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Neuroprotective Effects of Reactive Oxygen Species Mediated by BDNF-Independent Activation of TrkB

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Reactive oxygen species (ROS) have diverse biological consequences in the mammalian CNS, but the molecular targets mediating these pleiotropic effects are incompletely understood. Like ROS, the neurotrophin receptor, TrkB receptor tyrosine kinase, has diverse effects in the developing and mature mammalian brain. Our discovery that zinc can transactivate TrkB, together with the finding that ROS can trigger zinc release from cytosolic zinc binding proteins, led us to hypothesize that ROS can transactivate TrkB in CNS neurons by a zinc-dependent mechanism. We found that both exogenous H_2O_2 and endogenous ROS activate TrkB signaling by a Src family kinase-dependent but brain-derived neurotrophic factor-independent mechanism in cultured rat cortical neurons. Exogenous H_2O_2 enhances cytosolic zinc content in a metallothionein-3 (MT-3)-requiring manner. Both exogenous H_2O_2 and endogenous ROS mediated transactivation of TrkB requires intracellular zinc and MT-3. The ROS-triggered transactivation of TrkB exerts neuroprotective effects, because inhibition of TrkB kinase activity or uncoupling Shc signaling from TrkB exacerbates neuronal cell death induced by H_2O_2. Thus, we propose a molecular signaling event whereby ROS induce release of zinc from cytosolic MT-3, the increased cytosolic zinc transactivates TrkB, and the enhanced Shc signaling downstream from TrkB promotes prosurvival effects. We suggest that such neuroprotective effects mediated by ROS are operative in diverse acute and chronic neurological disorders.

Introduction

Cytotoxic effects of reactive oxygen species (ROS) have been extensively documented in diverse mammalian cells (Martindale and Bolbrook, 2002). Cytotoxic effects of ROS contribute to the death of neurons of the mammalian CNS in ischemic and traumatic brain injury acutely, as well as in chronic neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Uttara et al., 2009). Interestingly, ROS also function as signaling molecules underlying physiological processes including cell proliferation, migration, and survival (Rhee, 2006; Stone and Yang, 2006; Veal et al., 2007). A number of extracellular stimuli, including growth factors such as epidermal growth factor and platelet-derived growth factor as well as cytokines like tumor necrosis factor-α and interleukin-1, evoke increases of H_2O_2 (Rhee et al., 2000; Rhee, 2006). One molecular consequence of increased cytosolic ROS is inhibition of protein phosphatases, thereby regulating activity of diverse intracellular signaling pathways, including MAPK, PI3 kinase/Akt, JAK/STAT, and PLCγ1 (Trachootham et al., 2008; Groeger et al., 2009). The inhibition of protein tyrosine phosphatases in particular raises the possibility that tyrosine phosphorylation signaling activated by receptor tyrosine kinases (RTKs) may underlie some of the physiological and pathological effects of ROS.

The most extensively studied RTK of the mammalian CNS is the brain-derived neurotrophic factor (BDNF) receptor TrkB. Widely expressed in neurons of the mammalian CNS, TrkB contributes to diverse biological processes, including neuronal survival and differentiation as well as synaptic structure, function, and plasticity (Huang and Reichardt, 2001). We previously reported that extracellular zinc can activate synaptic TrkB in cultured cortical neurons by a mechanism independent of neurotrophins like BDNF (Huang et al., 2008, 2010). Importantly, ROS have been demonstrated to induce release of zinc from the cytosolic zinc-binding protein metallothionein (MT), in cultured mammalian cortical neurons (Aizenman et al., 2000; Maret, 2000; Hwang et al., 2008). Together, these observations led us to hypothesize that ROS activate TrkB by a zinc-dependent mechanism that is independent of BDNF. We report that both exogenous and endogenous ROS activate TrkB by an intracellular zinc-requiring mechanism independent of BDNF. We further demonstrate that one of the functional consequences of ROS mediated by transactivation of TrkB is enhanced cell survival of cultured neurons.

Materials and Methods

Reagents. The following reagents were purchased from the respective commercial sources: BDNF from Millipore Bioscience Research Reagents and Millipore; K252a from Tocris Bioscience; FluoZin-3-AM from Invitrogen; and PP1 from Calbiochem. SMI 11293 and CGP76030 were gifts from Signase and Novartis, respectively. 1NMPP1 was generously provided by Dr. David Ginty (Johns Hopkins University, Balti-

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more, MD). Remaining reagents were purchased from Sigma. Peptides were synthesized and purified by Tufts University Core Facility, Boston, MA.

Mice. *TrkB*^{F616A} mutant mice in C57BL/6 background carrying a point mutation of residue 616 (F⟶A) of TrkB was described previously (Chen et al., 2005). MT3-null mutant in 129/C57BL/6 mixed background was described previously (Erickson et al., 1997). *TrkB*$^{PLC/PLC}$ (p/p) mice in C57BL/6 background carrying a substitution of phenylalanine for tyrosine at residue 816 of TrkB was described previously (Minichiello et al., 2002). The genotype of each animal was verified by PCR of genomic DNA isolated from tail before and after experiments. Animals of either gender were handled according to National Institutes of Health *Guide for the Care and Use of the Laboratory Animals* and approved by the Duke University Animal Welfare Committee.

Cell culture and transfection. Dissociated neuronal cultures were prepared as described previously (Huang et al., 2008). In brief, cortical or hippocampal neuron/glia mixed cultures were prepared from embryonic day (E)18 embryonic Sprague Dawley rat pups (Charles River) or postnatal day (P)0 and P1 mouse pups. Neurons were cultured in Neurobasal medium with B27 supplement for 14–21 days *in vitro* (DIV). HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum. For transfection experiments, cells were transfected using Lipofectamine 2000 (Invitrogen). Culture medium was replaced with ACSF buffer (in mM: 150 NaCl, 3 KCl, 10 HEPES, 2 $CaCl_2$, 2 $MgCl_2$, 20 glucose, pH 7.4, osmolarity ~310) for 1–2 h at 37°C before initiation of the experiment, and cells were maintained in ACSF thereafter.

Immunoprecipitation and immunoblotting. Cortical neurons maintained *in vitro* for 12–14 days or HEK293 cells were lysed in modified RIPA buffer (in mM, 50 Tris-HCl, pH 7.4, 150 NaCl, 1% NP-40, 0.25% sodium deoxycholate, 5 EDTA, 3 sodium vanadate, 1 PMSF, and proteinase inhibitors); following centrifugation, the supernatant was used for experiments and referred to as cell lysate. Immunoprecipitation was performed as described previously (Huang et al., 2008). In brief, cell lysates (500 μg) were incubated with phosphotyrosine antibody (1 μg) and protein A/G beads at 4°C overnight. Cell lysates (10–20 μg) or immunoprecipitates were resolved by SDS-PAGE. The blots were incubated overnight with the indicated primary antibodies at 4°C followed by incubation with secondary antibodies (1:5000) for 1 h at room temperature. The antibodies and dilution used in this study are as follow: p-Trk (pY515, 1:500; Sigma), p-Trk (pY705/706, 1:1000; Santa Cruz Biotechnology), p-Src (Y416), p-Akt, p-Erk (1:1000; Cell Signaling Technology); TrkB (1:500; BD Transduction Laboratories; 1:500, Millipore Biotechnology); pan-Trk (Santa Cruz Biotechnology); phosphotyrosine (4G10, 1:1000; Millipore Biotechnology pY20, 1:1000; BD Transduction Laboratories); and β-actin (1:10000; Sigma). p-TrkB (pY816) (1:500) was kindly provided by Dr. Moses Chao (New York University, New York, NY). To assure equivalent protein on each lane, blots were probed with an antibody to β-actin. Immunoblots shown were representative of at least three independent experiments. For quantitative analyses, immunoblots were scanned with a digital scanner and optical band density was quantified with ImageJ analysis software. Optical densities were normalized to β-actin levels. Data are presented as mean ± SEM.

*Live-cell micros*copy. Hippocampal neurons (DIV 7–10) were incubated with 5 μM FluoZin-3-AM, a zinc fluorescent indicator in ACSF buffer (pH 7.4) at 37°C for 15 min in a 5% CO_2 incubator. After washing with ACSF buffer, the cells were incubated with ACSF buffer at 37°C for 30 min. Zinc fluorescence was excited with 488 nm epifluorescence illumination and emission was collected using a 512 nm filter set. FluoZin-3-AM was specific to zinc because preincubation with TPEN [*N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine], a cell-permeable zinc-selective chelator, virtually abolished both basal and H_2O_2-induced increases in fluorescence signals (Fig. 3*C* and data not shown). Live-cell imaging of zinc fluorescence was captured at 1 min intervals with a CoolSNAP CCD camera on a Zeiss Axio Observer microscope. After 5 min imaging of basal fluorescence, the cells were incubated with an equal volume of either vehicle or 0.5 mM H_2O_2 and imaged for the indicated periods of time. Fluorescence intensity was measured in arbitrary units using MetaMorph software. The increases in zinc concentrations were plotted by normalizing maximal zinc fluorescence over that of the 5 min baseline.

Cell death assay. Neuronal cell death was induced by withdrawal of the B27 growth supplement from healthy cultures of cortical neurons. *N*-methyl-D-aspartate receptor activation-dependent toxicity was prevented by the addition of MK-801 (1 μM) to the culture (Lee and Chao, 2001). Following B27 withdrawal from the cultures for 24 h, cell death was assessed by measuring lactate dehydrogenase (LDH) activity in culture supernatants by a spectrophotometric method (Whitney and McNamara, 2000). Total cell death was induced by incubation with hypotonic solution (Neurobasal medium/H_2O: 1:1) for 4 h. Data are presented as the means ± SEM of determinations made in six wells per condition from at least four independent experiments.

