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Some Forms of cAMP-Mediated Long-Lasting Potentiation Are Associated with Release of BDNF and Nuclear Translocation of Phospho-MAP Kinase

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Summary

Long-lasting forms of synaptic plasticity like the late phase of LTP (L-LTP) typically require an elevation of cAMP, the recruitment of the cAMP-dependent protein kinase (PKA), and ultimately the activation of transcription and translation; some forms also require brain-derived neurotrophic factor (BDNF). Both cAMP and BDNF can activate mitogen-activated protein kinase (MAPK/ERK), which also plays a role in LTP. However, little is known about the mechanisms whereby cAMP, BDNF, and MAPK interact. We find that increases in cAMP can rapidly activate the BDNF receptor TrkB and induce BDNF-dependent long-lasting potentiation at the Schaffer collateral-CA1 synapse in hippocampus. Surprisingly, in these BDNF-dependent forms of potentiation, which are also MAPK dependent, TrkB activation is not critical for the activation of MAPK but instead appears to modulate the subcellular distribution and nuclear translocation of the activated MAPK.

Introduction

Experience-dependent modifications of synaptic strength are thought to underlie a variety of brain processes, including both learning and memory and the activity-dependent refinement of synaptic connections during development. Studies in evolutionarily distant species, ranging from *Drosophila* and *Aplysia* to mouse, have demonstrated that synaptic plasticity, like memory, has at least two forms. Short-term synaptic plasticity generally involves rapid modifications of existing proteins. Long-lasting synaptic plasticity, in contrast, requires the initiation of gene transcription and protein synthesis and, in at least some cases, involves the growth of new synapses (Bailey et al., 1996; Bailey and Kandel, 1993). The synaptic events required for the early phase of LTP (E-LTP) are linked to the nuclear events required for the late phase of LTP (L-LTP) by complex spatially and temporally converging signals. We have here examined the interplay between three molecules implicated in the conversion of E-LTP to L-LTP at the Schaffer collateral-CA1 synapse of the hippocampus: cAMP, MAPK, and BDNF (for reviews, see Kandel and Abel, 1995; Impey et al., 1999; Sweatt, 2001; McAllister et al., 1999; Jankowsky and Patterson, 1999).

Using a combination of physiological and immunohistochemical methods, we have examined the idea that during the induction of L-LTP, the evoked increase in cAMP induces the acute release of BDNF, thereby activating the TrkB receptor signaling system, which then activates MAPK signaling. We find, as previously reported (Kang et al., 1997; Korte et al., 1998; Chen et al., 1999), that BDNF is required for some forms of cAMP-dependent long-lasting synaptic plasticity but not for others. Specifically, we find that BDNF is required for the L-LTP produced by theta burst stimulation but not for the L-LTP produced by a strong 4-train stimulation protocol. We also find that elimination of BDNF attenuates potentiation induced with forskolin, an adenylate cyclase activator that elevates intracellular cAMP and leads to transcription-dependent, long-lasting synaptic strengthening (Chavez-Noriega and Stevens, 1992; Huang et al., 1994; Wang et al., 1999). Consistent with these results, we observe that both forskolin- and theta burst-induced potentiation lead to a very rapid activation of TrkB, the BDNF receptor. In contrast, 4-train-induced LTP, which is largely independent of BDNF, appears to be less efficient in activating TrkB in this system and does not lead to detectable TrkB activation at the time point examined. The rapid activation of TrkB receptors can be blocked with a BDNF scavenger, which suggests that BDNF is released, and that this BDNF plays an instructive role.

Although the BDNF-dependent forms of long-lasting potentiation examined here are also MAPK dependent, we find that pharmacological blockade or genetic attenuation of the BDNF signaling does not significantly reduce the total activation of MAPK in response to potentiating stimulation. Instead of activating MAPK, BDNF appears to modulate the translocation of the activated MAPK from the dendrites to the soma and then to the nucleus. These results suggest that during the induction of some forms of long-lasting potentiation, cAMP may cause activation of the TrkB signaling system via released BDNF, which in turn induces translocation of activated MAPK to the nucleus, thereby modulating the access of MAPK to nuclear substrates (e.g., the transcription factor CREB) that contribute to the persistence of potentiation.

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Results

Different Patterns of Stimulation Induce BDNF-Dependent and -Independent Forms of L-LTP

BDNF knockout mice have a marked deficiency in E-LTP induced by two 100 Hz trains of stimuli (Patterson et al., 1996). When we examined L-LTP in these mice, we found, as previously reported (Kang et al., 1997; Korte et al., 1998; Chen et al., 1999), that BDNF contributes more to some forms of L-LTP than to others and that the pattern of stimulation determines the dependence of the resulting potentiation on BDNF. Specifically, we found that BDNF knockout mice had only a slight deficit in L-LTP produced by a protocol of four 100 Hz trains (Figure 1A; 4 hr after tetanus, percent baseline: homozygotes = 147% ± 8%; wild-types = 188% ± 22%; $p = 0.09$). However, BDNF knockout mice had a profound deficit in the response to a theta burst protocol (Figure 1B; 4 hr after tetanus, percent baseline: homozygotes = 109% ± 6%; wild-types = 158% ± 10%; $p < 0.006$). Pharmacological removal of BDNF also reduced the ability of a slice to respond to theta burst stimulation. Hippocampal slices from wild-type mice were pretreated with a TrkB-Fc fusion protein (the ligand binding domain of the native TrkB receptor coupled to the Fc fragment of human immunoglobulin) that scavenges unbound TrkB ligands (Shelton et al., 1995). This treatment dramatically reduced potentiation in response to theta burst (Figure 1C; 4 hr after tetanus, percent baseline: saline treated = 170% ± 13%, TrkB-Fc treated = 113% ± 7%; $p < 0.006$); TrkB-Fc treatment had no apparent effect on transmission in unpotentiated slices (after 4 hr of test stimuli, percent baseline: 97% ± 7%).

Forskolin-Induced Potentiation Is Also Reduced by a BDNF Scavenger

Both the L-LTP induced by 4-train stimulation (Huang et al., 1994; Abel et al., 1997) and that induced by theta burst stimulation (Nguyen and Kandel, 1997) require an elevation of cAMP and the activation of cAMP-dependent protein kinase (PKA). We therefore also examined a slowly developing, long-lasting form of potentiation induced with forskolin, which elevates cAMP concentrations by activating adenylyl cyclase (Chavez-Noriega and Stevens, 1992; Huang et al., 1994; Wang et al., 1999; Wong et al., 1999). Consistent with previous observations, we found that brief application (15 min) of forskolin to slices pretreated only with saline produced long-lasting potentiation, but application of 1,9-dideoxyforskolin (an analog that does not activate adenylyl cyclase) did not (Figure 2; 4 hr after onset of drug application, percent baseline: forskolin = 136% ± 13%, 1,9-dideoxyforskolin = 89% ± 2%; $p < 0.05$).

We next asked whether this cAMP-induced potentiation requires BDNF-dependent signaling. To test this idea, we pretreated the slices with TrkB-Fc to scavenge unbound ligand. We and others have found that BDNF scavenging reagents persist in the slice after pretreatment for at least 30 min (our unpublished data; Kang et al., 1997) and thus will also scavenge neurotrophins acutely released during forskolin application. We found that the potentiation produced by forskolin was blocked by TrkB-Fc (Figure 2; 4 hr after onset of forskolin application, percent baseline: saline + forskolin = 136% ± 13%, TrkB-Fc + forskolin = 100% ± 6%; $p < 0.006$). This result indicates that some forms of cAMP-induced potentiation require neurotrophin-mediated signaling.

TrkB Receptors Are Rapidly Activated by Stimuli Evoking BDNF-Dependent Forms of Long-Lasting Potentiation

The role of BDNF in L-LTP could be permissive, instructive, or both. If BDNF is permissive, constitutive release of BDNF may be required to modulate other factors that are more directly involved in the induction of plasticity, such as synaptic vesicle docking machinery (Pozzo-Miller et al., 1999) or the function of the NMDA receptor (Suen et al., 1997; Jarvis et al., 1997). Alternatively, rapid activation of TrkB signaling may be directly involved in the induction of some forms of long-lasting plasticity and thus be instructive. Several studies have suggested that hippocampal neurons can release neurotrophins or activate Trk receptors in response to prolonged or intense stimulation (Blochl and Thoenen, 1995, 1996; Goodman et al., 1996; Binder et al., 1999; Aloyz et al., 1999), but it is not clear whether this occurs normally in response to more physiological plasticity-inducing stimuli (reviewed in McAllister et al., 1999).

We therefore examined whether different stimulus protocols that evoke long-lasting potentiation led to activation of BDNF/TrkB signaling. Because of the difficulty of directly measuring release of endogenous neurotrophins, particularly on a short timescale, we measured activation of the BDNF receptor TrkB. When receptor tyrosine kinases like TrkB bind ligand, they dimerize, and this induces autophosphorylation (Guiton et al., 1994; Schlessinger and Ullrich, 1992). Thus, we examined TrkB receptor phosphorylation in stimulated slices (Figure 3).

We used 4-train, theta burst, and forskolin stimulation to induce potentiation at the Schaffer collateral-CA1 synapse. A 4-train stimulation protocol, which induces L-LTP that is not particularly dependent on BDNF, did not evoke a detectable increase in phosphorylation of TrkB (within 5 min of the last stimulus train). In contrast, theta burst stimulation, a stimulus protocol that induces BDNF-dependent L-LTP, evoked increased phosphorylation of the TrkB receptor very rapidly, within 5 min of the stimulus (theta versus untetanized, $p < 0.05$). Similarly, treating slices with forskolin for 15 min, a treatment that also produces a BDNF-dependent potentiation, increased levels of phosphorylated TrkB in area CA1 ($p < 0.05$). Application of 1,9-dideoxyforskolin did not significantly increase levels of phosphorylated TrkB (data not shown). Activation of TrkB by forskolin was significantly reduced by removal of TrkB ligands by pretreatment with TrkB-Fc ($p < 0.03$), demonstrating that released ligand is required for the observed TrkB activation.

