Neuron
Article

Zinc-Mediated Transactivation of TrkB Potentiates the Hippocampal Mossy Fiber-CA3 Pyramid Synapse

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DOI 10.1016/j.neuron.2007.11.026

SUMMARY

The receptor tyrosine kinase, TrkB, is critical to diverse functions of the mammalian nervous system in health and disease. Evidence of TrkB activation during epileptogenesis in vivo despite genetic deletion of its prototypic neurotrophin ligands led us to hypothesize that a non-neurotrophin, the divalent cation zinc, can transactivate TrkB. We found that zinc activates TrkB through increasing Src family kinase activity by an activity-regulated mechanism independent of neurotrophins. One subcellular locale at which zinc activates TrkB is the postsynaptic density of excitatory synapses. Exogenous zinc potentiates the efficacy of the hippocampal mossy fiber (mf)-CA3 pyramid synapse by a TrkB-requiring mechanism. Long-term potentiation of this synapse is impaired by deletion of TrkB, inhibition of TrkB kinase activity, and by CaEDTA, a selective chelator of zinc. The activity-dependent activation of synaptic TrkB in a neurotrophin-independent manner provides a mechanism by which this receptor can regulate synaptic plasticity.

INTRODUCTION

The neurotrophin receptor, TrkB, serves a pivotal role not only in neuronal survival and differentiation but also in synaptic structure, function, and plasticity (McAllister et al., 1999; Poo, 2001). TrkB signaling has also been implicated in diverse neurological and psychiatric disorders, including Alzheimer's disease, Huntington's disease, schizophrenia, depression, neuropathic pain, and epilepsy (Chao et al., 2006; Coull et al., 2005; McNamara et al., 2006). A detailed understanding of the mechanisms of activation of TrkB will provide insight into its pleiotropic functions. The external signals mediating activation of TrkB are the neurotrophins, 14 kDa proteins packaged in dense core vesicles of nerve terminals and released in an activity-dependent manner. Neurotrophins that activate TrkB include neurotrophin-4 (NT4) and the prototypic ligand, brain-derived neurotrophic factor (BDNF). These ligands bind to the ectodomain of TrkB and induce its activation by a mechanism that involves dimerization, increased intrinsic kinase activity, autophosphorylation of the receptor, and initiation of downstream signaling pathways (Huang and Reichardt, 2003).

Within cellular signaling networks, crosstalk among diverse stimuli has emerged as an important signaling mechanism combining and diversifying signal transduction pathways. Transactivation refers to the process whereby a given receptor and its downstream signaling is activated by a stimulus that does not interact directly with the receptor (Carpenter, 1999), a mechanism distinct from activation of TrkB by neurotrophins. Previous studies of animal models of epileptogenesis revealed enhanced tyrosine phosphorylation of TrkB (p-TrkB), a surrogate measure of its activation (Chen et al., 2005; Segal et al., 1996), in the mossy fiber pathway of hippocampus. Surprisingly, neither a null mutation of *NT4* nor a conditional deletion of *BDNF* prevented the increase of p-TrkB evident in the mossy fiber pathway during epileptogenesis (He et al., 2004, 2006). This led us to consider the possibility that a non-neurotrophin ligand might be capable of transactivating TrkB.

In search of such a ligand, several lines of evidence led us to hypothesize that the divalent cation, zinc, can transactivate TrkB. The epileptogenesis-induced activation of TrkB is localized to the mossy fiber pathway (Binder et al., 1999; He et al., 2002, 2004), and the mossy fiber axons contain the highest concentration of zinc in mammalian forebrain (Frederickson and Danscher, 1990). Zinc is packaged in synaptic vesicles together with the excitatory transmitter, glutamate, and is released with glutamate by physiological stimulation (Frederickson et al., 2005). Also, marked increases of synaptically released zinc are thought to occur in the mossy fiber pathway during seizures and ischemia (Frederickson et al., 2005). Zinc affects a diversity of ion channels and ion channel receptors (Frederickson et al., 2005). Here we demonstrate that zinc transactivates TrkB by a neurotrophin-independent and Src-dependent mechanism that is regulated by neuronal activity. We further demonstrate a functional consequence of zinc-mediated transactivation of TrkB, namely potentiation of the hippocampal mossy fiber-CA3 pyramid synapse.

RESULTS

Zinc, but Not Other Cations, Activates Trk Signaling in Cultured Neurons in a Concentration- and Time-Dependent Manner

To test whether zinc activates TrkB and its downstream signaling cascades, primary cultures of rat cortical neurons were

incubated with vehicle, BDNF (10 ng/ml), or exogenous zinc (100 μM) for 15 min, and western blots were prepared from culture lysates. As expected, BDNF increased tyrosine phosphorylation of Trk as evidenced by increased immunoreactivity of a 145 kDa band detected by each of two antibodies specific to phosphorylated tyrosine residues on each of the Trk receptors, pY515 and pY705/706 (in the mouse TrkB), respectively (Figure 1A) (Segal et al., 1996). Interestingly, brief incubation with exogenous zinc also increased the phosphorylation of Trk at each of these tyrosine residues (Figure 1A); henceforth, phosphorylation of Trk will be shown with one of these two antibodies. Both BDNF and zinc also enhanced phosphorylation of extracellular regulated kinase (Erk1/2), cAMP-response element binding protein (CREB), and phospholipase Cγ (PLCγ) (Figure 1A), suggesting activation of Trk receptor signaling in cultured neurons by each of these stimuli. The effects of a 15 min exposure to exogenous zinc were concentration dependent, as evidenced by increased phosphorylation of Trk (Figure 1B); the effective concentrations coincide with estimates of zinc concentration in the synaptic cleft ranging from 10 μM to greater than 100 μM following high-frequency stimulation of the mossy fibers (Vogt et al., 2000). Zinc increased p-Erk1/2 with the same concentration dependence as p-Trk, suggesting that activation of p-Erk1/2 is a consequence of Trk activation. The effects of exogenous zinc (100 μM) were also time dependent, with increases in p-Trk evident within 5 min of addition of zinc to the cultures (Figure 1C and see Figure S1A available online). The zinc-mediated increase of p-Trk immunoreactivity was maximal at 30–60 min and declined by 120 min, which is similar to that of BDNF (Figure 1C and Figure S1A), suggesting that zinc might activate Trk kinase. To test whether Trk kinase activity is required for zinc-mediated Trk activation, the effects of a Trk kinase inhibitor, K252a, were examined. Phosphorylation of Trk induced by both BDNF and zinc were inhibited by K252a (Figure 2A). K252a also inhibited both BDNF- and zinc-induced increased phosphorylation of Erk1/2, Akt, and PLCγ (Figure 2A), providing additional evidence of zinc-mediated activation of Trk receptor and its downstream signaling pathways.

The activation of Trk in response to zinc and BDNF led us to query how these stimuli interact in inducing Trk activation. To address this question, cultured neurons were incubated with BDNF alone, varying concentrations of zinc alone, or with BDNF combined with varying concentrations of zinc for 15 min. BDNF and zinc alone each led to increases of Trk phosphorylation and activation of downstream signaling effectors, and the results of simultaneous application appeared to be additive (data not shown). No evidence of synergy was detected.

To determine whether diverse cations are capable of activating Trk, cultured neurons were incubated with zinc, magnesium, calcium, cobalt, manganese, copper, nickel, cadmium, lithium, cesium, potassium, or sodium (100 μM of each) for 15 min. Zinc alone increased the phosphorylation of Trk (Figure S2A), thereby establishing the specificity of the zinc effect.

Zinc Activates TrkB in Cultured Neurons

Since both of the p-Trk antibodies (pY515 and pY705/706) recognize phosphorylation of all three Trk receptors (TrkA, TrkB, and TrkC) on corresponding tyrosine residues (Segal et al., 1996), whether zinc activates TrkB in particular was uncertain. To address this question, primary cultures of rat cortical neurons were treated with either BDNF (10 ng/ml) or zinc (100 μM) for 15 min. The phosphorylated Trk receptors were immunoprecipitated with either the pY515 antibody or a phosphotyrosine-specific antibody (4G10) (Figure 1D, top and middle, respectively); the presence of TrkB in the immunoprecipitates was detected by probing immunoblots with an antibody specific to TrkB. The amounts of TrkB were significantly increased in the immunoprecipitates following treatment with either BDNF or zinc (Figure 1D), thereby demonstrating that zinc activates TrkB in cultured neurons.

To further address whether zinc activates TrkB in cultured cortical neurons, an RNAi strategy was used to selectively reduce expression of TrkB. Initially, oligonucleotides encoding scrambled- or TrkB-shRNA were synthesized and cloned into a shRNA expression vector (pSUPER) under the control of an H1 promoter. To examine the efficacy and specificity of TrkB-shRNA, HEK293 cells were transfected with either TrkB or TrkC and cotransfected with a plasmid expressing either scrambled- (con) or TrkB-shRNA (TrkB). The expression of TrkB but not TrkC was inhibited by 50%–70% in TrkB-shRNA-transfected cells (Figure S2B), supporting the efficacy and specificity of the TrkB-shRNA. To obtain stable expression of shRNA in cultured neurons with high transfection efficiency, lentiviral stocks were prepared to express either LV-scrambled- (con) or LV-TrkB-shRNA (TrkB) and GFP driven by H1 and CMV promoters, respectively. Cultured cortical neurons were transduced with con- or TrkB-shRNA lentiviral vectors and 10–12 days later were incubated with either vehicle or zinc (100 μM) for 15 min. The LV-TrkB-shRNA reduced expression of TrkB, but not TrkC, by ~80% in comparison to LV-con-shRNA (Figure 1E and Figure S3A), supporting the efficacy and specificity of the LV-TrkB-shRNA. Brief incubation of zinc stimulated phosphorylation of Trk in LV-con-shRNA-treated neurons as detected by immunoblotting; by contrast, the zinc-stimulated phosphorylation of Trk was reduced by ~70% in LV-TrkB-shRNA-transduced neurons (Figure 1E and Figure S3B). Together with the immunoprecipitation experiments, the reduction of Trk activation by LV-TrkB-shRNA demonstrates that zinc activates TrkB in cultured cortical neurons.

Zinc Activates TrkB Signaling by a BDNF-Independent Mechanism

Zinc has been demonstrated to activate a metalloproteinase that cleaves heparin-binding-EGF (Wu et al., 2004) or pro-EGF (Le Gall et al., 2003), resulting in increased concentrations of mature EGF and the subsequent activation of the EGF receptor tyrosine kinase. While the current studies were underway, Hwang et al. (2005) reported that zinc increases Trk signaling by activating a metalloprotease to increase the production of mature BDNF. Three experiments were performed to determine whether zinc activates TrkB by simply increasing production of mature BDNF. If zinc activated TrkB signaling indirectly by promoting cleavage of pro-BDNF, then scavenging BDNF with TrkB-Ig would be expected to limit zinc-mediated activation of TrkB. Cultured neurons were pretreated with TrkB-Ig for 30 min followed by exposure to BDNF (10 ng/ml) or zinc (100 μM) for 15 min.

