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Glutamatergic Neurotransmission in the Prefrontal Cortex Mediates the Suppressive Effect of Intra-PFC Infusion of BDNF on Cocaine-Seeking

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Suppressive Effect of Intra-PFC Infusion of BDNF on Cocaine-Seeking

by

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DEDICATION

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I dedicate my dissertation work to my family for their endless love, emotional support, and encouragement. I give special thanks to my loving parents, Sang Woo Go and Mi Ja Youn, and my brother Young Bae Go, sister in-law Seong Hye Choe, niece Joon Seo Go, and nephew Dong Whee Go for being there for me throughout the entire doctorate program and my life.

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ABSTRACT

BOK SOON GO. Glutamatergic Neurotransmission in the Prefrontal Cortex Mediates the Suppressive Effect of Intra-PFC Infusion of BDNF on Cocaine-Seeking (Under the direction of JACQUELINE F. MCGINTY)

Infusion of BDNF into the dorsomedial prefrontal cortex (dmPFC) immediately after the last session of cocaine self-administration prevents cocaine-mediated malfunction of synaptic modulation in the dmPFC that mediates relapse to cocaine-seeking through both GluN2A subunit-containing NMDA receptors and GluN2B subunit-containing NMDA receptors (NMDARS). Intra-dmPFC BDNF is known to suppress cocaine-seeking by normalization of cocaine self-administration-induced disturbance of glutamatergic transmission in the nucleus accumbens (NAc) and by prevention of the cocaine self-administration-induced decrease in extracellular signal-regulated kinase (ERK) activity in the dmPFC through activation of tyrosine receptor kinase B (TrkB). Neuronal activity is required for BDNF-induced ERK activation and the interaction between TrkB receptors and synaptic NMDA receptors upregulates ERK activity. The current study found that infusion of a mixture of the AMPA receptor antagonist, CNQX, and the NMDA receptor antagonist, LY235959, (CNQX/LY235959), the GluN2A-containing NMDA receptor antagonist, TCN-201, or the GluN2B-containing NMDA receptor antagonist, Ro-25-6981, into the dmPFC of rats immediately after the final session of cocaine self-administration inhibited BDNF's

suppressive effect on cocaine-seeking during a context-induced relapse test after home-cage abstinence and during a conditioned cue-induced reinstatement test. We also found that during early withdrawal from cocaine self-administration, intra-dmPFC BDNF infusion prevented cocaine self-administration-induced reduction of phosphorylated ERK, GluN2A (Y1325) and GluN2B (Y1472) levels in the dmPFC. TCN-201 infusion into the dmPFC blocked the BDNF-mediated increase in pGluN2A (Y1325) and Ro-25-6981 infusion into the dmPFC suppressed the BDNF-induced elevation of pGluN2B (Y1472) expression and both GluN2 antagonists blocked BDNF-mediated ERK phosphorylation, indicating that both GluN2A- and GluN2B-containing NMDA receptors mediate BDNF-mediated ERK phosphorylation. Together, these findings indicate that BDNF-mediated co-activation of GluN2A- or GluN2B-containing NMDA receptors induces ERK activation in the dmPFC, which prevents relapse to cocaine-seeking.

CHAPTER1

INTRODUCTION

Drug addiction is a chronic disease showing persistent drug-seeking and taking despite its harmful effects and currently there is no cure. Cocaine is one of the most prevalent illegal drugs causing addiction. Cocaine is a strong central nervous psychostimulant that induces a feeling of euphoria at initial stages by activating brain regions involved in pleasure and reinforcement. However, during withdrawal or abstinence, this rewarding effect of cocaine is replaced by a negative emotional state that includes dysphoria, anxiety and depression. To avoid the negative withdrawal syndrome and to recover the reinforcing effect, people continuously use increasing amounts of cocaine, which results in persistent neuroadaptations in the reward pathways of brain, leading to development of chronic relapse even after prolonged abstinence which is a crucial feature of cocaine addiction. Neuroscience researchers have investigated the neural circuitries that are responsible for cocaine addiction in order to develop effective treatment approaches. However, despite these advances, there is no cure approved for cocaine addiction. Therefore, research on the molecular mechanisms underlying cocaine-mediated neuroadaptaions will enhance the

potential molecular targets for the treatment of cocaine addiction, which would facilitate therapeutic development for cocaine addicts. Further, neurobiological research could expand the therapeutic possibilities available to addicted individuals depending on their phase or severity of addiction as well as provide more cost-effective and safe options for treatment.

REVIEW OF RELEVANT LITERATURE

Cocaine induces neural adaptations in the PFC-NAc glutamatergic pathway associated with cocaine-seeking

Drug addiction is a brain disease characterized by chronic relapse to drug-seeking and use, which is mediated by long-lasting alteration of neuroplasticity in reward circuits (White and Kalivas, 2001; Koob and Volkow, 2010; Weiss et al., 2001; Kalivas and Volkow, 2005). The mesocorticolimbic dopaminergic pathway which arises from the ventral tegmental area (VTA) and projects to prefrontal cortex (PFC) and the nucleus accumbens (NAc) is the circuitry associated with drug-mediated reward and motivation. Cocaine acts on this mesocorticolimbic system as an indirect dopamine agonist by increasing synaptic dopaminergic concentrations (van Huijstee and Mansvelder, 2015). Cocaine-induced increase in dopamine transmission in the dorsal medial (dm) PFC but not in the NAc core drives cocaine-seeking in rats with cocaine self-administration (SA) history. After extinction training, infusion of the D1/D2 antagonist fluphenazine into the dmPFC, but not into the NA core, suppresses systemic cocaine prime-induced cocaine-seeking in rats with a cocaine SA history, suggesting that the dopaminergic pathway from the VTA to the dmPFC but not from VTA to the NA core is involved

in cocaine-seeking behavior (McFarland and Kalivas, 2001). Glutamatergic rather than dopaminergic neurotransmission in the NAc core contributes to cocaine prime-induced reinstatement. Although dopamine infusion into the NAc core induces reinstatement of cocaine-seeking after extinction training, intra-NAc core infusion of the dopamine receptor antagonist, fluphenazine, inhibits the reinstatement induced by intra-NAc core dopamine only, but infusion of the AMPA/kainate receptor antagonist, CNQX, into the NAc core blocks the reinstatement of cocaine-seeking mediated by intra-NAc core dopamine, systemic injection of cocaine, and intra-NAc core AMPA, respectively (Cornish and Kalivas, 2000). Moreover, infusion of the AMPA/KA receptor antagonist, LY293558, into the NAc core also suppresses cue-induced cocaine-seeking (Di Ciano and Everitt, 2001). The NAc core is densely innervated by glutamatergic axons of pyramidal cells in the dmPFC and glutamatergic neurotransmission in the dmPFC to NAc core pathway underlies reinstatement of cocaine-seeking after extinction by cocaine-conditioned context, cue, or cocaine-prime (Fuchs et al., 2005; McLaughlin and See, 2003; Park et al., 2002; McFarland et al., 2003). Cocaine-seeking induced by systemic cocaine injection following cocaine SA selectively enhances extracellular levels of glutamate but not dopamine in the rat NAc core and this cocaine-seeking behavior was suppressed by blockade of neuronal activity in dmPFC, suggesting that the glutamatergic pathway from the dmPFC to NAC core underlies this cocaine-seeking (McFarland et al., 2003). Both brain derived neurotrophic factor (BDNF), an important modulator of

neurotransmission and neural plasticity, and its receptor tyrosine kinase B (TrkB) are highly expressed in the dmPFC-NAc core neurons (Altar et al., 1997).

BDNF modulates synaptic plasticity and neurotransmission

While proBDNF (immature form) preferentially binds to the p75 (NTR), mature BDNF preferentially acts on TrkB receptors, and mature BDNF has an ability to promote neurotransmission. In the adult rat hippocampus, TrkB is expressed at both the presynaptic and postsynaptic regions of synapses (Drake et al., 1999), and the presynaptic TrkB may underlie BDNF-mediated enhancement of neurotransmitter release. In postnatal cortical neurons, BDNF increases glutamate release via PLC gamma and ERK, both of which are downstream components of TrkB signaling (Matsumoto et al., 2006). In addition, BDNF promotes neuronal activity-induced glutamate release from nerve terminals of rat and mouse cerebral cortices via ERK-mediated phosphorylation of synapsin I (Jovanovic et al., 2000).

BDNF binding to TrkB activates TrkB's tyrosine kinase activity, triggering the phosphorylation and activation of downstream signaling cascades, including mitogen-activated extracellular signal-regulated kinase (ERK), phosphoinositol 3-kinase (PI3K), and phospholipase C gamma PLCγ1 (Huang and Reichardt, 2003; Reichardt, 2006), leading to gene transcription via activation of cyclic AMP response element binding protein (CREB) (Finkbeiner et al., 1997). BDNF-mediated CREB activation may contribute to long-term neural plasticity through new protein expression required for the maintenance of synaptic plasticity during

extended periods. BDNF induces both the early and late phases of LTP in the hippocampus. BDNF/TrkB signaling induces the early phase of LTP by releasing existing presynaptic BDNF contents and modulation of existing proteins located at presynaptic and postsynaptic regions, but the late phases of LTP require BDNF-mediated new protein synthesis (Waterhouse and Xu, 2009). Therefore, dysregulation of BDNF/TrkB signaling and subsequent CREB activity may lead to long-lasting neural adaptations associated with persistent behavioral changes.

BDNF/TrkB signaling in the dmPFC and NAc core is associated with cocaine-seeking

Cocaine-mediated long-lasting dysregulation of synaptic function and structures in the reward circuit underlie the chronic relapse to cocaine-seeking. A previous study from our laboratory found that cocaine SA induces dephosphorylation and inactivation of ERK and CREB during early withdrawal in the rat dmPFC, and this cocaine-mediated dephosphorylation was inhibited by intra-dmPFC BDNF when infused immediately after the last session of cocaine SA via Trk receptor-ERK signaling in the dmPFC (Whitfield et al., 2011). In addition, cocaine SA induces reduction of BDNF mRNA 22 h after the last session of cocaine SA (McGinty et al., 2010). Cocaine-induced prefrontal hypoactivity during withdrawal may be linked to the cocaine-mediated reduction of BDNF/TrkB signaling in the dmPFC. In rats with a cocaine SA history, the basal activity of the dmPFC is suppressed (Sun and Rebec, 2006) and human cocaine addicts show suppressed basal levels of activity in the PFC (Bolla et al., 2004; Volkow et al., 1992; Volkow and Fowler, 2000), Neuronal activity upregulates BDNF expression in the cerebral

cortex and TrkB surface expression in cultured hippocampal neurons (Poo, 2001). Together, this supports the assumption that a cocaine-mediated decrease in BDNF/TrkB signaling in the dmPFC is due to hypofrontality. Berglind and colleagues (2009) previously showed that BDNF infusion into the dmPFC immediately after the last cocaine SA session normalized a cocaine-mediated disturbance in glutamatergic transmission in the NAc core and this BDNF infusion also decreased reinstatement of cocaine-seeking triggered by conditioned stimuli (CS), context, or cocaine (Berglind et al., 2007; Whitfield et al., 2011). Inhibition of BDNF-TrkB signaling in the NAc core before cocaine SA increases cue-induced cocaine seeking after 1d withdrawal from cocaine SA, suggesting basal levels of BDNF in the NAc core contributes to the suppression of cocaine seeking during early withdrawal (Li et al., 2013). Together, cocaine-mediated alterations of BDNF/TrkB signaling in the dmPFC and NAc core may underlie enduring relapse to cocaine-seeking.

NMDA receptors mediates long-term neuroplasticity and has a functional role in cocaine-seeking

The postsynaptic calcium influx through NMDA receptors plays an essential role in long-lasting synaptic plasticity like long-term potentiation (LTP) and long-term depression (LTD) (Kauer and Malenka, 2007; Lau and Zukin 2007). NMDARs are tetrameric assemblies of two obligatory GluN1 subunits and two modulatory GluN2 (A-D) or GluN3 (A-B) subunits and both GluN2A and GluN2B subunits show higher expression in the cerebral cortex compared to other modulatory subunits (Lau and Zukin 2007; Yashiro and Philpot, 2008). Both GluN2A and

GluN2B subunits of NMDARs are involved in the induction of LTP and LTD (Yashiro and Philpot, 2008; Sakimura et al., 1995; Miwa et al., 2008; Brigman et al., 2000). In the rat PFC, NMDAR stimulation induces long-term potentiation (LTP) and long-term depression (LTD) *in vitro* (Hirsch and Crepel, 1990). In addition, NMDAR induces LTP in the rat hippocampal-prefrontal cortex pathway in vivo (Jay et al., 1995). Dopamine (DA) also modulates rat PFC long-term plasticity, LTP and LTD (Otani et al., 2003). D1 but not D2 receptors in the rat PFC mediate LTP induction at hippocampal-prefrontal synapses (Gurden et al., 2000). Together, as cocaine acts as an indirect dopamine agonist in the dmPFC, these results suggest that cocaine may induce long-term synaptic plasticity in the dmPFC via DA receptors and/or through crosstalk between DA receptors and NMDARS. This dysregulation of neural plasticity in the dmPFC may also affect the synaptic plasticity in the NAc core through modulation of the dmPFC-NAc pathway. During withdrawal from cocaine SA, the ability to mediate LTP and LTD is compromised in the rat NAc core (Moussawi et al., 2009), which supports this idea. As cocaine addiction is characterized by its inability to adapt behavior in response to new stimuli associated with the persistent cocaine-seeking after extended periods of abstinence, this malfunctioning neural plasticity of the essential brain regions of dmPFC and NAc core in cocaine addiction may underlie cocaine-mediated long-lasting neuroadaptations linked to enduring relapse to cocaine-seeking.

There is also ample evidence indicating the role of NMDAR in the regulation of cocaine-seeking behaviors using NMDAR agonist/antagonists. A

partial NMDA receptor agonist, D-cycloserine (DCS), has been found to facilitate the extinction of conditioned place preference (CPP) of cocaine in rats (Botreau et al., 2006, Yang et al., 2013). In rats with a cocaine SA history, DCS also facilitates the extinction of cocaine-seeking and reduces reacquisition of cocaine SA (Thanos et al., 2011; Nic Dhonnchadha et al., 2010). In parallel with this, while an NMDA receptor antagonist CPP decreases the suppressive effect of extinction training on cocaine-primed reinstatement of cocaine seeking (Kelamangalath et al., 2007), d-serine, the NMDAR co-agonist, suppresses cocaine-primed reinstatement following extinction in rats with short and long access coc-SA history, respectively (Kelamangalath et al., 2009; Kelamangalath and Wagner, 2010). Together, as both BDNF and NMDARs are involved in synaptic plasticity as well as cocaine-seeking, finding a plausible relationship between TrkB and NMDARs in reward-related brain regions would expand our understanding of the mechanism underlying the roles of BDNF/TrkB signaling and NMDARs in cocaine addiction.

Interaction between BDNF/TrkB signaling and NMDA receptor function and its effect on ERK activity

Both BDNF and TrkB are highly expressed in the PFC and striatum (McGinty et al., 2010). In the adult rat cerebral cortex, TrkB and BDNF are localized discretely in postsynaptic densities (PSD) of excitatory glutamatergic synapses (Aoki et al., 2000) and BDNF can be anterogradely transported to excitatory axon terminals and released in subcortical targets like the NAc core (Altar et al., 1997) in an activity-dependent manner (Balkowiec and Katz, 2002, McGinty et al.,

2010). In cortical cultures, glutamatergic neuronal activity is involved in the maintenance of BDNF-mediated ERK activity and for BDNF-induced potentiation of glutamate transmission (Matsumoto et al., 2006). Both TrkB and synaptic NMDARs are involved in the upregulation of ERK activity. NMDARs have opposite roles in the regulation of ERK activity. While synaptic NMDARs up-regulate ERK activity, extrasynaptic NMDARs down-regulate it (Ivanov et al., 2006). GluN2A-containing NMDARs and GluN2B-containing NMDARs are localized in both the synaptic and extrasynaptic regions (Thomas et al. 2006). TrkB receptors are localized in postsynaptic densities (PSD) of excitatory glutamatergic synapses in the adult rat cerebral cortex (Aoki et al., 2000). In dissociated cultures of cortical neurons, the co-localization between the GluN1 and TrkB on the cellular surface is increased at glutamatergic synapses during development (Gomes et al., 2006).

GluN2A (Y1325) and GluN2B (Y1472) are the major tyrosine phosphorylation sites targeted by the Src family non-receptor tyrosine kinases (SFK), including Src and Fyn (Chen and Roche, 2007; Goebel-Goody et al., 2009), and phosphorylation of these sites positively modulates the function of NMDARs. In Fyn-mutant mice, tyrosine phosphorylation of GluN2A was decreased, and Fyn increases its tyrosine phosphorylation in cultured human embryonic kidney 293T cells (Tezuka et al., 1999). In cells transfected with GluN1 and GluN2A subunits, Src and Fyn promote the amplitude of glutamate-activated whole-cell currents through phosphorylation of tyrosines in the C-terminal domain of GluN2A without affecting the desensitization and deactivation

kinetics (Kohr and Seeburg 1996), and the Y1325 residue of GluN2A is located within the C-terminal domain (Zheng et al., 1998). In cultured PFC neurons, activation of D1 receptors increases GluN2B expression in the cellular membrane through Fyn kinase (Hu et al., 2010). In cultured postnatal rat PFC pyramidal neurons, phosphorylation of GluN2B (Y1472) increases surface expression of GluN2B (Gao and Wolf, 2008). Fyn and Src phosphorylate GluN2B (Y1472), leading to an increase in GluN2B synaptic expression (Nakazawa et al., 2001; Cheung and Gurd, 2001; Goebel-Goody et al., 2009). Together, SFK-mediated upregulation of synaptic GluN2A-contatining NMDARs and GluN2B-contatining NMDARs may contribute to ERK activity.

BDNF/TrkB can increase the functions of synaptic GluN2A-contatining NMDARs or GluN2B-contatining NMDARs through SFK activation. In the PSD of rat cerebral cortex, BDNF mediates phosphorylation of GluN2B (Y1472) via Trk receptors, SFK, ERK or PI3K (Xu et al., 2006). Activation of TrkB facilitates the association of TrkB with Src or Fyn (Huang and McNamara, 2010; Iwasaki et al., 1998). BDNF stimulation mediates Fyn activity (Narisawa-Saito et al., 1999) and tyrosine phosphorylation of GluN2B in the cortical PSD fraction of rats (Lin et al., 1998). BDNF increases single NMDA receptor channel activity through Fyn activation (Xu et al., 2006). Binding of SFK to PSD is required for the tyrosine phosphorylation of GluN2A or GluN2B subunits by Fyn or Src (Cheung and Gurd, 2001), suggesting that SFK-mediated phosphorylation of both GluN2 subunits preferentially occurs at the synaptic region. In the supraoptic nucleus of the rat, water deprivation promotes TrkB phosphorylation at Y515 and also the physical

interaction of TrKB with Fyn or with GluN2B. Phosphorylation of GluN2B at Y1472 is also increased after water deprivation in a TrkB-dependent way (Carreño et al., 2011). This suggests the role of TrkB activity in the association of TrkB-Fyn-GluN2B and TrkB-mediated phosphorylation of GluN2B at Y1472 via Fyn. BDNF also increases the total protein levels of the GluN1, GluN2A and GluN2B subunits in a Trk receptor-dependent way through up-regulation of mRNA levels of the GluN1, GluN2A and GluN2B in hippocampal neuronal cultures of rat (Caldeira et al., 2007). Inhibition of the GluN2B-containing NMDAR decreases the BDNF-mediated increase in the amplitude of glutamate-induced synaptic currents in hippocampal neurons, suggesting that BDNF-induced activation of GluN2B-containing NMDAR in the synaptic region underlies this effect of BDNF (Crozier et al., 1999).

Together, BDNF/TrkB/SFK-induced phosphorylation and upregulation of GluN2A- or GluN2B-containing NMDAR at the synaptic region may contribute to BDNF-induced ERK activity.

PURPOSE OF THE STUDY

Intra-dmPFC BDNF infusion immediately after the final session of cocaine SA decreased reinstatement of cocaine-seeking induced by conditioned stimuli (CS) or cocaine in a TrkB-ERK dependent way (Berglind et al., 2007; Whitfield et al., 2011). BDNF infusion into the dmPFC immediately after the last cocaine SA session normalized a cocaine-mediated disturbance in glutamatergic transmission in the NAc core associated with suppression of cocaine-seeking

(Berglind et al., 2009). In cortical cultures, glutamatergic neuronal activity is involved in the maintenance of BDNF-mediated ERK activity and for BDNF-induced potentiation of glutamate transmission (Matsumoto et al., 2006). To assess the involvement of synaptic activity in the dmPFC on the BDNF-mediated suppression of cocaine-seeking, immediately after the final session of cocaine SA, we infused a mixture of AMPAR/NMDAR antagonists (CNQX/LY235959) before BDNF to inhibit BDNF's suppression on cocaine-seeking during a post-abstinence relapse test after 6d of home-cage abstinence and a cue-induced reinstatement test after extinction. As the decreased phosphorylation of GluN2B, but not AMPAR subunit GluA1, was found during early withdrawal from short access cocaine SA, (Sun et al., 2013), after we examined the essential role of synaptic activity in BDNF's suppressive effects, we hypothesized more specifically that NMDAR activity may underlie the suppressive ability of intra-dmPFC BDNF on cocaine-seeking. To examine the role of modulatory GluN2 subunits in BDNF's suppressive effect on cocaine-seeking, the dmPFC of a separate group of rats was infused with the GluN2A-selective antagonist, TCN-201, or the GluN2B-selective antagonist, Ro-25-6981, 20 min before BDNF immediately after the final session of cocaine SA. Then, we hypothesized that GluN2A-contatining NMDARs or GluN2B-contatining NMDARs may underlie BDNF-induced phosphorylation of ERK in the dmPFC. To examine this hypothesis, immediately after the last session of cocaine SA we infused the GluN2A-selective antagonist, TCN-201, or the GluN2B-selective antagonist, Ro-25-6981, into the dmPFC before BDNF and then 2hr after intracranial infusions,

rats were euthanized for immunoblotting to assess the effect of cocaine SA and intra-dmPFC BDNF on phosphorylation of ERK, GluN2A (Y1325) and GluN2B (Y1472).

