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Elevated BDNF after Cocaine Withdrawal Facilitates LTP in Medial Prefrontal Cortex by Suppressing GABA Inhibition

Hui Lu,[1] Pei-lin Cheng,[1] Byung Kook Lim,[1] Nina Khoshnevisrad,[1] and Mu-ming Poo[1,*]
[1]Division of Neurobiology, Department of Molecular and Cell Biology, Helen Wills Neuroscience Institute, University of California, Berkeley, Berkeley, CA 94720, USA
*Correspondence: mpoo@berkeley.edu
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SUMMARY

Medial prefrontal cortex (mPFC) is known to be involved in relapse after cocaine withdrawal, but the underlying cellular mechanism remains largely unknown. Here, we report that after terminating repeated cocaine exposure in rats, a gradual increase in the expression of brain-derived neurotrophic factor (BDNF) in the mPFC facilitates activity-induced long-term potentiation (LTP) of excitatory synapses on layer V pyramidal neurons. This enhanced synaptic plasticity could be attributed to BDNF-induced suppression of GABAergic inhibition in the mPFC by reducing the surface expression of $GABA_A$ receptors. The BDNF effect was mediated by BDNF-TrkB-phosphatase 2A signaling pathway. Downregulating TrkB expression bilaterally in the mPFC reduced the locomotor hypersensitivity to cocaine 8 days after cocaine withdrawal. Thus, elevated BDNF expression after cocaine withdrawal sensitizes the excitatory synapses in the mPFC to undergo activity-induced persistent potentiation that may contribute to cue-induced drug craving and drug-seeking behavior.

INTRODUCTION

Understanding the neurobiological basis of cocaine addiction, including cellular mechanisms underlying drug craving and relapse after cocaine withdrawal, will help the development of new therapeutic approaches to cocaine addiction. Activation of mesolimbic dopamine circuits, including the ventral tegmental area (VTA) and limbic structures they innervate, is known to be involved in cocaine craving. Human brain imaging studies showed that activation of two limbic areas—amygdala and anterior cingulate cortex—was significantly increased when cocaine users experienced cue-induced cocaine craving (Childress et al., 1999). Single-unit recording from rats revealed that exposure to cocaine-associated cues after cocaine withdrawal triggered the activation of prefrontal cortex (PFC) (Rebec and Sun, 2005). The rat medial PFC (mPFC) receives dopaminergic innervation from the VTA (Oades and Halliday, 1987), and cocaine-induced synaptic changes in this area may thus contribute to drug craving after cocaine withdrawal.

Chronic drug exposure causes long-lasting cellular adaptations, including persistent synaptic changes, in the reward circuits that may underlie compulsive drug consumption and relapse, even after long periods of abstinence (Hyman et al., 2006; White and Kalivas, 1998). For example, single or repeated injection of cocaine in rodents induces potentiation of excitatory synapses on dopamine neurons in the VTA (Argilli et al., 2008; Mameli et al., 2009; Ungless et al., 2001). Repeated cocaine exposure also modifies the extent of activity-dependent plasticity, as shown by the facilitated long-term potentiation (LTP) induction in VTA dopamine neurons (Liu et al., 2005) and mPFC pyramidal neurons (Huang et al., 2007).

After withdrawal from repeated cocaine exposure in rats the expression of brain-derived neurotrophic factor (BDNF) increases in the VTA, nucleus accumbens (NAc), amygdala, hippocampus, and mPFC (Grimm et al., 2003; McGinty et al., 2010). Since BDNF modulates synaptic efficacy and plasticity (Poo, 2001), such increased BDNF expression after cocaine withdrawal may cause synaptic changes that contribute to the rat's enhanced responses to drug-associated cues and compulsive drug seeking behavior. Indeed, the increased BDNF expression in the VTA after cocaine withdrawal in rats sensitizes the excitatory synapses on dopamine neurons for activity-induced LTP (Pu et al., 2006). Furthermore, infusion of BDNF into the VTA promotes a transition from drug-naive to drug-dependent motivational state of the rat by inducing a switch of VTA GABAergic neurons from inhibitory to excitatory (Vargas-Perez et al., 2009). In contrast to the BDNF's action in the VTA, nothing is known about the potential BDNF effect on synaptic function and plasticity in mPFC neurons after cocaine withdrawal. In the present study, we found that elevated BDNF expression after cocaine withdrawal facilitates activity-induced LTP of excitatory synapses on mPFC pyramidal neurons by downregulating the surface level of $GABA_A$ receptors ($GABA_AR$), and this BDNF effect was mediated by postsynaptic TrkB signaling that increased protein phosphatase 2A (PP2A)-dependent de-phosphorylation of the $GABA_AR$. At the behavioral level, we found that downregulating TrkB expression bilaterally in the mPFC reduced the locomotor hypersensitivity to cocaine after withdrawal from

repeated cocaine exposure. These results provide new insights into cellular mechanisms underlying synaptic adaptation in the mPFC after cocaine withdrawal.

RESULTS

Delayed Facilitation of LTP Induction after Cocaine Withdrawal

Rats were given intraperitoneal injections of either saline (0.9% NaCl, 1ml kg^{-1}) or saline containing cocaine (15 mg kg^{-1} in 1ml kg^{-1} of saline) for 7 days from P18 to P24 in their home cage. We obtained PFC slices from rats 5–17 days after withdrawal from the above saline/cocaine treatment. We used extracellular stimulation at layer II/III of the prelimbic region and whole-cell recording from layer V pyramidal neurons of the mPFC to monitor excitatory postsynaptic potentials (EPSPs) under the current clamp at −70 mV, corresponding to the reversal potential of inhibitory postsynaptic currents (IPSCs) (Liu et al., 2005). These EPSPs were completely abolished by glutamate receptor antagonists CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, 10 μM) and APV (D-2-amino-5-phosphonopentanoic acid, 50 μM). To induce LTP, we used a modified theta burst stimulation (mTBS) protocol (Experimental Procedures; Lu et al., 2009). We found that mTBS failed to induce LTP in layer V pyramidal cells in slices obtained from rat 5–17 days after withdrawal from saline treatment (Figures 1A and 1C), whereas it induced a robust LTP in slices from cocaine-treated rats (Figures 1B and 1D). Similar experiments performed during the first 1–4 days after cocaine/saline withdrawal showed that LTP was largely absent in both groups (Figures 1E and 1F). The magnitude of LTP, defined as the mean normalized EPSP amplitude at 10–20 min after mTBS, was not different between the saline and cocaine groups during 1–4 days after withdrawal ($p = 0.08$, Kolmogorov-Smirnov or K-S test). However, beginning on day 5, the cocaine-exposed rats showed an enhanced LTP induced by the same mTBS (Figures 1G and 1H), significantly different with that found for the saline group ($p < 0.0001$, K-S test) and for the cocaine group during 1–4 days after withdrawal ($p < 0.001$, K-S test). There was no significant difference between the early (1–4 days) and late (5–17 days) saline groups ($p = 0.164$, K-S test). Thus, there was a delayed appearance of LTP facilitation after cocaine withdrawal.

We also examined the induction of LTD, using a spike timing stimulation protocol consisting of repetitive paired post-pre-stimulation with 8 ms interval (0.2 Hz, 80 pairs; see Figure S1A available online). In contrast to that found for LTP induction, no difference in LTD induction at these excitatory synapses was observed in rats 9–11 days after withdrawal between saline- and cocaine-treated rats (Figures S1B and S1C). Thus, there was a selective sensitization of these mPFC excitatory synapses to LTP induction after cocaine withdrawal, without apparent change in that to LTD induction.

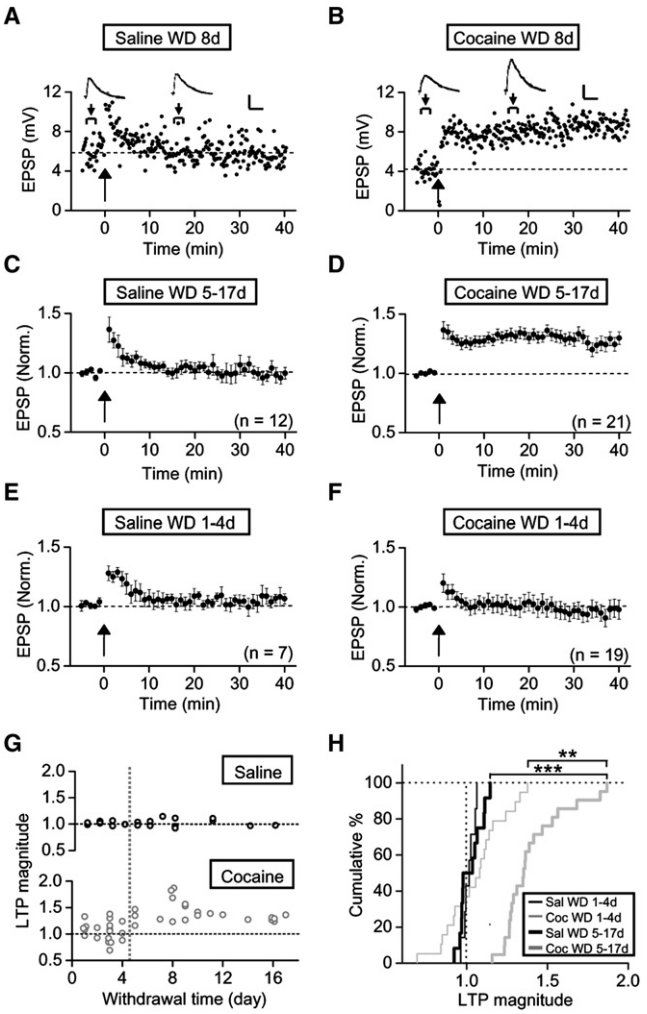

Figure 1. LTP Induction in mPFC Is Facilitated after Cocaine Withdrawal

(A and B) Example data on changes in the EPSP amplitude induced by modified theta burst stimulation (mTBS, arrow) recorded in mPFC layer V pyramidal cells in rat brain slice 8 days after withdrawal from 7-day saline (A) or cocaine (B) treatment. Sample traces above: averages of 10 EPSPs (arrowhead). "WD 8 days": 8 days after withdrawal. Scales: 4 mV, 50 ms.

