁺; B, CaEDTA (1 mM) was added following Zn²⁺ treatment for 15 min and the incubation was continued for another 15 min in the presence of both Zn²⁺ and CaEDTA; C, following treatment with Zn²⁺ for 30 min cells were maintained in its original medium for another 30 min; and D, following treatment with Zn²⁺ for 30 min cell lysates were incubated with alkaline phosphatase for another 30 min at 37 °C. E, neuronal cultures were treated with 1 μM, 10 μM or 100 μM Zn²⁺ in the presence or absence of pyrithione (20 μM) for 30 min. A–E, extracts with equal amounts of protein from each sample were analyzed with anti-STEP antibody (upper panel), and blots were re-probed with anti-β-tubulin antibody (lower panel). A—C, percentage phosphorylation of STEP61 band 2 and band 3 is represented as mean ± S.E. (n = 4), #, *p < 0.001 for phosphorylated STEP61 band 2 from untreated control; ¶, p < 0.0001 for phosphorylated STEP61 band 2 from untreated control; $, p < 0.001 for phosphorylated STEP61 band 2 from 100 μM or 300 μM Zn²⁺ treatment; %, p < 0.001 for phosphorylated STEP61 band 3 from 100 μM or 300 μM Zn²⁺ treatment.

Fig. 2E shows that, in contrast to extracellular Zn²⁺ application, approaches to preferentially increase intracellular Zn²⁺ concentrations did not increase STEP61 phosphorylation. Neurons were exposed to varying concentrations of Zn²⁺ (1, 10, and 100 μM) for 30 min in the presence of pyrithione (20 μM), an ionophore that facilitates Zn²⁺ entry into the cytosol (28). 1 or 10 μM of Zn²⁺ had no effect on the basal phosphorylation of STEP61, in the presence or absence of pyrithione. Treatment with Zn²⁺ (100 μM) in the absence of pyrithione led to increase in basal phosphorylation as well as hyperphosphorylation of STEP61 (Fig. 2E, lane 6), as shown earlier (Fig. 1). In contrast, exposure to Zn²⁺ (100 μM) in the presence of pyrithione led to a marked decrease in the basal phosphorylation of STEP61, as evident from the decrease in intensity of band 2 (phosphorylated form of STEP61) and concomitant increase in that of band 1 (dephosphorylated form of STEP61). Taken together these findings raise the possibility that hyperphosphorylation of STEP61 by exogenous Zn²⁺ is mediated through stimulation of cell surface receptors.