Statistical analysis. Data are presented as means ± SEM. Statistical significance was assessed with the Student's *t* test or one-way ANOVA *post hoc t* test.

Results

ROS activates TrkB signaling in cultured neurons

We previously discovered that endogenously released zinc is able to transactivate synaptic TrkB in cultured neurons (Huang et al., 2008). Evidence that ROS enhance zinc concentration in mammalian cells (Aizenman et al., 2000; Hwang et al., 2008) led us to hypothesize that ROS-induced release of intracellular zinc can transactivate TrkB. To test this hypothesis, we first asked whether hydrogen peroxide (H_2O_2), a stable form of ROS, is able to activate TrkB in cultured neurons. We used phosphorylation of TrkB (p-TrkB) as a surrogate measure of its activation (Huang and Reichardt, 2003). Primary cultures of rat cortical neurons were incubated with vehicle, BDNF (10 ng/ml), exogenous zinc (100 μM), or exogenous H_2O_2 (500 μM) for 15 min. Western blots prepared from neuronal cell lysates were probed with three distinct antibodies, each of which specifically recognize individual phosphorylated tyrosines of Trk (p-Trk 515, 705/706, and 816 respectively) (Huang et al., 2008 and data not shown). Consistent with our prior report (Huang et al., 2008), both BDNF and exogenous zinc enhanced tyrosine phosphorylation of Trk receptors as evidenced by increased immunoreactivity of a 145 kDa band detected by each of the three p-Trk antibodies (Fig. 1*A*). Brief incubation of exogenous H_2O_2 also induced increased phosphorylation of 145 kDa of Trk receptors at each of these tyrosine residues (Fig. 1*A*) and did so in a concentration and time-dependent manner (Fig. 1*B–E*). The H_2O_2-induced increased phosphorylation of Trk receptors was evident within 5 min, maximal at 15–30 min, and declined after 30 min of incubation of H_2O_2 (Fig. 1*D*,*E*). Notably, BDNF and H_2O_2 also induced increased phosphorylation of extracellular regulated kinase (Erk1/2) (p-Erk) (Fig. 1*A*,*B*,*D*,*F*), a signaling protein downstream of Trk receptors. The increased p-Erk was mediated by Trk activation, because pretreatment with K252a, a Trk kinase inhibitor, reduced BDNF and H_2O_2-induced increases of both p-Trk and p-Erk (Fig. 1*F*,*G*).

The finding that exogenous ROS in the form of H_2O_2 can activate Trk signaling in turn raised the question as to whether endogenous ROS are capable of activating Trk receptors in cultured neurons. To address this question, primary cultures of cortical neurons were incubated with paraquat (150 μM for 30 min), because paraquat can generate intracellular ROS in a variety of mammalian cells, including CNS neurons (Patel et al., 1996). Brief incubation of paraquat resulted in an increase of phosphorylation of 145 kDa Trk by about 50% (150 ± 28% over control, mean ± SEM, $n = 8$ independent experiments, $p < 0.05$) (Figs. 1*H*, 2*E*, 4*E*). Importantly, the phosphorylation of Erk was also increased in paraquat-treated neurons (Fig. 1*H*), thereby provid-

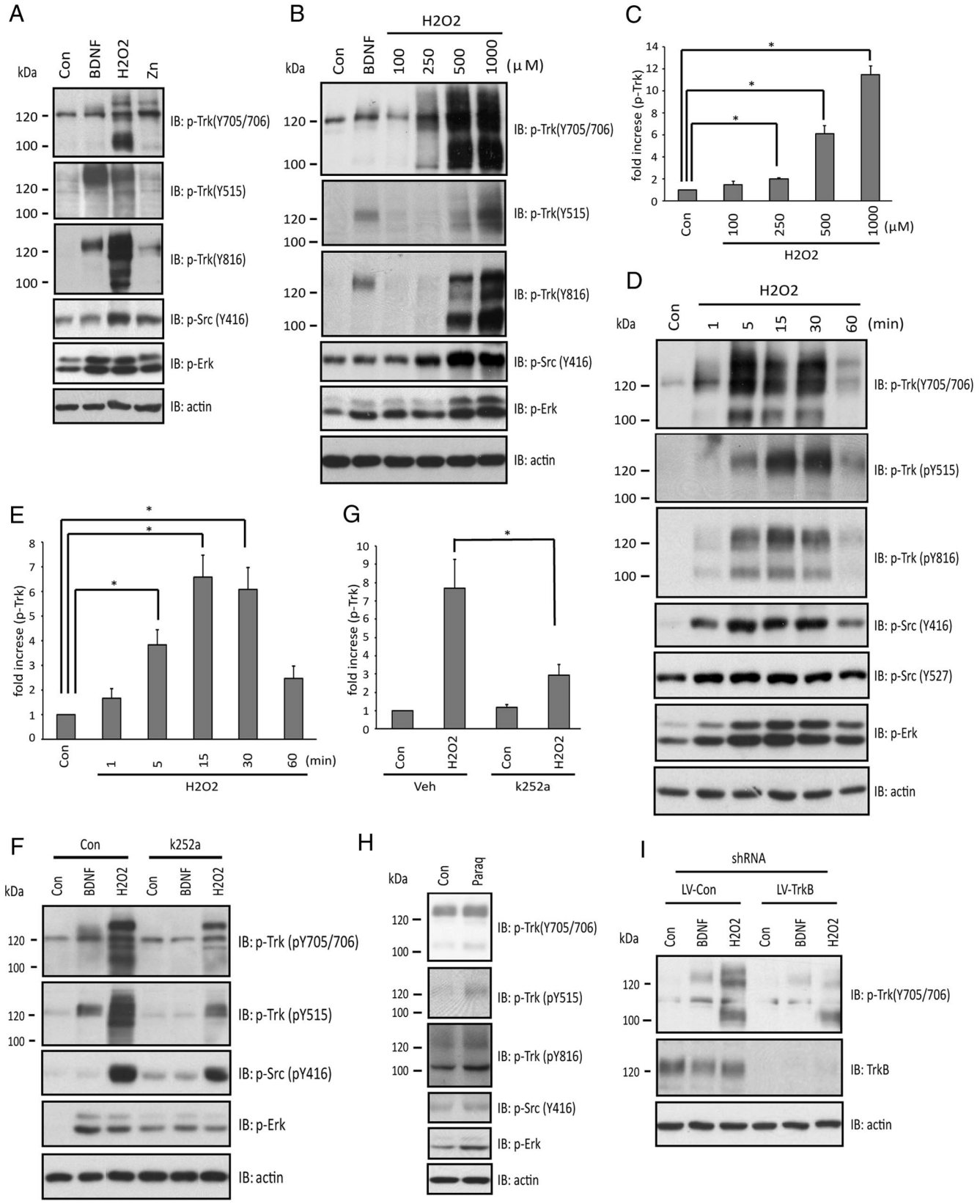

Figure 1. Both exogenous H_2O_2 and endogenous ROS can activate Trk signaling in cultured neurons. ***A***, Exogenous H_2O_2 activated Trk receptors. Cortical neurons were incubated with vehicle (Con, Control), BDNF (10 ng/ml), exogenous zinc (Zn; 100 μM), or exogenous H_2O_2 (500 μM) for 15 min. ***B***, Exogenous H_2O_2 activated Trk signaling in a concentration-dependent fashion. Cortical neurons were incubated with vehicle or H_2O_2 with varying concentrations for 15 min. ***C***, Quantitative analysis of p-Trk (p145) in ***A*** ($n = 4–5$ independent experiments). ***D***, Exogenous H_2O_2 activated Trk signaling in a time-dependent fashion. Cortical neurons were incubated with vehicle or H_2O_2 (500 μM) for indicated periods of time. ***E***, Quantitative analysis of p-Trk (p145) in ***D*** ($n = 3–8$ independent experiments). ***F***, The small molecule Trk inhibitor K252a reduced exogenous H_2O_2-induced Trk activation. Cortical neurons were preincubated with vehicle or K252a (200 nM) for 15 min; each of these treatments was continued for an additional 15 min in the presence of vehicle, BDNF (10 ng/ml), or H_2O_2 (500 μM). ***G***, Quantitative analysis of p-Trk (p145) (*Figure legend continues*.)

ing evidence that endogenous ROS are able to activate Trk signaling in neurons.

Since p-Trk antibodies recognize all three Trk receptors (TrkA, TrkB, and TrkC), whether H_2O_2 activates TrkB in particular was unclear. To address this question, a lentiviral vector expressing TrkB-specific shRNA was employed to selectively reduce the expression of TrkB protein. Consistent with our prior report (Huang et al., 2008), TrkB protein content was reduced in TrkB but not in scrambled shRNA-transduced neurons (Fig. 1*I*). Brief incubation of BDNF or H_2O_2 resulted in increased phosphorylation of 145 kDa Trk in scrambled treated neurons; by contrast, this increase was reduced in TrkB-shRNA-treated neurons (Fig. 1*I*), thereby supporting the conclusion that H_2O_2 activates TrkB in cultured neurons. Collectively, these findings demonstrate that ROS can activate TrkB and its downstream signaling in a time-dependent and concentration-dependent manner.

