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Cocaine-induced Modification of Synaptic Plasticity

in Rat Medial Prefrontal Cortex

by

Hui Lu

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Neuroscience

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Mu-ming Poo, Chair
Professor Ehud Isacoff
Professor Jonathan Wallis
Professor Qing Zhong

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Abstract

Cocaine-induced Modification of Synaptic Plasticity

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Professor Mu-ming Poo, Chair

Medial prefrontal cortex (mPFC) is involved in relapse after withdrawal for cocaine exposure, but changes in synaptic function and plasticity in the mPFC during the period of withdrawal remain largely unknown. After the termination of repeated cocaine treatments in rats, I observed a gradual enhancement in the susceptibility of excitatory synapses on layer V mPFC pyramidal neurons to activity-induced long-term potentiation (LTP). This enhanced synaptic plasticity could be attributed to a gradual increase in the expression of brain-derived neurotrophic factor (BDNF) and its suppression of GABAergic inhibition in the mPFC via reducing the surface expression of GABA$_A$ receptors. The BDNF effect is mediated by TrkB activation in these neurons and accompanied by elevated protein phosphatase 2A activity and increased de-phosphorylation of GABA$_A$ receptor β3 subunit in the mPFC. Thus, elevated BDNF expression during cocaine withdrawal sensitizes the excitatory inputs in the mPFC for activity-induced persistent synaptic potentiation that may contribute to cue-induced drug craving and seeking.

Prenatal cocaine-exposed new-born babies could be considered as undergoing withdrawal from cocaine exposure *in utero*. Previous studies have shown that prenatal cocaine exposure results in abnormal brain development and cognitive dysfunction, but the underlying cellular mechanism remains largely unclear. I proposed the hypothesis that prenatal cocaine exposure may cause similar modification of synaptic plasticity in the mPFC as that found in above cocaine withdrawal studies in juvenile rats. Thus, in the second part of my study, I examined synaptic functions in the mPFC of postnatal rats which were exposed to cocaine *in utero*, using whole-cell recording from mPFC layer V pyramidal neurons in acute brain slices. I found that cocaine exposure *in utero* also resulted in a facilitated LTP of excitatory synapses on these pyramidal neurons and an elevated neuronal excitability in postnatal rat pups after P15. This facilitated LTP could be largely attributed to the reduction of GABAergic inhibition. Biochemical assays of isolated mPFC tissue from postnatal rats further showed that cocaine exposure *in utero* caused a marked reduction in the surface expression of GABA$_A$ receptor subunits α1, β2, and β3, but had no effect on glutamate receptor subunit GluR1. Both facilitated LTP and reduced surface expression of GABA$_A$ receptors persisted in rats up to at least P42. Finally, the behavioral

consequence of cocaine exposure *in utero* was reflected by the reduction in the sensitivity of locomotor activity in postnatal rats to cocaine and the dopamine receptor agonist apomorphine. Since the mPFC plays important roles in cognitive functions, these findings offer new insights into the cellular mechanism underlying the adverse effects of cocaine exposure *in utero* on brain development and cognitive functions.

In summary, this thesis work showed that excitatory inputs to mPFC layer V pyramidal neurons are sensitized for activity-induced persistent synaptic potentiation due to the reduction of GABAergic inhibition after withdrawal from repeated cocaine exposure either *in utero* or after birth. These findings have increased our understanding of the neurobiological basis of cocaine addiction and may help to establish more thorough pharmacological treatments for cocaine addiction.

Dedication

To Mom, Dad, my brothers and my husband Xudong Li

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Acknowledgement

This dissertation would not have been possible without the help and support of many people. First of all, I would like to thank Dr. Mu-ming Poo, my advisor and mentor, for his inspiration, enthusiasm, encouragement, open-mindedness, unfailing support and generosity over the years, and for his teaching in science as well as many aspects of scientific life. I thank my thesis committee member Dr. Ehud Isacoff, Dr. Jonathan Wallis and Dr. Qing Zhong for their valuable advice to my thesis work. I would also like to thank members of the Poo lab and Dan lab, especially Byung Kook Lim, Pei-lin Chen, Hayan Yoon and Hongfeng Gao, for their collaboration, technical help, and stimulating discussions. Special thanks to Dr. Yan Dan for her kindness and guidance at the beginning of my study here at UC Berkeley. I thank Kati Markowitz of Neuroscience Graduate Program of Helen Wills Neuroscience Institute for her constant help and quick responses to my questions all the time. I would like to acknowledge Helen Wills Neuroscience Institute for offering me this opportunity to pursue my PhD here.

Finally, I would like to thank my husband Xudong Li for his sacrifice, patience, encouragement, and confidence that he has been giving me over the years. I wish to thank my parents and brothers for their faithful love and trust in me. I would also like to thank all of my dear friends for their support and encouragement during my PhD study.

Chapter 1 Introduction

Cocaine addiction has become a major health problem, with increasing prevalence in life-long abuse. Understanding the neurobiological basis of cocaine addiction, including cellular mechanisms underlying the drug craving and relapse during cocaine withdrawal, will help the development of new therapeutic approaches to cocaine addiction. Much attention has been focused on mesolimbic dopamine circuits, which include neurons in the ventral tegmental area (VTA) and limbic structures they innervate. Limbic activation 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 awake, unrestrained rats revealed that PFC activation was triggered by exposure to cocaine-associated cue during cocaine withdrawal but was unrelated to the hedonic effects of the drug (Rebec and Sun, 2005). As a part of PFC in the rat brain, the medial PFC (mPFC) receives dopaminergic innervation from the VTA and represents a component of the mesocortical dopamine system (Oades and Halliday, 1987). Therefore, studying cocaine-induced synaptic changes in the mPFC will help the understanding of neural circuit mechanisms underlying drug craving and seeking behaviors during cocaine withdrawal.

Previous studies have indicated that chronic drug exposure causes long-lasting molecular and cellular adaptations in the reward circuits that may underlie compulsive drug consumption and relapse, even after long periods of abstinence (Hyman and Malenka, 2001; Hyman et al., 2006; Kauer and Malenka, 2007; Nestler and Aghajanian, 1997; White and Kalivas, 1998). There is direct evidence for drug-induced persistent modification of excitatory synapses in these circuits. For example, single or repeated injection of cocaine in rodents induces a potentiation of AMPA (a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor (AMPAR)-mediated excitatory synaptic transmission onto dopamine (DA) 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).

Recent studies have shown that during the withdrawal period after repeated cocaine exposure in rats the expression of brain-derived neurotrophic factor (BDNF) increases in the VTA, nucleus accumbens (NAc), amygdala, hippocampus, and mPFC (McGinty et al., 2009). Since BDNF is known to modulate synaptic efficacy and plasticity (Poo, 2001), such increased BDNF expression during drug withdrawal may cause synaptic changes that contribute to the rat's enhanced responses to drug-associated cues and compulsive drug seeking behavior. A recent study from our laboratory has shown that the increased BDNF expression in the VTA during cocaine withdrawal in rats sensitizes the excitatory synapses on DA 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, very little is known about the potential BDNF effect on synaptic function and plasticity in mPFC neurons during cocaine withdrawal.

In the first part of my study, I examined the changes in synaptic efficacy and activity-dependent plasticity of excitatory synapses on mPFC layer V pyramidal neurons, the expression of BDNF and neurotransmitter receptors, phosphatase activity, and neuronal excitability at different times after cocaine withdrawal. My results show that BDNF facilitates activity-induced LTP of excitatory synapses on these mPFC pyramidal neurons by down-regulating the surface level of GABA$_A$ receptors, through postsynaptic TrkB signaling that increases protein phosphatase 2A (PP2A)-dependent de-phosphorylation of the GABA$_A$ receptor.

Prenatal cocaine-exposed new-born babies could be considered as undergoing withdrawal from cocaine exposure *in utero*. It would be of interest to explore whether similar synaptic changes occur in the mPFC of the offspring exposed to cocaine prenatally. The first NIDA National Pregnancy and Health survey conducted in 1992 reported that each year in the United States, 5.5% of all expectant mothers used an illicit drug at least once during their pregnancy (Mathias, 1995). Among those women surveyed, 1.1% had used cocaine at some time during pregnancy. Cocaine use during pregnancy results in retarded fetal brain growth (Zuckerman et al., 1989), leading to postnatal changes in brain functions (Salisbury et al., 2007; Shankaran et al., 2007). Children who are exposed to high-level cocaine are also likely to show dose-dependent postnatal growth impairment (Delaney-Black et al., 1996; Mirochnick et al., 1995) as well as deficits in postnatal motor function, attention and language skills (Azuma and Chasnoff, 1993; Chiriboga et al., 1995; Nulman et al., 1994). Previous animal studies have shown that prenatal cocaine exposure results in changes of cognitive and emotional development of the offspring, including learning and memory (Malanga et al., 2007; Thompson et al., 2005), but cellular and circuit mechanisms underlying these behavioral changes remain unclear. Because dopaminergic inputs to mPFC layer V pyramidal cells are involved in attention as well (Broersen et al., 1996), prenatal cocaine-induced synaptic and neuronal alterations in these neurons may contribute to cognitive impairments and behavioral deficits in postnatal animals.

In the second part of my study, I used a rat model to examine the effects of cocaine exposure *in utero* on activity-dependent synaptic plasticity in the mPFC. Pregnant rats were given daily intraperitoneal injections with either saline or cocaine for 7 d from E15 to E21. Postnatal rats were examined during P8-P42 for cocaine-induced alterations in the activity-induced long-term potentiation (LTP) and long-term depression (LTD), the expression of glutamate and GABA$_A$ receptors, and the excitability of mPFC layer V pyramidal neurons. Similar to that found after withdrawal from postnatal cocaine treatment, cocaine exposure *in utero* caused a reduction of GABAergic inhibition in layer V pyramidal neurons of rat mPFC, leading to an increased susceptibility of excitatory synapses to LTP induction as well as an elevated spiking activity in response to synaptic excitation or membrane depolarization in these neurons. At the behavioral level, rats exposed to cocaine *in utero* showed a reduced locomotor sensitivity to cocaine and the dopamine receptor agonist. Together, these results provided new evidence on the cellular mechanisms underlying the effects induced by cocaine exposure *in utero*.

Chapter 2   Experimental Methods

2.1 Animals and slice preparation

Acute fresh mPFC slices were prepared from Sprague Dawley rats (Charles River) as previously described (Mansvelder and McGehee, 2000). For examining the effect of prenatal cocaine exposure, pregnant animals 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 d from E15 to E21. For postnatal cocaine treatment, juvenile rats were given intraperitoneal injections of either saline or saline containing cocaine in the same manner as described above for 7 d from P18 to P24. Offsprings from the treated mother and the rats undergoing withdrawal from postnatal cocaine injection were anaesthetized with halothane and then killed by decapitation. Animal use procedure was approved by the Animal Care and Use Committee at the University of California, Berkeley. Coronal slices (250 μm thick) containing mPFC were cut with a vibratome (Vibratome Company) in a chamber filled with chilled (2–5 °C) cutting solution containing (in mM) 110 choline-chloride, 25 NaHCO$_3$, 25 D-glucose, 11.6 sodium ascorbate, 7 MgSO$_4$, 3.1 sodium pyruvate, 2.5 KCl, 1.25 NaH$_2$PO$_4$ and 0.5 CaCl$_2$. The slices were then incubated in artificial cerebrospinal fluid (ACSF, in mM) containing 119 NaCl, 26.2 NaHCO$_3$, 11 D-glucose, 2.5 KCl, 2.5 CaCl$_2$, 1.5 MgSO$_4$, 1.25 NaH$_2$PO$_4$ at the room temperature. The solutions were bubbled with 95% O$_2$ and 5% CO$_2$.

2.2 Electrophyisiology

Whole-cell recordings were made from layer V mPFC pyramidal neurons by using a patch clamp amplifier (MultiClamp 700B, Axon Instruments, Foster City, CA) under infrared differential interference contrast optics. Microelectrodes were made from borosilicate glass capillaries and had a resistance of 2.5-5 MΩ. Data acquisition and analysis were performed by using a digitizer and pClamp 9 software (DigiData 1322A, Axon instruments). To stimulate presynaptic fibers, a bipolar tungsten stimulation electrode (WPI Inc., Sarasota, FL) was placed in the layer II/III of the prelimbic region of the mPFC, and pulses of 50 μs duration were applied (at 0.1 Hz). Whole-cell recording was made at 30 ± 1 °C in a recording chamber, under the regulation of an automatic temperature controller (Warner Instr., Grand Haven, MI). Series resistance (15-30 MΩ) and input resistance (100-200 MΩ) were monitored throughout the recording. Data were discarded when the change in the series resistance was >20% during the course of the experiment. Mostly, monosynaptic excitatory postsynaptic potentials (EPSPs) were recorded at -70 mV in current-clamp mode. For recording inhibitory postsynaptic currents (IPSCs), neurons were held at -20 mV in voltage-clamp mode in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and D-2-amino-5-phosphonopentanoic acid (APV, 25 μM). The intrapipette solution for most whole-cell recordings contained (in mM) 140 potassium gluconate, 5 KCl, 10 HEPES, 0.2 EGTA, 2 MgCl$_2$, 4 MgATP, 0.3 Na$_2$GTP and 10 Na$_2$-phosphocreatine, pH 7.2 (with KOH). The BAPTA-containing intrapipette solution consisted of (in mM) 100 potassium gluconate, 5 KCl, 10 HEPES, 20 BAPTA, 2 MgCl$_2$, 4 MgATP, 0.3 Na$_2$GTP and 10 Na$_2$-phosphocreatine, pH 7.2 (with KOH). For recording biphasic responses (EPSC/IPSC sequences), neurons were voltage-clamped at the resting membrane potential (-54 mV). For this experiment, low Cl$^-$-containing intrapipette solution was used (in mM: 145 potassium gluconate, 10 HEPES, 0.2 EGTA, 1 MgCl$_2$, 4 MgATP, 0.3 Na$_2$GTP and 10

Na$_2$-phosphocreatine, pH 7.2 with KOH) in order to amplify the IPSCs to obtain biphasic response showed in Figure 3.2.7A. For measurements of AMPA/NMDA ratio, the recordings were made in the presence of 1 μM SR95531 to block GABAergic transmission and 10 μM glycine to coactivate NMDA receptor. First, the neuron was voltage-clamped at -70 mV to obtain stable AMPA receptor-mediated currents. Then the neuron was held at +40 mV, and responses were recorded first in the presence of CNQX (10 μM) and then in the presence of both CNQX (10 μM) and APV (25 μM). The NMDA receptor-mediated currents were obtained by subtracting the averaged responses obtained in the presence of both CNQX and APV from those obtained in the presence of CNQX only. The intrapipette solution contained (in mM) 120 CsCH$_3$SO$_3$, 20 HEPES, 0.4 EGTA, 5 TEA-Cl, 2 MgCl$_2$, 2.5 MgATP and 0.3 GTP, 10 Na$_2$-phosphocreatine, 1 QX-314·Cl, pH7.2 (with CsOH). For the treatment of SR95531, data were taken 10 min after the onset of the drug perfusion in order to allow drug penetration into the slices. Summary data are given as means ± s.e.m. For assaying EPSP-spike coupling, the initial slope of EPSPs was measured for the first 4-ms period of the rising phase (mV/ms). In all cases, data from one neuron were collected from each slice. Unless indicated otherwise, statistical tests were performed by using Student's *t* test or Kolmogorov-Smirnov test (for cumulative percentage plots).

2.3 Measurement of membrane receptor expression

For biotinylation of membrane proteins, mPFC tissues dissected from freshly isolated brain slices (pre-incubated for 2 hr in ACSF) were incubated in a solution containing sulfo-NHS-S-S-biotin (1 mg/ml, Pierce, Rockford, IL) for 30 min at 4°C. Unreacted biotinylation reagent was quenched by two successive 20 min washes in ACSF containing 100 mM glycine (or Quenching Solution), followed by two washes in ice-cold TBS (50 mM Tris, pH 7.5, 150 mM NaCl). The mPFC tissues were lysed in ice-cold homogenate buffer (50 mM Tris-HCl, 100 mM NaCl, 15 mM sodium pyrophosphate, 50 mM sodium fluoride, 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 2 mM benzamidine, 60 μg/ml aprotinin, and 60 μg/ml leupeptin) and homogenized. To precipitate biotinylated proteins, supernatants of cell lysate (2 mg of total protein) were mixed with immobilized neutravidin beads (Pierce) and kept in a rotator overnight at 4°C. The beads were washed five times with PBS and then eluted with SDS-PAGE sample buffer by boiling for 15 min. Both the total and biotinylated proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with specific antibodies for Glutamate receptor subunit GluR1 (Upstate Biotechnology, Inc., Charlottesville, VA) and for the GABA$_A$ receptor subunits α1 and β2 (Alpha Diagnostic International, San Antonio, TX), as well as β1 and β3 (Santa Cruz Biotechnology, Santa Cruz, CA). The HRP-tagged secondary antibody was used to visualize the specific signals by chemifluorescence substrate. The scanned digital images were quantified with Adobe photoshop software. For each experiment, tissue lysates from both prenatal cocaine- and saline-treated rats were loaded to the same gel for determining the relative levels of either biotinylated protein or total protein. Immunoblots of endogenous actin (see Fig. 3.2.8A) confirmed the effective isolation of the membrane fraction. The ratio of protein levels in the cocaine vs. saline samples were determined in three separate experiments and averaged ratios of biotinylated surface pools and of total protein expression were calculated as shown in Fig. 3.1.8B and Fig. 3.2.8B.