**Role of TrkB Receptor in the Phosphorylation of STEP61—**

Earlier studies have reported that exogenous Zn²⁺ can modulate the activation of several receptors, including tropomyosin-related kinase (Trk), NMDA, AMPA, and L-type voltage gated channels (8, 9, 29–33). To clarify the mechanism by which Zn²⁺ mediates STEP61 hyperphosphorylation we evaluated the effect of pharmacological inhibition of these receptors on STEP61. To determine the involvement of Trk receptors, neurons were incubated with Zn²⁺ (300 μM, 30 min) in the presence of K252a (100 nM), an inhibitor of Trk tyrosine kinases (34). Fig. 3A shows that K252a can effectively block the Zn²⁺-induced hyperphosphorylation of STEP61 (band 3) and the increase in basal phosphorylation of STEP61 (band 2). Since cultured neurons predominantly express TrkB receptors (12) we next wanted to confirm whether Zn²⁺ enhances tyrosine phosphorylation of TrkB, a surrogate marker of its activation (35, 36). Lysates from neurons treated with Zn²⁺ (300 μM, 30 min) in the absence or presence of K252a (100 nM) were immunoprecipitated with anti-phosphotyrosine (p-Tyr) antibody followed by immunoblot analysis with an anti-TrkB antibody. The results show that Zn²⁺ exposure markedly increased the phosphorylation of TrkB without altering its expression, while pre-incubation with K252a completely blocked Zn²⁺-induced TrkB phosphorylation (Fig. 3B, panels 1 and 2). To confirm that phosphorylation of TrkB led to its activation we further inves-
FIGURE 3. Role of Trk receptors in Zn\textsuperscript{2+}-induced phosphorylation of STEP. Neuronal cultures were pre-incubated with (A, B) K252a (100 nM) for 15 min before treatment with 300 \& Microsymbol M Zn\textsuperscript{2+} (C) MK801 (15 \& Microsymbol M), APV (200 \& Microsymbol M), or CNQX (20 \& Microsymbol M) for 15 min before treatment with 300 \& Microsymbol M Zn\textsuperscript{2+} for 30 min, or (D) nifedipine (15 \& Microsymbol M) for 15 min before treatment with 300 \& Microsymbol M Zn\textsuperscript{2+} for 30 min. A, C, D, samples were analyzed using anti-STEP antibody (upper panel), and the blots were re-probed with anti-\(\beta\)-tubulin antibody (lower panels). B, tyrosine-phosphorylated proteins were immunoprecipitated with anti-pTyr antibody. Immunoprecipitates were analyzed by SDS-PAGE and probed with anti-TRkB antibody (upper panel). In addition, total cell lysates from the same samples were analyzed with anti-TRkB to confirm equal TRkB level in all lysates (upper panel). Total lysates were also immunoblotted with anti-phospho-PLC\(\gamma\) antibody (middle panel), and blots were re-probed with anti-PLC\(\gamma\) antibody (lower panel). A, percentage phosphorylation of STEP\(_{61}\), band 2 and band 3 is represented as mean \(\pm\) S.E. (n = 4). A, * \(p < 0.001\) for phosphorylated STEP\(_{61}\) band 2 from untreated control; \# \(p < 0.0001\) for phosphorylated STEP\(_{61}\) band 3 from untreated control; \#, \(p < 0.001\) for phosphorylated STEP\(_{61}\) band 2 from 300 \& Microsymbol M Zn\textsuperscript{2+} treatment; \(\dagger\), \(p < 0.0001\) for phosphorylated STEP\(_{61}\) band 3 from 300 \& Microsymbol M Zn\textsuperscript{2+} treatment.

STEP Phosphorylation by Zn\textsuperscript{2+} and Trk Signaling
**FIGURE 4.** Zn\(^{2+}\)-mediated phosphorylation of ERK in neurons. Neuronal cultures were treated with (A) different concentrations of \(\text{Zn}^{2+}\) for 30 min or (B) 300 \(\mu\text{M}\) \(\text{Zn}^{2+}\) for the specified times. Phosphorylation of ERK2 was analyzed using the anti-phospho-ERK antibody (upper panel, pERK1/2). Total ERK2 was analyzed by re-probing the blot with anti-ERK2 antibody (lower panel). Quantification of phosphorylated ERK2 (p-ERK2) is represented as mean ± S.E. (\(n = 4\)). A and B, *, \(p < 0.01\) for phosphorylated ERK2 from untreated control. A, Δ, \(p < 0.001\) for phosphorylated ERK2 from 25 \(\mu\text{M}\) \(\text{Zn}^{2+}\) treatment; #, \(p < 0.0001\) for phosphorylated ERK2 from 50 \(\mu\text{M}\) \(\text{Zn}^{2+}\) treatment.