Molecular identity of 100 kDa p-Trk immunoreactivity induced by ROS

In addition to inducing increased phosphorylation of 145 kDa Trk, brief incubation of exogenous H_2O_2 resulted in a robust increase in immunoreactivity of a band migrating at ~100 kDa that was detected by all three p-Trk antibodies but more prominently by antibodies recognizing p-Trk Y705/706 and Y816 compared to Y515 (Fig. 1*A*,*B*,*D*). By contrast, only minimal increases of immunoreactivity migrating at 100 kDa were evident in response to BDNF or exogenous zinc (Fig. 1*A*). The question arose as to whether the 100 kDa band represents TrkB immunoreactivity. The fact that the band was recognized by three antibodies recognizing distinct phosphorylated tyrosine residues of Trk favored this possibility. Moreover, prior biochemical studies of Trk expressed in heterologous cells (Klein et al., 1989; Watson et al., 1999) demonstrated that the extent of glycosylation markedly affected the migration of Trk receptors in SDS-PAGE and revealed a species of Trk receptor with reduced glycosylation that migrated at ~100 kDa. To further investigate the molecular identity of the 100 kDa p-Trk band, three experiments were performed. First, we asked whether the 100 kDa band could be detected by TrkB antibodies following immunoprecipitation with phosphotyrosine antibodies. Primary cultures of cortical neurons were incubated with either vehicle, BDNF (10 ng/ml), zinc (100 μM), or H_2O_2 (500 μM) for 15 min. The presence of TrkB in the immunoprecipitates was revealed by probing the blots with an antibody specific to TrkB (Huang et al., 2008). As expected, the amount of 145 kDa TrkB immunoreactivity was significantly increased in BDNF or exogenous zinc-treated neurons (Fig. 2*A*), as well as following a brief incubation in exogenous H_2O_2 (Fig. 2*A*). Interestingly, the immunoreactivity migrating at ~100 kDa was also increased in H_2O_2-treated neurons as evident by immunoblotting with a TrkB-specific antibody (Fig. 2*A*); H_2O_2 also increased 100 kDa p-Trk immunoreactivity in TrkB-transfected HEK cells (see Fig. 5*C*). The presence of 100 kDa band of TrkB immunoreactivity was also found in phosphotyrosine immunoprecipitates of paraquat-treated neurons (Fig. 2*B*). Additionally, an increase of TrkB immunoreactivity migrating at 100 kDa was identified in immunoprecipitates of multiple p-Trk antibodies obtained from different sources (data not shown). Collectively, these findings support the assertion that 100 kDa of p-Trk immunoreactivity is TrkB protein that can undergo tyrosine phosphorylation in response to either exogenous or endogenous ROS in cultured neurons.

Next, we reasoned that if the 100 kDa protein contains phosphorylated TrkB, then inhibition of TrkB kinase would be expected to at least partly inhibit the H_2O_2-induced increase. We employed a chemical–genetic approach to test this idea. Engineering a point mutation (F616A) into the ATP binding pocket of the TrkB kinase domain renders the mutant TrkB ($TrkB^{F616A}$) sensitive to inhibition by a membrane-permeable, small molecule PP1 derivative, 1NMPP1 (Chen et al., 2005; Huang et al. 2008). Cortical neurons cultured from a *trkB*^{F616A} mutant mouse were incubated with either vehicle, BDNF or H_2O_2. In the presence of 1NMPP1, BDNF-induced increased p-TrkB was eliminated (Fig. 2*C*), thereby demonstrating the efficacy of inhibition of TrkB kinase by 1NMPP1. Importantly, increased phosphorylation of both 145 and 100 kDa bands mediated by H_2O_2 was reduced by 1NMPP1 (Fig. 2*C*), supporting the idea that H_2O_2 activates TrkB at both 145 and 100 kDa in cultured neurons. Likewise, increased p-Trk at both 145 and 100 kDa mediated by endogenous ROS was also reduced by 1NMPP1 (Fig. 2*E*). In contrast to neurons cultured from *trkB*^{F616A} mice, addition of 1NMPP1 to neurons cultured from wild-type (WT) mice did not inhibit the H_2O_2-mediated increases of p-Trk at 145 or 100 kDa induced by H_2O_2 (Fig. 2*D*), reinforcing the conclusion that 1NMPP1 produced its effect by inhibiting TrkB kinase *per se*.

Lastly, if the 100 kDa protein contains TrkB, then reducing TrkB protein content would be expected to reduce this immunoreactivity. We employed shRNA and genetic approaches to test this idea. Consistent with this idea, knockdown of TrkB protein content by shRNA reduced immunoreactivity of the 100 kDa band of p-Trk in H_2O_2-treated neurons (Fig. 1*I*). Whether the remaining p-Trk immunoreactivity reflects residual TrkB or belongs to TrkC is unclear. The dependence of the phosphorylated 100 kDa band on the presence of TrkB was further examined in neurons deficient in TrkB protein. Substitution of phenylalanine for tyrosine at residue 816 of TrkB (*trkB*$^{plc/plc}$, p/p) unexpectedly resulted in a marked reduction of TrkB protein content in cortical neurons cultured from mutant mice (P0) (Minichiello et al., 2002; Fig. 2*F*). The 100 kDa p-Trk immunoreactivity was almost eliminated in H_2O_2-treated mutant neurons (Fig. 2*F*), further supporting the idea that p-TrkB contributes to the p-Trk immunoreactivity migrating at 100 kDa.

H_2O_2 activates TrkB by an intracellular zinc requiring mechanism

To test whether H_2O_2 activates TrkB by a zinc-requiring mechanism, we asked two questions: (1) does addition of H_2O_2 to cultured neurons increase labile zinc concentration; and (2) does reducing H_2O_2-induced increase of zinc content inhibit H_2O_2-induced activation of TrkB? To address the first question, we monitored the effects of H_2O_2 on zinc content in cultured neurons with time-lapse imaging using a zinc-specific fluorescent indicator, FluoZin-3 AM. After loading hippocampal neurons with FluoZin-3 and recording basal intracellular zinc content (Fig. 3*A*), addition of H_2O_2 (250 μM) produced an enhanced zinc fluorescence in a time-dependent manner, with an increase approximating fourfold evident at 30 min (Fig. 3*A*,*B*), confirming

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(*Figure legend continued.*) in ***F*** ($n = 4$ independent experiments). ***H***, Endogenous ROS produced by paraquat (Paraq) was able to activate Trk signaling. Cortical neurons were incubated with vehicle or paraquat (150 μM) for 30 min. ***I***, Exogenous H_2O_2-induced phosphorylation of Trk receptors in a TrkB requiring manner. Cortical neurons (DIV 6) were transduced with lentiviral vectors expressing either control-shRNA or TrkB-shRNA and then maintained for additional 14 days. Cortical neurons were incubated with vehicle, BDNF (10 ng/ml), or exogenous H_2O_2 (500 μM) for 15 min. Asterisk (*) indicates $p < 0.05$. IB, Immunoblot.