Although BDNF is not the only neurotrophin that can bind the TrkB receptor and is therefore not the only ligand scavenged by TrkB-Fc, BDNF is likely to be the primary molecule involved in the processes we observe here. The other preferred TrkB ligand, NT-4/5, is in low abundance in the hippocampus (Timmusk et al., 1993; Friedman et al., 1998). The neurotrophin NT-3 can bind

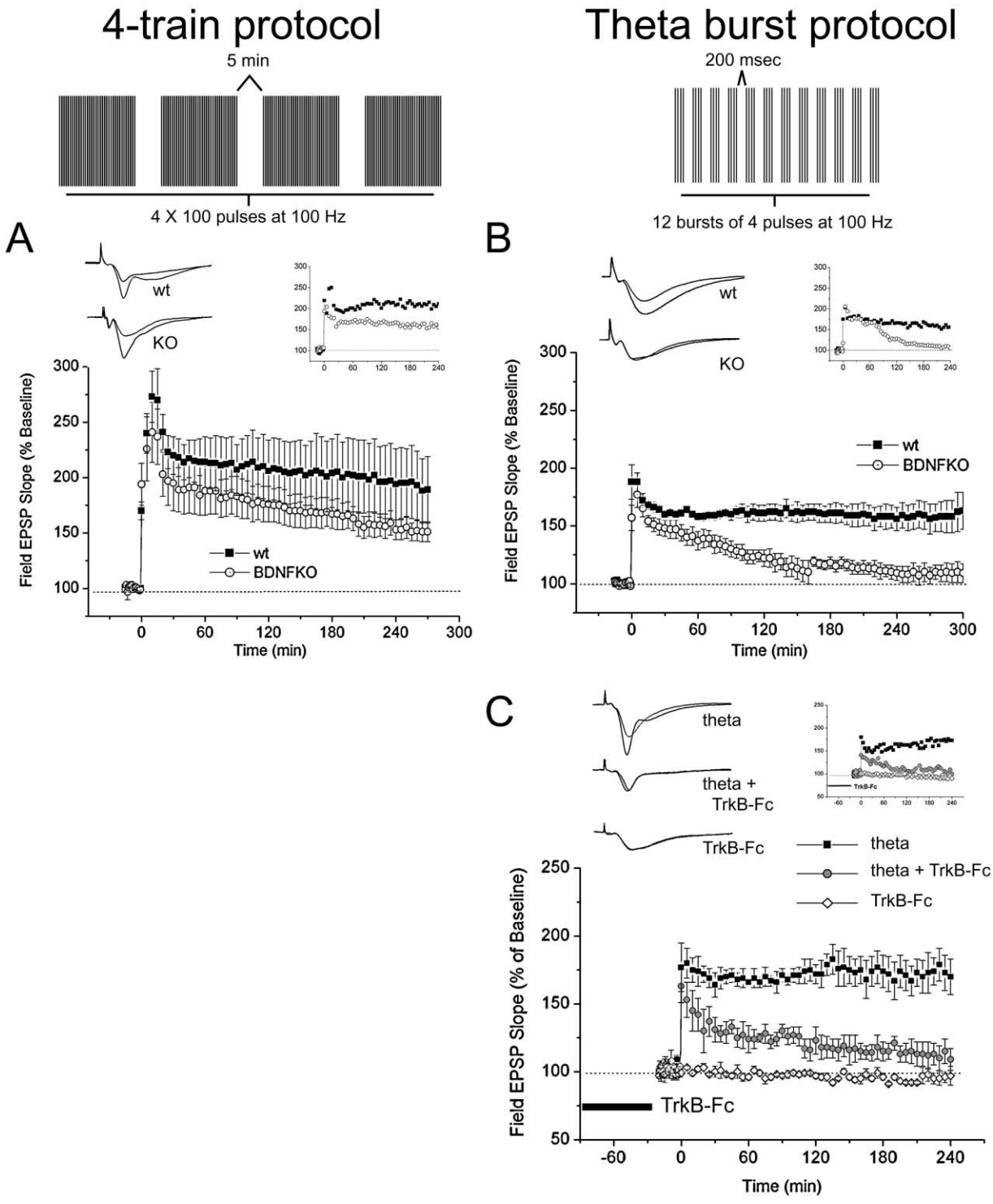

Figure 1. L-LTP Induced by Some Types of Stimulus Patterns Is Reduced in BDNF Knockout Mice

Field EPSP slopes plotted as a percentage of pretetanus baseline for slices from wild-type and homozygous BDNF mutant mice tetanized using one of two distinct protocols. (A) Four 1 s trains of stimuli, at 100 Hz, delivered 5 min apart or (B) 12 bursts of four pulses each, at 100 Hz, delivered 200 ms apart. Each point represents the mean ± SEM (n = 8 slices, 7 mice for wild-type, and n = 8 slices, 8 mice for homozygotes in [A]; n = 9 slices, 9 mice for wild-type, and n = 6 slices, 5 mice for homozygotes in [B]). (C) L-LTP induced by theta burst stimulation is also reduced in slices from wild-type mice by pretreatment with Trk-Fc, a BDNF scavenger. Slices were incubated in saline (n = 4 slices, 4 mice) or TrkB-Fc (n = 5 slices, 5 mice) for 1 hr prior to theta burst stimulation. TrkB-Fc control slices (n = 2 slices, 2 mice) received only test stimulation. In each panel, representative field potentials before and 4 hr after tetanization are shown on the top left; representative examples of individual experiments are shown on the top right.

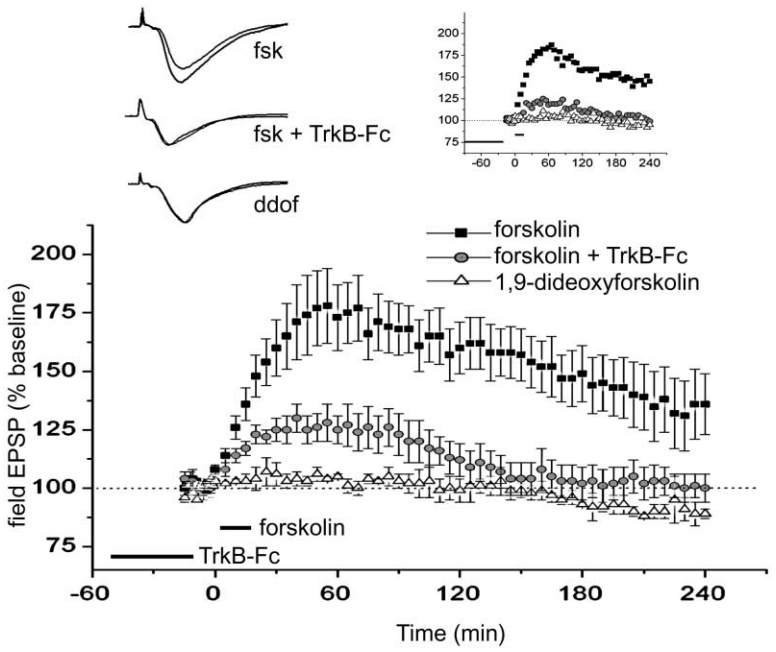

Figure 2. Forskolin-Induced Potentiation Is Reduced by Pretreatment with TrkB-Fc

Field EPSP slopes plotted as percentage of baseline. Slices were pretreated with saline or TrkB-Fc for 1 hr prior to treatment. Slices pretreated with saline were then treated with 1,9 dideoxyforskolin (n = 3 slices, 3 mice) or forskolin (n = 11 slices, 10 mice), and slices pretreated with TrkB-Fc were treated with forskolin (n = 6 slices, 6 mice). Representative field potentials before and 4 hr after forskolin application are shown on the top left; representative examples of individual experiments are shown on the top right. The slight rundown seen over time is attributable to the pretreatment of the slices in an incubation chamber (compare the forskolin-induced potentiation seen here with that seen in slices that were not preincubated in a separate incubation chamber, shown in Figure 4). Incubation in this separate chamber was necessary in these experiments because TrkB-Fc was not available in sufficient quantities to permit continuous perfusion throughout the 1 hr preincubation.

TrkB but less efficiently than BDNF, and, again, NT-3 levels in the CA1 region are comparatively low. Furthermore, the fact that several groups, including our own, have obtained very similar effects on plasticity with BDNF-blocking antibodies, TrkB-Fc treatment, and BDNF knockout strongly suggests that the majority of the effects described here are due specifically to a reduction of BDNF binding to TrkB (Chen et al., 1999).

Several mechanisms could account for the increased activation of TrkB. Rapid increases in the phosphorylation of TrkB could result either from acute, regulated release of BDNF or from rapid insertion of additional TrkB into the cell membrane (increasing the pool of receptors available for activation), or from some combination of the two. It is also possible that there are activity-dependent alterations in the activity of a phosphatase acting on TrkB. Both regulated release of BDNF and rapid insertion of additional TrkB into the cell membrane have been observed in cell culture (Blochl and Thoenen, 1995, 1996; Goodman et al., 1996; Binder et al., 1999, Aloyz et al., 1999; Balkowiec and Katz, 2000; Meyer-Franke et al., 1998; Du et al., 2000). We think it likely that at least some of the activation we observe results from acute release of BDNF, for two reasons. First, whereas significant TrkB receptor insertion has been observed 30–60 min after stimulation in cell culture (Meyer-Franke et al., 1998; Du et al., 2000), we observe a dramatic increase in TrkB activation within 5 min of theta burst stimulation, which seems very rapid for the insertion of new receptors (although little is currently known about the kinetics of TrkB receptor insertion). Second, we observe a 2- to 3-fold increase in phospho-TrkB after stimulation (Figure 3). For insertion of TrkB to explain this magnitude of increase, the insertion of TrkB would not only have to be very rapid, but also it would have to be equal to the entire amount of TrkB already present in the membrane. While regulated insertion of TrkB receptors is likely to be an important factor in the physiological regulation of BDNF signaling on somewhat longer timescales (we have observed intracellular clusters of TrkB in CA1 dendrites, which may represent pools of receptor available for insertion into the membrane [Drake et al., 1999]), our data suggest that some BDNF is acutely released during the induction of BDNF-dependent forms of plasticity.

Full Expression of BDNF-Dependent Forms of Long-Lasting Potentiation Requires MAPK, but BDNF Does Not Appear to Be a Major Activator of MAPK

Like cAMP and BDNF, MAPK plays a role in synaptic plasticity (English and Sweatt, 1996, 1997; Martin et al., 1997), and both cAMP and BDNF can lead to the activation of MAPK (Martin et al., 1997; Roberson et al., 1999; Meyer-Franke et al., 1998; Bonni et al., 1999), but the mechanisms by which MAPK is activated by stimuli that evoke long-lasting potentiation are unknown. We therefore explored whether BDNF activates MAP kinase in BDNF-dependent forms of long-lasting potentiation.

LTP induced by some protocols has been clearly shown to require MAPK (English and Sweatt, 1997), but this requirement has not been tested in the two forms of BDNF-dependent forms of plasticity examined here. We found that pretreatment of hippocampal slices with the MAPK kinase (MEK) inhibitor UO126, like pretreatment with the BDNF scavenger TrkB-Fc, attenuated theta burst-induced potentiation (Figure 4A; 4 hr after tetanus, percent baseline: saline + theta = 183% ± 17%, U0126 + theta = 125% ± 7%; $p < 0.02$) but had no effect on baseline transmission (after 4 hr of test stimuli, percent baseline: 106% ± 5%). U0126 pretreatment also attenuated potentiation by forskolin (Figure 4B; 4 hr after tetanus, percent baseline: saline + forskolin = 168% ± 18%, U0126 + forskolin = 123% ± 12%). This demonstrates that full expression of theta burst- and forskolin-induced potentiation, like certain other forms of cAMP-dependent plasticity (English and Sweatt, 1997), requires activation of MAPK.