**FIGURE 5.** Zn\(^{2+}\)-mediated sustained phosphorylation of ERK is Trk receptor-dependent. A–C, neuronal cultures were treated with 300 \(\mu\text{M}\) \(\text{Zn}^{2+}\) for 30 min. CaEDTA (1 mM) was added (A) along with \(\text{Zn}^{2+}\) or (B) following \(\text{Zn}^{2+}\) treatment for 15 min and the incubation was continued for another 15 min in the presence of both \(\text{Zn}^{2+}\) and CaEDTA. C, following treatment with \(\text{Zn}^{2+}\) for 30 min cells were maintained in its original medium for another 30 min. D–F, neuronal cultures were pre-incubated with (D) K252a (100 nM) for 15 min before treatment with \(\text{Zn}^{2+}\) (30 min); (E) MK801 (15 \(\mu\text{M}\)), APV (200 \(\mu\text{M}\)) or CNQX (20 \(\mu\text{M}\)) for 15 min before treatment with \(\text{Zn}^{2+}\) (30 min) or KCl (5 min); and (F) with nifedipine (15 \(\mu\text{M}\)) for 15 min before treatment with \(\text{Zn}^{2+}\) (30 min) or KCl (5 min). A–F, phosphorylation of ERK2 was analyzed using the anti-phospho-ERK antibody (upper panel, pERK1/2). Total ERK2 was analyzed by re-probing the blot with anti-ERK2 antibody (lower panel). A–D, quantification of phosphorylated ERK2 is represented as mean ± S.E. (\(n = 4\)). *, \(p < 0.0001\) for phosphorylated ERK2 from untreated control; ¶, \(p < 0.0001\) for phosphorylated ERK2 from 300 \(\mu\text{M}\) \(\text{Zn}^{2+}\) treatment.
intracellular pools could play a role in the hyperphosphorylation of STEP. Neurons were exposed to Zn\(^{2+}\) (300 μM, 30 min) in the absence of Ca\(^{2+}\) in the medium. Fig. 6A shows that lack of extracellular Ca\(^{2+}\) had no effect on the ability of Zn\(^{2+}\) to phosphorylate STEP. To deplete Ca\(^{2+}\) released from intracellular sources, in the next set of experiments neurons were pre-incubated with thapsigargin, prior to treatment with Zn\(^{2+}\). Fig. 6B demonstrates the effect of treatment with thapsigargin alone. Incubation with thapsigargin (1 μM, 15 min) led to a transient increase in ERK2 phosphorylation within 5 min of stimulation, which returned to basal levels by 15 min. Such transient increase in ERK2 phosphorylation following exposure to thapsigargin has been previously attributed to depletion of Ca\(^{2+}\) stores (21). Therefore, in subsequent experiments neurons were treated with thapsigargin (1 μM) for 15 min prior to Zn\(^{2+}\) exposure (300 μM, 30 min). Fig. 6C shows that such pretreatment with thapsigargin failed to alter the effect of Zn\(^{2+}\) on STEP phosphorylation (compare lane 2 with lane 4). Fig. 6D further shows that the Zn\(^{2+}\)-mediated increase in ERK2 phosphorylation correlates with the phosphorylation of STEP both in the absence or presence of thapsigargin (compare lane 2 with lane 4). Thus depletion of intracellular Ca\(^{2+}\) stores also has no effect on the ability of exogenous Zn\(^{2+}\) to increase the phosphorylation of STEP and ERK2.

**Role of PKA in the Phosphorylation of STEP**—The Src family of tyrosine kinases, protein kinase C (PKC) and cAMP-dependent protein kinase A (PKA) are known to play important roles in transducing TrkB receptor mediated intracellular signaling (14). To evaluate the role of these kinases in the phosphorylation of STEP and ERK2, neurons were treated with Zn\(^{2+}\) (300 μM, 30 min) in the presence or absence of inhibitors of Src, PKC and PKA. Fig. 7, A and B show that inhibition of Src tyrosine kinase with PP2 (0.4, 1.0, or 2.5 μM) and PKC with bisindolylmaleimide I (Bis; 1 or 3 μM) failed to inhibit Zn\(^{2+}\)-induced phosphorylation of STEP and ERK2. The efficacy of PP2 and Bis was confirmed in control studies, where neurons were treated with glutamate (100 μM, 5 min) in the presence of PP2; or phorbol ester (PMA; 10 ng/ml, 5 min) in the presence of Bis followed by analysis of ERK2 phosphorylation (Fig. 7, A and B). Fig. 7C shows that inhibition of PKA with H89 (40 μM) attenuated the Zn\(^{2+}\)-induced hyperphosphorylation (band 3) as well as the basal phosphorylation (band 2) of STEP. The PKA inhibitor also attenuated the phosphorylation of ERK2 (Fig. 7C). Using a phospo-specific antibody that recognizes STEP when phosphorylated at the PKA site within the KIM domain (21), we found that STEP was partially phosphorylated under basal condition (Fig. 7D, lane 1). The phosphorylation of this site increased following exposure to Zn\(^{2+}\) (Fig. 7D, lane 2). The recognition of the second STEP band at a higher molecular weight by the phospho-specific antibody (Fig. 7D, lane 2) confirmed the phosphorylation of STEP at additional sites by Zn\(^{2+}\), resulting in the mobility shift. The lack of detection of any STEP band with the phospho-specific STEP antibody in the presence of H89 (Fig. 7D, lane 3) further suggested that Zn\(^{2+}\)-induced phosphorylation of STEP at multiple PKA sites. The strong correlation between the phosphorylation of STEP at the PKA sites (bands 2 and 3) and the concomitant increase in ERK2 phosphorylation (Fig. 7C) suggests that the loss of affinity of phosphorylated STEP toward its substrate leads to sustained phosphorylation of ERK2.

