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The Role of BDNF within the Medial Prefrontal Cortex on Relapse to Cocaine-Seeking Behavior

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THE ROLE OF BDNF WITHIN THE MEDIAL PREFRONTAL CORTEX ON RELAPSE TO COCAINE-SEEKING BEHAVIOR

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A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the College of Graduate Studies.

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To my wife Jennifer, my best friend and my greatest love; and to my son William, my greatest joy. Without you, I am nothing.

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WILLIAM JOHN BERGLIND. The Role of Brain-Derived Growth Factor in the Prefrontal Cortex on Relapse to Cocaine Seeking. (Under the direction of JACQUELINE F. MCGINTY)

Glutamatergic pyramidal cells arising from the prefrontal cortex (PFC) are critical mediators of reinstatement to cocaine-seeking. A potent modulator of PFC activity is brain derived neurotrophic factor (BDNF), a molecule that has been implicated in the regulation of glutamatergic neurotransmission. In this study, the role of BDNF in the PFC was investigated in an animal model of cocaine addiction. First, a cocaine-induced decrease in BDNF mRNA within the PFC was documented following 22 h of abstinence, followed by an increase in BDNF protein expression within the PFC following 21 d of extinction training. Second, an intra-PFC infusion of BDNF attenuated relapse to cocaine seeking following drug abstinence and reinstatement of cocaine seeking after extinction training. Furthermore, the intra-PFC infusion of BDNF prevented cocaine-induced decreases in phospho-ERK expression in the nucleus accumbens (NAc), implicating the PFC-NAc pathway in BDNF's suppression of cocaine seeking. Disturbance in this pathway underlies the decrease in extracellular glutamate levels in the NAc during withdrawal from chronic cocaine and an increase in glutamate levels after cocaine-primed reinstatement of drug seeking. BDNF prevented the decrease in basal extracellular glutamate levels following cocaine self administration and the increase in cocaine prime-induced glutamate levels in the NAc. These data indicate that cocaine-induced neuroadaptations within the PFC-NAc pathway are normalized by intra-PFC BDNF treatment. Considering that 1) these effects are long-lasting and are the result of a single dose of BDNF and 2) the infusion is only efficacious when administered shortly after the end of cocaine self-administration, the possible therapeutic implications are

significant and serve as the impetus for future investigation into the mechanisms involved in the ability of intra-PFC BDNF infusions to suppress cocaine seeking behavior.

CHAPTER 1
INTRODUCTION

The enduring effects of cocaine abuse have had a devastating effect on our society. Since cocaine's introduction in the late nineteenth century, countless individuals have fallen victim to its spell, seduced by the paradox of immense pleasure ultimately causing immense suffering. The long-term behavioral effects of chronic cocaine abuse are characterized by endless cycles of cocaine use, abstinence, and then relapse to cocaine seeking. These interminable cycles cause catastrophic long-term changes in specific reward-associated brain circuitry. Furthermore, these long-term neuroadaptations only serve to perpetuate the cycle of addiction and lead to a deadly spiral from which there is little chance of escape. For this reason, researchers have for decades sought to determine what neuronal targets are critical in the long-term cocaine-induced neuroadaptations that underlie the development of addiction. As a result, there is much greater understanding of how the natural reward circuitry is hijacked and forced to be subservient to the pursuit of cocaine alone. These servile diseased brain regions slowly silence the beautiful symphony that is the human mind and trade it for a singular, shrieking drive that is the addicted brain. This horror does not happen overnight. Therefore, substance abuse research has sought to delineate the chronic neuroadaptations induced by cocaine that ultimately play a major role in driving relapse and addiction.

REVIEW OF LITERATURE

Adaptations Associated with Addiction and Relapse

The behavioral adaptations that characterize cocaine addiction are caused by drug-induced, long-term neuronal adaptations that alter neuronal circuits and substrates (Koob et al., 1997a; Wang and McGinty, 1997; White and Kalivas, 2001). Alterations in gene and protein expression are likely a significant component of the adaptations that lead to addictive behaviors characterized by craving and relapse (Torres and Horowitz; Nestler, 1997; Shalev et al., 2003). Relapse to cocaine seeking has been studied extensively using rodent models. These studies have utilized re-exposure to drug-associated contexts, discrete conditioned stimuli, stress, and cocaine itself to elicit reinstatement to cocaine-seeking behavior in these animal models (Gerber and Stretch, 1975; de Wit and Stewart, 1981; Allerweireldt et al., 2001; Crombag et al., 2002; Kalivas and McFarland, 2003; See et al., 2005). These studies, along with many others, have served to identify the brain areas that are altered by cocaine and are implicated in the reinstatement of drug-seeking behavior. These structures, which are naturally associated with reward are collectively called the motive circuit, and include the prefrontal cortex (PFC) and its downstream target, the nucleus accumbens (NAc). Both of these structures receive dopamine (DA) innervations from the ventral tegmental area (VTA) via the mesocorticolimbic DA ("reward") pathway. Many cocaine-induced neuroadaptations that are thought to drive reinstatement of cocaine-seeking behavior are manifested by alterations in the plasticity of the PFC-NAc pathway. These include, but are not limited to, alterations in the electrophysiological profile of glutamatergic pyramidal neurons

arising from the PFC (Trantham et al., 2002; Sun and Rebec, 2006) as well as changes in the extracellular levels of glutamate within the NAc (Pierce et al., 1996; McFarland et al., 2003). Alterations within the motive circuit may induce changes in the in excitatory neurotransmission and associated intracellular signaling of medium spiny neurons within the NAc (Xi et al., 2002; Baker et al., 2003). One molecule that has been implicated in driving changes in plasticity and is expressed within this pathway is brain derived neurotrophic factor (BDNF; Xiong, 2002). BDNF is a secreted neurotrophin that is associated with modulating plasticity (McAllister et al., 1995; McAllister et al., 1996). Furthermore, cortical pyramidal neurons arising from the PFC are the predominant source of BDNF within the NAc (Altar et al., 1997). Figure 1-1 illustrates the expression and transport of BDNF within the mesocorticolimbic "reward" pathway where it signals through the tyrosine kinase B (TrkB) receptor.

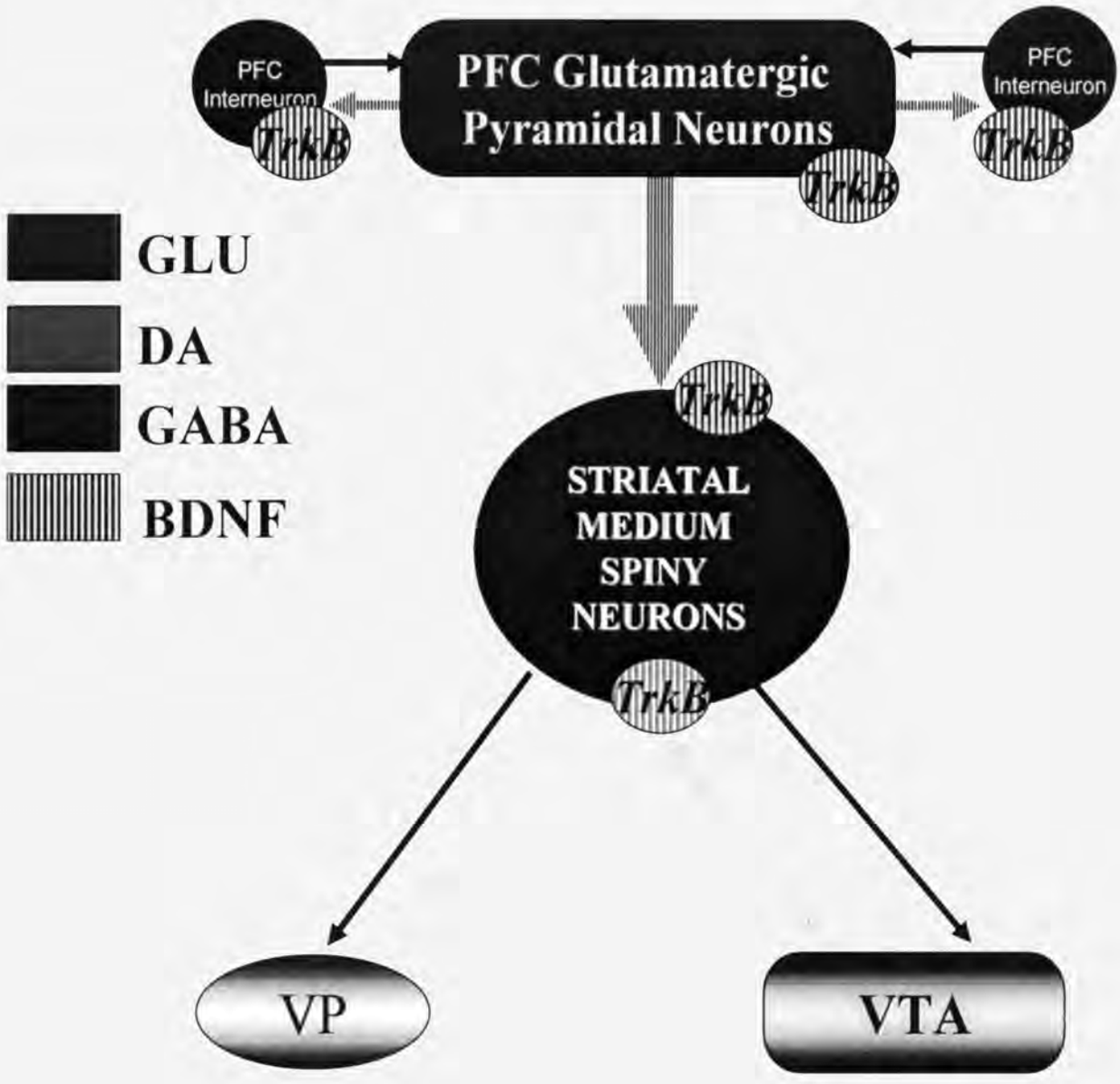

FIGURE 1-1. BDNF expression within the meso-corticolimbic pathway. BDNF is expressed in glutamatergic pyramidal neurons arising from the PFC as well as dopaminergic neurons arising from the VTA, both of which synapse on GABAaergic medium spiny neurons within the NAc. Furthermore, BDNF itself is transported anterogradely to the striatum where it binds to TrkB receptors on medium spiny neurons (Altar et al., 1997; Freeman et al., 2003). Labeling studies have demonstrated that a majority of BDNF expression within the NAc arises from the PFC, not the VTA.