Specific Aims

Specific Aim 1. Examine whether glutamatergic neuronal activity, more specifically, GluN2A or GluN2B-containing NMDAR activity underlies intra-dmPFC BDNF-mediated suppression of cocaine-seeking induced by context-cue or cocaine-associated cue.

Specific Aim 2. Assess whether GluN2A-containing NMDAR or GluN2B-containing NMDAR activity underlies intra-dmPFC BDNF-mediated normalization of cocaine-induced dephosphorylation in pERK, pGluN2A (Y1325) and pGluN2B (Y1472) in the dmPFC.

CHAPTER 2

NMDA RECEPTORS MEDIATE THE SUPPRESSIVE EFFECT OF AN INTRA-DMPFC BDNF INFUSION ON COCAINE-SEEKING

Introduction

Drug addiction is a chronic, relapsing brain disorder induced by long-lasting dysregulation of synaptic adaptation (Koob and Volkow, 2010; Weiss et al., 2001; Kalivas and Volkow, 2005). The glutamatergic projection of the dorsomedial prefrontal cortex (dmPFC) to the nucleus accumbens core (NAc core) is associated with reinstatement of cocaine-seeking after extinction by cocaine-conditioned context, cue, and cocaine-prime (Fuchs et al., 2005; McLaughlin and See, 2003; Park et al., 2002; McFarland et al., 2003). Brain-derived neurotrophic factor (BDNF) is expressed in the cortico-accumbens pathway where, as in other brain regions, it modulates synaptic plasticity and glutamatergic neurotransmission in an activity-dependent manner (McGinty et al., 2010; Korte et al., 1998; Figurov et al., 1996; Lessmann and Heumann, 1998; Kang and Schuman, 1995; Bramham and Messaoudi, 2005). BDNF binds to and activates tropomyosin-receptor kinase B (TrkB), leading to the activation of mitogen-activated extracellular signal-regulated kinase (ERK), phosphoinositol 3-kinase,

and phospholipase C gamma1 intracellular signaling (Huang and Reichardt, 2003; Reichardt, 2006). Of these kinase signaling cascades, ERK1/2-MSK2 activation was found to exclusively mediate BDNF's ability to phosphorylate CREB in cortical neurons (Simon et al., 2004). We previously showed that infusion of exogenous BDNF into the dmPFC immediately after the last session of cocaine SA effectively suppresses cocaine-seeking induced by cocaine-conditioned context, cue, or cocaine-prime through Trk receptor-mediated ERK activation (Berglind et al., 2007; Whitfield et al., 2011) and through normalization of the cocaine-mediated dysregulation in extracellular glutamate level in NAc core in rats with a cocaine SA history (Berglind et al., 2009).

Glutamatergic neuronal activity is essential for the maintenance of BDNF-mediated ERK activity and for BDNF-induced potentiation of glutamate transmission in cortical cultures (Matsumoto et al., 2006). Of the receptors which mediate glutamatergic neuronal activity, NMDARs are known to suppress cocaine-seeking. NMDAR co-agonist, D-serine, effectively extinguishes cocaine-seeking both in cocaine-induced conditioned place preference (CPP) and in a cocaine SA paradigm (Hammond et al., 2013; Kelamangalath et al., 2009; Kelamangalath and Wagner, 2010). In contrast, systemic injection of NMDAR antagonist MK-801 promotes reinstatement of cocaine-seeking (De Vries et al., 1998). GluN2 (A-D) are modulatory subunits of NMDARs and GluN2A and GluN2B subunits are highly expressed in the cerebral cortex (Lau and Zukin, 2007; Yashiro and Philpot, 2008). Given that glutamatergic neuronal activity underlie BDNF-mediated ERK activity and glutamate neurotransmission, both of

which are the underlying mechanisms of BDNF's suppressive effect on cocaine-seeking, we hypothesized that PFC synaptic activity contributes to BDNF-induced ERK activity in the dmPFC that underlies BDNF's ability to suppress cocaine-seeking. The initial study examined whether blocking all dmPFC synaptic activity with a mixture of AMPAR/NMDAR antagonists (CNQX/LY235959) would prevent an intra-dmPFC BDNF infusion immediately after the end of cocaine SA from suppressing subsequent cocaine-seeking. Because a previous study from our lab found changes in the phosphorylation of NMDAR, but not AMPAR, subunits during early withdrawal from short access cocaine SA (Sun et al., 2013), after assessing the essential role of synaptic activity on BDNF's suppressive effects, this study also examined the role of regulatory GluN2 subunits in BDNF's suppressive effect on cocaine-seeking. For this purpose, rats were infused with a GluN2A-selective antagonist, TCN-201, or a GluN2B-selective antagonist, Ro-25-6981, into the dmPFC before intra-dmPFC BDNF.

Materials and Methods

Experimental Design. Figure 2.1 illustrates the design of all experiments in which rats were trained to self-administer cocaine for 2 hr/day and received intracranial infusions immediately after the end of cocaine SA. In Experiments 1-3, after intracranial infusions of glutamate receptor antagonists prior to BDNF or PBS, rats underwent abstinence, a post-abstinence (PA) test under extinction conditions, followed by extinction of lever pressing, and a conditioned-cue test as

described below. A schematic flowchart of each experiment is shown in Figure 2.1.

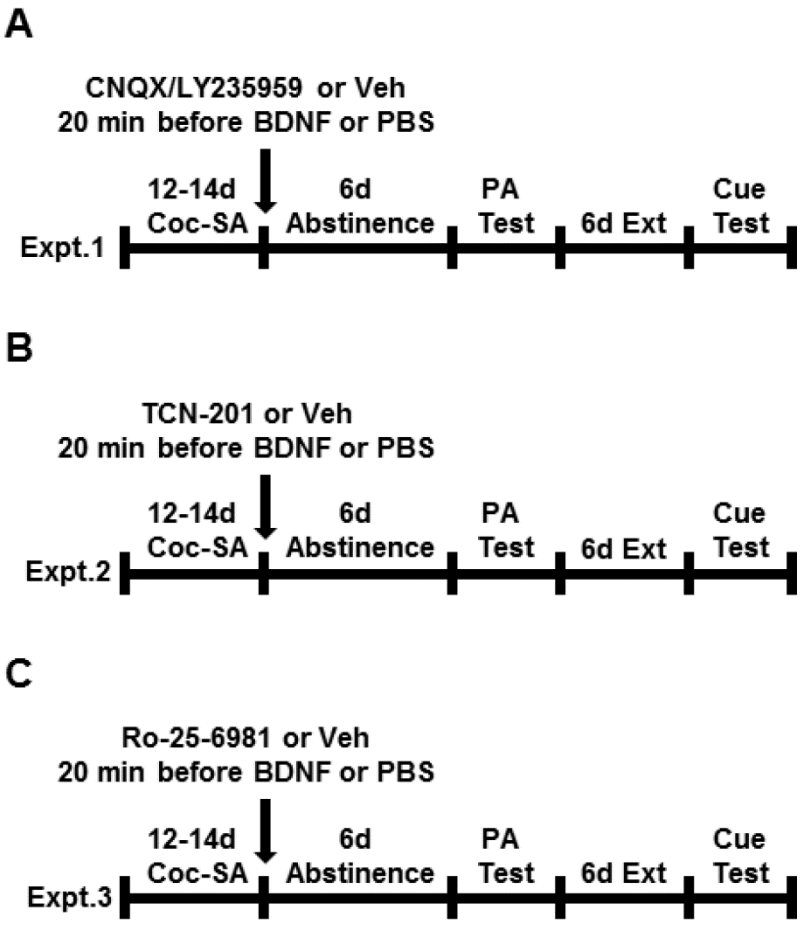

Figure 2.1. Schematic representation of the experimental designs for experiments 1-3.

Animals and surgery. Adult male Sprague Dawley rats (Charles River Laboratories, Raleigh, NC, USA; 275-300g on arrival) were individually placed in a temperature- and humidity-controlled room on a reversed light/dark cycle (lights off from 6AM-6PM). Rats were provided standard rodent chow with water

available *ad libitum.* All experimental procedures were conducted under the guidelines and approval of the Institutional Animal Care and Use Committee (IACUC) of the Medical University of South Carolina and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised 1996). Rats were housed singly in their home cages at least 4 days for acclimation before surgery.

On the day of surgery, rats were anesthetized with a mixture of ketamine (66 mg/kg, i.p.) and xylazine (1.33 mg/kg,i.p.), and then equithesin (0.5 ml/kg, i.p.) followed by ketorolac (2.0 mg/kg, i.p.). One end of a catheter was placed into the right jugular vein and the other end was connected to the back mount cannula pedestal (Med Associates) for intravenous infusions. After catheterization, 26 gauge bilateral guide cannulae (Plastics One) were placed 1 mm above the dmPFC target region [anterior-posterior (AP), +3.0; medial-lateral (ML), +/- 0.6; dorsoventral (DV),-1.6 relative to dural surface] (Paxinos and Watson 2005). Guide cannulae were affixed to the skull with cranioplastic cement and steel screws. Stylets the same length as guide cannulae (Plastics One) were inserted into the guide cannulae to avoid blockage throughout the experiment. Following surgery, rats were infused intravenously with 0.1 ml of cefazolin (33.3 mg/kg, i.p.) as well as an anti-microbial lock solution TCS (0.1ml; Norfolk Access Technologies) twice daily for 5 days.

Cocaine self-administration. After at least 5 days of surgical recovery, rats were subject to cocaine SA under a FR1 reinforcement schedule for 12-14 days

(2hr/day). Rats were provided with a moderately restricted amount of food (20g standard chow/day) starting 1d before SA training to the last day of cocaine SA. The SA chamber contained a lever on the left (inactive lever) and right (active lever) side of one wall, and a circular stimulus light above each lever. A cocaine infusion line was connected to a swivel (Instech Laboratories) that allowed the rats to freely move in the chamber. Rats pressing the active lever were infused with cocaine hydrochloride (0.2mg/infusion; National Institute on Drug Abuse, Research Triangle Park, NC) through a computer-controlled infusion pump paired with both light and tone cues (a white stimulus light above the active lever; 2 kHz, 15 dB above ambient noise). Following each cocaine infusion, there was a 20s timeout period when pressing on the active lever was counted, but no infusion of cocaine occurred. Pressing left (inactive) levers also was counted without any programmed consequences. The criterion for cocaine SA was more than 10 infusions per day.

Intracranial infusions into the dmPFC. Immediately after the final SA session in all experiments, a 33 gauge bilateral infusion injector (Plastics One) was inserted into the guide cannula so that it protruded 1 mm below the tip of the guide cannula, targeting the anterior cingulate/prelimbic cortex. All intracranial infusions were conducted using 10 ul Hamilton gas-tight syringes connected to an infusion pump (Harvard Apparatus) that was programmed to deliver a 0.5 ul volume per side (0.25 ul/min). Injectors remained in the guide cannula for 1 min before and after infusions. In Experiment 1, a mixture of the AMPAR antagonist, CNQX (0.1 nmol/0.5 ul/side; Tocris; Pierce et al., 1996) and the NMDAR antagonist,

LY235959 (10 ng/0.5 ul/side; Tocris; Kenny et al., 2009) or 1% DMSO in PBS, was infused into the dmPFC 20 min before recombinant human BDNF (0.75 ug/0.5 ul/side; R&D Systems) or PBS. In Experiment 2,, the GluN2A-selective NMDAR antagonist, TCN-201 (0.1 nmol/0.5 ul/side; Tocris;) or 2% DMSO in PBS, was infused into the dmPFC. In Experiment 3, the GluN2B-selective NMDAR antagonist, Ro-25-6981 (2 ug/0.5 ul/side; Tocris;) or PBS, was infused into the dmPFC.

Post-abstinence relapse test, extinction, and cue-induced reinstatement test. After dmPFC infusions, rats were placed back into their home cages for 6 days of abstinence before a 2-hr post-abstinence (PA) relapse test under extinction conditions. During this test, active (right) and inactive (left) lever presses were recorded but did not lead to any programmed consequences. Following the PA test, rats underwent further extinction training until meeting criteria (2hr/day, 6 or more days and less than 10 active lever presses during the last 2 consecutive days) before the cue-induced reinstatement test. During extinction training, responses on both active and inactive levers were recorded, but resulted in no programmed consequences. During the 2-hr cue-induced reinstatement test, both active and inactive lever presses were recorded but whereas pressing the active lever resulted in presentation of cocaine-paired cues (light plus tone), inactive lever pressing had no programmed consequences.

Verification of cannula placements. Cannula placements are shown in Figure 2.2. The rats were decapitated without anesthesia; their brains were immediately

frozen in isopentane (-20°C for 30 sec) and kept at -80°C before 40 µm sections were cut in a cryostat, and cannula placements within the dmPFC were confirmed by Nissl-staining. Only cannulae located within the target region of dmPFC, including the prelimbic or anterior cingulate cortex, were mapped and included in the data set for statistical analysis.

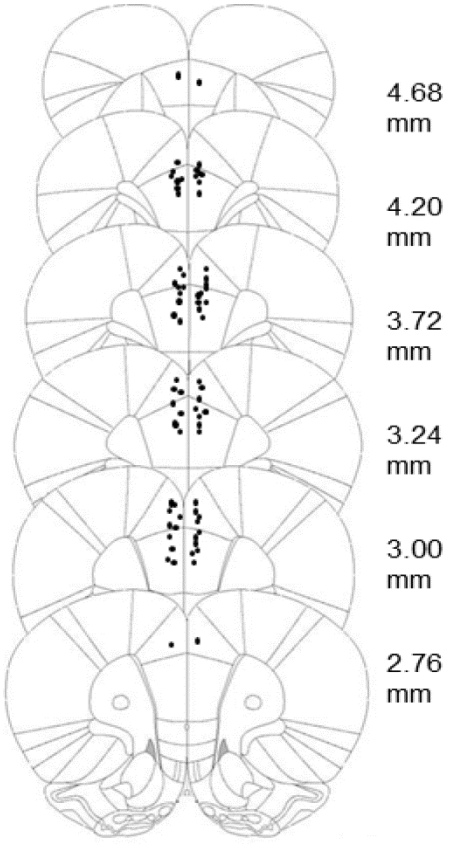

Figure 2.2. Cannula placements from Experiments 1-3. Graphical representation of the tip of the intracranial infusion cannula within the target area of the dmPFC (anterior cingulate and prelimbic cortex) (shown as small filled dots).

Statistical analysis. For the analysis of behavioral data one-way ANOVA followed by Student–Newman–Keuls (SNK) multiple-comparison tests were performed to

compare active lever presses of all four experimental groups during either last 3d of cocaine SA, PA relapse test, last 2d of extinction or cue-induced reinstatement test. To compare active lever presses of the same experimental group between two different time points (last 3d of cocaine SA vs PA relapse test, or last 2d of extinction vs cue-induced reinstatement test), within-sample paired t-tests were used for the analysis. When the p-value was <0.05, results were considered statistically significant.

Results

Histology

Cannula placements for rats in Experiments 1- 3 are shown in Figure 2.2. Cannula placements extended from AP 3.0-4.7 and punches extended from 2.76-4.7. Cannula placements that were located outside the dmPFC (n=2) were excluded from the schematic mapping and the data set for statistical analysis.

Experiment 1: The ability of BDNF to suppress cocaine-seeking requires ionotropic glutamate receptor-mediated neuronal activity in the dmPFC

To examine whether glutamate-mediated neuronal activity was required for the suppressive effect of intra-dmPFC BDNF on cocaine-seeking, rats were divided into four experimental groups that were subject to an intra-dmPFC infusion of a mixture of AMPAR/NMDAR antagonists (CNQX/LY235959) or 1% DMSO in PBS 20 min before BDNF or PBS infusion immediately after the final session of cocaine SA (see Fig. 2.1A). One-way ANOVA showed that during the last 3 days of cocaine SA before intracranial infusions, active (right) lever presses were not

significantly different among the four groups ($F_{(3,16)=}0.27$, p=0.85; Fig. 2.3A). Paired t-tests showed that during the PA relapse test, DMSO/PBS (t_4=5.96, p<0.01), CNQX/LY235959-PBS (t_4=2.78, p<0.05) and CNQX/LY235959-BDNF-infused rats (t_4=4.74, p<0.01) but not DMSO/BDNF-infused rats (t_4=2.10, p=0.10) showed significantly more active lever presses than during the last 3 days of cocaine SA.

A one-way ANOVA revealed that during the PA relapse test after intracranial infusions, active (right) lever presses were significantly different among the four groups ($F_{(3,16)=}$ 4.71, p=0.02 ; Fig. 2.3A). SNK multiple comparison tests revealed that rats infused with DMSO/BDNF exhibited significantly less active lever pressing than DMSO/PBS-infused rats (p<0.05) and CNQX/LY235959/BDNF-infused rats pressed the previously active levers more than DMSO/BDNF-infused rats (p<0.01), indicating that CNQX/LY235959 blocked the suppressive effect of BDNF on cocaine-seeking during the PA relapse test. SNK multiple comparison tests also showed that during the PA relapse test, there was no significant difference in active lever pressing between DMSO/PBS and CNQX/LY235959/PBS-infused rats (p>0.05), confirming that the dose of CNQX/LY235959 chosen for the study effectively blocked BDNF-mediated behavior changes only, leaving cocaine-induced behaviors unaffected.

After the PA relapse test, rats were subjected to extinction training. A one-way ANOVA showed that during the last two consecutive days of extinction (Fig. 2.3B), the average number of active (right) lever presses was not significantly

different among the four groups ($F_{(3,16)}$=0.03, p=0.99). Paired t-tests indicated that during the cue-induced reinstatement test, DMSO/PBS (t_4=10.65, p<0.001), DMOS/BDNF (t_4=4.14, p<0.05), CNQX/LY235959/PBS (t_4=4.29, p<0.05), and CNQX/LY235959/BDNF-treated rats (t_4=4.81, p<0.01) showed significantly greater active lever responses than during the last 2 days of extinction training.

A one-way ANOVA revealed that during the cue-induced reinstatement test after intracranial infusions, active (right) lever presses were significantly different among the four groups ($F_{(3,16)}$=4.58, p=0.02; Fig. 2.3B). SNK tests revealed that DMSO/BDNF-infused rats pressed the formerly active lever significantly less than DMSO/PBS-infused rats (p<0.001) and CNQX/LY235959/BDNF-infused rats responded significantly more on the active levers than DMSO/BDNF-infused rats (p < 0.001), indicating CNQX/LY235959 blocked the suppressive effect of BDNF on cue-induced reinstatement of cocaine-seeking. In addition, SNK multiple comparison tests showed that during the cue-induced reinstatement test, there was no significant difference in active lever pressing between DMSO/PBS and CNQX/LY235959/PBS-infused rats (p>0.05), indicating that the dose of CNQX/LY235959 chosen for the study effectively blocked BDNF-mediated behavior changes only, leaving cocaine-induced behaviors unaffected.