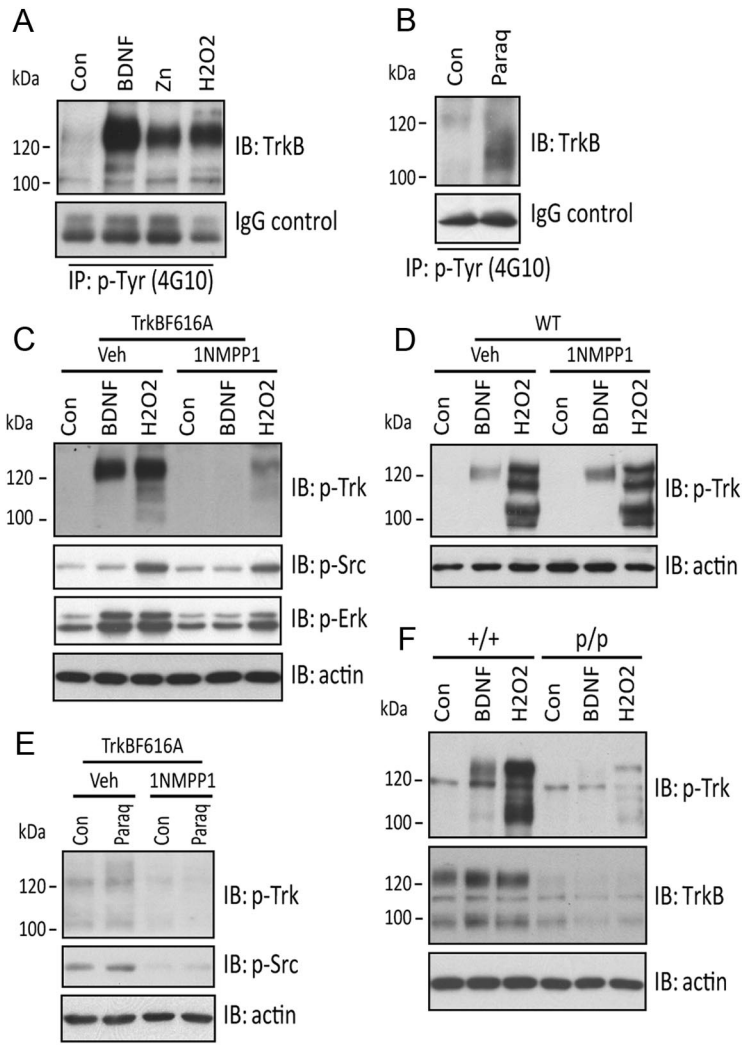

Figure 2. Both exogenous H_2O_2 and endogenous ROS enhance phosphorylation of 100 kDa TrkB in cultured neurons. Cortical neurons cultured from E18 rat pups (***A***, ***B***) or P0–P1 mouse pups (***C***–***F***) were maintained *in vitro* for 12–14 days. ***A***, Exogenous H_2O_2 enhanced phosphorylation of 100 kDa TrkB. Exogenous H_2O_2 activated Trk receptors. Cortical neurons were incubated with vehicle (Con, Control), BDNF (10 ng/ml), exogenous zinc (Zn; 100 μM), or exogenous H_2O_2 (500 μM) for 15 min. Cell lysates were immunoprecipitated (IP) with an anti-phosphotyrosine (4G10) antibody. The immunoprecipitates were subjected to immunoblotting (IB) with a monoclonal antibody to TrkB. ***B***, Endogenous ROS enhanced phosphorylation of 100 kDa TrkB. Cortical neurons were incubated with vehicle or paraquat (Paraq; 150 μM) for 30 min. Immunoprecipitation was performed with anti-phosphotyrosine (4G10) antibody followed by immunoblotting with a monoclonal TrkB antibody. ***C***, The small molecule TrkB inhibitor 1NMPP1 inhibited exogenous H_2O_2-induced Trk signaling in neurons cultured from *trkB*^{F616A} mice. Cortical neurons were preincubated with vehicle or 1NMPP1 (1 μM) for 15 min; each of these treatments was continued for an additional 15 min in the presence of vehicle, BDNF (10 ng/ml) or H_2O_2 (500 μM). ***D***, The small molecule TrkB inhibitor 1NMPP1 did not inhibit exogenous H_2O_2-induced Trk signaling in neurons cultured from WT mice. WT cortical neurons were incubated with vehicle, BDNF (10 ng/ml), exogenous zinc (100 μM), or exogenous H_2O_2 (500 μM) for 15 min. ***E***, The small molecule TrkB inhibitor, 1NMPP1, inhibited endogenous ROS-induced Trk signaling in neurons cultured from *trkB*^{F616A} mice. Cortical neurons cultured from *trkB*^{F616A} mice were preincubated with vehicle or 1NMPP1 (1 μM) for 15 min; each of these treatments was continued for an additional 30 min in the presence of vehicle or paraquat (150 μM). ***F***, Reduction of TrkB protein prevented increased p-TrkB induced by exogenous H_2O_2. Cortical neurons cultured from WT or *trkB*$^{p/p}$ mice bearing point mutation of TrkB Y816F were incubated with either vehicle, BDNF (10 ng/ml), or exogenous H_2O_2 (500 μM) for 15 min.

and extending a previous report (Hwang et al., 2008). Pretreatment with TPEN (1 μM), a cell membrane-permeable zinc selective chelator, eliminated the increased zinc fluorescence (Fig. 3*C*). By contrast, preincubation with CaEDTA (10–100 μM), a cell membrane impermeable zinc selective chelator, was ineffective (data not shown). The efficacy of TPEN but not CaEDTA supports the conclusion that H_2O_2 induces zinc release from intracellular compartments of cultured neurons. Notably, the peak increase of zinc fluorescence occurred later than the peak activation of TrkB by H_2O_2 (Fig. 1*D*,*E*). We suspect that insensitivity of the zinc reporter may limit accurate detection of the rise of intracellular zinc; it is also possible that the concentration of zinc required to activate TrkB may be far less than the maximum rise of zinc induced by H_2O_2.

To address the second question, cortical neurons were preincubated with vehicle or TPEN (1 μM), after which H_2O_2 (500 μM) was added for 15 min. The H_2O_2-induced increases of both 145 and 100 kDa bands of p-Trk was reduced by pretreatment of TPEN (Fig. 4*A*,*B*). By contrast, BDNF-mediated increases of p-TrkB were unaffected by pretreatment of TPEN (Fig. 4*C*). Importantly, pretreatment with the cell-impermeable zinc chelator CaEDTA was ineffective (Fig. 4*D*), thereby supporting the notion that H_2O_2 activates TrkB signaling by an intracellular zinc-dependent mechanism.

The evidence that exogenous ROS in the form of H_2O_2 activates TrkB by an intracellular zinc-dependent mechanism led us to ask whether endogenous ROS activate TrkB by a similar mechanism. To address this question, cortical neurons were pretreated with vehicle, CaEDTA, or TPEN and subsequently exposed to paraquat (150 μM) for 30 min. Pretreatment with TPEN but not CaEDTA inhibited paraquat-induced increased p-Trk (Fig. 4*E*). Collectively, these findings support the conclusion that both exogenous and endogenous ROS activate TrkB signaling by an intracellular zinc-dependent mechanism.

H_2O_2 activates TrkB by a BDNF-independent mechanism

The dependence of ROS-mediated activation of TrkB on intracellular zinc led us to query whether the mechanism also involved the prototypic ligand of TrkB, brain-derived neurotrophic factor, or BDNF. To address this issue, cortical neurons were pretreated with vehicle or TrkB-Ig (2 μg/ml), a recombinant protein that binds and scavenges neurotrophin ligands of TrkB, and subsequently exposed to vehicle, BDNF (10 ng/ml), or H_2O_2 (500 μM) for 15 min. Whereas TrkB-Ig eliminated the BDNF-induced increased p-Trk (Fig. 5*A*), TrkB-Ig did not inhibit H_2O_2-mediated increase of p-Trk (Fig. 5*B*), thereby demonstrating that H_2O_2 activates TrkB signaling independent of neurotrophins. We also asked whether H_2O_2 can activate TrkB in heterologous cells in which BDNF is not expressed. Incubation of heterologous cells stably expressing full-length TrkB (Huang et al., 2008) with vehicle, BDNF (10 ng/ml), NT-3 (10 ng/ml), or exogenous H_2O_2 (500 μM) for 15 min resulted in enhanced phosphorylation of TrkB and Erk in HEK cells (Fig. 5*C*,*D*), further demonstrating that

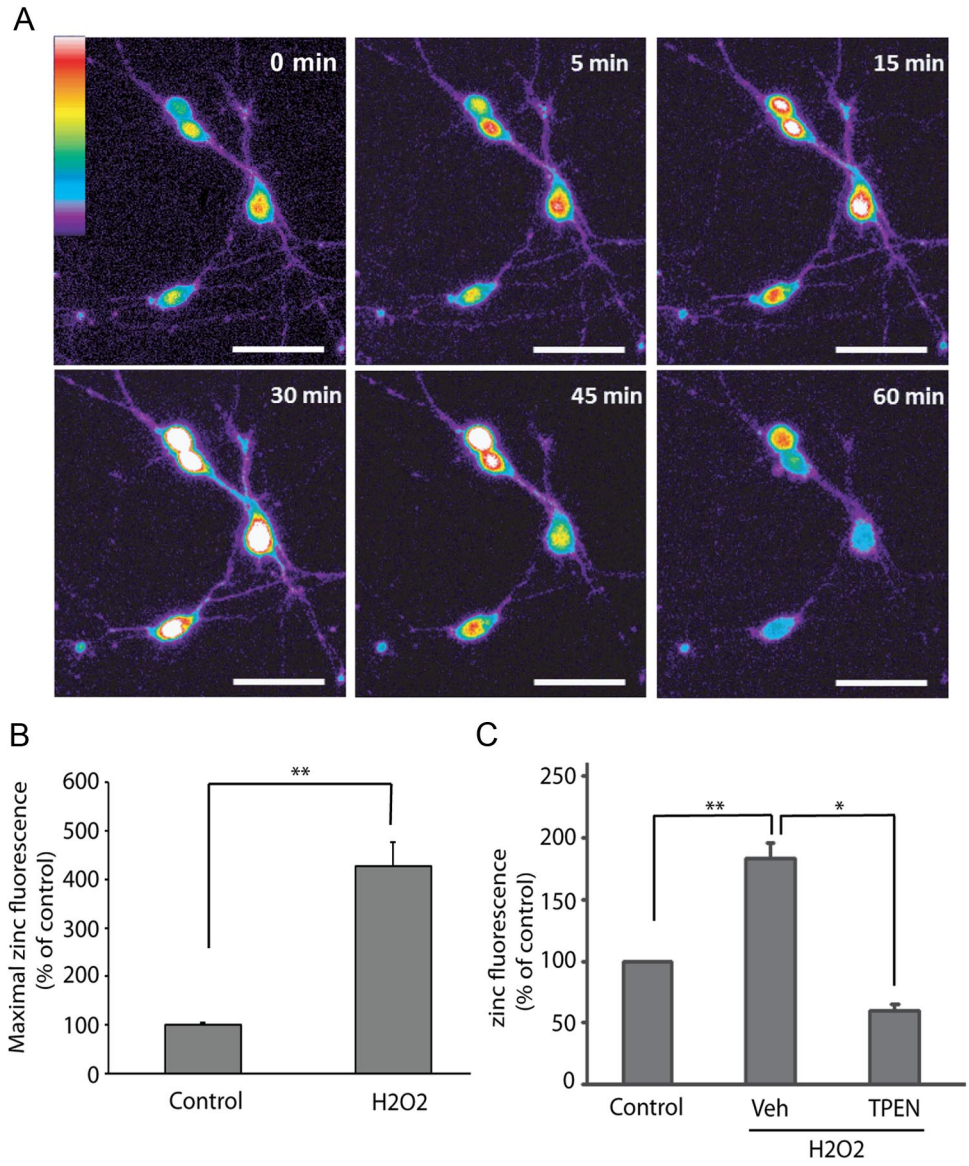

Figure 3. Exogenous H_2O_2 increases cytosolic zinc concentration in cultured neurons. ***A***, Hippocampal neurons cultured from E18 rat pups (DIV7–10) were incubated with vehicle or exogenous H_2O_2 (250 μM) in ACSF buffer for the indicated periods of time. Live cell images of zinc indicator FluoZin-3 fluorescence of cultured neurons were captured at indicated time points after addition of exogenous H_2O_2 (250 μM) to the ACSF buffer. Zinc fluorescence signals were increased in a time-dependent manner. Scale bars, 50 μM. ***B***, Quantitative analysis of maximal increases of zinc signals measured at ~30 min in ***A*** (191 cells, $n = 5$ independent experiments). ***C***, The zinc selective chelator TPEN reduced zinc fluorescence induced by exogenous H_2O_2. Hippocampal neurons cultured from E18 rat pups (DIV7–10) were pretreated with vehicle (VEH) or TPEN (1 μM) for 15 min and continued for an additional incubation with vehicle or exogenous H_2O_2 (250 μM) for 15 min (cell numbers for control, H_2O_2, and H_2O_2+TPEN groups were 115, 148, and 133, respectively; $n = 4$ independent experiments). Asterisks (* and **) indicate $p < 0.05$ and $p < 0.01$, respectively.