BDNF Expression is Associated with Changes in Neuronal Activity

BDNF expression and activity are extensively associated with synaptic plasticity and ultimately induce changes in receptor-mediated intracellular signaling and

transcription factor activity (Lu, 2003; Patapoutian and Reichart, 2001). BDNF mRNA and protein expression is induced by several different factors that induce neuronal activity (Shieh and Ghosh, 1999; Lu, 2003). These include cellular mechanisms, such as LTP-inducing tetanic stimulation (Morimoto et al., 1998), or more global mechanisms such as physical activity and enriched environments (Russo-Neustadt et al., 1999; Young et al., 1999). BDNF mRNA expression is induced via CREB activation in a calcium-dependent manner (Shieh et al., 1998). Following transcription, BDNF mRNA can be trafficked to the dendrites for translation and preferentially targets dendrites of active synapses via unknown mechanisms (Steward and Schuman, 2001; Tongiorgi et al., 1997). Release of BDNF via synapsin-associated mechanisms and subsequent TrkB receptor activation are associated with increased glutamatergic activity (Hartmann et al., 2001.; Jovanovic et al., 2000; Balkowiec and Katz, 2002). Furthermore, BDNF promotes both early and late-phase long-term potentiation (LTP) and increases dendritic spine formation (Bramham et al., 1996). This may be due the fact that BDNF promotes dendritic protein synthesis (Kang and Schuman, 1996). Specifically, BDNF regulates spine formation at the dendritic level by inhibiting miR-134, a micro-RNA which itself inhibits the translation of Limk1, a protein kinase that regulates spine formation by regulating actin filament activity (Schratt et al., 2006).

Taken together, this evidence suggests that alterations in BDNF protein levels may serve as an indicator of global changes in local activity. However, alterations in BDNF tissue levels cannot be interpreted unambiguously without also examining downstream BDNF/TrkB signaling. Such supporting evidence is necessary in order to determine whether BDNF is being released and impacting its targets or is being

sequestered pre-synaptically and is quiescent. Molecular evidence of BDNF activity includes examining the cell surface localization of the TrkB receptor as well as downstream evidence of increased TrkB signaling via protein phosphorylation.

BDNF-induced Activation of TrkB Receptors Induces Activation of Intracellular Signaling Cascades via Phosphorylation

Changes in plasticity or synaptic efficacy occur via a number of BDNF activity-dependent mechanisms, all of which depend on the interaction of BDNF with the TrkB receptor. Initially, BDNF binding to the TrkB receptor can rapidly induce release of intracellular calcium, like a fast-acting neurotransmitter (Rose et al., 2004). Upon Trk receptor activation, phosphorylation-dependent internalization of the receptor-ligand complex occurs and is trafficked within the intracellular space via signaling endosomes (Grimes et al., 1996; Watson et al., 1999). Therefore, BDNF binding reduces the cell surface expression of TrkB receptors which are replaced by intracellular stores of TrkB receptors that are trafficked to the cell surface in an activity dependent, but not ligand-dependent, manner (Meyer-Franke et al., 1998; Du et al., 2000). Following binding, the BDNF/TrkB dimer complex is auto-phosphorylated and can exert its effects within the post-synaptic neuron by activating key signaling pathways. These pathways include the extracellular-regulated MAP kinase (ERK MAPK) cascade, phosphatidylinositol 3'-kinase (PI3-K), and phospholipase Cγ (PLCγ) (Berhow et al., 1996; Patapoutian and Reichardt, 2001). Through these signaling pathways, BDNF/TrkB activation leads to increases in the phosphorylation state of transcription factors, like CREB and Elk-1, similar to effects observed following psychostimulants and NMDA receptor stimulation in cortical and striatal neurons (Arthur et al., 2004; Finkbeiner et al., 1997; Hardingham

et al., 1999; Simpson et al., 1995; Valjent et al., 2000; Shi and McGinty, 2007). Activity of PI3-K and ERK modulates BDNF-induced dopamine release and GLUR1 expression (Xiong, 2002; Goggi et al., 2004). PI3-K activity also is involved in the ability of BDNF to induce LTP within hippocampal neurons and reinforces the role of PI3-K in modulating BDNF-induced long-term neuroadaptations (Gottschalk et al., 1999). Most studies of the PI3-K pathway have evaluated the phosphorylation state of AKT, a target of PI3-K that is induced by TrkB receptor activity (Zhu et al., 2002). Together, this evidence provides a strong correlation between BDNF mRNA increases, TrkB receptor activation, and the activation of ERK and PI3-kinase during neuronal activity.

BDNF-associated Intracellular Signaling Pathways Are Altered by Cocaine

The ERK MAPK pathway regulates the post-synaptic response to glutamate and dopamine afferents in the striatum, which has implications for long-term cocaine-induced neuroadaptations. Striatal ERK signaling has been implicated in the development of behavioral sensitization and the rewarding efficacy of cocaine as well as in the regulation of cocaine-induced activation of transcription factors (Valjent et al., 2000; Valjent et al., 2004; Valjent et al., 2005). Furthermore, multiple intra-VTA infusions of BDNF decreased total ERK immunoreactivity without affecting ERK activity; however, these studies were performed prior to the availability of phospho-specific antisera (Berhow et al., 1996). More recently, it was found that ERK, and not PKA, activation mediates cocaine-induced CREB phosphorylation in the NAc of rats following repeated, passive administration of sensitizing doses of cocaine (Mattson et al., 2005). Additionally, the MEK inhibitor U0126 blocked the enhancement of reinstatement induced by a single

BDNF infusion into the VTA (Lu et al., 2004), implicating the ERK MAPK signaling pathway in BDNF's facilitory effects on cocaine seeking. The role of PI3-K and PLC-γ activation with respect to BDNF's facilitory effects on cocaine-induced neuro-adaptations is less well studied. However, it is known that blockade of PI3K and MAPK activity prevents BDNF mediated fear conditioning in the amygdala (Ou and Gean, 2006). Furthermore, inhibition of PI3-K decreases the expression of cocaine-induced behavioral sensitization (Izzo et al., 2002). Also, acute cocaine administration induced phospho-PLCγ expression within the NAc and this effect was abolished by prior infusion of anti-BDNF into NAc (Graham et al., 2007). Thus, BDNF can modulate cocaine-induced effects via all three BDNF-associated intracellular signaling cascades.

BDNF Expression Is Altered by Chronic Cocaine Self-administration

BDNF is induced following extended periods of abstinence from cocaine self-administration and BDNF administration regulates the reinstatement and reinforcing effects of chronic exposure to cocaine (Grimm et al., 2003; Lu et al., 2004; Graham et al., 2007). BDNF mRNA in the mPFC is transiently induced for 2-4 h following acute cocaine administration (Le Foll et al., 2005). This transient induction of BDNF mRNA within the PFC may lay the foundation for more long-term neuroadaptations within the PFC following chronic exposure to cocaine. For example, 22 hr following chronic cocaine self-administration, there is a decrease in BDNF mRNA within the mPFC (Berglind et al., 2007) that is followed by an increase in BDNF mRNA in the mPFC after one week of abstinence. In addition to changes in BDNF mRNA levels within the PFC following chronic cocaine, there are also changes in BDNF protein expression. For

example, BDNF protein expression is increased in the VTA, NAc, and amygdala of rats following 30 and 90 days of withdrawal from cocaine self-administration (Grimm et al., 2003).

BDNF Modulates Cocaine-induced Behavior

In addition to the effects of chronic cocaine on endogenous BDNF, exogenous BDNF can alter cocaine-induced biochemical and behavioral changes when administered directly into selected mesolimbic areas. Specifically, BDNF infusions into the VTA and NAc enhanced cocaine-induced behavioral sensitization (Horger et al., 1999). BDNF within the VTA increased cue-induced reinstatement of cocaine seeking, a long-term effect that lasts for up to 30 days of cocaine abstinence (Lu et al., 2004). Additionally, repeated high doses of intra-NAc BNDF increased cocaine self-administration and reinstatement of cocaine seeking (Graham et al., 2007). In contrast, injection of anti-BDNF directly into the NAc attenuated cocaine-induced reinstatement when it was infused repeatedly during chronic cocaine self-administration, and an inducible BDNF knock-down in the NAc decreased cocaine self-administration (Graham et al., 2007). Collectively, these studies have implicated VTA and NAc BDNF activity in the long-term enhancement of cocaine-induced behavior. Although the mechanisms of this enhancement have not been investigated, it is likely that enhancement of the VTA-NAc-VTA circuit is involved, considering the important role of dopamine receptor activation in reinstatement of cocaine-seeking behavior (Bachtell et al., 2005).

The PFC-NAc Glutamatergic Pathway is Critical to Cocaine-induced Reinstatement

Glutamatergic pyramidal cells in the PFC are critical mediators of reinstatement to cocaine-seeking. In rats whose cocaine seeking behavior has been extinguished, inactivation of the dPFC blocks cocaine-induced and context-induced reinstatement (McFarland and Kalivas, 2001; Fuchs et al., 2005). Cocaine-induced disturbance of the PFC-NAc glutamatergic pathway is implicated by studies that demonstrated a decrease in extracellular glutamate levels in the NAc during withdrawal from chronic cocaine contrasted by an increase in glutamate levels after a cocaine injection (Pierce et al., 1996; McFarland et al., 2003) and alterations in glutamatergic receptors in the NAc (Xi et al., 2002; Tang et al., 2004). Intra-NAc manipulation of AMPA receptor agonists and antagonists either exacerbate or block cocaine-induced reinstatement, respectively (Cornish and Kalivas, 2001a; Cornish et al., 1999). The combination of lowered basal levels and cocaine prime-induced spiking of glutamate in the NAc in rats with a cocaine self administration history characterizes a pathway that is hypoactive in the absence of stimuli and hyper-responsive to cocaine-related stimuli. Interestingly, corroborating evidence of this phenomenon has been demonstrated by electrophysiological studies within the PFC (Sun and Rebec, 2006). Their data clearly demonstrate that the basal activity of pyramidal neurons within the PFC is depressed after chronic cocaine whereas burst firing is increased after re-exposure to cocaine. This pattern parallels the changes observed in extracellular glutamate within the NAc, and altogether, suggest that presynaptic changes in pyramidal cell profiles within the PFC drive cocaine-induced alterations in the PFC-NAc pathway. However, a pivotal study by Baker and colleagues (2003) clearly demonstrates that cocaine alters the characteristics of the cystine/glutamate exchanger (xCT) located on glial cells and, consequently, the dynamics of glutamate re-

uptake within the NAc. Furthermore, normalization of xCT kinetics is sufficient to prevent the decrease in basal glutamate and cocaine-prime induced glutamate spiking, as well as reinstatement behavior (Baker et al., 2003). This finding has been reinforced to demonstrate the critical role that xCT plays in regulation of basal extracellular glutamate levels and cocaine-induced neuroadaptations within the NAc-PFC pathway (Melendez et al., 2005; Moran et al., 2005).