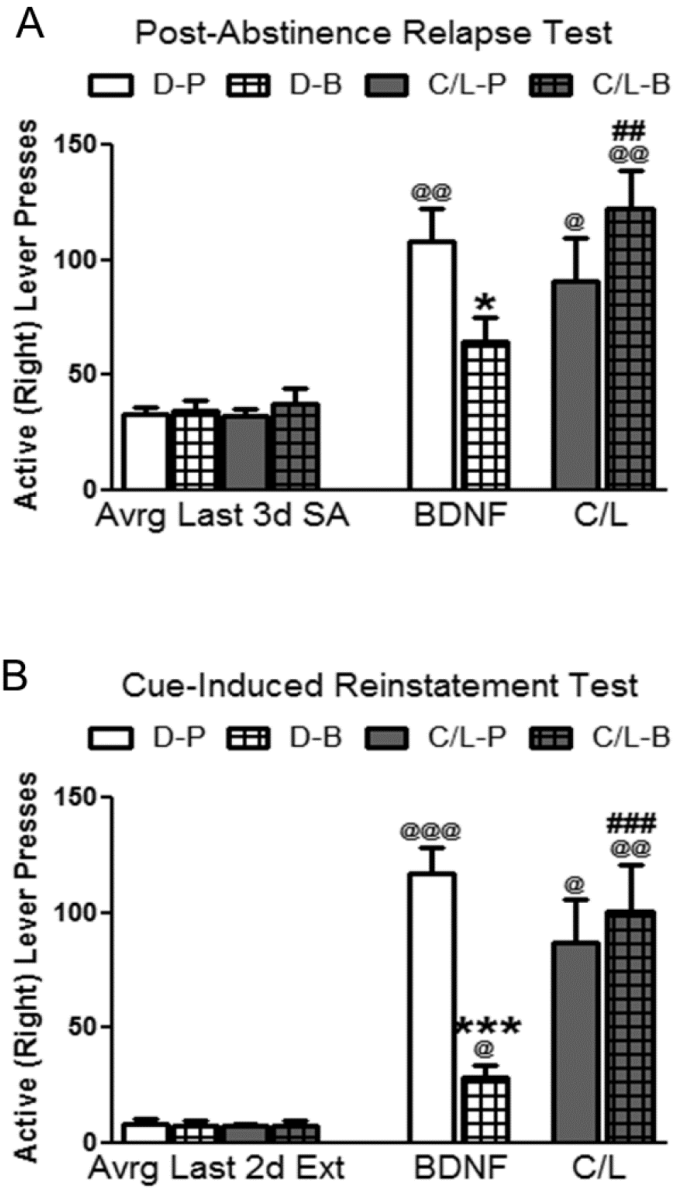

Figure 2.3. Infusion of CNQX/LY235959 into the dmPFC blocked BDNF's suppressive effect on cocaine-seeking during the post-abstinence (PA) relapse test and cue-induced reinstatement test. (A) C/L effects on PA test responding after one week of abstinence. Left: During the last 3d of cocaine SA, the average number of active lever presses was similar among groups (n= 5/group). Immediately after the last cocaine SA session, the rats were given intra-dmPFC infusions of C/L, and after one week of home cage abstinence, they were subjected to the PA test. Right: During the PA test, all other infusion groups except the D-B group showed significantly greater active lever pressing compared with responses during the last 3d of cocaine SA ($^{@}p < 0.05$, $^{@@}p < 0.01$). DMSO (D)-B rats showed significantly less active lever pressing than D-PBS (P) controls (*p < 0.05), but C/L infusion before BDNF (C/L-B) significantly inhibited the suppressive effect of BDNF on active lever pressing compared to the D-B group ($^{\#\#}p < 0.01$). **(B)** C/L effects on **c**ue-induced reinstatement after extinction training (n=5/group). Left: Rats were subject to extinction of active lever pressing until reaching a common response criterion. The average number of active lever presses during the last 2d of extinction training was not significantly different among groups. Right: During the cue-induced reinstatement test, each experimental group exhibited significantly greater active lever pressing compared with responses during the last 2d of extinction training ($^{@}p < 0.05$, $^{@@}p < 0.01$, $^{@@@}p < 0.001$). D-B rats pressed the formerly active lever significantly less than D-P controls (***p < 0.001). A C/L infusion before BDNF treatment significantly decreased the

inhibitory effect of BDNF on cocaine-seeking compared with the D-B group ($^{###}p < 0.001$). D-B=DMSO vehicle before BDNF, C/L-P=CNQX/LY235959 before PBS, C/L-B= CNQX/LY235959 before BDNF.

Experiment 2: Activity of GluN2A-containing NMDA receptors in the dmPFC is necessary for the suppressive effect of intra-dmPFC BDNF on cocaine-seeking

Once Experiment 1 established that synaptic activity was necessary for the suppressive effect of intra-dmPFC BDNF on cocaine-seeking, we reasoned that NMDA receptors were the likely drivers because our previous studies demonstrated that NMDA, but not AMPA, receptor functionality was altered by short access cocaine SA (Sun et al., 2013). To examine the role of the GluN2A subunit-containing NMDARs in the suppressive effect of BDNF on cocaine-seeking, rats were divided into four experimental groups that were subjected to intracranial infusion of the GluN2A-selective antagonist, TCN-201, or 2% DMSO in PBS 20 min before BDNF or PBS infusion immediately after the last session of cocaine SA (see Fig. 2.1B). A One-way ANOVA showed that during the last 3 days of cocaine SA before intracranial infusions, active (right) lever pressing was not significantly different among the four groups ($F_{(3,27)}=0.34$, $p=0.80$; Fig. 2.4A). Paired t-tests showed that during the PA relapse test, DMSO/PBS ($t_6=7.46$, $p<0.001$), TCN-201/PBS ($t_9=2.90$, $p<0.05$) and TCN-201/BDNF-infused rats ($t_6=3.97$, $p<0.01$) but not DMSO/BDNF-infused rats ($t_6=1.63$, $p=0.15$) showed significantly more active lever presses than during the last 3 days of cocaine SA.

A one-way ANOVA revealed that during the PA relapse test after intracranial infusions, active (right) lever presses were significantly different

among the four groups ($F_{(3,27)}$= 4.07, p=0.02 ; Fig. 2.4A). SNK multiple comparison tests revealed that rats infused with DMSO/BDNF pressed the active lever significantly less than DMSO/PBS-infused rats (p<0.01). TCN-201/BDNF-infused rats pressed the previously active levers significantly more than DMSO/BDNF-infused rats (p<0.01), indicating that TCN-201 blocked the suppressive effect of BDNF on cocaine-seeking during the PA test. SNK multiple comparison tests also showed that during the PA test, there was no significant difference in active lever pressing between DMSO/PBS and TCN-201/PBS-infused rats (p>0.05), confirming that the doses of TCN-201 chosen for the study effectively blocked BDNF-mediated behavior changes only, leaving cocaine-induced behaviors unaffected.

After the PA test, rats were subjected to extinction training. A one-way ANOVA showed that during the last two consecutive days of extinction (Fig. 2.4B), active (right) lever responses were not significantly different among the four groups ($F_{(3,25)}$=0.35, p=0.79). Paired t-tests indicated that during the cue-induced reinstatement test, DMSO/PBS (t_6=7.52, p<0.001), DMOS/BDNF (t_6=4.30, p<0.01), TCN-201/PBS (t_8=5.81, p<0.001), and TCN-201/BDNF-treated rats (t_5=10.25, p<0.001) showed significantly greater active lever responses than during the last 2 days of extinction training.

A one way ANOVA found that during the cue-induced reinstatement test after intracranial infusions, active (right) lever presses were significantly different among the four groups ($F_{(3,25)}$= 4.79, p=0.01; Fig. 2.4B). SNK tests revealed that

DMSO/BDNF-infused rats pressed the active lever less than DMSO/PBS-infused rats ($p<0.01$). TCN-201/BDNF-infused rats responded significantly more on the active levers than DMSO/BDNF-infused rats ($p < 0.05$), indicating TCN-201 blocked the suppressive effect of BDNF on cue-induced reinstatement of cocaine-seeking. In addition, SNK multiple comparison test showed that during the cue-induced reinstatement test, there was no significant difference in active lever pressing between DMSO/PBS and TCN-201/PBS-infused rats ($p>0.05$), indicating that the dose of TCN-201 chosen for the study effectively blocked BDNF-mediated behavior changes only, leaving cocaine-induced behaviors unaffected.

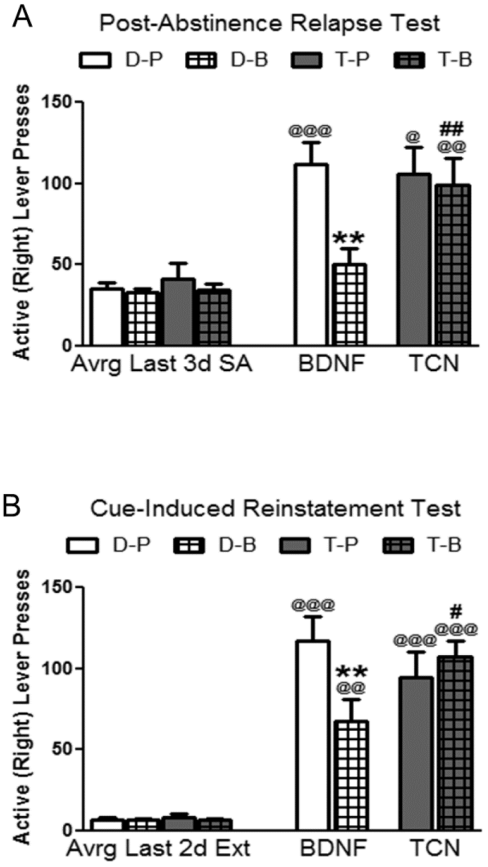

Figure 2.4. Infusion of TCN-201 (T) into the dmPFC blocked BDNF's (B) suppressive effect on cocaine-seeking during the post-abstinence (PA) relapse test and cue-induced reinstatement test. (A) TCN-201 effects on PA test responding after one week of abstinence. Left: During the last 3d of coc-SA, the average number of active lever presses was similar among groups (n=10 in T-P group; n=7 in other groups). Immediately after the last cocaine SA session, the rats were given intra-dmPFC infusions of TCN-201, and after one week of home cage abstinence, they were subjected to the PA test. Right: During the PA test, all groups except the D-B group pressed the formerly active lever significantly less than during the last 3d of cocaine SA ($^{@}p < 0.05$, $^{@@}p < 0.01$, $^{@@@}p < 0.001$). The D-B group showed significantly less active lever pressing compared with D-P controls ($^{**}p < 0.01$). However, TCN-201 pretreatment before BDNF (T-B) significantly decreased the suppressive effect of BDNF on active lever

pressing compared with the D-B group ($^{\#\#}p < 0.01$). **(B)** TCN-201 effects on **c**ue-induced reinstatement after extinction training (n=9 in T-P group; n=6 in T-B group; n=7 in other groups). Left: All rats were given extinction training until reaching a common response criterion. The average number of active lever presses during the last 2 d of extinction training was not significantly different among groups. Right: During the cue-induced reinstatement test, all four experimental groups showed significantly greater active lever pressing test than the responses during the last 2d of extinction training ($^{@@}p < 0.01$, $^{@@@}p < 0.001$). D-B treated rats exhibited significantly less active lever pressing than D-P controls (**p < 0.01). Infusion of TCN-201 before BDNF significantly inhibited the ability of BDNF to suppress cocaine-seeking compared to the D-B group ($^{\#}p < 0.05$). D-P=DMSO vehicle before PBS, T-P=TCN-201 before PBS, T-B=TCN-201 before BDNF.

Experiment 3: Activity of GluN2B-containing NMDA receptors in the dmPFC is essential for the inhibitory effect of intra-dmPFC BDNF on cocaine-seeking

To examine the role of the GluN2B subunit-containing NMDARs in the suppressive effect of BDNF on cocaine-seeking behavior, rats were divided into four experimental groups that were subject to intracranial infusion of the GluN2B-selective antagonist, Ro-25-6981, or PBS 20 min before BDNF or PBS infusion immediately after the last session of cocaine SA (see Fig. 2.1C). One-way ANOVA showed that during the last 3 days of cocaine SA before intracranial infusions, active (right) lever pressing was not significantly different among the four groups ($F_{(3,21)}=0.53$, p=0.67; Fig. 2.5A). Paired t-tests showed that during the PA relapse test, PBS/PBS ($t_4=3.87$, $p<0.05$), PBS/BDNF ($t_6=2.45$, $p<0.05$), Ro-25-6981/PBS ($t_5=10.46$, $p<0.001$), and Ro-25-6981/BDNF-infused rats ($t_6=5.52$, $p<0.01$) showed significantly more active lever presses than during the last 3 days of cocaine SA.

A one way ANOVA revealed that during the PA relapse test after intracranial infusions, active (right) lever presses were significantly different among the four groups ($F_{(3,21)}$= 4.88, p=0.01; Fig. 2.5A). SNK tests revealed that during the PA relapse test, rats infused with PBS/BDNF exhibited significantly less active lever pressing than PBS/PBS-infused rats (p<0.001) and Ro-25-6981/BDNF-infused rats pressed the previously active levers more than PBS/BDNF-infused rats (p<0.001), indicating that Ro-25-6981 blocks the suppressive effect of BDNF on cocaine-seeking during the PA relapse test. SNK multiple comparison test followed by one way ANOVA also showed that during the PA relapse test, there was no significant difference in active laver pressing between PBS/PBS Ro-25-6981/PBS-infused rats (p>0.05), confirming that the doses of Ro-25-6981 chosen for the study effectively blocked BDNF-mediated behavior changes only, leaving cocaine-induced behaviors unaffected.

After the PA relapse test, rats were subject to extinction training. One-way ANOVA showed that during the last two consecutive days of extinction (Fig. 2.5B) active (right) lever presses were not significantly different among the four groups ($F_{(3,19)}$=0.58, p=0.64). Paired t-tests indicated that during the cue-induced reinstatement test, PBS/PBS (t_4=6.21, p<0.01), PBS/BDNF (t_5=8.96, p<0.001), Ro-25-6981/PBS (t_5=8.15, p<0.001), and Ro-25-6981/BDNF-treated rats (t_5=4.94, p<0.01) showed significantly greater active lever responses than during the last 2 days of extinction training.

A one way ANOVA revealed that during the cue-induced reinstatement test after intracranial infusions, active (right) lever presses were significantly different among the four groups ($F_{(3,19)}$= 4.92 , p=0.01; Fig. 2.5B). SNK tests revealed that during the cue-induced reinstatement test PBS/BDNF-infused rats showed significantly less active lever presses than PBS/PBS-infused rats ($p<0.01$) and Ro-25-6981/BDNF-infused rats showed significantly more responses on the active levers than PBS/BDNF-infused rats ($p < 0.001$), indicating Ro-25-6981 blocked the suppressive effect of BDNF on cue-induced reinstatement of cocaine-seeking. In addition, SNK multiple comparison test followed by one way ANOVA also showed that during the cue-induced reinstatement test, there was no significant difference in active laver presses between PBS/PBS and Ro-25-6981/PBS-infused rats ($p>0.05$), indicating that the dose of Ro-25-6981 chosen for the study effectively blocked BDNF-mediated behavior changes only, leaving cocaine-induced behaviors unaffected.

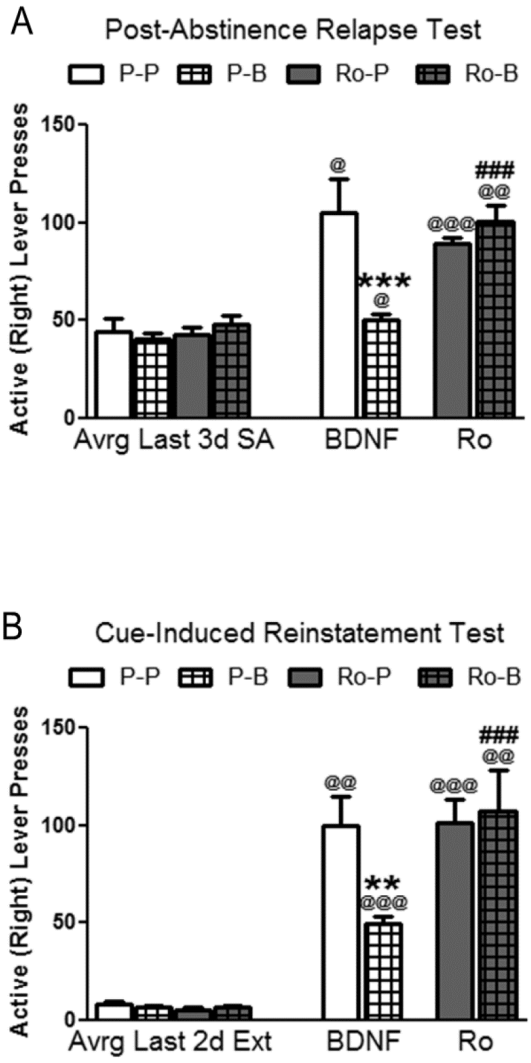

Figure 2.5. Infusion of Ro-25-6981 (Ro) into the dmPFC blocked BDNF's (B) suppressive effect on cocaine-seeking during the post-abstinence (PA) relapse test and cue-induced reinstatement test. (**A**) Ro effects on PA test responding. Left: During the last 3d of cocaine SA, the average number of active lever presses was similar among groups (n=5 in P-P; n=6 in Ro-P; n=7 in other groups). Immediately after the last cocaine SA session, the rats were given intra-dmPFC infusions of Ro, and after one week of home cage abstinence, they were subjected to the PA relapse test. Right: During the PA test, all four groups pressed the formerly active lever significantly more than during last 3d of cocaine SA ($^{@}p < 0.05$, $^{@@}p < 0.01$, $^{@@@}p < 0.001$). The P-B group showed significantly less active lever pressing compared with P-P controls (***p< 0.001). However, Ro pretreatment before BDNF (R-B) significantly blocked the suppressive effect of BDNF on active lever pressing compared with the P-B group ($^{###}p < 0.001$). **(B)** Ro effects on cue-induced reinstatement after extinction training (n=5 in P-P; n=6 in other groups). Left: All rats were given extinction training until reaching a common response criterion. The average number of active lever presses during the last 2 d of extinction training was not significantly different among groups. Right: During the cue-induced reinstatement test, all four experimental groups pressed the active lever significantly more than during the last 2d of extinction training ($^{@@}p<0.01$, $^{@@@}p < 0.001$). P-B treated rats exhibited significantly less active lever pressing than P-P controls (**p < 0.01). Infusion of Ro before BDNF significantly inhibited the ability of BDNF to suppress cocaine-seeking compared to the P-B group ($^{###}p < 0.001$). The bar graphs indicate the mean ± SEM. P-P=PBS before PBS, P-B=PBS before BDNF, Ro-P= Ro-25-6981 before PBS, Ro-B= Ro-25-6981 before BDNF.

Discussion

CNQX/LY235959, TCN-201, or Ro-25-6981 inhibited BDNF-mediated suppression of cocaine-seeking.

Infusion of TCN-201, or Ro-25-6981 into the dmPFC blocked the suppressive effect of BDNF on cocaine-seeking during a post-abstinence relapse test and a cocaine-conditioned cue-induced reinstatement test after extinction without affecting cocaine-seeking in PBS-infused rats. These findings indicate that the activity of ionotropic glutamate receptors, specifically GluN2A- or GluN2B-containing NMDARs, in the dmPFC is required for BDNF to suppress cocaine-seeking. A previous study supports the functional role of NMDARs in the suppressive effect of BDNF on cocaine-seeking. In the infralimbic cortex, TrkB activation promotes extinction learning of a cocaine-induced conditioned place preference (CPP) via GluN2B-containing NMDARs (Otis et al., 2014).

Intra-dmPFC BDNF infusion attenuates cocaine-seeking induced by cocaine-conditioned context, cue, or cocaine-prime via Trk receptor-mediated ERK activation in the dmPFC (Whitfield et al., 2011). BDNF upregulates the open probability of NMDAR through TrkB *in vitro* (Levine et al., 1998). In hippocampal cultures, BDNF increases NMDAR activity (Crozier et al., 2008). TrkB induces tyrosine phosphorylation of GluN2B-contatining NMDA receptors via Fyn in the hippocampus (Mizuno et al., 2003). Fyn-mediated phosphorylations of tyrosines in the C-terminal domain of GluN2A upregulate the amplitude of glutamate-activated whole-cell currents (Kohr and Seeburg, 1996). Fyn-induced phsophorylation of GluN2B (Y1472) enhances GluN2B synaptic (Nakazawa et al.,

2001; Cheung and Gurd, 2001; Goebel-Goody et al., 2009) and surface expression (Gao and Wolf, 2008). NMDAR activity contributes to ERK activation in rat anterior cingulate cortex (Cao et al., 2009) and CaMKIIα underlies synaptic GluN2B-mediated ERK activity in mouse cultured cortical neurons (El Gaamouch et al., 2012). Therefore, BDNF may activate NMDAR via TrkB-SFK signaling, and this BDNF-mediated NMDAR activity may contribute to ERK activation, which supports the role of NMDARs in the suppressive effect of BDNF on cocaine-seeking.

Intra-dmPFC infusion of BDNF normalizes a cocaine SA-mediated decrease in the basal level of extracelluar glutamate and a cocaine-prime induced increase in extracelluar glutamate level in NAc core of rats, which is associated with suppression of cocaine-seeking (Berglind et al., 2009). In cortical neurons, NMDAR activity is required for BDNF-potentiated glutamate release (Matsumoto et al., 2006). Together, this indicates that NMDAR activity may underlie BDNF-mediated suppression of cocaine-seeking through its potentiation of BDNF-mediated glutamatergic neurotransmission.

Infusion of CNQX/LY235959, TCN-201 or Ro-25-6981 in the absence of BDNF did not alter cocaine-seeking compared to vehicle-infused rats (D-P vs C/L-P; D-P vs T-P; P-P vs Ro-P), indicating that CNQX/LY235959, TCN-201 or Ro-25-6981 treatment itself has no effect on cocaine-seeking. As cocaine SA is known to reduce basal activity of the dmPFC (Sun and Rebec, 2006), NMDA receptor activity in the dmPFC may be highly suppressed after cocaine SA

because of voltage-sensitive magnesium ion blockade of NMDARs (MacDonald and Nowak, 1990). Therefore, due to the suppressed NMDAR activity in the dmPFC after cocaine SA, either CNQX/LY235959, TCN-201 or Ro-25-6981 infusion itself may not be effective to further reduce AMPAR/NMDAR, GluN2A- or GluN2B-containing NMDAR activity in the dmPFC, exerting no significant effect on cocaine-seeking compared to its vehicle. This idea is supported by our findings of the cocaine SA-mediated decrease in phosphorylation of GluN2A (Y1325) or GluN2B (Y1472) which will be described in the next chapter. Because SFK-induced phosphorylation of GluN2A (Y1325) or GluN2B (Y1472) is known to upregulate NMDAR functions (Gao and Wolf, 2008; Taniguchi et al., 2009; Nakazawa et al., 2001; Cheung and Gurd, 2001; Goebel-Goody et al., 2009), cocaine SA-mediated reduction in phosphorylation of GluN2A (Y1325) and GluN2B (Y1472) in the dmPFC during early withdrawal is an indirect indicator of NMDAR hypofuntion, leading to subsequent cocaine-seeking.