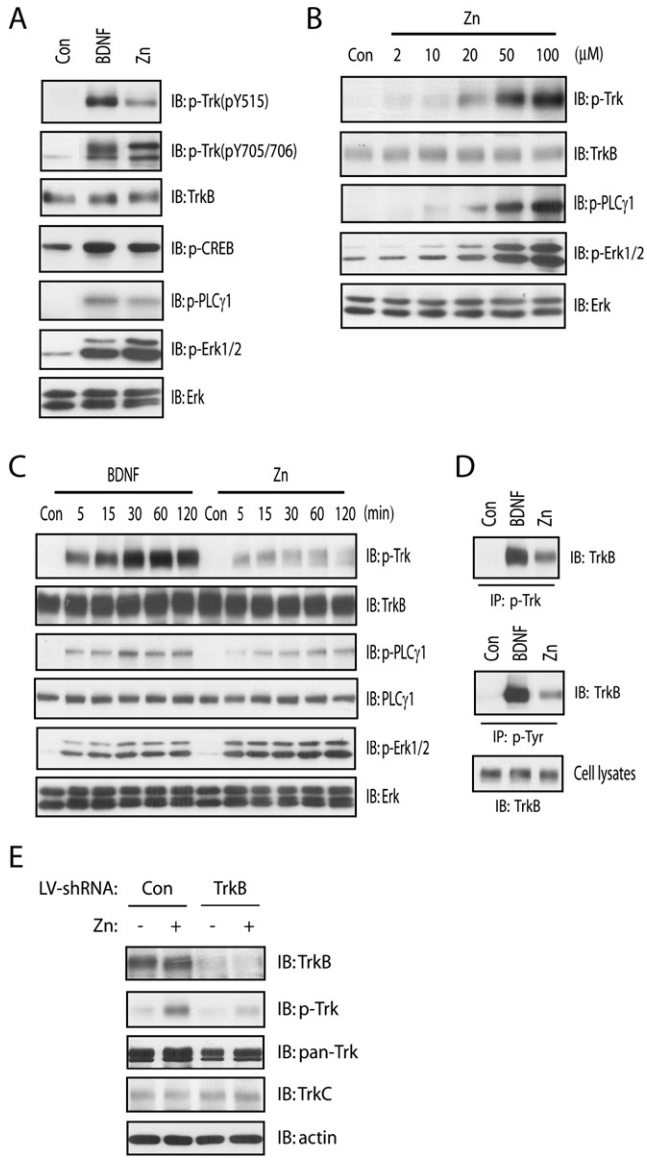

Figure 1. Zinc Activates TrkB in Cultured Cortical Neurons

In all of the experiments below, cortical neurons cultured from E18 rat pups were maintained in vitro for 12–14 days. Unless specified otherwise, cell lysates were subjected to immunoblotting with the indicated antibodies after the treatments described.

(A) Exogenous zinc induces Trk activation. Cortical neurons were incubated with vehicle, BDNF (10 ng/ml), or zinc (100 μM) for 15 min.

(B) Exogenous zinc activates Trk signaling in a concentration-dependent manner. Cortical neurons were incubated with varying concentrations of exogenous zinc for 15 min.

(C) Exogenous zinc activates Trk signaling in a time-dependent manner. Cortical neurons were incubated with BDNF (10 ng/ml) or zinc (100 μM) for the indicated periods of time.

(D) Exogenous zinc activates TrkB. Cortical neurons were incubated with vehicle, BDNF (10 ng/ml), or zinc (100 μM) for 15 min. Cell lysates were immunoprecipitated with anti-p-Trk (pY515) and anti-phosphotyrosine (4G10) antibodies, respectively. The immunoprecipitates were subjected to immunoblotting with an antibody specific to TrkB.

(E) TrkB-shRNA specifically reduces TrkB protein and zinc-induced TrkB activation in cultured cortical neurons. Cortical neurons (DIV5) were transduced with lentiviral stocks expressing scrambled -shRNA (Con) or TrkB-shRNA (TrkB) for 10–12 days, followed by incubation with vehicle or zinc (100 μM) for 15 min.

Pretreatment with TrkB-Ig for 30 min abolished BDNF-induced phosphorylation of TrkB as well as downstream signaling effectors including Erk1/2, Akt, and PLCγ (Figure 2A); by contrast, TrkB-Ig had no effect on zinc-induced phosphorylation of TrkB or its downstream signaling effectors, suggesting that the action of zinc is independent of BDNF. Likewise, addition of TrkB-Ig to hippocampal slices acutely isolated from wild-type (WT) mice prevented BDNF, but not zinc, mediated increase of p-Trk (Figure 2B). Next, we asked whether zinc could activate TrkB in neurons cultured from mice carrying a null mutation of *BDNF*. Addition of zinc (100 μM) for 15 min to cortical neurons cultured from *BDNF* mutant mice resulted in increased activation of TrkB similar to that of WT controls (Figure 2C). Finally, we asked whether addition of zinc is sufficient to activate TrkB in heterologous cells expressing TrkB. Briefly (15 min) treating HEK293 cells stably expressing recombinant TrkB with zinc (500 μM) or BDNF resulted in increased p-TrkB as detected in immunoprecipitation experiments (Figure 2D). Taken together, these findings demonstrate that zinc activates TrkB by a BDNF-independent mechanism in cultured cortical neurons, hippocampal slices, and heterologous cells.

Zinc Ionophore Facilitates Zinc-Mediated TrkB Activation

The evidence that zinc activates TrkB by a BDNF-independent mechanism together with the fact that extracellular zinc can permeate neurons (Frederickson et al., 2005) raised the possibility that bath-applied zinc gains access to an intracellular locale in order to activate TrkB. If so, inclusion of a zinc ionophore should enhance activation of TrkB by bath-applied zinc. Inclusion of the zinc ionophore, sodium pyrithione, enhanced zinc-mediated activation of TrkB (Figure 2E); these effects were virtually eliminated by inclusion of the cell-impermeable selective chelator of zinc, CaEDTA (Koh et al., 1996), thereby supporting the conclusion that the mechanism of pyrithione potentiation involves zinc. Addition of zinc to PC12 cells stably expressing TrkB was not sufficient to activate TrkB; by contrast, addition of zinc in the presence of the zinc ionophore markedly enhanced TrkB activation (Figure S4). Together these findings support the conclusion that zinc permeates these neurons and accesses an intracellular locale in order to activate TrkB.

Neuronal Activity Facilitates TrkB Activation by Zinc

The evidence that zinc permeates neurons to enhance TrkB activation suggested that increased neuronal activity may facilitate the effects of zinc. That is, a subset of endogenous zinc is packaged in synaptic vesicles of presynaptic terminals of excitatory neurons in the CNS and is released with membrane depolarization (Frederickson et al., 2005). In addition to promoting release, increased neuronal activity facilitates zinc permeation of cortical neurons by promoting activation of NMDA receptors, AMPA receptors lacking the GluR2 subunit, and voltage-gated calcium channels (VGCC), each of which can serve as a route of entry for zinc (Frederickson et al., 2005). We first asked whether

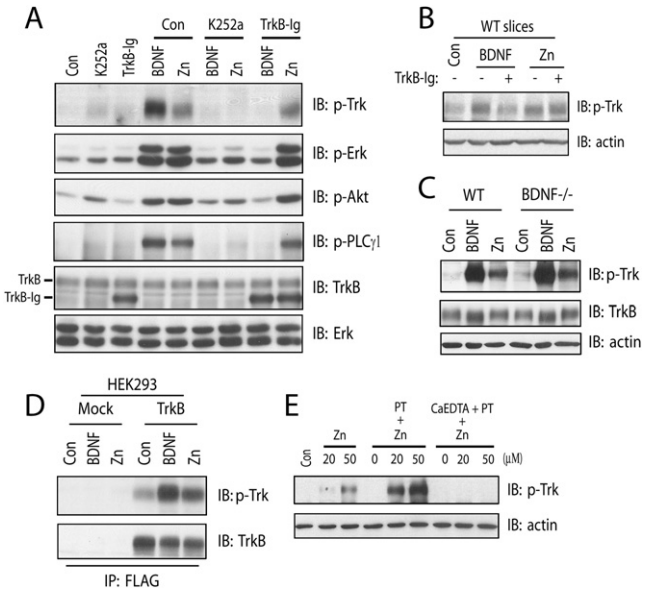

Figure 2. Zinc Activates TrkB Signaling by a BDNF-Independent Mechanism

In the experiments below, cortical neurons were cultured from E18 rat pups (A and E) or mouse P1 pups (C) and maintained in vitro for 12–14 days. Unless specified otherwise, cell lysates were subjected to immunoblotting with the indicated antibodies after the treatments described.

(A) BDNF- but not zinc-induced activation of TrkB signaling is inhibited by neurotrophin scavenger, TrkB-Ig in cultured neurons. Cortical neurons were preincubated with vehicle, TrkB-Ig (2 μg/ml), or K252a (100 nM) for 30 min, followed by a 15 min incubation with vehicle, BDNF (10 ng/ml), or zinc (100 μM).

(B) BDNF- but not zinc-induced activation of TrkB signaling is inhibited by TrkB-Ig in hippocampal slices. Hippocampal slices acutely isolated with WT mice were preincubated with vehicle or TrkB-Ig (2 μg/ml) for 2 hr, followed by a 20 min incubation with vehicle, BDNF (10 ng/ml), or zinc (100 μM).

(C) Zinc activates Trk in cortical neurons of *BDNF*$^{-/-}$ mice. Cortical neurons cultured from WT and *BDNF* null mutant pups (P1) were incubated with vehicle, BDNF (10 ng/ml), or zinc (100 μM) for 15 min.

(D) Zinc activates TrkB in heterologous cells. HEK293 cells stably expressing C-terminal FLAG-epitope-tagged TrkB were incubated with vehicle, BDNF (10 ng/ml), or zinc (500 μM) for 15 min. Cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with p-Trk antibody.

(E) Zinc ionophore facilitates the zinc-induced tyrosine phosphorylation of TrkB in cultured neurons. Cortical neurons were incubated with vehicle, varying concentrations of zinc, or varying concentrations of zinc together with its specific ionophore pyrithione (PT, 20 μM), or varying concentrations of zinc together with both PT (20 μM) and zinc chelator, CaEDTA (1 mM), for 15 min.

neuronal depolarization might promote TrkB activation in cultured cortical neurons in the absence of exogenous zinc. Addition of 50 mM KCl to these cells resulted in a modest increase of Trk phosphorylation (Figure 3A and Figure S5); this increase was eliminated by inclusion of CaEDTA, implying that the effect required zinc. By contrast, addition of TrkB-Ig had little effect on the KCl-induced increase in phosphorylation of Trk, implying that BDNF or TrkB-binding neurotrophins were not required (Figure 3A and Figure S5). The effect of KCl was inhibited by nifedipine and APV but not CNQX, suggesting that endogenous zinc gained access to these neurons through voltage-gated calcium channels and NMDA but not AMPA receptors (Figure 3A and Figure S5). Notably, the magnitude of TrkB activation mediated by either BDNF or zinc endogenous to these cultures was modest by comparison to the effects of exogenous BDNF or zinc. Whether these modest effects are due to low content of BDNF and vesicular zinc in these cultures, rapid diffusion from the site of release into the culture media, or some other factor is uncertain.