Forskolin treatment has been shown to lead to activation of MAPK, but the mechanism is unknown (Martin et al., 1997; Roberson et al., 1999). If the forskolin-induced

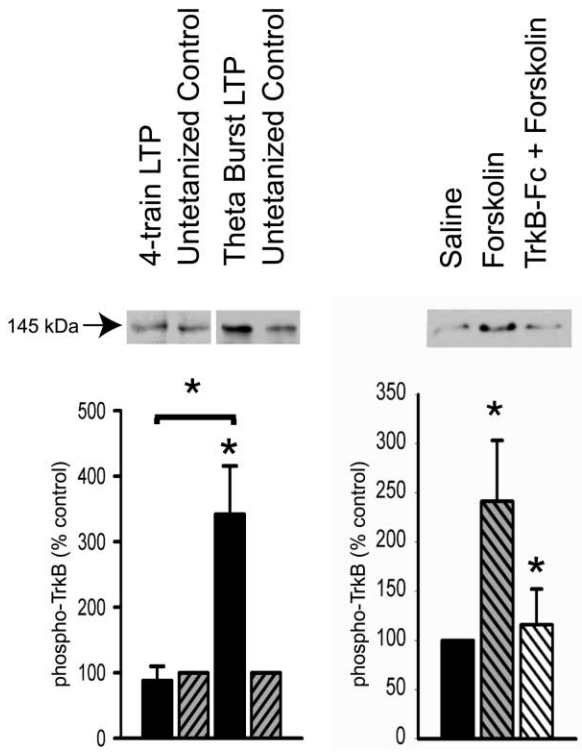

Figure 3. TrkB Receptors Are Rapidly Activated by Stimuli Evoking BDNF-Dependent Forms of Long-Lasting Potentiation

Extracts of hippocampal slices immunoprecipitated with phosphotyrosine antiserum and immunoblotted with an antibody specific for TrkB. In each experiment, the CA1 regions of six to ten hippocampal slices were microdissected and pooled to make each sample. All experiments were repeated at least four times. Pretreatment with the BDNF scavenger TrkB-Fc was used to block forskolin-induced activation of TrkB. 4-train versus 4-train control, $p > 0.62$, paired t test; theta burst versus theta burst control, $p < 0.05$, paired t test; theta burst versus 4-train, $p < 0.05$, t test; $n = 4$ experiments. Forskolin versus saline, $p < 0.05$, paired t test; $n = 4$ forskolin, 4 saline, 2 dideoxyforskolin experiments. Forskolin versus TrkB-Fc + forskolin, $p < 0.05$, paired t test; $n = 4$ experiments.

increase in cAMP uses released BDNF to activate MAPK, then removing free BDNF/TrkB ligands from the slice should attenuate this activation. To test this idea, we prepared protein extracts from hippocampal slices treated with forskolin, vehicle control, or TrkB-Fc plus forskolin and analyzed them on Western blots using an antibody specific for phosphorylated, and therefore active, MAPK (Figure 5). We found that levels of phospho-MAPK were increased by forskolin (forskolin versus DMSO vehicle, $p < 0.015$; forskolin versus 1,9-dideoxyforskolin, $p < 0.04$), consistent with the observation that MAPK activation is required for this form of potentiation. However, to our surprise, the forskolin-induced increase in phospho-MAPK was not significantly reduced by treatment with concentrations of TrkB-Fc that attenuated the forskolin-induced plasticity (2 μg/ml; $p > 0.21$). Higher concentrations of TrkB-Fc (5 and 10 μg/ml) were similarly ineffective (data not shown). This suggests that, while MAPK is recruited in BDNF-dependent forms of plasticity, the principal role of BDNF in this context is not direct activation of a significant percentage of the recruited MAPK.

In BDNF-Dependent Forms of Plasticity, BDNF Modulates the Subcellular Distribution of Activated MAPK

If BDNF does directly influence MAPK signaling, as opposed to modulating plasticity at some point downstream of or parallel to MAPK, it must affect some other aspect of the MAPK signaling process. One possibility is modulation of the subcellular distribution of MAPK. Activated MAPK translocates to the nucleus and is required for long-term facilitation in *Aplysia*, and phospho-MAPK is increased in the nucleus after forskolin treatment in mouse hippocampus (Martin et al., 1997). By analogy, translocation of activated MAPK into the nucleus may be required for L-LTP.

To investigate the possibility that BDNF regulates the subcellular distribution of activated MAPK, we used immunohistochemistry to study the distribution of phospho-MAPK in hippocampal neurons at various times after the induction of potentiation. Because forskolin provides strong, relatively homogeneous stimulation to many synapses in a slice, making it easier to detect subtle effects, we began by examining phospho-MAPK in forskolin-potentiated slices. We first asked whether reducing levels of BDNF altered the amount of phospho-MAPK in the dendrites. As shown in Figure 6 and in agreement with the results of the Western blots for MAPK, the initial forskolin-induced activation of MAPK did not appear to be greatly dependent upon the presence of BDNF. Phospho-MAPK levels were clearly increased after 15 min of forskolin application as compared to baseline (Figure 6, compare panels 6A and 6B with 6C and 6D). At this time point, both the distribution and levels of forskolin-evoked phospho-MAPK immunoreactivity appeared comparable in slices pretreated with vehicle and in slices pretreated with TrkB-Fc (Figure 6, compare panels 6C and 6D); there was no significant difference in the number of dendrites stained for phospho-MAPK (Figure 6K, $p > 0.94$) or the overall area occupied by stained dendrites (Figure 6L, $p > 0.35$).

Thirty minutes after the beginning of the forskolin treatment, a very different pattern emerged. In slices containing normal levels of BDNF, the spatial distribution of forskolin-evoked phospho-MAPK had shifted, with less activated MAPK present in the dendrites and more in the soma (Figure 6E). This shift was much less apparent in slices that lacked BDNF as a result of treatment with TrkB-Fc (Figure 6F); more labeled dendrites were present after TrkB-Fc treatment than in the control (Figure 6, panels 6K and 6L, $p < 0.05$ by both measures). These differences in distribution remained but became less apparent at later time points (Figure 6, compare panels 6G and 6I with 6H and 6J) as levels of phospho-MAPK declined.

To independently confirm our observation about the consequences of removal of BDNF, we performed the same experiment at the 30 min time point in slices from the BDNF knockout mice. Phospho-MAPK was considerably increased by forskolin relative to the inactive analog 1,9 dideoxyforskolin in both wild-type (Figures 7A and 7D) and knockout animals (Figures 7B and 7E); however, in the knockouts, more of the activated MAPK remained in the dendrites (Figures 7A and 7B). Both the number of immunoreactive dendrites in stratum radiatum (Figure 7C, $p < 0.01$) and the overall area occupied

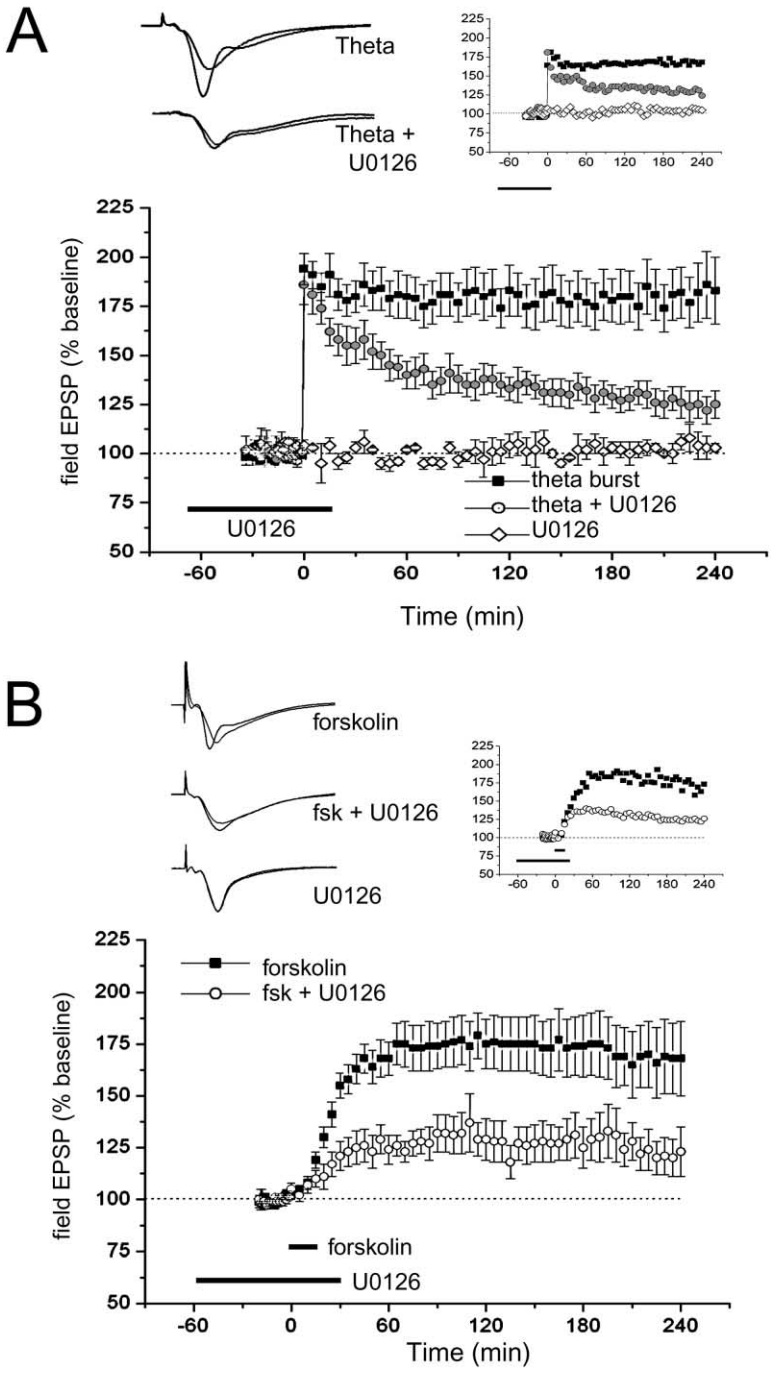

Figure 4. A MAPK Kinase Inhibitor Reduces Two Forms of TrkB-Dependent Long-Lasting Potentiation

(A) Field EPSP slopes plotted as percentage of baseline for slices incubated in saline (n = 6 slices, 6 mice) or UO126 (n = 5 slices, 5 mice) for 1 hr prior to theta burst stimulation. (B) Field EPSP slopes plotted as percentage of baseline before treatment for slices treated with forskolin alone (n = 9 slices, 9 mice) and for slices treated with UO126 followed by forskolin (n = 6 slices, 6 mice). Representative field potentials before and 4 hr after stimulation are shown on the top left of each panel; representative examples of individual experiments are shown on the top right.

by stained dendrites (Figure 7F, $p < 0.05$) were greater in the knockout.

As noted above, phospho-MAPK translocates to the nucleus and is required for long-lasting plasticity in *Aplysia*; it may play a similar role in plasticity in the hippocampus (Martin et al., 1997). When the distribution of phospho-MAPK within the cell bodies was examined 30 min after forskolin treatment, the phospho-MAPK appeared to be excluded from the nucleus more often in slices treated with TrkB-Fc (Figures 6E and 6F) and slices from BDNF knockouts (Figures 7A and 7B) than in controls. This apparent exclusion formed a "nuclear ghost," where high signal in the cytoplasm outlined a nucleus with little or no signal. To analyze the possibility that BDNF influences the nuclear translocation of MAPK, we turned to paraffin-embedded, thin-sectioned potentiated slices, which provide better preservation of cytoarchitecture (Patterson et al., 1992).