CHAPTER 3

ACTIVITY OF BOTH GLUN2A SUBUNIT-CONTAINING NMDA RECEPTORS AND GLUN2B SUBUNIT-CONTAINING NMDA RECEPTORS IS ESSENTIAL FOR BDNF'S EFFECT ON ERK, GLUN2A AND GLUN2B PHOSPHORYLATION

Introduction

In the cortico-accumbens pathway BDNF and its receptor TrkB are highly expressed and regulate neural plasticity and glutamatergic neurotransmission in an activity-dependent manner (McGinty et al., 2010; Korte et al., 1998; Figurov et al., 1996; Lessmann and Heumann, 1998; Kang and Schuman, 1995; Bramham and Messaoudi, 2005). The previous studies from our lab found that intra-dmPFC BDNF-mediated ERK activity and normalization of cocaine-induced dysregulation of glutamatergic neurotransmission in the NAc core underlie the suppressive effect of BDNF on cocaine seeking induced by context cue, cocaine-conditioned cue or cocaine-prime (Berglind et al., 2007; Whitfield et al., 2011). Glutamatergic synaptic activity is essential for the maintenance of BDNF-induced ERK activity and for BDNF-potentiated glutamate transmission in cortical cultures (Matsumoto et al., 2006). Further, co-activation of D1 and NMDA receptors synergistically activates ERK1/2 in PFC (Sarantis et al., 2009).

BDNF upregulates the open probability of NMDARs in a TrkB-dependent way, increasing glutamate-induced synaptic transmission *in vitro* (Levine et al., 1998). Brief stimulation of synaptic NMDARs mediates ERK activation whereas sustained stimulation of extrasynaptic NMDARs underlies ERK inactivation (Ivanov et al., 2006) and BDNF reverses this effect (Mulholland et al., 2008). Regulation of ERK activity is mediated by GluN2A- and GluN2B-containing NMDARs that are both located in synaptic and extrasynaptic membranes (Thomas et al., 2006) and are more highly expressed in the cerebral cortex than other NMDAR regulatory subunits (Yashiro and Philpot, 2008).

BDNF activation of TrkB promotes the association of TrkB with Src family kinases (SFK), leading to SFK activation and tyrosine phosphorylation of GluN2A and GluN2B (Goebel-Goody et al., 2009; Huang and McNamara, 2010; Iwasaki et al., 1998; Narisawa-Saito et al., 1999). SFK-mediated phosphorylation of GluN2 subunits increases the expression of both subunits in postsynaptic density-enriched fractions (Nakazawa et al., 2001; Cheung and Gurd, 2001; Goebel-Goody et al., 2009). Moreover, SFK-mediated phosphorylation of GluN2A (Y1325) leads to the potentiation of NMDA-stimulated whole cell currents (Taniguchi et al., 2009) whereas SFK-mediated phosphorylation of GluN2B (Y1472) enhances GluN2B surface expression (Gao and Wolf, 2008) and synaptic expression (Nakazawa et al., 2001; Cheung and Gurd, 2001; Goebel-Goody et al., 2009). Synaptic GluN2B-containing NMDARs mediate ERK activation via CaMKIIα in mouse cultured cortical neurons (El Gaamouch et al., 2012).

Therefore, we hypothesized that BDNF/TrkB/SKF-mediated stimulation of synaptic GluN2A-containing NMDARs or GluN2B-contatining NMDARs underlies the BDNF-induced phosphorylation and activation of ERK in the dmPFC via CaMKIIα. In this experiment, the effect of BDNF on the phosphorylation of GluN2A (Y1325) or GluN2B (Y1472) in the dmPFC was examined 2 hr after the end of cocaine SA sessions. Further, the effect of a GluN2A-selective antagonist, TCN-201, or a GluN2B-selective antagonist, Ro-25-6981 on BDNF's effects was investigated. The reason for choosing the 2 hr timepoint is that previous study from our lab found that cocaine SA decreases pERK 2 h after the final session of cocaine SA and intra-dmPFC BDNF increases pERK level in the dmPFC of cocaine-naive rat at 0.5 h and 2 h, but not 6 h postinfusion and intra-dmPFC BDNF infused immediately after the last session of cocaine SA prevents the cocaine SA-mediated reduction in pERK.

Materials and Methods

Experimental Design. Figure 3.1 illustrates the design of all experiments in which rats were trained to self-administer cocaine for 2 hr/day and received intracranial infusions immediately after the end of cocaine SA. In Experiment 4, BDNF or PBS was infused without antagonists into cocaine SA or yoked-saline rats that were decapitated 2 hr later to investigate the effect of cocaine on GluN2A (Y1325) and GluN2B (Y1472) and whether BDNF would prevent such effects while confirming BDNF's ability to prevent cocaine SA-induced ERK dephosphorylation. In Experiments 5-6, rats were euthanized 2 hr after intra-PFC infusions of

glutamate receptor antagonists prior to BDNF or PBS in cocaine SA or yoked-saline rats to investigate whether GluN2A and/or GluN2B antagonists would block BDNF's ability to prevent cocaine SA-induced protein dephosphorylation, leading to cocaine-seeking.

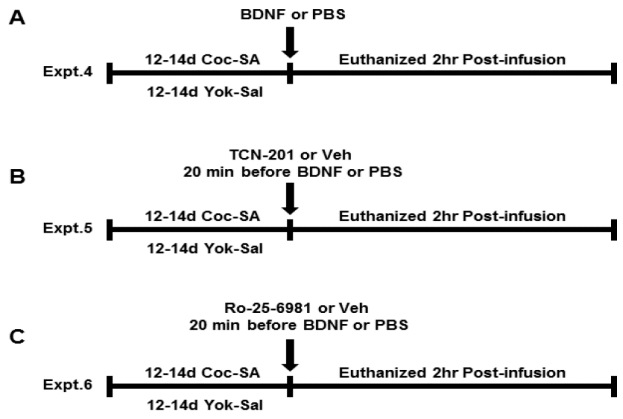

Figure 3.1. Schematic representation of the experimental designs for experiments 4-6.

Animals and surgery. Adult male Sprague Dawley rats (Charles River Laboratories, Raleigh, NC, USA; 275-300g on arrival) were individually placed in a temperature- and humidity-controlled room on a reversed light/dark cycle (lights off from 6AM-6PM). Rats were provided standard rodent chow with water available *ad libitum.* All experimental procedures were conducted under the guidelines and approval of the Institutional Animal Care and Use Committee (IACUC) of the Medical University of South Carolina and National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised 1996). Rats were housed singly in their home cages at least 4 days for acclimation before surgery.

On the day of surgery, rats were anesthetized with a mixture of ketamine (66 mg/kg, i.p.) and xylazine (1.33 mg/kg,i.p.), and then equithesin (0.5 ml/kg, i.p.) followed by ketorolac (2.0 mg/kg, i.p.). One end of a catheter was placed into the right jugular vein and the other end was connected to the back mount cannula pedestal (Med Associates) for intravenous infusions. After catheterization, 26 gauge bilateral guide cannulae (Plastics One) were placed 1 mm above the dmPFC target region [anterior-posterior (AP), +3.0; medial-lateral (ML), +/- 0.6; dorsoventral (DV),-1.6 relative to dural surface] (Paxinos and Watson 2005). Guide cannulae were affixed to the skull with cranioplastic cement and steel screws. Stylets the same length as guide cannulae (Plastics One) were inserted into the guide cannulae to avoid blockage throughout the experiment. Following surgery, rats were infused intravenously with 0.1 ml of cefazolin (33.3 mg/kg, i.p.) as well as an anti-microbial lock solution TCS (0.1ml; Norfolk Access Technologies) twice daily for 5 days.

Cocaine self-administration. After at least 5 days of surgical recovery, rats were subject to cocaine SA under a FR1 reinforcement schedule for 12-14 days (2hr/day). Rats were provided with a moderately restricted amount of food (20g standard chow/day) starting 1d before SA training to the last day of cocaine SA. The SA chamber contained a lever on the left (inactive lever) and right (active lever) side of one wall, and a circular stimulus light above each lever. A cocaine infusion line was connected to a swivel (Instech Laboratories) that allowed the rats to freely move in the chamber. Rats pressing the active lever were infused with cocaine hydrochloride (0.2mg/infusion; National Institute on Drug Abuse,

Research Triangle Park, NC) through a computer-controlled infusion pump paired with both light and tone cues (a white stimulus light above the active lever; 2 kHz, 15 dB above ambient noise). Following each cocaine infusion, there was a 20s timeout period when pressing on the active lever was counted, but no infusion of cocaine occurred. Pressing left (inactive) levers also was counted without any programmed consequences. The criterion for cocaine SA was more than 10 infusions per day. In Experiments 4-6, each rat in the yoked-saline group was matched to a rat in the cocaine SA group and was infused with 0.9% saline instead of cocaine when its paired rat received a cocaine infusion.

Intracranial infusions into the dmPFC. Immediately after the final SA session in all experiments, a 33 gauge bilateral infusion injector (Plastics One) was inserted into the guide cannula so that it protruded 1 mm below the tip of the guide cannula, targeting the anterior cingulate/prelimbic cortex. All intracranial infusions were conducted using 10 ul Hamilton gas-tight syringes connected to an infusion pump (Harvard Apparatus) that was programmed to deliver a 0.5 ul volume per side (0.25 ul/min). Injectors remained in the guide cannula for 1 min before and after infusions. In Experiment 5, the GluN2A-selective NMDAR antagonist, TCN-201 (0.1 nmol/0.5 ul/side; Tocris;) or 2% DMSO in PBS, was infused into the dmPFC. In Experiment 6, the GluN2B-selective NMDAR antagonist, Ro-25-6981 (2 ug/0.5 ul/side; Tocris;) or PBS, was infused into the dmPFC.

Verification of cannula placements and dmPFC punches. At the 2 hr time point after intracranial infusion in Experiments 4-6, rats were decapitated without

anesthesia, their brains were rapidly removed and frozen in isopentane (-20°C for 30 sec), and stored at -80°C. Brains were blocked in a cryostat, and 2 mm punches were taken in the dmPFC at the level of AP= 2.8-4.7. Cannula placements from rats in Experiments 4-6 are shown in Figure 3.2. Cannula placements were verified to determine that the dmPFC was punched and the AP extent of the punch was noted, but the precise placements were not mapped.

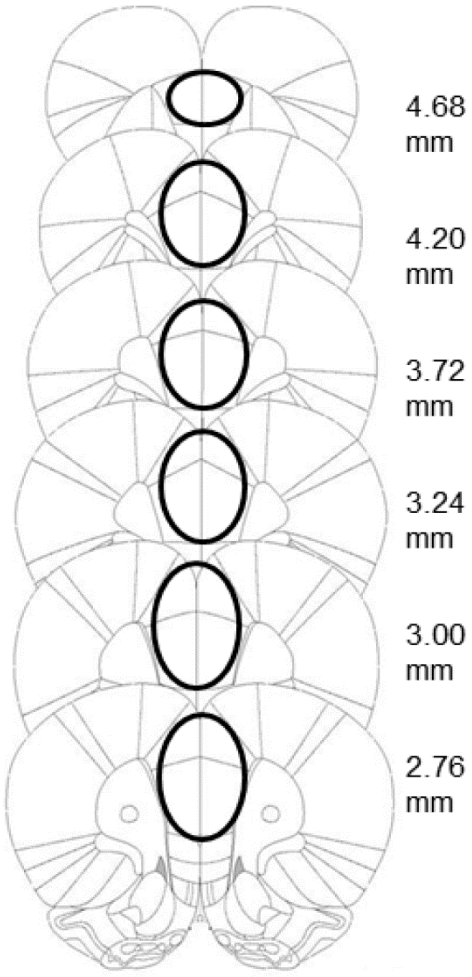

Figure 3.2. Areas punched from Experiments 4-6. Cannula placements within the dmPFC were verified by inspection during brain punching. Punch target was the anterior cingulate-prelimbic cortical areas (shown as large, unfilled circles).

Immunoblotting. For cell lysis and protein extraction, punched dmPFC tissue was sonicated in RIPA buffer and then centrifuged for 20 min at 10,000 g at 4°C.The resulting supernatant was collected and protein concentrations were measured using the BCA protein assay kit (Thermo Scientific). The same volume of protein sample was mixed with Laemmli buffer and heated for 5 minutes at 95°C and then loaded onto a 7.5% polyacrylamide gel (Bio-Rad). After separation of proteins using SDS-PAGE, gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Life Technologies). After 1 hr blocking in 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature, membranes were incubated overnight at 4°C with primary antibodies against pERK (1:4000, Cell Signaling Technology), pGluN2A (Y1325) (1:500, Abcam), and pGluN2B (Y1472) (1:500, Sigma-Aldrich) in 5% non-fat milk in TBST (pERK, pGluN2B) or in 5% bovine serum albumin (BSA) in TBST (pGluN2A). After three washes with 5% non-fat milk in TBST for 10 min each, membranes were incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000 for pERK, 1:3000 for pGluN2A, 1:1000 for pGluN2B; Millipore) for 1 hr at room temperature. After washing in TBST three times for 10 min, membranes were immersed in ECL Plus (Pierce) for 3-5 min followed by exposure to Hyperfilm (GE Healthcare Life Sciences) and then developed. After 10-15 min of stripping with ReBlot Plus Mild Antibody Stripping Solution (Millipore), membranes were re-probed for total proteins with primary antisera against total ERK (1:6000, Cell Signaling Technology), GluN2A (1:2000, Millipore), and GluN2B (1:500, BD Biosciences). The integrated density for each

protein was measured using Image J software (National Institutes of Health). To confirm whether equal amounts of protein were loaded in each lane, the samples were incubated with anti-calnexin antibody (1:6000, Enzo Life Sciences), and the ratio of total protein/calnexin (CNX) in each group was calculated and compared among groups. As there was no difference in total protein/CNX among groups, the ratio of each phosphoprotein/total protein was used for statistical analysis.

Statistical analysis. Two-way or one-way ANOVAs followed by Student–Newman–Keuls (SNK) multiple-comparison tests, or unpaired t-tests were used for the analysis of immunoblotting data. When the p-value was <0.05, results were considered statistically significant.

Results

Histology

The AP extent of the 2 mm punches in Experiments 4-6 are shown in Figure 3.2. Cannula placements extended from AP 3.0-4.7 and punches extended from 2.8-4.7. All cannula placements were located within the dmPFC and included in the schematic mapping and the data set for statistical analysis.

Experiment 4: intra-PFC BDNF prevented cocaine SA-induced reduction of pERK, pGluN2A and pGluN2B levels in the dmPFC during early withdrawal

To detect the effect of BDNF on phosphoproteins including pERK, pGluN2A (Y1325) and pGluN2B (Y1472) in the dmPFC, the levels of protein expression were measured at the 2hr time point after infusion of BDNF or PBS into the dmPFC in rats with a cocaine SA or yoked-saline history (see Fig.3.1A). Two-

way ANOVA found that the drug (cocaine vs saline; $F(1,25)=4.61$, $p<0.05$) and the intracranial infusion (BDNF vs PBS; $F_{(1,25)} =14.61$, $p<0.001$) had a significant main effect on pERK (Fig. 3.3A), but there was no interaction between drug and infusion ($F_{(1,25)=}1.01$, $p=0.32$). SNK multiple comparison tests revealed that, as previously reported (Whitfield et al., 2011), cocaine SA caused a profound reduction in pERK expression in the dmPFC of PBS-infused rats compared to yoked-saline treated rats infused with PBS (S-P vs C-P, $p<0.05$) and BDNF infusion not only prevented this decrease in cocaine SA rats (C-P vs C-B, $p<0.01$) but augmented the level of pERK in yoked-saline-treated rats (S-P vs S-B, $p<0.05$).

Two-way ANOVA found that the drug (cocaine vs saline; $F_{(1,26)} = 4.73$, $p < 0.05$) and intracranial infusion (BDNF vs PBS; $F_{(1,26)} = 20.33$, $p < 0.001$) had a significant main effect on pGluN2A (Y1325), (Fig. 3.3B), but there was no interaction between drug and infusion ($F_{(1,26)=}0.12$, $p=0.74$). SNK multiple comparison tests revealed that cocaine SA caused a profound reduction in pGluN2A (Y1325) expression in the dmPFC of PBS-infused rats compared to yoked-saline treated rats infused with PBS (S-P vs C-P; $p<0.05$) and BDNF infusion not only prevented this decrease in cocaine SA rats (C-P vs C-B, $p<0.05$) but augmented the level of pGluN2A (Y1325) in yoked-saline-treated rats (S-P vs S-B, $p<0.01$).

Two-way ANOVA found that the drug (cocaine vs saline; $F_{(1,26)} = 7.75$, $p < 0.05$) and intracranial infusion (BDNF vs PBS; $F_{(1,26)} = 21.45$, $p < 0.001$) had a

significant main effect on pGluN2B (Y1472) expression, (Fig. 3.3C), but there was no interaction between drug and infusion ($F_{(1,26)}$=0.60, p=0.44). SNK multiple comparison tests revealed that cocaine SA caused a profound reduction in pGluN2B (Y1472) expression in the dmPFC of PBS-infused rats compared to yoked-saline treated rats infused with PBS (S-P vs C-P, p<0.05) and that BDNF infusion not only prevented this decrease in cocaine SA rats (C-P vs C-B, p<0.01) but augmented the level of pGluN2B (Y1472) in yoked-saline-treated rats (S-P vs S-B, p<0.05). One way ANOVA found that the expression levels of tERK ($F_{(3,25)}$=1.85, p=0.16), tGluN2A ($F_{(3,26)}$=2.44, p=0.09) and tGluN2B ($F_{(3,26)}$ =0.79, p=0.51) were not significantly different among the four experimental groups when normalized to the calnexin loading control (data not shown).

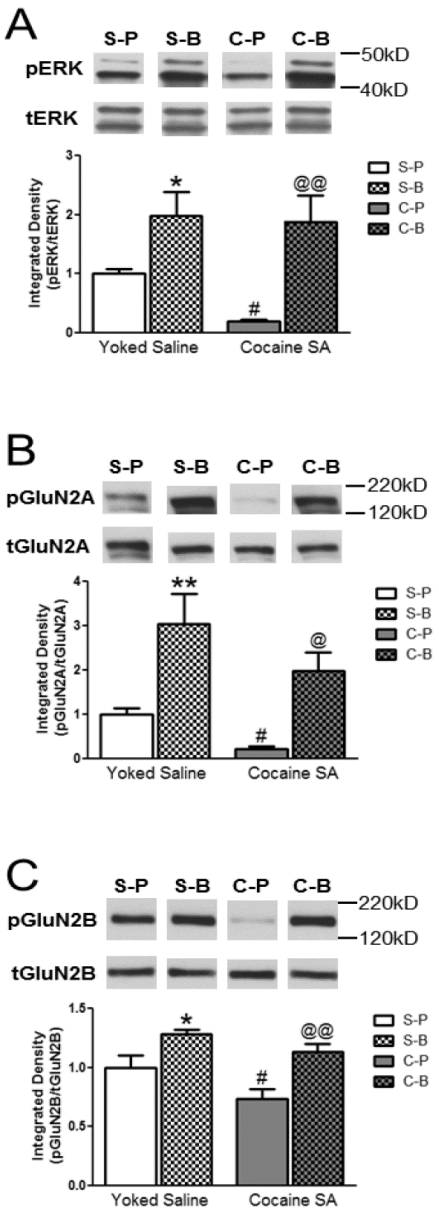

Figure 3.3. The effect of BDNF on the cocaine-induced reduction of pERK, pGluN2A (Y1325) and pGluN2B (Y1472) level, 2 hr after intra-dmPFC infusion. In Experiment 4, (A) pERK, (B) pGluN2A (Y1325), and (C) pGluN2B (Y1472) levels were significantly decreased in the cocaine SA (C-P) group compared to the yoked-saline group ($^{\#}p<0.05$). BDNF increased (A) pERK, (B) pGluN2A (Y1325), and (C) pGluN2B (Y1472) levels in the yoked-saline group (S-B) vs. the yoked-saline-PBS (S-P) group (*p<0.05, **p<0.01). BDNF prevented the decrease in (A) pERK, (B) pGluN2A (Y1325), and (C) pGluN2B (Y1472) levels in the cocaine SA groups ($^{@}p<0.05$, $^{@@}p<0.01$). The bar graphs indicate the mean ± SEM. S=Yoked-Sal, C=Cocaine SA, P=PBS, B=BDNF (n=5-9/group).

<u>Experiment 5: Activity of GluN2A subunit-containing NMDA receptors in the dmPFC is required for the effect of BDNF on pERK and pGluN2A but not pGluN2B level</u>.

In Experiment 5, all rats were pretreated with the GluN2A-selective antagonist, TCN-201, 20 min before infusion of BDNF or PBS into the dmPFC of rats with a cocaine SA or yoked-saline history and then decapitated 2 hr after infusion to investigate the involvement of GluN2A subunit-containing NMDA receptor activity on the ability of BDNF to prevent cocaine SA-mediated reduction in phospho-protein level in the dmPFC (see Fig. 3.4B). A two-way ANOVA found that TCN-201, had no effect on the ability of cocaine to decrease pERK levels in the dmPFC; there was

still a significant main effect of cocaine (cocaine vs saline; $F_{(1,25)=}$100.90, p<0.001; Fig. 9A). However, TCN-201 prevented the ability of BDNF to reverse the cocaine-induced decrease in P-ERK levels; there was no significant main effect of intracranial infusion (BDNF vs PBS) on pERK ($F_{(1,25)}$=0.17, p=0.68; Fig 3D). SNK multiple comparison tests found that, in the presence of TCN-201, cocaine SA caused a profound reduction in pERK level in the dmPFC of PBS-infused rats compared to yoked-saline treated rats infused with PBS (S-P vs C-P, p<0.001) and that TCN-201 effectively blocked BDNF's ability to increase pERK level in both yoked-saline treated rats infused with PBS (S-P vs S-B; p>0.05) and cocaine SA rats infused with PBS (C-P vs C-B; p>0.05), suggesting GluN2A subunit-containing NMDAR activity is essential for BDNF-mediated pERK level.