To verify that addition of KCl induced phosphorylation of TrkB in particular, lysates were prepared following incubation of cultures with 50 mM KCl or vehicle for 15 min followed by immunoprecipitation and were subjected to SDS-PAGE. Probing these immunoblots with an antibody specific to TrkB revealed that 50 mM KCl increased phosphorylation of TrkB, an effect eliminated by CaEDTA and partially reduced by APV (Figure 3B). In sum, these findings support the conclusion that depolarization induced by high potassium leads to a modest activation of TrkB by zinc endogenous to these cultures, an effect requiring NMDA receptors and/or voltage-gated calcium channels.

Synaptic NMDA Receptors Contribute to Neuronal-Activity-Regulated Activation of TrkB Activation by Zinc

To further explore the route by which neuronal activity promotes zinc permeation of neurons to activate TrkB, we studied the effects of membrane depolarization on TrkB activation in the presence of exogenous zinc. We first asked whether the effects of exogenous zinc were enhanced by KCl. Addition of 50 mM KCl markedly enhanced zinc-mediated phosphorylation of TrkB (Figure 3C), an effect reduced by antagonists of NMDA (MK801) or AMPA (CNQX) receptors or a VGCC blocker (nifedipine) (Figure 3D). Importantly, the KCl-mediated enhancement of zinc-induced p-Trk was not reduced by inclusion of TrkB-Ig, demonstrating its independence of neurotrophin ligands of TrkB (Figure S6B). Interestingly, addition of bicuculline, a $GABA_A$ receptor antagonist, enhanced KCl-mediated effects of exogenous zinc (Figure 3E), suggesting that synaptic activity may regulate zinc-induced TrkB activation. To test whether blockade of synaptic NMDA receptors attenuated these effects of exogenous zinc, synaptic NMDA receptors were first blocked by preincubation with bicuculline and MK801 (Hardingham et al., 2002), and the cultured neurons were subsequently exposed to zinc in the presence of 50 mM KCl for 15 min. These experiments confirmed that 50 mM KCl in the presence of bicuculline enhanced zinc-mediated activation of TrkB (compare c with b in Figure 3F). Preincubation with MK801 markedly reduced the zinc-mediated activation of Trk in the presence of 50 mM KCl (compare d with c in Figure 3F), implying that activation of synaptic NMDA receptors mediate some of the enhancing effects of 50 mM KCl.

If zinc is permeating synaptic NMDA receptors to activate TrkB, then one locale within the cell at which zinc activates TrkB might be the postsynaptic densities (PSD) of excitatory synapses. To test this possibility, cultured cells were incubated with zinc (200 μM) for 15 min, homogenized, and subjected to subcellular fractionation and sucrose density centrifugation. The presence of synaptophysin and PSD-95 in the presynaptic and PSD fractions, respectively, validated the separation procedures (Figure 3G, bottom two panels). Both full-length and truncated TrkB were present in the synaptosomal and presynaptic fractions, but full-length TrkB alone was detected in the PSD fraction

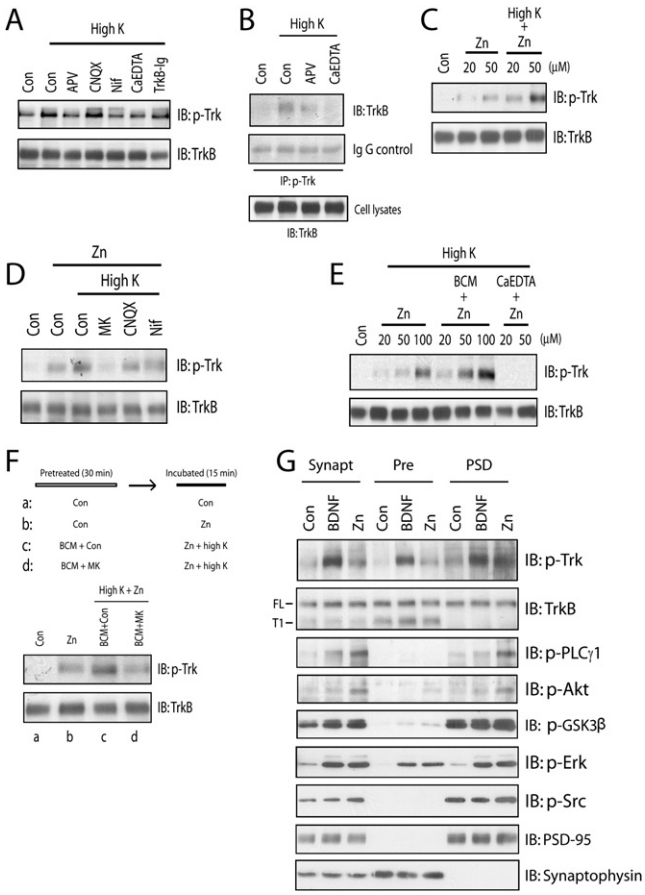

Figure 3. Neuronal Activity Facilitates Zinc-Induced Activation of TrkB

In the experiments below, cortical neurons cultured from E18 rat pups were maintained in vitro for 12–14 days unless specified otherwise. Cell lysates were subjected to immunoblotting with the indicated antibodies after the treatments described.

(A and B) Activity facilitates endogenous zinc-induced TrkB activation. Cortical neurons were preincubated with APV (50 μM), CNQX (50 μM), nifedipine (10 μM), CaEDTA (1 mM), or TrkB-Ig (2 μg/ml) for 30 min; each of these treatments was continued for an additional 30 min in the presence of a high (50 mM) K^+ buffer. TrkB-Ig eliminated BDNF-mediated increase of p-Trk in the presence of elevated KCl (50 mM) (data not shown), thereby demonstrating the efficacy of TrkB-Ig under these experimental conditions.

(C) Activity facilitates exogenous zinc-induced TrkB activation. Cortical neurons were incubated with varying concentrations of zinc in the presence of buffer containing normal (3 mM) or high (50 mM) K^+ for 15 min.

(D) NMDA receptors (NMDARs), AMPA receptors, and voltage-gated calcium channels (VGCC) contribute to activity-regulated zinc-induced TrkB activation. Cortical neurons were preincubated with MK801 (10 μM), CNQX (50 μM), or nifedipine (10 μM) for 30 min; each of these treatments was continued for an additional 15 min in the presence of zinc (50 μM) in a buffer containing normal (3 mM) or high K^+ (50 mM).

(E) Bicucculine enhances Trk activation induced by zinc in the presence of high (50 mM) K^+. Cortical neurons were incubated with high K^+ buffer and varying concentrations of zinc in the presence of vehicle, bicucculine (BCM, 50 μM), or CaEDTA (1 mM) for 15 min.

(F) Synaptic NMDARs contribute to activity-regulated zinc-induced TrkB activation. Synaptic NMDARs were blocked by preincubation of cortical neurons in BCM in the presence of MK801 (10 μM) for 30 min prior to the addition of exogenous zinc (50 μM) in the presence of normal (3 mM) or high (50 mM) K^+ for 15 min.

(G) Zinc activates TrkB signaling at excitatory synapses of cortical neurons. Cortical neurons (DIV21) were incubated with vehicle, BDNF (10 ng/ml), or zinc (200 μM) for 15 min. The synaptosome (Synapt), presynaptic membrane (Pre), and PSD fractions were isolated from cortical neurons incubated with vehicle, BDNF, or zinc; and 2 μg of protein of each fraction was analyzed by immunoblotting.

(Figure 3G, top panel) (Wu et al., 1996). Addition of exogenous zinc increased p-Trk immunoreactivity in the PSD fraction and also activated downstream signaling cascades locally in the PSD, as evident in the increased p-Erk, p-Akt, p-Src, p-PLCγ, and p-GSK3β immunoreactivity (Figure 3G). Taken together, these data support the conclusion that neuronal depolarization facilitated permeation of neurons by zinc in part through synaptic NMDA receptors and activates TrkB locally in the spines of these synapses. The zinc-mediated increase of p-Trk immunoreactivity in the presynaptic fraction (Figure 3G) suggests that zinc may also permeate presynaptic terminals and activate TrkB presynaptically as well as postsynaptically.

Zinc-Mediated Transactivation of TrkB Requires Src Family Kinase

Evidence that zinc acts at an intracellular locale to activate TrkB provided a clue to the molecular mechanism of the transactivation. We considered an Src family kinase (SFK) in particular because zinc can activate another receptor tyrosine kinase, the EGFR, through SFK (Wu et al., 2002). Src and Fyn are two SFKs that are highly expressed in brain and in the PSD in particular (Salter and Kalia, 2004). We therefore asked whether zinc activated SFK in these cultured neurons. Brief (15 min) treatment of cultured neurons with zinc (100 μM) resulted in increased phosphorylation of the catalytic site (pY416 in the prototypical chicken c-Src) of SFK, a surrogate measure of SFK activation (Cooper and Howell, 1993) (Figure 4A, second panel, and Figure S7B), implying that zinc activates SFK. Addition of the zinc ionophore, pyrithione, further increased the zinc-induced phosphorylation (pY416) of SFK, suggesting that increasing zinc concentration within the cells facilitated its activation of SFK (Figure 4A and Figure S7B). Inclusion of CaEDTA abolished the zinc-induced SFK activation (Figure 4A and Figure S7B), thereby implicating zinc in SFK activation. The activation of SFK by zinc is both time and concentration dependent (second panel of Figures 4B and 4C, respectively, and Figures S8A and S9A) and correlates with zinc-mediated phosphorylation of Trk (first panel of Figures 4B and 4C, respectively).

The correlation between the zinc-mediated activation of SFK and Trk led us to hypothesize that SFK is required for zinc-induced transactivation of TrkB in neurons. Two experimental approaches were employed to test this hypothesis. First, addition of the selective inhibitors of SFK, PP1 and PP2, virtually eliminated the effects of zinc on phosphorylation of both SFK and Trk in a concentration-dependent fashion (Figure 4D). The effects of PP1 and PP2 were likely mediated by inhibition of SFK because PP3, a structural analog lacking SFK inhibitory activity, had little effect (Figure 4D). Next, we examined zinc-mediated transactivation of TrkB in a mutant fibroblast cell line (SYF cells) in which the SFKs Src, Yes, and Fyn had been deleted. The effects of zinc were compared in control and SYF cells, each of

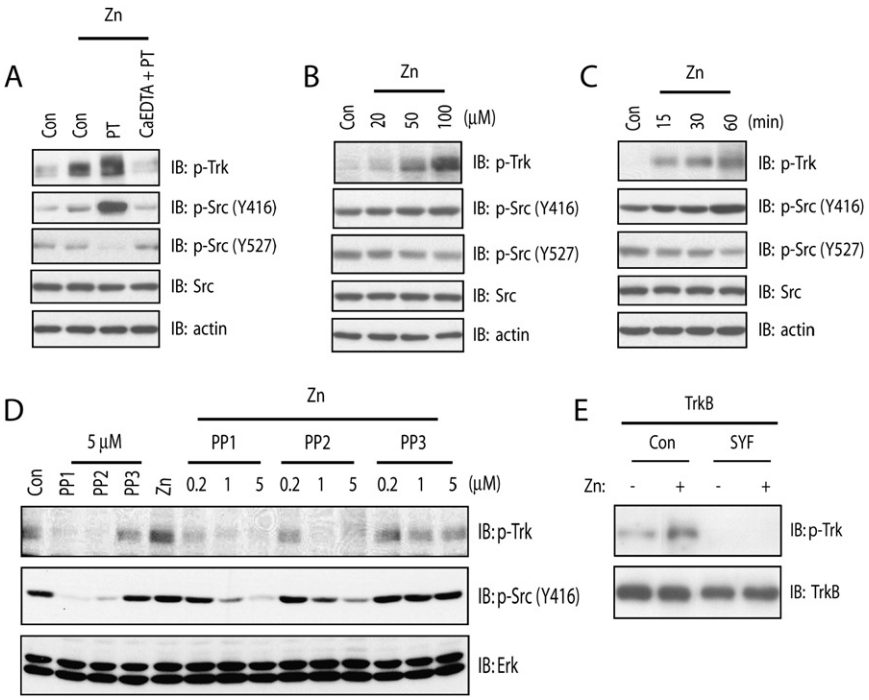

Figure 4. Src Family Kinase Is Required for Zinc-Induced TrkB Activation

In the experiments below, cortical neurons cultured from E18 rat pups were maintained in vitro for 12–14 days. Cell lysates were subjected to immunoblotting with the indicated antibodies after the treatments described. Tyrosine phosphorylation of Src was measured by immunoblotting with p-Src (pY416) or p-Src (pY527) antibodies.