H_2O_2 is able to activate TrkB by a mechanism independent of BDNF.

H_2O_2-mediated transactivation of TrkB requires Src family kinases

The H_2O_2-mediated activation of TrkB will subsequently be referred to as transactivation, because it involves a neurotrophin-independent mechanism. We next sought to explore the signaling mechanism by which H_2O_2 transactivates TrkB. We previously discovered that exogenous zinc transactivates TrkB by a Src family kinase (SFK)-requiring mechanism (Huang et al., 2008). The evidence that H_2O_2 transactivates TrkB by a mechanism requiring endogenous intracellular zinc led us to investigate whether SFKs are also required for this H_2O_2 effect. Brief (15 min) incubation of cultured neurons in exogenous H_2O_2 (500 μM) induced increased phosphorylation of pY416 (the catalytic site of SFKs) (Figs. 1, 6), a surrogate measure of SFK activation (Cooper and Howell, 1993), in a concentration-dependent (Fig. 1*B*) and time-dependent manner (Fig. 1*D*), implying that H_2O_2 activates SFKs. H_2O_2-induced SFK activation requires intracellular zinc, because pretreatment with TPEN but not CaEDTA reduced H_2O_2-mediated phosphorylation of Y416 (Fig. 4*D*). Endogenous ROS also activated SFK by an intracellular zinc-requiring mechanism because the addition of paraquat (150 μM) induced enhanced pY416 (Figs. 1*H*, 4*E*), which was eliminated by preincubation with TPEN but not CaEDTA (Fig. 4*E*). Collectively, these results demonstrate that both exogenous and endogenous ROS activate SFKs in neurons by an intracellular zinc-dependent mechanism.

The evidence that ROS activate SFKs by an intracellular zinc-requiring mechanism led us to query whether SFKs are required for H_2O_2-induced transactivation of TrkB in neurons. To test this

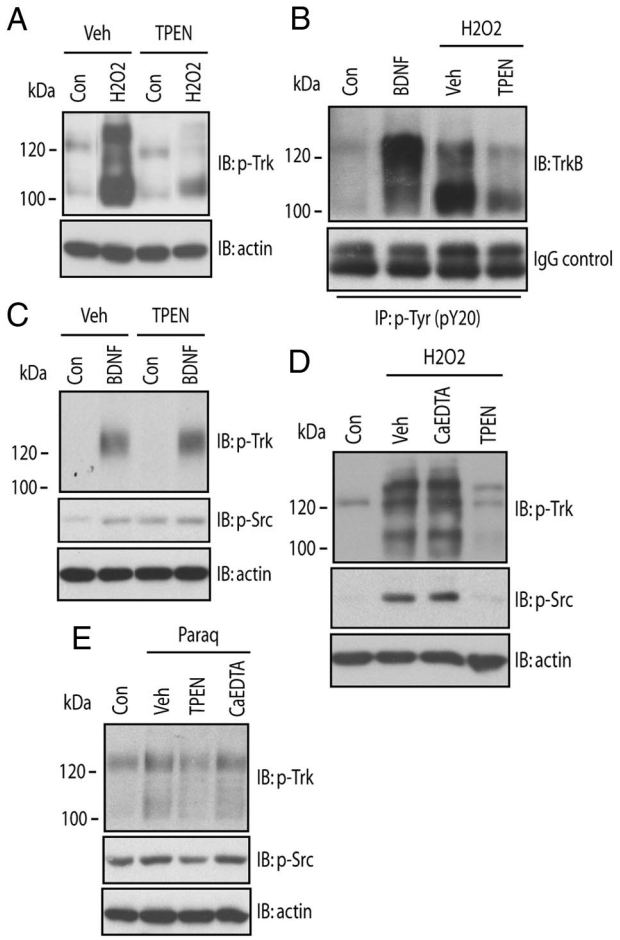

Figure 4. Both exogenous H_2O_2 and endogenous ROS activate TrkB in an intracellular zinc-requiring manner. ***A***, The zinc-selective chelator TPEN reduced exogenous H_2O_2-induced phosphorylation of Trk receptors. Cortical neurons were preincubated with vehicle (Veh) or TPEN (1 μM) for 15 min; each of these treatments was continued for an additional 15 min in the presence of vehicle (Con, Control) or H_2O_2 (500 μM). ***B***, The zinc chelator TPEN reduced H_2O_2-induced p-Trk. Cortical neurons were preincubated with vehicle or TPEN (1 μM) for 15 min; each of these treatments was continued for an additional 15 min in the presence of H_2O_2 (500 μM). Cell lysates were immunoprecipitated (IP) with an anti-phosphotyrosine (pY20) antibody. The immunoprecipitates were subjected to immunoblotting (IB) with a polyclonal antibody to TrkB. ***C***, TPEN did not affect BDNF-induced phosphorylation of TrkB. Cortical neurons were preincubated with vehicle or TPEN (1 μM) for 15 min; each of these treatments was continued for an additional 15 min in the presence of vehicle or BDNF (10 ng/ml). ***D***, The extracellular zinc chelator CaEDTA failed to inhibit exogenous H_2O_2-induced p-Trk. Cortical neurons were preincubated with vehicle, CaEDTA (100 μM), or TPEN (1 μM) for 15 min; each of these treatments was continued for an additional 15 min in the presence of H_2O_2 (500 μM). ***E***, The zinc-selective chelator TPEN inhibited endogenous ROS-induced p-Trk. Cortical neurons were preincubated with vehicle, TPEN (1 μM), or CaEDTA (100 μM) for 15 min; each of these treatments was continued for an additional 30 min in the presence of paraquat (Paraq; 150 μM).

idea, cortical neurons were pretreated with each of three structurally distinct SFK-selective inhibitors, PP1 (Fig. 6*A*,*B*), SMI (Fig. 6*C*,*D*), and CGP (Fig. 6*D*). Each of these compounds reduced the H_2O_2-mediated increases of p-Trk and pY416 (Fig. 6). Importantly, pretreatment with PP3, the inactive analog of PP1, was ineffective (Fig. 6*B*). Residual increases of p-Src and p-Trk in SFK inhibitor-treated neurons might be due to partial inhibition of SFK activity and/or inability of these small molecules to inhibit all SFK family members. Together, these findings support the conclusion that ROS-mediated activation of SFKs is a critical component of the mechanism by which ROS transactivate TrkB.

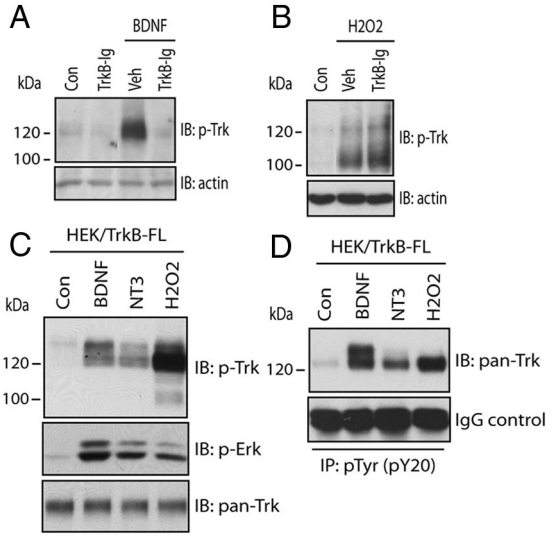

Figure 5. Exogenous H_2O_2 activates TrkB in a BDNF-independent manner. ***A***, The neurotrophin scavenger TrkB-Ig prevented BDNF-mediated phosphorylation of TrkB. Cortical neurons were preincubated with vehicle (Veh) or TrkB-Ig (2 μg/ml) for 15 min; each of these treatments was continued for an additional 15 min in the presence of BDNF (10 ng/ml). ***B***, TrkB-Ig did not affect H_2O_2-mediated increased p-Trk. Cortical neurons were preincubated with vehicle or TrkB-Ig (2 μg/ml) for 15 min; each of these treatments was continued for an additional 15 min in the presence of H_2O_2 (500 μM). ***C***, ***D***, Exogenous H_2O_2-induced phosphorylation of full-length TrkB. HEK cells stably expressing TrkB were incubated with vehicle (Con, Control), BDNF (10 ng/ml), NT3 (10 ng/ml), or H_2O_2 (500 μM) for 15 min. ***C***, Western blotting analysis of cell lysates. ***D***, Western blotting analysis of immunoprecipitates. IB, Immunoblotting; IP, immunoprecipitation.

A question arose from these findings demonstrating a requirement of intracellular zinc for SFK-dependent activation of TrkB; that is, what is the signaling mechanism by which intracellular zinc released by ROS activates SFK activity? Our prior work provided evidence that extracellular zinc activates SFK activity by inhibiting C-terminal Src family kinase (Csk), a conclusion based upon reduced pY527 of SFKs that is thought to result in disinhibiting SFK activity (Huang et al., 2008). We therefore asked whether intracellular zinc activates SFKs by a same mechanism. Consistent with our previous findings (Huang et al., 2008), brief incubation of exogenous zinc (100 μM) produced a significant reduction of pY527 (Fig. 6*E*,*F*). By contrast, addition of exogenous H_2O_2 (500 μM) to cultured neurons resulted in a modest increase of pY527 (Figs. 1*D*, 6*E*,*F*), providing evidence for a Csk-independent mechanism.