2.4 BDNF immunoassay

Rats were decapitated 1 d, 3 d, 8 or 14 d after the termination of 7 d cocaine or saline treatment (n = 4 per group). Bilateral mPFC tissue were rapidly collected from the cortical brain slices and put into liquid nitrogen. Prior to the assay, wet samples were removed from the liquid nitrogen and weighed. Lysis buffer contains (in mM): 100 PIPES (pH 7), 500 NaCl, 0.2 % Triton X-100, 0.1 % NaN$_3$, 2% BSA, 2 EDTA·Na$_2$·2H$_2$O, 0.2 PMSF, 0.01 leupeptin, 0.0003 aprotinin, and 0.001 pepstatin. Samples were homogenized in iced Lysis buffer and centrifuged at 13,000 × g for 30 min at 4°C. The BDNF proteins in the supernatants were determined using the BDNF Emax ImmunoAssay Systme Kit (Promega, Madison, WI) according to manufacturer's instruction. Optical absorbance was read at 450 nm with a Model 680 Microplate Reader (Bio-Rad Laboratories, Hercules, CA). BDNF contents were interpolated from the standard curve which was determined for each plate (linear range of 7.8 - 500 pg/ml). BDNF levels were presented in picograms of peptide per microgram of wet weight tissue (pg/mg ± s.e.m).

2.5 Lentivirus-based short hairpin RNA (shRNA) expression

VSVg pseudotyped lentiviruses were produced by triple trasfection of 293 FT cells (Invirtrogen) with psPAX2, pMD2.G and pLenti using lipofectamine 2000. The Lentiviral production protocol were the same as previously described (Boyden et al., 2005; Dull et al., 1998). After harvest, viruses were concentrated by centrifuging in a SW28 rotor (Beckman Coulter) at 25,000 rpm for 30 min at 4 $^{\circ}$C. The pellet was resuspended with cold PBS and restored at -80 $^{\circ}$C. The viral titer was examined by FACS to be between 5 X 10^8 and 1 X 10^9 infectious unit per ml.

Trk constructs were subcloned into the pEF-GM expression vector (Paquin et al., 2005). The two TrkB shRNA constructs targeted two different regions on the TrkB mouse mRNA sequence. The sequence for TrkB shRNA was 5'-CCTTGTAGGAGAAGATCAATTCAAGAG-ATTGATCTTCTCCTACAAGG-3'. The double-stranded shRNA template oligonucleotide was cloned into pSIH-H1-copGFP lentivector (System Biosciences, CA) through *Bam*H1 and *Eco*RI sites located downstream of an H1 promoter. The silencing effect of the shRNAs to TrkB expression in cortical cultures was examined using Western blotting 5 day after virus infection. The silencing efficiency of two TrkB shRNAs was expressed as ~80% reduction in TrKB expression relative to the level from control shRNA experiment and normalized with infection efficiency in cultures.

2.6 *In vivo* infection of cells in the mPFC

Sprague Dawley rats (aged at P18) were anesthetized with an i.p. injection of ketamine (70 mg/kg). Animals were kept deeply anesthetized as assessed by monitoring pinch withdrawal, eyelid reflex, corneal reflex, respiration rate, and vibrissae movements. Body temperature was maintained at 37°C by using a heating blanket (Watlow). Two craniotomies, ~300–400 μm in diameter, were drilled above the prefrontal cortex. Viral stock was slowly injected, by using an ultraprecise animal stereotaxic apparatus (Kopf Instruments, Tujunga, CA) at two sites (300 nl per site, one site per hemisphere) with the following coordinates: anterioposterior = - 2.9 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.). Postsurgical rats were given cocaine injection for 7

d (P18 - P24) as described above and sacrificed 7-12 d after discontinuation of cocaine treatment for electrophysiological analysis as described above. Fluorescent dye Alexa594 (0.5 μM) was added into pipette solution to label the recorded neurons. After recording, the slices were put in 4% paraformaldehyde (PFA) for 30 min and then transferred into PSB solution containing 30% sucrose for 24 hr. Finally the slices were stored in PBS solution for imaging analysis.

2.7 Phosphotase assay, Western blotting, and Immunoprecipitation

Medial PFC tissue was taken from the rat brain and rapidly frozen on liquid nitrogen and kept in -80ºC until being used. For Western blotting, mPFC tissues were homogenized in the RIPA buffer (Sigma Aldrich) supplemented with protease inhibitors. Samples were spin down and supernatant was used for Western blotting with antibodies against PP2A (1:10,000, BD Biosciences). With the mPFC sample collected following above procedure, phosphotase assay for PP2A activity was performed following manufacture's protocol (Promega) and that described before (Maeda et al., 2006).

For the $GABA_A$ receptor β3 phosphorylation assay, lysate extracted from brain slices was prepared with RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1 % SDS; pH 7.6) containing phosphatase inhibitor (PhosSTOP) and 1x EDTA-free complete proteaseinhibitor cocktail (Roche, Indianapolis, IN). The lysate (1 mg total each) was subjected to immunoprecipitation with $GABA_A$ receptor β3-specific antibody (C-20; santa cruz biotechnology, CA) conjugated to Protein G-sepharose beads (Amersham, Piscataway, NJ) at 4 ºC for 4 hr. The precipitates were immunoblotted for the phosphorylation level with anti-phosphoserine antibody (clone 4A4; Millipore, MA).

2.8 Locomotion test

The rat pups exposed to cocaine or saline *in utero* were born at the same day and transferred to the same non-treated mother, with the left (saline) or right (cocaine) front foot marked. At P20, the rats were placed in the activity chamber immediately after a single injection of saline (0.9% NaCl, 1ml kg^{-1}) to habituate the chamber for 20 min for 2 d. At the third day, two rats, each from either the saline or cocaine group, were put into the test chamber at the same time for monitoring locomotor activity in ten (Fig. 3.2.11A) or four (Fig. 3.2.11B) 10-min sessions, respectively, with a video tracking system (Med. Associates). After the first session, rats were both given an intraperitoneal injection of cocaine (15 mg kg^{-1} in 1 ml kg^{-1} of saline) or subcutaneous injection of Apomorphine–HCl (2 mg kg^{-1} body weight, Sigma, St. Louis, MO, USA) dissolved in 0.1% ascorbate/saline in 1 mg ml^{-1}.

Chapter 3 Results

Part I. Elevated BDNF during cocaine withdrawal facilitates LTP
in medial prefrontal cortex by suppressing GABA inhibition

3.1.1 Delayed facilitation of LTP induction during cocaine withdrawal

We injected rats intraperitoneally with either saline or cocaine-containing saline daily for 7 d and obtained PFC slices from rats at 5-17 d after terminating the 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 the membrane potential of -70 mV, which corresponds 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 (see Method; (Lu et al., 2009). As shown by example of rats at 8 d after withdrawal, we found that mTBS failed to induce LTP in layer V pyramidal neurons in slices from the saline-exposed rat (Fig. 3.1.1A), but a robust LTP from the cocaine-treated rat (Fig. 3.1.1B). The magnitude of LTP was defined as the ratio of the mean EPSP amplitude at 10-20 min after mTBS to the mean EPSP amplitude prior to mTBS. During the first 1-4 d of cocaine/saline withdrawal, the magnitude of LTP found in the cocaine withdrawal group (n = 19) was not significantly different from that of the saline withdrawal group (n = 7; $p = 0.08$, Kolmogorov-Smirnov test). However, beginning on day 5, the cocaine-exposed rats showed an increased susceptibility of the cocaine group (n = 21) to LTP induction by the same mTBS (Fig. 3.1.1E-H), significantly different with that found for the saline group (n = 12; $p < 0.0001$, Kolmogorov-Smirnov test), indicating a delayed sensitization of these synapses to activity-induced LTP during 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; Fig. 3.1.2A). In contrast to that found for LTP induction, we observed no difference in the induction of LTD at these excitatory synapses in rats 9-11 d after withdrawal between the saline- or cocaine-treated rats (Fig. 3.1.2B,C). Thus, during cocaine withdrawal, there was a selective increase in the susceptibility of these mPFC excitatory synapses to LTP induction, without apparent effect on that for LTD induction.

3.1.2 BDNF elevation during cocaine withdrawal could facilitate LTP

Given previous findings that BDNF signaling is essential for LTP induction and stabilization in other brain regions (Du and Poo, 2004; Pang et al., 2004; Rex et al., 2007; Shen et al., 2006; Yano et al., 2006), and that BDNF expression is elevated the reward system during cocaine withdrawal, we investigated whether elevated BDNF is responsible for the delayed facilitation of LTP in the mPFC during cocaine withdrawal. We first examined the time course of changes in BDNF expression in the mPFC, and found that after terminating the repeated daily cocaine treatment (for 7 d) enzyme-linked immunosorbent assay (ELISA) showed that the level of BDNF in mPFC tissue lysates was significantly higher after 1 week but not 1- or 3-d of
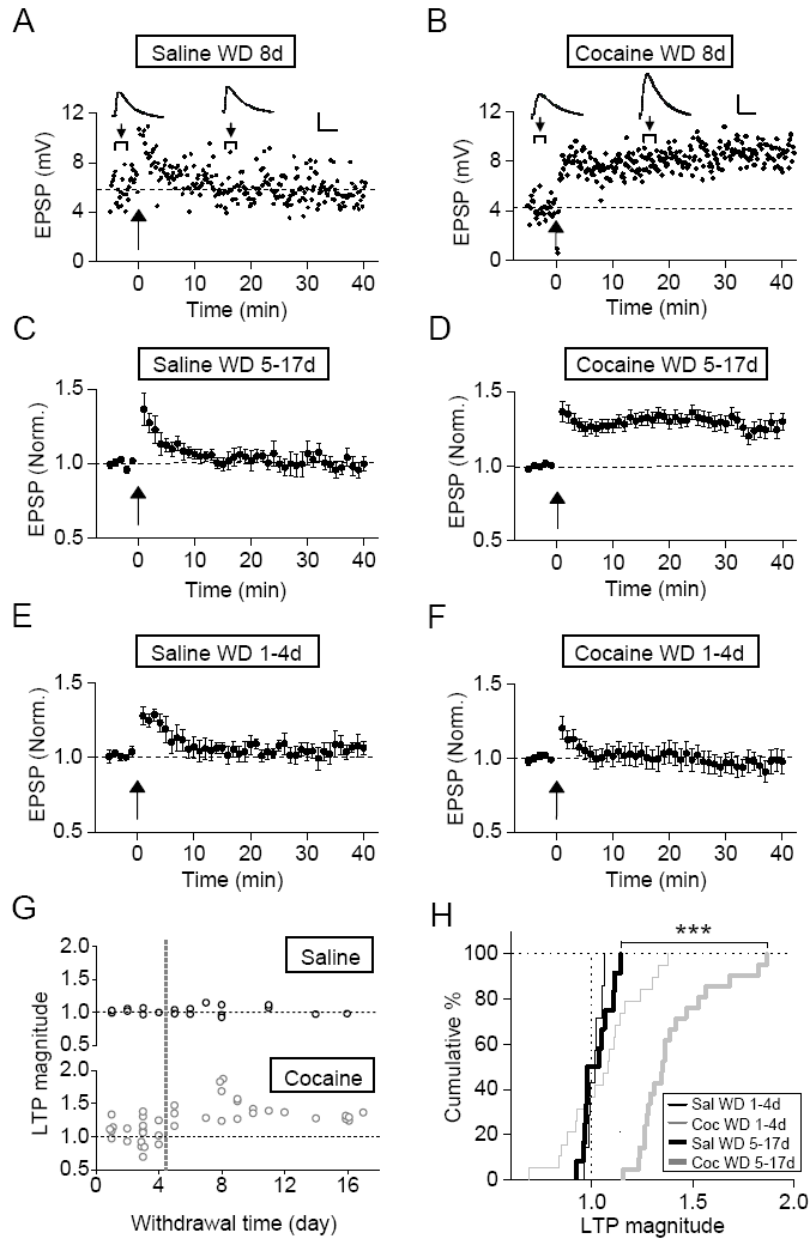
Figure 3.1.1 Cocaine withdrawal time-dependently facilitated LTP induction in layer V pyramidal neurons of rat medial prefrontal cortex (mPFC). *A,B*, An example EPSP amplitude before and after application of modified theta burst stimulation (mTBS) (at the time marked by arrow) for a rat at 8 d after withdrawal from repeated saline (*A*) or cocaine (*B*) treatment. Sample traces above represent averages of 10 EPSPs at the marked time (arrowhead). Scales: 4 mV, 50 ms. *C,D*, Summary of normalized EPSP amplitude before and after the application of mTBS for rats 5-17 d after withdrawal from saline (*C*, n = 12) or cocaine (*D*, n = 21) treatment. Error bars = s.e.m. *E,F*, Summary of normalized EPSP amplitude before and after the application of mTBS for rats undergoing 1-4 d withdrawal from repeated either saline (*E*, n = 7) or cocaine (*F*, n = 19) treatment. *G*, Summary of the magnitude of LTP induced in mPFC pyramidal neurons on different withdrawal day (1 − 17 d). LTP magnitude is obtained by dividing the averaged amplitude of EPSPs at 10-20 min after mTBS with that recorded before mTBS. *H*, Cumulative percentage plot of the distribution of LTP magnitude for the data set shown in (*G*). (*, *p* < 0.0001; Kolmogorov-Smirnov test). "Sal WD" and "Coc WD": saline and cocaine withdrawal, respectively.
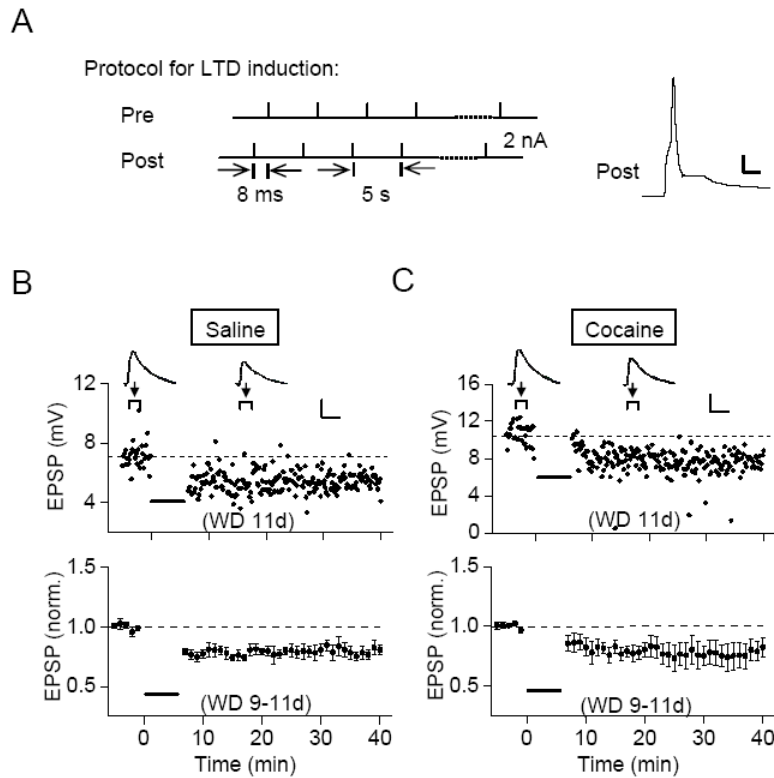
Figure 3.1.2 Cocaine withdrawal did not affect LTD induction in layer V pyramidal neurons of the mPFC. *A*, Left, the protocol for LTD induction consisting of 80 pairs of pre- and postsynaptic stimulation at 0.2 Hz, with postsynaptic spiking induced at 8 ms before each presynaptic stimulation. A typical postsynaptic response is shown on the right. Scales, 20 mV, 5 ms. *B*,*C*, Top, examples of LTD induction in the mPFC of rats at 11 d after withdrawal from repeated saline (*B*) or cocaine (*C*) treatment. Bottom, summarized results from all 6 experiments on rats at 9-11 d after withdrawal from repeated saline (*B*) or cocaine (*C*) treatment. Data presented in the same manner as in Fig. 3.1.1. Horizontal bar: the time of paired stimulation. Scales: 4 mV, 80 ms.

cocaine withdrawal, as compared to that found for the mPFC tissue from corresponding saline-injected rats (Fig. 3.1.3A). This time course of BDNF elevation was similar to that found for the facilitation of LTP induction during cocaine withdrawal (Fig. 3.1.1E).

To further determine whether the facilitation of LTP in cocaine-withdrawn rats is due to the presence of elevated BDNF level, we exogenously applied BDNF (200 ng/ml) to mPFC slices from saline-treated rats 9 d after the termination of the treatment. We found that the amplitude of EPSPs in mPFC layer V pyramidal cells was not affected by the applied BDNF, but the presence of exogenous BDNF resulted in robust LTP induction by mTBS (Fig. 3.1.3B). Thus, BDNF had increased the susceptibility of these mPFC excitatory synapses to LTP induction without affecting the efficacy of basal transmission. As shown in Fig. 3.1.3C, exogenous BDNF caused a similar extent of LTP facilitation in slices from saline-treated rat as that found in slices from cocaine-treated rats. Thus, endogenous BDNF elevation during cocaine withdrawal may facilitate LTP induction at mPFC excitatory synapses.