As we had observed in Figures 6E and 6F, somatic phospho-MAPK was clearly detectable 30 min after the onset of forskolin treatment (Figures 8B and 8C). As before, nuclear ghosts were more frequently observed in TrkB-Fc-treated slices than in slices treated only with forskolin (Figure 8, compare 8C and 8F with 8B and

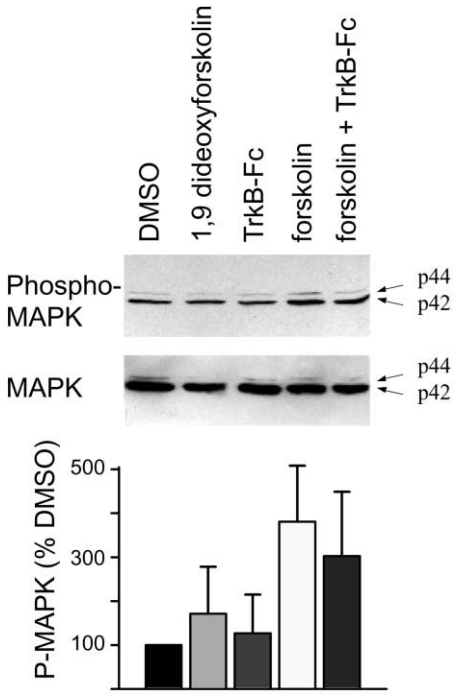

Figure 5. Forskolin-Induced Phosphorylation of MAP Kinase Is Not Reduced by TrkB-Fc

Representative anti-phospho-MAPK and anti-MAPK (detecting both the phosphorylated and unphosphorylated forms) Western blots of extracts made from three to five pooled hippocampal slices. Slices were pretreated with saline or TrkB-Fc for 1–2 hr. Slices were then treated with vehicle, 1,9-dideoxyforskolin, TrkB-Fc, forskolin, or TrkB-Fc + forskolin for 15 min. Parallel Western blots with an antibody detecting total MAPK served as loading controls. $p = 0.01$ forskolin versus ACSF; $p < 0.05$ forskolin versus 1,9-dideoxyforskolin; $p > 0.21$ forskolin versus TrkB-Fc + forskolin; paired t test. $n = 5$ forskolin, 6 TrkB-Fc + forskolin, 5 DMSO, 3 TrkB-Fc, 3 1,9-dideoxyforskolin.

8E). Forskolin increased the amount of phospho-MAPK immunoreactivity in the nuclei (Figure 8, compare 8A and 8D with 8B and 8E). This increase was attenuated by treatment with the BDNF scavenger TrkB-Fc (Figure 8, compare 8B and 8E with 8C and 8F). These effects were statistically significant when differences in background between independent experiments were taken into account either in a two-way ANOVA with condition and experiment as the independent measures (Figure 8J) or by normalizing to saline, in those experiments in which all three conditions were represented (Figures 8K and 8L).

This difference in levels of nuclear phospho-MAPK could derive from a difference in nuclear translocation or from a cell-wide reduction in activated MAPK in the absence of BDNF due to a subtle difference in phosphorylation or dephosphorylation. Our Phospho-MAPK Western blot data (Figure 5) suggest that the differences we observe are probably not the result of a cell-wide reduction in activated MAPK. However, to further distinguish between these possibilities, we also quantitated total phospho-MAPK activity in the pyramidal cell body layer for each image; this measure includes phospho-MAPK in both the nucleus and the somatic cytoplasm. Treatment with TrkB-Fc did not produce any significant difference in total phospho-MAPK in the cell body layer (arbitrary immunofluorescence intensity units: forskolin = 118.2% ± 11.8; forskolin + TrkB-Fc = 108.5% ± 4.7, $p > 0.45$, t test). However, the slightly lower levels of phospho-MAPK present in the cell body layer of the TrkB-Fc-treated slices were consistent with more phospho-MAPK remaining in the dendrites of these slices. The ratio of nuclear to total pyramidal cell body layer phospho-MAPK was significantly reduced in TrkB-Fc-treated slices, consistent with a disruption of nuclear translocation (ratio of nuclear to total pyramidal P-MAPK signal intensity: forskolin = 105% ± 3%; forskolin + TrkB-Fc = 93% ± 4%, $p = 0.01$, t test). However, it is important to note that phospho-MAPK containing nuclei were sometimes seen in slices with reduced levels of BDNF, and some nuclear ghosts were present in slices with normal levels of BDNF. This implies that, while TrkB activation is an important determinant of phospho-MAPK localization, other, overlapping regulatory mechanisms probably exist.

Redistribution of Phospho-MAPK after Theta Burst Potentiation Is Similarly Dependent on TrkB Ligands

While forskolin potentiates large numbers of synapses simultaneously and thus serves as a useful screen for detecting normally subtle events associated with plasticity, we also wanted to know whether TrkB ligands modulate the subcellular distribution of phospho-MAPK in an electrically induced form of BDNF-dependent potentiation.

Paraffin-embedded sections provide excellent preservation of both cytoarchitecture and marks left by electrodes, making it possible to identify the stimulated subregion of potentiated slices. Theta burst potentiation activated MAPK over untetanized levels (Figure 9, compare 9A with 9B and 9C). More nuclear phospho-MAPK was seen after theta burst stimulation than in control slices which received only test stimuli (Figure 9, compare 9A and 9D with 9B and 9E); this demonstrates that the observed increase in phospho-MAPK was due to the potentiating stimulation rather than to the test stimuli or electrode damage. Furthermore, the increase in nuclear phospho-MAPK evoked by theta burst, like the forskolin-evoked increase, was reduced in TrkB-Fc-treated slices (Figure 9, compare 9B and 9E with 9C and 9F). Again, when interexperiment differences in background were taken into account either in a two-way ANOVA (with condition and experiment as the independent factors; Figure 9J) or by normalization to unpotentiated controls (Figures 9K and 9L), both effects were significant.

In theta burst-stimulated slices, as in forskolin-stimulated slices, the ratio of nuclear to total pyramidal cell body layer phospho-MAPK was significantly reduced in TrkB-Fc-treated slices, consistent with a disruption of nuclear translocation (ratio of nuclear to total pyramidal P-MAPK signal intensity: theta = 107% ± 4%; theta + TrkB-Fc = 98% ± 1%, $p < 0.025$, t test). This finding strongly suggests that in theta burst potentiation, as in forskolin-induced potentiation, BDNF modulates the access of activated MAPK to nuclear substrates.

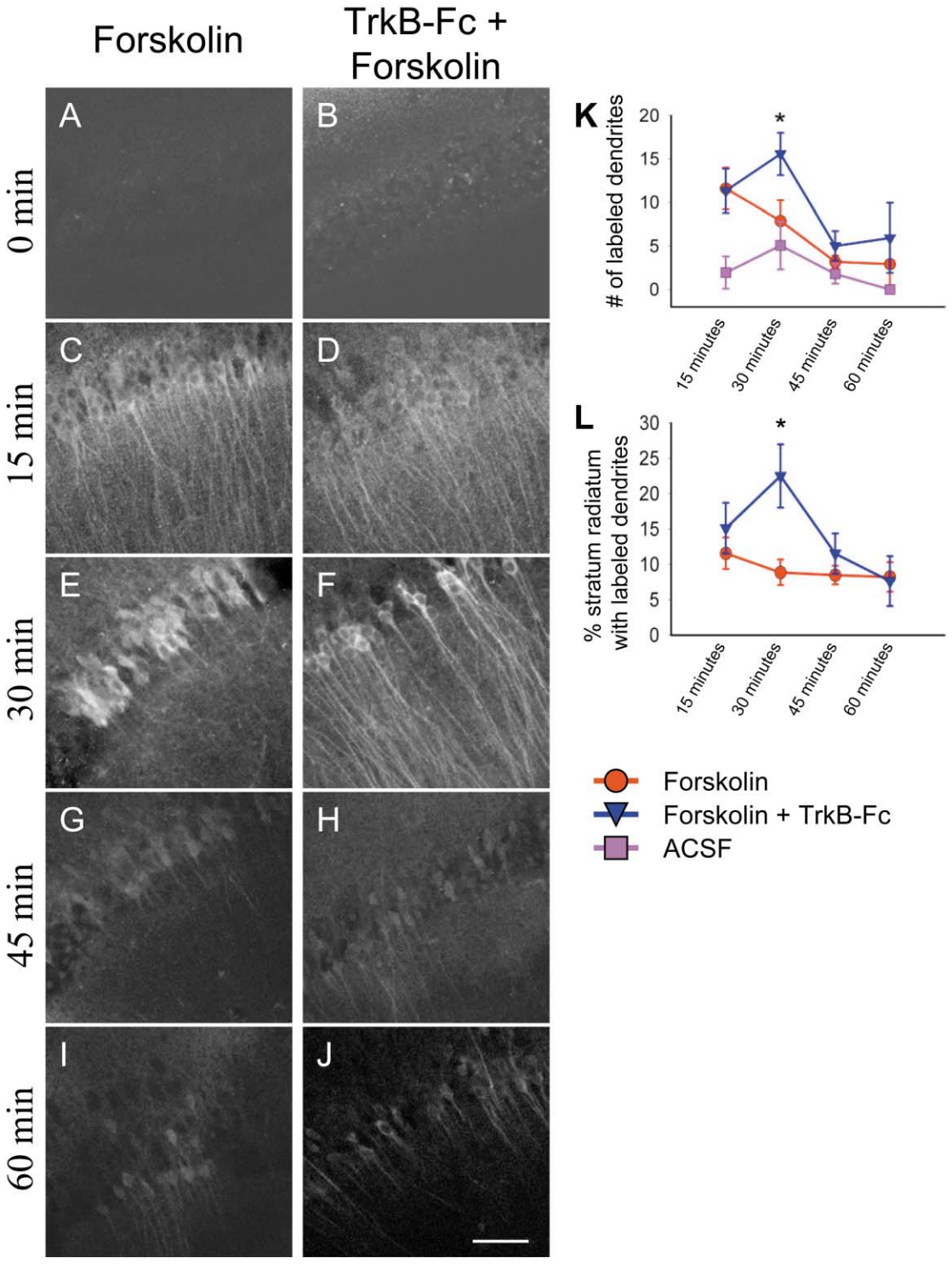

Figure 6. The Subcellular Distribution of Activated MAPK Is Altered in the Absence of TrkB Ligands

Slices were pretreated with physiological saline or TrkB-Fc and then stimulated with forskolin for 15 min. Immunoreactivity for phospho-MAPK was examined in saline-treated slices (A and B) and in forskolin-treated slices 15 min (C and D), 30 min (E and F), 45 min (G and H), and 60 min (I and J) after the start of the treatment. After 15 min (C and D), TrkB-Fc caused little difference in phospho-MAPK distribution, but, at 30 min, there was a marked redistribution of phospho-MAPK signal to the cell body in the forskolin-treated slices (E) that was absent in the TrkB-Fc/forskolin slices (F). Dendrites containing phospho-MAPK were quantitated by an observer (blind to treatment) using two different methods (see Experimental Procedures for details): the number of labeled dendrites in stratum radiatum were counted for each image (K); and the percentage of stratum radiatum occupied by labeled dendrites was quantitated using Metamorph (L). Using both measures, there was a significant difference between ACSF-forskolin and TrkB-Fc-forskolin-treated slices over time ($p < 0.04$; ANOVA with repeated measures). Post-hoc analysis to determine the points at which this difference was significant confirmed that there was more phospho-MAPK in the