H_2O_2 transactivates TrkB by a metallothionein-3-dependent mechanism

The finding that ROS transactivate TrkB by an intracellular zinc-requiring mechanism led us to seek the molecular source of intracellular zinc. Intracellular zinc homeostasis in mammalian cells is tightly regulated by zinc transporters and zinc-binding proteins (Frederickson et al., 2005). Metallothioneins (MTs) are thought to be the major proteins involved in buffering intracellular zinc through an interexchange of disulfide and thiol (Maret, 2000). MTs are sensitive to redox perturbation and release zinc to cytosol. Oxidative stress such as H_2O_2 has been shown to compromise the cellular redox state and induce zinc release in mammalian cells (Hwang et al., 2008). Therefore, we hypothesized that MT-3, a brain-specific form of MTs, is involved in H_2O_2-induced transactivation of TrkB in cultured neurons. This hypothesis leads to the following predictions: (1) that MT-3 is

required for ROS-mediated increase of intracellular zinc; and (2) that MT-3 is required for ROS-mediated activation of SFK and of Trk. To test these predictions, cortical neurons cultured from pups of wild-type or MT-3-knockout mice were examined.

Time-lapse imaging revealed that H_2O_2-induced increases of zinc fluorescence were markedly reduced in neurons from MT-3 knockout compared to WT mice (Fig. 7*A*). The zinc fluorescence signals in vehicle-treated neurons were indistinguishable between WT and MT-3 mutant neurons (in arbitrary units, WT: 11.6 ± 0.8, 223 cells; MT-3$^{-/-}$: 11.1 ± 0.6, 87 cells; mean ± SEM, $n = 3$–4 independent experiments, $p > 0.05$). Biochemical studies revealed that H_2O_2-induced increases of p-Trk (at both 145 and 100 kDa) and pY416 of SFKs in WT neurons are also markedly reduced in neurons cultured from MT3-null mutants compared to WT mice (Fig. 7*B*,*C*). By contrast, the BDNF-mediated increase of p-Trk was similar in neurons cultured from WT and MT-3-null mice (Fig. 7*D*). Notably, modest residual increases of FluoZin3 fluorescence (Fig. 7*A*) and p-Trk and pY416 immunoreactivity were evident in MT3-null neurons following H_2O_2 treatment (Fig. 7*B*,*C*), suggesting that ROS also induces intracellular zinc release and activation of TrkB by an MT-3-independent mechanism. Consistent with this idea, prior work revealed that H_2O_2 can induce zinc release from lysosomes in cultured mouse hippocampal neurons (Hwang et al., 2008).

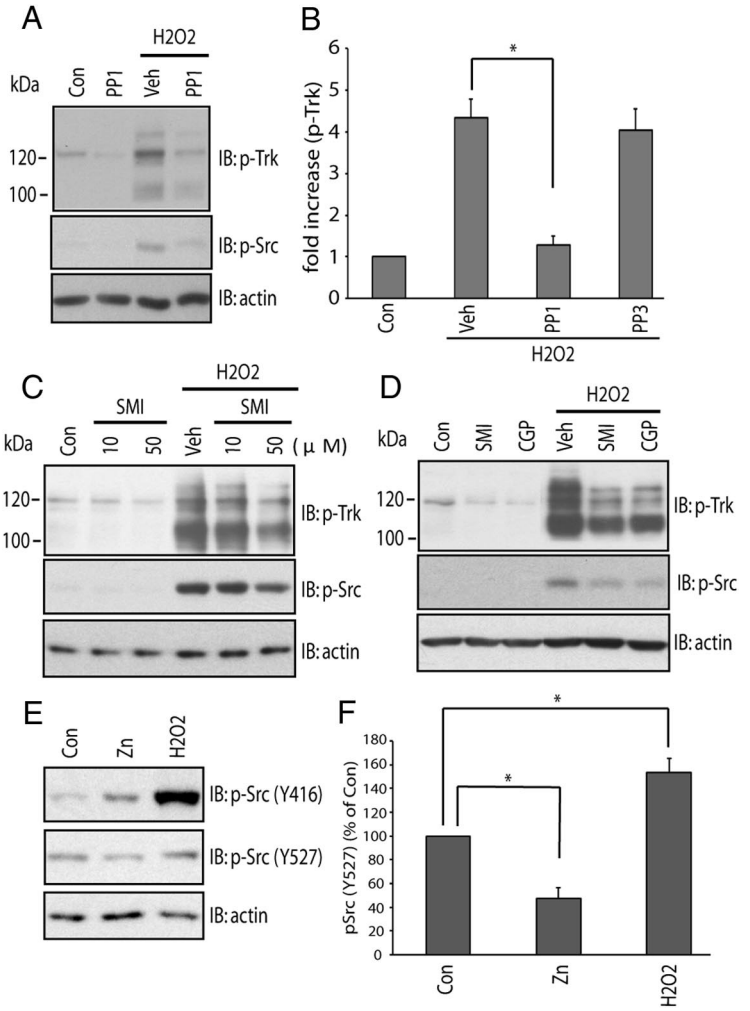

Figure 6. Src family kinases (SFKs) are required for exogenous H_2O_2-induced phosphorylation of Trk. ***A***, The small molecule SFK inhibitor PP1 reduced exogenous H_2O_2-mediated phosphorylation of Trk. Cortical neurons were preincubated with SFK inhibitor, PP1 or the inactive analog PP3 (not shown), for 15 min; each of these treatments was continued for an additional 15 min in the presence of H_2O_2 (500 μM). ***B***, Quantitative analysis of p-Trk (p145) in ***A*** ($n = 10$ independent experiments). * indicates $p < 0.05$. ***C***, ***D***, The structurally distinct small molecule SFK inhibitors SMI11293 and CGP76030 inhibited exogenous H_2O_2-induced phosphorylation of Trk. Cortical neurons were preincubated with SFK inhibitors SMI11293 (SMI; 10 nM) (***C***, ***D***) or CGP76030 (CGP; 100 nM) (***D***) for 15 min; each of these treatments was continued for an additional 15 min in the presence of H_2O_2 (500 μM). ***E***, Exogenous zinc but not H_2O_2 reduced pY527 of SFKs. Cortical neurons were incubated with vehicle (Con, control), zinc (Zn; 100 μM), or H_2O_2 (500 μM) for 30 min. ***F***, Quantitative analysis of pY527 in ***E*** ($n = 4$ independent experiments). Asterisk (*) indicates $p < 0.05$. Con, Control; IB, immunoblotting; IP, immunoprecipitation.

H_2O_2 exerts a TrkB-dependent neuroprotective effect

We next asked whether ROS-mediated transactivation of TrkB may have neuroprotective effects. The destructive consequences of ROS in mammalian cells and its contribution to cell death in acute and chronic insults of the CNS are well documented, suggesting that ROS exert neurotoxic effects on cultured neurons. Interestingly, prosurvival consequences of ROS have also been identified in non-neuronal mammalian cells (Martindale and Bolbrook, 2002; Trachootham et al., 2008; Groeger et al., 2009), raising the possibility of neuroprotective as well as neurotoxic effects of ROS in the CNS. Evidence of cell survival effects of TrkB led us to query whether ROS-mediated transactivation of TrkB may promote neuronal survival. To address these questions, we implemented an assay of cell death in which cortical neurons were exposed to H_2O_2 (250 μM) for 15 min followed by incubation in culture medium lacking the B27 growth supplement for 24 h; cell survival was assessed by measuring LDH release into the culture media (Fig. 8*A*) (Whitney and McNamara, 2000). For this experiment, B27 growth supplement was withdrawn from healthy cultures of cortical neurons, which results in cell death that can be rescued by BDNF (Lee and Chao, 2001; data not shown), thereby demonstrating a TrkB-dependent mechanism in neuronal cell survival. Withdrawal of B27 from cultured medium for 24 h produced significant cell death as evidenced by increased LDH in the media (44 ± 9 units/L, mean ± SEM, $n = 8$ independent experiments) and ~20–30% of total LDH released by hypotonic solution (Neurobasal medium/H_2O: 1:1 for 4 h) (232 ± 10 units/L, mean ± SEM, $n = 5$ independent experiments), consistent with previous findings (Lee and Chao, 2001). Neurotoxic effects of H_2O_2 were evident in the increased LDH following brief exposure to H_2O_2 (Fig. 8*B*,*C*,*E*). To test whether ROS-mediated transactivation of TrkB also exerts neuroprotective effects, we performed three experiments. We first asked whether pharmacological inhibition of Trk signaling would exacerbate H_2O_2-induced cell death. Pretreatment of K252a, a Trk kinase inhibitor, reduced H_2O_2-mediated TrkB activation (Fig. 1*F*,*G*) and resulted in more cell death as evidenced by further increase in H_2O_2 mediated LDH compared