To establish more directly that phosphorylation of STEP at multiple PKA sites regulates ERK2 activation we carried out a number of studies in HeLa cells. Based on an earlier study that identified Ser-160 and Ser-221 as the two putative PKA phosphorylation sites in STEP (22) we generated three mutants of STEP where Ser-160 and Ser-221 were mutated to alanine (V5-tagged STEP S160A/S221A) and in the third mutant both Ser-160 and Ser-221 were converted to alanine (V5-tagged STEP S160A/S221A). HeLa cells transfected with V5-STEP wild type (WT) or one of these mutants were treated with or without the PKA agonist, forskolin (40 μM), followed by immunoblot analysis with anti-V5 antibody. Fig. 8B shows that in the absence of forskolin the mobility of the protein bands of STEP mutants remained unchanged. Fig. 8C shows that treatment with forskolin resulted in an upward shift in mobility of V5-STEP WT,
V5-STEP61 S160A, and V5-STEP61 S221A, whereas V5-STEP61 S160A/S221A did not show any change in mobility, suggesting that phosphorylation of at least one of the sites is necessary for the upward shift in mobility of STEP61. The upward shift in mobility of V5-STEP61 WT was also relatively higher than V5-STEP61 S160A or V5-STEP61 S221A, suggesting that concomitant phosphorylation of both the sites (Ser-160 and Ser-221) resulted in the hyperphosphorylation of STEP61.

To evaluate the consequence of phosphorylation of STEP61 at Ser-160, Ser-221, or both Ser-160 and Ser-221 in terms of their ability to bind to and dephosphorylate ERK MAPK we generated four mutant forms of STEP61. In the first mutant both Ser-160 and Ser-221 were converted to alanine to mimic the non-phosphorylated form of STEP61 (V5-STEP61 S160A/S221A). In the second mutant Ser-160 was converted to alanine and Ser-221 was converted to glutamic acid (V5-STEP61 S160A/S221E), whereas in the third mutant Ser-160 was converted to glutamic acid and Ser-221 was converted to alanine (V5-STEP61 S160E/S221A) to mimic partially phosphorylated forms of STEP61. In the fourth mutant both Ser-160 and Ser-221 were converted to glutamic acid (V5-STEP61 S160E/S221E) to mimic a completely phosphorylated form of STEP61. In initial studies HeLa cells were treated with phorbol ester (10 ng/ml) for varying time periods (0, 5, 10, or 15 min) to determine the temporal profile of ERK MAPK phosphorylation. Immunoblot analysis showed that phosphorylation of ERK MAPK was detectable within 5 min of phorbol ester application that peaked at 15 min (Fig. 8D). HeLa cells expressing V5-STEP61 S160A/S221A, V5-STEP61 S160A/S221E, V5-STEP61 S160E/S221A, or V5-STEP61 S160E/S221E were then treated with phorbol ester (15 min) followed by immunoprecipitation of ERK and immunoblot analysis. The data show almost complete dephosphorylation of ERK MAPK in cells transfected with V5-STEP61 S160A/S221A (Fig. 8E, lane 1). In cells transfected with either V5-STEP61 S160A/S221E or V5-STEP61 S160E/S221A a significant reduction in ERK MAPK phosphorylation was observed (Fig. 8E, lanes 2 and 3), whereas in cells transfected with V5-STEP61 S160E/S221E did not show any reduction in ERK MAPK phosphorylation (Fig. 8E, lane 4). The incomplete dephosphorylation of ERK MAPK with the partially phosphorylated forms of STEP61 (V5-STEP61 S160A/S221E and V5-STEP61 S160E/S221A) and the inability of the completely phosphorylated form of STEP61 (V5-STEP61 S160E/S221E) to reduce ERK MAPK phosphorylation indicate that in intact cells both the Ser-160 and Ser-221 are involved in interacting with and dephosphorylating ERK2. The efficacy of V5-STEP61 S160E/S221A in dephosphorylating ERK MAPK more efficiently than V5-STEP61 S160A/S221E further suggests a differential substrate binding ability of the two PKA sites with Ser-221 playing a greater role in substrate binding than Ser-160. These findings in conjunction with the observations in Figs. 1 and 4 indicate that Zn$^{2+}$-induced progressive increase in the magnitude of ERK MAPK phosphorylation is regulated by sequential phosphorylation of STEP61 at the two PKA sites.