3.1.3 Postsynaptic TrkB is required for LTP facilitation

To test whether the high-affinity BNDF receptor TrkB is required for the facilitation of LTP induction in the mPFC pyramidal neurons during cocaine withdrawal, we used lentiviral expression of specific short hairpin RNAs (shRNA) against TrkB (Bartkowska et al., 2007) to down-regulate TrkB expression in a subpopulation of mPFC neurons (see Methods). One type of TrkB-shRNA and a control EGFP were prepared and tested for their efficacy in down-regulating TrkB expression in cultured cortical cells, using Western blot analysis of cell lysates (Fig. 3.1.4). The lentiviruses expressing the effective TrkB shRNA and EGFP or those expressing EGFP only were stereotactically and bilaterally injected into the deep layers of mPFC of P18 rats. As shown in Fig. 3.1.5A, effective expression of EGFP was found in cells of layer V and VI when the PFC was sectioned and examined at P33. 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 mark the recorded cell (Fig. 3.1.5B). In neurons expressing EGFP and TrkB shRNA, we found that mTBS failed to induce LTP at the excitatory synapses on mPFC layer V pyramidal neurons in slices obtained from virus-injected rat at 9 d (P33) after cocaine withdrawal, whereas robust LTP was induced in non-fluorescent (control) neurons from the same area of the same rat brain or from fluorescent cells in slices from cocaine-withdrawn rats injected with lentiviruses expressing EGFP only (Fig. 3.1.5C,D). Thus, BNDF-TrkB signaling in the postsynaptic pyramidal cells is required for LTP facilitation during cocaine withdrawal.

3.1.4 BDNF-TrkB signaling causes reduction of GABAergic inhibition

Previous studies have shown that LTP induction can be facilitated in many brain regions by reducing GABAergic inhibition of postsynaptic neurons (Davies et al., 1991; Meredith et al., 2003; Paulsen and Moser, 1998; Wigstrom and Gustafsson, 1983). We thus examined whether GABAergic inhibition is reduced during cocaine withdrawal and whether BDNF elevation contributesto this reduced inhibition. Whole-cell recording from layer V pyramidal neurons in the mPFC of either saline- or cocaine-withdrawn rats was made to measure the maximal amplitude of IPSCs evoked by extracellular stimulation at layer II/III. We evoked IPSCs at -20 mV in the presence of 10 μM CNQX and 25 μM APV, with the stimulation intensity gradually
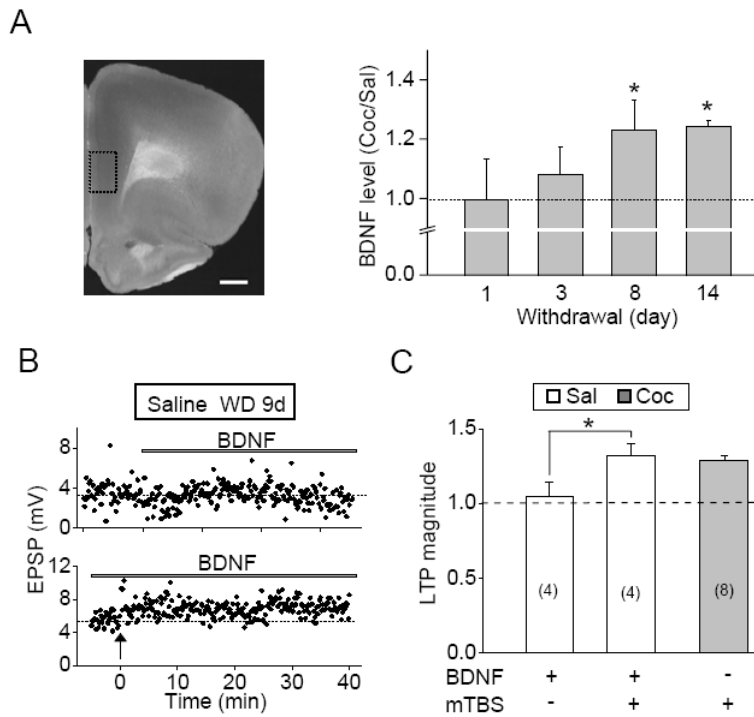
Figure 3.1.3 Elevated BDNF level during cocaine withdrawal facilitated LTP induction in the mPFC. *A*, Left, a bright field picture of a fresh isolated slice containing mPFC. The box indicated the area of mPFC tissue collected for ELISA. Scale: 1 mm. Right, summary of the ratio of BDNF level in the mPFC of cocaine-withdrawn rats to that of saline-withdrawn rats. (n = 3; *, $p < 0.05$, *t* test). *B*, Top, BDNF (200 ng/ml) had no effect on the amplitude of EPSP in a slice from a rat at 9 d after saline withdrawal. Bottom, robust LTP was induced in another slice from the same brain in the presence of BDNF (200 ng/ml). Arrow indicated the application of LTP induction protocol, mTBS. *C*, Summary of LTP magnitude in the mPFC obtained from the rats 9-12 d after withdrawal from repeated saline (Sal) or cocaine (Coc) treatment. (*, $p < 0.05$, *t* test). The total number of neurons examined is shown in parentheses.
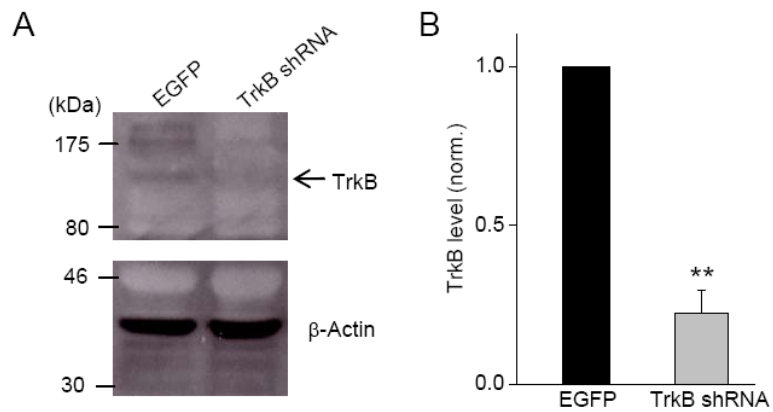
Figure 3.1.4 Effectiveness of TrkB shRNA tested in cultured cortical neurons. *A*, Examples of Western blotting for TrkB and β-actin in the same set of lysate sampls. *B*, Summarized data for the level of TrkB protein in EGFP-only or EGFP-TrkB shRNA transfected cultured cortical neurons. The level of TrkB protein in EGFP-TrkB shRNA transfected cultured cortical neurons was normalized to that in EGFP-only transfected neurons of the same set of lysate sampls. (n = 3; **, $p < 0.01$; *t* test)

Figure 3.1.5 Lentivirus-based TrkB shRNA expression showed the requirement of TrkB for LTP facilitation after cocaine withdrawal. *A*, Examples of lentivirus-based EGFP expression in the pyramidal neurons in deep layers of 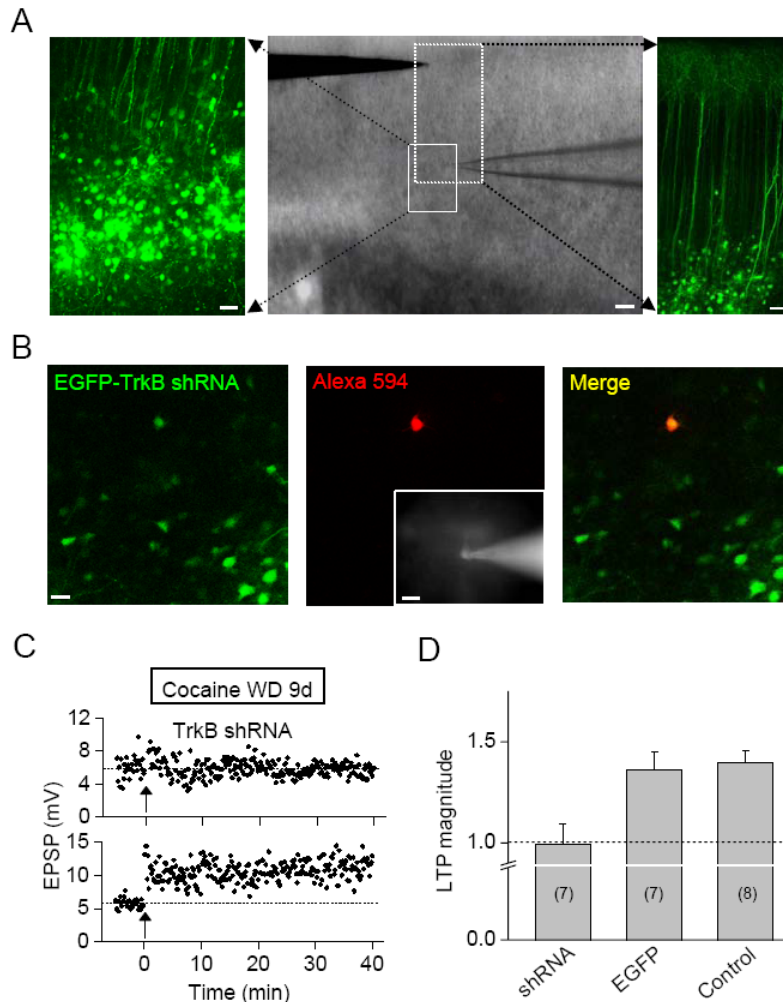the mPFC. The pictures with EGFP florescence were taken under confocal microscope with a fixed slice after recording as showed in the middle picture which was taken under an upright microscope during recording on a fresh slice. Scales: 30 μm (left), 100 μm (middle), 50 μm (right). *B,* Examples of lentivirus-based EGFP-TrkB shRNA expression in the mPFC layer V pyramidal neurons and a recorded neuron recognized by red florescence of Alexa 594 filled into the neuron during recording. The pictures with EGFP and red florescence were taken under confocal microscope with a fixed slice. The insert was taken under an upright microscope during recording under green light. Scales: 20 μm. *C*, Example of LTP obtained from lentivirus-based EGFP-TrkB shRNA infected neuron (top) and a non-infected neuron (bottom) of the same brain. *D*, Summary of LTP amplitude of EGFP-TrkB shRNA or EGFP-only infected and non-infected neurons. The total number of neurons examined is shown in parentheses.

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increased until the peak IPSC amplitude reached the maximum. We found that the mean amplitude of maximal IPSCs in layer V pyramidal neurons was indeed reduced during cocaine withdrawal with a time course similar to that found for the increase of BDNF expression and the facilitation of LTP – no reduction at 1 or 3 d but significant reduction after 1 week of cocaine withdrawal (Fig. 3.1.6A). Furthermore, this reduction of the IPSC amplitude was mediated by BDNF-TrkB signaling, because mPFC pyramidal cells expressing TrkB-shRNA showed higher amplitude of maximal IPSCs than that found in control non-fluorescent cells in the same slices from cocaine-withdrawn rats (Fig. 3.1.6B). Thus, postsynaptic BDNF-TrkB signaling is required for the reduction of GABAergic inhibition during cocaine withdrawal. Consistent with the idea that reduction of IPSC amplitude is due to post- rather than pre-synaptic changes, we found that the paired-pulse ratios of IPSCs after 1 week (8-14 d) of withdrawal were the same in both cocaine- and saline-treated rats (data not shown).

Further studies were performed to examine whether BDNF-TrkB signaling is sufficient to reduce GABAergic inhibition in layer V pyramidal cells of the mPFC. We found that bath application of BDNF (200 ng/ml) to PFC slices from rats withdrawn from saline-treatment (for 9-11 d) resulted in changes in the mean amplitude of IPSCs 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 (Fig. 3.1.6C). This result is fully consistent with the findings in cultured cortical and hippocampal neurons (Jovanovic et al., 2004; Kanematsu et al., 2006). In contrast, in slices obtained from rats cocaine-withdrawn for a similar period (9-11 d), no effect of exogenous BNDF on IPSCs was observed, suggesting endogenous BDNF had already suppressed the GABAergic transmission and this suppression completely 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), but not by its less potent analog K-252b (Fig. 3.1.6D). Taken together, these results showed that BDNF-TrkB signaling is both necessary and sufficient to account for the reduction of GABAergic inhibition of mPFC layer V pyramidal neurons observed during cocaine withdrawal.

3.1.5 Reduction of GABAergic inhibition mediates LTP facilitation

To test whether reduced GABAergic inhibition is sufficient to facilitate LTP induction at excitatory synapses on layer V mPFC pyramidal neurons during cocaine withdrawal, we applied the $GABA_A$ receptor specific inhibitor SR95531 during the LTP induction. While mTBS normally failed to induce LTP in slices obtained from saline-treated rats (at 7-11 d of withdrawal), the presence of 0.5 µM SR95531 (which reduced IPSCs in these mPFC pyramidal cells by ~ 60%, (Lu et al., 2009) resulted in a robust LTP (Fig. 3.1.7A,C,D). 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 ($p = 0.14$, Kolmogorov-Smirnov test), indicating that the cocaine withdrawal effect had occluded that caused by reducing GABA inhibition with SR95531 (Fig. 3.1.7B-D). This suggests that cocaine withdrawal effect shares the same mechanism as reducing GABA inhibition in facilitating LTP. Consistent with this idea, we found that preventing the reduction of GABA inhibition by enhancing $GABA_A$ receptor function with diazepam (DIA, a benzodiazepine agonist known to enhance the activation of $GABA_A$ receptors, (Eghbali et al., 1997) dose-dependently abolished the LTP facilitation in cocaine-
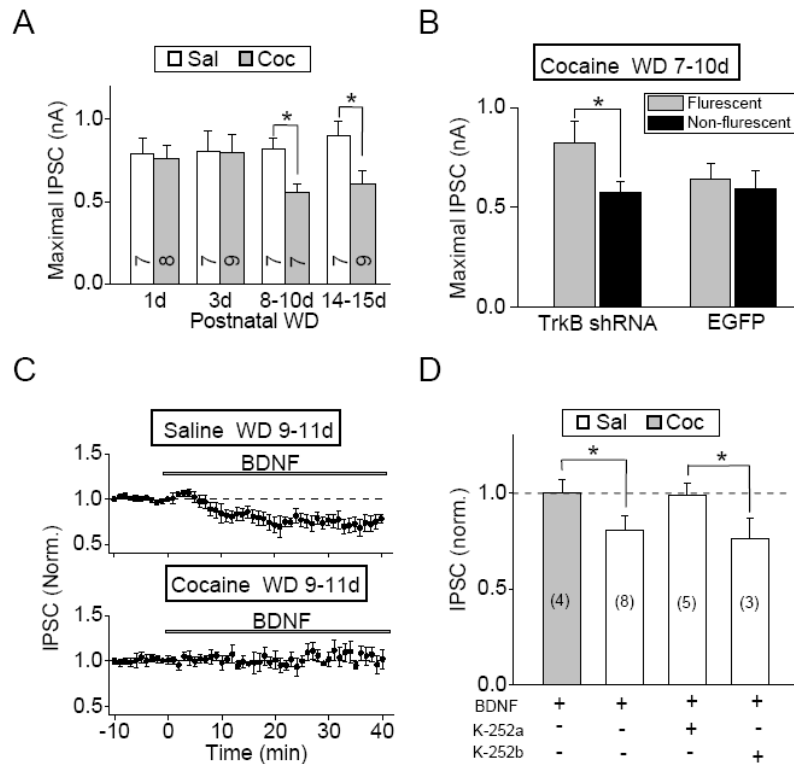
Figure 3.1.6 Elevated BDNF down-regulated GABAergic transmission through TrkB activation. *A*, The maximal amplitude of IPSCs evoked in the pyramidal neurons by extracellular stimulation was compared between rats at 1, 3, 8-10 and 14-15 d after withdrawal from repeated cocaine or saline treatment. For each group, the results were obtained on the same day from the rats in the same litter. (*, $p < 0.05$, *t* test). Number associated with the histogram refers to the total number of cells recorded. *B*, The comparison of maximal amplitude of IPSCs evoked in the pyramidal neurons expressing EGFP only or EGFP-TrkB shRNA with corresponding non-florescent neurons during 7-10 d after cocaine withdrawal. (n = 6-8;*, $p < 0.05$, *t* test). *C*, BDNF effect on IPSCs (normalized) recorded from the slices obtained from the rats at 9-11 d after saline (top, n = 8) or cocaine (bottom, n = 4) withdrawal. *D*, Summary of the BDNF effect on IPSCs recorded from the slices obtained from the rats at 9-11 d after saline or cocaine withdrawal in the absence and presence of K252a (200 nM) or K252b (200 nM) in the extracellular solution ACSF. The magnitude of IPSC (norm.) was calculated as the averaged amplitude of IPSCs from 20-30 min after the application of BDNF, normalized to the mean amplitude before the application of BDNF. The total number of neurons examined is shown in parentheses. (*, $p < 0.05$, *t* test).
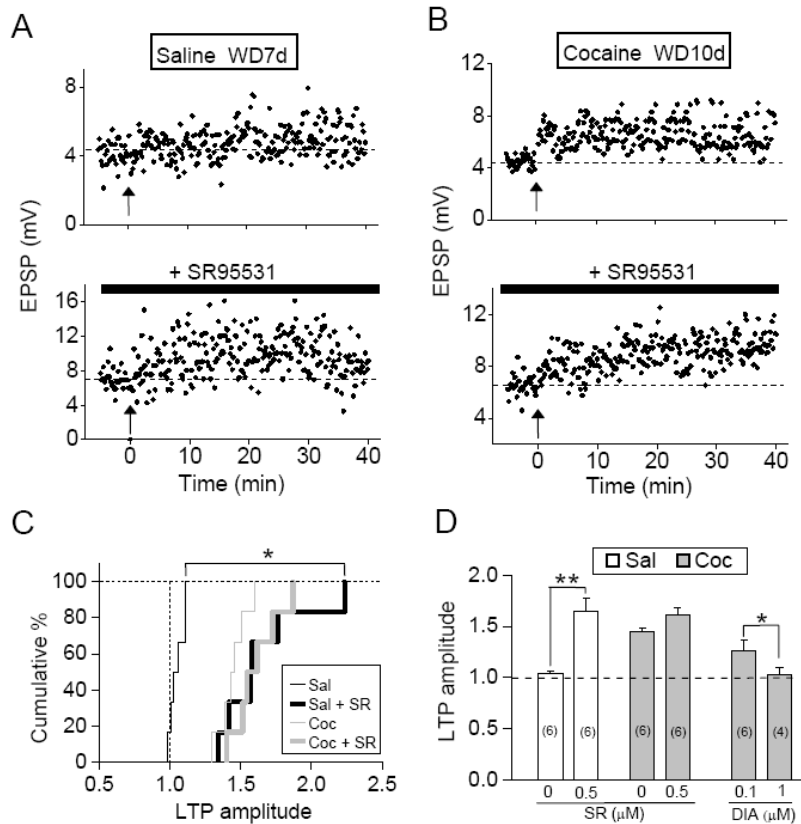
Figure 3.1.7 Reduction of GABA$_A$ receptor-mediated inhibition facilitated LTP induction. *A*, Top, LTP could not be induced by mTBS in a slice from a rat at 7 d after saline withdrawal. Bottom, robust LTP was induced in another slice from the same brain in the presence of a specific GABA$_A$ receptor antagonist SR95531 ("SR", 0.5 μM). *B*, same as *A* except that the slices were from a rat at 10 d after cocaine withdrawal. *C*, Cumulative percentage for the distribution of the LTP magnitude of all experiments similar to those shown in (*A*) and (*B*) for rats 7-11 d after saline (n = 6) or cocaine (n = 6) withdrawal. *D*, Summary of the effects of SR95531 and diazepam on LTP induction in rats 7-11 d after saline (open bars) or cocaine (grey bars) withdrawal. (**, $p < 0.01$, *, $p < 0.05$, *t* test). The total number of neurons examined is shown in parentheses.

withdrawn rats (Fig 3.1.7D). Thus, the reduction of GABAergic inhibition during cocaine withdrawal is necessary and sufficient for LTP facilitation in the mPFC during cocaine withdrawal.