(C and D) Summary of all experiments similar to those in A and B, showing EPSP amplitude (mean ± SEM, normalized by the mean baseline amplitude) before and after mTBS for rats 5–17 days after withdrawal from treatment with saline (C, n = 12 cells, 9 rats) or cocaine (B, n = 21 cells, 15 rats).

(E and F) Same as (C) and (D), for rats 1–4 days after withdrawal from saline (E, n = 7 cells, 4 rats) or cocaine (F, n = 19 cells, 11 rats).

(G) Summary of the magnitude of LTP induced in mPFC pyramidal cells on different days after withdrawal from saline or cocaine treatment (for 7 days).

(H) Cumulative percentage plot of the distribution of LTP magnitude, for data shown in G. (***$p < 0.0001$; **$p < 0.001$; Kolmogorov-Smirnov or K-S test). Error bars = SEM. See also Figures S1 and S2.

BDNF Elevation after Cocaine Withdrawal Facilitates LTP

The induction and stabilization of LTP require BDNF signaling in many brain regions (Du and Poo, 2004; Pang et al., 2004; Rex et al., 2007) and BDNF expression is elevated in the reward system after cocaine withdrawal (Grimm et al., 2003; McGinty et al., 2010). We thus examined whether elevated BDNF is responsible for the facilitation of LTP induction in the mPFC after cocaine withdrawal. Enzyme-linked immunosorbent assay (ELISA) showed that the level of BDNF in rat mPFC tissue lysates

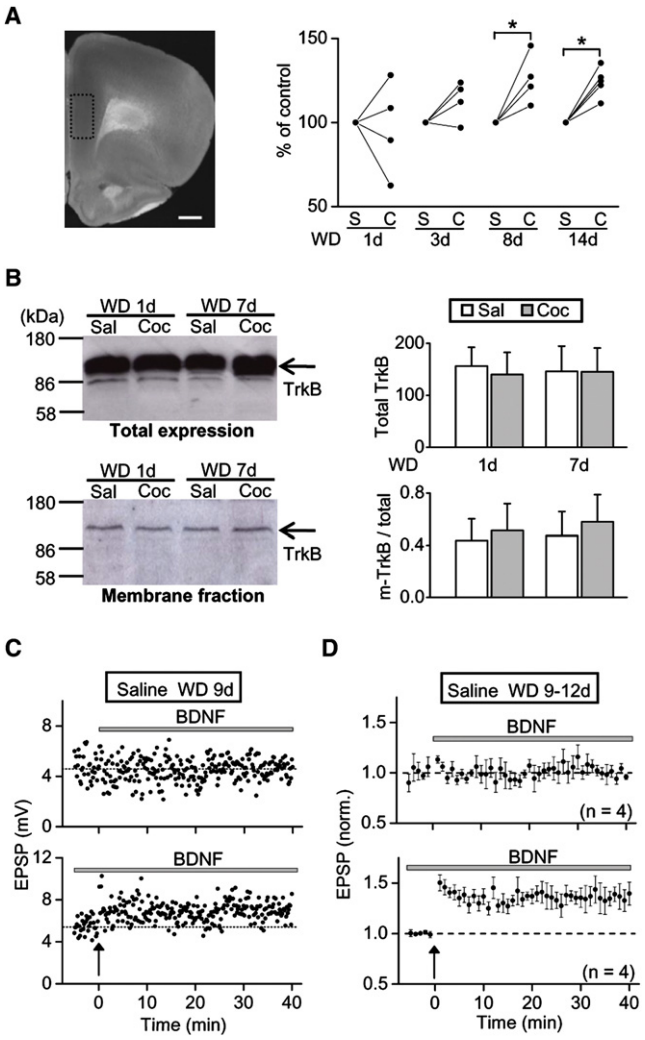

Figure 2. Elevated BDNF Level after Cocaine Withdrawal Facilitates LTP

(A) Left, image of a freshly isolated slice containing mPFC, with the box indicating mPFC tissue collected for ELISA. Scale: 1 mm. Right, the BDNF level in the mPFC tissue from cocaine-withdrawn normalized by that of saline-withdrawn paired littermates (n = 4–5; *p < 0.05, paired t test).

(B) Left, example immunoblot of the lysates of rat mPFC tissues obtained from littermates 1 and 7 days after withdrawal from saline or cocaine, showing the level of total protein (upper panel) and membrane-bound fraction for TrkB (lower panel). Right, densitometric measurements of band densities for membrane-bound and total levels of TrkB. (n = 3; p > 0.05, paired t test).

(C) Example data showing that exogenous BDNF (200 ng/ml) had no effect on the EPSP amplitude in a slice from 9-day saline-withdrawn rat (Top), but mTBS (arrow) induced LTP in the presence of BDNF (bottom).

(D) Summary of all experiments similar to those in (C), for rats 9–12 days after saline withdrawal (n = 4 cells from 4 rats for both top and bottom).

Error bars = SEM. See also Figure S2.

was significantly higher over 1 week but not 1 or 3 days after cocaine withdrawal, as compared to that found for the mPFC tissue from parallel saline-injected littermates (Figure 2A). This time course of BDNF elevation matched that found for the facilitated LTP induction (Figure 1G), suggesting that these two phenomena may be related. No change were found on the surface expression and total level of the high-affinity BDNF receptor TrkB, either 1 day or 7 days after cocaine withdrawal, comparing to those found in corresponding saline-treated control littermates (Figure 2B).

To further test whether BDNF elevation is sufficient to facilitate LTP induction at excitatory synapses on mPFC layer V pyramidal cells, we exogenously applied BDNF (200 ng/ml) to mPFC slices from saline-treated rats 9 days after withdrawal. We found that the bath-applied BDNF did not affect the EPSP amplitude, but caused a robust LTP induction by mTBS (Figures 2C, 2D, and S2A; p = 0.0002, for BDNF versus BDNF + mTBS, K-S test), which was ineffective in LTP induction in the absence of exogenous BDNF (Figure 1C). We also found that bath-applied BDNF had no effect on LTD induction (Figures S1D and S1E; p = 0.19, for Sal versus Sal + BDNF, K-S test). Thus, BDNF had selectively increased the susceptibility of mPFC excitatory synapses to LTP induction without affecting the efficacy of basal transmission. Moreover, in the presence of exogenous BDNF the magnitude of LTP induced in slices from saline-treated rats was similar to that found in slices from cocaine-treated rats (Figure S2A; p = 0.62, K-S test), suggesting that LTP induction in cocaine-treated rats may be attributed to the elevated expression of endogenous BDNF in the mPFC after cocaine withdrawal (Figure 2A).

Postsynaptic TrkB Is Required for LTP Facilitation

To test whether the high-affinity BNDF receptor TrkB is required for LTP facilitation in the mPFC pyramidal cells after cocaine withdrawal, we used lentiviral expression of specific short hairpin RNAs (shRNA) against *TrkB* to downregulate TrkB expression in a subpopulation of mPFC neurons (see Experimental Procedures). One effective sequence of *TrkB* shRNA and a control scrambled shRNA were prepared and their efficacy in downregulating TrkB expression in cultured cortical cells was confirmed by Western blot analysis of cell lysates (Figure S3A) and by imaging Ca^{2+} elevation induced by BDNF in single infected cultured neurons (Figures S3C and S3D). Lentiviral constructs expressing EGFP alone or EGFP together with *TrkB* shRNA or scrambled shRNA were stereotactically and bilaterally injected into the deep layers in the prelimbic region of mPFC of P18 rats.