(A) Zinc increases activity of SFK. Cortical neurons were incubated with zinc alone (100 μM), zinc together with zinc ionophore (PT, 20 μM), zinc plus PT (20 μM), and the zinc chelator, CaEDTA (1 mM), for 15 min.

(B) Exogenous zinc activates SFK in a concentration-dependent manner. Cortical neurons were incubated with varying concentrations of exogenous zinc for 15 min.

(C) Exogenous zinc activates SFK in a time-dependent manner. Cortical neurons were incubated with zinc (100 μM) for the indicated periods of time.

(D) SFK activation is required for zinc-induced phosphorylation of Trk. Cortical neurons were preincubated with varying concentrations of Src inhibitors, PP1 or PP2, or the inactive analog, PP3, for 30 min; each of these treatments was continued for an additional 15 min in the presence of vehicle or zinc (100 μM).

(E) Zinc-mediated phosphorylation of TrkB is impaired in SYF fibroblast cells. The control and SYF cells were transfected with TrkB. Twenty-four hours after transfection, the cells were incubated with vehicle or zinc (500 μM) for 15 min.

which had been transfected with TrkB. Addition of zinc increased phosphorylation of TrkB in control, but not SYF cells (Figure 4E), thereby demonstrating the requirement for SFK for zinc-induced transactivation of TrkB.

The requirement of SFK for zinc-mediated transactivation of Trk led us to investigate the mechanism by which zinc activates SFK. Prior studies demonstrated that SFK is inactivated by the interaction of its C-terminal tyrosine residue (Y527) and N-terminal SH2 domain in an intramolecular fashion (Cole et al., 2003) . This autoinhibition requires phosphorylation of Y527, which is mediated by another protein tyrosine kinase, C-terminal Src kinase (Csk) (Cole et al., 2003). The autoinhibition of this conformation of SFK is relieved by dephosphorylation of Y527, leading to activation of SFK, reflected by an increase of phosphorylation of Y416 (Cooper and Howell, 1993). Interestingly, zinc binds to Csk with high affinity and potently inhibits its kinase activity (IC_{50}, 0.5 μM) (Sun and Budde, 1999) . If zinc activates SFK by inhibition of Csk activity, this predicts that addition of zinc will reduce phosphorylation of Y527 and subsequently increase phosphorylation of Y416. In accord with this prediction, brief (15 min) addition of zinc to cultured neurons produced a modest reduction of pY527, paralleled by a modest increase of pY416, changes that were markedly enhanced by inclusion of the zinc ionophore (Figure 4A, panels two and three, respectively, and Figures S7B and S7C). The zinc-mediated reduction of pY527 and increase of pY416 were both concentration (Figure 4B, panels two and three, and Figure S8) and time (Figure 4C, panels two and three, and Figure S9) dependent. Importantly, these zinc-mediated modifications of pY527 and pY416 correlated with increased pTrk content (Figures 4A–4C and Figure S7A), further implicating SFK in zinc-mediated activation of Trk. Together these findings suggest a signaling mechanism by which zinc transactivates TrkB. That is, increased intracellular zinc inhibits Csk, which results in dephosphorylation of the C-terminal inhibitory tyrosine; relief of this autoinhibition of SFK leads to activation of SFK and subsequent phosphorylation and activation of TrkB.

Zinc Potentiates Mossy Fiber-CA3 Pyramid Synapse by a TrkB-Requiring Mechanism Independent of BDNF

One property of neurons regulated by TrkB is the plasticity of excitatory synapses, including the Schaffer collateral-CA1 synapse of hippocampus (McAllister et al., 1999; Poo, 2001). Whether TrkB signaling regulates plasticity of the mossy fiber-CA3 pyramid synapse is unknown. The biochemical evidence that zinc promotes activation of TrkB at excitatory synapses in an activity-dependent manner raised the question as to whether zinc might regulate the function of an excitatory synapse in a TrkB- and activity-dependent manner. We focused on the mossy fiber axons of the dentate granule cells because endogenous zinc is highly enriched in mossy fiber terminals, and our earlier study suggested that TrkB undergoes transactivation in the mossy fiber pathway during epileptogenesis in vivo. The mossy fibers provide excitatory glutamatergic synaptic input to both CA3 pyramids and inhibitory interneurons in stratum lucidum, the latter providing powerful feedforward inhibition of the CA3 pyramids.

We selected the mf-CA3 pyramid synapse because the PSD is a component of excitatory synapses on dendritic spines, a structural specialization common to principal cells.

The prior finding that bath-applied zinc induced long-lasting potentiation of the mf-CA3 pyramid synapse in hippocampal slices isolated from adult rats (Li et al., 2001) led to our initial focus on the effects of bath-applied zinc. A brief (20 min) application of zinc (100 μM) to the solution bathing hippocampal slices acutely isolated from an adult mouse was sufficient to enhance Trk activation, as evidenced by enhanced p-Trk content detected in immunoblots (Figure 5C). Moreover, bath application of zinc increased the efficacy of the mf-CA3 pyramid synapse, as evident in a 37% increase of the mf-evoked fEPSP in comparison to vehicle-treated control slices (vehicle: 99% ± 4.2%, n = 6; Zn: 135% ± 4.5%, n = 8; $p < 0.01$, t test; Figure 5A), thereby confirming and extending prior findings in slices of rats (Li et al., 2001). Importantly, the zinc-mediated enhanced efficacy of the mf-CA3 pyramid synapse required TrkB because it was virtually eliminated in hippocampal slices isolated from *TrkB* conditional null mutant mice (WT: 132% ± 4.5%, n = 10; $TrkB^{-/-}$: 100% ± 6%, n = 8; $p < 0.001$, t test; Figure 5B).

Use of a chemical genetic approach revealed that the potentiating effects of zinc on the mf-CA3 pyramid synapse required not only TrkB but also TrkB kinase activity. Introduction of a phenylalanine-to-alanine substitution within kinase subdomain V ($TrkB^{F616A}$) of *TrkB* exon 14 in the region of the TrkB kinase ATP binding pocket (Chen et al., 2005) renders $TrkB^{F616A}$ sensitive to specific inhibition by a membrane-permeable, small-molecule PP1 derivative, 1NMPP1 (Bishop et al., 2000). Addition of 1 μM 1NMPP1 to hippocampal slices isolated from $TrkB^{F616A}$ mice reduced the zinc-mediated enhancement of mf-CA3 fEPSP by ~50% (vehicle: 125% ± 4.8%, n = 9; 1NMPP1: 111% ± 2.5%; n = 10, $p < 0.05$, t test; Figure 5D). The partial reduction of the zinc-mediated enhancement of the mf-CA3 fEPSP by 1NMPP1 in slices isolated from the $TrkB^{F616A}$ mice was paralleled by a partial reduction of zinc-mediated increase of p-Trk immunoreactivity (Figure 5C) as revealed by immunoblotting. Importantly, 1NMPP1 (1 μM) did not inhibit the zinc-mediated enhancement of mf-CA3 fEPSP in slices isolated from *TrkB* WT mice (vehicle: 127% ± 2%, n = 6; 1NMPP1: 123% ± 7.1%; n = 6, $p > 0.05$, t test; Figure 5E).

Studies of hippocampal slices from *BDNF* mutant mice revealed that these effects of zinc did not require the prototypic TrkB ligand, BDNF. That is, bath application of zinc enhanced the fEPSP of the mf-CA3 pyramidal cell synapse to a similar extent in slices from WT and *BDNF* conditional null mutant mice (WT: 122% ± 3%, n = 13; $BDNF^{-/-}$: 124% ± 4%; n = 12, $p > 0.05$, t test; Figure 5F). Together these findings establish a functional consequence of zinc-mediated transactivation of TrkB, namely potentiation of the mf-CA3 pyramid synapse.

TrkB Is Required for LTP of Mossy Fiber-CA3 Pyramid Synapse

The fact that TrkB is required for zinc-mediated potentiation of the mf-CA3 pyramid synapse raised the question as to whether TrkB is also required for high-frequency stimulation (HFS) induced LTP of this synapse. To address this question, two distinct approaches were employed, namely elimination of the TrkB protein itself in conditional null mutants or selective inhibition of kinase activity intrinsic to TrkB in $TrkB^{F616A}$ mice. Analyses of the mf-CA3 pyramid synapse in slices from *TrkB* conditional null mutants revealed paired-pulse facilitation (PPF) of the fEPSP similar to that found in slices of WT mice (PPF: WT, 2.04 ± 0.1, n = 10; $TrkB^{-/-}$, 2.11 ± 0.08, n = 10, $p = 0.54$, t test). By contrast, the HFS-induced increase of the mf-CA3 pyramid fEPSP was reduced by ~60% in slices from *TrkB* conditional null mutant mice compared to WT littermate controls. (WT: 161% ± 10.6%, n = 12; $TrkB^{-/-}$: 124% ± 7.4%, n = 9; $p < 0.01$, t test; Figure 6A), thereby demonstrating a requirement for TrkB in mf-CA3 LTP. Selective inhibition of TrkB kinase activity also inhibited HFS-induced LTP of the mf-CA3 pyramid synapse. HFS of the mossy fibers increased the amplitude of the mf-CA3 fEPSP in vehicle-treated slices of $TrkB^{F616A}$ mice by ~60%; inclusion of 1NMPP1 (1 μM) prevented this increase (vehicle: 158% ± 4.8%, n = 7; 1NMPP1: 104% ± 9.3%, n = 8; $p < 0.001$, t test; Figure 6B). Importantly, the effect of 1NMPP1 was specific to the $TrkB^{F616A}$ mice because inclusion of 1NMPP1 had no effect on LTP of the mf-CA3 pyramid synapse in slices from WT mice (vehicle: 163% ± 5.5%, n = 7; 1NMPP1: 173% ± 13.2%, n = 7; $p > 0.05$, t test; Figure 6C). Inclusion of 1NMPP1 did not modify paired-pulse facilitation of the mf-CA3 pyramid synapse in either $TrkB^{F616A}$ or WT mice (PPF: vehicle, 1.95 ± 0.05, n = 9; 1NMPP1, 2.03 ± 0.08, n = 8, $p = 0.42$, t test). Moreover, these effects were specific to the mf-CA3 pyramid synapse in that 1 μM 1NMPP1 did not inhibit HFS-induced LTP of the Schaffer collateral-CA1 synapse in slices of $TrkB^{F616A}$ mice (vehicle: 200% ± 22.4%, n = 7; 1NMPP1: 193% ± 22.8%, n = 7; $p > 0.05$, t test; Figure 6D), underscoring the selectivity of contribution of TrkB signaling in regulating the plasticity of mf-CA3 pyramid synapse. Together these findings establish the requirement for TrkB and, in particular, TrkB kinase activity for LTP of the mf-CA3 pyramid synapse.