3.1.6 PP2A-dependent downregulation of surface GABA$_A$ receptors

The above pharmacological experiments suggest that postsynaptic GABA$_A$ receptors may be a downstream target of BDNF-TrkB signaling during cocaine withdrawal. We thus compared the surface expression of α1 subunit of GABA$_A$ receptors and GluR1 subunit of AMPA receptors in the mPFC between saline- and cocaine-withdrawn rats. Using biotinylation technique to isolate the protein expressed on the cell surface and Western blotting for the membrane pool (M) and total protein (T) in the cell, we found that the surface expression of GABA$_A$ receptor α1 subunit was reduced significantly at 8 d and 14 d after cocaine withdrawal, relative to the level found in corresponding saline groups (Fig. 3.1.8). In contrast, the total level of this protein in the mPFC cells was not different between the cocaine and saline groups (Fig. 3.1.8). Furthermore, this reduction of surface pool of GABA$_A$ receptor α1 subunit was not observed at 1- and 3-d after the withdrawal. The change of α1 subunit could represent the change of GABA$_A$ receptor because it is the most abundant subunit that is required to form GABA$_A$ receptor in the cerebral cortex (Farrant and Kaila, 2007; Pirker et al., 2000). This effect on GABA$_A$ receptors is selective, because no difference between cocaine and saline groups was found for either the surface pool or the total level of GluR1 in these mPFC cells at all four time points during withdrawal (Fig. 3.1.8).

Previous studies have shown that BNDF suppresses the surface expression of GABA$_A$ receptors on cultured hippocampal 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 at different days after cocaine withdrawal, and found that the PP2A activity was significantly elevated at 8 and 14 d ($p < 0.05$, t test; n = 3) but not at 1 and 3 d ($p > 0.05$, t test; n = 3), relative to that found in corresponding saline group (Fig. 3.1.9A). 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 (Fig. 3.1.9B). As expected for a higher activity of PP2A, we found that the phosphorylation of GABA$_A$ receptor β3 subunit, the main phosphorylated subunit of most cortical GABA$_A$ receptors (Brandon et al., 2002), was significantly decreased during cocaine withdrawal, as shown by Western blots using phospho-serine antibodies on immunoprecipitates of GABA$_A$ receptor subunit β3 from the mPFC tissue obtained from rats at 7-8 d (but not 1 d) after saline/cocaine withdrawal (Fig. 3.1.9C). Thus, elevation of PP2A activity during cocaine withdrawal appears to be responsible for mediating the reduction of surface expression of GABA$_A$ receptors.

Further evidence for the role of PP2A activity in mediating the BDNF effect on GABA$_A$ receptors was provided by the finding that in the presence of the PP2A inhibitor okadaic acid (0.5 μM, OA) in the pipette solution completely prevented the depression of GABAergic transmission by BDNF in the mPFC of the rat 7-12 d after saline withdrawal (Fig. 3.1.9D,E). Overall, elevation of BDNFexpression during cocaine withdrawal leads to the reduction of GABA$_A$ receptor expression on postsynaptic membrane via TrkB-PP2A signaling pathway.
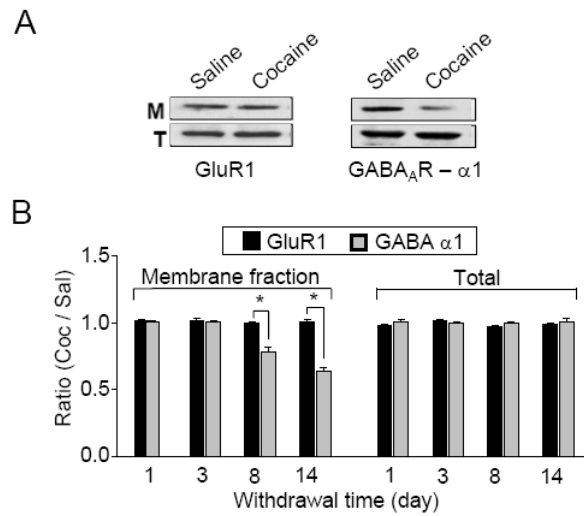
Figure 3.1.8 The surface expression of GABA$_A$ receptor subunits in the mPFC was reduced after cocaine withdrawal. *A*, Example immunoblots of the lysates of mPFC tissues obtained from rats at 8 d after saline or cocaine withdrawal, showing the level of biotinylated membrane-bound fraction (M) and of the total protein (T) for GluR1, GABA$_A$ receptor α1subunit. *B*, Densitometric measurements of the band densities of immunoblots for membrane-bound and total levels of various receptor subunits in the mPFC. The data are presented as the ratio of densities measured for cocaine- vs. saline-treated rats in the same immunoblot (n = 3; *, $p < 0.05$; *t* test).

Figure 3.1.9 PP2A mediated the down-regulation of surface expression of GABA_A receptors by BDNF. **A**, Summarized result of phosphatase assay to show the change of PP2A activity during cocaine withdrawal. The data a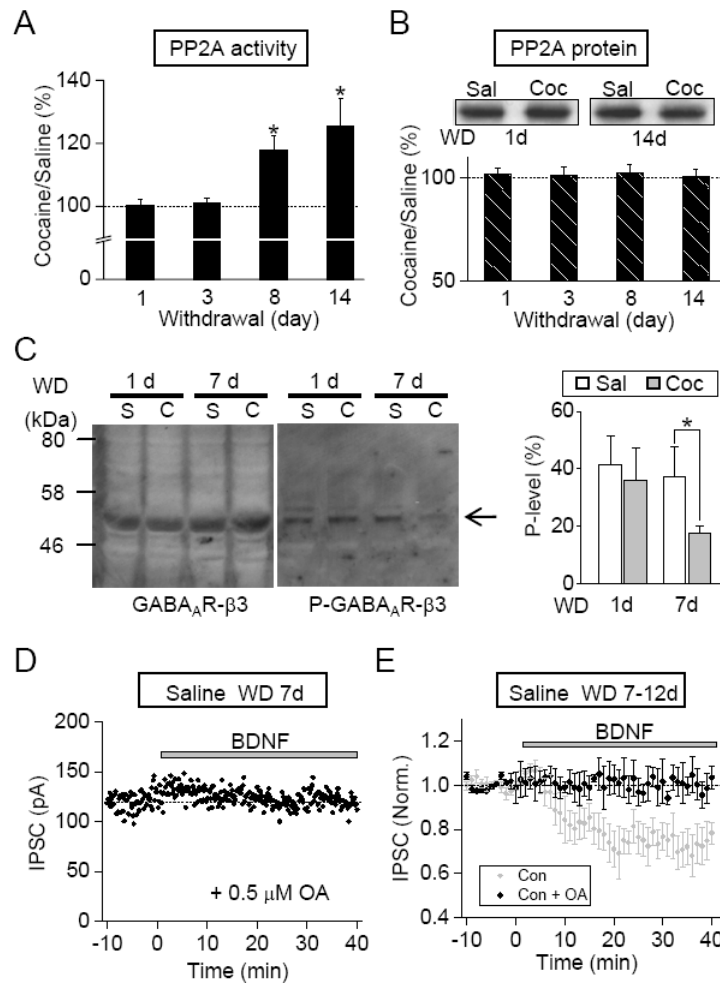re presented as the ratio of the enzyme activity calculated in the same group of tests for cocaine- vs. saline-withdrawn rats (n = 3; *, $p < 0.05$; t test). **B**, Comparison of densitometric measurements of the band densities of immunoblots for PP2A protein from the same lysate sample as in (**A**). Top, example immunoblots for PP2A protein in the mPFC obtained from rats at 1 and 14 d after saline or cocaine withdrawal. The data are presented as the ratio of densities measured in the same blots of tests for cocaine- vs. saline-withdrawn rats (n = 3; $p > 0.05$, t test). **C**, De-phosphorylation level of GABA_A receptor (GABA_AR) β3 subunit at 1 and 7 d after saline (S) or cocaine (C) withdrawal. Left, examples of Western blotting for GABA_AR β3 subunit (GABA_AR-β3) and phosphorylated GABA_AR β3 (P- GABA_AR-β3) in the same set of lysate sampls. (n = 3; *, $p < 0.05$; t test) **D**, **E**, An example (**D**) and summary (**E**) of BDNF effect on IPSCs recorded from the slices obtained from the rats at 7-12 d after saline withdrawal in the presence of PP2A bloker, okadaic acid (OA, 0.5 μM) (n = 5). The horizontal bar represents the application of BDNF (200 ng/ml). The summarized result for BDNF effect on IPSCs in the absence of OA (Con) is from the same data set as in Fig. 3.1.6*C*.

3.1.7 Elevated excitability of layer V pyramidal neurons during cocaine withdrawal

At 8-14 d after cocaine withdrawal, the reduction of $GABA_A$ receptor-mediated inhibition of mPFC layer V pyramidal neurons should lead to elevated excitability of these neurons. We first examined the excitation of these mPFC pyramidal cells at 8-11 d after cocaine withdrawal by measuring EPSP-spike (E-S) coupling (Lu et al., 2000). Whole-cell recording from these pyramidal cells was used to determine neuronal spiking probability vs. a range of presynaptic stimulation intensity that induced EPSPs of different slopes (Fig. 3.1.10A). Fitting the data with a sigmoid function yielded the value of the EPSP slope ($E_{0.5}$) that corresponded to the spiking probability of 0.5. We found that at 8-11 d after cocaine withdrawal the mean value of $E_{0.5}$ (Fig. 3.1.10B) and the threshold membrane potential for spiking (Fig. 3.1.10C) were significantly lower than those found for corresponding saline-withdrawn rats. Furthermore, the elevated excitability of mPFC pyramidal neurons is also reflected by the increased number of APs initiated by depolarizing current pulses injected into these neurons in the slices obtained from the rat 8-14 d after cocaine withdrawal (Fig. 3.1.10D), indicating a facilitation in evoked spiking during cocaine withdrawal. Together, supportive to the result of reduced inhibition to layer V mPFC pyramidal neurons, the excitation of these neurons by both presynaptic neurotransmitter release and injection of depolarizing current was promoted 8-14 d after cocaine withdrawal.

3.1.8 Presynaptic change of excitatory transmission during cocaine withdrawal

Many studies have shown that BDNF increases presynaptic release of glutamate (Madara and Levine, 2008; Poo, 2001). To estimate the presynaptic change of excitatory synapse during cocaine withdrawal, we performed whole-cell voltage-clamp recording of EPSCs at -70 mV that were evoked by two sequential trains of stimuli - 14 pulses at 50 Hz, followed by 5 pulses at 1.67 Hz (Fig. 3.1.11A). These two trains were used respectively to examine synaptic fatigue of excitatory synapses by high-frequency use that depletes the pre-primed vesicles in the presynaptic terminal and to determine the recovery of the excitatory synaptic response by recruitment of synaptic vesicles. We found that the characteristic decay time of EPSCs during the first train is significantly smaller in the cocaine than the saline group (Fig. 3.1.11B), whereas the plateau values of EPSC amplitudes showed no significant difference between the two groups (Fig. 3.1.11C). The paired-pulse ratio for sequential EPSCs (at an interval of 20 ms) was reduced markedly during cocaine withdrawal (Fig. 3.1.11B), suggesting a significant increase of the probability of presynaptic glutamate release (Abrahamsson et al., 2005; Hanse and Gustafsson, 2001). Finally, the characteristic time for the recovery of the EPSC amplitude was not different between two groups, although the extent of recovery was lower in the cocaine group, suggesting persistent depression of basal transmission (Fig. 3.1.11D,E). Overall, in addition to the facilitation of LTP induction, there are presynaptic changes at these excitatory synapses on layer V mPFC pyramidal neurons during cocaine withdrawal, as reflected by the reduced PPR, faster rate synaptic fatigue during high-frequency transmission, and incomplete recovery from the fatigue.
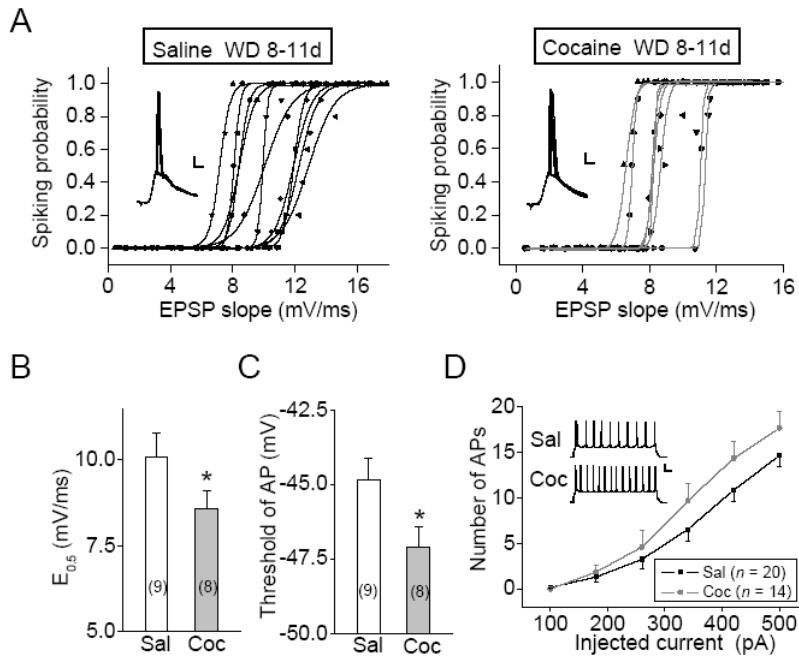
Figure 3.1.10 Cocaine withdrawal increased the EPSP-spike (E-S) coupling and excitability of mPFC layer V pyramidal neurons. *A*, E-S coupling studies on rats at 8-11 d after saline or cocaine withdrawal. The curves represent the best-fit with the sigmoidal function. Inserts, sample traces of EPSC with the firing probability between 0 and 1. Scales: 10 mV, 5 ms. *B*, Comparison of the values of $E_{0.5}$ for same data sets as in (*A*). $E_{0.5}$ represents the efficacy of E-S coupling, as defined by the EPSP slope that initiates spiking with a probability of 0.5. (*, $p < 0.05$, *t* test). The total number of neurons examined is shown in parentheses. (Sal: saline; Coc: cocaine). *C*, Summary of the threshold potential for action potential (AP) initiation. The threshold was determined by the membrane potential at the peak of the maximal subthreshold EPSP. (*, $p < 0.05$; *t* test, the same data set as in *B*). *D*, Changes of the excitability of mPFC layer V pyramidal neurons after cocaine withdrawal, as shown by the number of APs triggered by injection of depolarizing currents of various amplitudes (duration 800 ms). Data were obtained from the recording on the slices obtained from rats at 8-14 d after saline (Sal) or cocaine (Coc) withdrawal. Insert, examples of APs elicited by a constant depolarizing current (500 pA, 800 ms) in mPFC neurons in the rats undergoing 9-d saline or cocaine withdrawal. Scales, 20 mV, 80 ms.
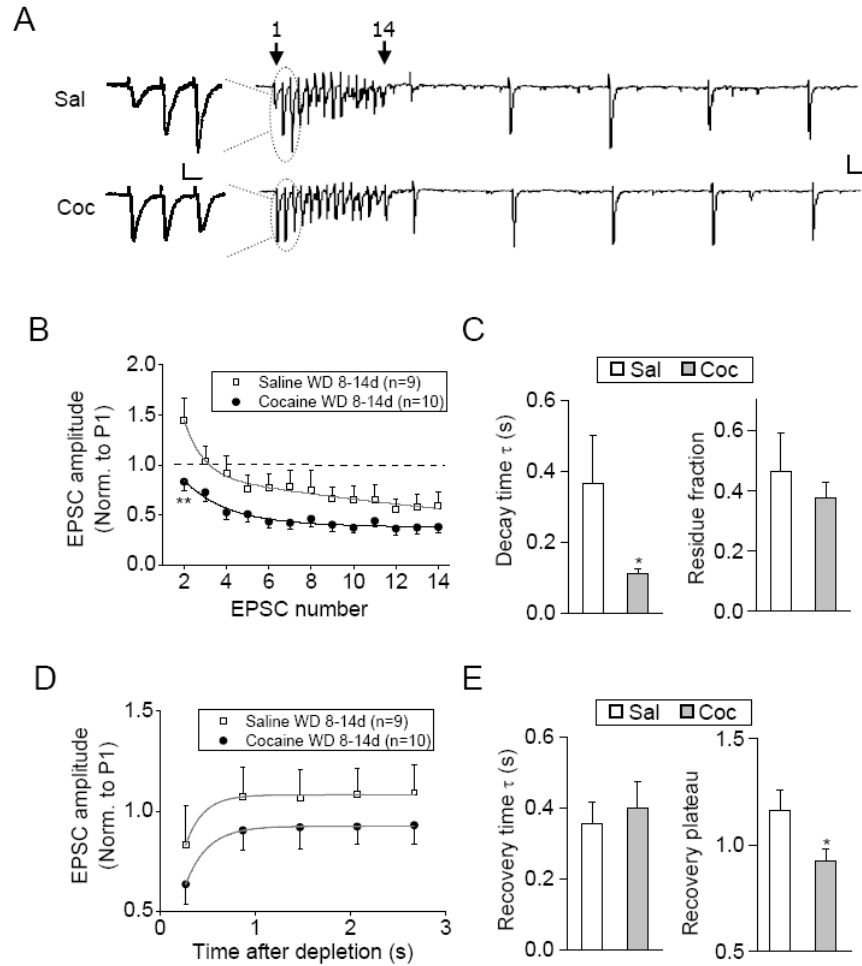
Figure3.1.11 Elevated BDNF increased presynaptic glutamate release and decreased the release recovery extend. *A*, Sample traces of EPSCs evoked by two train stimulations (14 impulses in 50 Hz, followed by 5 impulses in 1.67 Hz with 0.27 sec interval) in the slice obtained from rats at 9 d after saline (Sal) or cocaine (Coc) withdrawal. Scales, 100 pA, 50 ms. *B,* Comparison of EPSC fatigue in mPFC layer V pyramidal neurons between saline or cocaine groups during 8-14 d withdrawal. The x axis represents the number of the 14 peaks of EPSC evoked by the first 50 Hz train stimulation as shown in (*A*). The y axis represents amplitude of each peak normalized to peak 1 (P1). The curve is the fitting of an exponential decay function. *C*, Comparison of the decay time and residue fraction of EPSCs of the same data set as in (*B*). The decay time was obtained by fitting the plot of each case with an exponential decay function. The residue fraction was counted as the amplitude of peak 14. (Sal: saline; Coc: cocaine. *, $p < 0.05$, t test). *D*, Comparison of the recovery of EPSCs evoked by the second 5-pulse train stimulation as shown in (*A*). The y axis is same as that in (*B*). The time after depletion was counted as the time after pulse 14 of the first train stimulation. The curve is the fitting of an exponential rising function. *E*, Comparison of the recovery time and plateau of EPSCs of the same data set as in *D*. The recovery time was obtained by fitting the plot of each case with an exponential rising function. The recovery plateau was counted as the averaged amplitude of the last two peaks. (Sal: saline; Coc: cocaine. *, $p < 0.05$, t test).