Effective expression of EGFP was found in cells of layer V and VI when the PFC was sectioned and examined at P33 (Figure 3A). Whole-cell recording from EGFP-expressing cells was made using a patch pipette containing the fluorescent dye Alexa 594, which was used to guide pipette positioning and to mark the recorded cell (Figure 3B). In neurons with TrkB downregulated by *TrkB* shRNA, mTBS failed to induce LTP at the excitatory synapses on mPFC layer V pyramidal neurons in slices from virus-injected rat (P33) 9 days after cocaine withdrawal treatment (during P18–P24), whereas robust LTP was induced in nonfluorescent (control) neurons in the same mPFC area of the same rat brain slices (Figures 3C, 3D, and S2B; p < 0.0001, for *TrkB* shRNA versus noninfected, K-S test) or in fluorescent cells from cocaine-withdrawn rats injected with lentiviruses expressing either EGFP/scrambled shRNA (Figures 3E and

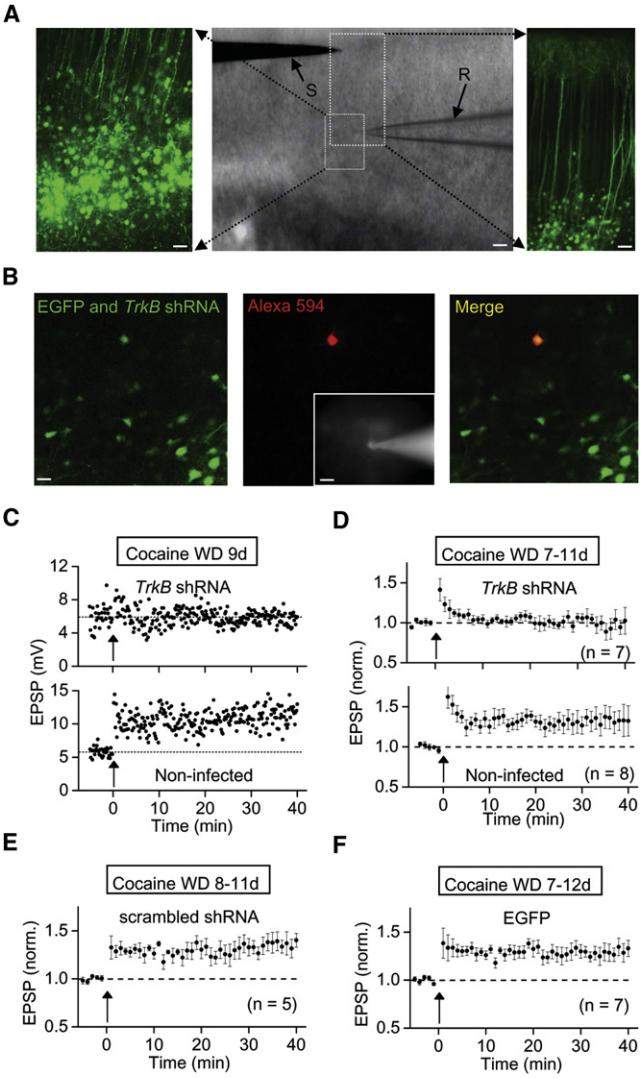

Figure 3. *TrkB* shRNA Expression in Pyramidal Cells Prevents LTP Facilitation

(A) Bright-field image shows the position of stimulating (S) and recording (R) electrodes, and fluorescence images shows lentivirus-based expression of EGFP in pyramidal cells in deep layers of the mPFC (of boxed areas), taken after recording. Scales (μm): 30 (left), 100 (middle), 50 (right).

(B) Image of mPFC neurons expressing EGFP and *TrkB* shRNA, with one recorded pyramidal cell loaded with Alexa 594 (red), taken from a fixed slice after recording. Insert shows Alexa 594 loading during recording. Scales: 20 μm.

(C) Examples of the effect of mTBS in a pyramidal cell expressing EGFP and *TrkB* shRNA (top) and a noninfected control cell (bottom) from two slices of the same rat brain 9 days after cocaine withdrawal.

(D) Summary of all experiments similar to those in (C), from neurons expressing *TrkB* shRNA (n = 7 cell, 6 rats) and noninfected neurons (n = 8 cells, 6 rats).

(E and F) Summary of experiments on LTP induction for mPFC pyramidal cells expressing EGFP and scrambled *TrkB* shRNA (E, n = 5 cells, 4 rats) or EGFP alone (F, n = 7 cells, 5 rats).

Error bars = SEM. See also Figures S2 and S3.

S2B; $p < 0.0001$, for *TrkB* shRNA versus SC shRNA, K-S test) or EGFP alone (Figures 3F and S2B; $p < 0.0001$, for *TrkB* shRNA versus EGFP, K-S test). Thus, BNDF-TrkB signaling in the postsynaptic pyramidal cells is required for LTP facilitation after cocaine withdrawal.

BDNF-TrkB Signaling Reduces GABAergic Inhibition

Next, we examined whether elevated BDNF had any effect on GABAergic inhibition in layer V pyramidal cells of the mPFC, by recording inhibitory postsynaptic currents (IPSCs) in these neurons evoked by extracellular stimulation at layer II/III in the presence of glutamate receptor blockers (see Experimental Procedures). We found that bath application of BDNF (200 ng/ml) to PFC slices from rats 9–11 days after withdrawal from the saline treatment resulted in changes in the mean IPSC amplitude in two phases—a transient but small elevation (1.05 ± 0.03 at the peak, normalized to mean baseline value, n = 8) followed by a prolonged and marked reduction after ~7 min (Figure 4A), consistent with findings in cultured neurons (Jovanovic et al., 2004; Kanematsu et al., 2006). In contrast, in slices obtained from rats withdrawn from cocaine for a similar period (9–11 days), no effect of exogenous BNDF on the IPSC amplitude was observed (Figures 4B and S2C; $p = 0.0028$, K-S test), suggesting that elevated endogenous BDNF may have already suppressed GABAergic synapses and thus occluded the effect of exogenous BDNF. Furthermore, the suppressive effect of exogenous BDNF on the IPSC amplitude required TrkB signaling, because it was abolished by the presence of the tyrosine kinase inhibitor K-252a (200 nM; Figures 4C and S2C; $p = 0.013$, K-S test), but not by its less potent analog K-252b (Figures 4D and S2C; $p = 0.42$, K-S test).

To further investigate the suppressive effect of BDNF on GABAergic inhibition in the mPFC, we examined whether GABAergic inhibition of layer V pyramidal cells exhibits a reduction after cocaine withdrawal. Whole-cell recording from these neurons was made to measure the maximal amplitude of IPSCs evoked by extracellular stimulation at layer II/III in slices from either saline- or cocaine-withdrawn rats. We evoked IPSCs under voltage clamp at −20 mV, with the stimulation intensity gradually increased until the IPSC amplitude reached the maximum. The mean amplitude of maximal IPSCs recorded was indeed reduced after cocaine withdrawal, with a time course similar to that found for the increase of BDNF expression and the facilitation of LTP: significant reduction was observed over 1 week (but not 1 or 3 days) after cocaine withdrawal (Figure 4E; $p < 0.05$, t test). Furthermore, this reduction of the IPSC amplitude was mediated by BDNF-TrkB signaling, because mPFC pyramidal cells expressing *EGFP-TrkB* shRNA showed higher amplitude of maximal IPSCs than that found in control nonfluorescent cells in the same slices from cocaine-withdrawn rats (Figure 4F). Finally, consistent with the idea that the reduction of the IPSC amplitude is due to postsynaptic (rather than presynaptic) changes, the mean amplitude of miniature IPSCs (mIPSCs) was significantly lower in cocaine- than saline-treated rats over 1 week (8–10 days) after withdrawal ($p < 0.05$, t test), but the frequency and decay time of mIPSC were not significantly different (Figure 4G). The mean paired-pulse ratios of IPSCs over 1 week (8–14 days) after withdrawal were also not significant between two groups of rats (Figure S4). Taken together, these results showed that BDNF-TrkB signaling is both necessary and sufficient to account for

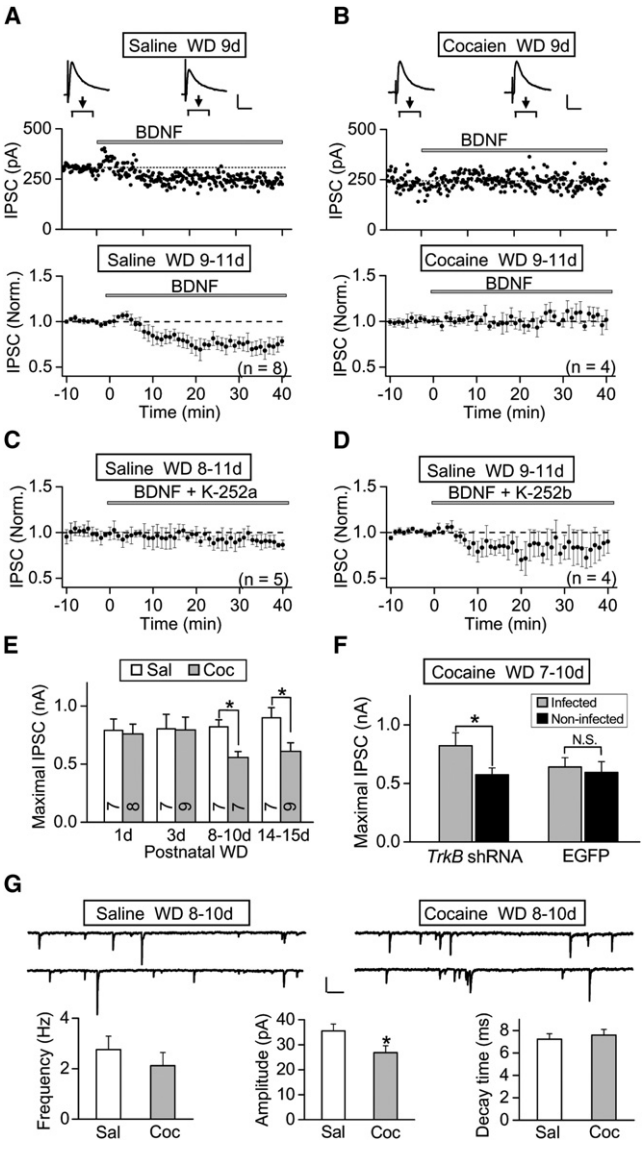

Figure 4. Elevated BDNF Downregulates GABAergic Activity via TrkB Activation

(A and B) Top, examples showing EPSP amplitude before and after bath application of BNDF (200 ng/ml) for rats on 9 days after saline (A) or cocaine (B) withdrawal. Sample traces, averages of 30 IPSCs (arrowhead). Scales: 100 pA, 50 ms. Bottom, summary of all experiments for rats 9–11 days after withdrawal from saline (A, n = 8 cells, 5 rats) or cocaine (B, n = 4 cells, 3 rats).