Therefore, there is substantial evidence to support both a PFC (pre-synaptic) and NAc (post-synaptic) based mechanism for the long-term neuroadaptations induced by cocaine within the PFC-NAc pathway. Furthermore, within the context of cocaine-induced disturbances in this pathway, the role of BDNF must be considered. BDNF plays a critical role in maintaining homeostatic balance within local cortical circuits that implicates its involvement in both presynaptic and postsynaptic regulation (Rutherford et al., 1998). First, there is evidence that BDNF directly induces glutamate release from pre-synaptic stores by indirectly activating vesicular trafficking machinery (Jovanovic et al., 2000). Moreover BDNF exerts control on postsynaptic regulation of GluRs and ion channels (Rose et al., 2004). Finally, delivery of exogenous BDNF into the NAc enhances reinstatement behavior as well as intracellular signaling (Graham et al., 2007). However, few studies have investigated the role of BDNF within the PFC-NAc pathway, and until this dissertation research, none had investigated the role of BDNF in the PFC following cocaine self administration.

Purpose of the Study

The critical importance of the PFC-NAc pathway in reinstatement to cocaine seeking, the prominence of BDNF in this pathway, and its role in neuroplasticity, together served as the impetus to examine the role of exogenous BDNF within the PFC on reinstatement to cocaine seeking. Therefore, the purpose of this research was to investigate (1) the effects of cocaine self administration on BDNF mRNA and protein expression (Callahan et al., 1997), (2) the effects of exogenous BDNF infused into the PFC on relapse to cocaine-seeking behavior and associated molecular alterations within the NAc and (3) the effects of intra-PFC BDNF infusions on basal levels and cocaine-prime-induced levels of glutamate in the NAc in rats with a history of cocaine self administration.

Specific Aims

Specific Aim 1. Twenty-two h or 21 d after the end of cocaine self-administration or after a cocaine prime, evaluate the levels of BNDF mRNA and protein expression in the NAc and PFC.

Specific Aim 2. Evaluate the effect of a single bilateral intra-PFC BDNF micro-infusion on cue and cocaine-induced relapse to drug-seeking after the end of cocaine self-administration.

Specific Aim 3. Evaluate the effects of a single bilateral intra-PFC BDNF infusion after the end of cocaine self-administration on cocaine-induced changes in the expression of phosphorylated ERK in the NAc.

Specific Aim 4: Evaluate the effect of a single bilateral intra-PFC BDNF micro-infusion on basal and cocaine-induced levels of extracellular glutamate within the NAc following extinction.

CHAPTER 2

SUB-CHRONIC COCAINE SELF-ADMINISTRATION ALTERS BDNF MRNA AND PROTEIN EXPRESSION IN THE PFC AND NAC

Introduction

Behavioral adaptations that characterize cocaine addiction are thought to be caused by drug-induced, long-term neuronal adaptations that alter neuronal circuits and substrates (Koob et al., 1997; White and Kalivas, 1998; Wang and McGinty, 1999). Alterations in gene expression and subsequent protein synthesis are likely a significant component of these adaptations that lead to addictive behaviors such as craving and relapse to drug seeking (Nestler, 1997; Torres and Horowitz, 1999; Shalev et al., 2002). Notably, gene expression changes seem to be integral to the development of behavioral sensitization (Sorg and Ulibarri, 1995; Bibb et al., 2001). Cocaine administration also alters the expression of genes associated with key neuronal signaling pathways including kinases, phosphatases and transcription factors (Kelz et al., 1999; Bibb et al., 2001; Yuferov et al., 2003). These pathways are integral to the regulation of plasticity and establishing connections between transcription and translation events. Cocaine administration disrupts these signaling pathways, causing alterations in downstream gene expression. A key signaling molecule closely associated with plasticity and regulation of gene expression is BDNF.

BDNF expression and activity is associated with neuronal activity and synaptic plasticity (Patapoutian and Reichart, 2001). Specifically, studies within the hippocampus have demonstrated that long-term potentiation (LTP) induces BDNF mRNA expression (Bramham et al., 1996). In contrast, mice with deficient BDNF expression show reductions in LTP (Bartoletti et al., 2002; Abidin et al., 2006). Interestingly, BDNF-induced BDNF mRNA induction is biphasic; early expression occurring after 1-3 hours while delayed expression is manifested after 24 or more hours (Yasuda et al., 2007). Following transcription, BDNF mRNA is preferentially translated within the dendrites of active synapses (Sterward and Schuman, 2001; Tongiorgi et al., 1997). In addition, BDNF is stored in large dense core vesicles in axons until it is released via tightly-regulated secretory pathways (Wu et al., 2004). BDNF release from synaptic stores and binding to TrkB receptors on post-synaptic targets is activity dependent (Kohara et al., 2001).

Chronic cocaine alters BDNF protein expression in select nuclei within the mesolimbic pathway after extended but not early abstinence (Grimm et al., 2003). These areas include the NAc, BLA, and VTA. However, BDNF is anterogradely transported and released in a Ca^{++}-dependent manner in the NAc by dopaminergic neurons arising in the VTA as well as from cortical glutamatergic pyramidal neurons (Altar et al., 1997). The striatum, including the NAc, expresses levels of BDNF mRNA that are only detectable by PCR amplification. In fact, most of the BDNF protein expression in the striatum arises from anterograde transport from the cortex, rather than from midbrain dopamine neurons (Altar et al., 1997). Moreover, little is known about the role of cortico-striatal BDNF neurotransmission following different durations of withdrawal

from sub-chronic cocaine self-administration. The present study examined the effect of 10 days of cocaine self-administration on BDNF expression within the PFC, NAc, and CPu following 22 h or 21 d of withdrawal with or without a cocaine priming injection.

Materials and Methods

Subjects and experimental design

Male Sprague Dawley rats were used in both experiments. Figure 2-1 illustrates the experimental design. Rats in experiment one underwent either cocaine self-administration or yoked-saline followed by either 22 hours or 21 days of abstinence. At both 22 hours and 21 days of abstinence, rats were euthanized and tissue was harvested for in situ hybridization histochemistry. Rats in experiment two underwent cocaine self-administration or received yoked-saline infusions. After self- administration, rats in experiment two underwent either 22 hours of abstinence followed by a 30 min contextual reinstatement test or 21 days of extinction training followed by a 2 hour cocaine-prime reinstatement test.

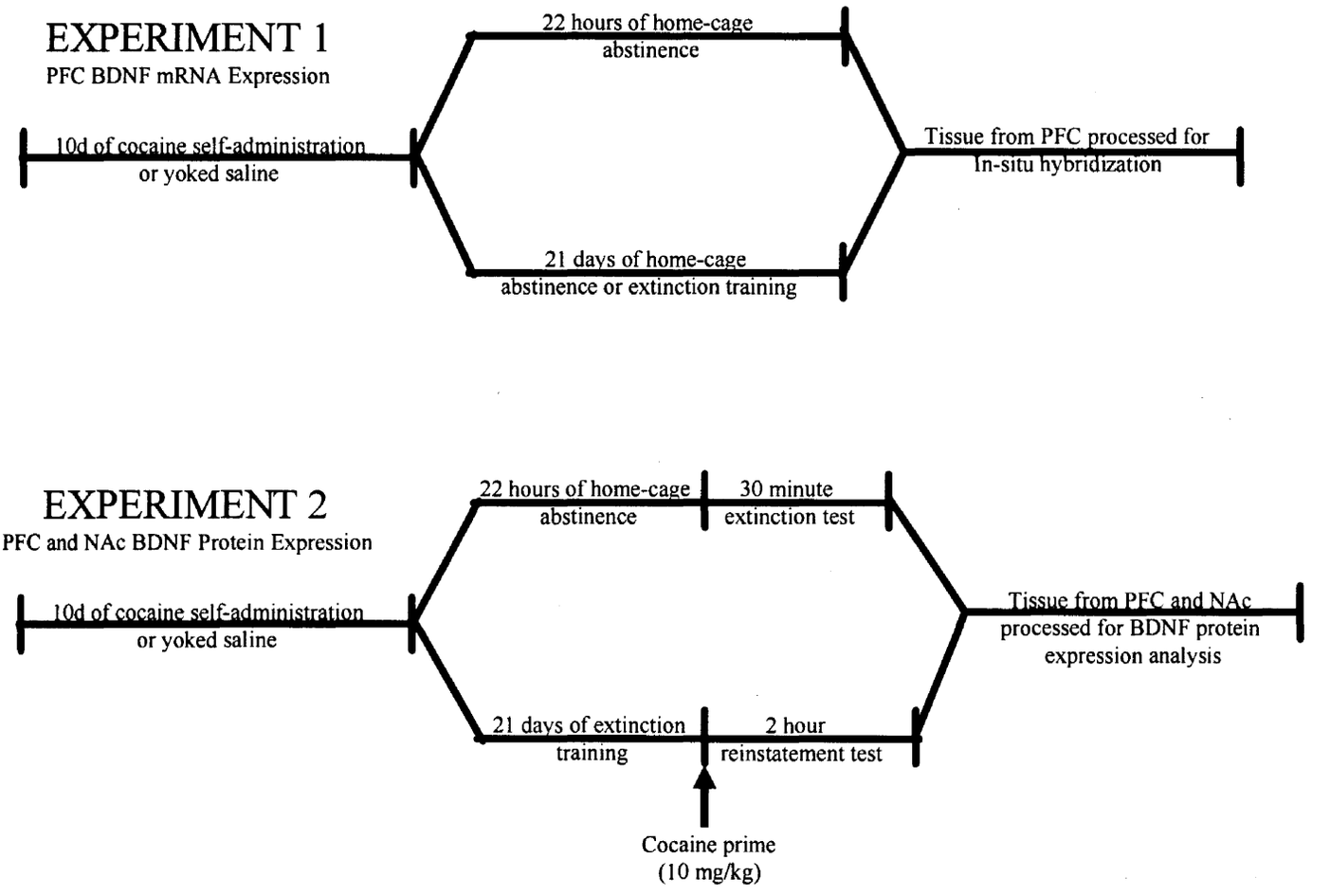

Figure 2-1: Experimental design. Experiment 1 measured BDNF mRNA expression via in-situ hybridization 22 hours or 21 days after 10 days of cocaine self-administration or yoked-saline. Experiment 1 measured BDNF protein expression via ELISA 22 hours or 21 days after 10 days of cocaine self-administration or yoked saline.