**Part II. Cocaine exposure *in utero* alters synaptic plasticity
in the medial prefrontal cortex of postnatal rats**

3.2.1 Prenatal cocaine exposure facilitates LTP induction

Whole-cell recordings were made from layer V pyramidal cells of the prelimbic (PL) region of mPFC to monitor monosynaptic excitatory postsynaptic potentials (EPSPs) in response to extracellular stimulation of axon fibers in layer II/III (Fig. 1A). We first examined the induction of LTP in these pyramidal neurons of fresh brain slices prepared from the offspring of pregnant rats that were intraperitoneally injected daily with either normal saline or cocaine-containing saline for 7 d (E15-E21). We monitored EPSPs at the membrane potential of -70 mV (under current clamp), which is close to the reversal potential of inhibitory postsynaptic currents (IPSCs) determined by the Cl⁻ concentration of the extracellular and intrapipette solution (Liu et al., 2005). The identity of EPSPs was confirmed by their disappearance following bath application of CNQX (10 μM) and APV (50 μM), the antagonist for the a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) subtype of glutamate receptors, respectively. We found that repetitive stimulation of presynaptic inputs with a modified theta burst stimulation (mTBS) protocol (Fig. 3.2.1B) induced a persistent increase in the amplitude of EPSPs in layer V pyramidal cells in slices from P16 – P42 rats that were exposed to cocaine *in utero*. As shown by the example recording for a P25 rat (Fig. 3.2.1C) and the summary of results from 40 rats in the age range of P16 - P42 (Fig. 1D), enhanced EPSPs persisted for at least 40 min after application of mTBS. In contrast, the same mTBS only induced a short-term (~5 min) potentiation in slices obtained from prenatal saline-treated rats of the age range of P16 – P42 (Fig. 3.2.1E,F). The synaptic potentiation observed for prenatal cocaine-treated rats is similar to LTP found in many brain areas (Dan and Poo, 2004; Malenka and Bear, 2004). It depended on the activation of NMDA receptors and intracellular Ca^{2+}, since no synaptic potentiation was induced in slices from prenatal cocaine-treated rats in the presence of APV or BAPTA (Fig. 3.2.2).

In contrast to the effect on LTP induction, prenatal cocaine exposure had no effect on the induction of LTD at these excitatory synapses in P20 - P25 rats by a spike timing stimulation protocol, involving repetitive presynaptic stimulation following postsynaptic spiking with 8-ms delay (0.2 Hz, 80 pairs; Fig. 3.2.3). In slices obtained from both prenatal cocaine- and saline-exposed rats, we found that the above paired stimulation caused a similar reduction in the EPSP amplitude ($p = 0.79$, Kolmogorov-Smirnov test). Thus, the cocaine exposure *in utero* had selectively modified the susceptibility to the induction of LTP in the mPFC, without apparent effect on LTD induction.

3.2.2 Delayed appearance of LTP facilitation

When LTP induction was performed on slices obtained from younger rats during the age of P8 - P15, we found substantial induction of LTP following mTBS in both prenatal cocaine- and saline-treated groups (Fig. 3.2.4A-D). To further examine the magnitude of LTP quantitatively, we defined the LTP magnitude as the ratio of the average EPSP amplitude during the 20 - 30 min period after mTBS to that during the 5-min control period prior to mTBS. Examination of the changes in the LTP magintude during the first 6 postnatal weeks showed that LTP became
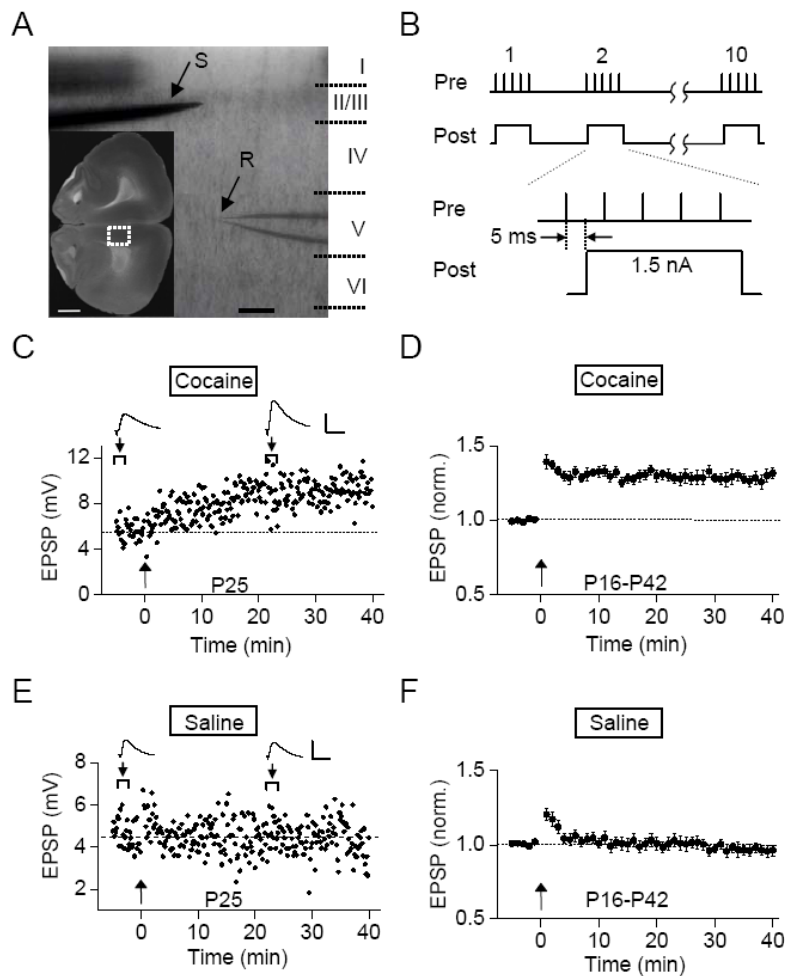
Figure 3.2.1 Prenatal cocaine exposure facilitated LTP induction in layer V pyramidal neurons of rat medial prefrontal cortex (mPFC). *A*, Images of an acutely isolated coronal slice of P20 rat brain, showing the extracellular stimulating electrode (S) at layer II/III and the whole-cell recording electrode (R) at layer V at the mPFC region (marked by the white box in the insert). Scales, 1 mm (box), 200 μm. *B*, Stimulation protocol for LTP induction (termed "modified theta burst stimulation, mTBS), consisting of presynaptic activation of 10 bursts (each with 5 pulses at 100 Hz) spaced at 200 ms and 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. *C*, An example of LTP induction in a slice prepared from a P25 rat that was exposed to cocaine *in utero* for 7d. The data points represent the amplitude of EPSPs recorded before and after application of mTBS (at the time marked by arrow). Sample traces above represent averages of 10 EPSPs at the marked time (arrowhead). Scales: 6 mV, 50 ms. *D*, Summary of data from all experiments similar to that in C, showing normalized EPSP amplitudes before and after LTP induction in slices from P16 – P42 rats that were prenatally exposed to cocaine (for 7 d from E15 to E21, n = 40). The EPSP amplitudes over 1-min intervals were normalized by the mean amplitude before induction. Error bars = s.e.m. *E,F*, Same as *C* and *D* except that the results were obtained from slices of prenatal saline-treated rats (*F*, n = 21).
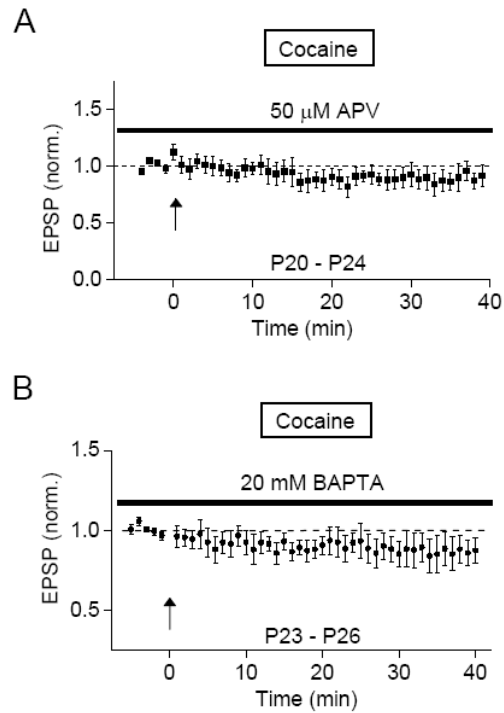
Figure 3.2.2 Dependence of LTP on NMDA receptor activation and intracellular Ca2+. *A*, Normalized EPSPs before and after mTBS (arrow) in mPFC slices obtained from P20 - P24 rats in the presence of APV (50 μM), an NMDA receptor antagonist. The rats were exposed to cocaine prenatally during E15-E21. Error bars indicate s.e.m. (n = 5). *B*, Normalized EPSPs before and after mTBS (arrow) in mPFC slices obtained from P23 - P25 rats, recorded with BAPTA (20 mM) in the intrapipette solution. The rats were exposed to cocaine prenatally during E15-E21. Error bars indicate s.e.m. (n = 5).
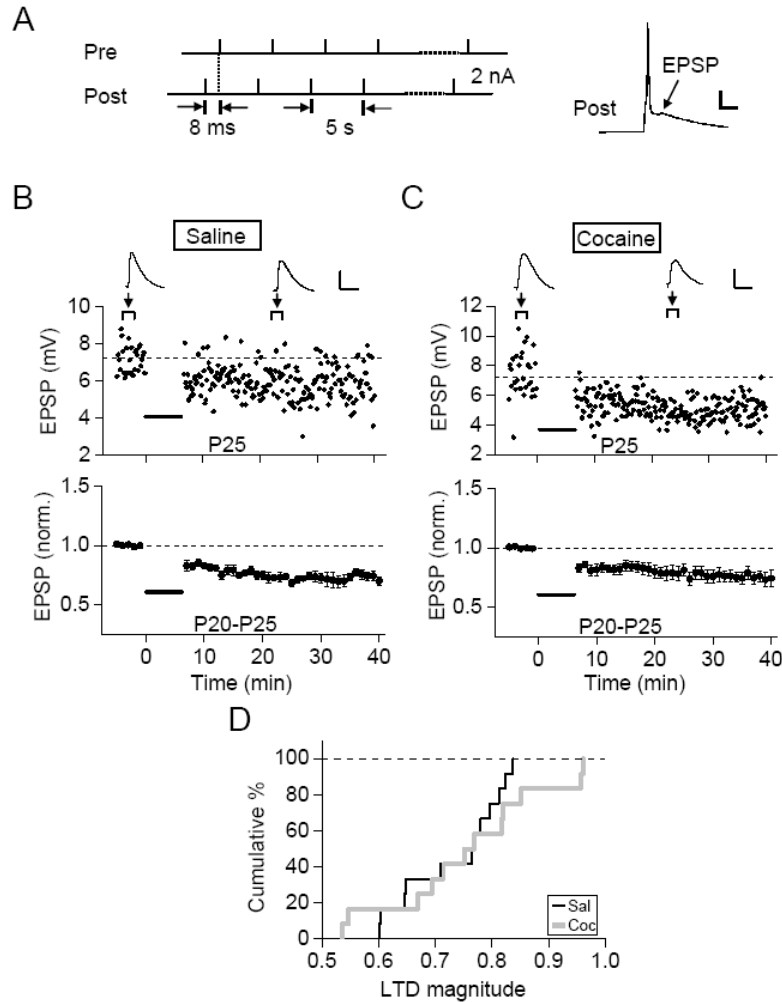
Figure 3.2.3 Prenatal cocaine exposure did not affect LTD induction in layer V pyramidal neurons of the mPFC. *A*, Left, the protocol for LTD induction consisting of 80 pairs of pre- and postsynaptic stimulation at 0.2 Hz, with postsynaptic spiking induced at 8 ms before each presynaptic stimulation. A typical postsynaptic response is shown on the right. Scales, 20 mV, 5 ms. *B,C*, Top, examples of LTD induction in the mPFC of P25 rats. Bottom, summarized results from all 12 experiments on rats treated prenatally with saline (*B*) or cocaine (*C*) for 7 d (E15-E21). Data presented in the same manner as in Fig. 3.2.1. Horizontal bar: the time of paired stimulation. Scales: 4 mV, 80 ms. *D*, Cumulative percentage plot of the distribution of the LTD magnitude, as determined by the mean amplitude of EPSCs (normalized by the mean baseline value of each cell) at 20-30 min after the onset of pre- and postsynaptic stimulation for the data set shown in *B* and *C*. "Sal" and "Coc": prenatally exposed to saline and cocaine, respectively. The two sets of data are not significantly different ($p = 0.79$; Kolmogorov-Smirnov test).

progressively reduced and essentially disappeared after P16 in prenatal saline-treated rats. In contrast, the LTP exhibited progressive increase over the same 6 weeks period for prenatal cocaine-treated rats (Fig. 3.2.4E). This opposite trend resulted in the prominent difference in synaptic plasticity between the two groups after P16, when developmental transition of the GABA action from excitation to inhibition is completed (Cancedda et al., 2007; Ganguly et al., 2001; Owens et al., 1996). As shown by the cumulative percentage plot of the LTP magnitude for prenatal saline- and cocaine-treated groups over two distinct postnatal periods of P8 - P15 and P16 – P42 (Fig. 3.2.4F), the distribution of LTP magnitude showed no significant difference between the two groups of rats during P8 - P15 (p = 0.83, Kolmogorov-Smirnov test), but became significantly different during P16 – P42 (p < 0.0001, Kolmogorov-Smirnov test). Thus, facilitation of LTP induction by prenatal cocaine exposure occurred only after P16. All following experiments were performed for rats in the age group of P16 – P42 unless indicated otherwise.