(C and D) Summary of all experiments for the effect of BNDF (in the presence of 200 nM K-252a or K-252b) on IPSCs for rats 8–11 days after saline withdrawal (C, n = 5 cells, 4 rats; D, n = 4 cells, 3 rats).

(E) Maximal amplitude of IPSCs evoked in mPFC layer V pyramidal cells by extracellular stimulation at layer II/III was compared between rats on different days after withdrawal from cocaine and saline (*$p < 0.05$, t test). Number associated with the histogram refers to the total number of cells recorded (2–3 rats for each group).

(F) Comparison of the maximal IPSC amplitudes evoked in pyramidal cells expressing EGFP only or EGFP and *TrkB* shRNA and in nonfluorescent (control) cells of the same slices 7–10 days after cocaine withdrawal (n = 6–8 cells, 2–3 rats; *$p < 0.05$, paired t test).

(G) Comparison of the mean frequency, amplitude and decay time of mIPSCs from rats 8–10 days after cocaine or saline withdrawal. Traces above show two sample recordings of mIPSCs (n = 12 cells, 6 rats for each group; *$p < 0.05$, t test). Scale, 20 pA, 20 ms.

Error bars = SEM. See also Figures S2 and S4.

the reduction of GABAergic inhibition of mPFC layer V pyramidal cells observed after cocaine withdrawal.

Reduction of GABAergic Inhibition Mediates LTP Facilitation

Induction of LTP at excitatory synapses can be facilitated in many brain regions by reducing GABAergic inhibition of postsynaptic neurons (Davies et al., 1991; Paulsen and Moser, 1998; Wigström and Gustafsson, 1983). We thus tested whether reduced GABAergic inhibition is sufficient to facilitate LTP induction at the excitatory synapses on mPFC layer V pyramidal cells after cocaine withdrawal. We first applied the $GABA_AR$-specific inhibitor SR95531 during LTP induction. While mTBS normally failed to induce LTP in slices obtained from saline-treated rats (7–11 days of withdrawal), the presence of 0.5 μM SR95531 (which reduced IPSCs in these mPFC pyramidal cells by ~60%; see Lu et al., 2009) resulted in a robust LTP (Figures 5A, 5C, 5D, and 5F). Importantly, the same SR95531 (0.5 μM) application did not cause significant change in the facilitated LTP induction found in slices from cocaine-withdrawn rats ($p = 0.14$, K-S test), indicating that the cocaine withdrawal effect had occluded that caused by reducing GABA inhibition with SR95531 (Figures 5B–5D and 5F). This suggests that cocaine withdrawal effect shares the same mechanism as reducing GABA inhibition in facilitating LTP.

The above experiments showed that reduction of GABAergic inhibition is sufficient to facilitate LTP induction. We further showed that preventing the reduction of GABA inhibition by enhancing $GABA_AR$ function with diazepam (DIA), a coagonist that enhances $GABA_AR$ activation (Eghbali et al., 1997), dose-dependently abolished the facilitation of LTP induction in cocaine-withdrawn rats (Figures 5E, 5F, and S2D). Thus, the reduction of GABAergic inhibition is also necessary for LTP facilitation in the mPFC after cocaine withdrawal.

Elevated PP2A Activity Downregulates Surface $GABA_AR$

The above experiments suggest that postsynaptic $GABA_ARs$ may be a downstream target of BDNF-TrkB signaling after cocaine withdrawal. We thus compared the surface expression of α1 subunit of $GABA_ARs$ and GluR1 subunit of AMPA (a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors in the mPFC between saline- and cocaine-withdrawn littermates. Using biotinylation technique to isolate the protein expressed on the cell surface (see Experimental Procedures) and western blotting for the membrane pool (M) and total protein (T) in the cell, we found that the surface expression of $GABA_AR$ α1 subunit was reduced significantly 8 days and 14 days after cocaine withdrawal, relative to the level found in parallel saline-treated groups (Figure 6A). In contrast, the total level of this protein in the mPFC cells was not different between the two groups (Figure 6A). Furthermore, this reduction of surface pool of $GABA_AR$ α1 subunit was not observed 1 and 3 days after cocaine withdrawal. The change of α1 subunit could represent that of $GABA_AR$ because in the cerebral cortex it is the most abundant α subunit,

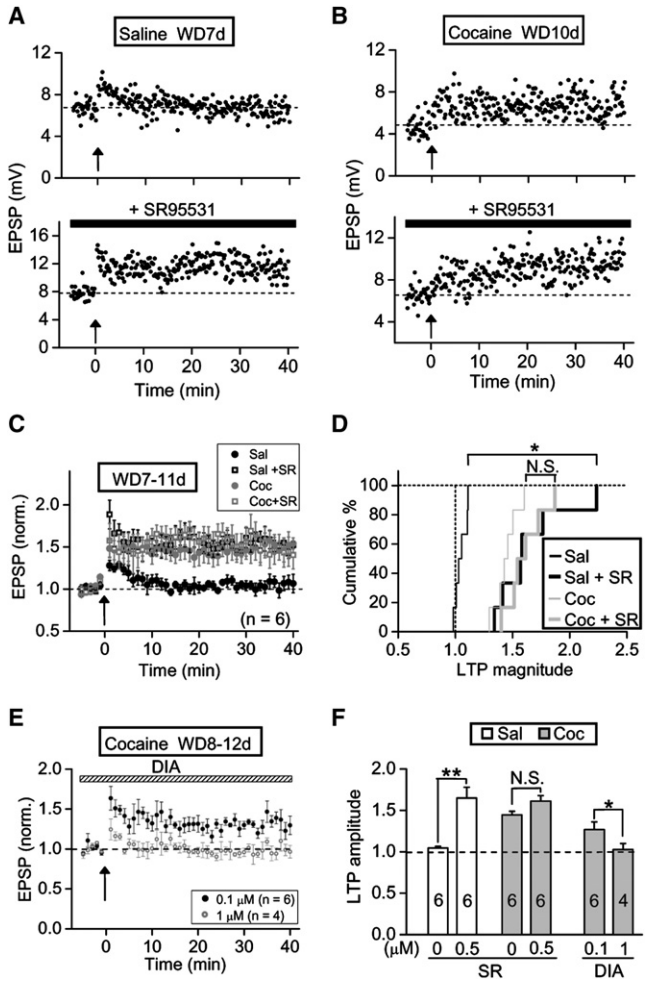

Figure 5. Reduction of GABAergic Inhibition Facilitates LTP Induction

(A and B) Top, example recordings showing that mTBS failed to induce LTP in a slice from 7-day saline-withdrawn rat, but induced robust LTP in that from a 10-day cocaine-withdrawn rat. Bottom, similar example recordings as in top but in the presence of $GABA_AR$ antagonist SR95531 ("SR," 0.5 μM).

(C) Summary of all experiments similar to those shown in (A) and (B), for rats 7–11 days after saline or cocaine withdrawal (n = 6 cells, 5 rats each).

(D) Cumulative percentage plot for the distribution of the LTP magnitude observed in all experiments shown in (C) (*$p < 0.001$; N.S., $p > 0.05$; K-S test).

(E) Summary of experiments on the effect of diazepam ("DIA," at 0.1 and 1 μM) on LTP induction from rats 8–12 days after cocaine withdrawal. "n," total number of cells recorded, from 4 (0.1 μM) or 2 (1 μM) rats.

(F) Summary of the effects of SR95531 and diazepam on LTP induction in rats 7–11 days after withdrawal from saline or cocaine (**$p < 0.01$; *$p < 0.05$; N.S., $p > 0.05$; t test).

Error bars = SEM. See also Figure S2.

which is essential for the $GABA_AR$ (Farrant and Kaila, 2007; Pirker et al., 2000). This effect on $GABA_AR$ is selective, because no difference between cocaine- and saline-treated littermates was found for either the surface pool or the total level of GluR1 as well as the amplitude and decay time of mEPSCs (Figure S5F) in the mPFC at all four time points after withdrawal from the treatment (Figure 6A).