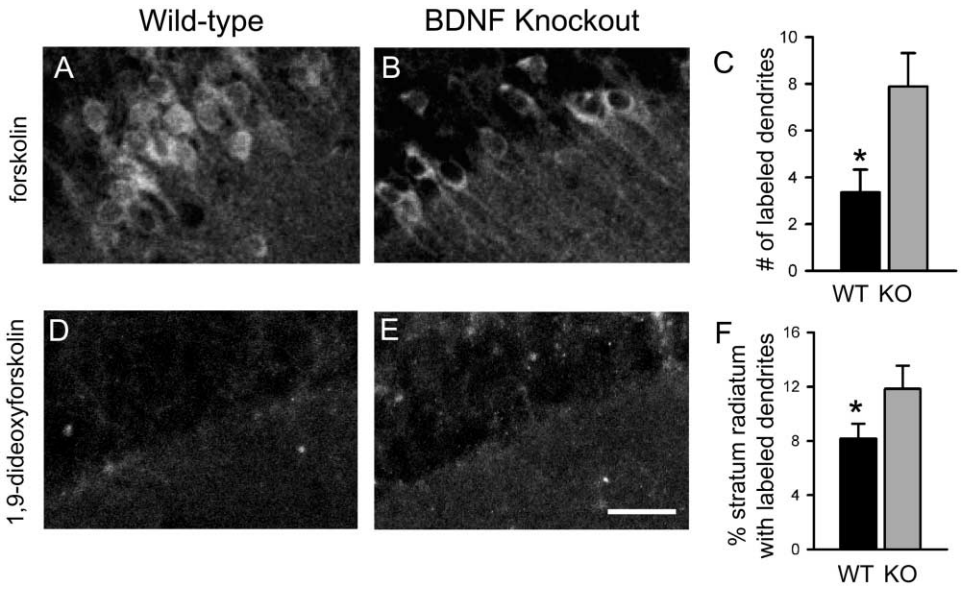

Figure 7. The Subcellular Distribution of Activated MAPK Is Altered in BDNF Knockout Mice

Slices from mice homozygous for the BDNF knockout and wild-type littermates were treated with forskolin or 1,9-dideoxyforskolin for 15 min. Immunoreactivity for phospho-MAPK was examined 30 min after the start of the treatment and quantitated as described in Figure 6. More phospho-MAPK immunoreactivity was visible in dendrites 30 min after forskolin application in knockout mice (B) than in wild-type controls (A). In animals of both genotypes, minimal activation was produced by 1,9-dideoxyforskolin (D and E). Scale bar = 25 μm. Quantitation confirmed that there was a significant difference in dendritic phospho-MAPK between wild-types and knockouts (Experimental Procedures as in Figure 6). The number of labeled dendrites in stratum radiatum was greater in knockouts than in wild-types (panel [C], $p < 0.01$, t test; n = 5 experiments, 18 slices, 5 mice for homozygotes, n = 21 slices, 6 mice for wild-types), as was the overall area occupied by stained dendrites (panel [F], $p < 0.05$, t test; n = 5 experiments, 16 slices, 5 mice for homozygotes, n = 15 slices, 6 mice for wild-types).

Both Presynaptic Terminals and Postsynaptic Spines Contain TrkB Receptors

Since the activation and translocation of MAPK in tetanus-evoked L-LTP occurs postsynaptically, our results are consistent with a postsynaptic action of BDNF. However, other studies have suggested that BDNF can also contribute to LTP through presynaptic mechanisms (Lessmann et al., 1994; Kang and Schuman, 1995; Gottschalk et al., 1998; Li et al., 1998; Pozzo-Miller et al., 1999). These two possibilities are not mutually exclusive; our data do not rule out an additional presynaptic contribution of BDNF to L-LTP.

Indeed, we have previously found that full-length TrkB immunoreactivity in the rat hippocampus is present both presynaptically and postsynaptically, consistent with a role for BDNF or other TrkB ligands in both components of the synapse (Drake et al., 1999). However, the subcellular localization of some molecules differs significantly between rats and mice (e.g., Herkenham and McLean, 1988). We therefore used immuno-EM to examine the localization of full-length TrkB in our mice and again found that TrkB immunoreactivity is present both pre- and postsynaptically at the Schaffer collateral-CA1 synapse (Figure 10). Qualitative examination of the mouse tissue suggested that more of the total TrkB label was postsynaptic in the mouse than in the rat. This suggests the interesting possibility that the relative contribution of BDNF to pre- and postsynaptic mechanisms may differ somewhat between rat and mouse. Such differences might partially explain reported differences in response to BDNF at the Schaffer collateral-CA1 synapse (reviewed in McAllister et al., 1999).

Discussion

We have examined the interplay of three signaling pathways known to play a role in long-term synaptic plasticity and long-term memory storage in the hippocampus: cAMP, MAPK, and BDNF (for reviews, see Kandel and Abel, 1995; Impey et al., 1999; Sweatt, 2001; McAllister et al., 1999; Jankowsky and Patterson, 1999; and see Minichiello et al., 1999). Our results suggest a model of BDNF-dependent forms of long-lasting synaptic plasticity (Figure 11) in which stimuli like theta burst and forskolin recruit cAMP, which here serves two functions. First, elevations in cAMP lead to the activation of MAPK by an unknown mechanism. Second, elevations in cAMP can lead to the release of BDNF and activation of TrkB signaling systems, which in turn promote the translocation of MAPK to the nucleus, providing access to sub-

dendrites of TrkB-Fc-forskolin-treated slices 30 min after forskolin (Duncan's test, $p < 0.05$ for both measures). For panel (K), forskolin-treated slices, n = 15, 13, 10, and 3 for 15, 30, 45, and 60 min, respectively; TrkB-Fc + forskolin-treated slices, n = 12, 11, 8, and 4; ACSF, n = 4, 3, 5, and 1; $p < 0.035$ at 30 min; t test. For panel (L), n values differ slightly because of different exclusion criteria during scoring (as detailed in Experimental Procedures); n = 15, 13, 10, and 3 for forskolin treatment; n = 12, 11, 6, and 6 for forskolin + TrkB-Fc; $p < 0.04$ at 30 min; t test. Scale bar = 50 μm.

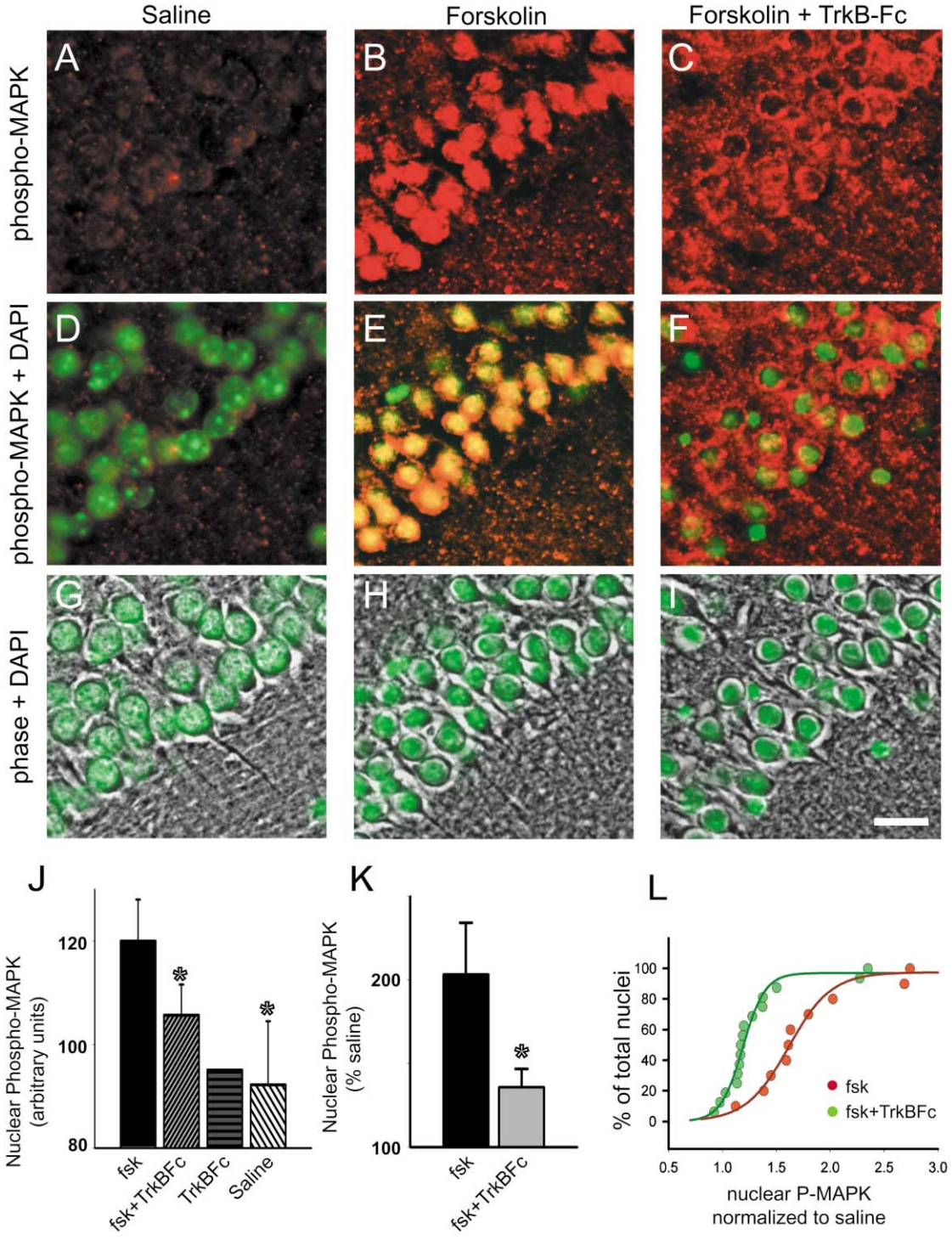

Figure 8. The Absence of TrkB Ligands Attenuates cAMP-Evoked Increases in Nuclear Phospho-MAPK

Slices were pretreated with physiological saline or TrkB-Fc and then stimulated with forskolin for 15 min. Immunoreactivity for phospho-MAPK (30 min after the start of the forskolin treatment) was examined in paraffin-embedded, thin-sectioned slices. More phospho-MAPK signal was apparent in nuclei in saline-pretreated, forskolin-treated slices (B and E) than in TrkB-Fc-pretreated, forskolin-treated slices (C and F). To quantitate this effect, an observer blind to experimental condition manually outlined well-isolated and morphologically clear nuclei in phase images with fluorescent nuclear counterstaining (G, H, and I), creating a template. The template was then superimposed on the phospho-MAPK image, and the intensity of immunoreactivity within these outlined nuclei was quantitated using Universal Imaging Metamorph software (see Experimental Procedures). (J) Raw nuclear phospho-MAPK (arbitrary fluorescence units, mean ± SEM). More nuclear phospho-MAPK was present in forskolin-treated slices than in TrkB-Fc-pretreated/forskolin-treated slices (main effect of TrkB-Fc treatment, $p < 0.02$, two-way ANOVA for TrkB-Fc treatment and experiment [to control for differences in background between experiments]: five experiments, containing a total of nine forskolin-treated and nine TrkB-Fc/forskolin-treated slices). More nuclear phospho-MAPK was also present in forskolin-treated

strates known to contribute to the persistence of potentiation (e.g., the transcription factor CREB—for a review of candidate substrates, see Impey et al., 1999).

This model is based on three important results. First, stimuli that induce forms of long-lasting synaptic plasticity dependent on both cAMP and BDNF evoke very rapid BDNF-dependent activation of TrkB receptors, suggesting that BDNF actively contributes to the induction of these forms of late-phase plasticity. Second, increases in cAMP can induce this activation, suggesting that one of the ways in which cAMP can contribute to long-lasting potentiation is by triggering the release of BDNF and the activation of TrkB receptors. Finally, in these forms of potentiation, TrkB activation is associated with modulation of the subcellular distribution and nuclear translocation of activated MAPK rather than with its activation.

The BDNF Receptor TrkB Is Rapidly Activated by Stimuli that Induce Neurotrophin-Dependent Forms of Long-Lasting Potentiation

Although BDNF is required for full expression of certain forms of plasticity, it has not been clear how it contributes to the signaling cascade leading to L-LTP. Is BDNF's role primarily permissive or instructive—is constitutive release required for maintenance of the cellular systems critical for the induction of plasticity, or, alternatively, is BDNF signaling activated transiently and rapidly as a component of the signal that induces L-LTP? If BDNF is instructive, are its actions upstream, downstream, or in parallel to those of cAMP and MAPK?