**STEP Is a Regulator of ERK2 Phosphorylation**—To confirm that the loss of function of STEP61 through hyperphosphoryla-
tion is indeed responsible for Zn\(^{2+}\)/Trk receptor mediated increase in ERK2 phosphorylation, neuronal cultures obtained from both WT and STEP KO mice were treated with Zn\(^{2+}\) (300 \(\mu\)M). Fig. 9A shows a time-dependent increase in ERK2 phosphorylation in the neuronal lysates obtained from the WT mice embryos that correlated with the temporal profile of STEP\(_{61}\) phosphorylation. In neuronal cultures obtained from the STEP KO mice embryos, basal phosphorylation of ERK2 was significantly elevated as compared with the WT controls (Fig. 9B, lane 1). Moreover, within 5 min of exposure to Zn\(^{2+}\) a large increase in ERK2 phosphorylation was also observed in the KO mice cultures that remained unchanged over time (Fig. 9B, lanes 2–4). This rapid increase in ERK2 phosphorylation in STEP KO mouse cultures within minutes of exposure to Zn\(^{2+}\) that remained unaltered thereafter establishes the role of STEP\(_{61}\) as a regulator of Zn\(^{2+}\)-mediated ERK2 phosphorylation. The lack of expression of STEP\(_{61}\) in the STEP KO mouse cultures was confirmed by immunoblotting with anti-STEP antibody (Fig. 9B).

Zn\(^{2+}\)/TrkB-mediated STEP\(_{61}\) Phosphorylation Is BDNF Dependent—Earlier studies have shown that Zn\(^{2+}\) can activate TrkB receptor either by activation of matrix metalloproteinases (MMPs), which release pro-BDNF from cells and convert it to mature BDNF (12) or through intracellular activation of Src tyrosine kinase (33). The inability of the Src tyrosine kinase inhibitor, PP2, to attenuate Zn\(^{2+}\)-mediated increase in STEP\(_{61}\) phosphorylation (Fig. 7A) led us to hypothesize a role of extracellular BDNF in Zn\(^{2+}\)/TrkB receptor mediated STEP\(_{61}\) phosphorylation. Two different experimental approaches were taken to test this possibility. Fig. 10A shows that pharmacological inhibition of MMPs with GM6001 substantially reduced BDNF-induced STEP\(_{61}\) and ERK2 phosphorylation. Fig. 10B shows that competitive inhibition of BDNF-TrkB receptor interaction using a function-blocking antibody against BDNF also attenuated Zn\(^{2+}\)-dependent increase in STEP\(_{61}\) and ERK2 phosphorylation. Consistent with these findings, a dose and time-dependent increase in the phosphorylation of STEP\(_{61}\) and ERK2 was also observed following treatment with BDNF (Fig. 10, C and D). The decrease in the level of STEP\(_{61}\) observed at higher concentrations of BDNF (Fig. 10D, lanes 4 and 5) further suggested that BDNF regulates both STEP\(_{61}\) phosphorylation and protein level. Pharmacological studies further demonstrated that inhibition of Trk receptors with K252a attenuated BDNF-induced STEP\(_{61}\) and ERK2 phosphorylation (Fig. 10E).