Similarly, a two-way ANOVA found that TCN-201 did not prevent the significant main effect of drug (cocaine vs saline; $F_{(1,25)}$=322.52 , p<0.001) on pGluN2A (Y1325) level (Fig. 3.4B). However, TCN-201 prevented the ability of BDNF to reverse the cocaine-induced decrease in pGluN2A levels; there was no significant main effect of intracranial infusion (BDNF vs PBS) on pGluN2A levels ($F_{(1,25)}$=0.20, p=0.66). SNK multiple comparison tests found that in the presence of TCN-201, pGluN2A level was significantly suppressed in the PBS-infused cocaine SA group compared to the PBS-infused yoked-saline group (S-P vs C-P, p <0.001) and that TCN-201 infusion blocked the ability of the BDNF to increase pGluN2A level compared to the PBS-infused rats in both yoked-saline (S-P vs S-B; p>0.05) and cocaine SA groups (C-P vs C-B; p>0.05), suggesting GluN2A

subunit-containing NMDARs activity is essential for BDNF-mediated pGluN2A level.

To confirm the specificity of TCN-201 on GluN2A subunit-containing NMDARs, the effect of TCN-201 on pGluN2B (Y1472) level was investigated. Two-way ANOVA found that when TCN-201 was infused before BDNF or PBS (Fig. 3.4C), the drug (cocaine vs saline; $F_{(1,25)}$=17.49, p<0.001) and the intracranial infusion (BDNF vs PBS; $F_{(1,25)}$=54.24, p<0.001) had a significant main effect on pGluN2B (Y1472) but there was no interaction between the drug and the intracranial infusion ($F_{(1,25)}$=3.59, p=0.07). SNK multiple comparison tests found that, in the presence of TCN-201, cocaine SA caused a profound reduction in pGluN2B (Y1472) level in the dmPFC of PBS-infused rats compared to yoked-saline treated rats infused with PBS (S-P vs C-P, p<0.001) and that BDNF infusion not only prevented this decrease in cocaine SA rats (C-P vs C-B, p<0.01) but augmented the level of pGluN2B (Y1472) in yoked-saline-treated rats (S-P vs S-B, p<0.001). As TCN-201 pretreatment before BDNF did not inhibit the ability of BDNF to enhance pGluN2B level in both yoked-saline and cocaine SA groups, this suggest the dose of TCN-201 used in the current study selectively blocked GluN2A subunit-containing NMDARs but not GluN2B subunit-containing NMDARs.

A one way ANOVA found that, in the presence of TCN-201, the level of tERK ($F_{(3,25)}$=2.70, p=0.07), tGluN2A ($F_{(3,25)}$=2.45, p=0.09) or tGluN2B ($F_{(3,25)}$=2.12, p=0.12) was not significantly different among experimental groups.

To confirm whether TCN-201 infusion into the dmPFC affected the basal level of pERK, pGluN2A (Y1325), or pGluN2B (Y1472) level, yoked saline-treated rats infused with PBS from experiment 4 were compared to yoked saline-treated rats infused with TCN-201 before PBS from experiment 5. An unpaired t-test found that there was no significant difference between these groups for pERK (t_{10}=0.27, p=0.79), pGluN2A (t_{10}= 1.42, p=0.19), and pGluN2B (t_{10}=1.26, p=0.24), respectively.

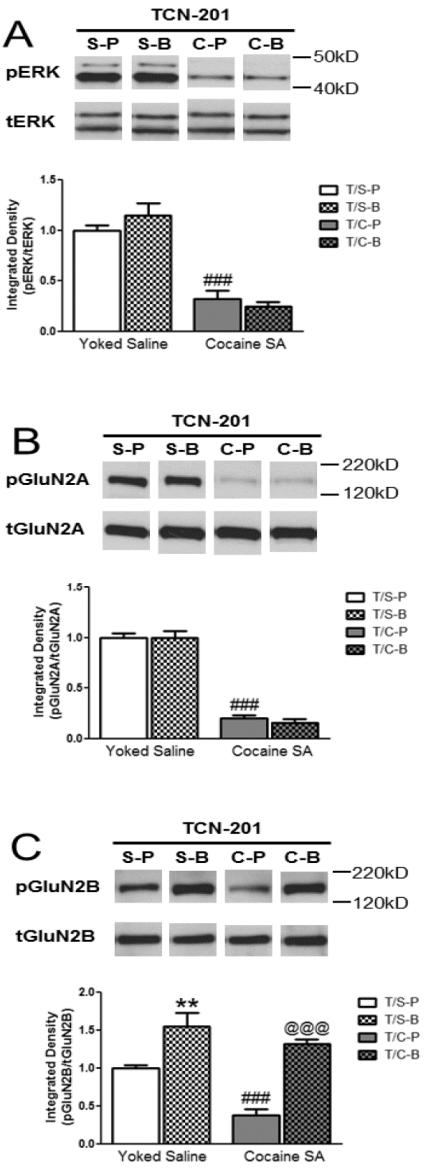

Figure 3.4. The effect of BDNF in the presence of TCN-201 on the cocaine-induced reduction of pERK, pGluN2A (Y1325) and pGluN2B (Y1472) levels, 2 hr after intra-dmPFC infusion. In Experiment 5, intra-dmPFC TCN-201 prevented the ability of BDNF to increase (D) pERK and, (E) pGluN2A (Y1325), but not (F) pGluN2B (Y1472) levels in the dmPFC of yoked-saline rats. TCN-201 did not prevent the ability of cocaine SA to decrease (D) pERK, (E) pGluN2A (Y1325), and (F) pGluN2B (Y1472) levels in the dmPFC vs. the yoked-saline group ($^{###}p<0.001$). TCN-201 reversed the ability of BDNF to prevent the cocaine SA–induced decrease in (D) pERK and, (E) pGluN2A (Y1325), but not (F) pGluN2B (Y1472) levels in the dmPFC. The bar graphs indicate the mean ± SEM. S=Yoked-Sal, C=Cocaine SA, P=PBS, B=BDNF, T=TCN-201 (n=7-8/group).

Experiment 6: Activity of GluN2B subunit-containing NMDA receptors in the dmPFC is required for the effect of BDNF on pERK and pGluN2B but not pGluN2A level

In this experiment, all rats were pretreated with the GluN2B-selective antagonist, Ro-25-6981, 20 min before infusion of BDNF or PBS into the dmPFC in rats with a cocaine SA or yoked-saline history and then decapitated 2 hrs after infusions to check the involvement of GluN2B subunit-containing NMDARs activity in the ability of BDNF to recover pERK and pGluN2B levels in the dmPFC (see Fig. 3.1C). A two-way ANOVA found that Ro-25-6981, had no effect on the ability of cocaine to decrease P-ERK levels in the dmPFC; there was still a significant main effect of cocaine (cocaine vs saline; $F_{(1,22)}$=11.12, p<0.01; Fig. 3.5A). However, Ro-25-6981 prevented the ability of BDNF to reverse the cocaine-induced decrease in P-ERK levels; there was no significant main effect of intracranial infusion (BDNF vs PBS) on pERK ($F_{(1,22)}$=1.05, p=0.32). SNK multiple comparison tests found that in the presence of Ro-25-6981 cocaine SA caused a profound reduction in pERK level in the dmPFC of PBS-infused rats compared to yoked-saline treated rats infused with PBS (S-P vs C-P, p<0.01) and that Ro-25-6981 effectively blocked BDNF's ability to increase pERK level in both yoked-saline treated rats infused with PBS (S-P vs S-B; p>0.05) and cocaine SA rats infused with PBS (C-P vs C-B; p>0.05), suggesting GluN2B subunit-containing NMDARs activity is essential for BDNF-mediated pERK up-regulation.

Similarly, a two-way ANOVA found that Ro-25-6981 did not prevent the significant main effect of drug (cocaine vs saline; $F_{(1,22)}$= 15.31, p<0.01) on pGluN2B (Y1472) level (Fig. 3.5B). However, Ro-25-6981 prevented the ability of

BDNF to reverse the cocaine-induced decrease in pGluN2B levels; there was no significant main effect of intracranial infusion (BDNF vs PBS) on pGluN2B levels ($F_{(1,22)}$= 0.76, p=0.39). SNK multiple comparison tests found that in the presence of Ro-25-6981, pGluN2B level was significantly suppressed in the PBS-infused cocaine SA group compared to the PBS-infused yoked-saline group (S-P vs C-P, p <0.01) and that Ro-25-6981 infusion blocked the ability of the BDNF to increase pGluN2B level compared to the PBS-infused rats in both yoked-saline (S-P vs S-B; p>0.05) and cocaine SA groups (C-P vs C-B; p>0.05), suggesting GluN2B subunit-containing NMDARs activity is essential for BDNF-mediated pGluN2B level.

To confirm the specificity of Ro-25-6981 on GluN2B subunit-containing NMDARs, the effect of Ro-25-6981 on pGluN2A (Y1325) level was investigated. Two-way ANOVA found that when TCN-201 was infused before BDNF or PBS (Fig. 3.5C), the intracranial infusion (BDNF vs PBS; $F_{(1,22)}$=28.73, p<0.001), but not the drug (cocaine vs saline; $F_{(1,22)}$ =0.01, p=0.94) had a significant main effect on pGluN2B (Y1472). SNK multiple comparison tests found that, in the presence of Ro-25-6981, cocaine SA caused a profound reduction in pGluN2A (Y1325) level in the dmPFC of PBS-infused rats compared to yoked-saline treated rats infused with PBS (S-P vs C-P, p<0.05) and that BDNF infusion not only prevented this decrease in cocaine SA rats (C-P vs C-B, p<0.001) but augmented the level of pGluN2B (Y1472) in yoked-saline-treated rats (S-P vs S-B, p<0.05). As Ro-25-6981 pretreatment before BDNF did not inhibit the ability of BDNF to enhance pGluN2A level in both yoked-saline and cocaine SA groups,

this suggest the dose of Ro-25-6981 used in the current study selectively blocked GluN2B subunit-containing NMDARs but not GluN2A subunit-containing NMDARs.

A one way ANOVA found that, in the presence of Ro-25-6981, the level of tERK ($F_{(3,22)}$=2.05, p=0.14), tGluN2A ($F_{(3,22)}$ =2.21, p=0.12) and tGluN2B ($F_{(3,22)}$ =2.35, p=0.10) was not significantly different among experimental groups. To confirm whether Ro-25-6981 infusion into the dmPFC affected the basal level of pERK, pGluN2A (Y1325), or pGluN2B (Y1472), yoked saline-treated rats infused with PBS from experiment 4 were compared to yoked saline-treated rats infused with TCN-201 before PBS from experiment 5. An unpaired t-test found that there was no significant difference between these groups for pERK (t8= 0.11, p=0.91), pGluN2A (t8=0.69, p=0.51), and pGluN2B (t8=1.54, p=0.16), respectively (data not shown).

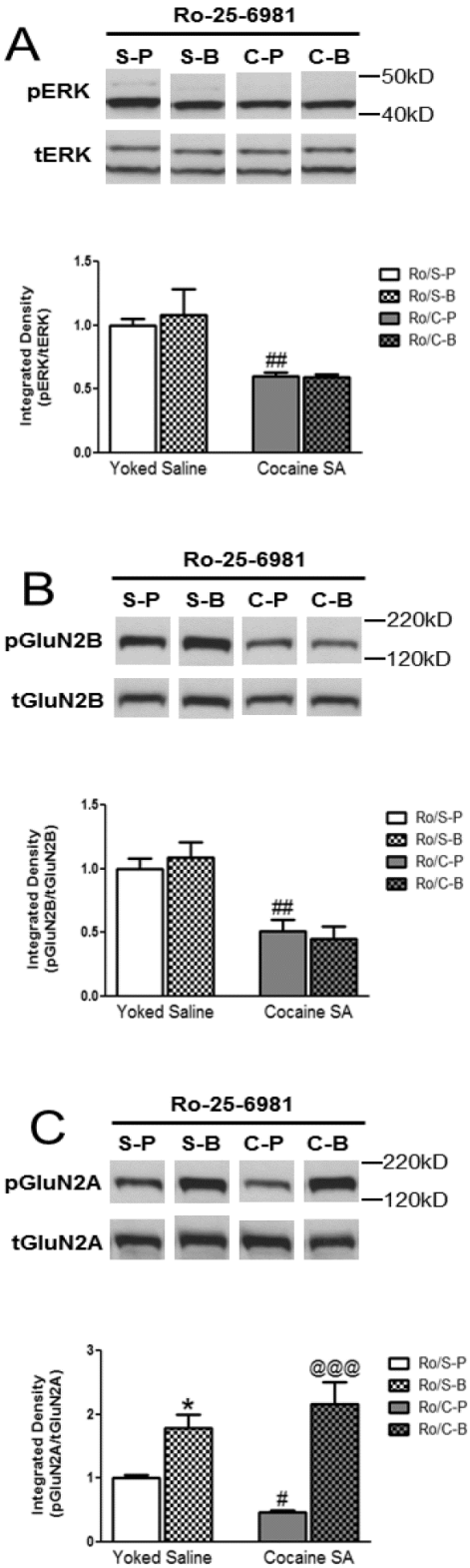

Figure 3.5. The effect of BDNF in the presence of Ro-25-6981 on the cocaine-induced reduction of pERK, pGluN2A (Y1325) and pGluN2B (Y1472) levels**, 2 hr after intra-dmPFC infusion.** In Experiment 6, intra-dmPFC Ro-25-6981 prevented the ability of BDNF to increase (D) pERK and, (E) pGluN2B (Y1472), but not (F) pGluN2A (Y1325) levels in the dmPFC of yoked-saline rats. Ro-25-6981 did not prevent the ability of cocaine SA to decrease (D) pERK, (E) pGluN2A (Y1325), and (F) pGluN2B (Y1472) levels in the dmPFC vs. the yoked-saline group ($^{\#}p,0.05$, $^{\#\#}p<0.01$). Representative Western blot images for each protein are shown above graphs. The positions and sizes of the protein molecular weight markers (kDa) are indicated on the right. The bar graphs indicate the mean ± SEM. S=Yoked-Sal, C= Cocaine SA, P=PBS, B=BDNF, Ro=Ro-25-6981 (n=5-8 per group)**.**

Discussion

Cocaine SA suppressed the level of pERK, pGluN2A, and pGluN2B in the dmPFC.

We recapitulated cocaine SA-induced ERK dephosphorylation in the dmPFC (Whitfield et al., 2011) and showed

concurrent suppression of pGluN2A (Y1325) and pGluN2B (Y1472) levels 2 hr after the last cocaine SA session. Although the mechanisms underlying these dephosphorylation events are not yet defined, our preliminary evidence points to the activation of striatal-enriched tyrosine phosphatase (STEP) in the dmPFC 2 hr after the end of cocaine SA (Sun et al., 2013). STEP dephosphorylates and inactivates ERK and GluN2A/B directly as well as indirectly through dephosphorylation and inactivation of SFKs (Paul et al., 2003; Pulido et al., 1998; Nguyen et al., 2002; Iwasaki et al., 1998; Huang and McNamara, 2010). STEP-mediated dephosphorylation of GluN2B at Tyr1472 induces endocytosis (Snyder et al., 2005), leading to the inhibition of NMDAR-mediated ERK phosphorylation in cortical neuronal cultures (Braithwaite et al., 2006). The Y1325 residue of GluN2A is located within the C-terminal domain (Zheng et al., 1998) and truncation of the GluN2A subunit lacking the C-terminal domain decreases the basal level of ERK2 activity (Moody et al., 2011). Suppression of pERK, pGluN2A (Y1325) and pGluN2B (Y1472) levels in the dmPFC within 2 hr of the end of cocaine SA suggests a profound cocaine-mediated hypofunction of dmPFC. This discovery is consistent with reduced basal activity of the dmPFC (Sun and Rebec, 2006) and reduced BDNF and immediate early gene mRNA levels within 24 hr after 10 days of cocaine SA (McGinty et al., 2010).

Cocaine SA-induced reduction of pERK, pGluN2A, or pGluN2B was not associated with a reduction in the total levels of these proteins 2 hr after the last session of cocaine SA in this study, suggesting that the tyrosine dephosphorylation and reduced functional impact of both GluN2 subunits and

ERK, but not state-independent total protein expression, are critical for triggering subsequent cocaine-seeking. Similarly, SFK-mediated tyrosine phosphorylation of synaptic GluN2A did not affect the total GluN1 and GluN2A expression in adult rat hippocampal slices (Goebel-Goody et al., 2009). However, previous studies have reported that a decrease in total GluN1 and GluN2B as well as NMDAR binding in the dmPFC occurs during early withdrawal from short access cocaine SA (Ben-Shahar et al., 2007; Sun et al., 2013).

BDNF infusion into the dmPFC prevents cocaine-mediated dephosphorylation events.

In the current study, the intra-dmPFC BDNF infusion immediately after the last session of cocaine SA normalized a cocaine-mediated reduction in pGluN2A (Y1325) and pGluN2B (Y1472). BDNF also prevented the cocaine-induced decrease in the levels of pERK previously reported to be mediated by TrkB (McGinty et al., 2010; Whitfield et al., 2011). BDNF-TrkB signaling phosphorylates and activates ERK and GluN2A/B directly or indirectly through phosphorylation and activation of SFKs. BDNF stimulates the association of TrkB with Src or Fyn, a member of SFK, leading to SFKs activation and subsequent activation of TrkB signaling including ERK (Iwasaki et al., 1998; Huang and McNamara, 2010; Narisawa-Saito et al., 1999). SFKs phosphorylate GluN2A (Y1325), leading to potentiation of NMDA-stimulated whole cell currents (Taniguchi et al., 2009). SFKs also phosphorylate GluN2B (Y1472), resulting in an increase in GluN2B synaptic expression (Nakazawa et al., 2001; Cheung and

Gurd, 2001; Goebel-Goody et al., 2009) and surface expression (Gao and Wolf, 2008).

In the present study, TCN-201 pretreatment before BDNF was effective in inhibiting the BDNF-mediated increase in pERK and pGluN2A, but not pGluN2B levels, whereas Ro-25-6981 blocked BDNF-mediated up-regulations of pERK and pGluN2B, but not pGluN2A. As the activity of NMDARs is required for the association between Fyn and GluN2A subunits in rat hippocampus (Hou et al., 2002), the results of the present study suggest that the dose of TCN-201 and Ro-25-6981 used in the current study is selective for GluN2A and Glu2B, respectively, and that both GluN2A-contatining NMDARs and GluN2B-contatining NMDARs are essential for the BDNF-mediated pERK augmentation. The inhibitory effect of TCN-201 and ifenprodil (Ro-25-6981 is a derivative of ifenprodil) on GluN1/GluN2A and GluN1/GluN2B diheteromers of NMDARs is much higher than their inhibitory effect on GluN1/GluN2A/GluN2B triheteromers of NMDARs (Hansen et al., 2014). Therefore, BDNF-mediated upregulation of pERK, pGluN2A and pGluN2B is likely to occur through GluN1/GluN2A diheteromers or GluN1/GluN2B diheteromers, not triheteromers.

Stimulation of NMDARs has opposite effects on ERK activity depending on their relative locations at the synapse. Synaptic NMDAR activity mediates ERK activation but activation of extrasynaptic NMDARs induces ERK inactivation (Ivanov et al., 2006) and BDNF reverses the extrasynaptic NMDAR-mediated ERK inactivation (Mulholland et al., 2008). TrkB receptors are localized in postsynaptic densities (PSD) of excitatory glutamatergic synapses in the adult rat

cerebral cortex (Aoki et al., 2000) and the co-localization between the TrkB and GluN1 on the cellular surface is increased at glutamatergic synapses during development in dissociated cultures of cortical neurons (Gomes et al., 2006). The SFKs, Fyn and Src, are expressed higher in synaptic regions than in extrasynaptic regions in adult rat hippocampal slices (Goebel-Goody et al., 2009) and binding of SFKs to PSD through the SH2-domain of SFK is required for the tyrosine phosphorylation of GluN2A and GluN2B by Fyn and Src (Cheung and Gurd, 2001). PSD-95, also known as SAP-90 (synapse-associated protein 90) (Kornau et al., 1995), a synaptic scaffolding protein highly expressed in the PSD (Cho et al., 1992) mediates the association of GluN2A with Fyn/Src and facilitates Fyn/Src-mediated tyrosine phosphorylation of GluN2A (Tezuka et al., 1999; Ma and Zhang, 2003; Hou et al., 2003; Chen et al., 2003) Furthermore, inhibition of SFK decreases the tyrosine phosphorylation of GluN2A in synaptic but not extrasynaptic regions in adult rat hippocampal slices, and BDNF enhances tyrosine phosphorylation of GluN2B in rat cortical PSD fractions (Lin et al., 1998; Goebel-Goody et al., 2009). Together, these data suggest that GluN2A-containing NMDARs and GluN2B-containing NMDARs, both of which are located at synaptic regions, may underlie BDNF-mediated ERK phosphorylation and activation through SFKs.