LTP of Mossy Fiber-CA3 Pyramid Synapse Is Partially Inhibited by Conditional Deletion of BDNF and by Zinc Chelator

The requirement of TrkB for LTP of the mf-CA3 pyramid synapse in turn raised the question as to which ligand(s) activated TrkB under these conditions. We addressed the contribution of BDNF by study of slices isolated from WT and *BDNF* conditional null mutant mice. The magnitude of the increase of the fEPSP induced by HFS of the mossy fibers was reduced by about 40% in slices of *BDNF* null mutant compared to WT mice (WT: 154% ± 8%, n = 18; $BDNF^{-/-}$: 134% ± 4%, n = 17; t test, $p < 0.05$; Figure 7A). To assess the contribution of zinc, we examined the effects of CaEDTA, a selective chelator of zinc (Koh et al., 1996); we selected a concentration (7.5 mM) similar to that which prevents increased zinc content in CA3 pyramids evoked by mossy fiber stimulation (Li et al., 2001). Inclusion of CaEDTA in the ACSF partially inhibited LTP induced by HFS of the mossy fibers (vehicle: 156% ± 12.3%, n = 7; CaEDTA: 117% ± 6.3%, n = 8; $p < 0.05$, t test; Figure 7B). Importantly, paired pulse facilitation of the mf-CA3 pyramid synapse was not altered by CaEDTA or in slices from *BDNF* mutant mice (PPF: vehicle, 2.17 ± 0.15, n = 8; CaEDTA, 2.04 ± 0.07, n = 7, $p = 0.46$, t test; WT, 2.18 ± 0.10, n = 21; $BDNF^{-/-}$, 2.31 ± 0.20, n = 17, $p = 0.94$,

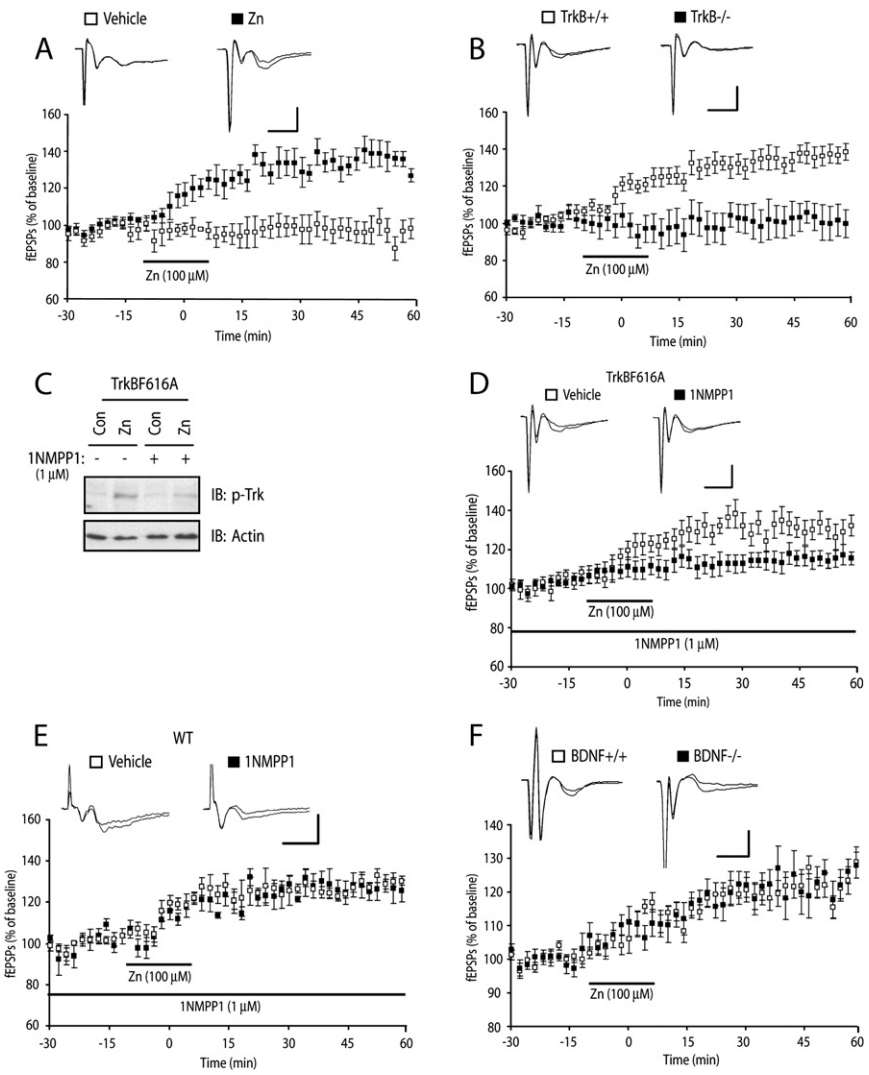

Figure 5. Zinc-Induced Transactivation of TrkB Potentiates Hippocampal Mossy Fiber-CA3 Synapse

In the experiments below, hippocampal slices were isolated from WT or mutant mice, and mossy fiber (mf)-evoked fEPSPs were recorded. In (A), (B), (D), (E), and (F), graphs represent mean ± SEM of the responses evoked compared to baseline. Traces of representative experiments are shown above each graph. Scale bar: 0.5 mV, 5 ms.

(A) Exogenous zinc induced long-lasting potentiation of fEPSPs at mf-CA3 pyramid synapse. Incubation of slices from WT mice with zinc (100 μM) (n = 8) for 20 min induced a significant increase in the amplitude of the mf-CA3 fEPSP in comparison to vehicle controls (n = 6, $p < 0.01$).

(B) Zinc enhances mf synaptic responses in a TrkB-requiring manner. Slices isolated from WT (+/+) or *TrkB* conditional knockout (−/−) mice were incubated with zinc (100 μM) for 20 min.

(C) 1NMPP1 reduced zinc-induced tyrosine-phosphorylation of TrkB. Hippocampal slices isolated from $TrkB^{F616A}$ mice were preincubated with vehicle or 1NMPP1 (1 μM) for 2 hr, followed by addition of exogenous zinc (100 μM) for 20 min. The slice homogenates were resolved on SDS-PAGE and subjected to immunoblotting with p-Trk antibody.

(D) Inhibition of TrkB kinase activity reduced zinc-induced potentiation of mf synaptic response. Slices isolated from $TrkB^{F616A}$ mice were preincubated with vehicle or 1NMPP1 for 2 hr, at which time zinc (100 μM) was added for 20 min. Incubation in 1NMPP1 was continued for the remainder of the recording procedure.

(E) 1NMPP1 did not impair zinc-induced potentiation of mf-evoked synaptic response in WT mice. Slices isolated from WT mice were preincubated with vehicle or 1NMPP1 for 2 hr, at which time zinc (100 μM) was added for 20 min. Incubation in 1NMPP1 was continued for the remainder of the experiment.

(F) BDNF is not required for zinc-induced enhancement of fEPSPs at mf-CA3 pyramid synapse. Slices isolated from WT (+/+) or *BDNF* conditional knockout (−/−) were incubated with zinc (100 μM) for 20 min.

t test), demonstrating that these perturbations selectively inhibited LTP of this synapse. Moreover, the effect of CaEDTA (7.5 mM) was specific to the mf-CA3 pyramidal cell synapse because CaEDTA (7.5 mM) did not inhibit HFS-induced LTP of the Schaffer collateral-CA1 pyramidal cell synapse (vehicle: 204% ± 19.8%, n = 6; CaEDTA: 218% ± 18.7%, n = 6; $p > 0.05$, t test; Figure 7C). Collectively, these findings support the conclusion that both BDNF and zinc serve as endogenous ligands of TrkB and contribute to LTP of the mf-CA3 pyramid synapse.

DISCUSSION

We used biochemical and electrophysiological studies of genetically modified mice to test the hypothesis that a divalent cation, zinc, can transactivate TrkB. The results reveal that zinc can transactivate synaptic TrkB by a neuronal activity-regulated and SFK-dependent mechanism. One consequence of zinc-mediated transactivation of TrkB is potentiation of the mf-CA3 pyramid synapse. The activity-dependent activation of synaptic TrkB in a neurotrophin-independent manner provides a mechanism by which this receptor can regulate synaptic plasticity.

Prior work of Moses Chao and colleagues demonstrated the transactivation of TrkB in cultured hippocampal neurons by two G protein-coupled receptor ligands, adenosine and pituitary adenylate cyclase-activating peptide (PACAP) (Lee and Chao, 2001; Lee et al., 2002; Rajagopal et al., 2004). Transactivation of Trk receptors by adenosine or PACAP differs in several respects from activation of TrkB by BDNF or zinc. Whereas BDNF and zinc activate TrkB within a few minutes, only minimal activation of Trks is evident even 1 hr after addition of an adenosine analog or PACAP to cultured neurons (Lee and Chao, 2001; Lee et al., 2002). Whereas BDNF and zinc activate mature, full-length TrkB, these G protein-coupled receptor ligands activate an immature form of Trk (Rajagopal et al., 2004). Whereas

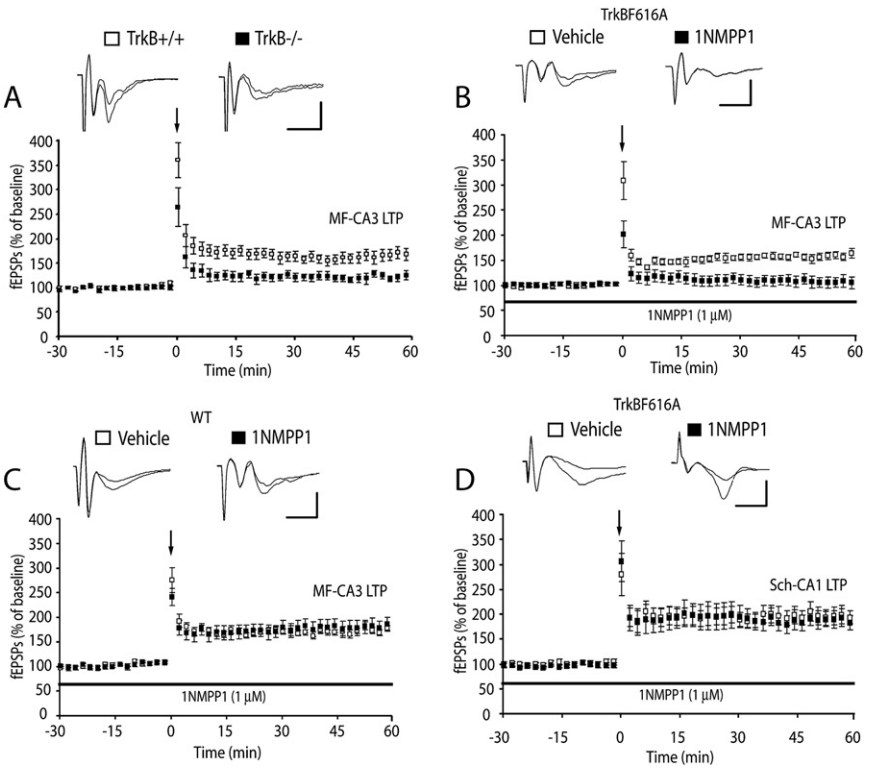

Figure 6. TrkB Is Required for HFS-Induced Hippocampal Mossy Fiber LTP

In the experiments below, hippocampal slices were isolated from WT or mutant mice, and mf-evoked fEPSPs were recorded. Slices were preincubated with vehicle or 1NMPP1 (1 μM) for 2 hr before recording and maintained in vehicle or 1NMPP1 throughout the experiment. Graphs represent mean ± SEM of the responses evoked compared to baseline. Traces of representative experiments are shown above each graph. Arrow indicates HFS. Scale bar in (A), (B), and (C): 0.5 mV, 5 ms. Scale bar in (D): 1.5 mV, 5 ms.