Our data indicate that BDNF is likely to be instructive for some forms of long-lasting synaptic plasticity. Both electrical and pharmacological stimuli that produce BDNF-dependent potentiation lead to rapid activation of TrkB. In addition, we show that TrkB activation after forskolin-induced potentiation is blocked by TrkB-Fc, demonstrating that it requires released BDNF (and possibly other TrkB ligands). Interestingly, the 4-train stimulus protocol, which induces L-LTP that is not particularly dependent on BDNF, does not produce detectable activation of TrkB at the time point examined. This indicates that rapid activation of TrkB is not a sign of pathology in the slices after tetanus or a general consequence of activity. Instead, the correlation between the BDNF dependence of different forms of long-lasting plasticity and the efficiency of TrkB activation suggests that the activation of TrkB reflects the recruitment of a specific subset of the available signaling cascades for specific forms of long-lasting plasticity. There is precedent for such a qualitative dissociation—for example, voltage-sensitive calcium channels (VSCCs) and N-Methyl-D-aspartate (NMDA) receptor/channels underlie two mechanistically distinct forms of LTP, are differentially recruited by different electrical stimulus protocols, and in turn recruit different downstream kinases (Grover and Teyler, 1990, 1995; Cavus and Teyler, 1996).

cAMP May Utilize TrkB Signaling Systems for Some Forms of Long-Lasting Plasticity

Stimuli inducing long-lasting forms of plasticity typically recruit cAMP and the cAMP-dependent protein kinase PKA (Huang and Kandel, 1994; Nguyen and Kandel, 1997), which may contribute to long-lasting forms of plasticity in several ways (Bacskai et al., 1993; Blitzer et al., 1995; Winder et al., 1998). For example, in long-term facilitation in *Aplysia*, which resembles some aspects of L-LTP in the hippocampus, elevations of cAMP activate PKA and lead to translocation of its catalytic subunit into the nucleus, where it may phosphorylate and activate transcription factors critical for long-lasting facilitation (Bacskai et al., 1993). Cyclic AMP can also activate other signal transduction cascades, like the MAPK cascade, in both *Aplysia* and rodents (Martin et al., 1997; Vossler et al., 1997; Impey et al., 1998). Our data suggest an additional mechanism by which cAMP may produce some of its actions in long-lasting plasticity—by rapidly recruiting the BDNF/TrkB signaling system.

Both BDNF and MAPK may act downstream of and in parallel to cAMP. Neurotrophins and cAMP synergistically promote both growth and survival in PC12 cells and in cultured cortical neurons (Gunning et al., 1981; Heidemann et al., 1985; Ghosh et al., 1994; Meyer-Franke et al., 1995). BDNF can induce potentiation at frog neuromuscular synapses in culture; this effect is blocked by inhibitors and enhanced by activators of cAMP signaling (Boulanger and Poo, 1999). Similarly, MAPK interacts synergistically with cAMP to promote neuronal differentiation in PC12 cells (Yao et al., 1995). Given that BDNF activates MAPK in retinal ganglion (Meyer-Franke et al., 1998) and cerebellar granule cells (Bonni et al., 1999), it seemed likely that cAMP might release BDNF to activate MAPK, perhaps as a part of a coincidence detection system, in some forms of long-lasting potentiation in the hippocampus.

MAPK Activation and Its Translocation Are Regulated Separately

If cAMP releases BDNF to activate MAPK in BDNF-dependent forms of synaptic plasticity, then both theta burst stimulation and forskolin treatment should lead to the activation of MAPK. One would also predict that treatment with the MAPK kinase (MEK) inhibitor UO126 or the BDNF scavenger TrkB-Fc should reduce both theta burst and forskolin-induced potentiation. This is what we observed. Thus, we were surprised to find that attenuation of BDNF signaling did not significantly reduce the activation of MAPK in response to forskolin. In this system, elevations of cAMP must lead to MAPK activation by other or additional means. For example, elevations in cAMP might trigger the activation of the small GTPase Rap1 (Vossler et al., 1997; de Rooij et al., 1998; Kawaski et al., 1998) or the switching of coupling

slices than in saline-treated slices (main effect of forskolin treatment, $p < 0.0025$; two-way ANOVA for forskolin treatment and experiment: three experiments, containing a total of seven forskolin-treated, four saline-treated slices). (K) Values for forskolin-stimulated slices normalized to unstimulated saline controls; $p = 0.05$. Data are plotted only for the subset of experiments for which saline controls were available (some slices were lost during processing). (L) Cumulative probability histogram of normalized nuclear phospho-MAPK in individual sections. The ordinate represents the total probability that nuclei will contain phospho-MAPK signal falling within a particular range.

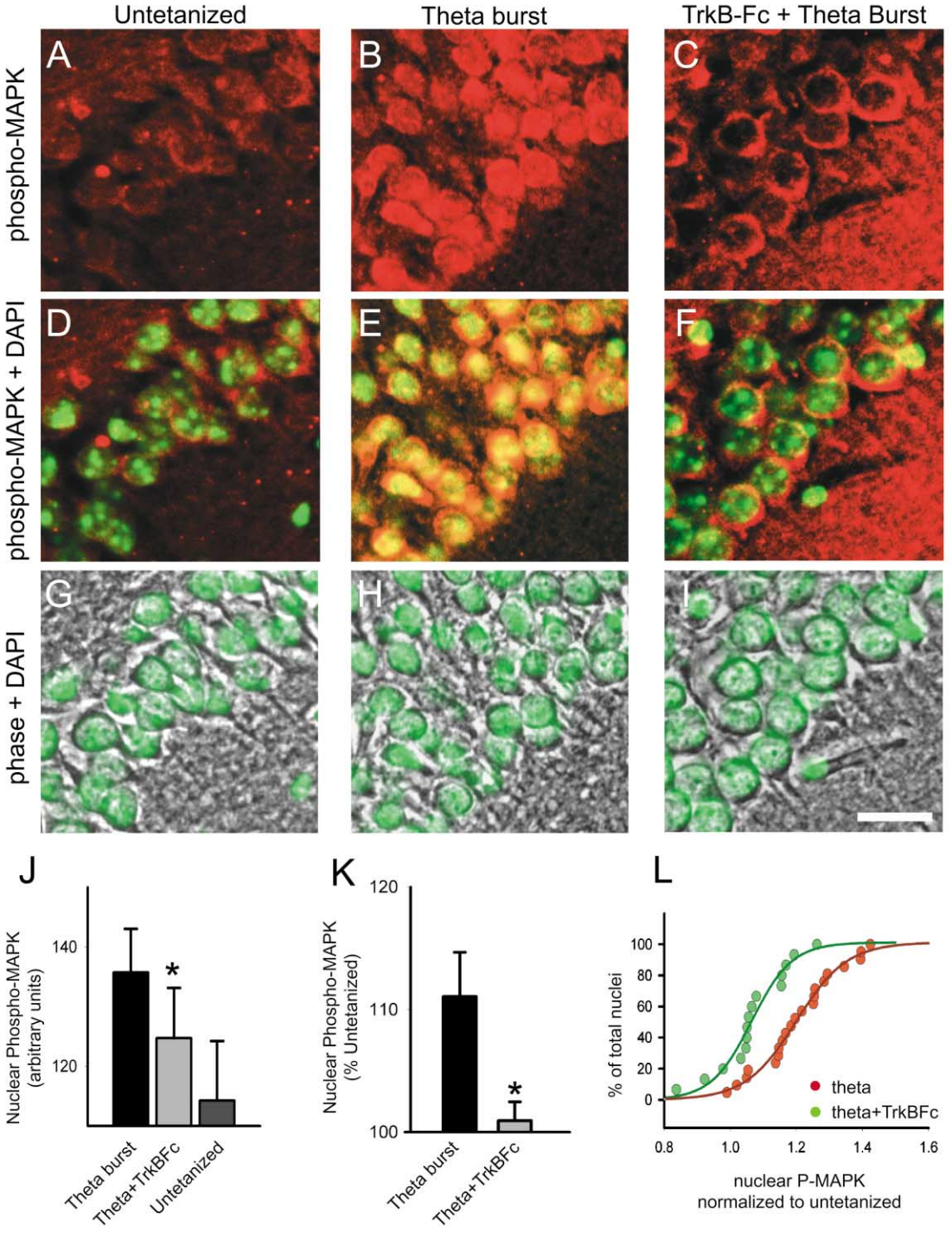

Figure 9. Increases in Nuclear Phospho-MAPK Evoked by Theta Burst Are Also Reduced by TrkB-Fc

Phospho-MAPK immunoreactivity was examined in paraffin-embedded, thin-sectioned slices. Theta-stimulated slices (B and E) showed more nuclear phospho-MAPK than TrkB-Fc-pretreated theta-stimulated slices (C and F) or slices subjected to only test stimuli (A and D). As in Figure 8, phase contrast images and nuclear staining (superimposed in [G], [H], and [I]) were used to select and define the outlines of the nuclei from which nuclear phospho-MAPK signal was quantitated. (J) Raw nuclear phospho-MAPK (arbitrary fluorescence units, mean $\pm$ SEM). TrkB-Fc pretreatment markedly reduced the nuclear localization of phospho-MAPK following tetanus (main effect of treatment, $p = 0.005$, two-way ANOVA for treatment and experiment; by Duncan's post-hoc test: $p = 0.0025$ for theta versus TrkB-Fc/theta; $p < 0.01$ for theta versus unstimulated; $p = 0.2$ for TrkB-Fc/theta versus unstimulated; four experiments, containing a total of eight theta slices, five TrkB-Fc/theta slices, four unstimulated slices). (K) Values for tetanized slices normalized to untetanized controls. $p < 0.005$. (L) Cumulative probability histogram of normalized nuclear phospho-MAPK in individual sections. The ordinate represents the total probability that nuclei will contain phospho-MAPK signal falling within a particular range. *, $p < 0.05$ compared to theta burst.

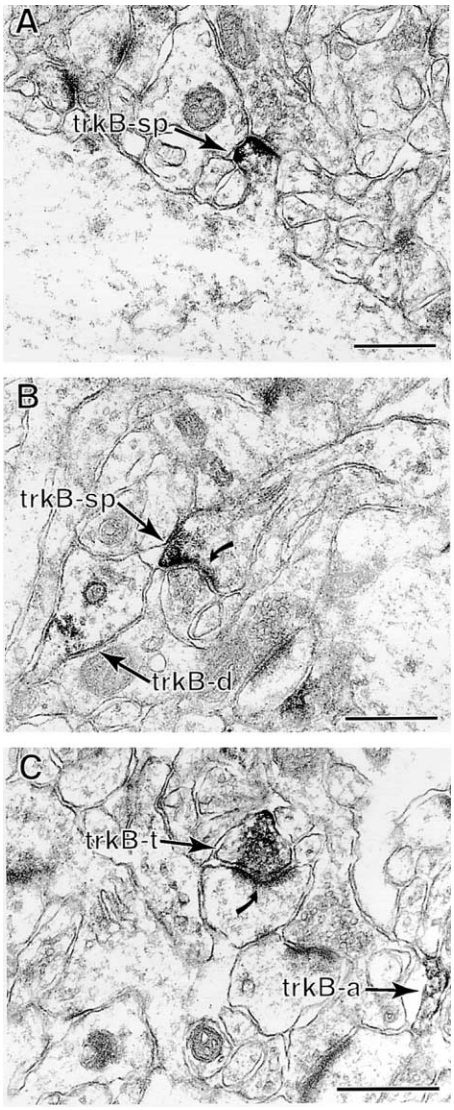

Figure 10. TrkB Immunoreactivity Is Present in Both Axon Terminals and Dendritic Spines

(A) A TrkB-labeled dendritic spine (TrkB-sp) emerges from an unlabeled dendritic shaft in stratum radiatum.