In the present study, TCN-201 or Ro-25-6981 infusion without BDNF did not affect the levels of pERK, pGluN2A, and pGluN2B in yoked-saline rats compared to vehicle-infused rats (S-P vs T/S-P; S-P vs Ro/S-P), indicating that the TCN-201 or Ro-25-6981 treatment had no effect on basal phosphoprotein

levels. NMDAR activity is highly inhibited under normal neuronal activity because the NMDAR channel is blocked by magnesium. Therefore, TCN-201 or Ro-25-6981 under basal conditions without BDNF would have no effect on GluN2A- or GluN2B-containing NMDAR activity or on tyrosine phosphorylation of GluN2 subunits targeted by SFK because the activity of NMDARs is required for the association between SFK and GluN2 subunits, as demonstrated in rat hippocampus (Hou et al., 2002). Further, it is likely that TCN-201 or Ro-25-6981 infusion in the absence of BDNF did not alter the cocaine-induced reductions in pGluN2A and pGluN2B levels compared to vehicle-infused rats (D-P vs T-P; P-P vs Ro-P) because cocaine SA decreased the phosphorylation state of these receptors. However, our results show that the doses of TCN-201 and Ro-25-6981 used in the current study were effective because they reversed BDNF's ability to restore pGluN2B and pGluN2A levels. Therefore, neither TCN-201 nor Ro-25-6981 infusion had an effect on the phosphorylation state of GluN2A- or GluN2B-containing NMDARs in the cocaine SA group in the absence of BDNF and, likewise, exerted no significant effect on cocaine-seeking.

In contrast, in the present study BDNF infusion into the dmPFC increased phosphorylation of GluN2A and GluN2B at SFK target sites compared to PBS-infused rats in both yoked-saline and cocaine SA groups and effectively suppressed cocaine-seeking in rats with a cocaine SA history. As discussed above, functional activity of NMDARs is likely to be low in both yoked-saline and cocaine SA groups due to voltage-sensitive Mg^{2+} block and the activity of NMDARs is required for the association between SFK and GluN2 subunits (Hou

et al., 2002). Therefore, the current finding of BDNF-mediated phosphorylation of GluN2A and GluN2B at SFK target sites is surprising. However, in adult cocaine-naïve rats, BDNF infusion into the rat NAc core increases cell surface expression of the GluA1 subunit of AMPA receptors in an ERK- or protein synthesis-dependent manner within 30 min post-injection (Li and Wolf, 2011). This suggests that intra-dmPFC BDNF could effectively remove the magnesium block from NMDARs in the dmPFC within 2hr of its infusion by increasing functional AMPA receptors on the cellular surface of the dmPFC, allowing interaction between SFK and GluN2 subunits, which results in phosphorylation of GluN2A and GluN2B at SFK target sites. As the phosphorylation of GluN2A (Y1325) and GluN2B (Y1472) increases single NMDAR channel activity (Xu et al., 2006) and/or synaptic (Nakazawa et al., 2001; Cheung and Gurd, 2001; Goebel-Goody et al., 2009) and surface expression (Gao and Wolf, 2008) and as either AMPAR or NMDAR contributes to the depolarization of spine heads (Grunditz et al., 2008), the BDNF-mediated phosphorylation of GluN2A and GluN2B would further activate NMDARs by both the phosphorylation of GluN2A and GluN2B at SFK sites and the removal of the magnesium block and restoring synaptic activity in the dmPFC.

CHAPTER 4

SUMMARY, DISCUSSION, AND FUTURE DIRECTIONS

Summary and Significance

New signaling cascades underlying the suppressive effect of intra-dmPFC BDNF on cocaine seeking in rats with a cocaine SA history have been discovered. In this dissertation I showed that two hr after the last session of cocaine SA, pERK, pGluN2A (Y1325) and pGluN2B (Y1472) levels in the dmPFC were decreased. BDNF infusion into the dmPFC immediately following the last session of cocaine SA prevented this reduction of phospho-proteins, suggesting that intra-dmPFC BDNF ameliorates cocaine SA-induced hypofunction in the dmPFC during early withdrawal from cocaine. Inhibition of GluN2A-containing NMDARs in the dmPFC with TCN-201 blocked the BDNF-mediated increase in pERK and pGluN2A (Y1325), but not pGluN2B (Y1472), and blockade of GluN2B-containing NMDARs in the dmPFC with Ro-25-6981 suppressed BDNF-mediated elevation

of pERK and pGluN2B (Y1472), but not pGluN2A (Y1325). The effective inhibition of intra-dmPFC BDNF's suppression of cocaine-seeking either by TCN-201 or Ro-25-6981 indicates that either inhibition of GluN2A-containing or GluN2B-containing NMDARs is enough to block BDNF-induced enhancement of pERK and BDNF's suppression of cocaine-seeking. These findings also suggest that co-activation of GluN2A-containing NMDARs and GluN2B-containing NMDARs is required for BDNF-induced ERK activation and BDNF's suppressive effect on cocaine-seeking because TCN-201 or Ro-25-6981 infusion before BDNF did not prevent BDNF-mediated pGluN2B or pGluN2A levels, respectively. However, either drug effectively blocked BDNF's suppression of cocaine-seeking. Together, the current body of work suggests that BDNF-mediated co-activation of both GluN2A-containing NMDA receptors and GluN2B-containing NMDA receptors underlie ERK activity in the dmPFC, and prevents relapse to cocaine-seeking.

These studies provide novel and important findings that enhance our understanding of the molecular mechanisms underlying the relapse to cocaine-seeking. First, the cocaine SA-mediated decrease in GluN2 phosphorylation in the dmPFC provides new evidence that the dmPFC is hypofunctional during early withdrawal, leading to subsequent cocaine-seeking. Second, exogenous BDNF-mediated phosphorylation of both GluN2A and GluN2B in the rat dmPFC is notable considering that there are few *in vivo* studies showing the role of the BDNF/TrkB signaling pathway in the phosphorylation of GluN2 subunits and subsequent modulation of NMDAR activity. Third, BDNF-mediated activation of

NMDARs containing GluN2A or GluN2B subunits in the dmPFC is critical to the enduring ability of BDNF to suppress cocaine-seeking. Fourth, the fact that both GluN2A-containing and GluN2B-containing NMDARs mediate the BDNF-induced ERK activation in the dmPFC which underlies the suppressive effect of BDNF on cocaine-seeking sheds light on GluN2 regulation of NMDARs in the dmPFC as a potential molecular target linked to cocaine-seeking. Finally, although inhibition of either GluN2A-containing NMDARs or GluN2B-containing NMDARs is sufficient to inhibit BDNF-mediated increase in pERK level and BDNF's suppression of cocaine-seeking, co-activation of GluN2A-containing NMDARs and GluN2B-containing NMDARs is necessary for BDNF-induced ERK activation and BDNF's suppressive effect on cocaine-seeking. Given that NMDARs are important modulators of cocaine-mediated neuroadaptations, a further discussion to explain plausible reasons why activation of both GluN2A-containing and GluN2B-containing NMDARs is required for BDNF's effect on ERK activity and on suppression of cocaine-seeking will be provided. Moreover, the homeostatic role of BDNF in the regulation of synaptic plasticity in the dmPFC linked to the modulation of presynaptic release of glutamate into the NAc core will be discussed.

Inhibition of either GluN2A-containing NMDARs or GluN2B-containing NMDARs blocks BDNF's suppressive effect on cocaine-seeking in a similar way

We initially hypothesized that blockade of GluN2A-containing NMDARs or GluN2B-containing NMDARs would inhibit BDNF's suppressive effect on cocaine-seeking differently because of their dissimilar characteristics, but we

found in the current study that either inhibition of GluN2A-containing NMDARs or GluN2B-containing NMDARs similarly inhibits BDNF's suppressive effect on cocaine-seeking.

GluN2A-containing NMDARs and GluN2B-containing NMDARs have different ion channel kinetics. GluN2 subunit compositions of NMDARs regulate the kinetic properties of NMDARs. GluN2A-containing NMDARs have a higher open probability than GluN2B-containing NMDARs in response to brief application of glutamate. In contrast, as GluN2B-containing NMDARs open longer than GluN2A-containing NMDARs, they sustain a greater activation for longer periods. Together, GluN2A-containing NMDARs show a quicker and sharper increase in open probability which lasts a shorter time and GluN2B-containing NMDARs show a slower increase in opening probability that endures for longer periods. Theses kinetic properties also affect their responses to stimulation at different frequencies. Under high frequency tetanic stimulation (100 Hz, a protocol for inducing LTP), GluN2A-containing NMDARs transfer more current but when stimulated at low frequencies (1Hz, a protocol for inducing LTD) GluN2B-contatining NMDARs pass more current (Erreger et al., 2005; Vicini et al., 1998; Cull-Candy et al., 2001). In the dendritic spines of rat CA1 pyramidal neurons, small-volume spines have more GluN2B-containing NMDARs that contribute to longer current and more calcium per unit of current than those of large-volume spines (Sobczyk et al., 2005). Together, this suggests that GluN2A-containing NMDARs and GluN2B-containing NMDARs contribute to ERK activity to a different degree, because synaptic NMDAR-mediated Ca^{2+} influx induces

phosphorylation of ERK and CREB in cultured hippocampal neurons (Hardingham et al., 2001).

In addition, many previous studies suggest opposite roles of GluN2A-containing NMDARs and GluN2B-containing NMDARs in ERK activity due to their different locations relative to the synapse. During development, synaptic GluN2B-containing NMDARs are replaced by GluN2A-containing NMDARs and transferred to extrasynaptic regions, leading to predominant expression of GluN2A-containing NMDARs at the synaptic regions and GluN2B-containing NMDARs at extrasynaptic regions (Dumas, 2005). Synaptic NMDARs up-regulate ERK activity but extrasynaptic NMDARs down-regulate it (Ivanov et al., 2006). However, a recent study shows that both GluN2A-containing NMDARs and GluN2B-containing NMDARs are localized in both the synaptic and extrasynaptic regions (Thomas et al. 2006). Moreover, most studies indicating the discrete localization of GluN2A-containing NMDARs and GluN2B-containing NMDARs relative to the synapse are derived from primary sensory cortices such as visual cortex not PFC. In addition, the functional neurotransmission via GluN2B-containing NMDARs emerges only after the late adolescent period in the rat medial PFC including the prelimbic and infralimbic regions and this emergence of GluN2B-mediated neurotransmission is essential for the ventral hippocampal-induced LTP in the dmPFC of P50–80 mice (Flores-Barrera et al., 2014). Further, in the telencephalon of mice, phosphorylation of Y1472 GluN2B, which is associated with the synaptic expression of GluN2B-containing NMDA receptors (Goebel-Goody et al., 2009), is gradually enhanced during postnatal

development (P3-P56) (Nakazawa et al., 2001). Together, these results support our findings in the current study that either inhibition of GluN2A-containing NMDARs or GluN2B-containing NMDARs is similarly effective to block BDNF's suppressive effect on cocaine-seeking.

Activation of both GluN2A-containing NMDARs and GluN2B-containing NMDARs is required to mediate BDNF's suppressive effect on cocaine-seeking

Although we found in the current study that inhibition of either GluN2A-containing NMDARs or GluN2B-containing NMDARs is sufficient to block BDNF-mediated pERK enhancement and BDNF's suppressive effect on cocaine-seeking, activation of either GluN2A-containing NMDARs or GluN2B-containing NMDARs is not enough to mediate BDNF-induced pERK up-regulation and BDNF's suppressive effect on cocaine-seeking. Instead, activity of both GluN2A-containing NMDARs and GluN2B-containing NMDARs is necessary to mediate BDNF's effects. In the present study infusion of the GluN2A-selective antagonist, TCN-201, before BDNF did not prevent BDNF-mediated pGluN2B enhancement, suggesting that TCN-201 infusion before BDNF did not affect BDNF-induced enhancement of GluN2B-containing NMDAR function; however, the TCN-201 pretreatment effectively blocked BDNF-induced pERK up-regulation and BDNF's suppression of cocaine-seeking. Together, this suggests that activation of GluN2B-containing NMDARs is not sufficient to mediate BDNF's effects and that co-activation of GluN2A-containing NMDARs and GluN2B-containing NMDARs is required to mediate BDNF's effects. Similarly, the GluN2B-selective antagonist, Ro-25-6981, before BDNF did not prevent BDNF-enhanced pGluN2A level;

however, the Ro-25-6981 pretreatment effectively blocked BDNF-induced pERK level and BDNF's suppression of cocaine-seeking, suggesting that activation of GluN2A-containing NMDARs is not sufficient to mediate BDNF's effects and that co-activation of GluN2A-containing NMDARs and GluN2B-containing NMDARs is required to mediate BDNF's effects. The requirement of co-activation of GluN2A-containing NMDARs and GluN2B-containing NMDARs for BDNF's effects may be explained by their co-localization in the same pyramidal neurons of dmPFC.

GluN2A and GluN2B are expressed and distributed similarly both at apical and basal dendrites in layer V neurons in the mPFC of mice (postnatal days 21–33) (Balsara et al., 2014). In dendritic spines of rat CA1 pyramidal neurons, small-volume spines contain more GluN2B-containing NMDARs that contribute to longer current and more calcium per unit of current than those of large-volume spines (Sobczyk et al., 2005). GluN2A-containing NMDARs are involved in basolateral amygdala (BLA)-induced LTP whereas GluN2B-containing NMDARs are essential for ventral hippocampal-induced LTP in the dmPFC of P50–80 mice (Flores-Barrera et al., 2014). Together, this indicates that both GluN2A-containing NMDARs and GluN2B-containing NMDARs may be located in the same pyramidal neurons of dmPFC and that while GluN2A-containing NMDARs may be located preferentially at large-volume spines where glutamatergic afferents from the ventral hippocampus (vHipp) make synaptic connection, GluN2B-containing NMDARs may be located preferentially at small-volume spines where glutamatergic afferents from the BLA make synaptic contact.

Selective inhibition of neuronal activity in the ventral hippocampus (Rogers and See, 2007), BLA, or dmPFC (McLaughlin and See, 2003) immediately before a reinstatement test suppresses cocaine-seeking induced by cocaine-associated cues. Further, selective inhibition of neuronal activity in the ventral hippocampus (Lasseter et al., 2010), BLA, or dmPFC (Fuchs et al., 2005) immediately before a reinstatement test decreases context-induced cocaine-seeking. Together, this suggests that although inhibition of either BLA or the ventral hippocampus immediately before a reinstatement test is sufficient to suppress cocaine-seeking, activation of either BLA or the ventral hippocampus is not enough to mediate reinstatement of cocaine-seeking. Instead, activity of both BLA and the ventral hippocampus is necessary to mediate reinstatement of cocaine-seeking. This also indicates that dmPFC, which receives glutamatergic input from both vHipp and BLA, may play a functional role as a common gate in the neural circuitry underlying cocaine-seeking. If we assume that GluN2A-containing NMDARs and GluN2B-containing NMDARs may be located in the same pyramidal neuron rather than in different subpopulations of pyramidal neurons of dmPFC, we could explain better the reason for the requirement of both BLA and ventral hippocampus activity to mediate reinstatement of cocaine-seeking.

Although GluN2A-contatining NMDARs and GluN2B-contatining NMDARs may be segregated on different spines of the same pyramidal neurons in dmPFC that are innervated by discrete glutamatergic from different brain regions, a convergence of inputs from both BLA and vHipp onto the same pyramidal neurons of dmPFC may be required to reach a threshold level of intracellular

Ca^{2+} concentration that is required for the full and sustained activation of ERK. This convergence would explain why co-activation of GluN2A-containing NMDARs and GluN2B-containing NMDARs are required for the effects of BDNF on pERK enhancement and on suppression of cocaine-seeking.

The relatively small differences in Ca^{2+} influx via GluN2A-containing NMDARs and/or GluN2B-containing NMDARs could produce dramatic changes in downstream signaling of the $Ca^{2+/}$Calmodulin (CaM) complex. Due to the limited amount of $Ca^{2+/}$CaM complex, many of its targets compete for binding to $Ca^{2+/}$CaM. CaMKII and calcineurin (PP2B) are prominent targets of the $Ca^{2+/}$CaM complex (Kennedy et al., 2005). Modulation of CaMKII activity depending on the intracellular level of Ca^{2+} could explain why co-activation of GluN2A-containing and GluN2B-containing NMDARs is required for the effects of BDNF on pERK up-regulation and on suppression of cocaine-seeking. BDNF induces autophosphorylation of alpha-CaMKII (CaMKIIα) at Thr286 and long-lasting activation of it (Blanquet et al., 2003). CaMKII inhibition blocks group I metabotropic glutamate receptor (mGluR)-mediated increase in pERK and pCREB in the rat dorsal striatum (Choe and Wang, 2001), and CaMKII underlies nicotine-induced ERK phosphorylation in cultured mouse cortical neurons (Steiner et al., 2007). Together, this suggests that CaMKII activity may underlie BDNF-induced pERK enhancement in the dmPFC via NMDAR containing GluN2A or GluN2B. Under the relatively low level of intracellular Ca^{2+}, CaMKII activity is dependent on Ca^{2+}/CaM complex binding to it; however, under relatively greater amounts of intracellular Ca^{2+}, CaMKII undergoes

autophosphorylation at Thr286, which renders CaMKII long-lasting Ca^{2+}/CaM-independent (autonomous) activity without altering its maximal Ca^{2+}/CaM-stimulated activity (Miller and Kennedy, 1986; Hudmon and Schulma, 2002). Together, activation of either GluN2A-containing NMDAR or GluN2B-containing NMDAR would not be enough to induce autophosphorylation of CaMKII, however, co-activation of GluN2A-containing NMDAR and GluN2B-containing NMDAR by intra-dmPFC BDNF could amplify Ca^{2+} influx, leading to the autophosphorylation and long-lasting activation of CaMKII and subsequently sustained ERK activity in the dmPFC.

Activation of PP2B, another prominent target of the $Ca^{2+/}$CaM complex, is linked to dephosphorylation and activation of STEP (Paul et al., 2003). STEP is a protein phosphatase which dephosphorylates and inactivates ERK and GluN2A/B directly as well as indirectly through dephosphorylation and inactivation of SFKs (Paul et al., 2003; Pulido et al., 1998; Nguyen et al., 2002; Iwasaki et al., 1998; Huang and McNamara, 2010). A previous study from our lab found that STEP is activated in the dmPFC 2 hr after the end of cocaine SA compared to yoked-saline controls, suggesting STEP may be the phosphatase responsible for cocaine SA-mediated dephosphorylation of GluN2A, GluN2B and ERK (Sun et al., 2013). A delayed larger increase in intracellular Ca^{2+} via GluN2B-containing NMDARs is required to mediate STEP dephosphorylation and activation as well as ERK inactivation in primary neuronal cultures (Paul and Connor, 2010). However, in the present study we showed that two hr after the last session of cocaine SA, pERK, pGluN2A (Y1325) and pGluN2B (Y1472)

levels in the dmPFC were decreased, suggesting NMDAR hypofunction. Together, during early withdrawal from cocaine SA, STEP and Ca^{2+}/CaM-dependent CaMKII activity could be mediated but induction of CaMKII autophosphorylation would fail because of the relatively low concentration of intracellular Ca^{2+} due to NMDAR hypofunction. Under this condition, the effect of STEP on ERK dephosphorylation would overcome the effect of CaMKII on ERK phosphorylation, leading to cocaine SA-induced ERK shut-off through STEP. In contrast, BDNF-induced phosphorylation of both GluN2A and GluN2B may induce a local, relatively high concentration of Ca^{2+}, sites with a restricted high Ca^{2+} concentration in the immediate vicinity of synaptic NMDARs, which would be sufficient for the induction of autophosphorylation and autonomous activity of CaMKII and STEP activity. In this condition, the sustained effect of CaMKII on ERK phosphorylation would surpass the effect of STEP on ERK dephosphorylation, which may contribute to BDNF-mediated ERK activity. However, activation of either GluN2A-contatining or GluN2B-contatining NMDARs may not be enough to induce CaMKII autophosphorylation due to relatively lower Ca^{2+} influx via either of them compared to the Ca^{2+} influx through both of them.

While weak presynaptic stimulation of presynaptic neurons leads to accumulation of intracellular Ca^{2+} within single postsynaptic spines not in the dendritic shaft, stronger stimulation increases intracellular Ca^{2+} levels both in a single postsynaptic spine and the parent dendrite in an NMDAR-dependent way (Müller and Connor, 1991), which can diffuse and increase intracellular

concentration of Ca^{2+} further in the soma. The increase also would help to remove the magnesium block of NMDARs by increasing neuronal activity, which further enhances functional NMDAR activity and subsequent Ca^{2+} influx via NMDAR. Therefore, intracellular concentration of Ca^{2+} in the soma would be amplified and prolonged by the co-activation of GluN2A-containing NMDARs and GluN2B-containing NMDARs. Together, BDNF-mediated co-activation of GluN2A-containing NMDARs and GluN2B-containing NMDARs would be required to effectively remove the magnesium block of NMDARs by increasing synaptic neuronal activity, which further promotes subsequent BDNF-mediated NMDAR activity followed by ERK activation. As both BDNF and NMDARs are important modulators of synaptic plasticity, intra-dmPFC BDNF-induced ERK activity in the dmPFC through both GluN2A- and GluN2B-containing NMDARs may contribute to recovery of cocaine SA-mediated hypofrontality. GluN2B-contatining NMDAR activity is required for the sustained ERK1/2 phosphorylation, which is linked to enhancement of synaptic AMPAR expression as well as spine volume (El Gaamouch et al., 2012), thus supporting this idea.