Training and surgery

Rats learned to press a lever to obtain a reward by undergoing a food training session in which active lever presses (right lever, fixed ratio 1 schedule) resulted in the delivery of a single 45 mg food pellet (Formula A/I; Research Diets Inc., New Brunswick, NJ) in the absence of any presented stimuli. Food training sessions took place overnight following a 24 h period of food deprivation. For the duration of the experiment, rats were maintained at approximately 90% of their ad libitum weight in a climate-controlled room on a reverse light/dark cycle. The day following successful completion of food training, the rats were anesthetized with ketamine (64.68 mg/kg) and equithesin (1ml/kg) and catheters were implanted into the right jugular vein (as described by See et al., 2003).

Following surgery, animals received daily intravenous infusions (0.1 ml) containing the antibiotic cefazolin (100 mg/ml) and the anticoagulant heparin (70 U/ml) until the experiment was complete.

Self-administration

Following three days of recovery from surgery, rats were placed into standard operant chambers (30 x 24 x 30 cm, Med Associates, St Albans, VT) for 120 min sessions for 10 consecutive days. Each chamber was equipped with two levers (7 cm above the floor), and a circular stimulus light 7 cm above each lever. The infusion line was attached to a liquid swivel (Instech, Plymouth Meeting, PA) mounted on a suspended counterbalance. Half of the animals received response- (active, right lever presses) contingent infusions of cocaine hydrochloride (0.2 mg/infusion; National Institute on Drug Abuse, Research Triangle Park, NC) delivered by a computer-controlled infusion pump with a 20 sec timeout period following each infusion. Responses on left (inactive) levers had no programmed consequences, but were recorded. The other half of the animals received yoked-saline infusions, dependent upon the adjacent self-administration box. These animals had access to the levers and presses were recorded but had no programmed consequences.

Abstinence

Rats in experiment one underwent 22 hours of home cage abstinence following 10 days of cocaine self-administration (N=8) or yoked-saline infusions (N=7). Other rats from experiment one underwent 21 days of abstinence following 10 days of cocaine self-

administration (N=9) or yoked-saline infusions (N=7). During abstinence, all rats remained singly housed in their respective home cages. To adjust for effects of handling and altered scheduling, rats were handled daily and their home cages were moved and placed into a different room during the time of the day that they had previously undergone self-administration. Rats received the same amount of food (90% of their ad libitum weight) during the abstinence period.

Extinction Training

Some rats in experiment one underwent 21 days of extinction training following cocaine self-administration (N=9) or yoked-saline infusions (N=9). These rats were euthanized immediately following the final daily 2 h extinction session. Rats in experiment two underwent either a single 30 min "extinction test" 22 hours following 10 days of cocaine self-administration (N=6) or yoked saline (N=6) or 21 days of extinction training following 10 days of either cocaine self-administration (N=11) or yoked-saline infusions (N=10). Extinction training sessions were held at the same time of day as the self-administration sessions had been. During the extinction session, responding on the active and inactive levers was recorded but had no programmed consequences.

Reinstatement testing

In experiment one, rats were sacrificed following either 22 h or 21 d of home-cage abstinence or 21 days of extinction training, but they did not return to the self-administration environment for any type of reinstatement testing. However, in experiment two, rats that underwent 21 days of extinction training were given a cocaine-

primed reinstatement test for which each rat was injected with cocaine (10 mg/kg, i.p.) or saline at the beginning of the test. During the cocaine-primed reinstatement test, lever presses were recorded, but had no programmed consequences. At the end of this test, the brains were harvested for BDNF protein analysis.

Tissue extraction

All rats were sacrificed by rapid decapitation. Rats that underwent reinstatement testing were sacrificed immediately following the completion of the 2 h session. In experiment one, brains were removed immediately and one hemisphere was frozen in isopentane in preparation for in situ hybridization. In experiment two, whole brains were removed and immediately frozen in isopentane for ELISA assays. Brains were sectioned in a cryostat in a rostral to caudal then a caudal to rostral direction until the PFC, NAc, and CPu were isolated and removed via a 13 gauge (2.1 mm i.d.) needle and immediately weighed and stored at -80 °C.

In situ hybridization

The oligodeoxynucleotide probes were synthesized and purified by Invitrogen (San Diego, CA). The tissue was processed and analyzed as described (Gonzalez-Nicolini et al., 2003). Probes (5 μM) were labeled at the 3' end using alpha-[^{35}S]-deoxyadenosine triphosphate (> 1000 ci/mmol, New England Nuclear, Boston, MA) and terminal deoxynucleotidyl transferase (Roche Diagnostics Corp., Indianapolis, IN) in 5x tailing buffer for 5 min. at 37°C to a specific activity of 7-12 x 10^5 cpm/μl. Ten mM Tris-1 mM EDTA and tRNA were added to stop the reaction. The sample was then

centrifuged and 10 mM TE and 1 mM EDTA and 1 M DTT were added to a total volume of 100 µl. A 1µl aliquot in 5 ml Ecoscint A (National Diagnostics) was counted in a Beckman Coulter liquid scintillation counter. Frozen 12 µm sections were cut in a cryostat and mounted onto gelatin-chromium-coated slides. Following fixation with 4% paraformaldehyde and defatting in ethanol, each section was hybridized with $1x10^6$ dpm of a probe in hybridization buffer at 37°C for 20 hours. Following four washes in sterile saline citrate/50% formamide at 40°C and two washes in sterile saline citrate, the sections were dried. Slides were then placed into an x-ray film cassette along with ^{14}C standards (American Radiolabeled Chemicals, St. Louis, MO) and Kodak Biomax MR (Rochester, NY) film was applied in a dark room at room temperature. After 3-4 weeks of development films were removed and hybridization levels were quantified using NIH Image (W. Rasband, NIMH) v. 1.6 as described (Wang and McGinty, 1996). Briefly, ^{14}C standards were measured in order to construct a calibration curve by plotting these values against known d.p.m./mg. A "blank field" was created to eliminate uneven illumination and background was subtracted. Using density slice optimization, the hybridization signal was measured in the PFC of three adjacent coronal sections for each animal. Changes were quantified by measuring both the number of labeled pixels per area and the mean density of tissue in d.p.m./mg and these measurements were multiplied together to determine the integrated density. The mean ± S.E.M. of each of these measures was calculated for each rat by averaging the values in three adjacent sections.

BDNF ELISA

BDNF levels were assessed using enzyme linked immunosorbent assay (ELISA-E_{max} Immunassay System, Promega Corp., Madison, WI) kits, according to the manufacturer's protocol. Flat-bottom plates were coated with an anti-BDNF monoclonal antibody (1:1000) overnight. After blocking nonspecific binding, immobilized anti-BDNF monoclonal antibody was incubated with brain tissue samples containing BDNF protein or BDNF standards serially-diluted to prepare a standard curve, followed by anti-human BDNF polyclonal antibody (1:500). The complex was bound using an IgY antibody conjugated to horseradish peroxidase as a tertiary reactant. After repeatedly washing unbound conjugate, plates were incubated with tetramethylbenzidine chromagenic substrate, and color change was measured in an ELISA plate reader at 450 nm. Using this kit, BDNF can be quantified in the range 7.8-500 pg/ml. Measurements given by the plate reader were calibrated against a standard curve prepared with human BDNF protein from Promega plotted in serial dilution with only $R^2 > 0.95$ accepted. Adjusted concentrations of BDNF expression were normalized to the average concentration of the control condition.

Statistics

Analysis of variance (ANOVA) was used to analyze cocaine intake (mg/kg) and active and inactive lever presses during self-administration, extinction and reinstatement testing. Significant interactions were further analyzed using Tukey's honestly significant difference (HSD) tests. For the gene and protein expression data, an ANOVA was performed on the mean integrated density values or calibrated concentration respectively.

Significant interactions were further analyzed using Tukey's honestly significant difference (HSD) tests. The probability level at which the null hypothesis was rejected was $p<0.05$.

Results

Experiment 1

Operant responding during self-administration

Rats that self-administered cocaine demonstrated significant differences in active lever presses over the last three days of self-administration compared to saline-yoked controls (Figure 2-2, $F=11.032$, $p=0.004$). However, there was no significant difference in inactive lever pressing between groups during the last three days of self-administration ($F=0.571$, $p=0.461$), demonstrating a clear, learned association for the active lever. There was no significant change in the number of cocaine infusions received over the ten days of cocaine self-administration.

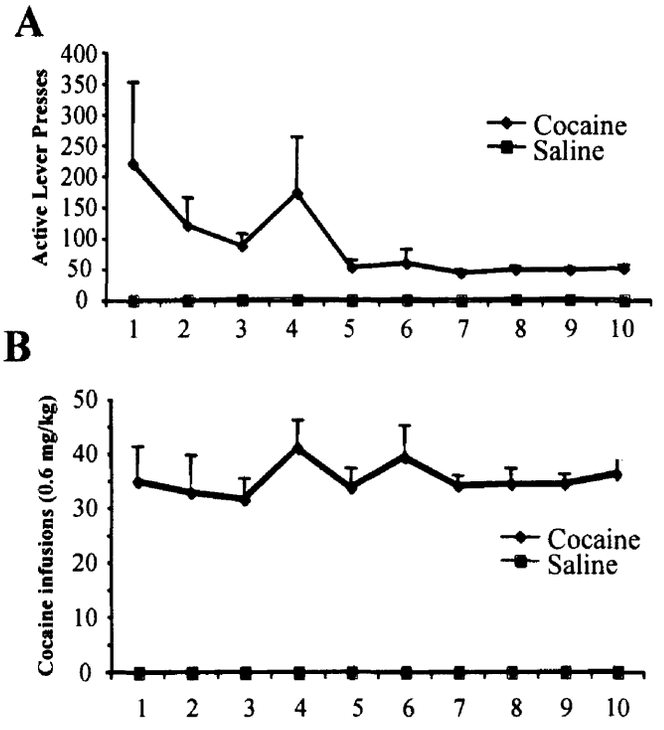

Figure 2-2. Self-administration Behavior. Responding on the active lever (A) and the number of cocaine infusions (B) received during the 10 days of cocaine self-administration or yoked–saline infusions.