(B) In a labeled spine (TrkB-sp), TrkB is concentrated along the plasma membrane near the synapse (curved arrow), while TrkB labeling in a nearby dendritic shaft (TrkB-d) is primarily around intracellular organelles.

(C) Presynaptic TrkB labeling in an excitatory-type axon terminal (TrkB-t) that forms an asymmetric synapse (curved arrow) with an unlabeled spine. A neighboring axon is also labeled (TrkB-a). Scale bar = 0.5 μm.

of the β2 adrenergic receptor (Daaka et al., 1997) or the release of growth factors other than BDNF.

How might BDNF contribute to long-lasting potentiation in the hippocampus, if not by activation of MAPK? In addition to being regulated by phosphorylation, the actions of MAPK in cultured cells can be regulated by modulation of its subcellular distribution (Lenormand et al., 1993; Widmann et al., 1999). For example, its participation in the induction of long-lasting facilitation in *Aplysia* apparently requires its translocation to the nucleus (Martin et al., 1997). Consistent with the idea that BDNF might regulate the translocation rather than the activation of MAPK, we found that pharmacological or genetic removal of BDNF reduced the redistribution of forskolin-activated MAPK from the dendrites to the cell body. Similarly, treatment with the BDNF scavenger TrkB-Fc reduced the increase in nuclear phospho-MAPK evoked by forskolin or theta burst stimulation. Together, our data strongly suggest that TrkB signaling is an important regulator of phospho-MAPK redistribution during the induction of certain forms of plasticity. Interestingly, the cAMP-dependent protein kinase PKA is required for nuclear translocation of activated MAPK in PC12 cells (Impey et al., 1998). Thus, it is possible that PKA sometimes recruits BDNF to modulate the nuclear translocation of activated MAPK.

How might BDNF regulate the subcellular distribution of activated MAPK? Perhaps BDNF mediates subtle differences in phosphorylation of MAPK, which in turn modulate the subcellular distribution of the activated kinase. It has been hypothesized that MEK, the upstream activator of p42/p44 MAPK, might form part of a cytoplasmic anchoring complex (Lenormand et al., 1998). BDNF might disrupt such an anchoring complex, freeing the activated kinase to translocate. Alternatively, there could be spatially distinct pools/complexes of MAPK, with activation of one leading to phosphorylation of cytoplasmic substrates, and activation of the other, phosphorylation of nuclear substrates. In yeast, MAPK pathways form modules held together by protein-protein interactions; these modules appear to provide spatial regulation of MAPK pathways (Widmann et al., 1999). Thus, BDNF might shift activation to a more translocatable pool of MAPK. It is also possible that TrkB signaling in some way promotes the retention of activated MAPK in the nucleus. Studies in fibroblasts suggest that nuclear accumulation of p42/p44 MAPK is dependent on the synthesis of short-lived proteins, possibly nuclear anchors, induced by activation of p42/p44 MAPK (Lenormand et al., 1998).

There Are Multiple Forms of L-LTP with Distinct Molecular Mechanisms

Our model of BDNF-dependent forms of cAMP-dependent synaptic plasticity has several important implications. First, complex cellular processes such as synaptic plasticity result not from simple linear molecular cascades but from the integration of networks of multiple signaling systems. Second, there are multiple forms of L-LTP, so experiments using different protocols and different experimental preparations may not converge on the same set of molecular mechanisms (Sanes and Lichtman, 1999), although there will undoubtedly be many commonalities. Third, it will be important to identify which specific mechanisms are recruited under physiological conditions and by different learning paradigms, in order to appreciate which forms of experimentally induced plasticity have the most relevance for particular forms of learning and memory. Finally, the existence of different forms of L-LTP suggests that they may be invoked under different circumstances—perhaps regulated by modulatory transmitters or growth factors, behavioral state, attention, or other variables. Such a rich

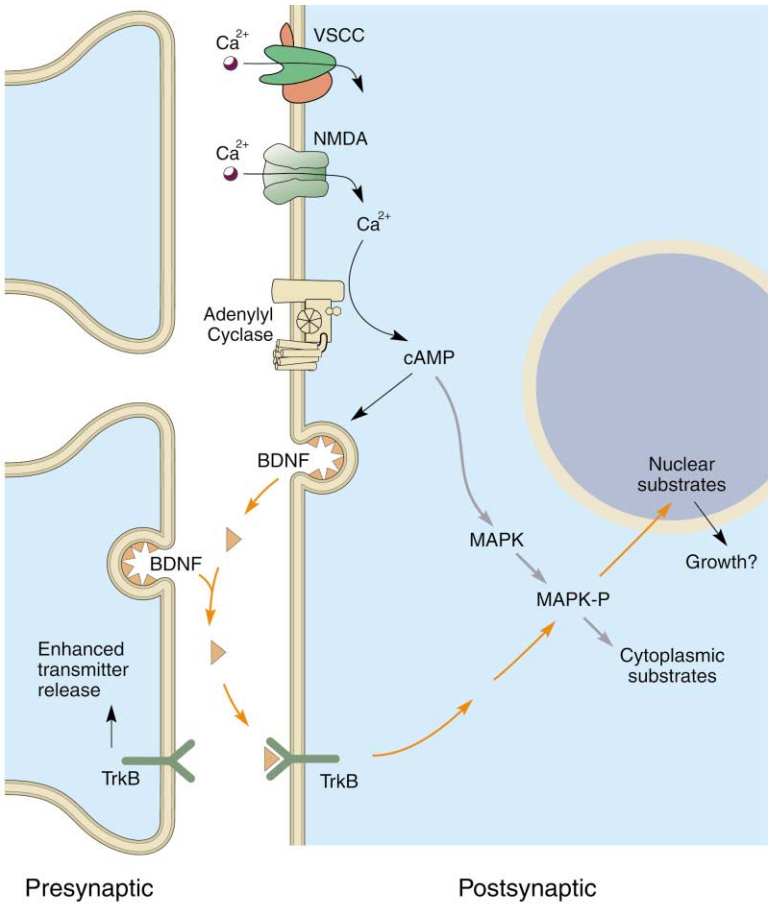

Figure 11. A Model Whereby BDNF May Contribute to Some Forms of Long-Lasting Potentiation

Levels of cAMP are elevated in response to stimuli evoking BDNF-dependent forms of long-lasting potentiation. The increase in cAMP activates the MAPK pathway by an unknown mechanism, as discussed in the text. The elevation of intracellular cAMP may also trigger a rapid increase in activation of TrkB through the rapid release of BDNF (or other TrkB ligands), the rapid insertion of additional TrkB receptors into the membrane for activation, or both. BDNF is likely to have presynaptic effects, such as modulation of transmitter release. However, BDNF also has postsynaptic actions, modulating nuclear translocation of the activated MAP kinase, which may also contribute to long-lasting potentiation. In the presence of BDNF, therefore, activated MAPK may act on nuclear targets (e.g., the transcription factor CREB) important for the persistence of some forms of potentiation; in the absence of BDNF, activated MAPK may have reduced access to the nucleus.

repertoire of molecular mechanisms may greatly increase the computational power and flexibility of learning algorithms.

Experimental Procedures

BDNF Knockout Mice

The BDNF mutant mice were bred in our colony, which is derived from heterozygote mutant mice provided by P. Ernfors and R. Jaenisch (Ernfors et al., 1994). The original BDNF knockout was maintained on a mixed 129/ter SV and BALB/c genetic background (Ernfors et al., 1994). All of the BDNF mutant and wild-type mice used in these studies were descended from mice produced by crossing heterozygous BDNF knockout mice with the original mixed 129/ter SV and BALB/c genetic background with wild-type C57BL/6J mice. The phenotype of the BDNF knockout on the new genetic background was similar to the phenotype of the BDNF knockout on the original mixed 129/ter SV and BALB/c genetic background (Patterson et al., 1996). However, the survival time of the homozygotes was improved (from ~3.5 weeks on the original background to ~5 weeks on the new background). Wild-type and homozygote BDNF mutant mice were examined between 2.5 weeks, soon after the slices become capable of producing LTP, and 4.5 weeks, before the death of the homozygotes. Animals were genotyped using a PCR-based assay (Ernfors et al., 1994).

Slice Preparation

Hippocampi were collected from mice after cervical dislocation. Conventional techniques were used to prepare transverse hippocampal slices (400 μm) (Patterson et al., 1996). Slices used for physiology were maintained in an interface chamber at 28°C and perfused with an oxygenated saline solution (in mM: 124.0 NaCl, 4.4 KCl, 26.0 $NaHCO_3$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 10 glucose). Slices were permitted to recover from cutting for at least 90 min before recordings were begun. To record field excitatory postsynaptic potentials in the CA1 region of the hippocampus, the stimulating and recording electrodes were both placed in the stratum radiatum of CA1.

Stimulation Protocols for Physiology

Because the BDNF mutant mice used in these studies had a deficit in basal synaptic transmission (Patterson et al., 1996), in experiments using both BDNF mutant and wild-type mice, stimuli were delivered at intensities that evoked field EPSP slopes equal to 1mV/ms in all slices. For all other physiology experiments, stimuli were delivered at intensities that evoked field EPSP slopes equal to 1/3 of the

maximum in each slice. Test stimuli were delivered every 2 min, and test responses were recorded for 15–30 min prior to beginning the experiment to assure stability of the response. Slices were tetanized using one of two protocols: either four trains of stimuli, each 1 s, at 100 Hz, delivered 5 min apart; or 12 bursts, of four pulses at 100 Hz, delivered 200 ms apart. Both of these protocols induce L-LTP in wild-type mice of this genetic background. The same stimulus intensity was used for tetanization and evoking test responses. Slices were potentiated pharmacologically by perfusing with 50 μM/ml forskolin in DMSO (0.1% of final volume) for 15 min. Responses were recorded for 4 hr at 0.01 Hz after tetanization or stimulation with forskolin.

Treatment with TrkB-Fc

Freshly cut slices from 3.5- to 4.5-week-old wild-type mice were incubated submerged in either oxygenated saline alone or 2 μg/ml TrkB-Fc (generous gift of Regeneron) in oxygenated saline, at 28°C for 1–1.5 hr prior to use for physiology or immunochemistry. Slices for physiology were then moved to the interface recording chamber and allowed 10 min to recover prior to starting recordings. Some slices for immunochemistry were treated for 15 min with 50 μM/ml forskolin (CalbioChem) in DMSO, or controls including 50 μM/ml 1,9 dideoxyforskolin (CalbioChem) in DMSO, DMSO in saline, or saline solution alone. Some slices for immunochemistry were stimulated using theta burst or test stimuli alone (see above).

Treatment with MAPK Kinase Inhibitors

Slices were pretreated in the interface recording chamber for 1 hr with a membrane-permeable MAPK kinase (MEK) inhibitor, UO126 at 20 μM (Research Biochemicals International) dissolved in DMSO (0.1% of final volume) then diluted in ACSF. UO126 treatment was continued during the 15 min forskolin treatment and maintained for 15 min after the start of the forskolin washout.