Previous studies have shown that BNDF suppresses the surface expression of $GABA_ARs$ on cultured neurons and activation of protein phosphatase 2A (PP2A) is required for this BDNF effect (Jovanovic et al., 2004). We have performed a phosphatase assay of the PP2A activity in the mPFC tissue on different days after cocaine withdrawal, and found that the PP2A activity was significantly elevated 8 and 14 days ($p < 0.05$, t test) but not 1 and 3 days ($p > 0.05$, t test) after cocaine withdrawal, relative to corresponding saline littermates (Figure 6B). This elevated PP2A activity was not due to any increase in the total PP2A protein level, as shown by western blotting of the same mPFC tissue (Figure 6C). As expected for a higher activity of PP2A, the phosphorylation of $GABA_AR$ β3 subunit, the main phosphorylated subunit of most cortical $GABA_ARs$ (Brandon et al., 2002), was significantly decreased after cocaine withdrawal. This was shown by western blots using phosphoserine antibodies on immunoprecipitates of $GABA_AR$ β3 subunit from the mPFC tissue obtained from rats 7–8 days (but not 1 day) after saline/cocaine withdrawal (Figure 6D). Consistent with previous finding that increased phosphorylation of β3 subunit promoted the internalization of the $GABA_ARs$ (Jovanovic et al., 2004), we found that the surface expression of β3 subunit was also reduced 7 days (but not 1 day) after cocaine withdrawal (Figure 6E). Thus, elevation of PP2A activity after cocaine withdrawal appears to be responsible for mediating the reduction of surface expression of $GABA_ARs$.

The above idea was further supported by the finding that adding the PP2A inhibitor okadaic acid (0.5 μM, OA) in the recording pipette completely prevented the suppression of GABAergic transmission (Figures 6F and S2C) and the facilitation of LTP induction at excitatory synapses by exogenously applied BDNF (Figures 6G and S2A) in the mPFC 7–12 days after saline withdrawal. Taken together, elevation of BDNF expression after cocaine withdrawal leads to the reduction in the surface expression of postsynaptic $GABA_ARs$ via BDNF-TrkB-PP2A signaling pathway.

Elevated Excitability of Layer V Pyramidal Neurons

Reduction of $GABA_AR$-mediated inhibition may lead to elevated excitability of mPFC pyramidal neurons. We examined this idea first by measuring EPSP-spike (E-S) coupling (Lu et al., 2000) in these neurons 8–11 days after cocaine withdrawal. Whole-cell recording was used to determine neuronal spiking probability in response to a range of presynaptic stimulation intensity that induced EPSPs of different slopes (Figure 7A). Fitting the data with a sigmoid function yielded the value of the EPSP slope corresponding to the spiking probability of 0.5 ($E_{0.5}$). We found that the mean value of $E_{0.5}$ (Figure 7B) and the threshold membrane potential for spiking (Figure 7C) 8–11 days after cocaine withdrawal were significantly lower than those found for parallel saline-treated rats. Furthermore, the elevated excitability of mPFC pyramidal cells was also reflected by the increased number of action potential (APs) initiated by depolarizing current pulses injected into these neurons 8–14 days after cocaine withdrawal (Figure 7D), indicating a facilitation in evoked spiking after cocaine withdrawal. Together, these results showed that the excitation of these mPFC neurons by either presynaptic

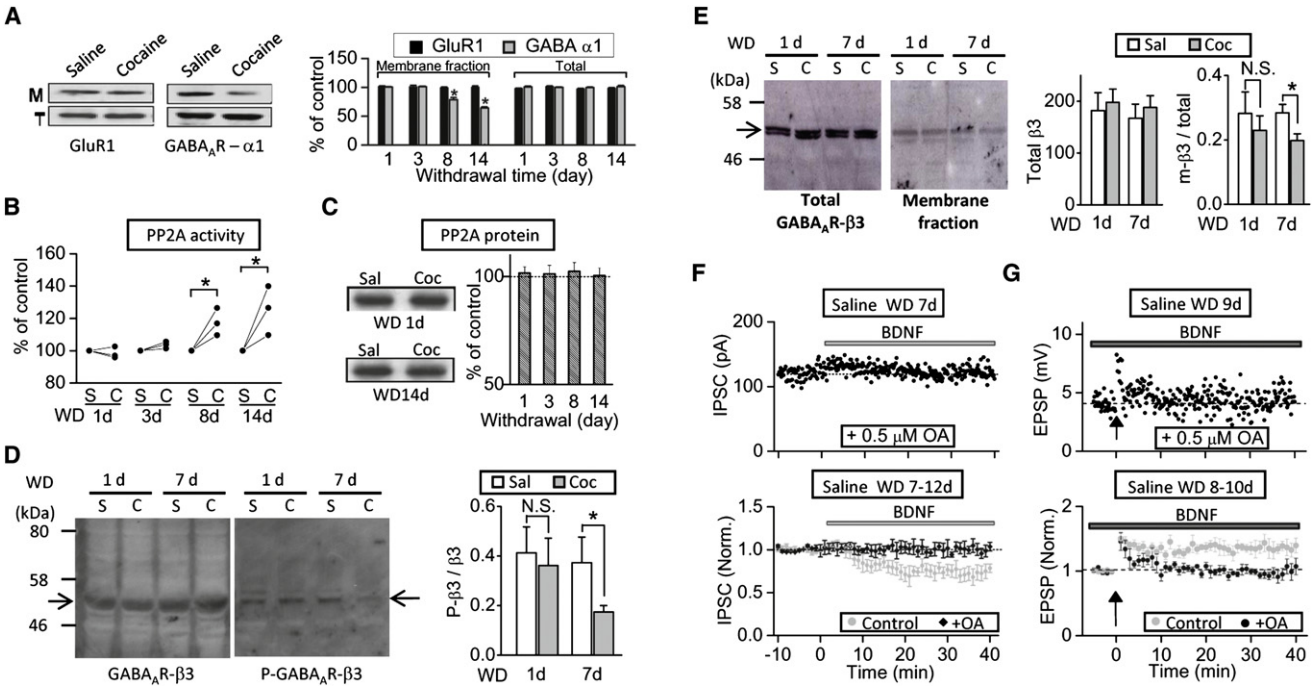

Figure 6. Elevated PP2A Activity Mediates BDNF-Induced Downregulation of Surface GABA$_A$Rs

(A) Left, example immunoblots of mPFC tissue lysates obtained from littermate rats 8 days after saline or cocaine withdrawal, showing the level of biotinylated membrane-bound fraction (M) and of the total protein level (T) for GluR1 and GABA$_A$R α1 subunit. Right, densitometric measurements of band densities for the membrane-bound and total protein level. Data are presented as the percentage of the level measured for the saline-treated (control) rats in the same immunoblot ($n = 3$; $^*p < 0.05$, paired t test).

(B) Summary of the results of phosphatase assay for PP2A activity on different days after cocaine withdrawal. Data are presented as the percentage of the enzyme activity measured from saline-withdrawn (control) littermate rats ($n = 3$; $^*p < 0.05$, paired t test).

(C) Comparison of densitometric measurements of the band densities of immunoblots for the total PP2A protein from the same lysate sample as in (B). Left, example immunoblots for PP2A protein in the mPFC obtained from rats 1 and 14 days after saline or cocaine withdrawal. Data are presented as the percentage of densities measured in the same blots for saline-withdrawn (control) littermate rats ($n = 3$; $p > 0.05$, paired t test).

(D) Phosphorylation level of GABA$_A$R β3 subunit 1 and 7 days after withdrawal from saline (S) or cocaine (C). Left, examples of western blots for total β3 and phosphorylated β3 in the same lysate samples ($n = 3$; $^*p < 0.05$, paired t test). Right, summary of the average ratio of band densities for P-GABA$_A$R-β3 versus GABA$_A$R-β3.

(E) Left, example immunoblots of mPFC tissue lysates obtained from rats 1 and 7 days after saline or cocaine withdrawal, showing the level of total protein and biotinylated membrane-bound fraction for GABA$_A$R β3 subunit. Right, band densities of the total β3 level and the ratio of membrane-bound versus total level of β3. ($n = 3$; $p > 0.05$, paired t test).

(F) An example (top) and summary (bottom, n = 5 cells, 4 rats) of experiments showing that loading PP2A blocker okadaic acid (0.5 μM) in the recorded cell abolished the effect of exogenous BDNF (200 ng/ml, bar) on the IPSC amplitude, for rats 7–12 days after saline withdrawal. Data set for the control ("Con") is the same as that shown in Figure 4C.

(G) An example (top) and summary (bottom, n = 5 cells, 3 rats) showing that loading OA in the recorded cell blocked the effect of exogenous BDNF in facilitating LTP induction in slices from 8–10 days saline-withdrawn rats. Data set for the control ("Con") is the same as that shown in Figure 2D.

Error bars = SEM. See also Figure S2.

transmitter release or postsynaptic injection of depolarizing currents was promoted 8–14 days after cocaine withdrawal.