Cocaine self-administration induced a significant decrease in BDNF mRNA expression within the mPFC after 22 h of abstinence

BDNF mRNA expression within the mPFC was analyzed using in situ hybridization histochemistry 22 hours after 10 days of cocaine self-administration or yoked-saline infusions. A unpaired, Student's t-test demonstrated a significant decrease in BDNF mRNA expression within the mPFC of rats that self-administered cocaine compared to yoked-saline controls (Figure 2-3, $F=9.688$, $p=0.010$).

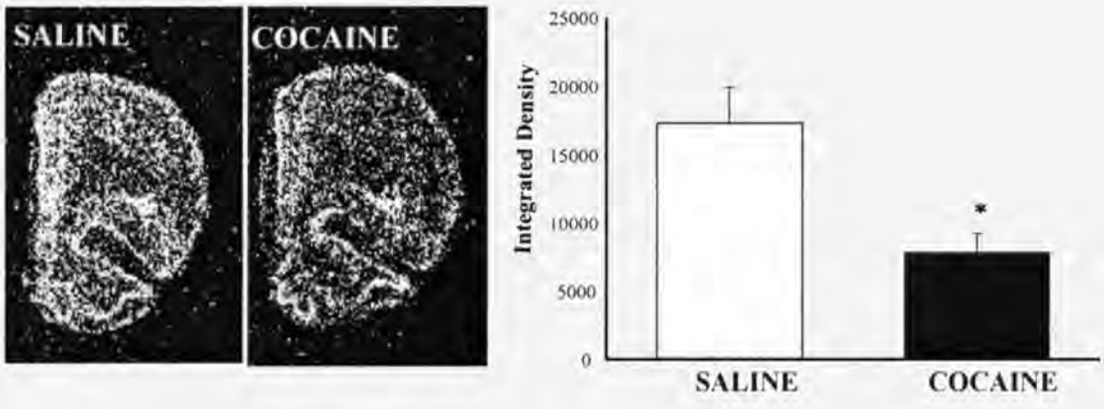

Figure 2-3. BDNF mRNA Expression Following 22 Hours of Abstinence. Twenty-two hours following 10 days of cocaine self-administration, BDNF mRNA is significantly decreased in the mPFC of rats that self-administered cocaine compared with yoked-saline controls. *$p<0.05$.

Following 21 days of abstinence or extinction training from 10 days of cocaine self-administration there was no change in BDNF mRNA within the mPFC]

Following 21 d of either home-cage abstinence or extinction training at the end of 10 d of cocaine self-administration or yoke- saline infusions, tissue from the PFC was processed for in situ hybridization. No significant difference in BDNF mRNA expression was observed within the PFC of rats that had a history of cocaine self-administration compared to yoked saline. Furthermore, there was no significant effect of

extinction training on BDNF mRNA expression compared to rats that underwent home-cage abstinence (Figure 2-4).

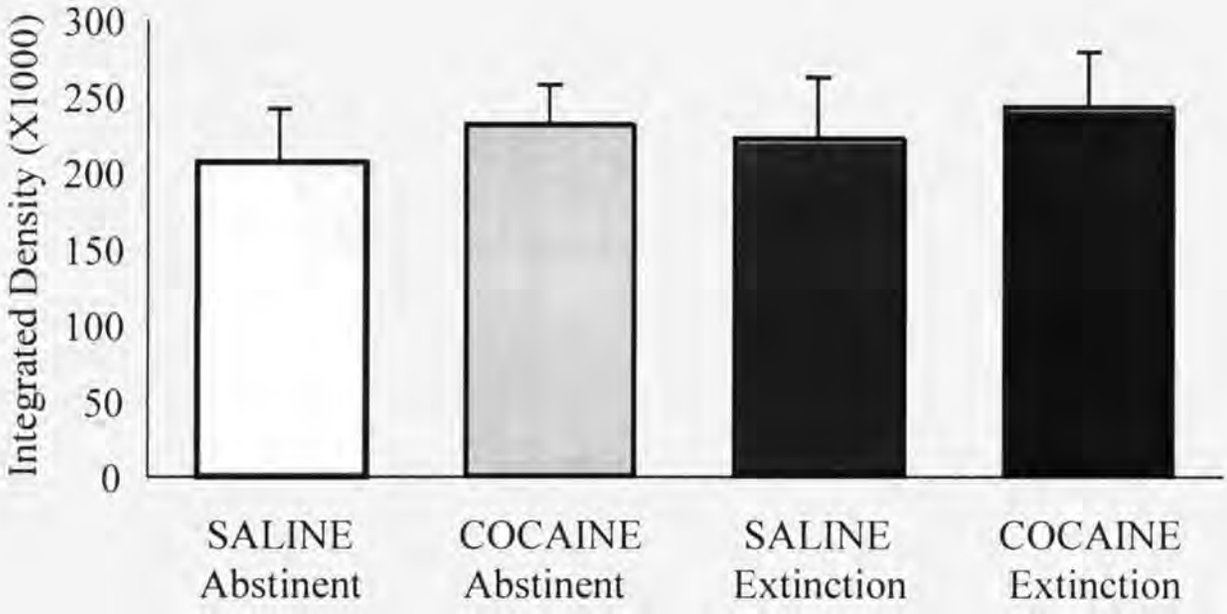

Figure 2-4. PFC BDNF mRNA Expression Following 21 Days. Twenty-one days after cocaine self-administration or yoked-saline infusions, there was no change in BDNF mRNA expression within the PFC among any treatment groups. Saline abstinent=7, cocaine abstinent=9, saline extinction=9, cocaine extinction=9.

Experiment 2

Self-administration and extinction responding on day 1

There were significant differences in active lever responding over the last three days between rats that underwent either cocaine self-administration or yoked saline (figure 2-5; $F_{1,11}=7.89$, $p<0.05$). Twenty-two hours after the 10th self-administration session, a 30 min extinction test was conducted after which the brains were harvested for BDNF protein expression measurements. Rats with a cocaine history exhibited significantly greater active lever ($F_{1,11}=5.76$, $p<0.05$) and inactive lever (data not shown; $F_{1,11}=4.41$, $p<0.05$) pressing during the 30 min test than those with a yoked-saline history.

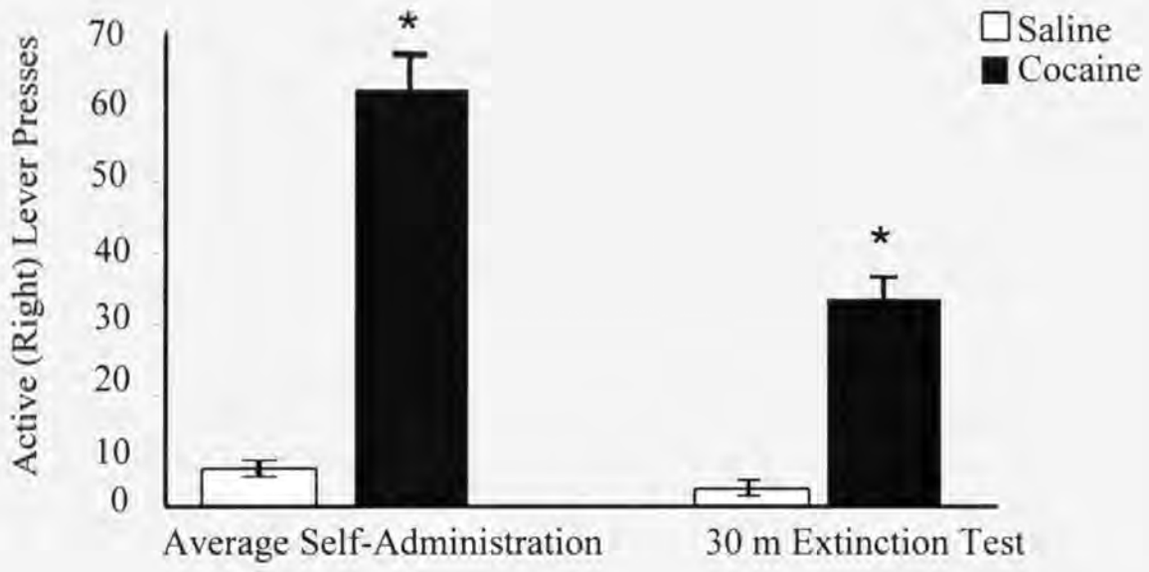

Figure 2-5. Self-administration and Extinction Behavior. Self-administration and responding on the active lever in rats that self-administered cocaine and underwent a 30 min extinction test after 22 h of abstinence. The average self-administration was calculated by averaging the active lever responding during the last three days of self-administration. Cocaine self-administering rats had significantly higher rates of active lever responding during self-administration and during the 30 min extinction test compared to yoked saline (* $p<0.05$).

Self-Administration and Reinstatement Behavior

One-way ANOVA analysis revealed a significant effect of group on active lever pressing over the last three days of cocaine self-administration (Figure 2-6, $F=6.22$, $p<0.05$). Tukey's HSD test revealed a significant increase in responding in both groups of rats that self-administered cocaine compared to yoked-saline counterparts ($p<0.05$). One-way ANOVA analysis revealed a significant effect of group on active lever pressing during the 2 h cocaine-prime induced reinstatement test ($F=5.961$, $p<0.05$). Tukey's HSD test analysis revealed a significant increase in responding in rats from the cocaine prime group compared to all other groups ($p<0.05$ in each case), but there was no significant difference between any of the other groups. sal-coc $F=14.421$, $p<0.05$). A one-way ANOVA revealed no significant effect of group on inactive lever responding during the

last three days of self-administration (F=0.938, p>0.05), during extinction or during the 2 h cocaine-prime induced reinstatement test.