3.2.3 LTP facilitation is related to reduced GABAergic inhibition

GABAergic activity normally suppresses LTP induction in mature excitatory synapses (Davies et al., 1991; Meredith et al., 2003; Paulsen and Moser, 1998; Wigstrom and Gustafsson, 1983), after the developmental transition (between P10 - P14 in rat) of the GABA action from excitation to inhibition. Our finding that significant LTP could be induced after P16 for prenatal cocaine-exposed rats (Fig. 3.2.4E,F) suggests that reduced GABAergic inhibition may account for the facilitated LTP induction, similar to that found in rat VTA dopaminergic neurons (Liu et al., 2005) and mPFC pyramidal neurons (Huang et al., 2007) after repeated *postnatal* cocaine treatment. To determine whether *prenatal* cocaine exposure also causes the reduction of GABAergic inhibition during the postnatal period, we bath-applied the specific inhibitor of GABA$_A$ receptor SR95531 during LTP induction. In brain slices from prenatal saline-treated rats on P25, mTBS failed to induce LTP (Fig. 3.2.1E, 3.2.5A). However, in the presence of SR95531 (0.5 μM), which reduced the amplitude of GABA$_A$ receptor-mediated IPSCs by 60% (Fig. 3.2.6), mTBS induced a robust LTP in mPFC layer V pyramidal neurons of prenatal saline-treated rat (Fig. 3.2.5B). This result is consistent with the notion that LTP induction is normally suppressed at mature excitatory synapses due to the presence of strong GABAergic inhibition (Davies et al., 1991; Meredith et al., 2003; Paulsen and Moser, 1998; Wigstrom and Gustafsson, 1983). In contrast, for prenatal cocaine-exposed rats, we found that the presence of SR95531 (0.5 μM) resulted in rightward shifts of the cumulative percentage distribution of LTP magnitude in both saline- and cocaine-treated rats (Fig. 3.2.5D), showing that the reduction of inhibition indeed elevated the magnitude of LTP. Importantly, the increase in the magnitude of LTP caused by SR95531 was much larger in prenatal saline-treated rats than that in cocaine-treated rats (Fig. 3.2.5C,D), and the SR95531 treatment led to a same extent of LTP elevation in both groups of rats (p = 0.093, Kolmogorov-Smirnov test). Thus, cocaine exposure *in utero* had resulted in cellular changes similar to the effect induced by SR95531, suggesting that the reduction of GABAergic inhibition underlies the facilitated LTP found in the mPFC of prenatal cocaine-exposed rats.

3.2.4 Measurements of GABAergic synaptic transmission

To test whether prenatal cocaine exposure reduces the GABA-mediated inhibition postnatally, we applied weak stimuli of different intensities to the layer II/III of the mPFC and
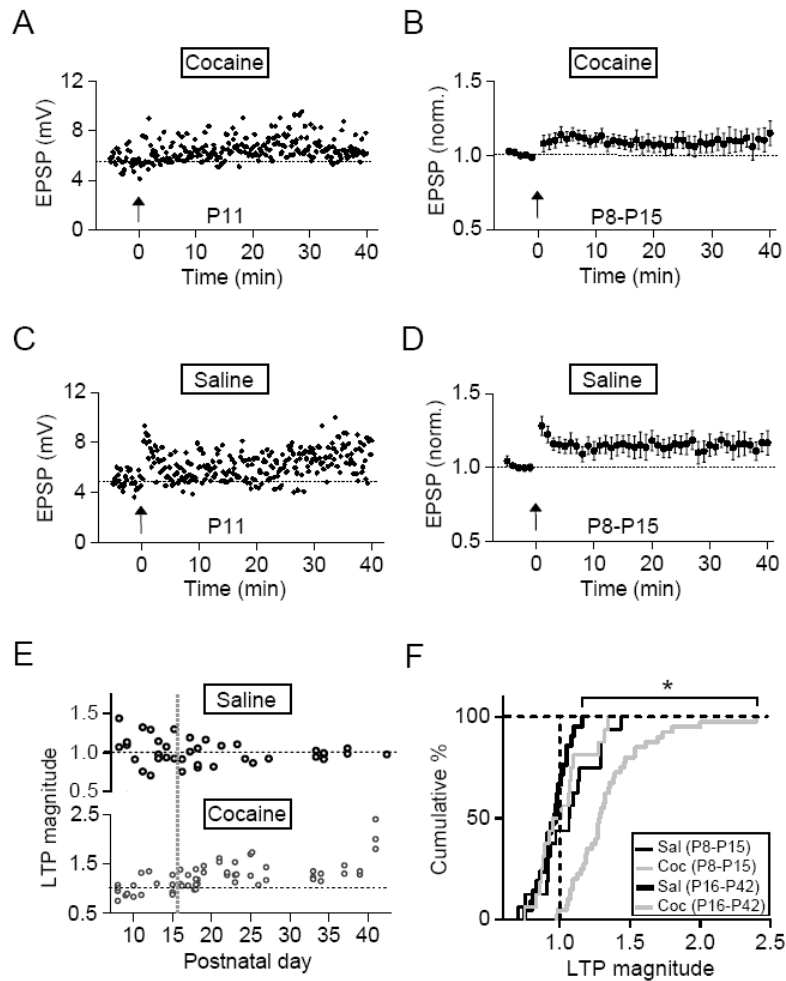
Figure 3.2.4 The effect of prenatal cocaine exposure on LTP induction appeared during the late postnatal period (after P16). *A*,*B*, An example and summarized results (n = 20) on LTP induction in slices prepared from an early postnatal rats (P8 - P15) that was exposed to cocaine *in utero* for 7d (E15 - E21). *C*,*D*, Same as *A*,*B* except that the rats were prenatally exposed to saline (n = 20). *E*, Summary of the magnitude of LTP induced in mPFC pyramidal neurons on different postnatal days (P8 – P42), as indicated by the LTP magnitude, which is obtained by dividing the averaged amplitude of EPSPs at 20-30 min after mTBS with that recorded before mTBS. *F*, Cumulative percentage plot of the distribution of LTP magnitude for the data set shown in *E*. (*, $p < 0.0001$; Kolmogorov-Smirnov test). "Sal" and "Coc": prenatally exposed to saline and cocaine, respectively.
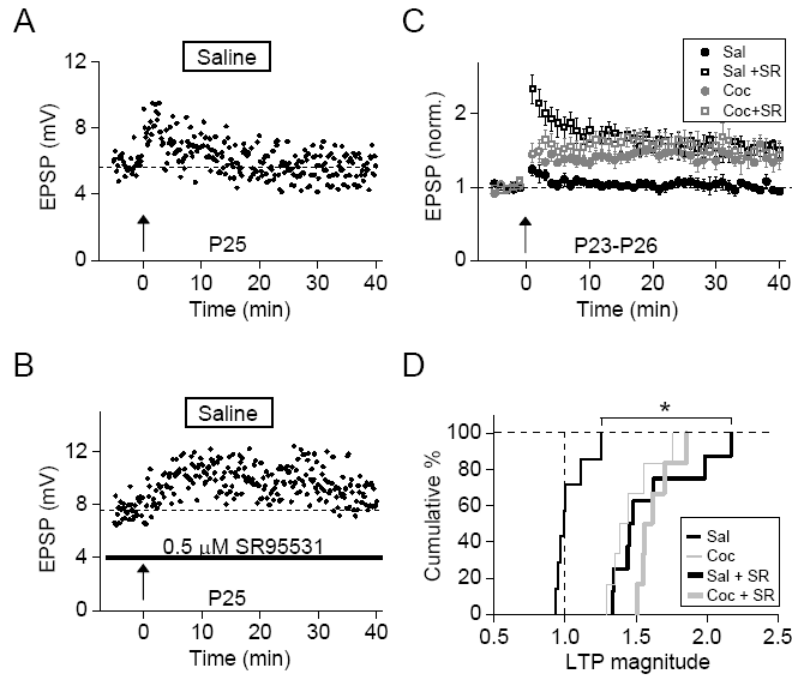
Figure 3.2.5 Reduction of GABA$_A$ receptor-mediated inhibition facilitated LTP induction. *A*, LTP could not be induced by mTBS in a slice from a P25 rat prenatally treated with saline. *B*, Robust LTP was induced in another slice from the same brain as that in *A*, in the presence of a specific GABA$_A$ receptor antagonist SR95531 ("SR", 0.5 μM). *C*, Summary of results on LTP induction from all experiments similar to those shown in *A,B* for rats prenatally treated with saline (n = 6) or cocaine (n = 8). *D*, Cumulative percentage for the distribution of the LTP magnitude for the data set shown in *C*. (*, *p* < 0.0001; Kolmogorov-Smirnov test).
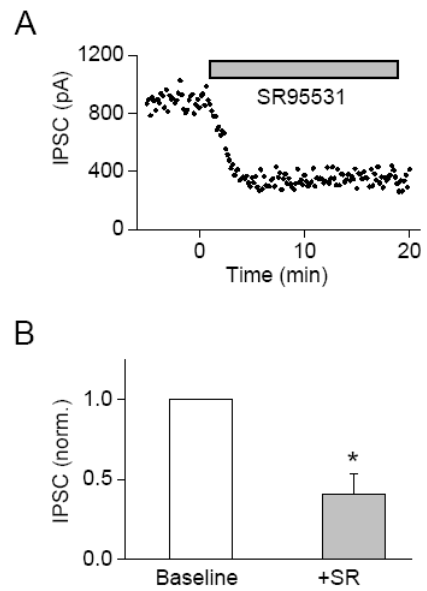
Figure 3.2.6 The effect of SR95531 on IPSCs. Data points depict the amplitude of maximal IPSCs before and after bath-application of SR95531 (0.5 μM), an antagonist of $GABA_A$ receptors, recorded from an mPFC pyramidal neuron in a slice from prenatal saline-treated rat (P24). Bar graphs show the summary of the results from 5 recordings. ("*", $p < 0.05$; t test).

measured the amplitude of IPSCs of the disynaptic inhibition (Fig. 3.2.7A), by holding the layer V pyramidal neurons at the resting membrane potential (-54 mV). The input-out relationship was determined by plotting the data as stimulus intensity vs. IPSC amplitude (Fig. 3.2.7B). The slope of the line with best linear fit of the data for prenatal cocaine-treated rats was significantly lower than that for control saline-treated rats, in either the absence ($p = 0.017$, Kolmogorov-Smirnov test) or the presence ($p = 0.017$, Kolmogorov-Smirnov test) of CNQX (10 μM) and APV (25 μM) (Fig. 3.2.7C). In addition, we found that prenatal cocaine exposure increased the slope of the input-output curve for EPSCs ($p = 0.021$, Kolmogorov-Smirnov test) (Fig. 3.2.7D). These results showed that prenatal cocaine exposure weakens the synaptic strength of inhibitory transmission in the pathway where LTP was induced, supporting the hypothesis that the reduction of GABAergic inhibition underlies the facilitated LTP found in the mPFC of prenatal cocaine-exposed rats.

In addition to the measurements of IPSCs in the disynaptic responses, we also examined whether the effect of prenatal cocaine exposure is reflected by the maximal IPSC amplitude inducible in these pyramidal neurons. We recorded monosynaptic IPSCs (delay of onset < 3 ms) at -20 mV in the presence of CNQX (10 μM) and APV (25 μM) in response to extracellular stimulation at layer II/III, with stimulus intensity gradually increased to a level that elicited IPSCs with the maximal amplitude. We found that the mean maximal amplitude of IPSCs in slices from rats exposed to cocaine *inutero* was significantly smaller than that found for rats exposed to saline ("Sal", 0.96 ± 0.08 nA, $n = 20$; "Coc", 0.63 ± 0.04 nA, $n = 21$; $p < 0.05$, t test) (Fig. 3.2.7E). This finding on the reduction of monosynaptic response agrees with that found for disynaptic inhibition, indicating the reduction of GABAergic inhibition of layer V mPFC pyramidal neurons caused by prenatal cocaine exposure. Furthermore, we found that the paired-pulse depression of IPSCs (at an interpulse interval of 50 ms) was not significantly different between prenatal saline- and cocaine-treated rats ("Sal", 0.72 ± 0.02, $n = 40$; "Coc", 0.76 ± 0.03, $n = 49$; $p = 0.36$, t test) (Fig. 3.2.7F), suggesting postsynaptic rather than presynaptic changes as the cause of reduced inhibition.

3.2.5 Reduced surface expression of GABA$_A$ receptors

To directly assess potential postsynaptic changes associated with reduced GABAergic inhibition, we examined the expression of GABA$_A$ receptors in the mPFC tissue from postnatal rats at different stages. The amount of surface pool and the total protein level of various GABA$_A$ receptor subunits were determined by Western blotting in combination with the biotinylation method (see Materials and Methods). As shown in Figure 3.2.8A, we found that at P27 the surface expression of GABA$_A$ receptor α1, β2, and β3 subunits in prenatal cocaine-treated rats were significantly lower than that found in the prenatal saline-treated controls, whereas the total protein levels of these subunits were the same in both groups. Similar reduction was also observed for β1subunit (data not shown). In contrast to GABA$_A$ receptor subunits, the levels of the surface pool andthe total protein of the AMPA receptor subunit GluR1 were the same in both groups (Fig. 3.2.8A,B). Furthermore, when we examined the change in GABA$_A$ receptor subunit α1 expression at five different postnatal ages, we found significant reduction of the surface pool on P17, P27, P33 and P41, but not on P10 (Fig. 3.2.8B). Finally, we found that such reduction of surface GABA$_A$ receptor subunits was absent in the primary visual cortex (V1) of the same brain tissue used in the above assays for the mPFC at P10, P17 and P27 (Fig. 3.2.8C). Thus, cocaine
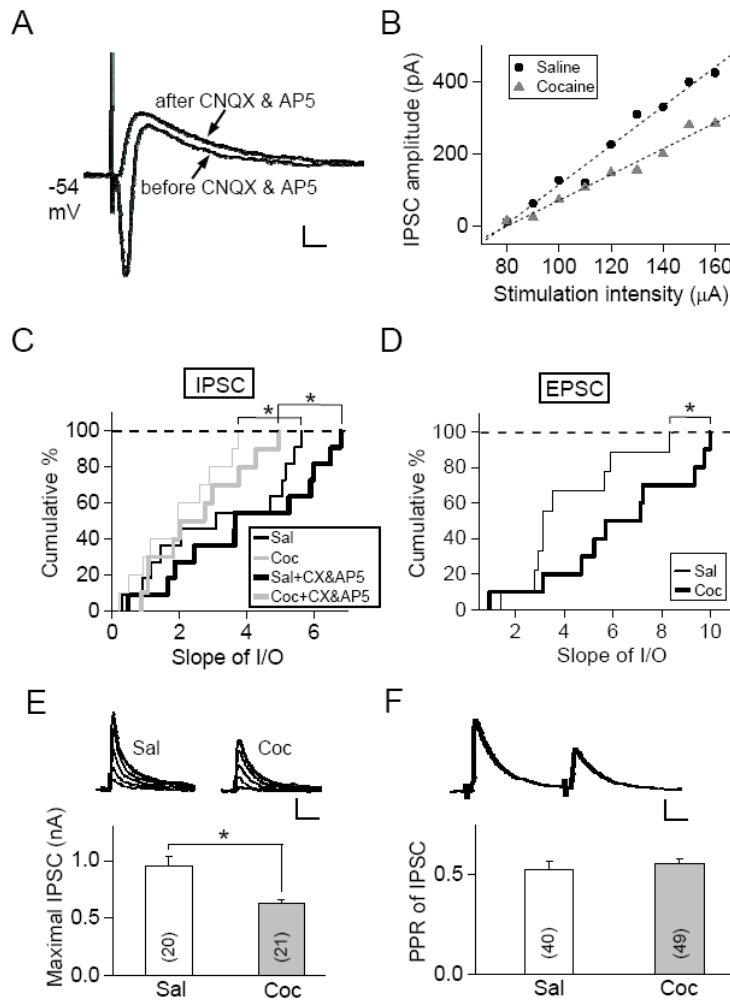
Figure 3.2.7 Prenatal cocaine exposure reduced GABAA receptor-mediated inhibition to mPFC layer V pyramidal neurons. *A*, Examples of synaptic currents before and after the application of CNQX (10 μM) and AP5 (25 μM) in response to extracellular stimulation in the mPFC slice from P24 prenatal cocaine-treated rat. Scale, 50 pA, 10 ms. *B*, Examples of input-output plot for IPSCs elicited by stimuli of different intensities, from P24 rats prenatally exposed to cocaine and saline, respectively. *C*, Cumulative percentage for the distribution of the slopes of best fit line for the input-out plots illustrated in (B), before and after the application of CNQX (10 μM) and AP5 (25 μM), from P23 – P26 rats prenatally exposed to saline (n = 11) and cocaine (n = 10) (*, $p = 0.017$; Kolmogorov-Smirnov test). *D*, Cumulative percentage for the distribution of the slopes of input-output plots for EPSCs elicited by stimuli of different intensities from the same group of rats as those in *C*. (*, $p = 0.021$; Kolmogorov-Smirnov test). *E*, The maximal amplitude of IPSCs evoked in the pyramidal neuron by extracellular stimulation was compared between rats (P24 - P25) exposed prenatally with either cocaine or saline (*, $p < 0.05$, t test). Number associated with the histogram refers to the total number of cells recorded. Scale, 200 pA, 50 ms. *F*, Comparison of paired-pulse ratio (PPR, IPSC amplitude of the second divided by the first). The IPSCs were evoked by two sequential presynaptic stimuli at an interval of 50 ms, with a sample of IPSC pair (average of 15) shown above. Scale, 100 pA, 10 ms. Data were obtained from P20 - P25 rats prenatally treated with cocaine or saline. ($p = 0.36$, t test).
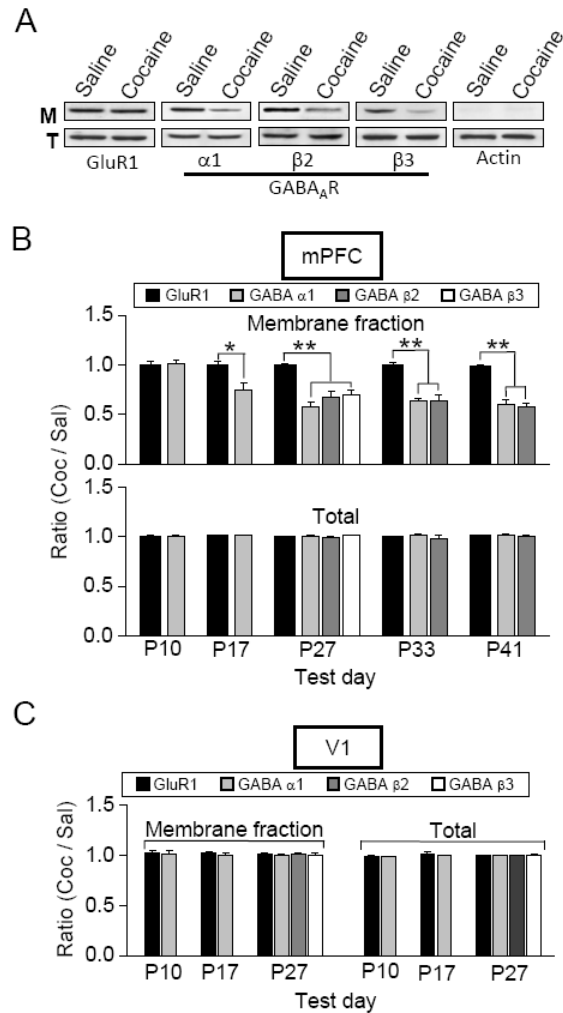
Figure 3.2.8 Prenatal cocaine exposure reduced the surface expression of GABA$_A$ receptor subunits in the mPFC. **A**, Example immunoblots of the lysates of mPFC tissues obtained from P27 rats that were treated prenatally with saline or cocaine, showing the level of biotinylated membrane-bound fraction (M) and of the total protein (T) for GluR1, GABA$_A$ receptor subunits α1, β2 and β3, and actin. **B**, Densitometric measurements of the band densities of immunoblots for membrane-bound and total levels of various receptor subunits in the mPFC. The data are presented as the ratio of densities measured for cocaine- vs. saline-treated rats in the same immunoblot (n = 3; *, $p < 0.05$; **, $p < 0.01$; t test). **C**, Same as (**B**) except the tested tissue is from V1 region.