Downregulation of TrkB Reduces Locomotor Hypersensitivity to Cocaine

Cocaine sensitization in locomotor activity of rats reflects cocaine-induced neural circuit changes that may be related to relapse in human cocaine addicts (Robinson and Berridge, 2001). Medial PFC is a part of mesocorticolimbic dopamine system that plays a major role in behavioral sensitization to cocaine (Di Chiara, 1995; Feltenstein and See, 2008; Kalivas and Nakamura, 1999). Rat is known to exhibit locomotor hypersensitivity to cocaine challenge 1 week after cocaine withdrawal (Park et al., 2010). We have performed locomotion test in our system and found that rat showed increasing locomotor sensitization to cocaine during daily injection with cocaine but not with saline, and this sensitization to cocaine reached a plateau after 3 days of cocaine exposure (Figure 8A). Furthermore, 8 days after the withdrawal from daily cocaine or saline injections, cocaine-treated rats showed significantly higher locomotor activity in response to a single cocaine injection than that found in their saline-treated littermates (Figure 8A), indicating cocaine-induced behavioral hypersensitivity 8 days after cocaine withdrawal, consistent with the previous report (Park et al., 2010).

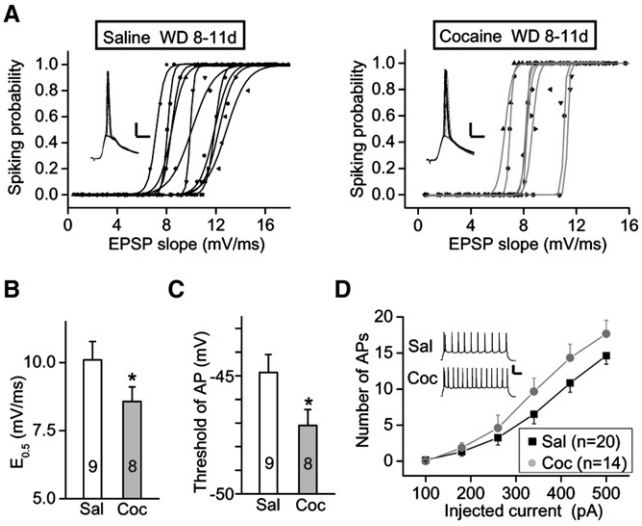

Figure 7. Enhanced EPSP-Spike Coupling and Neuronal Excitability

(A) EPSP-spike coupling in mPFC layer V pyramidal cells was measured by plotting spiking probability versus the slope of EPSPs evoked by extracellular stimuli of different intensities, for rats 8–11 days after withdrawal from treatment with saline (9 cells, 5 rats) or cocaine (8 cells, 4 rats). Curves, best-fit sigmoidal function, each for one cell. Inserts, 10 sample traces of EPSCs with spiking probability between 0 and 1. Scales: 20 mV, 10 ms.

(B and C) Comparison of the values of $E_{0.5}$ and of the AP initiation threshold for same data sets as in A (*$p < 0.05$, t test).

(D) Changes of the neuronal excitability, as measured by the number of APs triggered by injection of various depolarizing currents (duration, 800 ms), for rats 8–14 days after withdrawal from treatment with saline (20 cells, 14 rats) or cocaine (14 cells, 10 rats). Insert, APs elicited by a depolarizing current pulse (500 pA, 800 ms) in a pyramidal cell for a rat 9 d after saline or cocaine withdrawal. Scales, 40 mV, 100 ms.

Error bars = SEM.

To test whether BDNF-TrkB signaling is involved in the locomotor hypersensitivity after cocaine withdrawal, we used adenoassociated virus (AAV) to deliver EGFP and *TrkB* shRNA or EGFP-only plasmid bilaterally into the deep layers of the prelimbic mPFC of P20 rats (Figure 8C). We first confirmed that AAV-delivered RNA showed stable expression on 15 days after injection (Figure S6). On the average, about half of AAV injections resulted in detectable expression of EGFP in the mPFC, and locomotion data were used only when post hoc analysis showed clear AAV expression in the rat mPFC. On P21 (one day after AAV injection), rats were subjected to the same locomotor activity test as described above. We found that AAV-injected rats exhibited increasing sensitization to cocaine during daily cocaine injection, reaching a sensitivity level similar to that found in rats not subjected to AAV injection at the end of 7 days cocaine treatment (Figures 8A and 8B). The lower activity exhibited during the first few days of cocaine treatment may be attributed to a gradual recovery from surgery associated with AAV injections. Furthermore, 8 days after cocaine withdrawal, rats injected with AAV-*TrkB* shRNA in the mPFC exhibited significantly lower locomotor activity in response to a single cocaine injection than that found in rats injected with AAV - EGFP (Figure 8B). Since rats injected with AAV-EGFP showed similar hypersensitivity to cocaine as those cocaine-treated rats without AAV injection, the surgery and AAV infection did not affect locomotor sensitization to cocaine. Taken together, these results indicate that downregulation of TrkB in the mPFC reduced locomotor hypersensitivity after cocaine withdrawal, suggesting the involvement of elevated BDNF-TrkB signaling in behavioral modification after cocaine withdrawal.

DISCUSSION

In this study, we have examined the alteration of synaptic plasticity in the mPFC during the period of cocaine withdrawal and explored the underlying cellular mechanisms. We showed that the susceptibility of excitatory synapses of layer V pyramidal neurons to LTP induction was increased significantly over 5 days but not 1–4 days after withdrawal from repeated cocaine treatment. This delayed appearance of facilitated LTP induction could be attributed to a gradual elevation of BDNF expression in the mPFC that was shown to suppress GABAergic inhibition of these pyramidal neurons. This suppressing effect of BDNF correlated with the reduction of surface expression of $GABA_AR$ and required the postsynaptic activation of TrkB. Furthermore, we showed that BDNF elevates the activity of PP2A in the mPFC, leading to an increased dephosphorylation of $GABA_AR$ β3 subunit, which is known to cause internalization of $GABA_ARs$ (Jovanovic et al., 2004). Finally, we showed that downregulation of TrkB in the mPFC reduced locomotor hypersensitivity toward cocaine that is known to reflect behavioral sensitization after cocaine withdrawal. In short, we have described a BDNF-dependent sensitization of mPFC excitatory synapses to LTP induction after cocaine withdrawal, and elucidated its underlying cellular mechanisms and potential behavioral relevance.

Elevation of BDNF Expression after Cocaine Withdrawal

After cocaine withdrawal, there is an increased sensitivity to drug-associated environmental cues that trigger cocaine craving (Childress et al., 1999). Studies using rat addiction model showed that lever pressing and cue-induced reinstatement of cocaine seeking were progressively increased over 90 days or longer after cocaine withdrawal (Grimm et al., 2003). During the period after cocaine withdrawal, significant neuroadaptation associated with behavioral sensitization (Pierce and Kalivas, 1997) occurs in the reward circuit at molecular, cellular, and morphological levels (Hyman et al., 2006; Nestler, 2001; White and Kalivas, 1998). Along with the increased cocaine craving after withdrawal, the BDNF level rises gradually in the VTA, NAc, and amygdala (Grimm et al., 2003). Intra-VTA infusion of BDNF enhances cocaine seeking after cocaine withdrawal (Lu et al., 2004). These findings suggest that the elevation of BDNF expression after cocaine withdrawal may mediate circuit modification underlying drug craving and seeking behaviors. In the present study, we found a *delayed* BDNF upregulation in the mPFC 8 or 14 days, but not 1 and 3 days after withdrawal from the repeated cocaine treatment. In the mouse dorsomedial PFC, a similar extent (20%–30%) of BDNF elevation was also found 21 days after the termination of cocaine self-administration (McGinty et al., 2010). Given that BDNF could be transported

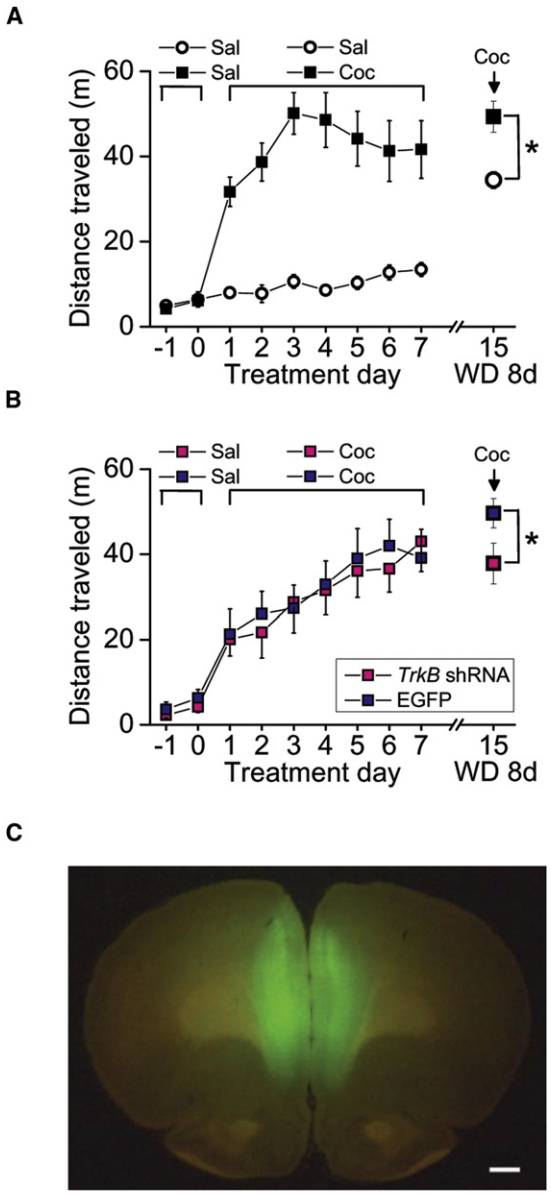

Figure 8. TrkB Downregulation Reduces Locomotor Hypersensitivity

(A) Locomotor sensitization caused by repeated cocaine injection. Littermate rats received daily injections of either saline (n = 11) or cocaine (n = 11) for 7 days (after 2 days habituation with saline injection), and the distance traveled during 20 min period after cocaine/saline injection was measured. Eight days after cocaine/saline withdrawal, locomotor activity following a single cocaine injection was again tested for rats from both groups (*$p < 0.05$, paired t test).