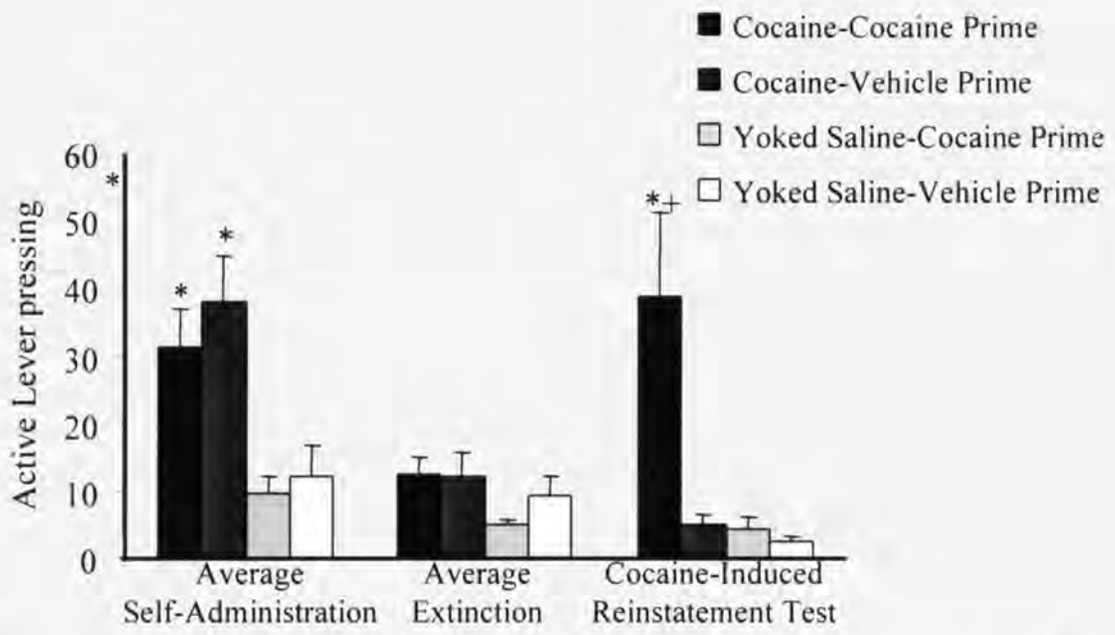

Figure 2-6. Self-administration, extinction, and reinstatement responding on the active levers. Left: Rats that self-administered cocaine (N=11) pressed the active lever significantly more than yoked-saline rats (N=10) (* $p<0.05$). Middle: Responding during extinction was not different among rats that had self-administered cocaine or received yoked-saline. Right: Only rats that previously self-administered cocaine and also received a cocaine prime (N=6) (10 mg/kg, i.p.) prior to the reinstatement session demonstrated a significant increase in active lever responding compared with extinction values (+ $p<0.05$) and all three of the other treatment groups (* $p<0.05$).

BDNF protein expression within the PFC and NAc is unchanged 22 h after cocaine self-administration

BDNF protein expression was evaluated using ELISA in tissue punches from the PFC that were harvested immediately following a 30 m extinction test 22 h after 10 d of cocaine self-administration or yoked-saline infusions. BDNF expression within the PFC was not significantly altered 22 h after cocaine self-administration compared to yoked saline controls (Figure 2-7, $p>0.05$). BDNF expression within the NAc also was not

significantly altered by cocaine self-administration compared to yoked saline controls (Figure 2-7, $F=1.507$, $p>0.05$).

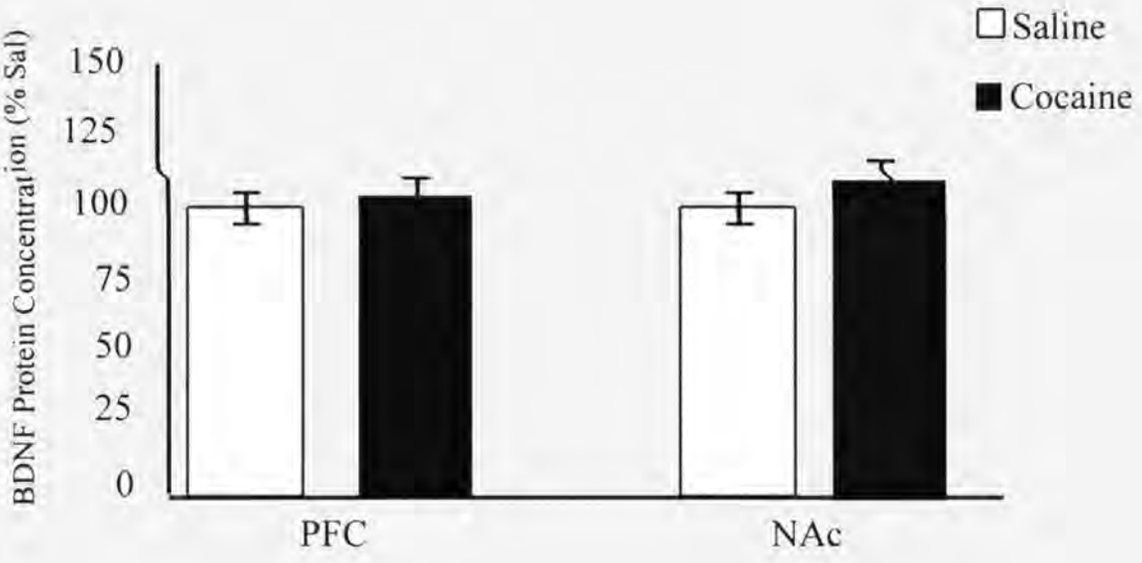

Figure 2-7. BDNF Protein Expression After 22 Hours. BDNF protein expression is unaltered following 22 h of abstinence from cocaine self-administration or yoked-saline infusions.

BDNF protein expression in the PFC and NAc following cocaine-prime induced reinstatement

BDNF protein expression was evaluated in tissue punches from the PFC and NAc of rats that self-administered cocaine or yoked saline and underwent 21 d of extinction training before a single cocaine prime-induced reinstatement session. Immediately following the 2 h session, rats were euthanized and the tissue was stored for ELISA assays. An ANOVA revealed a significant interaction between groups on BDNF protein expression levels within the PFC ($F_{3,36}=3.92$, $p=0.016$). A Tukey's HSD test revealed that rats that self-administered cocaine, regardless of whether they received a cocaine prime or not, demonstrated a significant increase in BDNF protein expression within the PFC

compared to yoked-saline controls (Figure 2-8). Finally, there was no significant effect of the cocaine prime alone on BDNF protein expression levels within the PFC. In contrast, cocaine self-administration alone failed to alter BDNF protein expression within the NAc (Figure 2-9). Furthermore, the cocaine prime (10 mg/kg) failed to produce significant changes in BNDF protein expression within the NAc of rats that had cocaine self-administration experience, although there was a trend towards an increase compared ($p=0.083$) to an acute cocaine exposure in yoked-saline rats. A one-way ANOVA revealed a trend towards an effect of group on BDNF protein expression (expressed as percent sal-sal) ($F=6.76$, $p>0.05$).

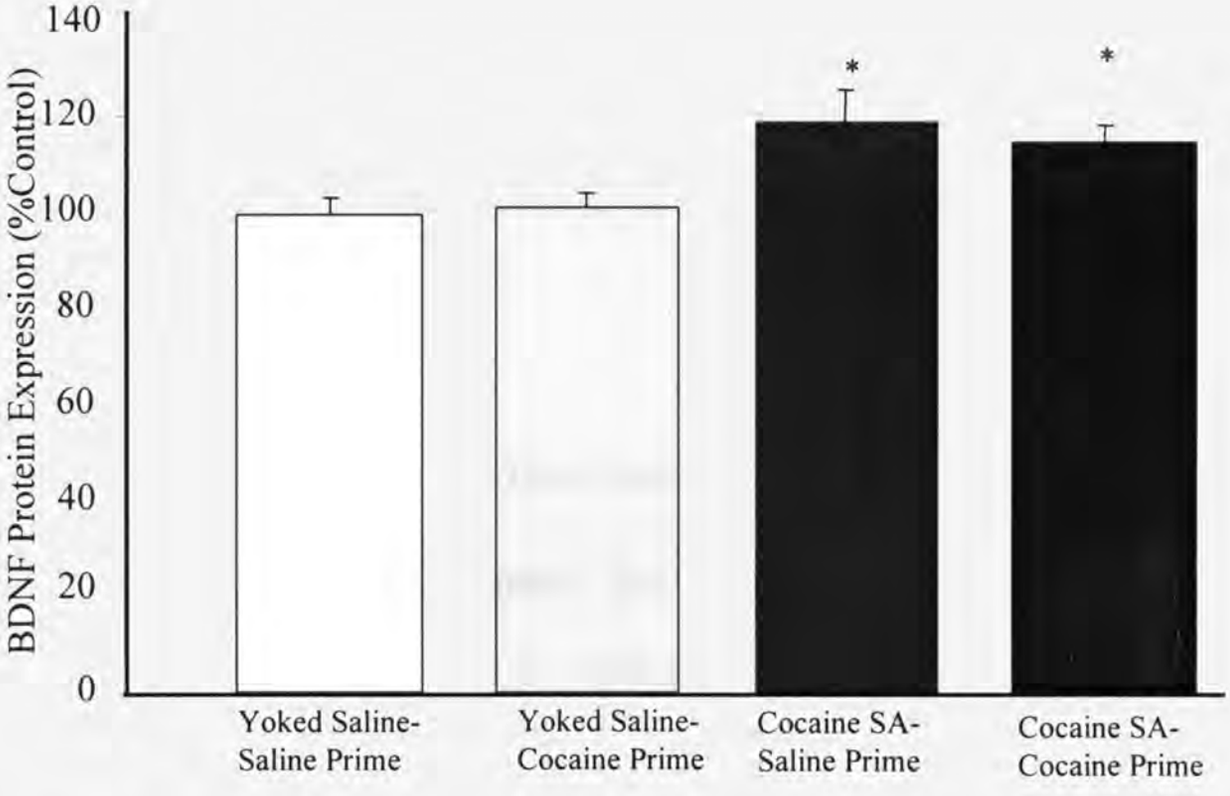

Figure 2-8. PFC BDNF Protein Expression after 21 Days. BDNF protein expression was significantly increased in the PFC in rats that self-administered cocaine for 10 d compared to yoked saline controls after 21 d of extinction training (* $p<0.05$). There was no significant acute effect of the cocaine prime on BDNF protein expression levels in the PFC.