exposure *in utero* had selectively reduced the surface expression of $GABA_A$ receptors in the mPFC during late postnatal period, leading to reduced GABAergic inhibition of layer V pyramidal neurons.

3.2.6 Enhanced excitatory transmission and neuronal excitability

To address whether prenatal cocaine exposure affects the baseline excitatory glutamatergic transmission, we compared the properties of miniature EPSCs (mEPSCs), as well as the paired-pulse ratio and the AMPA/NMDA ratio of EPSCs in layer V mPFC pyramidal neurons between prenatal saline- and cocaine-treated groups. The mean frequency of mEPSCs in the mPFC of prenatal cocaine-treated rats (recorded in the presence of 0.5 μM of tetrodotoxin) was higher than that in prenatal saline-treated rats ("Sal", 1.8 ± 0.7 Hz, $n = 12$; "Coc", 3.6 ± 0.7, $n = 12$; $p = 0.016$, t test), but the mean amplitude and decay time of mEPSCs did not show any significant difference (Fig. 3.2.9A; amplitude: "Sal", 10.7 ± 0.9 pA, $n = 12$; "Coc", 12.7 ± 1.6 pA, $n = 12$; $p = 0.056$, t test; decay time: "Sal", 5.3 ± 0.7 ms, $n = 12$; "Coc", 5.3 ± 0.9 ms, $n = 12$; $p = 0.73$, t test). Furthermore, for sequentially elicited EPSC pairs at an interval of 50 ms, the paired-pulse ratio of EPSCs from prenatal cocaine-treated rats was smaller than that in prenatal saline-treated rats, suggesting the increase of the probability of presynaptic glutamate release (Fig. 3.2.9B; "Sal", 1.2 ± 0.2, $n = 30$; "Coc", 0.9 ± 0.1, $n = 29$; $p = 0.034$, t test). The postsynaptic effect of prenatal cocaine exposure on glutamatergic synapses was also examined by measuring the AMPA/NMDA ratio of EPSCs in these mPFC pyramidal neurons. As shown in Figure 3.2.9C, prenatal cocaine exposure did not alter the AMPA/NMDA ratio ("Sal", 2.7 ± 0.5, $n = 15$; "Coc", 3.1 ± 0.7, $n = 14$; $p = 0.12$, t test).

The composition of NMDARs in forebrain is known to be developmentally regulated, with the ratio of NR2A to NR2B increased during early postnatal development (Yashiro and Philpot, 2008). To examine the possibility that prenatal cocaine exposure causes alteration of the postnatal development of NMDARs, we analyzed the decay kinetics of NMDAR-mediated EPSCs, which is known to reflect the composition of the NMDARs – with fast and slow decay for NMDARs containing NR2A and NR2B, respectively. By fitting the decay phase of NMDAR-mediated EPSCs with a double exponential function, we found no difference in the composition of fast and slow components of the decay phase between prenatal saline- and cocaine-treated rats (the percentage of slow component is 40.3 ± 6.2 % and 40.1 ± 5.4 % for prenatal saline- and cocaine-treated rats, respectively; $n = 14$ - 15; $p = 0.98$, t test). Therefore, the facilitation of LTP induction by prenatal cocaine exposure is unlikely to involve alteration in the subunit composition of NMDARs. Taken together, prenatal cocaine exposure appeared to have increased presynaptic release of excitatory synapses in the mPFC without changing postsynaptic properties.

Reduced inhibition and enhanced excitatory transmission of mPFC layer V pyramidal neurons may cause an elevated excitation of these neurons by excitatory synaptic inputs, known as EPSP-spike (E-S) coupling (Lu et al., 2000). Such elevated excitation may in turn facilitate the induction of LTP by mTBS. We have thus examined the E-S coupling by measuring the probability of spike initiation in response to EPSPs of different initial slopes (see Materials and Methods), as elicited by extracellular stimuli of different intensities. As shown by example recordings in Fig. 3.2.10A, the curve for E-S coupling was left shifted in a P25 rat that was
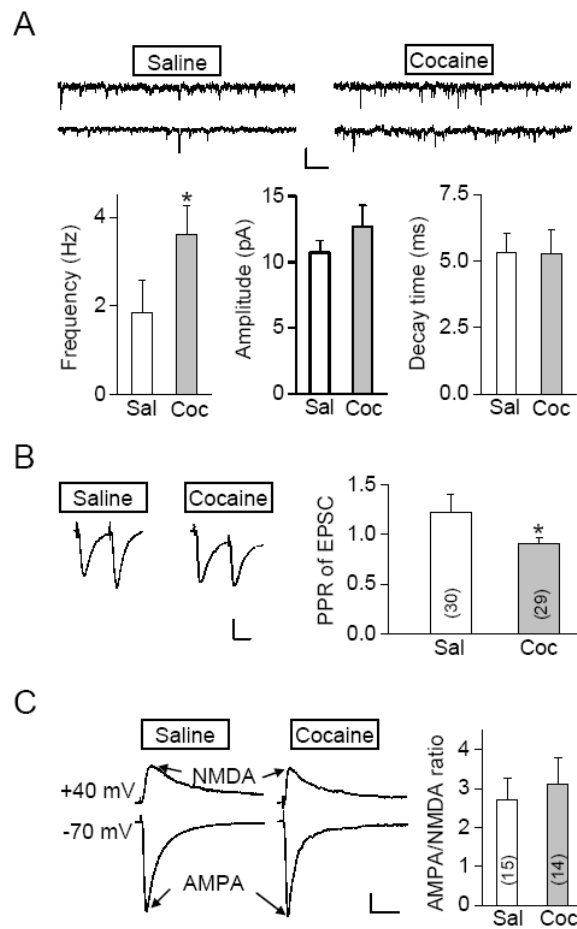
Figure 3.2.9 Prenatal cocaine exposure enhanced excitatory glutamatergic transmission of mPFC layer V pyramidal neurons. *A*, Comparison of the frequency, and the averaged amplitude and decay time of mEPSCs from P23 - P26 rats prenatally treated with cocaine or saline, with two sample recordings of mEPSCs for each group shown above (n = 12 for each group; *, $p = 0.016$, t test). Scale, 10 pA, 20 s. *B*, Left, Sample traces of paired EPSCs (average of 15) in response to two sequential presynaptic stimuli at an interval of 50 ms. Scale, 100 pA, 25 ms. Right, Summary of PPR (EPSC amplitude of the second divided by the first). Data were obtained from P23 - P26 rats prenatally treated with cocaine or saline. Number associated with the histogram refers to the total number of cells recorded. (*, $p = 0.034$, t test). *C*, Left, Sample traces of glutamatergic currents mediated by AMPA and NMDA receptors, recorded at -70 and +40 mV, respectively. Recordings were from P25 – P26 rats prenatally treated with saline or cocaine. Scale, 50 pA, 50 ms. Right, Average AMPA/NMDA ratio obtained by dividing the peak amplitude of AMPA and NMDA receptor-mediated EPSCs. Data were obtained from P24 - P26 rats. Number associated with the histogram refers to the total number of cells recorded.
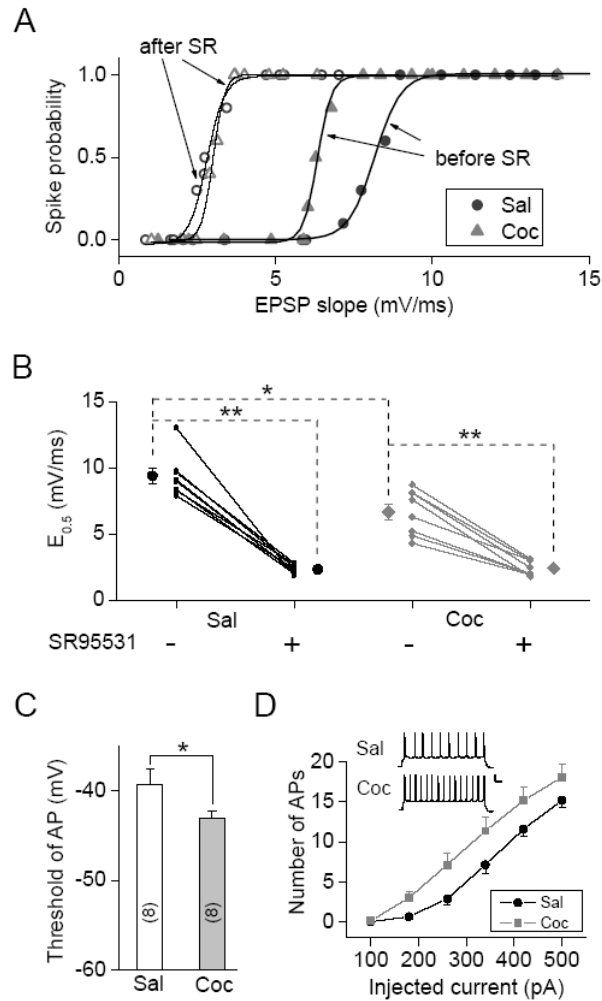
Figure 3.2.10 Prenatal cocaine exposure increased the EPSP-spike (E-S) coupling and excitability of mPFC layer V pyramidal neurons. *A*, E-S coupling studies on rats prenatally exposed to saline (Sal) or cocaine (Coc) in the absence ("before") and presence ("after") of SR95531 ("SR", 0.5 μM). The curves represent the best-fit with the sigmoidal function. *B*, The values of E50 before and after bath-application of SR95531 (0.5 μM). $E_{0.5}$ represents the efficacy of E-S coupling, as defined by the EPSP slope that initiates spiking with a probability of 0.5. Data obtained from the same neuron were connected by the line. Recording were performed in the slices obtained from P24 - P27 rats. (*, $p < 0.05$; **, $p < 0.01$, *t* test). *C*, Summary of the threshold potential for action potential (AP) initiation. The threshold was determined by the membrane potential at the peak of the maximal subthreshold EPSP. (*, $p < 0.05$; *t* test, n = 9, the same data set as in *B*). *D*, Changes of the excitability of mPFC layer V pyramidal neurons resulting from prenatal cocaine exposure, as shown by the number of APs triggered by injection of depolarizing currents of various amplitudes (duration 800 ms). Data were obtained from the recording on the slices obtained from P19 - P27 rats. Insert, examples of APs elicited by a constant depolarizing current (500 pA, 800 ms) in mPFC neurons from prenatal saline- and cocaine-treated P25 rats. (n = 17-24). Scales, 20 mV, 80 ms. Error bars = s.e.m.

prenatally exposed to cocaine, ascompared to that of a prenatal saline-exposed P25 rat. Fitting the data with a sigmoid function yielded the value of the EPSP slope ($E_{0.5}$) that corresponded to the spiking probability of 0.5 (Fig. 3.2.10B). The average value of $E_{0.5}$ was significantly different between prenatal cocaine- and saline-treated rats. Moreover, the enhanced E-S coupling caused by cocaine exposure *in utero* is consistent with a reduced GABAergic inhibition on these pyramidal cells, since blocking GABAergic inhibition with SR95531 (0.5 μM) resulted in a further leftward shift of the E-S coupling curve to the same level for both groups of rats (Fig. 3.2.10A,B). Finally, the elevated excitability of mPFC pyramidal neurons is also reflected by the lower threshold of membrane potential for initiating action potentials (APs) in rats treated with cocaine *in utero* than those treated with saline (Fig. 3.2.10C), and by the increased number of APs initiated by depolarizing current pulses injected into these neurons (Fig. 3.2.10D). Thus prenatal exposure to cocaine had modified the excitability of mPFC layer V pyramidal neurons, an effect that could be largely attributed to the reduction of GABAergic inhibition (see Discussion).

3.2.7 Decreased locomotor sensitivity to cocaine and apomorphine

Layer V mPFC pyramidal neurons provide the main excitatory inputs to VTA dopaminergic neurons (Gabbott et al., 2005), which in turn send dopaminergic projections to the mPFC (see reviews by (Steketee, 2003; Steketee, 2005). Thus higher excitability of these mPFC pyramidal neurons leads to increased excitation of VTA dopaminergic neurons and more dopamine release. This is supported by the finding that the basal dopamine level in mesocorticolimibic area is higher in rat pups exposed to cocaine *in utero* than prenatal saline-treated control group (Keller and Snyder-Keller, 2000). Given higher basal dopamine level, the prenatal cocaine-exposed rats may become less sensitive to the cocaine challenge when tested postnatally. To examine this idea, we performed locomotion test using P22 rats. The locomotor activity prior to the *i.p.* cocaine injection showed no difference between prenatal saline- and cocaine-treated groups during the first 10-min session (Fig. 3.2.11A, $p = 0.49$, *t* test). However, as shown in Figure 3.2.11B, cocaine-induced increase in locomotor activity was higher in prenatal saline-treated group and lasted for a longer period than that found in the prenatal cocaine-treated group. This result is consistent with that obtained previously by using a mouse model of prenatal cocaine exposure (Crozatier et al., 2003). To further test the hypothesis that the reduced locomotor sensitivity to cocaine in prenatal cocaine-treated rats is related to dopamine system, we examine the effect of apomorphine, a dopamine receptor agonist, on the locomotor activity. Similar to that found for cocaine challenge, prenatal cocaine-treated rats were less sensitive to the apomorphine challenge than prenatal saline-treated rats (Fig. 3.2.11C), supporting the hypothesis that the reduced locomotor sensitivity to cocaine is due to higher basal dopamine level caused by prenatal cocaine exposure.
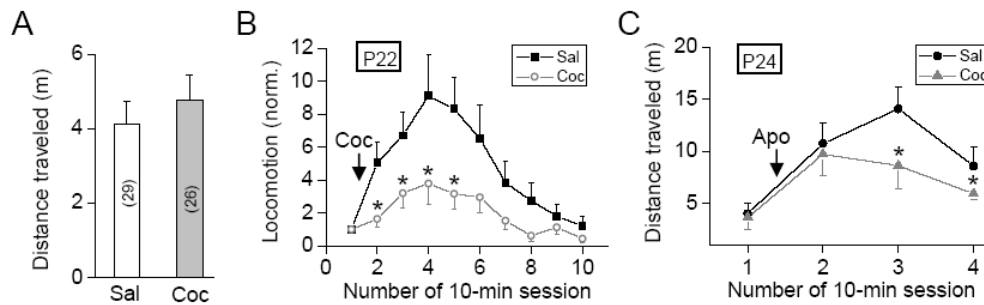
Figure 3.2.11 Prenatal cocaine exposure decreased the sensitivity of the locomotor activity to cocaine and apomorphine in postnatal rats. *A*, Average horizontal distances traveled by P22 - P24 rats spontaneously were measured for 10 min. Number associated with the histogram refers to the total number of rats tested. *B*, Normalized horizontal distances traveled by the rat were measured during ten 10-min sessions at P22. The horizontal distance traveled of each session was normalized to that of the first session. Cocaine (Coc) challenge was given after the first session. (n = 6-16) *C*, Average horizontal distances traveled by the P24 rat were measured during four 10-min sessions. Apomorphine (Apo) challenge was given after the first session. (n = 11 -13). Significant difference between "Sal" and "Coc" of the same session is marked by "*" ($p < 0.05$, t test).