(B) Locomotor sensitization induced by daily cocaine injections during 7 days cocaine treatment and 8 days after cocaine withdrawal were measured as in (A), except that rats tested had been previously injected bilaterally in the mPFC with either AAV-EGFP and *TrkB* shRNA (red, n = 10) or AAV-EGFP (blue, n = 8) (*$p < 0.05$, t test).

(C) EGFP fluorescence of a fixed slice that bilaterally expressed EGFP and *TrkB* shRNA in the mPFC, on 18 days after bilateral injection of the AAV vector. Scale: 1 mm.

Error bars = SEM. See also Figure S6.

from the PFC to the NAc through the cortical glutamatergic projections (Altar et al., 1997), a pathway that regulates relapse to drug-seeking (McFarland et al., 2003), BDNF upregulation in the mPFC may play a critical role in cocaine-induced neuroadaptive response of the reward circuit.

It remains unclear whether the elevated BDNF results from elevated BDNF synthesis locally by mPFC neurons or through anterograde/retrograde transport to mPFC via afferent/efferent axons. It is possible that the delay in the appearance of BDNF elevation in mPFC after cocaine withdrawal is due to the time required for BDNF transport from other brain regions, e.g., NAc, VTA, and amygdala, where high expression of BDNF has been found following repeated cocaine exposure. Further studies on the time course of BDNF expression, the existence and properties of anterograde/retrograde transport of BDNF, as well as activity-dependent uptake and secretion of BDNF in various parts of the reward circuit are required to fully understand the function of BDNF after cocaine withdrawal.

BDNF-Induced Changes in Synaptic Plasticity in the Reward Circuit

After withdrawal from repeated cocaine exposure to rats, measurements in midbrain slices in the presence of picrotoxin (that blocks GABAergic inhibition) showed that excitatory synapses onto VTA dopamine neurons become highly sensitive to LTP induction (Pu et al., 2006). This BDNF-dependent facilitation of LTP in the VTA is mediated by an enhancement of presynaptic glutamate release that depends on postsynaptic NMDA receptors and TrkB activation. We showed here that, although there was presynaptic elevation of glutamate release (see Figure S5), LTP facilitation at the excitatory synapses onto mPFC layer V pyramidal neurons could be largely accounted for by BDNF-induced reduction of postsynaptic GABAergic inhibition that elevates postsynaptic excitation during LTP induction. This conclusion is based on the following lines of evidence: First, blocking GABAergic inhibition with the specific $GABA_AR$ antagonist SR95531 (Figures 5A, 5C, 5D, and 5F) and the application of BDNF (Figures 2C and 2D) both facilitated LTP induction to a similar level in slices from control rats withdrawn from the saline treatment and this level was similar to that found in cocaine-withdrawn rats. Second, SR95531 treatment of cocaine-withdrawn rats produced no additional LTP facilitation. This occlusion of LTP facilitation suggests that cocaine withdrawal and GABAergic suppression share a common mechanism, an idea further supported by the finding that diazepam-induced enhancement of GABA inhibition completely abolished the LTP facilitation in cocaine-withdrawn rats (Figures 5C, 5D, and 5F). Third, direct measurements of the maximal amplitude of IPSCs evoked by extracellular stimulation showed substantial reduction (30%–40%) in the total GABAergic inhibition in these neurons (Figure 6A). Fourth, the surface expression of the $\alpha 1$ and $\beta 3$ subunits of $GABA_AR$ was reduced after cocaine withdrawal (Figure 6A). Fifth, both the facilitation of LTP induction (Figure 3D) and the reduction of IPSCs in cocaine-withdrawn rats were not found in mPFC neurons in which TrkB was downregulated (Figure 4F). Finally, the time courses for LTP facilitation

(Figure 1E), BDNF elevation (Figure 2A), and the reduction of IPSCs (Figure 4E) and surface pool of $GABA_A$Rs (Figure 6A) were all similar during the period after cocaine withdrawal. We also noted that exogenous BDNF application failed to suppress IPSCs in the mPFC of rats 7 days after cocaine withdrawal (Figure 4B), implying that BDNF's suppressing effect on IPSCs is saturated after 1 week of cocaine withdrawal. Interestingly, similar LTP facilitation was found at excitatory synapses on rat VTA dopamine neurons following repeated cocaine exposure, through the reduction of $GABA_A$R-mediated inhibition. However, the latter LTP facilitation was not mediated by the elevated BDNF, because the reduction of GABAergic inhibition and the facilitation of LTP induction occur prior to the elevation of BDNF expression in the VTA (Liu et al., 2005; Pu et al., 2006). How inhibitory synapses on VTA dopaminergic neurons become suppressed during repeated cocaine exposure remains to be examined.

Cocaine administration resulted in changes at multiple sites of the BDNF/TrkB signaling cascade. For instance, acute or chronic exposure to cocaine significantly increases ERK phosphorylation in the VTA, NAc, and PFC (Jenab et al., 2005; Sun et al., 2007; Valjent et al., 2005). Repeated cocaine exposure also increases phosphatidylinositol-3-kinase activity in the NAc shell, but decreases its activity in the NAc core (Zhang et al., 2006). Cocaine self-administration in rats also triggers TrkB-mediated activation of phospholipase Cγ in the NAc (Graham et al., 2007). Our present results imply that TrkB receptor signaling mediates the reduction of GABAergic inhibition in the mPFC after cocaine withdrawal. First of all, downregulation of TrkB abolished the suppression of IPSCs (Figure 4F) and prevented LTP facilitation after cocaine withdrawal (Figure 3D). Second, blocking the activity of PP2A, a downstream target of BDNF-TrkB signaling, impaired the effect of BDNF on IPSCs (Figure 6F) and facilitated LTP induction in layer V pyramidal neurons (Figure 6G). Finally, phosphatase assay revealed that the PP2A activity was increased 8 and 14 days but not 1 and 3 days after cocaine withdrawal, a time course similar to that of BDNF elevation. Furthermore, the action of PP2A on the dephosphorylation of $GABA_A$R β3 subunit, a process that promotes internalization of these receptors (Jovanovic et al., 2004), was shown to occur 1 week after cocaine withdrawal (Figure 6D).

Important Role of mPFC in Relapse

The relapse triggered by stress, conditioned stimuli, and drugs is known to be mediated by activities in the PFC. Pharmacological studies showed that inactivation of dorsal mPFC blocks cue-induced (McLaughlin and See, 2003), cocaine-primed (McFarland and Kalivas, 2001), and stress-induced (Capriles et al., 2003) drug reinstatement in animal models of addiction. More importantly, cocaine-primed reinstatement involves increased glutamate level in the NAc core, and inactivation of the PFC prevented this increase (Baker et al., 2003; McFarland et al., 2003). The layer V pyramidal neurons of the mPFC represent the main source of excitatory inputs to the VTA and NAc. Thus, facilitation of LTP of excitatory inputs (from other brain areas) to these neurons may lead to their higher outputs in the VAT and NAc, and promote drug reinstatement.

Results of the present study implicate a role of BDNF-TrkB signaling in facilitating LTP induction in excitatory synapses in the rat mPFC after withdrawal from repeated cocaine exposure. Moreover, we found that downregulation of BDNF-TrkB signaling reduces both the synaptic sensitization to LTP induction as well as behavioral sensitization in rats, as reflected by locomotor hypersensitivity to cocaine, although it remains debatable whether cocaine-induced locomotor sensitization in rats reflects human behaviors involved in drug craving and relapse. Whether BDNF-TrkB signaling also affects the withdrawal effects in other models besides locomotor hypersensitivity, e.g., cocaine self-administration, requires further study. It has been suggested that persistence of neural circuit sensitization leaves addicts susceptible to relapse even long after the discontinuation of drug use (Robinson and Berridge, 2001). Prolonged BDNF-dependent sensitization of excitatory synapses in the mPFC after cocaine withdrawal reported here may represent one of many potential mechanisms of circuit sensitization involved in triggering relapse.

EXPERIMENTAL PROCEDURES

Animal Protocols and Slice Preparation

Acute fresh mPFC slices were prepared from Sprague-Dawley rats (Charles River) as previously described (Lu et al., 2009). For repeated cocaine or saline treatment, juvenile rats (body weight 45–50 g at P18) were given intraperitoneal injections of either saline (0.9% NaCl, 1ml kg^{-1}) or saline containing cocaine (15 mg kg^{-1} in 1 ml kg^{-1} of saline) for 7 days from P18 to P24 in their home cage. Animal protocols were approved by the Animal Care and Use Committee of UC Berkeley. Coronal slices (250 μm thick) containing mPFC were cut with a vibratome, in a solution containing (in mM) 110 choline Cl, 25 $NaHCO_3$, 25 D-glucose, 11.6 Na ascorbate, 7 $MgSO_4$, 3.1 Na pyruvate, 2.5 KCl, 1.25 NaH_2PO_4, and 0.5 $CaCl_2$. Before use, slices were incubated in artificial cerebrospinal fluid containing (in mM) 119 NaCl, 26.2 $NaHCO_3$, 11 D-glucose, 2.5 KCl, 2.5 $CaCl_2$, 1.5 $MgSO_4$, and 1.25 NaH_2PO_4 at the room temperature, with the solution bubbled with 95% O_2 and 5% CO_2.