BDNF-mediated recovery of neuronal activity in the dmPFC leads to normalization of glutamatergic neurotransmission in the PFC-NAc pathway associated with suppression of cocaine-seeking

Dopamine modulates rat PFC long-term plasticity, LTP and LTD (Otani et al., 2003). D1 receptors in the rat PFC induce LTP at hippocampal-prefrontal synapses (Gurden et al., 2000). Together, as cocaine acts as an indirect dopamine agonist in the dmPFC, these results suggest that cocaine may induce long-lasting synaptic plasticity in the dmPFC via DA receptors and this cocaine-

mediated dysregulation of neural plasticity in the dmPFC may also affect synaptic plasticity in the NAc core through modulation of the dmPFC-NAc pathway. During withdrawal from cocaine SA, the ability to mediate LTP and LTD is compromised in the rat NAc core (Moussawi et al., 2009), which supports this idea.

Cocaine addiction is characterized by the addict's inability to adapt behavior in response to new stimuli, which is associated with persistent cocaine-seeking after extended periods of withdrawal or abstinence. Maladaptation in critical brain regions, such as dmPFC and NAc core, may underlie enduring relapse to cocaine-seeking. The present study showed that intra-dmPFC BDNF blocks cocaine SA-mediated dephosphorylation of ERK, GluN2A (Y1325) and GluN2B (Y1472) in the dmPFC during early withdrawal. This result is consistent with a previous study from our lab (Whitfield et al., 2011) showing that BDNF prevented cocaine-SA induced CREB dephosphorylation in the dmPFC during early withdrawal, suggesting that intra-dmPFC BDNF rescues cocaine SA-induced disruption of normal neuronal activity within the rat dmPFC. Rats with a cocaine SA history show reduced basal activity of the dmPFC (Sun and Rebec, 2006) and cocaine addicts also show decreased basal metabolic activity in the PFC (Bolla et al., 2004; Volkow et al., 1992; Volkow and Fowler, 2000). In the rat PFC, while a single injection of cocaine enhances BDNF mRNA and protein expression 24 hr after injection, repeated non-contingent injections of cocaine decrease BDNF protein expression 2 or 72 h after the last injection (Fumagalli et al., 2007). Moreover, BDNF and immediate early gene mRNA are also reduced 22 h after the last session of cocaine SA (McGinty et al., 2010), indicating PFC

hypofunction during early withdrawal from cocaine SA. However, a clinical study shows that administration of methylphenidate, a drug that has similar pharmacological effects as cocaine (Volkow et al., 1995), induces PFC hyperactivity, which is associated with cocaine craving (Volkow and Fowler, 2000) and a pre-clinical study also shows that cocaine infusion following 2-3 weeks of cocaine SA induces hyperexcitability in the rat dmPFC (Sun and Rebec, 2006). Together, these data suggest that a chronic cocaine-mediated reduction in basal activity of dmPFC may lead to dmPFC hyperexcitability in response to cocaine as a compensatory mechanism, which results in cocaine craving and seeking in response to cocaine. Intra-dmPFC BDNF infusion immediately after the last session of cocaine SA would block this transition of hypofrontality to hyperexcitability in the dmPFC by reversing hypofrontality during early withdrawal from cocaine SA. Intra-dmPFC BDNF-mediated SFK or ERK activation may upreguate AMPAR functions. In cultured rat neocortical neurons, BDNF enhances the total level of GluR1 or GluR2/3 via SFK (Narisawa-Saito et al., 1999). In cultured neurons, NMDAR stimulation increases the surface expression of the GluR1 subunit of AMPARs through ERK activation (Kim et al., 2005). In adult drug-naïve rats, BDNF infusion into the rat NAc core increases cell surface expression of GluA1 in an ERK- or protein synthesis-dependent manner 30 min post-injection but not 3h or 24h post-injection. As the surface level of GluA2 or GluA3 is not altered by this BDNF infusion, this result suggests that BDNF upregulates calcium-permeable AMPA receptors (CP-AMPARs) on the cell surface in the rat NAc core (Li and Wolf, 2011). Taken together, BDNF-induced

recovery of phosphoprotein levels in the dmPFC may normalize PFC neuronal excitability during early withdrawal, which prevents cocaine SA-mediated disruptions in the normal activity of dmPFC during extended withdrawal from cocaine SA.

It is possible that intra-dmPFC BDNF-mediated recovery of hypofrontality alters glutamatergic transmission in the dmPFC-NAc core pathway. BDNF-mediated normalization of neuronal excitablilty in the dmPFC would also normalize the dysfunctional synaptic plasticity in the NAc core induced by cocaine SA. Cocaine-seeking after cocaine SA mediated by context-cue or discrete cues increases expression of activity-regulated genes, including *c-fos* and *bdnf* in the dmPFC (Ciccocioppo et al., 2001; Hearing et al., 2008), indicating an increase in neuronal activity in the dmPFC by cocaine-associated cues. Further, tetrodotoxin (TTX) infusion-mediated inactivation of dmPFC immediately before a reinstatement test suppresses discrete cue-mediated cocaine-seeking (McLaughlin and See, 2003), suggesting neuronal activity elicited by discrete cues in the dmPFC underlies cocaine-seeking. Furthermore, infusion of dopamine or cocaine into dmPFC induces cocaine-seeking after cocaine SA by increasing AMPAR-mediated glutamate transmission in the nucleus accumbens (McFarland and Kalivas 2001; Park et al. 2002) and inhibition of dmPFC neuronal activity immediately before reinstatement suppresses cocaine-prime induced cocaine-seeking and the associated increase in extracellular glutamate levels in the NAc core of rats with a cocaine SA history (McFarland et al., 2003). Intra-dmPFC infusion of BDNF normalizes a cocaine SA-mediated decrease in

the basal level of extracelluar glutamate and a cocaine prime-induced increase in extracelluar glutamate level in NAc core of rats (Berglind et al., 2009), which is associated with suppression of cocaine-seeking. The BDNF-induced normalization of cocaine-mediated suppression in the basal level of extracellular glutamate may recover cocaine-induced reduction of presynaptic mGluR2/3 autoreceptor activity (Moran et al., 2005) in the NAc, core, which would inhibit further release of glutamate into the NAc core by a cocaine priming injection. Together, these studies support the idea that BDNF-induced phosphorylation of both GluN2A and GluN2B in the dmPFC may prevent hypofrontality during early withdrawal, which may normalize extracellular glutamate tone in the NAc core by increasing glutamate release into the NAc core. Recovery of extracellular glutamate levels in the NAc core would further prevent excessive glutamate release into the NAc core induced by cocaine-associated cues or cocaine itself, leading to suppression of cocaine-seeking.

Collectively, cocaine SA may reduce BDNF expression and its subsequent downstream signaling activity in the dmPFC during early withdrawal, resulting in hypofrontality. Intra-dmPFC BDNF may prevent cocaine- or cocaine-associated cue-mediated PFC hyperexcitability as well as subsequent abnormal glutamatergic neurotransmission in the dmPFC-NAc core pathway that is associated with cocaine craving and seeking.

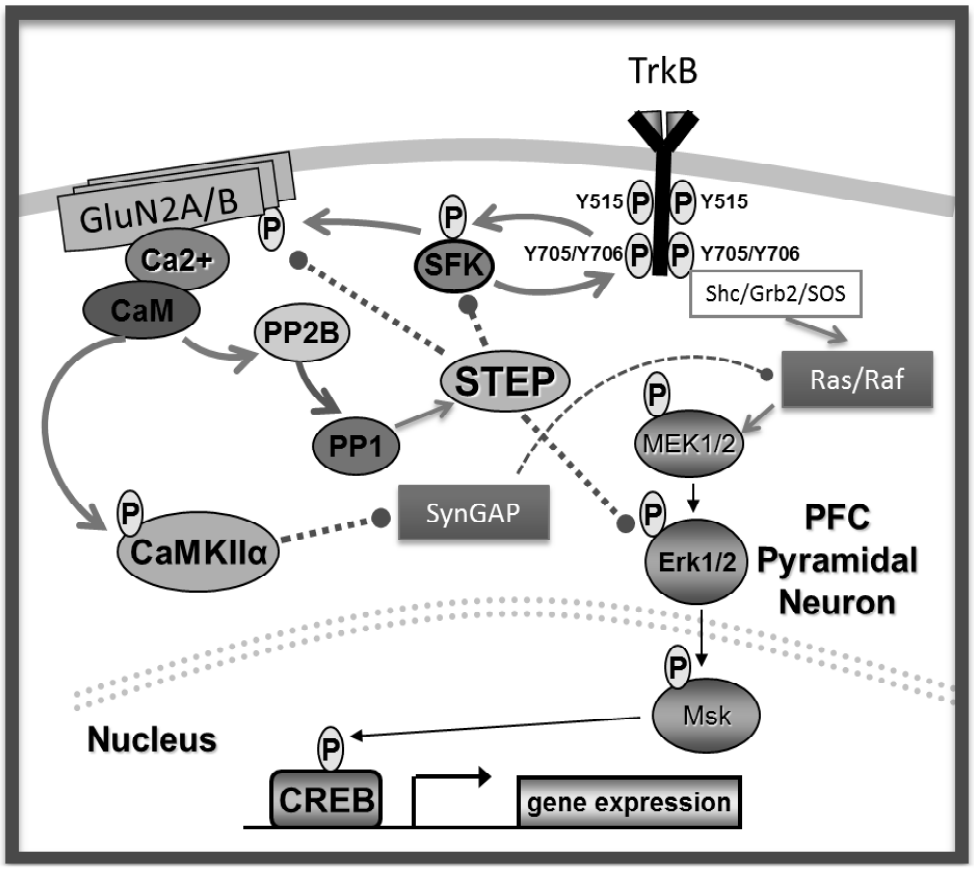

Figure 4.1. Proposed model of the crosstalk between TrkB and NMDARs leading to ERK activation in the pyramidal neurons of the dmPFC. During early withdrawal, basal neuronal activity of the dmPFC is highly suppressed and under the condition Mg^{2+} blocker of NMDAR blocks NMDAR, inhibiting Ca^{2+} influx through NMDAR. Subsequently, concentration of intracellular Ca^{2+} is very low, resulting in binding of Ca^{2+}/CaM complex to PP2B and CaMKIIα, leading to STEP dephosphorylation and activation and Ca^{2+}/CaM-dependent CaMKIIα activity. Active STEP directly or indirectly dephosphorylates and inactivates ERK, SFK, and GluN2A/2B, all of which result in inhibition of ERK phosphorylation and CaMKIIα-mediated ERK phosphorylation does not overcome STEP-induced ERK dephosphorylation. BDNF binding to TrkB induces autophosphorylation of TrkB at Y705/Y706, leading to TrkB kinase activation and subsequent phosphorylation at Y515. This phosphorylation at Y515 induces activation of TrkB downstream signaling pathway of ERK. Autophosphorylated and activated TrkB also phosphorylates and activates SFK and active SFK promotes phosphorylation of

TrkB at Y705/Y706, leading to further activation of TrkB-ERK signlaing. SFK also phosphorylates GluN2A and GluN2B, which results in the upregulation of synaptic GluN2A-containing NMDARs and GluN2B-containing NMDARs, respectively. As BDNF-mediated ERK signaling also enhances basal neuronal activity of the dmPFC via AMPAR insertion into the cellular surface, now Mg^{2+} blocker of NMDAR is released. Both SFK-mediated phosphorylation of NMDAR at GluN2A/GluN2B and Mg^{2+} block release from NMDAR leads to the functional NMDAR activation and subsequent increase in Ca^{2+} influx through NMDAR, which leads to enhancement of the intracellular Ca^{2+} level. Under the condition, CaMKIIα is autophosphorylated, leading to Ca^{2+}/CaM-independent CaMKIIα activity. Long-lasting CaMKIIα activation induces sustained ERK activation and surpasses STEP-mediated ERK dephosphorylation and inactivation. In this way, CaMKIIα activation via synaptic NMDAR is positively linked to ERK activity.

Future Directions

Although our findings of BDNF-mediated co-activation of GluN2A- and GluN2B-containing NMDARs have expanded our knowledge of the molecular mechanisms by which BDNF induces ERK activation in the dmPFC associated with cocaine-seeking, additional studies including investigation of the roles of CaMKIIα, STEP and SFK in the BDNF's effect would further advance our understating of the molecular mechanisms underlying BDNF's effect in the cortico-accumbens glutamate afferents. Those additional studies are as follows:

1) Infuse the SFK inhibitor PP2 before BDNF into the dmPFC to examine whether SFK inhibition blocks the ability of BDNF to normalize dmPFC phospho-proteins and suppress cocaine seeking.
2) Infuse the CaMKII selective inhibitor KN-93 before BDNF into the dmPFC to examine whether CaMKII inhibition blocks the ability of BDNF to normalize dmPFC pERK level and suppress cocaine seeking.

3) Infuse TAT-STEP WT fusion protein into the dmPFC before BDNF to assess whether increasing STEP level will block the ability of BDNF to normalize dmPFC phospho-proteins and suppress cocaine seeking.
4) Quantify cocaine SA or intra-dmPFC BDNF-induced changes in expression of GluN2A- and GluN2B-containing NMDARs in terms of cellular surface versus intracellular receptors using biotin cell surface labeling. This would clarify whether cocaine or BDNF-mediated alteration in tyrosine phosphorylation of GluN2 subunits would affect their surface membrane expression.
5) Quantify cocaine SA or intra-dmPFC BDNF-induced changes in expression of GluN2A- and GluN2B-containing NMDARs in terms of extrasynaptic versus synaptic regions using Triton X-100 extraction as synaptic membranes are insoluble in Triton-X 100. This would clarify whether cocaine or BDNF-mediated alteration in tyrosine phosphorylation of GluN2 subunits would affect their synaptic and extrasynaptic expression.
6) Examine whether intra-dmPFC TCN-201 or Ro-25-6981 inhibit the effect of BDNF to normalize cocaine SA-induced decrease and cocaine prime-induced increase in extracellular glutamate levels in the NAc core using in vivo no-net flux microdialysis.

Significance Statement

The novel findings of BDNF-mediated co-activation of GluN2A- and GluN2B-containing NMDARs expand our knowledge of the molecular mechanisms by which BDNF induces ERK activation in the dmPFC. These studies will impact the field of cocaine addiction research by improving the understanding of the underlying molecular mechanisms of BDNF/TrkB signaling in the dmPFC during early withdrawal that modulate relapse to cocaine-seeking. Understanding how BDNF normalizes excitatory synaptic transmission in the dmPFC during early withdrawal will expand the potential molecular targets for the treatment of cocaine addiction, which would facilitate therapeutic development for cocaine addicts. Early intervention during early withdrawal from cocaine would be significantly more advantageous than treatment after prolonged abstinence by avoiding or reducing additional cocaine-seeking and taking over the course of treatment. Moreover, early intervention could expand the therapeutic possibilities available to addicted individuals depending on their phase or severity of addiction.

References

Altar CA, Cai N, Bliven T, Juhasz M, Conner JM, Acheson AL, Lindsay RM, Wiegand SJ (1997) Anterograde transport of brain-derived neurotrophic factor and its role in the brain. Nature 389:856-860.

Aoki C, Wu K, Elste A, Len G, Lin S, McAuliffe G, Black IB (2000) Localization of brain-derived neurotrophic factor and TrkB receptors to postsynaptic densities of adult rat cerebral cortex. J Neurosci Res 59:454-63.

Balkowiec A, Katz DM (2002) Cellular mechanisms regulating activity-dependent release of native brain-derived neurotrophic factor from hippocampal neurons. J Neurosci 22:10399-407.

Balsara RD, Ferreira AN, Donahue DL, Castellino FJ, Sheets PL (2014) Probing NMDA receptor GluN2A and GluN2B subunit expression and distribution in cortical neurons. Neuropharmacology 79:542-9 ..

Ben-Shahar O, Keeley P, Cook M, Brake W, Joyce M, Nyffeler M, Heston R, Ettenberg A (2007) Changes in levels of D1, D2, or NMDA receptors during withdrawal from brief or extended daily access to IV cocaine. Brain Res. 1131:220-8.

Berglind WJ, See RE, Fuchs RA, Ghee SM, Whitfield TW Jr, Miller SW, McGinty JF (2007) A BDNF infusion into the medial prefrontal cortex suppresses cocaine seeking in rats. Eur J Neurosci 26:757-66.

Berglind WJ, Whitfield TW Jr, LaLumiere RT, Kalivas PW, McGinty JF (2009) A single intra-PFC infusion of BDNF prevents cocaine-induced alterations in extracellular glutamate within the nucleus accumbens. J Neurosci 29:3715–3719.

Blanquet PR, Mariani J, Derer P (2003) A calcium/calmodulin kinase pathway connects brain-derived neurotrophic factor to the cyclic AMP-responsive transcription factor in the rat hippocampus. Neuroscience 118:477-90.

Bolla K, Ernst M, Kiehl K, Mouratidis M, Eldreth D, Contoreggi C, Matochik J, Kurian V, Cadet J, Kimes A, Funderburk F, London E (2004) Prefrontal cortical dysfunction in abstinent cocaine abusers. J Neuropsychiatry Clin Neurosci 16:456-64.

Botreau F, Paolone G, Stewart J (2006) d-Cycloserine facilitates extinction of a cocaine-induced conditioned place preference. Behav Brain Res 172:173–178.

Braithwaite SP, Adkisson M, Leung J, Nava A, Masterson B, Urfer R, Oksenberg D, Nikolich K (2006) Regulation of NMDA receptor trafficking and function by striatal-enriched tyrosine phosphatase (STEP). Eur J Neurosci 23:2847–2856.

Bramham CR, Messaoudi E (2005) BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. Prog Neurobiol 76:99-125.

Brigman JL, Wright T, Talani G, Prasad-Mulcare S, Jinde S, Seabold GK, Mathur P, Davis MI, Bock R, Gustin RM, Colbran RJ, Alvarez VA, Nakazawa K, Delpire E, Lovinger DM, Holmes A (2000) Loss of GluN2B-containing NMDA receptors in CA1 hippocampus and cortex impairs long-term depression, reduces dendritic spine density, and disrupts learning. J Neurosci 30:4590-600.

Caldeira MV, Melo CV, Pereira DB, Carvalho RF, Carvalho AL, Duarte CB (2007) BDNF regulates the expression and traffic of NMDA receptors in cultured hippocampal neurons. Mol Cell Neurosci. 35:208–219

Cao H, Cui YH, Zhao ZQ, Cao XH, Zhang YQ (2009) Activation of extracellular signal-regulated kinase in the anterior cingulate cortex contributes to the induction of long-term potentiation in rats. Neurosci Bull 25:301-8.

Carreño FR, Walch JD, Dutta M, Nedungadi TP, Cunningham JT (2011) Brain-derived neurotrophic factor-tyrosine kinase B pathway mediates NMDA receptor NR2B subunit phosphorylation in the supraoptic nuclei following progressive dehydration. J Neuroendocrinol. 23:894-905.

Chen BS, Roche KW (2007) Regulation of NMDA receptors by phosphorylation. Neuropharmacology 53: 362–368.

Cheung HH, Gurd JW (2001) Tyrosine phosphorylation of the N-methyl-d-aspartate receptor by exogenous and postsynaptic density-associated Src-family kinases. J Neurochem 78:524–534.

Cho KO, Hunt CA, Kennedy MB (1992) The rat brain postsynaptic density fraction contains a homolog of the Drosophila discs-large tumor suppressor protein. Neuron 9:929-42.

Choe ES, Wang JQ (2001) Group I metabotropic glutamate receptors control phosphorylation of CREB, Elk-1 and ERK via a CaMKII-dependent pathway in rat striatum.Neurosci Lett 313:129-32.

Ciccocioppo R, Sanna PP, Weiss F (2001) Cocaine-predictive stimulus induces drug-seeking behavior and neural activation in limbic brain regions after multiple months of abstinence: reversal by D(1) antagonists. Proc Natl Acad Sci U S A 98:1976-81.

Cornish JL, Kalivas PW (2000) Glutamate transmission in the nucleus accumbens mediates relapse in cocaine addiction. J Neurosci 20:RC89.

Crozier RA, Bi C, Han YR, Plummer MR (2008) BDNF modulation of NMDA receptors is activity dependent. J Neurophysiol 100:3264-74.

Crozier RA, Black IB, Plummer MR (1999) Blockade of NR2B-containing NMDA receptors prevents BDNF enhancement of glutamatergic transmission in hippocampal neurons. Learn Mem 6:257-66.

Cull-Candy S, Brickley S, Farrant M (2001) NMDA receptor subunits: diversity, development and disease. Curr Opin Neurobiol 11:327–335.

De Vries TJ, Schoffelmeer AN, Binnekade R, Mulder AH, Vanderschuren LJ (1998) MK-801 reinstates drug-seeking behaviour in cocaine-trained rats. Neuroreport 9:637-40.

Di Ciano P, Everitt BJ (2001) Dissociable effects of antagonism of NMDA and AMPA/KA receptors in the nucleus accumbens core and shell on cocaine-seeking behavior. Neuropsychopharmacology 25:341–360.

Drake CT, Milner TA, Patterson SL. Ultrastructural localization of full-length trkB immunoreactivity in rat hippocampus suggests multiple roles in modulating activity-dependent synaptic plasticity (1999) J Neurosci 19:8009–8026.

Dumas TC (2005) Developmental regulation of cognitive abilities: Modified composition of a molecular switch turns on associative learning. Prog Neurobiol 76:189–211.

El Gaamouch F, Buisson A, Moustié O, Lemieux M, Labrecque S, Bontempi B, De Koninck P, Nicole O (2012) Interaction between αCaMKII and GluN2B controls ERK-dependent plasticity. J Neurosci 32:10767-79.

Erreger K, Dravid SM, Banke TG, Wyllie DJ, Traynelis SF (2005) Subunit-specific gating controls rat NR1/NR2A and NR1/NR2B NMDA channel kinetics and synaptic signalling profiles. J Physiol 563:345-58.

Figurov A, Pozzo-Miller LD, Olafsson P, Wang T, Lu B (1996) Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 381:706-9.