**Discussion**

A key finding of the present study is that exposure of cultured neurons to high concentrations of exogenous Zn\(^{2+}\) leads to hyperphosphorylation of STEP\(_{61}\) involving multiple PKA sites. Mutational studies in cell lines further demonstrate that Ser-160 and Ser-221 are involved in the PKA-dependent hyperphosphorylation of STEP\(_{61}\). The differential abilities of the non-
**STEP Phosphorylation by Zn\(^{2+}\) and Trk Signaling**

**FIGURE 9. Role of STEP in the Zn\(^{2+}\)-dependent phosphorylation of ERK.** Neuronal cultures obtained from (A) wild-type and (B) STEP KO mice were treated with 300 \(\mu M\) Zn\(^{2+}\) for the specified times. Phosphorylation of ERK2 was analyzed by immunoblotting with anti-phospho-ERK antibody (upper panels, pERK1/2). Total ERK2 or STEP was analyzed by reprobing the blots with either anti-ERK2 (middle panels) or anti-STEP antibody (lower panels). Quantification of phosphorylated ERK2 is represented as mean \(\pm\) S.E. (\(n=4\)). A, \(p<0.001\) for phosphorylated ERK2 from untreated control in WT cultures; B, \(p<0.01\) for phosphorylated ERK2 from untreated control in STEP KO cultures.

In vivo administration of Zn\(^{2+}\) to dephosphorylate ERK2 suggests that one of the functional consequences of such multi-site phosphorylation is to regulate the affinity of STEP to its substrate. The findings further suggest that the progressive loss of function of STEP through phosphorylation of the two PKA sites regulates the magnitude of Zn\(^{2+}\)-induced ERK MAPK phosphorylation.

Our findings further show that Zn\(^{2+}\)-induced hyperphosphorylation of STEP to attenuate K252a, suggesting a role of Trk receptor-dependent signaling in the modulation of STEP phosphorylation by exogenous Zn\(^{2+}\). In vivo studies have further demonstrated that chronic Zn\(^{2+}\) administration increases BDNF gene expression in the cerebral cortex resulting in increased BDNF level (42–45), indicating a potential role of STEP in modulating Zn\(^{2+}\)-induced ERK MAPK phosphorylation.

In addition to our findings, two earlier studies have also addressed the regulation of ERK MAPK by extracellular Zn\(^{2+}\) (12, 13). The study by Hwang et al. (12) was performed in mixed cultures (neuron and astrocytes) and demonstrated TrkB-mediated increase in ERK MAPK phosphorylation using 10 \(\mu M\) Zn\(^{2+}\). In contrast, our study was carried out in primary cortical neuron cultures and did not show increase in ERK MAPK phosphorylation with Zn\(^{2+}\) concentrations below 50 \(\mu M\), suggesting that the increase in ERK MAPK phosphorylation observed by Hwang et al. (12) could be triggered, at least in part, by Zn\(^{2+}\) signaling in astrocytes. Since astrocytes do not express STEP, the observed increase in ERK MAPK phosphorylation by Hwang et al. (12) could not be attributed to the loss of function of STEP. On the other hand, the study by He et al. (13) reported Zn\(^{2+}\)-induced neuronal ERK MAPK phosphorylation in post-zinc exposure time periods. Our findings now reveal an important additional phase of ERK MAPK phosphorylation during Zn\(^{2+}\) exposure and provide a molecular basis for such sustained activation of ERK MAPK. We also observed that inhibition of Trk receptor signaling attenuated Zn\(^{2+}\)-induced phosphorylation of ERK2. Complementary studies in cells treated with BDNF showed increases in ERK MAPK phosphorylation, which was attenuated by TrkB receptor inhibition. In contrast, Zn\(^{2+}\)-induced ERK MAPK phosphorylation was not blocked either by inhibition of channels/receptors that facilitates entry of Zn\(^{2+}\) inside the neurons or by inhibition of Src kinase that transactivates TrkB in a BDNF-independent manner. Taken together, these findings suggest that by stimulating BDNF pro-
duction, extracellular Zn\(^{2+}\) may facilitate ERK MAPK phosphorylation through TrkB receptor signaling.