Electrophysiology

Whole-cell recording was made from layer V pyramidal neurons in the mPFC using a patch clamp amplifier (MultiClamp 700B, Axon Instruments, Foster City, CA) under infrared DIC optics at 30 (±1)°C. Data was acquired with a digitizer (DigiData 1322A, Axon Instruments) and analyzed with pClamp 9 software (Axon Instruments). Extracellular stimulation pulses (50 μs duration at 0.1 Hz) were applied with a bipolar electrode (WPI Inc.) placed in layer II/III of the prelimbic region of mPFC. EPSPs were recorded at −70 mV in current clamp, and IPSCs recorded in voltage clamp at −20 mV in the presence of CNQX (10 μM) and AP5 (25 μM). The intracellular solution for whole-cell recording contained (in mM) 140 K gluconate, 5 KCl, 10 HEPES, 0.2 EGTA, 2 $MgCl_2$, 4 MgATP, 0.3 Na_2GTP and 10 Na_2-phosphocreatine (at pH 7.2 with KOH). Data were discarded when the series resistance changed by >20% during the course of the experiment. Stimulation protocol for LTP induction (termed "modified theta burst stimulation," mTBS) consisted of presynaptic activation of 10 bursts (each with 5 pulses at 100 Hz, spaced at 200 ms), repeated 3 times at 10 s intervals, and postsynaptic injection of a depolarizing current pulse (1.5 nA, 40 ms) during each burst, with a 5 ms interval between the onset of pre- and postsynaptic stimulation.

Expression Assays for Transmitter Receptor, TrkB, and BDNF

Slices of mPFC were biotinylated with 1 mg/ml sulfo-NHS-S-S-biotin (Pierce) for 30 min at 4°C, and supernatants of cell lysates in RIPA buffer (Sigma, supplemented with protease inhibitors) were mixed with immobilized neutravidin beads (Pierce) and rotated overnight at 4°C to precipitate biotinylated proteins. The beads were washed 5 times with PBS and then eluted with SDS-PAGE

sample buffer by boiling for 15 min. Both total and biotinylated proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with specific antibodies for GluR1, α1, and β3 $GABA_A$ subunits, or TrkB. The HRP-tagged secondary antibodies were used to visualize specific signals by chemifluorescence substrate. The digital images of scanned immunoblots were quantified with Adobe Photoshop software. For BDNF assay, bilateral mPFC tissue were rapidly collected from the cortical brain slices and put into liquid nitrogen and stored at −80°C. Prior to the assay, wet samples were weighed before homogenized in iced RIPA buffer. The supernatant collected after centrifugation at 13,000 × g (for 30 min at 4°C) was analyzed for the amount of using BDNF Emax ImmunoAssay System Kit (Promega, Madison, WI) according to manufacturer's instruction.

Lentivirus and AAV-Based Short Hairpin RNA Expression

The lentiviral production protocol were the same as previously described (Boyden et al., 2005), and VSVg pseudotyped lentiviruses were produced by triple transfection of 293 FT cells (Invitrogen) with psPAX2, pMD2.G, and pLenti using lipofectamine 2000. The viral titer was examined by FACS to be between 0.5–1 × 10^9 infectious unit per ml. *TrkB* shRNA construct targeted to *TrkB* mRNA sequence was subcloned into the pEF-GM expression vector. The sequence for *TrkB* shRNA was 5′- GGGCAGCTAATAAATACTTCCTGTCA GATATTTATTAGCTGCCCGCA-3′. Control scrambled shRNA was 3′-TTCTCC GAACGTGTCACGTTTCAAGAGAACGTGACACGT-TCGGAGAA-3′ (Bartkowska et al., 2007). The double-stranded *TrkB* shRNA template oligonucleotide was cloned into pSIH-H1-copGFP lentivector (System Biosciences, CA) through BamH1 and EcoRI sites located downstream of an H1 promoter. The silencing effect of the shRNA to TrkB expression in cortical cultures was confirmed using western blotting 5 days after virus infection of cultured cortical neurons and Ca^{2+} imaging of single infected cultured hippocampal neurons (Figure S3). For AAV-based shRNA expression, AAV1 vector was constructed by subcloning the PCR-amplified fragment covering copGFP and shRNA sequence of pSIH-H1-copGFP constructs into the pAAV-EF1a-double floxed-hCH2(H134R)-EYFP-WPFP-HGHpA through XbaI and ClaI sites. The AAV serotype 1 virus was generated and purified by University of North Carolina Vector Core Facility.

In Vivo Infection of mPFC cells

For infection using lentivirus, Sprague-Dawley rats (P18) were anesthetized with an i.p. injection of Ketamine (70 mg kg^{-1}) and kept deeply anesthetized using isoflurane, with body temperature maintained at 37°C by a heating blanket (Watlow). Two craniotomies, (300–400 μm in diameter) were drilled above the prefrontal cortex. Viral stock solution was slowly injected, using a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) at two sites (300 nl per site, one site per hemisphere) with the following coordinates: anterioposterior = − 2.8 mm from bregma; lateral = ± 0.5 mm; ventral = 3.5 mm. The injection speed is controlled at 10 nl/min by a microinjection pump (WPI Inc.). For AAV infection of rats for locomotion test, the procedure was the same as above, except that mPFC of each hemisphere received 2 injections (200 nl each, 1 × 10^{12} virus particles/ml) along the dorsal-ventral axis (ventral = 3.2 and 3.7 mm). AAV injections were done at P20, one day before the locomotion test.

Phosphotase Assay, Western Blotting, and Immunoprecipitation

For western blotting, mPFC tissues were homogenized in the RIPA buffer (Sigma) supplemented with protease inhibitors. Supernatant was blotted with antibodies against PP2A (1:10,000, BD Biosciences). The same mPFC lysates were used for phosphotase assay of PP2A activity following manufacture's protocol (Promega). For $GABA_AR$ β3 subunit phosphorylation assay, lysate extracted from brain slices was prepared with RIPA buffer containing phosphatase inhibitor (PhosSTOP) and 1× EDTA-free complete protease inhibitor cocktail (Roche, Indianapolis, IN). The lysate (1 mg total each) was subjected to immunoprecipitation with $GABA_AR$ β3-specific antibody (C-20; Santa Cruz Biotechnology, CA) conjugated to Protein G-Sepharose beads (Amersham, Piscataway, NJ) at 4°C for 4 hr. The precipitates were immunoblotted for the phosphorylation level with anti-phosphoserine antibody (clone 4A4; Millipore, MA).

Locomotion Test

Beginning on P21, rats were habituated in the activity chamber for 20 min each day immediately after a single injection of saline (0.9% NaCl, 1ml kg^{-1}) in the first 2 days (day "−1" and "0"). Beginning on the third day, rats were given daily intraperitoneal injections of either saline or cocaine (saline, 0.9% NaCl, 1 ml kg^{-1}; cocaine, 15 mg kg^{-1} in 1 ml kg^{-1} of saline; during 10:00–12:00) for 7 days. Two littermate rats, each from the saline or cocaine group, were put into the test chamber at the same time for monitoring locomotor activity for 20 min with a video tracking system (Med. Associates) immediately after the injection. 8 d after withdrawal from the cocaine or saline treatment, rats were given an intraperitoneal injection of cocaine (15 mg kg^{-1} in 1 ml kg^{-1} of saline), and their locomotor activity was monitored for 20 min. Same procedure was performed using rats with AAV injected into the prelimbic region of the mPFC, except that two groups were both given 7 days daily cocaine treatment as described above. For AAV-infected rats, rats were perfused after locomotion test with 4% paraformaldehyde (PFA). Coronal slices (100 μm thick) were collected from the brains stored in 4% PFA for at least 24 hr to examine the location and effectiveness of AAV infection in mPFC, as indicated by the EGFP fluorescence (see Figures 8C and S6).

Statistical Analyses

For electrophysiological data on LTP magnitude and changes in IPSCs by applied BNDF (Figures 1, 2C, 2D, 3C–3F, 4A–4D, 5, 6F, and 6G), we used the standard nonparametric Kolmogorov-Smirnov (K-S) test. For biochemical data, the paired t test was chosen because experiments were always performed in parallel for two groups of littermates under the same experimental condition for each time point. For the measurement of maximal IPSCs (Figure 4E), Student's t test was used also because the data were collected in parallel from littermates for each time point after withdrawal from the treatment—two rats treated at the same time in the same litter (one treated with cocaine, the other with saline) were sacrificed together and data were collected on the same day. For the measurement of maximal IPSCs for lentiviral infected neurons (Figure 4F), paired t test was used because paired measurements from infected and non-infected neurons were obtained from each slice.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with your article online at doi:10.1016/j.neuron.2010.08.012.

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