Finkbeiner S, Tavazoie SF, Maloratsky A, Jacobs KM, Harris KM, Greenberg ME (1997) CREB: a major mediator of neuronal neurotrophin responses. Neuron 19:1031–1047.

Flores-Barrera E, Thomases DR, Heng LJ, Cass DK, Caballero A, Tseng KY (2014) Late adolescent expression of GluN2B transmission in the prefrontal cortex is input-specific and requires postsynaptic protein kinase A and D1 dopamine receptor signaling. Biol Psychiatry 75:508-16.

Fuchs RA, Evans KA, Ledford CC, Parker MP, Case JM, Mehta RH, See RE (2005) The role of the dorsomedial prefrontal cortex, basolateral amygdala, and dorsal hippocampus in contextual reinstatement of cocaine seeking in rats. Neuropsychopharmacology 30:296-309.

Fumagalli F, Di Pasquale L, Caffino L, Racagni G, RivaMA (2007) Repeated exposure to cocaine differently modulates BDNF mRNA and protein levels in rat striatum and prefrontal cortex. Eur J Neurosci 26:2756 –2763.

Gao C, Wolf ME (2008) Dopamine receptors regulate NMDA receptor surface expression in prefrontal cortex neurons. J Neurochem 106:2489-501.

Goebel-Goody SM, Davies KD, Alvestad Linger RM, Freund RK, Browning MD (2009) Phospho-regulation of synaptic and extrasynaptic N-methyl-d-aspartate receptors in adult hippocampal slices. Neuroscience 158:1446-59.

Gomes RA, Hampton C, El-Sabeawy F, Sabo SL, McAllister AK (2006) The dynamic distribution of TrkB receptors before, during, and after synapse formation between cortical neurons. J Neurosci 26:11487-500.

Grunditz A, Holbro N, Tian L, Zuo Y, Oertner TG (2008) Spine neck plasticity controls postsynaptic calcium signals through electrical compartmentalization. J Neurosci 28:13457-66.

Gurden H, Takita M, Jay TM (2000) Essential role of D1 but not D2 receptors in the NMDA receptor-dependent long-term potentiation at hippocampal-prefrontal cortex synapses in vivo. J Neurosci 20:RC106.

Hammond S, Seymour CM, Burger A, Wagner JJ (2013) D-Serine facilitates the effectiveness of extinction to reduce drug-primed reinstatement of cocaine-induced conditioned place preference.Neuropharmacology. 64:464-71.

Hansen KB, Ogden KK, Yuan H, Traynelis SF (2014) Distinct functional and pharmacological properties of Triheteromeric GluN1/GluN2A/GluN2B NMDA receptors. Neuron 81:1084-96.

Hardingham GE, Arnold FJ, Bading H (2001) A calcium microdomain near NMDA receptors: on switch for ERK-dependent synapse-to-nucleus communication. Nat Neurosci 4:565-6.

Hearing MC, Miller SW, See RE, McGinty JF (2008) Relapse to cocaine seeking increases activity-regulated gene expression differentially in the prefrontal cortex of abstinent rats. Psychopharmacology (Berl) 198:77-91.

Hirsch JC, Crepel F (1990) Use-dependent changes in synaptic efficacy in rat prefrontal neurons in vitro. J Physiol 427:31–49.

Hou XY, Zhang GY, Yan JZ, Chen M, Liu Y (2002) Activation of NMDA receptors and L-type voltage-gated calcium channels mediates enhanced formation of Fyn-PSD95-NR2A complex after transient brain ischemia. Brain Res 955:123-32.

Hou XY, Zhang GY, Zong YY (2003) Suppression of postsynaptic density protein 95 expression attenuates increased tyrosine phosphorylation of NR2A subunits

of N-methyl-D-aspartate receptors and interactions of Src and Fyn with NR2A after transient brain ischemia in rat hippocampus. Neurosci Lett. 343:125-8.

Hu JL, Liu G, Li YC, Gao WJ, Huang YQ (2010) Dopamine D1 receptor-mediated NMDA receptor insertion depends on Fyn but not Src kinase pathway in prefrontal cortical neurons.Mol Brain.3:20.

Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 72:609–642.

Huang YZ, McNamara JO. (2010) Mutual regulation of Src family kinases and the neurotrophin receptor TrkB. J Biol Chem 285:8207–8217.

Hudmon A, Schulman H (2002) Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. Biochem J 364:593-611.

Ivanov A, Pellegrino C, Rama S, Dumalska I, Salyha Y, Ben-Ari Y, Medina I (2006) Opposing role of synaptic and extrasynaptic NMDA receptors in regulation of the extracellular signal-regulated kinases (ERK) activity in cultured rat hippocampal neurons. J Physiol 572:789–798.

Iwasaki Y, Gay B, Wada K, Koizumi S (1998) Association of the Src family tyrosine kinase Fyn with TrkB. J Neurochem 71:106-11.

Jay TM, Burette F, Laroche S (1995) NMDA receptor-dependent long-term potentiation in the hippocampal afferent fibre system to the prefrontal cortex in the rat. Eur J Neurosci 7:247-50.

Jovanovic JN, Czernik AJ, Fienberg AA, Greengard P, Sihra TS. Synapsins as mediators of BDNF-enhanced neurotransmitter release (2000) Nat Neurosci. 3:323–329.

Kalivas PW, Volkow ND (2005) The neural basis of addiction: a pathology of motivation and choice. Am J Psychiatry 162:1403–1413.

Kang H, Schuman EM (1995) Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. Science 267:1658-62.

Kauer JA, Malenka RC (2007) Synaptic plasticity and addiction. Nat Rev Neurosci 8:844-58.

Kelamangalath L, Seymour CM, Wagner JJ (2009) d-serine facilitates the effects of extinction to reduce cocaine-primed reinstatement of drug-seeking behavior. Neurobiol Learn Mem, 92:544–551.

Kelamangalath L, Swant J, Stramiello M, Wagner JJ (2007) The effects of extinction training in reducing the reinstatement of drug-seeking behavior: involvement of NMDA receptors. Behav Brain Res, 185:119–128.

Kelamangalath L, Wagner JJ (2010). D-serine treatment reduces cocaine-primed reinstatement in rats following extended access to cocaine self-administration. Neuroscience 169: 1127–1135.

Kennedy MB, Beale HC, Carlisle HJ, Washburn LR (2005) Integration of biochemical signalling in spines. Nat Rev Neurosci 6:423-34.

Kenny PJ, Chartoff E, Roberto M, Carlezon Jr WA, Markou A (2009) NMDA receptors regulate nicotine-enhanced brain reward function and intravenous nicotine self-adminstration: role of the ventral tegmental area and central nucleus of the amygdala. Neuropsychopharmacol 34:266-281.

Kim MJ, Dunah AW, Wang YT, Sheng M (2005) Differential roles of NR2A- and NR2B-containing NMDA receptors in Ras-ERK signaling and AMPA receptor trafficking.Neuron.46:745-60.

Kohr G, Seeburg PH (1996) Subtype-specific regulation of recombinant NMDA receptor-channels by protein tyrosine kinases of the src family.J Physiol.492:445-52.

Koob GF, Volkow ND (2010) Neurocircuitry of addiction. Neuropsychopharmacology 35:217-38.

Kornau HC, Schenker LT, Kennedy MB, Seeburg PH (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. Science 269:1737-40.

Korte M, Kang H, Bonhoeffer T, Schuman E (1998) A role for BDNF in the late-phase of hippocampal long-term potentiation. Neuropharmacology 37:553-9.

Lasseter HC, Xie X, Ramirez DR, Fuchs RA (2010) Sub-region specific contribution of the ventral hippocampus to drug context-induced reinstatement of cocaine-seeking behavior in rats. Neuroscience 171:830-9.

Lau CG, Zukin RS (2007) NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. Nat Rev Neurosci 8:413-26.

Lessmann V, Heumann R (1998) Modulation of unitary glutamatergic synapses by neurotrophin-4/5 or brain-derived neurotrophic factor in hippocampal microcultures: presynaptic enhancement depends on pre-established paired-pulse facilitation. Neuroscience 86:399-413.

Levine ES, Crozier RA, Black IB, Plummer MR (1998) Brain-derived neurotrophic factor modulates hippocampal synaptic transmission by increasing N-methyl-D-aspartic acid receptor activity. Proc Natl Acad Sci USA 95:10235–10239.

Li X, DeJoseph MR, Urban JH, Bahi A, Dreyer JL, Meredith GE, Ford KA, Ferrario CR, Loweth JA, Wolf ME (2013) Different roles of BDNF in nucleus

accumbens core versus shell during the incubation of cue-induced cocaine craving and its long-term maintenance. J Neurosci 33:1130-42.

Li X, Wolf ME (2011) Brain-derived neurotrophic factor rapidly increases AMPA receptor surface expression in rat nucleus accumbens. Eur J Neurosci. 2011 34:190-8.

Lin SY, Wu K, Levine ES, Mount HT, Suen PC, Black IB (1998) BDNF acutely increases tyrosine phosphorylation of the NMDA receptor subunit 2B in cortical and hippocampal postsynaptic densities. Brain Res Mol Brain Res 55:20-7.

Ma J, Zhang GY (2003) Lithium reduced N-methyl-D-aspartate receptor subunit 2A tyrosine phosphorylation and its interactions with Src and Fyn mediated by PSD-95 in rat hippocampus following cerebral ischemia. Neurosci Lett.348:185-9.

MacDonald JF, Nowak LM (1990) Mechanisms of blockade of excitatory amino acid receptor channels. Trends Pharmacol Sci 11:167-72.

Matsumoto T, Numakawa T, Yokomaku D, Adachi N, Yamaguchi S, Numakawa Y, Kunugi H, Taguchi T (2006) Brain-derived neurotrophic factor-induced potentiation of glutamate and GABA release:Different dependency on signaling pathways and neuronal activity. Mol Cell Neurosci 31:70-84.

McFarland K, Kalivas PW (2001) The circuitry mediating cocaine-induced reinstatement of drug-seeking behavior. J Neurosci 21:8655-63.

McFarland K, Lapish CC, Kalivas PW (2003) Prefrontal glutamate release into the core of the nucleus accumbens mediates cocaine-induced reinstatement of drug-seeking behavior. J Neurosci 23:3531–3537.

McGinty JF, Whitfield TW Jr, Berglind WJ (2010) Brain-derived neurotrophic factor and cocaine addiction. Brain Res 1314:183-93.

McLaughlin J, See RE (2003) Selective inactivation of the dorsomedial prefrontal cortex and the basolateral amygdala attenuates conditioned-cued reinstatement of extinguished cocaine-seeking behavior in rats. Psychopharmacology (Berl)168:57-65.

Miller SG, Kennedy MB (1986) Regulation of brain type II Ca2+/calmodulin-dependent protein kinase by autophosphorylation: A Ca2+-triggered molecular switch. Cell 44:861–870.

Miwa H, Fukaya M, Watabe AM, Watanabe M, Manabe T (2008) Functional contributions of synaptically localized NR2B subunits of the NMDA receptor to synaptic transmission and long-term potentiation in the adult mouse CNS. J Physiol 586:2539-50

Mizuno M, Yamada K, He J, Nakajima A, Nabeshima T (2003) Involvement of BDNF receptor TrkB in spatial memory formation. Learn Mem 10:108-15.

Moody TD, Watabe AM, Indersmitten T, Komiyama NH, Grant SG, O'Dell TJ (2011) Beta-adrenergic receptor activation rescues theta frequency stimulation-induced LTP deficits in mice expressing C-terminally truncated NMDA receptor GluN2A subunits. Learn Mem. 18:118-27.

Moran MM, McFarland K, Melendez RI, Kalivas PW, Seamans JK (2005) Cystine/glutamate exchange regulates metabotropic glutamate receptor presynaptic inhibition of excitatory transmission and vulnerability to cocaine seeking. J Neurosci 25:6389–6393.

Moussawi K, Pacchioni A, Moran M, Olive MF, Gass JT, Lavin A, Kalivas PW (2009) N-Acetylcysteine reverses cocaine-induced metaplasticity. Nat Neurosci 12:182-189.

Mulholland PJ, Luong NT, Woodward JJ, Chandler LJ (2008) Brain-derived neurotrophic factor activation of extracellular signal-regulated kinase is autonomous from the dominant extrasynaptic NMDA receptor extracellular signal-regulated kinase shutoff pathway. Neurosci 151:419-427.

Müller W, Connor JA (1991) Dendritic spines as individual neuronal compartments for synaptic Ca2+ responses. Nature 354:73-6.

Nakazawa T, Komai S, Tezuka T, Hisatsune C, Umemori H, Semba K, Mishina M, Manabe T, Yamamoto T (2001) Characterization of Fyn-mediated tyrosine phosphorylation sites on GluR epsilon 2 (NR2B) subunit of the N-methyl-D-aspartate receptor. J Biol Chem 276:693–699.

Narisawa-Saito M, Silva AJ, Yamaguchi T, Hayashi T, Yamamoto T, Nawa H (1999) Growth factor-mediated Fyn signaling regulates alpha-amino-3- hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor expression in rodent neocortical neurons. Proc Natl Acad Sci U S A. 96:2461-6.

Nguyen TH, Liu J, Lombroso PJ (2002) Striatal enriched phosphatase 61 dephosphorylates Fyn at phosphotyrosine 420. J Biol Chem 277:24274–24279.

Nic Dhonnchadha BA, Szalay JJ, Achat-Mendes C, Platt DM, Otto MW, Spealman RD, Kantak KM (2010) D-cycloserine deters reacquisition of cocaine self-administration by augmenting extinction learning. Neuropsychopharmacology 35: 357–367

Otani S, Daniel H, Roisin MP, Crepel F (2003) Dopaminergic modulation of longterm synaptic plasticity in rat prefrontal neurons. Cereb Cortex 13:1251–1256.

Otis JM, Fitzgerald MK, Mueller D (2014) Infralimbic BDNF/TrkB enhancement of GluN2B currents facilitates extinction of a cocaine-conditioned place preference. J Neurosci 34:6057-64.

Park WK, Bari AA, Jey AR, Anderson SM, Spealman RD, Rowlett JK, Pierce RC (2002) Cocaine administered into the medial prefrontal cortex reinstates cocaine-seeking behavior by increasing AMPA receptor-mediated glutamate transmission in the nucleus accumbens. J Neurosci 22:2916-25.

Paul S, Connor JA (2010) NR2B-NMDA receptor-mediated increases in intracellular Ca2+ concentration regulate the tyrosine phosphatase, STEP, and ERK MAP kinase signaling. J Neurochem 114:1107-18.

Paul S, Nairn AC, Wang P, Lombroso PJ (2003) NMDA-mediated activation of the tyrosine phosphatase STEP regulates the duration of ERK signaling. Nat Neurosci 6:34–42.

Pierce RC, Bell K, Duffy P, Kalivas PW (1996) Repeated cocaine augments excitatory amino acid transmission in the nucleus accumbens only in rats having developed behavioral sensitization. J Neurosci 16:1550-1560.

Poo MM (2001) Neurotrophins as synaptic modulators. Nat Rev Neurosci 2:24-32.

Pulido R, Zuniga A, Ullrich A (1998) PTP-SL and STEP protein tyrosine phosphatases regulate the activation of the extracellular signal-regulated kinases ERK1 and ERK2 by association through a kinase interaction motif. EMBO J 17:7337–7350.

Reichardt LF (2006) Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci 361:1545–1564.

Rogers JL, See RE (2007) Selective inactivation of the ventral hippocampus attenuates cue-induced and cocaine-primed reinstatement of drug-seeking in rats. Neurobiol Learn Mem 87:688-92.

Sakimura K, Kutsuwada T, Ito I, Manabe T, Takayama C, Kushiya E, Yagi T, Aizawa S, Inoue Y, Sugiyama H, Mishina M (1995) Reduced hippocampal LTP and spatial learning in mice lacking NMDA receptor epsilon 1 subunit. Nature 373:151-5.

Sarantis K, Matsokis N, Angelatou F (2009) Synergistic interactions of dopamine D1 and glutamate NMDA receptors in rat hippocampus and prefrontal cortex: involvement of ERK1/2 signaling. Neuroscience 163:1135-45.

Simon J, Arthur C, Fong AL, Dwyer JM, Davare M, Reese E, Obrietan K, Impey S (2004) Mitogen- and stress-activated protein kinase I mediates cAMP response element-binding protein phosphorylation and activation by neurotrophins. J Neurosci 24:4324-4332.

Snyder EM, Nong Y, Almeida CG, Paul S, Moran T, Choi EY, Nairn AC, Salter MW, Lombroso PJ, Gouras GK, Greengard P (2005) Regulation of NMDA receptor trafficking by amyloid-beta. Nat Neurosci 8:1051–1058.

Sobczyk A, Scheuss V, Svoboda K (2005) NMDA receptor subunit-dependent [Ca2+] signaling in individual hippocampal dendritic spines. J Neurosci 25:6037-46.

Steiner RC, Heath CJ, Picciotto MR (2007) Nicotine-induced phosphorylation of ERK in mouse primary cortical neurons: evidence for involvement of glutamatergic signaling and CaMKII. J Neurochem 103:666-78.

Sun W, Rebec GV (2006) Repeated cocaine self-administration alters processing of cocaine-related information in rat prefrontal cortex. J Neurosci 26:8004–8008.

Sun WL, Zelek-Molik A, McGinty JF (2013) Short and long access to cocaine self-administration activates tyrosine phosphatase STEP and attenuates GluN expression but differentially regulates GluA expression in the prefrontal cortex. Psychopharmacology 229:603-13.

Taniguchi S, Nakazawa T, Tanimura A, Kiyama Y, Tezuka T, Watabe AM, Katayama N, Yokoyama K, Inoue T, Izumi-Nakaseko H, Kakuta S, Sudo K, Iwakura Y, Umemori H, Inoue T, Murphy NP, Hashimoto K, Kano M, Manabe T, Yamamoto T (2009) Involvement of NMDAR2A tyrosine phosphorylation in depression-related behaviour. EMBO J 28:3717-29.

Tezuka T, Umemori H, Akiyama T, Nakanishi S, Yamamoto T (1999). PSD-95 promotes Fyn-mediated tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit NR2A. Proc Natl Acad Sci U S A 96:435-40.

Thanos PK, Bermeo C, Wang GJ, Volkow ND (2011) D-cycloserine facilitates extinction of cocaine self-administration in rats. Synapse 65:938-44.

Thomas CG, Miller AJ, Westbrook GL (2006) Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. J Neurophysiol 95:1727–1734.

Van Huijstee AN, Mansvelder HD (2015) Glutamatergic synaptic plasticity in the mesocorticolimbic system in addiction.Front Cell Neurosci 8:466. Adinoff B (2004) Neurobiologic processes in drug reward and addiction. Harv Rev Psychiatry 12:305-20

Vicini S, Wang JF, Li JH, Zhu WJ, Wang YH, Luo JH, Wolfe BB, Grayson DR (1998) Functional and pharmacological differences between recombinant N-methyl-D-aspartate receptors. J Neurophysiol 79:555-66.

Volkow ND, Ding Y-S, Fowler JS, Wang GJ, Logan J, Gatley SJ, Dewey SL, Ashby C, Lieberman J, Hitzemann R, Wolf AP (1995) Is methylphenidate like cocaine? Studies on their pharmacokinetics and distribution in human brain. Arch Gen Psychiat 52:456–463.

Volkow ND, Fowler JS (2000) Addiction, a disease of compulsion and drive: involvement of the orbitofrontal cortex. Cereb Cortex 10:318-25.

Volkow ND, Hitzemann R, Wang GJ, Fowler JS, Wolf AP, Dewey SL, Handlesman L (1992) Long-term frontal brain metabolic changes in cocaine abusers. Synapse 11:184–190.

Waterhouse EG, Xu B (2009) New insights into the role of brain-derived neurotrophic factor in synaptic plasticity. Mol Cell Neurosci 42:81-9.

Weiss F, Ciccocioppo R, Parsons LH, Katner S, Liu X, Zorrilla EP, Valdez GR, Ben-Shahar O, Angeletti S, Richter RR (2001) Compulsive drug-seeking behavior and relapse. Neuroadaptation, stress, and conditioning factors. Ann N Y Acad Sci 937:1-26.

White FJ, Kalivas PW (2001) Neuroadaptations involved in amphetamine and cocaine addiction. Drug Alcohol Depend 51:141-153.

Whitfield TM, Shi X, Sun WL, McGinty JF (2011) The Suppressive Effect of an Intra-Prefrontal Cortical infusion of BDNF on cocaine-Seeking is Trk receptor and extracellular signal-regulated protein kinase mitogen-activated protein kinase dependent. The Journal of Neuroscience 31:834–842.

Xu F, Plummer MR, Len GW, Nakazawa T, Yamamoto T, Black IB, Wu K (2006) Brain-derived neurotrophic factor rapidly increases NMDA receptor channel activity through Fyn-mediated phosphorylation. Brain Res 1121:22-34.

Yang FY, Lee YS, Cherng CG, Cheng LY, Chang WT, Chuang JY, Kao GS, Yu L (2013) D-cycloserine, sarcosine and D-serine diminish the expression of cocaine-induced conditioned place preference. J Psychopharmacol 27:550-8.

Yashiro K, Philpot BD (2008) Regulation of NMDA receptor subunit expression and its implications for LTD, LTP, and metaplasticity. Neuropharmacology 55:1081-94.

Zheng F, Gingrich MB, Traynelis SF, Conn PJ (1998) Tyrosine kinase potentiates NMDA receptor currents by reducing tonic zinc inhibition.Nat Neurosci.1:185-91.