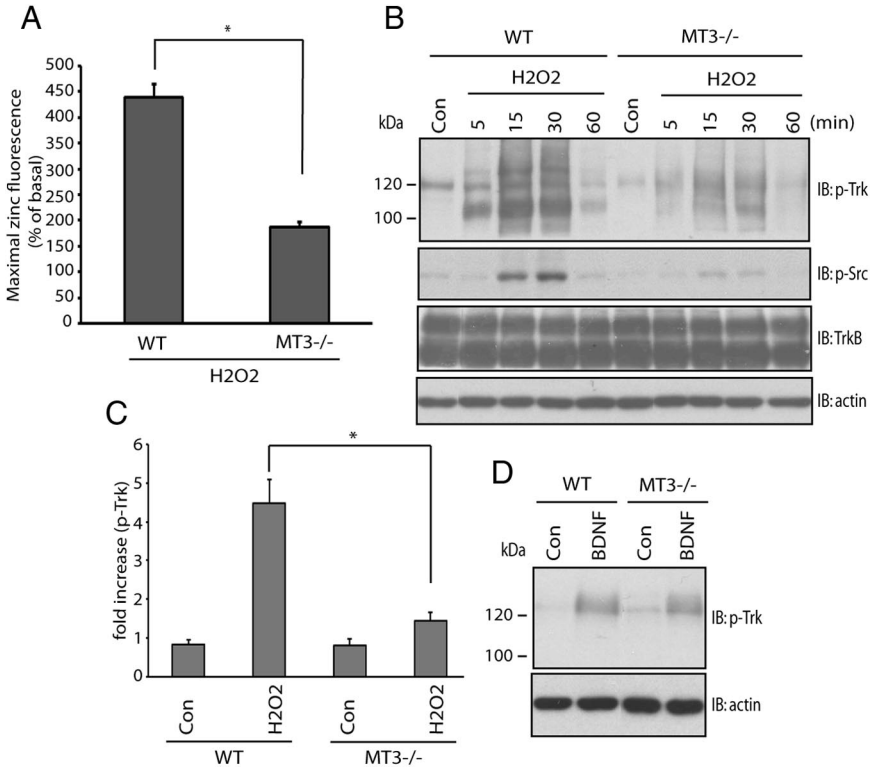

Figure 7. Exogenous H_2O_2 enhanced zinc concentration and phosphorylation of Trk by a metallothionein-3 (MT-3)-dependent mechanism. In all of the experiments below, cortical neurons cultured from P0–P1 pups of either WT or MT-3-null mice were maintained *in vitro* for 7–10 days. ***A***, Exogenous H_2O_2-evoked increased zinc fluorescence was reduced in MT-3 deficient neurons. Cortical neurons cultured from WT or MT-3-null pups were incubated with zinc indicator FluoZin-3 followed by incubation of exogenous H_2O_2 (250 μM). Maximal increases of zinc fluorescence were compared between WT and MT-3 deficient neurons (cell numbers for WT and MT-3 mutant neurons were 157 and 182, respectively; $n = 4–5$ independent experiments). ***B***, Exogenous H_2O_2-evoked increased p-Trk was reduced in MT-3 deficient neurons. WT or MT-3 knockout neurons were incubated with vehicle (Con, control) or H_2O_2 (500 μM) for the indicated periods of time. ***C***, Quantitative analysis of p-Trk (p145) at 15 min in ***B*** ($n = 4$ independent experiments). ***D***, BDNF-mediated increases of p-Trk were not affected in MT-3-deficient neurons. WT or MT-3 knockout neurons were incubated with vehicle (Con, control) or BDNF (10 ng/ml) for 15 min. Asterisk (*) indicates $p < 0.05$. Con, Control; IB, immunoblotting; IP, immunoprecipitation.

with that of H_2O_2 alone (Fig. 8*B*). Next, we used a chemical genetic approach to ask whether inhibition of TrkB kinase resulted in enhanced cell death. Here, TrkB kinase activity was specifically inhibited by 1NMPP1 in cortical neurons cultured from *trkB*^{F616A} mice (Chen et al., 2005). Preincubation of 1NMPP1 inhibited H_2O_2-induced TrkB activation (Fig. 2*C*) and also resulted in enhanced cell death induced by H_2O_2 (Fig. 8*C*). Importantly, 1NMPP1 did not affect H_2O_2-induced increased LDH in cortical neurons cultured from WT mouse (data not shown). Finally, we used a pharmacological approach to uncouple TrkB from its prosurvival signaling pathway and asked whether this uncoupling exacerbated H_2O_2-induced cell death. Phosphorylation of the adaptor protein, Shc, and the subsequent activation of the signaling pathway downstream of Shc, including p-Erk and p-Akt, is known to contribute to the prosurvival effects of TrkB (Huang and Reichardt, 2001, 2003). To selectively uncouple TrkB from the Shc pathway, we designed a phosphopeptide (pY515, IENPQpYFGI, corresponding to amino acids 510–518 of mouse TrkB) that includes the sequence of TrkB that is critical for the binding of Shc. To facilitate access of the pY515 peptide across cell membranes, the protein transduction domain of the viral TAT protein (YGRKKRRQRRR, corresponding to HIV-1 Tat peptide 47–57) was fused to the N terminus of pY515 peptide. A TAT peptide fused with scrambled amino acids of pY515 peptide served as a control. The fusion peptides containing TAT sequence have been demonstrated to permeate plasma membranes of cells *in vitro* in addition to permeating the blood–brain barrier *in vivo* (Aarts et al., 2002). Preincubation of cultured neurons in pY515 peptide (10 μM) but not scrambled peptide reduced H_2O_2-induced activation of the downstream signaling proteins p-Akt and p-Erk induced by H_2O_2 (Fig. 8*D*), thereby demonstrating the efficacy of this peptide for inhibition of TrkB-Shc signaling. Likewise, preincubation of cultured neurons in pY515 peptide resulted in enhanced H_2O_2-induced cell death evident as LDH release as compared to scrambled peptide (Fig. 8*E*). Collectively, these results support the conclusion that ROS-mediated transactivation of TrkB and the subsequent activation of the Shc signaling pathway in turn underlie the prosurvival effects of ROS.

Discussion

We used biochemical, pharmacological, and functional studies of primary cultures of rodent cortical neurons to test whether ROS can transactivate TrkB. The results reveal that both exogenous and endogenous ROS can transactivate TrkB by an intracellular zinc-dependent and SFK-dependent mechanism. One consequence of ROS-mediated transactivation of TrkB is enhanced survival of cultured cortical neurons, revealing a heretofore unrecognized biological effect of ROS in mammalian CNS neurons. We propose a model whereby ROS induce release of intracellular zinc in the cytosol from MT-3, leading to activation of SFK, which phosphorylates and activates TrkB by a neurotrophin-independent mechanism.

ROS transactivate TrkB by an intracellular zinc-dependent mechanism

ROS have diverse biological consequences in physiological and pathological conditions, but the molecular targets mediating these pleiotropic effects are incompletely understood. The neurotrophin receptor TrkB is highly expressed in the developing and mature mammalian brain and, like ROS, has been implicated in diverse physiological and pathological processes (Huang and Reichardt, 2001). Several facts led us to hypothesize that ROS may activate TrkB: (1) ROS can transactivate another RTK, the epidermal growth factor receptor (Goldkorn et al., 1998); (2) ROS can induce zinc release from cytosolic zinc-binding proteins like MT-3 by a redox-regulated mechanism (Aizenman et al., 2000; Maret, 2000; Hwang et al., 2008); and (3) zinc itself can transactivate TrkB (Huang et al., 2008). Our results support the idea that ROS can activate TrkB by an intracellular zinc-requiring mechanism that is independent of BDNF: (1) ROS induce increases of phosphorylation of p145 Trk receptors in cortical neurons in the presence of a BDNF scavenger and in HEK cells that lack endogenous BDNF, implicating a BDNF-independent mechanism; (2) the ROS-induced increase of p-Trk is reduced by

a cell-permeable (TPEN) but not cell-impermeable (CaEDTA) zinc chelator, thereby revealing a requirement for intracellular zinc; (3) the ROS-induced increase of 145 kDa p-Trk is reduced in neurons cultured from mice lacking MT-3, thereby identifying the molecular source of the intracellular zinc. Collectively, these results support a model whereby increasing ROS induces release of zinc from MT-3 protein into the cytosol, leading to transactivation of p145 TrkB in CNS neurons.

Interestingly, in addition to p145 Trk receptors, ROS enhanced phosphorylation of p100 Trk receptors in cortical neurons. Like p145 Trk receptors, ROS-mediated increases of phosphorylation of p100 Trk receptors are dependent on both intracellular zinc and MT-3, but independent of BDNF. Prior studies by multiple investigators reported that reducing glycosylation of Trk receptors expressed in heterologous cells results in migration at ~100 kDa on SDS-PAGE (Klein et al., 1989; Watson et al., 1999), thereby suggesting that the p100 band induced by ROS reflects TrkB protein. We used biochemical, shRNA, and genetic approaches to test the proposal that the increased p100 induced by ROS contains TrkB protein. First, ROS induced increased immunoreactivity to a band migrating at ~100 kDa as detected by distinct antibodies recognizing different phosphorylated tyrosine residues of TrkB protein. Second, following ROS treatment, Western blot analyses of p-Trk immunoprecipitates reveal p100 immunoreactivity using two antibodies specific to TrkB, reinforcing the idea that the p100 band contains TrkB. Third, reduction of TrkB content by shRNA knockdown or in neurons cultured from Y816F TrkB mutant mice results in reduced p100 immunoreactivity following ROS treatment of cortical neurons. Taken together, these findings support the conclusions that the p100 band contains TrkB and that ROS transactivate both p145 and p100 TrkB by an intracellular zinc-dependent mechanism. Enhanced p100 Trk is a feature common to diverse transactivating stimuli. That is, transactivation of TrkB in heterologous cells and cultured neurons by various non-neurotrophin ligands, including adenosine and PACAP (pituitary adenylate cyclase-activating polypeptide) (Lee and Chao, 2001; Jeanneteau and Chao, 2006), results in increased p-Trk immunoreactivity at 100 kDa in SDS-PAGE. Likewise, treatment of both WT mice and mice rendered deficient in BDNF with serotonin selective reuptake inhibitors (SSRIs; e.g., fluoxetine) *in vivo* (Rantamäki et al., 2011) results in increased p-Trk immunoreactivity at 100 kDa. We suspect that the p100 TrkB represents an immature, less glycosylated form of TrkB receptor in neurons.