(A) High-frequency stimulation (HFS)-induced mf LTP is impaired in *TrkB* knockout mice. Slices were isolated from WT (+/+) and *TrkB* knockout (−/−) mice.

(B) Inhibition of TrkB kinase activity prevents HFS-induced mf LTP. Slices were isolated from $TrkB^{F616A}$ mice.

(C) 1NMPP1 does not affect mf LTP in WT mice. Slices were isolated from WT mice.

(D) 1NMPP1 does not affect Schaffer collateral-LTP in $TrkB^{F616A}$ mice. Slices were isolated from $TrkB^{F616A}$ mice, and LTP of CA1 pyramidal cell fEPSP was induced by HFS of Schaffer collaterals (Sch-CA1 LTP).

BDNF and zinc activate TrkB localized to the PSD of excitatory synapses, the G protein-coupled receptor ligands activate Trks localized to an intracellular pool associated mainly with Golgi membranes (Rajagopal et al., 2004). Whereas zinc endogenous to these cultured neurons can activate TrkB in the presence of elevated potassium, whether GPCR ligands endogenous to primary neurons or hippocampal slices can transactivate Trks is unclear. Although transactivation of Trks by exogenously applied adenosine or PACAP can promote survival of primary hippocampal neurons, whether GPCR ligands endogenous to cultured neurons can promote neuronal survival or affect other biological consequences in a Trk-dependent manner has yet to be shown. A particularly striking feature of zinc-mediated transactivation of TrkB is its dependence upon neuronal activity, a property due in part to the activity-dependence of zinc release from synaptic vesicles and in part to the activity dependence of activation of the channels through which zinc gains access to its intracellular site of action.

The requirement of SFK for zinc-mediated transactivation of Trk led us to investigate the mechanism by which zinc activates SFK. Prior insights into the mechanism of Src activation provided a context to address this question. The dephosphorylation of Y527 leads to increased phosphorylation of Y416 and subsequent activation of Src and other SFK (Cooper and Howell, 1993). The present studies revealed that addition of zinc induced in parallel a decrease of pY527 and an increase of pY416, both of which correlated with increased pTrk content. In principle, the zinc-induced dephosphorylation of pY527 could be mediated by inhibition of a kinase and/or activation of a phosphatase. The protein kinase mediating phosphorylation of pY527 has been identified, namely C-terminal Src kinase (Csk) (Cole et al., 2003). Interestingly, analyses of the requirement of divalent metal cations for Csk activity in a cell-free system revealed that zinc potently inhibits its kinase activity (IC_{50}, 0.5 μM) (Sun and Budde, 1999). It thus seems plausible that increased intracellular zinc inhibits Csk, which leads to the dephosphorylation of pY527. Alternatively, zinc may somehow increase activity of a phosphatase, but this seems less likely because brief addition of zinc potently inhibits, rather than stimulates, protein tyrosine phosphatase activity (Brautigan et al., 1981). A parsimonious explanation, which we favor, is that increased intracellular zinc inhibits Csk, leading to the dephosphorylation of pY527 and relief of autoinhibition of SFK. The resulting activation of SFK in turn leads to the phosphorylation and activation of Trk.

The biochemical evidence that zinc can transactivate TrkB raised the question as to whether this might have functional consequences. Among the diverse biological functions regulated by TrkB, the neuronal activity-dependence of the zinc effects provided the rationale for our study of LTP of excitatory synapses. This led to demonstration of a pivotal role of TrkB in LTP of the mf-CA3 pyramid synapse and that zinc-mediated transactivation of TrkB potentiates this synapse. How might zinc released from a mossy fiber bouton access TrkB and promote LTP of the mf-CA3 pyramid synapse? Extensive studies of the mechanisms of LTP of the mf-CA3 pyramid synapse (Nicoll and Schmitz, 2005) provide a framework for addressing this question. A consensus exists that the mechanism underlying expression of LTP of this synapse is exclusively presynaptic, due to the increased probability of release of glutamate from the mossy fiber bouton. By contrast, whether pre- and/or postsynaptic signaling

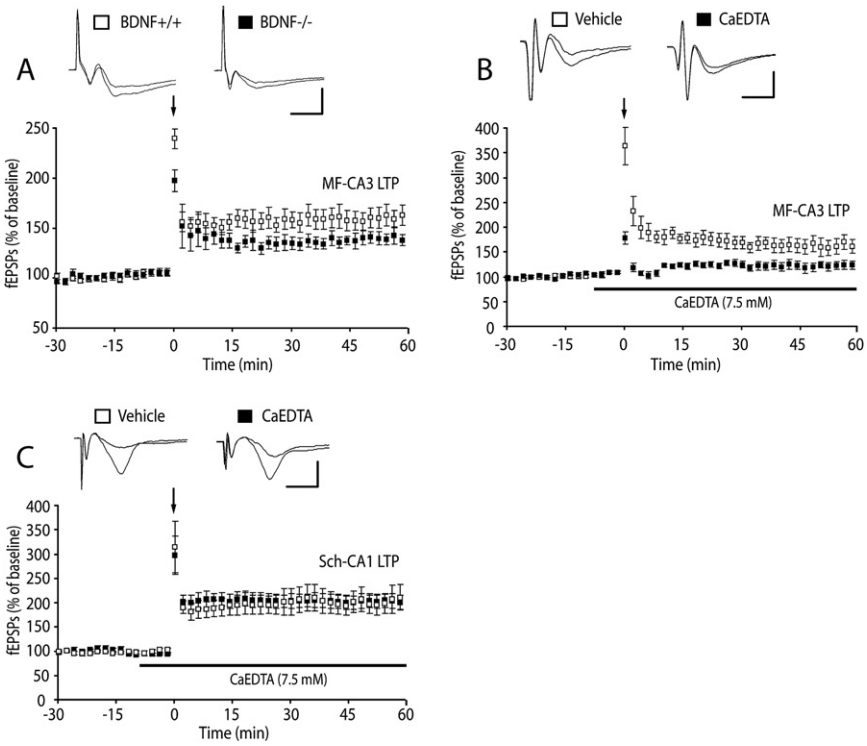

Figure 7. Both BDNF and Endogenous Zinc Are Required for Hippocampal Mossy Fiber LTP

In the experiments below, hippocampal slices were isolated from WT or mutant mice, and mf-evoked fEPSPs were recorded. Graphs represent mean ± SEM of the responses evoked compared to baseline. Traces of representative experiments are shown above each graph. Arrow indicates HFS. Scale bar: 0.5 mV, 5 ms in (A) and (B); 1.5 mV, 5 ms in (C).

(A) Hippocampal mf LTP is reduced in *BDNF* knockout mice. Slices were isolated from WT (+/+) and *BDNF* knockout (−/−) mice.

(B) Endogenous zinc is required for hippocampal mf LTP. Slices isolated from WT mice were incubated with vehicle or CaEDTA (7.5 mM) beginning 10 min before HFS and continually during the entire recording procedure.

(C) Schaffer collateral LTP is not affected by CaEDTA (7.5 mM). Slices isolated from WT mice were incubated with vehicle or CaEDTA (7.5 mM), and Schaffer collateral LTP was examined.

mediates induction of LTP of this synapse is controversial (Nicoll and Schmitz, 2005). Interestingly, analyses of cultured neurons have demonstrated that activation of TrkB results in increased release of glutamate (Poo, 2001; Lu, 2003). One model (Figure 8) suggested by our findings is that zinc released from a mossy fiber bouton permeates the thorny excrescences of CA3 pyramid dendrites by one of multiple routes, including VGCCs, NMDARs, GluR2-lacking AMPA receptors, or others yet to be defined. Once within the spine, the zinc locally activates SFK, leading to activation of TrkB and downstream signaling likely involving PLCγ1, a critical signaling cascade involving LTP of Schaffer collateral-CA1 synapse (Minichiello et al., 2002). This would require generation of a retrograde signal to the presynaptic bouton, resulting in increased glutamate release. This proposal is consistent with evidence that zinc is released upon stimulation of the mossy fibers (Qian and Noebels, 2005; Vogt et al., 2000) and that zinc can permeate neurons through each of these routes in an activity-regulated mechanism (Frederickson et al., 2005). That said, our findings (Figure 3G) are also consistent with the alternative that zinc released from a mossy fiber bouton reenters the presynaptic terminal where it activates SFK and subsequently TrkB and downstream signals, resulting in increased glutamate release and LTP of this synapse. In either case, how TrkB signaling interacts with identified molecules required for LTP of the mf-CA3 pyramid synapse including GluR6, EphB2 receptor, Rab3a, PKA, and other signaling proteins (Nicoll and Schmitz, 2005) remains to be elucidated.

The partial inhibition of LTP in slices from *BDNF* conditional mutant mice implicates BDNF as one ligand activating TrkB in this plasticity. The partial inhibition of LTP by inclusion of the selective zinc chelator, CaEDTA, implicates zinc as well. Insight into how BDNF and zinc interact to promote TrkB activation and LTP of this synapse will require knowledge of the cellular locale and temporal pattern of release of these ligands and the patterns of neuronal activity that govern their release. Zinc is packaged with glutamate in clear synaptic vesicles of the giant boutons of mf axons (Frederickson et al., 2005) and is coreleased with glutamate, a single action potential being sufficient to effect release (Qian and Noebels, 2005). We are unaware of evidence localizing vesicular zinc to the thorny excrescences, the unique structure of spines of the CA3 pyramids. BDNF has been localized to both the giant boutons of the mossy fiber axons and a subset of thorny excrescences of CA3 pyramids (Danzer and McNamara, 2004). Moreover, BDNF can be released from both axons and dendrites of cultured neurons in a depolarization-dependent manner (Poo, 2001; Lu, 2003). Whether the source of BDNF promoting LTP of the mf-CA3 pyramid synapse is pre- and/or postsynaptic is uncertain because the conditional *BDNF* mutant analyzed in the present studies reduced BDNF expression in both dentate granule cells and CA3 pyramids (He et al., 2004). It seems plausible that the availability of two distinct ligands capable of activating TrkB would provide increased flexibility for control of the function of this synapse under diverse conditions. Indeed the present findings raise the possibility that a homeostatic response involving BDNF may contribute to the persistence of mf-CA3 pyramid LTP in slices from *mocha* mice that lack ZnT_3 (Vogt et al., 2000), a molecule that transports zinc into synaptic vesicles (Cole et al., 1999).