Chapter 4 Discussion and Conclusion

In the first part of my study, I have examined the alteration of synaptic plasticity in the mPFC during the period of cocaine withdrawal and explored the underlying cellular mechanisms. I showed that the susceptibility of excitatory synapses of layer V pyramidal neurons to LTP induction was increased significantly by 5 d but not 1-4 d after cocaine withdrawal. This delayed facilitation of 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$_A$ receptor and required the activation of TrkB in these pyramidal neurons. Consistent with previous studies *in vitro*, we showed that BDNF elevates the activity of PP2A in the mPFC, leading to an increased de-phosphorylation of GABA$_A$ receptor β3 subunit, which is known to cause internalization of GABA$_A$ receptors (Jovanovic et al., 2004).

In the second part of my study, I tested the neurophysiological consequence of cocaine exposure of rat *in utero* and obtained evidence for altered synaptic plasticity in the mPFC of the offspring during the postnatal period. I showed that 7-d repeated prenatal cocaine exposure facilitated activity-induced LTP at excitatory synapses on layer V pyramidal neurons in the mPFC of the rat offspring during the postnatal period P16 - P42, whereas no LTP could be induced at these synapses in prenatal saline-treated control rats of the same age unless GABAergic inhibition was reduced. Further studies showed that this facilitated LTP in prenatal cocaine-exposed rat could be attributed to postnatal reduction of IPSCs and the surface expression of GABA$_A$ receptor subunits in the mPFC.

Together, the excitatory inputs to mPFC layer V pyramidal neurons are sensitized for activity-induced persistent synaptic potentiation due to the reduction of GABAergic inhibition in the mPFC after withdrawal from repeated either pre- or postnatal cocaine exposure. These studies provided some of the underlying cellular mechanisms which would increase our understanding of the neurobiological basis of cocaine addiction and help to develop more effective pharmacological therapy for treating cocaine addiction and preventing brain dysfunctions of the fetus caused by drug exposure during pregnancy.

4.1 Elevation of BDNF expression during cocaine withdrawal

It is known that during cocaine withdrawal, there is an increase in the sensitization 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 withdrawal period, significant neuroadaptation associated with behavioral sensitization (Pierce and Kalivas, 1997) occurs in the reward circuit, at the molecular, cellular, and morphological changes (Hyman and Malenka, 2001; Hyman et al., 2006; Kauer and Malenka, 2007; Nestler and Aghajanian, 1997; White and Kalivas, 1998). Along with the increased cocaine craving after withdrawal, BDNF levels 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 during the withdrawal period may mediate circuit modification underlying drug

craving and seeking behaviors. In the present study, we found a *delayed* BDNF up-regulation in the mPFC at 8 or 14 d, but not at 1 and 3 d after withdrawal from repeated cocaine treatment. In the mouse dorsomedial PFC, a similar extent (20-30%) of BDNF elevation was also found at 21 d after the termination of cocaine self-administration (McGinty et al., 2009). Given that BDNF could be transported from the PFC to the NAc through the cortical glutamatergic projections (Altar et al., 1997), a pathway that regulates relapse to drug-seeking (Fuchs et al., 2004; McFarland et al., 2003), BDNF up-regulation may constitute a critical component of cocaine-induced neuroadaptive response in cortical neurons.

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 highly expression of BDNF has been found following repeated cocaine exposure. Further studies on the time course of BDNF expression in various brain regions, the existence and properties of anterograde/retrograde transport of BDNF in the reward circuits, as well as the activity-dependent uptake and secretion of BDNF in various brain regions are required to fully understand the action of BDNF during the period of cocaine withdrawal.

4.2 BDNF-induced changes in synaptic plasticity during cocaine withdrawal

During the period of withdrawal from repeated cocaine exposure to rats, the excitatory synapses onto dopamine neurons in the VTA are highly sensitive to LTP induction by weak presynaptic stimuli, when the measurements were done in midbrain slices in the presence of picrotoxin that completely abolished GABA inhibition (Pu et al., 2006). This BDNF-dependent facilitation of LTP in the VTA was mediated by a postsynaptic NMDA receptor- and TrkB-dependent enhancement of presynaptic glutamate release. We showed here that, although there was presynaptic alteration of glutamate release machinery (see Fig. 3.1.11), 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 $GABA_A$ receptor antagonist SR95531 (Fig. 3.1.7A,C) and the application of BDNF (Fig. 3.1.3B,C) both facilitated LTP induction to a similar level in slices from control saline-withdrawn rats and this level is similar to that found in the cocaine-withdrawn rats (Fig. 3.1.3D, 3.1.7D). Second, SR95531 treatment of cocaine-withdrawn rats produced no additional LTP facilitation. This occlusion of LTP facilitation suggests that cocaine withdrawal shares the same mechanism as GABAergic suppression, an idea further supported by the finding that diazepam-induced enhancement of GABA inhibition completely abolished the LTP facilitation in cocaine-withdrawn rats (Fig. 3.1.7D). 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 (Fig. 3.1.6A). Fourth, the surface expression of the $\alpha 1$ subunit of the $GABA_A$ receptor was reduced during the cocaine withdrawal (Fig. 3.1.8). Fifth, both the facilitation of LTP induction and the reduction of $GABA_A$ receptor- mediated IPSC in cocaine-withdrawn rats were not found in mPFC neurons in which TrkB was down-regulated (Fig. 3.1.5D and Fig. 3.1.6B). Finally, the time courses for LTP

facilitation (Fig 1E), BDNF elevation (Fig. 3.1.3A), and the reduction of IPSCs (Fig. 3.1.6A) and surface pool of GABA$_A$ receptors (Fig. 3.1.8B) were all similar during the period of cocaine withdrawal. We also noted that exogenous BDNF application failed to suppress IPSCs in the mPFC of cocaine-withdrawn rats after 7 d of withdrawal from cocaine (Fig. 3.1.6C), 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$_A$ receptor-mediated inhibition, but this LTP facilitation was not mediated by elevated BDNF. This is because the reduction of GABA$_A$ receptor-mediated inhibition and the facilitation of LTP induction occurs right after the cocaine withdrawal, prior to BDNF elevation (Liu et al., 2005; Pu et al., 2006). Thus, synapses on VTA dopaminergic neurons and mPFC pyramidal neurons are differentially modulated during repeated cocaine exposure and after cocaine withdrawal, through mechanisms that remain to be further clarified.

Previous findings have shown that 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., 2004; 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 during cocaine withdrawal. First of all, down-regulation of TrkB abolished the suppression of IPSCs and prevented LTP facilitation during cocaine withdrawal (Fig. 3.1.6B). Secondly, blocking the activity of PP2A, a downstream target of BDNF-TrkB signaling, impaired the suppressing effect of BDNF on IPSCs (Fig. 3.1.9E). Finally, phosphatase assay revealed that the PP2A activity was increased at 8 and 14 d but not 1 and 3 d after cocaine withdrawal, a time course similar to that BDNF elevation. The action of PP2A on GABA$_A$ receptor β3 subunit de-phosphorylation, which promotes internalization of these receptors (Jovanovic et al., 2004), was shown to occur 1 week after cocaine withdrawal (Fig. 3.1.9C). All these findings are consistent with previous studies on cultured cortical neurons (Jovanovic et al., 2004).

4.3 The role of mPFC in relapse

The relapse triggered by stress, conditioned stimuli and drugs is known to be mediated by activities in PFC. Phamacological 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 PFC prevented this increase (Baker et al., 2003; McFarland et al., 2003). The layer V pyramidal neurons of mPFC represent the main resources of excitatory inputs to the VTA and NAc. Facilitation of LTP of excitatory inputs (from other brain areas) to these neurons leads their higher outputs in the VAT and NAc, promoting drug reinstatement. Thus, BDNF-induced facilitation of LTP may play a key role in triggering relapse during the period of drug withdrawal.

4.4 Molecular and cellular changes caused by *in utero* cocaine exposure

Cocaine exposure *in utero* results in molecular and cellular changes in several brain areas known to be involved in memory and attention, such as caudate nucleus (Harvey et al., 2001), prefrontal cortex (Morrow et al., 2002; Morrow et al., 2007) and hippocampus (Harvey et al., 2001; Little and Teyler, 1996; Morrow et al., 2002; Morrow et al., 2007). For example, cocaine exposure *in utero* results in anatomical changes in the prefrontal cortex of postnatal rats, including a loss of inhibitory projections from parvalbumin-containing GABAergic local circuit neurons to prelimbic pyramidal neurons (Morrow et al., 2005), deficient inhibitory axo-axonic synapses on pyramidal cells (Morrow et al., 2003), and an increased number of spine synapses (Morrow et al., 2007). These structural changes could result in stronger excitation of pyramidal neurons of the prefrontal cortex in response to a given stimulus.

Our study provided strong evidence for another cellular mechanism that could contribute to an elevated excitability of mPFC pyramidal neurons – the reduced GABAergic inhibition. We found a significant reduction in both the amplitude of IPSCs recorded in these pyramidal neurons (Fig. 3.2.7E) and the surface expression of GABA$_A$ receptor subunits in mPFC tissue of prenatal cocaine-treated rats (Fig. 3.2.8A,B). This reduced GABAergic inhibition could elevate the excitability of mPFC pyramidal neurons, leading to disruption of physiological cortical rhythms that depend on balanced inhibition and excitation (Shu et al., 2003). In slices from prenatal cocaine-treated rats, an elevated excitation was shown by the higher probability of firing of pyramidal cells in response to membrane depolarizations (Fig. 3.2.10), an effect that could largely be attributed to a reduced GABAergic inhibition and could enhance LTP of excitatory synapses.

We have also obtained evidence that prenatal cocaine exposure results in elevated presynaptic release probability at excitatory synapses, as indicated by the increased frequency of mEPSCs and reduced paired-pulse ratio of EPSCs. However, the facilitated LTP appeared to be caused mainly by a reduced GABAergic inhibition rather than enhanced glutamate release. This is based on the following observations: First, blocking GABAergic inhibition with the specific GABA$_A$ receptor antagonist SR95531 facilitated LTP induction in slices from prenatal saline-treated control rats (Fig. 3.2.5A,B). Second, SR95531 treatments increased the magnitude of LTP of the saline-treated group to the same level as that found for prenatal cocaine-treated rats, and it has no effect on the LTP in the latter group (Fig. 3.2.5C,D), indicating that prenatal cocaine exposure had occluded the effect of SR95531 in facilitating LTP. Similarly, SR95531-induced enhancement of EPSP-spike coupling completely occluded the enhanced EPSP-spike coupling found in prenatal cocaine-exposed rats (Fig. 3.2.10A). Third, direct measurements of the maximal amplitude of IPSCs in these neurons evoked by extracellular stimulation showed a ~40% reduction of the total inhibitory input, as compared to that found for control rats (Fig. 3.2.7E). Fourth, surface biotinylation and Western blotting analysis of mPFC tissues of prenatal saline- or cocaine-treated rats showed that the surface expression of GABA$_A$ receptor subunits were significantly reduced in the prenatal cocaine-treated group, with a time course in parallel with that of the LTP facilitation (Fig. 3.2.8B). Finally, the effect on LTP was found after P16, when the transition of the GABA action from excitation to inhibition has been completed (Cancedda et al., 2007; Ganguly et al., 2001; Owens et al., 1996) (Fig. 3.2.4E). Interestingly, we found that the augmented LTP and the reduced surface expression of GABA$_A$ receptor persisted

in young rats during P16 - P42, suggesting that prenatal cocaine exposure has a long-lasting effect on synaptic plasticity in the mPFC of postnatal rats. This is consistent with previous animal studies showing long-term effects of prenatal cocaine exposure on brain structure and behaviors (see review (Chae and Covington, 2009). We note that short-term plasticity (within 10 min after LTP induction) was different between prenatal saline- and cocaine-treated rats (Fig. 3.2.5C). This may result from the effect of prenatal cocaine exposure on presynaptic glutamate release mechanisms, an aspect not addressed in this study. For assaying the magnitude of LTP, we focused on the mean EPSP amplitude at 20-30 min after LTP induction, after short-term plasticity had subsided. In the postnatal (P30 - P40) rabbit hippocampus, prenatal cocaine exposure has been shown to facilitate LTP induction (Little and Teyler, 1996), although whether GABAergic inhibition is altered has not been examined.

Interestingly, the reduction of expression of $GABA_A$ receptor $\alpha 1$ subunit caused by prenatal cocaine exposure was observed after P17, but not at P10. There is a progressive increase in the expression of $\alpha 1$ subunit during postnatal development (Fritschy et al., 1994). The change in the expression caused by cocaine exposure *in utero* may not be detectable at P10 when the normal expression level of $\alpha 1$ subunit is low. Alternatively, the effect of prenatal cocaine exposure takes time to accumulate. The latter idea is supported by the observation that similar reduction in the surface expression of $\alpha 1$ subunit was observed in the mPFC at 1 week (but not on 1 and 3 d) after withdrawal from daily cocaine treatment for 7 d in juvenile rats (Fig. 3.1.8).

Although cocaine exposure in juvenile rats is known to increase mPFC neuronal excitability by modifying the properties of Ca^{2+} and K^+ channels (Dong et al., 2005; Nasif et al., 2005a; Nasif et al., 2005b), there is yet no evidence for such changes in rats exposed to cocaine *in utero*. Based on our results, the elevation of the excitability of the mPFC pyramidal neurons by cocaine exposure *in utero* may be related to both enhanced baseline excitatory transmission and reduced GABAergic inhibition. However, the latter represents the predominant cause since SR95531 caused a leftward shift of the E-S curve to the same level for both prenatal cocaine- and saline-treated rats (Fig. 3.2.10).

4.5 Decreased locomotor sensitivity to cocaine

Microdialysis analysis (Keller and Snyder-Keller, 2000) showed that prenatal cocaine treatment caused an elevation of the basal dopamine level in the NAc (of postnatal animals), which receives dopaminergic projections from the VTA. Our finding of the elevated excitability of mPFC layer V pyramidal neurons, which provide the main excitatory inputs to dopaminergic neurons in the VTA (Gabbott et al., 2005), suggests a potential mechanism underlying the higher dopamine release by VTA in these prenatal cocaine-exposed animals. Furthermore, elevated basal dopamine level in these animals may cause desensitization of the reward circuit to postnatal cocaine challenge, since the extent of the dopamine increase in NAc due to postnatal cocaine challenge, when normalized by the basal level, is lower in prenatal cocaine-treated rats than in saline-treated rats (Keller and Snyder-Keller, 2000). As the NAc is the primary brain region responsible for the expression of cocaine sensitization (Robinson and Berridge, 2003; Steketee, 2003), the reduced extent of cocaine-induced dopamine elevation in the NAc of the animals exposed to cocaine *in utero* may account for their weaker locomotor sensitivity to cocaine (Fig. 3.2.11) (Crozatier et al., 2003). Our finding that the dopamine receptor agonist

apomorphine exerted similar effect on the locomotor activity as cocaine further supports the involvement of dopamine system.

Cocaine exposure *in utero* may also alter the sensitivity of the reward system to cocaine through long-term alteration of dopamine receptors. For examples, prenatal cocaine exposure results in a sustained impairment in the coupling between D_1 dopamine receptors and G -protein in cortical neurons (Friedman and Wang, 1998; Friedman et al., 1996; Jones et al., 2000; Stanwood and Levitt, 2007; Wang et al., 1995; Zhen et al., 2001), leading to the reduced D_1 receptor functions. Such reduction of D1 receptor functions may also contribute to the reduced locomotor sensitivity to postnatal cocaine challenge.

In conclusion, my results offer new insights into cellular changes caused by repeated cocaine exposure during pregnancy. The finding of the reduced inhibitory input and resultant elevated excitability of mPFC pyramidal neurons may explain the deficits in attention shown by prenatal cocaine-exposed human (Richardson et al., 1996), rats (Garavan et al., 2000) and rabbits (Romano and Harvey, 1996), and support the hypothesis that disruption of excitation/inhibition balance in the prefrontal cortex results in ADHD (Barkley, 1997; Barkley et al., 1992; Casey et al., 1997; Mattes, 1980). Further study in identifying the mechanisms underlying these changes may help the development of effective approaches for preventing brain dysfunctions of the fetus caused by drug exposure during pregnancy.

Thus, an important prediction of my studies is that the elevated expression of BDNF in the mPFC after cocaine withdrawal may result in an increased secretion of endogenous BDNF in the other targeting regions, such as VTA and NAc, which in turn sensitizes the synapses to potentiation by cue-associated excitatory inputs to VTA dopamine neurons, contributing to compulsive drug seeking and relapse. It is highly possible that prenatal cocaine exposure may be through similar mechanism to sensitize the excitatory synapses in the mPFC as well. To further determine the causal linkage between BDNF and aspects of enduring synaptic potentiation, such as AMPAR subunit distribution, actin cycling or spine morphology will not only increase our understanding of the neurobiological basis of cocaine addiction, but also help with establishing more thorough assays for assessing the neurological impact and validity of emerging pharmacological treatments for cocaine addiction.

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