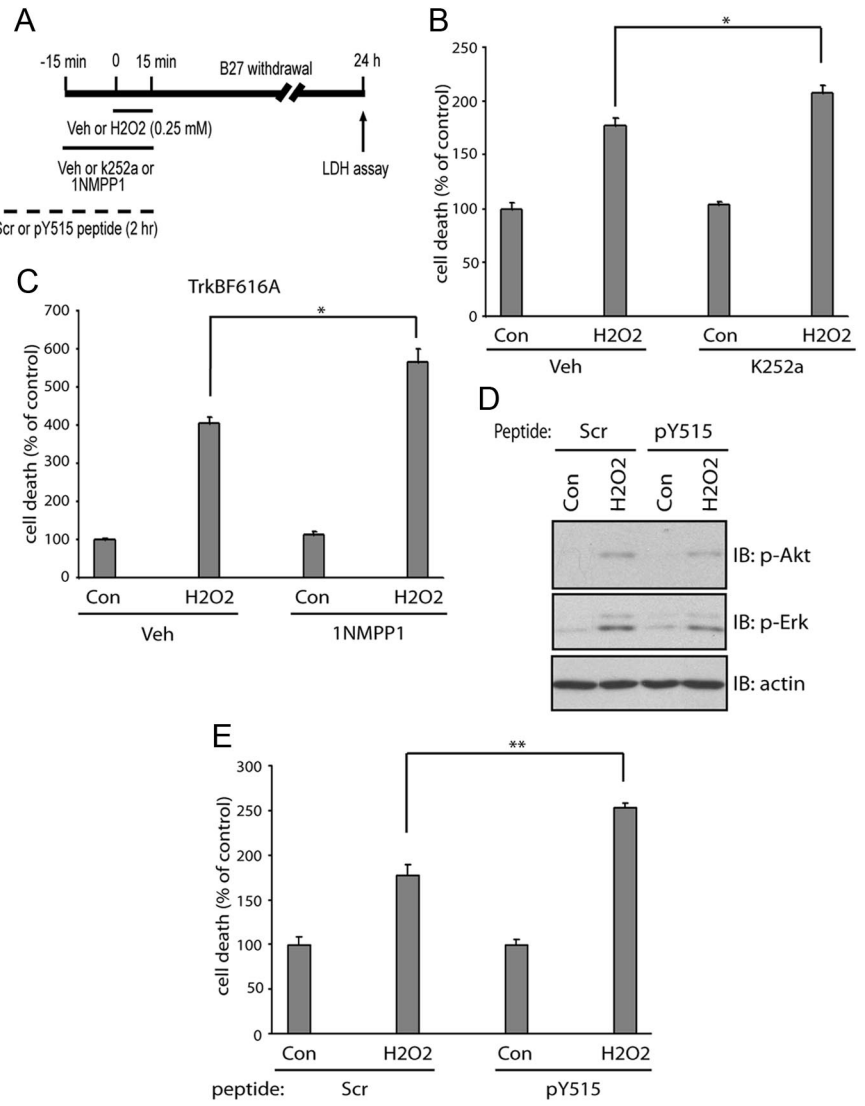

Figure 8. Exogenous H_2O_2-induced activation of TrkB promotes cell survival of cortical neurons in culture. Cortical neurons cultured from E18 rat pups (***B***, ***D***, ***E***) or P0–P1 pups of *trkB*^{F616A} mice (***C***) were maintained *in vitro* for 14–21 and 10–14 days, respectively. ***A***, Schematic of experimental paradigm for assessing neuronal cell death. In brief, cortical neurons were briefly treated with either vehicle (Veh) or H_2O_2 (250 μM) for 15 min and returned to Neurobasal medium lacking B27 supplement. LDH was measured at 24 h after treatments. ***B***, The Trk inhibitor K252a exacerbated exogenous H_2O_2-induced cell death. Cortical neurons were pretreated with either vehicle or K252a (200 nM) for 15 min; each of these treatments were continued for an additional 15 min in the presence of vehicle or H_2O_2 (250 μM) ($n = 4–6$ independent experiments). (***C***) The TrkB inhibitor 1NMPP1 exacerbated exogenous H_2O_2-induced cell death. Cortical neurons were pretreated with either vehicle (Con, Control) or 1NMPP1 (1 μM) for 15 min; each of these treatments was continued for an additional 15 min in the presence of vehicle or H_2O_2 (250 μM; $n =$ 4 independent experiments). ***D***, pY515 peptide reduced H_2O_2-mediated activation of TrkB-Shc signaling. Cortical neurons were pretreated with either scrambled (Scr) or pY515 (10 μM) peptide for 90 min; each of these treatments was continued for an additional 15 min in the presence of vehicle or H_2O_2 (500 μM). Cell lysates were subjected to SDS-PAGE followed by immunoblotting (IB) analysis using the indicated antibodies. ***E***, Uncoupling TrkB-Shc signaling by pY515 peptide exacerbated exogenous H_2O_2-induced cell death. Cortical neurons were pretreated with either scrambled or pY515 (10 μM) peptide for 90 min; each of these treatments was continued for an additional 15 min in the presence of vehicle or H_2O_2 (250 μM; $n = 5–6$ independent experiments). Asterisk (*) indicates $p < 0.05$.

TrkB signaling is activated by a zinc signaling network

The present findings confirm and extend our initial report that zinc can transactivate TrkB in cultured cortical neurons. In mammalian brain, one pool of zinc within cortical neurons is labile or "free" zinc. The labile zinc is packaged in synaptic vesicles (SVs) in subsets of glutamatergic neurons, a process that is tightly con-

trolled by ZnT3, a brain-specific zinc transporter (Cole et al., 1999). Vesicular zinc is released with glutamate into the synaptic cleft by action potentials invading presynaptic terminals, triggering fusion of SVs with plasma membrane (Frederickson et al., 2005). We previously discovered that increased extracellular zinc induced by chemical depolarization of cultured cortical neurons can transactivate synaptic TrkB (Huang et al., 2008). In contrast to free zinc within SVs, a large pool of zinc is bound to cytosolic proteins like MT-3, thereby serving as a "reservoir," and is released from the MT-3 protein by various stimuli, including ROS. In contrast to neuronal depolarization triggering release of zinc into a synaptic cleft, here we showed that regulating cellular redox state enhances zinc content within the cytosol by an MT-3-requiring mechanism that in turn leads to TrkB activation. The TrkB activation evoked by these distinct stimuli and different sources of zinc within CNS neurons has distinct biological consequences. While we previously discovered that synaptic vesicular zinc enhances the efficacy of the hippocampal mossy fiber-CA3 pyramidal synapse (Huang et al., 2008; Pan et al. 2011), here we showed that cytosolic zinc promotes survival of cultured cortical neurons. Collectively, these discoveries reveal that distinct sources of zinc released by distinct cellular and molecular events can transactivate TrkB in CNS neurons, thereby demonstrating the flexibility and complexity of TrkB signaling transactivated by zinc.

One feature common to the transactivation of TrkB by zinc residing in SVs or bound to MT-3 within the cytosol is the requirement for SFKs. A question arises as to whether these two sources of zinc activate SFKs by the same mechanism. Our previous study suggests that extracellular zinc enhances SFK activity through inhibition of Csk activity as evidenced by reduced phosphorylation of Y527 (pY527) of SFKs (Huang et al., 2008; Huang and McNamara, 2010), thereby disinhibiting SFK activity. Indeed, activated Src can directly phosphorylate recombinant TrkB protein in a cell-free system (Huang and McNamara, 2010). In contrast to extracellular zinc, intracellular zinc-dependent activation of SFK induced by ROS seems independent of Csk activity as evidenced by a modest increase of pY527 of SFKs. The mechanism by which ROS activate SFKs in neurons is not clear. One possibility is that ROS might activate SFKs by direct oxidation of SFKs as demonstrated in non-neuronal cells (Giannoni et al., 2005). Whether such a mechanism is operative in CNS neurons needs further investigation.

Potential role of TrkB signaling in physiological and biological consequences of ROS

The present findings reveal that one consequence of increased ROS is enhanced activation of TrkB signaling. These findings raise the question as to what biological consequences of ROS signaling may be mediated by TrkB activation. Increasing evidence suggests that ROS, H_2O_2 in particular, act as signaling molecules in diverse physiological processes of mammalian cells, including cell proliferation, differentiation, migration, and survival (Rhee, 2006; Stone and Yang, 2006; Veal et al., 2007). Yet, the molecular targets by which H_2O_2 mediates these biological consequences are incompletely understood. TrkB signaling itself has been implicated in many of these same biological processes. It will be interesting to determine whether ROS-induced transactivation of TrkB underlies some of these ROS effects.

Excessive levels of ROS are produced and thought to contribute to neuronal injury in diverse acute and chronic neurological disorders, including brain ischemia, status epilepticus, and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Uttara et al., 2009). Whereas the maladaptive consequences of excess ROS, namely oxidative stress and cell injury, are well documented, beneficial consequences of ROS in CNS neurons are underappreciated. The fact that inhibition of TrkB kinase exaggerated cell death induced by H_2O_2 demonstrates a prosurvival effect of TrkB signaling induced by ROS. Moreover, the TrkB-dependent prosurvival effect is mediated at least in part by the Shc pathway, because a phosphopeptide that uncouples TrkB from Shc blocked the neuroprotective effects of TrkB activation. Interestingly, genetic deletion of MT-1/2 or MT-3 in a mutant superoxide dismutase mouse model (G93A SOD1) of familial amyotrophic lateral sclerosis (ALS) resulted in reduced neuronal survival and accelerated disease progression (Puttaparthi et al., 2002), findings consistent with a neuroprotective effect of ROS *in vivo*. Collectively, these findings demonstrate that the well documented neurotoxic consequences of ROS are paralleled by neuroprotective consequences. Moreover, one molecular mechanism of the neuroprotective effect of ROS involves its release of zinc from cytosolic proteins like MT-3, culminating in transactivation of TrkB and activation of Shc signaling. One consideration in pharmacological approaches aimed at neuroprotection in diverse CNS disorders is selectively inhibiting the neurotoxic while preserving the neuroprotective consequences of ROS.

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