In contrast to the requirement of TrkB for LTP of the mf-CA3 pyramid synapse, inhibiting TrkB signaling failed to inhibit LTP of the Schaffer collateral-CA1 pyramid synapse, a puzzling result in light of prior work. That is, both genetic and pharmacological

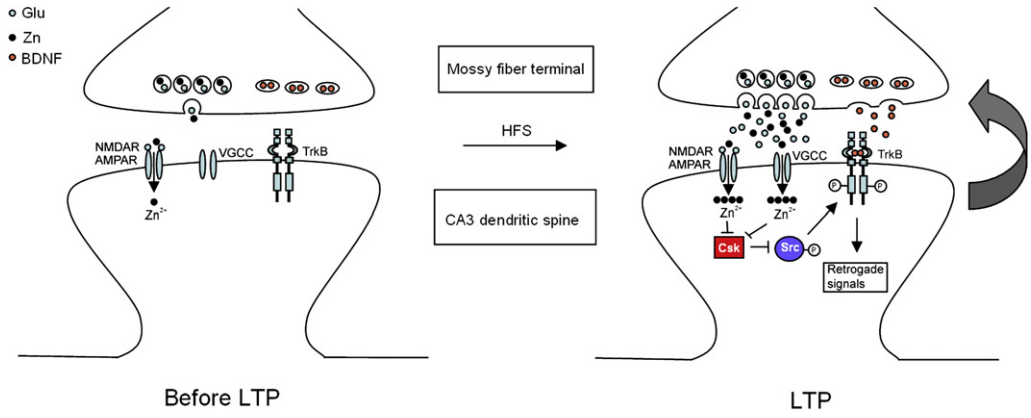

Figure 8. Model for TrkB-Dependent Potentiation of Mossy Fiber-CA3 Pyramid Synapse

Under normal conditions, a single action potential releases a relatively small amount of glutamate and zinc into synaptic cleft. By contrast, during HFS, larger amounts of glutamate and zinc are released from mossy fiber terminals into the synaptic cleft, resulting in zinc influx into the spine of a CA3 pyramid through NMDA receptors, AMPA receptors lacking the GluR2 subunit, VGCCs, or possibly other routes. The local increase of intracellular zinc inhibits Csk activity, leading to SFK activation by reduced phosphorylation of Y527 and increased phosphorylation of Y416 and subsequent phosphorylation and activation of TrkB. The transactivation of TrkB produces unidentified retrograde signals to presynaptic sites, which in turn potentiates release probability of glutamate from mossy fiber terminals. Importantly, the release of BDNF is also regulated by activity, resulting in activation of TrkB and contributing to potentiation of this synapse. An equally plausible mechanism (not pictured here) is that zinc and BDNF activate TrkB directly on the presynaptic terminal.

perturbations of TrkB signaling partially inhibit LTP of the Schaffer collateral-CA1 pyramid synapse induced by HFS of the Schaffer collaterals (McAllister et al., 1999; Poo, 2001). Yet in the present study, inclusion of 1 μM 1NMPP1 eliminated HFS-induced LTP of the mf-CA3 pyramid synapse in slices of *TrkB*^{F616A} mice but had no effect on LTP of the Schaffer collateral-CA1 synapse. The ineffectiveness of 1NMPP1 on LTP of the Schaffer collateral-CA1 pyramid synapse is not due to impaired effectiveness of the drug, because a subset of experiments examining both synapses in the same slice revealed that 1NMPP1 inhibited LTP of the mf-CA3 but not Schaffer collateral-CA1 synapse. Likewise, inclusion of CaEDTA partially inhibited LTP of the mf-CA3 pyramid synapse yet had no effect on LTP of the Schaffer collateral-CA1 synapse. Importantly, an identical paradigm was employed for induction of LTP at the two synapses in the present study. One explanation for these unexpected results is that higher concentrations of BDNF and zinc in the mossy fiber compared to the Schaffer collateral terminals (Conner et al., 1997; Yan et al., 1997) confer a greater contribution of TrkB to LTP of the mf-CA3 pyramid synapse. Alternatively, the stimulation paradigm used for induction of LTP in the present study may induce LTP in which expression of LTP of the Schaffer collateral-CA1 synapse excludes a presynaptic mechanism. In this regard, Zakharenko et al. (Zakharenko et al., 2003) detected impairment of LTP of the Schaffer collateral-CA1 pyramid synapse in *BDNF* mutant mice only in those forms of LTP that recruited a presynaptic component of expression as revealed by increased synaptic vesicle cycling detected with FM-143 imaging. Perhaps the induction paradigm used in the present study resulted in a form of Schaffer collateral-CA1 LTP with an exclusively postsynaptic locus of expression. The idea that TrkB is particularly important for forms of LTP in which expression has a presynaptic locale is consistent with the present findings implicating TrkB in LTP of the mf-CA3 pyramid synapse. Finally, LTP of the Schaffer collateral-CA1 synapse is NMDA receptor dependent, in contrast to the mf-CA3 pyramid synapse, which is NMDA receptor independent (Nicoll and Malenka, 1995), raising the possibility that TrkB signaling may contribute preferentially to NMDA receptor independent forms of LTP.

The discovery that TrkB is required for LTP of the mf-CA3 pyramid synapse has implications for mechanisms of epilepsy, in particular a mechanism by which TrkB promotes development of epilepsy (epileptogenesis). That is, a conditional deletion of *TrkB* prevents epileptogenesis in the kindling model (He et al., 2004); together with evidence of increased p-TrkB content (Binder et al., 1999; He et al., 2002, 2004) in the mossy fiber pathway in multiple models, this suggests that enhanced activation of TrkB promotes limbic epileptogenesis. The present findings suggest that enhanced activation of TrkB in the mossy fiber pathway would strengthen the efficacy of the mf-CA3 pyramid synapse. Regulation of the strength of the mf-CA3 pyramid synapse is an appealing mechanism of epileptogenesis because the local circuit including the CA3 pyramids of a normal brain are poised to seize (Traub et al., 1989), a consequence of their extensive recurrent excitatory synaptic connections combined with their ability to fire in bursts. Normally, the low release probability of glutamate from a mossy fiber bouton (Lawrence and McBain, 2003; von Kitzing et al., 1994) likely contributes to the inability of single action potentials evoked in granule cells to activate a synaptically coupled CA3 pyramid (Henze et al., 2002). HFS-induced LTP of the mf-CA3 pyramid synapse increases the release probability from a mf (Nicoll and Schmitz, 2005); this would be expected to increase the likelihood that afferent input from granule cells could discharge CA3 pyramids and thereby enhance the propensity for seizure, the hallmark of the epileptic brain. Indeed, prior studies demonstrated that the mf-CA3 pyramid synapse has undergone LTP during limbic epileptogenesis in vivo (Goussakov et al., 2000). The enhanced p-TrkB content in the mossy fiber pathway together with the present studies raise the possibility that the LTP of the mf-CA3 pyramid synapse occurring

during epileptogenesis in vivo is caused by enhanced activation of TrkB. Preventing LTP of the mf-CA3 pyramid synapse may be one mechanism by which the conditional deletion of *TrkB* prevents epileptogenesis in the kindling model (He et al., 2004). Zinc-mediated transactivation of TrkB may contribute to the increased p-TrkB content in the mossy fiber pathway evident during epileptogenesis in WT mice as well as in *BDNF* conditional and *NT4* null mutant mice (He et al., 2004, 2006). Finally, a prominent feature of the chronically epileptic brain is formation of aberrant recurrent excitatory synapses among dentate granule cells mediated by massive sprouting of zinc-rich mossy fibers (Nadler, 2003); transactivation of TrkB may contribute to the increased epileptiform activity mediated by synaptically released zinc from sprouted mossy fibers under these conditions (Timofeeva and Nadler, 2006).

In sum, this study establishes a mechanism of TrkB activation, namely its transactivation by the divalent cation, zinc, and its independence of neurotrophins combined with its regulation by neuronal activity. One functional consequence of this transactivation is the regulation of a neuronal activity-dependent synaptic plasticity, namely strengthening of the hippocampal mossy fiber-CA3 pyramid synapse. The functional convergence of zinc and TrkB signaling at the synapse provides a new perspective as to how these well-studied molecules can impact nervous system function in health and disease.

EXPERIMENTAL PROCEDURES

Reagents, cell culture, and antibodies are described in Supplemental Data.

Mice

BDNF null mutant mice were generated by crossing male and female heterozygotes (Jackson laboratory). *BDNF* and *TrkB* conditional mutant mice were generated as described previously (He et al., 2004). *TrkB*^{F616A} mutant mice were provided by Dr. David Ginty (Chen et al., 2005). The strains of mice listed below were generously provided by the following investigators: floxed *BDNF* and floxed *TrkB* by Dr. Luis Parada; *Synapsin I-Cre* mice by Dr. Jamey Marth. The genotype of each animal was verified twice using PCR of genomic DNA isolated from the tail before and after experiments.

Biochemistry

Cell lysates and subcellular fractions were prepared using methods as described in Supplemental Data. Immunoprecipitation and western blotting analysis were performed as described previously (Huang et al., 2000). See Supplemental Data for details.

Electrophysiology

fEPSPs were recorded on hippocampal slices acutely isolated from mouse (P28–P42). Detailed descriptions and the criteria required to be considered a mossy fiber-CA3 pyramid fEPSP are available in Supplemental Data. LTP of the mossy fiber-CA3 pyramid synapse and Schaffer collateral-CA1 synapse was induced by applying a total of four trains of high-frequency stimulation (HFS) (each train consisting of 200 μs pulses at 100 Hz and intensity sufficient to induce maximum fEPSP amplitude and intertrain interval of 10 s). LLP was induced by bath application of zinc (100 μM) for 20 min beginning after 20 min baseline recording. To assure objectivity, the individual performing all experiments with *BDNF* and *TrkB* conditional null mutant mice was blinded as to genotype.

shRNA and Lentiviral Vectors

The target sequence of the TrkB for shRNA was 5′ CAAGCTGACGAGTTTGTCC 3′ (corresponding to nt 976–994 of mouse *trkB* [accession number: NM008745]). The lentiviral vectors expressing scrambled or TrkB-shRNA were prepared by Tranzyme Co (RTP, NC). Cultured cortical neurons (DIV5) were transduced with lentiviral vectors (MOI: 2) and cultured for an additional 10–12 days.

SUPPLEMENTAL DATA

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/57/4/546/DC1/.

ACKNOWLEDGMENTS

This work was supported by NS056217 from the National Institute of Neurological Disease and Stroke (J.O.M.) and Epilepsy Foundation postdoctoral fellowship (Y.Z.H). We thank Drs. Raymond Dingledine, Victor Nadler, and Anne West for their comments on this manuscript. We also thank the members of McNamara lab for valuable discussion of this project.