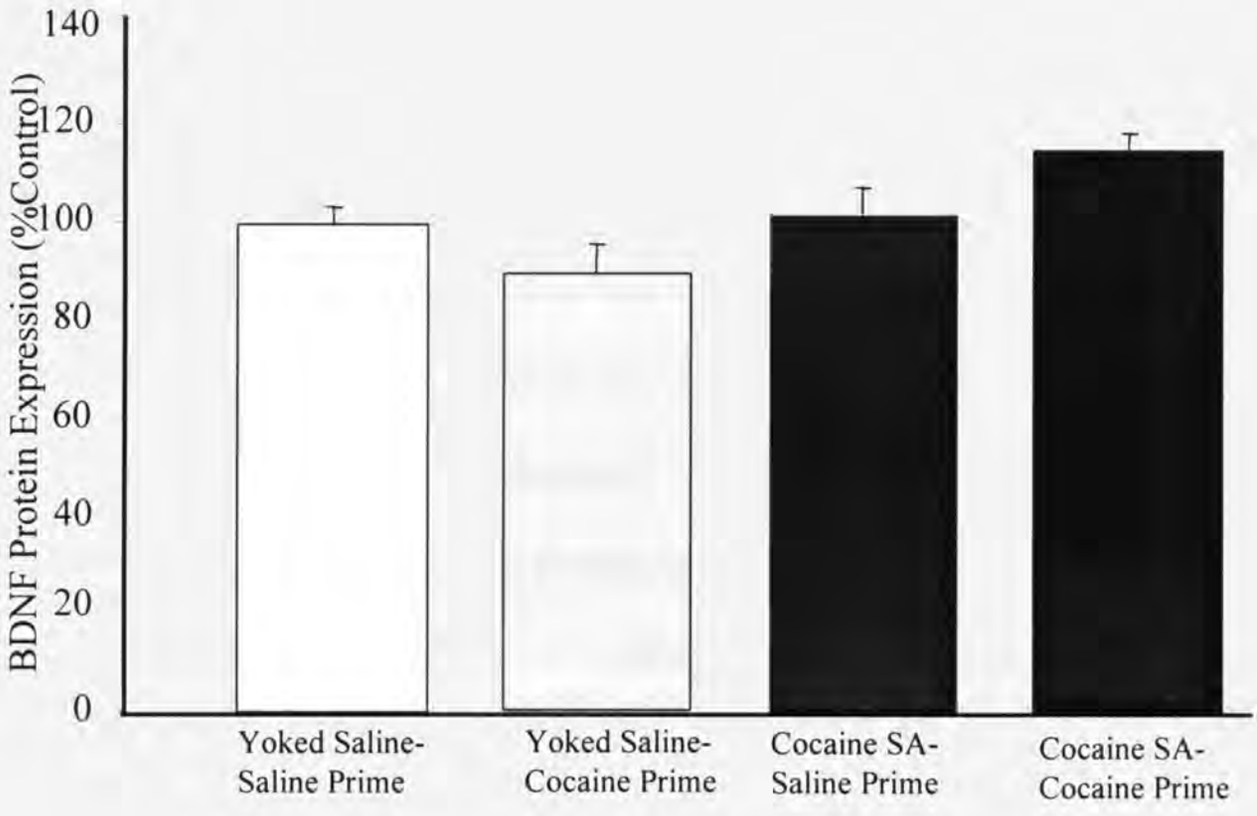

Figure 2-9. NAc BDNF Protein Expression after 21 Days. BNDF protein expression within the NAc was not significantly altered by cocaine self-administration following 21 d of extinction training, nor was it altered 2 h after a cocaine prime (10 mg/kg, i.p.).

Discussion

The present study demonstrated the effect of sub-chronic cocaine self-administration on the mRNA and protein expression of BDNF in the PFC and NAc after 22 h or 21 d of abstinence or extinction training with or without a cocaine priming injection. BDNF mRNA expression within the PFC was suppressed 22 h following 10 d of cocaine self-administration, suggesting a depression in cortical neuronal activity in early abstinence. However, this suppression in BDNF mRNA recovered after 21 d of abstinence or extinction training. In contrast, after 21 d of extinction training, there was

a significant increase in BDNF protein expression within the PFC of rats that had self-administered cocaine, independent of whether they received a single cocaine-priming injection or not. This result sharply contrasts with a lack of change in BDNF expression within the NAc after 21 d of extinction training with or without a cocaine prime. This indicates that BDNF protein expression within the PFC is increasing over extended durations of extinction as the result of cocaine self-administration. Together, this study demonstrates that the effect of sub-chronic cocaine self-administration on BDNF expression remains a dynamic process throughout abstinence or extinction training. This emphasizes the point that long-term neuroadaptations induced by chronic exposure to cocaine continue to be manifested even in the absence of cocaine.

The evidence from the present study demonstrates that BDNF mRNA levels within the PFC are initially depressed but return to normal levels by 21 days. BDNF mRNA is highly correlated with activity-dependent cortical plasticity (Lu 2003; Shieh and Ghosh, 1999). Induction of LTP is associated with increases in BDNF mRNA within the cortex (Bramham et al., 1996; Morimoto et al., 1998). Furthermore, depression of cortical BDNF mRNA expression is associated with pathological states such as schizophrenia and Alzeimer's disease (Connor et al., 1997; Holsinger et al., 2000; Weickert et al., 2005). Additionally, alterations in BDNF mRNA expression have been observed following cocaine treatment in other studies. Specifically, acute and repeated exposure to non-contingent cocaine transiently increases BDNF mRNA expression within the PFC at 2 hours following cocaine administration (Le Foll et al., 2005; Fumagalli et al., 2007). This increase is not preserved, and does not necessarily contradict the present findings. Rather, it demonstrates the very dynamic nature of BDNF mRNA

expression within the PFC following cocaine exposure. It is possible that the present reductions in BDNF mRNA within the PFC may be a compensatory response to the short-term increases in mRNA expression induced by cocaine. Also, decreases in BDNF mRNA are not observed 21 days after cocaine self-administration, suggesting that this effect may be only a "snapshot" in a fluctuating system with impaired homeostatic balance. Although cocaine-induced changes in BDNF mRNA expression may be transient, considering the significant role BDNF plays in the regulation of PFC homeostasis (Rutherford et al., 1998; Desai et al., 1999; Leslie et al., 2001) cocaine-induced oscillations during early withdrawal could lead to more long-term neuro-adaptations in the PFC.

In contrast to the seemingly transient and dynamic changes in BDNF mRNA expression within the PFC, the evidence from the present study suggests that cocaine-induced increases in BDNF protein expression within the PFC occur more gradually over longer durations of withdrawal. Considering that we did not observe parallel changes in both BDNF mRNA and protein expression within the PFC at either time-point, the changes in BDNF protein expression are likely to be the result of post-transcriptional mechanisms. An increase in BDNF protein expression within the PFC could result from a number of different mechanisms and could have a number of different consequences. Post-transcriptional alterations in BDNF expression within the PFC could be caused by increases in trafficking of BDNF from efferent sources, a decrease in synaptic release and uptake within local cortical efferents, an increase in the conversion of pro-BDNF to mature BDNF, or a decrease in BDNF degradation. One must consider the depressed state of PFC pyramidal neurons following withdrawal from repeated cocaine self-

administration, a state commonly termed hypo-frontality (Sun and Rebec, 2006 ; Goldstein and Volkow, 2002). Considering that BDNF is released normally from pre-synaptic stores in an activity dependent manner associated with increased glutamatergic activity, during withdrawal a decrease in the local release of BDNF within the PFC may result in an increase in BDNF that is sequestered pre-synaptically (Balkowiec and Katz, 2002; Hartmann et al,. 2001; Jovanovic et al., 2000). This may serve to "prime" the neurons within the PFC to elicit an enhanced response to drug-associated stimuli and ultimately perpetuate the behavior that underlies addiction.

Increases in BDNF protein expression corresponding to increasing durations of abstinence following cocaine has been demonstrated in other brain areas, including the NAc, VTA, and BLA (Grimm et al., 2003). Although we were unable to replicate this finding within the NAc, a number of methodological differences in the self-administration paradigm's used may account for this. Specifically, in the study by Grimm et al., rats were given extended access to cocaine (6-8 hours) with a higher dose of cocaine (1 mg/kg) compared to the 2 hour limited access paradigm at 0.6 mg/kg in the present study. Also, in the Grimm et al study, changes in BDNF protein expression were observed after 30 and 90 days of home-cage abstinence with the greatest changes seen after 90 days. In the present study BDNF protein expression was measured following only 21 days of extinction training. These differences suggest that increases in BDNF protein expression within the NAc may be correlated with both the amount of cocaine that was received as well as the duration of abstinence. Also, although we failed to see a change in BDNF protein it is possible that the pro form of BDNF rather than the mature form was altered within the NAc following cocaine (Fumagalli et al., 2007).

There is overwhelming evidence that the molecular alterations within the PFC, specifically BDNF, may underlie the increase in the salience of cocaine-associated stimuli, which drive cocaine-seeking behavior and relapse. The present study lends credence to this idea by demonstrating both the transient and persistent changes in endogenous BDNF mRNA and protein expression within the PFC. It is still speculative, but the perseverative response to cocaine-associated stimuli may be the direct result of long-term changes in synaptic plasticity and morphology which are mediated by BDNF. Very few, if any, studies have measured what effect directly altering BDNF activity within the PFC would have on reinstatement. Therefore, in the next phase of this study, we measured the effect of exogenous BDNF infusions within the PFC on reinstatement of cocaine-seeking behavior.