Immunoprecipitations with Phosphotyrosine Antibodies and Western Blotting for TrkB

Slices of mouse hippocampus were prepared as described above. In experiments involving tetanized slices, the slices were removed from the recording chamber 5 min after the last stimulus was delivered. In experiments involving drugged slices, slices were removed 15 min after the onset of the drug treatment. Slices were then rapidly frozen on dry ice, and the CA1 region was microdissected out. Each sample in each of these experiments was made from six to ten pooled slices. All samples were homogenized in a plastic Dounce homogenizer in lysis buffer (1× PBS, 0.5% Triton X-100, 2 mM Na-Orthovanadate, 2 mM Na-floride). Protein concentration in the extracts was measured using a Pierce Micro BCA Protein Assay Kit.

Hippocampal extracts were immunoprecipitated with phosphotyrosine antisera (BD Transduction Laboratories). The extracts and a bovine serum albumin control were incubated with the antisera at 4°C for 1 hr. Protein A coupled Agarose beads (BD Transduction Laboratories) in PBS with phosphotyrosyl-phosphatase inhibitors (2 mM Na-Orthovanadate, 2 mM Na-floride) were added to the extracts, and the mixture was incubated at 4°C for 1 hr. The beads were then washed three times (1× PBS, 0.5% Triton X-100, 0.2 mM Na-Orthovanadate, 0.2 mM Na-floride). The washed beads were then resuspended in 2× sample loading buffer and boiled for 5 min. The supernatant was then size fractionated on an 8% Tris-glycine SDS-polyacrylamide gel. The proteins were transferred to Nitrocellulose membranes, and the blots were blocked with BLOTTO (PBS, 0.1% Tween 20, 5% nonfat dry milk) for 30–60 min at room temperature. The blots were then incubated overnight at 4°C with rabbit anti-TrkB (BD Transduction Laboratories) diluted 1:1000 in BLOTTO. The blots were washed in BLOTTO at room temperature and then incubated for 1–2 hr at room temperature with a horseradish peroxidase-labeled goat anti-Rabbit IgG (New England Biolabs). Bands were visualized using enhanced chemiluminescence (Pierce). Densiometric analysis of TrkB immunoreactivity was conducted using a Molecular Dynamics Personal Densitometer SI and Molecular Dynamics ImageQuant software.

Western Blotting for MAPK and Phospho-MAP Kinase

Five slices from each treatment were homogenized in 300 μl of a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.5 mM sodium vanadate, 0.1% 2-mercaptoethanol, 1% Triton X-100, 50 mM sodium fluoride, 5 mM sodium phosphate, 10 mM glycerophosphate, 0.1 mM PMSF. Protein (25 μg) from each lysate was separated in 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with polyclonal antibodies specific to either double-phosphorylated p44 and p42 MAPK (New England Biolabs) or to total p44 and p42 MAPK (New England Biolabs) following manufacturer's protocol. HRP-anti-rabbit conjugate (New England Biolabs) was used as secondary antibodies at a 1:5000 dilution. The MAPK bands were visualized by chemiluminescence using Lumi-GLO reagent (New England Biolabs). Densiometric analysis of immunoreactivity for phosphorylated p44 and p42 MAPK or for total p44 and p42 MAPK was conducted using a Molecular Dynamics Personal Densitometer SI and Molecular Dynamics ImageQuant software. Phospho-MAPK immunoreactivity was normalized to total MAPK immunoreactivity.

Statistical Analysis

All statistical analysis was performed using Statistica (Statsoft, 1996). Comparisons of two groups were done by t test (paired where indicated). Three or more group comparisons were conducted using ANOVA with Duncan's post-hoc tests.

Immunocytochemistry and Confocal Microscopy

Slices were fixed in 4% paraformaldehyde. Phosphatase inhibitors (1 mM sodium orthovanadate and 1 mM sodium fluoride) were included in all subsequent solutions. Slices were permeabilized with 0.2% Triton X-100, quenched with 50 mM NH_4Cl, and blocked with 10% goat serum. Slices labeled with phospho-MAP kinase antibodies (New England Biolabs) were incubated overnight with the primary antisera (1:100 in 10% goat serum in PBS). After extensive washing in PBS, slices were incubated at 4°C overnight with the secondary antibody conjugated to Cy3 (1:200 in 10% goat serum in PBS). The slices were washed 4–6 hr in PBS before imaging. Images were taken using a BioRad MRC 1000 confocal microscope with a 15mV Krypton/Argon laser attached to a Zeiss Axiovert 100 with a 40×, 0.75 n.a. long working distance objective. Kalman-averaged images of eight scans were analyzed for Cy3 fluorescence at 568 nm with 10% laser light using BioRad's imaging software Comos. The same settings were used for all images.

Quantitation of Dendrites in Confocal Images

Dendrites positive for Phospho-MAPK were quantitated by an observer blind to treatment using two techniques.

- To count "number of labeled dendrites" (see Figures 6K and 7C), images were digitally contrast stretched for maximum resolution of phospho-MAPK-positive dendrites. Dendrites entering a band in stratum radiatum parallel to, of about the same width as, and about two cell body diameters away from stratum pyramidale were counted for each image. Generally, four to eight images of each slice were averaged to determine a value for the slice. Images were excluded if background was unacceptably high or if the tissue was obviously damaged. Images where cell bodies were visible but dendrites were not were counted as zero.
- To determine the "percentage of stratum radiatum occupied by labeled dendrites" (see Figures 6L and 7F), images were analyzed using Metamorph (version 4.1.3, Universal Imaging Corporation, 2000). Stratum radiatum was selected using anatomical criteria, excluding obvious artifacts. Images were thresholded to maximize the contrast of dendrites within the selected region. The reported measure represents the percentage area of stratum radiatum occupied by phospho-MAPK-positive dendrites (i.e., above threshold). It was computed by dividing the area within stratum radiatum in which the signal was above threshold by the total selected area. Again, where multiple images were available for a single slice, they were averaged. Images with no visible dendrites, unusually high background, or obvious anatomical damage were excluded.

Immunocytochemistry of Paraffin Sections

Sections were embedded in paraffin and analyzed by a variation on the method of Patterson et al. (1992). Hippocampal slices (400 μm) were prepared and potentiated in the presence or absence of TrkB-

Fc, as described above. Slices were collected and fixed overnight in methyl carnoy (60% methanol, 30% choloroform, 10% acetic acid). Slices were dehydrated through graded alcohols, cleared overnight in methyl salicylate (Sigma), embedded in paraffin, sectioned at 8 μm on a microtome, floated onto microscope slides (Fisher, SuperFrost Plus), and dried overnight at 37°C. Slides were deparafinized in d-Limolene (Stephens Scientific), rehydrated through graded ethanols, incubated in PBS, blocked in PBS/10% goat serum (Sigma), and immunostained with anti-phospho MAPK (NEB/Cell Signaling Technologies, 1:1000 in PBS/goat serum). Slides were washed in PBS, stained in secondary antibody (1:200, Cy3-conjugated goat anti-rabbit, Jackson Immunoresearch; 1–2 hr), washed, and coverslipped with Vecatshield containing the nuclear stain DAPI (Vector, 1:5 dilution of Vectashield-DAPI with Vectashield).

Quantitation of Nuclear Phospo-MAPK

An observer blind to treatment viewed the slides using a Nikon Optiphot microscope. Four sections on each slide were examined, and three images—a phase image, a blue fluorescence image (DAPI), and a red fluorescence image (Cy3)—were captured for each section. To quantitate nuclear phospho-MAPK, combined phase and DAPI images were used to select clear, in-focus, well-isolated nuclei, and to define the outlines of the nuclei. Seven to ten nuclei were manually outlined for each section, and then the intensity of immunoreactivity for Phospho-MAPK within the outlined nuclei (Figure 8E) was quantified using Universal Imaging Metamorph software. Values from all images for a slice were averaged for analysis. To quantitate signal throughout the pyramidal cell layer, the pyramidal cell layer was digitally outlined on the basis of anatomical criteria using Photoshop, excluding areas of significant damage and obvious artifacts. The signal in the pyramidal cell layer was then measured using Metamorph software. Ratios of nuclear signal (as determined above) to pyramidal signal were computed for each image and averaged for each slice.

For theta burst, slices were prepared, embedded, and sectioned identically. Intensity of phospho-MAPK signal and marks left by the electrode were used to determine which side of the slice and which region of CA1 had been stimulated. Two adjacent sections from the stimulated region of each slice were imaged. Nuclear phospho-MAPK was quantitated as above; values for each slice were averaged for analysis. Within each experiment, nuclear phospho-MAPK was normalized to values obtained from slices subjected to test pulses but not to tetanization.

Immunocytochemistry and Electron Microscopy

A polyclonal rabbit antiserum recognizing full-length TrkB was generously supplied by Dr. David Kaplan (Montreal Neurological Institute, McGill University). This antiserum was raised against a synthetic peptide corresponding to amino acids 482–501 in the predicted cytoplasmic domain of full-length TrkB. This antiserum was previously shown to recognize the full-length TrkB receptor but not the truncated TrkB receptor or full-length TrkA or TrkC receptors (Fryer et al., 1996) and has been shown to be specific in rat hippocampus using the labeling procedures described here (Drake et al., 1999).

Two 4.5-week-old wild-type mice were deeply anesthetized with sodium pentobarbital and were perfused sequentially with 2% paraformaldehyde and 3.75% acrolein (Polysciences, Warrington, PA) in 0.1 M phosphate buffer (PB; pH 7.4) followed by 2% paraformaldehyde in PB. Coronal blocks of the brain were postfixed in the last fixative for 30 min. Coronal sections (40 μm thick) were cut through the hippocampal formation on a vibrating microtome (Leica) and collected in PB. Free-floating sections were selected from the mid-septotemporal portion and pretreated with 1% sodium borohydride in PB as described (Drake et al., 1999).

Sections were immunolabeled for TrkB as previously described (Drake et al., 1999). In brief, sections were "freeze thawed" then incubated sequentially in (1) a 1/2000 dilution of TrkB antiserum in 0.1 M Tris-saline buffer (TS; pH 7.6) + 0.1% bovine serum albumen (BSA) for 36–40 hr at 4°C; (2) biotinylated goat anti-rabbit IgG (Vector; Burlingame, CA) diluted 1/400 in TS + 0.1% BSA for 30 min at room temperature; and (3) avidin-biotin peroxidase complex (Vectastain Elite kit; Vector) at twice the recommended dilution in TS for 30 min at room temperature. Tissue was rinsed in TS between incubations. Immunolabeling was visualized by incubating sections in PB containing 3,3′-diaminobenzidine (DAB, 0.22%) and hydrogen peroxide (0.00003%).

Labeled sections were fixed in 2% osmium tetroxide in PB for 1 hr, rinsed in PB and dehydrated through a series of ethanols and propylene oxide, and flat embedded in EMBed between two sheets of Aclar plastic. Immunolabeled regions of the CA1 region of hippocampus were glued onto Epon blocks. Ultrathin (~60 nm) sections were cut on an ultratome and collected on copper grids. Sections were counterstained with uranyl acetate and lead citrate.

Thin sections were examined and photographed on a Philips CM10 electron microscope. EM analysis was conducted using standardized definitions of somata, dendrites, axons, axon terminals, or glia (Peters et al., 1991). Some terminals possessed distinctive features (shape, size, vesicle content, and synaptic morphology) that allowed their categorization as "excitatory type" or "inhibitory type," based on previous descriptions of glutamatergic and GABAergic terminals, respectively (Bramham et al., 1990; Peters et al., 1991).

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