Received: June 15, 2007
Revised: October 19, 2007
Accepted: November 28, 2007
Published: February 27, 2008

REFERENCES

Binder, D.K., Routbort, M.J., and McNamara, J.O. (1999). Immunohistochemical evidence of seizure-induced activation of trk receptors in the mossy fiber pathway of adult rat hippocampus. J. Neurosci. *19*, 4616–4626.

Bishop, A.C., Ubersax, J.A., Petsch, D.T., Matheos, D.P., Gray, N.S., Blethrow, J., Shimizu, E., Tsien, J.Z., Schultz, P.G., Rose, M.D., et al. (2000). A chemical switch for inhibitor-sensitive alleles of any protein kinase. Nature *407*, 395–401.

Brautigan, D.L., Bornstein, P., and Gallis, B. (1981). Phosphotyrosyl-protein phosphatase. Specific inhibition by Zn. J. Biol. Chem. *256*, 6519–6522.

Carpenter, G. (1999). Employment of the epidermal growth factor receptor in growth factor-independent signaling pathways. J. Cell Biol. *146*, 697–702.

Chao, M.V., Rajagopal, R., and Lee, F.S. (2006). Neurotrophin signaling in health and disease. Clin. Sci. *110*, 167–173.

Chen, X., Ye, H., Kuruvilla, R., Ramanan, N., Scangos, K.W., Zhang, C., Johnson, N.M., England, P.M., Shokat, K.M., and Ginty, D.D. (2005). A chemical-genetic approach to studying neurotrophin signaling. Neuron *46*, 13–21.

Cole, T.B., Wenzel, H.J., Kafer, K.E., Schwartzkroin, P.A., and Palmiter, R.D. (1999). Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene. Proc. Natl. Acad. Sci. USA *96*, 1716–1721.

Cole, P.A., Shen, K., Qiao, Y., and Wang, D. (2003). Protein tyrosine kinases Src and Csk: a tail's tale. Curr. Opin. Chem. Biol. *7*, 580–585.

Conner, J.M., Lauterborn, J.C., Yan, Q., Gall, C.M., and Varon, S. (1997). Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. J. Neurosci. *17*, 2295–2313.

Cooper, J.A., and Howell, B. (1993). The when and how of Src regulation. Cell *73*, 1051–1054.

Coull, J.A., Beggs, S., Boudreau, D., Boivin, D., Tsuda, M., Inoue, K., Gravel, C., Salter, M.W., and De Koninck, Y. (2005). BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. Nature *438*, 1017–1021.

Danzer, S.C., and McNamara, J.O. (2004). Localization of brain-derived neurotrophic factor to distinct terminals of mossy fiber axons implies regulation of both excitation and feedforward inhibition of CA3 pyramidal cells. J. Neurosci. *24*, 11346–11355.

Frederickson, C.J., and Danscher, G. (1990). Zinc-containing neurons in hippocampus and related CNS structures. Prog. Brain Res. *83*, 71–84.

Frederickson, C.J., Koh, J.Y., and Bush, A.I. (2005). The neurobiology of zinc in health and disease. Nat. Rev. Neurosci. *6*, 449–462.

Goussakov, I.V., Fink, K., Elger, C.E., and Beck, H. (2000). Metaplasticity of mossy fiber synaptic transmission involves altered release probability. J. Neurosci. *20*, 3434–3441.

Hardingham, G.E., Fukunaga, Y., and Bading, H. (2002). Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat. Neurosci. *5*, 405–414.

He, X.P., Minichiello, L., Klein, R., and McNamara, J.O. (2002). Immunohistochemical evidence of seizure-induced activation of trkB receptors in the mossy fiber pathway of adult mouse hippocampus. J. Neurosci. *22*, 7502–7508.

He, X.P., Kotloski, R., Nef, S., Luikart, B.W., Parada, L.F., and McNamara, J.O. (2004). Conditional deletion of TrkB but not BDNF prevents epileptogenesis in the kindling model. Neuron *43*, 31–42.

He, X.P., Butler, L., Liu, X., and McNamara, J.O. (2006). The tyrosine receptor kinase B ligand, neurotrophin-4, is not required for either epileptogenesis or tyrosine receptor kinase B activation in the kindling model. Neuroscience *141*, 515–520.

Henze, D.A., Wittner, L., and Buzsaki, G. (2002). Single granule cells reliably discharge targets in the hippocampal CA3 network in vivo. Nat. Neurosci. *5*, 790–795.

Huang, E.J., and Reichardt, L.F. (2003). Trk receptors: roles in neuronal signal transduction. Annu. Rev. Biochem. *72*, 609–642.

Huang, Y.Z., Won, S., Ali, D.W., Wang, Q., Tanowitz, M., Du, Q.S., Pelkey, K.A., Yang, D.J., Xiong, W.C., Salter, M.W., et al. (2000). Regulation of neuregulin signaling by PSD-95 interacting with ErbB4 at CNS synapses. Neuron *26*, 443–455.

Hwang, J.J., Park, M.H., Choi, S.Y., and Koh, J.Y. (2005). Activation of the Trk signaling pathway by extracellular zinc. Role of metalloproteinases. J. Biol. Chem. *280*, 11995–12001.

Koh, J.Y., Suh, S.W., Gwag, B.J., He, Y.Y., Hsu, C.Y., and Choi, D.W. (1996). The role of zinc in selective neuronal death after transient global cerebral ischemia. Science *272*, 1013–1016.

Lawrence, J.J., and McBain, C.J. (2003). Interneuron diversity series: containing the detonation–feedforward inhibition in the CA3 hippocampus. Trends Neurosci. *26*, 631–640.

Le Gall, S.M., Auger, R., Dreux, C., and Mauduit, P. (2003). Regulated cell surface pro-EGF ectodomain shedding is a zinc metalloprotease-dependent process. J. Biol. Chem. *278*, 45255–45268.

Lee, F.S., and Chao, M.V. (2001). Activation of Trk neurotrophin receptors in the absence of neurotrophins. Proc. Natl. Acad. Sci. USA *98*, 3555–3560.

Lee, F.S., Rajagopal, R., Kim, A.H., Chang, P.C., and Chao, M.V. (2002). Activation of Trk neurotrophin receptor signaling by pituitary adenylate cyclase-activating polypeptides. J. Biol. Chem. *277*, 9096–9102.

Li, Y., Hough, C.J., Frederickson, C.J., and Sarvey, J.M. (2001). Induction of mossy fiber–> Ca3 long-term potentiation requires translocation of synaptically released Zn2+. J. Neurosci. *21*, 8015–8025.

Lu, B. (2003). BDNF and activity-dependent synaptic modulation. Learn. Mem. *10*, 86–98.

McAllister, A.K., Katz, L.C., and Lo, D.C. (1999). Neurotrophins and synaptic plasticity. Annu. Rev. Neurosci. *22*, 295–318.

McNamara, J.O., Huang, Y.Z., and Leonard, A.S. (2006). Molecular signaling mechanisms underlying epileptogenesis. Sci. STKE *2006*, re12.

Minichiello, L., Calella, A.M., Medina, D.L., Bonhoeffer, T., Klein, R., and Korte, M. (2002). Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron *36*, 121–137.

Nadler, J.V. (2003). The recurrent mossy fiber pathway of the epileptic brain. Neurochem. Res. *28*, 1649–1658.

Nicoll, R.A., and Malenka, R.C. (1995). Contrasting properties of two forms of long-term potentiation in the hippocampus. Nature *377*, 115–118.

Nicoll, R.A., and Schmitz, D. (2005). Synaptic plasticity at hippocampal mossy fibre synapses. Nat. Rev. Neurosci. *6*, 863–876.

Poo, M.M. (2001). Neurotrophins as synaptic modulators. Nat. Rev. Neurosci. *2*, 24–32.

Qian, J., and Noebels, J.L. (2005). Visualization of transmitter release with zinc fluorescence detection at the mouse hippocampal mossy fibre synapse. J. Physiol. *566*, 747–758.

Rajagopal, R., Chen, Z.Y., Lee, F.S., and Chao, M.V. (2004). Transactivation of Trk neurotrophin receptors by G-protein-coupled receptor ligands occurs on intracellular membranes. J. Neurosci. *24*, 6650–6658.

Salter, M.W., and Kalia, L.V. (2004). Src kinases: a hub for NMDA receptor regulation. Nat. Rev. Neurosci. *5*, 317–328.

Segal, R.A., Bhattacharyya, A., Rua, L.A., Alberta, J.A., Stephens, R.M., Kaplan, D.R., and Stiles, C.D. (1996). Differential utilization of Trk autophosphorylation sites. J. Biol. Chem. *271*, 20175–20181.

Sun, G., and Budde, R.J. (1999). Substitution studies of the second divalent metal cation requirement of protein tyrosine kinase CSK. Biochemistry *38*, 5659–5665.

Timofeeva, O., and Nadler, J.V. (2006). Facilitation of granule cell epileptiform activity by mossy fiber-released zinc in the pilocarpine model of temporal lobe epilepsy. Brain Res. *1078*, 227–234.

Traub, R.D., Miles, R., and Wong, R.K. (1989). Model of the origin of rhythmic population oscillations in the hippocampal slice. Science *243*, 1319–1325.

Vogt, K., Mellor, J., Tong, G., and Nicoll, R. (2000). The actions of synaptically released zinc at hippocampal mossy fiber synapses. Neuron *26*, 187–196.

von Kitzing, E., Jonas, P., and Sakmann, B. (1994). Quantal analysis of excitatory postsynaptic currents at the hippocampal mossy fiber-CA3 pyramidal cell synapse. Adv. Second Messenger Phosphoprotein Res. *29*, 235–260.

Wu, K., Xu, J.L., Suen, P.C., Levine, E., Huang, Y.Y., Mount, H.T., Lin, S.Y., and Black, I.B. (1996). Functional trkB neurotrophin receptors are intrinsic components of the adult brain postsynaptic density. Brain Res. Mol. Brain Res. *43*, 286–290.

Wu, W., Graves, L.M., Gill, G.N., Parsons, S.J., and Samet, J.M. (2002). Src-dependent phosphorylation of the epidermal growth factor receptor on tyrosine 845 is required for zinc-induced Ras activation. J. Biol. Chem. *277*, 24252–24257.

Wu, W., Samet, J.M., Silbajoris, R., Dailey, L.A., Sheppard, D., Bromberg, P.A., and Graves, L.M. (2004). Heparin-binding epidermal growth factor cleavage mediates zinc-induced epidermal growth factor receptor phosphorylation. Am. J. Respir. Cell Mol. Biol. *30*, 540–547.

Yan, Q., Rosenfeld, R.D., Matheson, C.R., Hawkins, N., Lopez, O.T., Bennett, L., and Welcher, A.A. (1997). Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. Neuroscience *78*, 431–448.

Zakharenko, S.S., Patterson, S.L., Dragatsis, I., Zeitlin, S.O., Siegelbaum, S.A., Kandel, E.R., and Morozov, A. (2003). Presynaptic BDNF required for a presynaptic but not postsynaptic component of LTP at hippocampal CA1-CA3 synapses. Neuron *39*, 975–990.