Although BDNF-TrkB receptor mediated intracellular signaling is mainly regarded as a contributor to synaptic development and plasticity (48), this pathway has also been shown to play a role in neurodegeneration (49) and promote cell death in vitro (50, 51). In addition, several studies have indicated a role of BDNF-TrkB receptor signaling in the development of epilepsy (52–56). One explanation for this differential response to BDNF-induced TrkB receptor stimulation is that potentiation of synaptic efficacy requires a transient release of BDNF that elicits a signaling response, which is different from that observed following sustained BDNF release. Consistent with this hypothesis a recent study showed that acute and sustained BDNF application elicited different patterns of surface TrkB receptor expression and ERK MAPK phosphorylation (57).

Acute administration of BDNF reduced surface TrkB receptor expression and induced transient increase in ERK MAPK phosphorylation. In contrast, progressive increase in BDNF application had little effect on surface expression of TrkB receptors, and induced a slow and sustained increase in ERK MAPK phosphorylation. Previous studies have also demonstrated that the small GTPases Ras and Rap1 differentially control the kinetics of ERK MAPK phosphorylation (57, 58). Ras-dependent signaling to ERK MAPK is transient following acute BDNF treatment, whereas Rap1-dependent signaling to ERK MAPK is sustained following prolonged exposure to BDNF. Our findings now indicate that TrkB receptor mediated activation of the cAMP/PKA leads to progressive increase in phosphorylation of STEP at multiple sites resulting in loss of function of STEP. The temporal profile of Zn\(^{2+}\)-mediated increase in ERK MAPK phosphorylation also strongly correlates with the pattern of STEP\(_{61}\) phosphorylation. Thus it appears that simultaneous activation of the Rap1 signaling pathway and inhibition of the tyrosine phosphatase STEP may be essential for the sustained phosphorylation of ERK MAPK. While the Rap1 signaling pathway is involved in triggering ERK MAPK phosphorylation, the loss of function of STEP through phosphorylation facilitates maintenance of sustained ERK MAPK phosphorylation. This interpretation is further supported by our studies in neuronal cultures obtained from STEP KO mice demonstrating a rapid increase in the magnitude of ERK MAPK phosphorylation within minutes of exposure to Zn\(^{2+}\) that remained unchanged over time. These findings provide a molecular basis for the sustained activation of ERK MAPK in Zn/BDNF/Trk receptor mediated signaling.

In conclusion, our findings present the first evidence that in addition to the neurotransmitters dopamine and glutamate, transition metal ions and neurotrophins can also modulate the function of STEP\(_{61}\). The study also establishes the signaling mechanism underlying Zn\(^{2+}\)-induced regulation of STEP\(_{61}\).

**FIGURE 10. Role of BDNF in Zn\(^{2+}\)-dependent phosphorylation of STEP and ERK.** Neuronal cultures were (A) pre-incubated with GM6001 (50 mM) for 15 min before treatment with 300 \(\mu\)M Zn\(^{2+}\) for 30 min (B) treated with 300 \(\mu\)M Zn\(^{2+}\) for 30 min in the absence or presence of anti-BDNF antibody (C) treated with different concentrations of BDNF for 30 min, (D) treated with BDNF (1 ng/ml) for the specified times, or (E) pre-incubated with K252a (100 nM) for 15 min before treatment with BDNF (1 ng/ml) for 30 min. Blots were probed with anti-STEP (upper panels) or anti-phospho-ERK (middle panels), pERK1/2 antibody. Total ERK2 was analyzed by re-probing with anti-ERK2 antibody (lower panels). Each experiment was repeated at least four times.
function and provides a novel mechanism of regulating the magnitude of ERK MAPK activation by STEP. Evaluation of the phosphorylation status of STEP may therefore help determine whether targeting STEP may attenuate ERK-MAPK-dependent progression of brain damage in neurodegenerative disorders.

Author Contributions—R. P. and S. P. conceived and coordinated the study, analyzed the data, prepared the figures and wrote the paper. R. P. and S. R. performed the experiments. C. W. S. analyzed the data, provided important intellectual input and revisited the manuscript. All authors approved the final version of the manuscript.

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