CHAPTER 3

A BDNF INFUSION INTO THE MEDIAL PREFRONTAL CORTEX SUPPRESSES COCAINE SEEKING IN RATS

Introduction

Exposure to contexts or cues previously associated with drug taking, or re-introduction of the drug itself, can elicit reinstatement of drug seeking in animal models of relapse (Spealman et al., 1999; Alleweireldt et al., 2001; Shaham et al., 2003; See, 2005). The susceptibility to drug relapse and other addictive behaviors is thought to depend on long-term neuroadaptations in mRNA, proteins and phospho-proteins (Kalivas and Volkow, 2005). One of the proteins that has been implicated in the reinstatement of cocaine seeking in rats is brain-derived neurotrophic factor (Lu et al., 2004; Liu et al., 2006). BDNF protein expression increases in the ventral tegmental area (VTA), nucleus accumbens (NAc) and amygdale of rats following 30 or 90 days of withdrawal from cocaine self-administration (Grimm et al., 2003). Furthermore, BDNF infusions into the VTA or NAc enhance cocaine-induced behavioral sensitization (Horger et al., 1999), and increase cue- and cocaine-induced reinstatement, a long-term effect that is present for at least 30 days of withdrawal (Lu et al., 2004). Conversely, repeated administration of BDNF antiserum into the NAc during chronic cocaine self-administration increases cocaine-induced reinstatement (Graham et al., 2007). Collectively, these studies implicate

BDNF activity in the VTA and NAc in long-term modulation of cocaine-induced behavior. Cocaine-induced neuroadaptations are commonly manifested by alterations in the postsynaptic plasticity of medium spiny neurons in the NAc. These alterations are likely related to adaptations in prefrontal cortex (PFC) glutamatergic and VTA dopaminergic afferents, both of which are critical mediators of reinstatement to cocaine seeking triggered by conditioned stimuli (CS), context or cocaine itself (McFarland et al., 2003; McLaughlin & See, 2003; Fuchs et al., 2004, 2005; Kalivas, 2004; Bachtell et al., 2005; Schmidt et al., 2005). Withdrawal from cocaine self-administration elicits long-term decreases in NAc basal extracellular glutamate levels and associated intracellular signaling (Pierce et al., 1996; Xi et al., 2002; Baker et al., 2003). Thus, in addition to neuroplasticity in the mesoaccumbens dopamine system, long-term adaptations in the PFC–NAc circuitry are critical for the reinstatement of cocaine seeking. Cortical pyramidal neurons are the predominant source of BDNF in the striatum (Altar et al., 1997), including the NAc. However, the significance of BDNF in the PFC to cocaine-induced motivated behavior has not been investigated. Based on the importance of the cortico-accumbens pathway for reinstatement and the significant role of BDNF in the activity of this pathway, in the current study we evaluated the effects of a BDNF infusion into the PFC on several forms of cocaine-seeking behavior, on food-seeking behavior, as well as on the expression of BDNF and phospho-extracellular-regulated kinase (ERK), a major BDNF-TrkB signaling protein, in the NAc and caudate putamen. We hypothesized that infusion of BDNF into the medial (m)PFC would enhance reinstatement of drug seeking, based on findings that a bilateral BDNF infusion into the VTA immediately after the final cocaine self-administration session augmented cocaine seeking (Lu et al., 2004).

However, we found the opposite, that intra-PFC BDNF, when administered immediately after the final cocaine self-administration session but not 6 days later, suppressed cocaine seeking triggered by CS, context or cocaine itself. These data expand our understanding of the complex role of BDNF in the regulation of addictive behavior.

Materials and Methods

Animals

Male Sprague–Dawley rats (n = 84; Charles River Laboratories, Wilmington, MA, USA), weighing 275–325 g at the time of surgery, were housed individually on a reverse light : dark cycle. Rats were maintained on 20–25 g of rat chow per day, with water available ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina, and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80-23, revised 1996).

Lever response training

Rats were trained to lever press on a fixed ratio 1 schedule of food reinforcement (45 mg pellets; Noyes, Lancaster, NH, USA) in sound-attenuated operant conditioning chambers (30 · 20 · 24 cm high; Medical Associates, St Albans, VT, USA) during a 16 h overnight training session. The chambers were equipped with two retractable levers, a stimulus light above each lever, a food pellet dispenser between the levers, a house light on the wall opposite the levers, and a speaker connected to a tone generator (ANL-926,Medical Associates). During the session, each lever press on the active lever resulted

in delivery of a food pellet only. Lever presses on the inactive lever had no programmed consequences. Following food training, food pellet dispensers were removed from the chambers.

Surgery

At 48 h after food training, rats were anesthetized using a mixture of ketamine hydrochloride (66 mg / kg i.p.; Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine (1.33 mg / kg i.p.; Bayer, Shawnee Mission, KS, USA) followed by equithesin (0.5 mL / kg). Catheters constructed of silastic laboratory tubing (0.64 mm i.d., 1.19 mm o.d.; Dow Corning, Midland, MI, USA) were implanted into the right jugular vein of rats, and were anchored to the vein with silk suture and a hardened silicon gel ball (silicon rubber sealant; General Electric, Waterford, NY, USA) placed 33 mm from the anterior end of the catheter. The silastic tubing ran directly under the skin to an exit point located in the mid-scapular region. The open end of the catheter cannula was enclosed with a short piece of polyethylene tubing that had been heat sealed, and the threaded region of the cannula was covered with a threaded plastic cap when not in use. Immediately following catheter implantation, rats were mounted onto a stereotaxic device (Stoelting, Wood Dale, IL, USA), and bilateral stainless steel cannulae (26 gauge, Plastics One, Roanoke, VA, USA) were implanted 1 mm above the PFC target region (AP=+3.0; ML= +0.6; DV=1.6), relative to bregma, Paxinos & Watson, 1998). Following implantation, guide cannulae were secured to the skull with cranioplastic cement and three steel machine screws. Stylets (Plastics One) were placed into the guide cannulae to prevent blockage. Following surgery, rats were infused i.v. with 0.1 mL each of cefazolin (100

mg/mL) and heparinized saline (70 U/mL) once daily during a 5 day recovery period. Catheter patency was verified by infusing 0.1 mL of methohexital sodium (20 mg/mL, i.v.; Eli Lilly, Indianapolis, IN, USA), which produces a rapid loss of muscle tone only when administered intravenously.

Cocaine self-administration

Cocaine self-administration was conducted in standard self-administration operant chambers (30 X 20 X 24 cm; Medical Associates). Each chamber contained two retractable levers (7 cm above the chamber floor) on each side of the front wall. A white circular stimulus light (2.5 W, 24 V bulb) was located on the panel 7 cm above the active (right) lever, and a red house light (2.5 W, 24 V bulb) was located on the wall on the opposite end of the chamber. The infusion line was tethered to a liquid swivel (Instech, Plymouth Meeting, PA, USA) mounted on a suspended counterbalance. The self-administration apparatus was enclosed in a sound-attenuating chamber (Medical Associates). Cocaine hydrochloride (4 mg/mL; National Institute on Drug Abuse/RTI International, Research Triangle Park, NC, USA) was delivered using a computer-controlled infusion pump located outside the chamber. The entire system was computer controlled using Medical PC for Windows. Rats self-administered cocaine on 10 consecutive days during 2 h daily sessions. Figure 1 illustrates the experimental design of each of the experiments. Rats received 0.1 mL of heparinized saline (10 U/mL, i.v.) prior to each self-administration session. Animals were then connected to the infusion tether and each session began immediately, signaled by the movement of both levers into the chamber. With the exception of the yoked-saline rats from Experiment 1, cocaine

reinforcement was available on a fixed ratio 1 schedule of reinforcement explicitly paired with a compound CS consisting of illumination of a white stimulus light located above the active lever and a tone (2 kHz, 15 dB above ambient noise). Responses on the active (right) lever resulted in the delivery of cocaine (0.2 mg / 0.05 mL bolus) over 2 s, and each infusion was followed by a 20 s 'time-out' period. During the time-out, active lever presses were recorded, but had no programmed consequences. Responses on the inactive (left) lever were also recorded, but had no programmed consequences. Yoked-saline rats (Experiment 1) see below) received an infusion of saline (0.05 mL bolus) over 2 s, contingent upon the cocaine infusion received by the self-administering rat in the adjacent self-administration chamber. Rats underwent 10 days of cocaine self-administration prior to a single intracranial infusion.

Intracranial infusions

We adapted the single infusion protocol and chose the dose of BDNF (0.75 μg / side) that Lu et al. (2004) previously used in vivo. For BDNF or vehicle (10 mM phosphate-buffered saline) (PBS) infusions, infusion cannulae (33 gauge; Plastics One) were inserted bilaterally into the guide cannulae such that 1 mm of the infusion cannulae extended past the end of the guide cannulae. Human recombinant BDNF (R & D Systems, Minneapolis, MN, USA) or vehicle (sterile PBS) was infused using gas-tight Hamilton syringes (10 μL) and an infusion pump (Harvard Apparatus, Holliston, MA, USA). A volume of 0.5 μL was infused over 2 min, and the infusion cannulae remained in the guide cannulae for 1 min before and after the infusion.

Abstinence, extinction and reinstatement

Figure 1 illustrates the experimental designs for all the studies. Experiment 1 was conducted to determine if infusion of BDNF immediately following the last self-administration session would alter the first day of extinction responding and to harvest tissue for BDNF and phospho-ERK measurements. In Experiment 1a, uncannulated rats self-administered cocaine or received yoked-saline for 10 days. After 22 h abstinence, they were placed back into the self-administration environment for a 30 min extinction test during which the rats were allowed to press on either the active or inactive lever without any programmed consequences. At the end of the 30 min test, they were decapitated, and the NAc and caudate putamen were harvested for phospho-protein and BDNF protein measurements. In Experiment 1b, cannulated rats received an intra-PFC infusion of either BDNF or vehicle immediately following the final (10th) cocaine self-administration session. Rats in Experiment 1b were also placed back into the self-administration environment for a 30 min extinction test following 22 h of abstinence from cocaine self-administration. At the end of the 30 min test, they were decapitated and the brains were harvested for histological verification of cannulae sites (PFC), and phospho-protein and BDNF protein measurements (NAc and caudate putamen). Tissue from Experiments 1a and 1b was processed, assayed and analyzed together for comparative purposes.

Experiment 2 was performed to investigate whether BDNF would alter: (a) relapse to cocaine seeking elicited by discrete light + tone cues in the self-administration context in abstinent rats; (b) reinstatement of cocaine seeking elicited by the light + tone cues in rats that underwent 6 days of extinction training; and (c) reinstatement of cocaine

seeking elicited by a cocaine-priming injection in the same rats that underwent an additional 6 days of extinction training which was sufficient to reach extinction criterion again. Cannulated rats received an intra-PFC infusion of either BDNF or vehicle immediately following the final (10th) self-administration session. The rats remained in the home cage for 6 days following the last self-administration session. On abstinence day 7, rats underwent a 2 h test in the self-administration chambers, in which each active lever press resulted in a contingent 5 s presentation of the light + tone CS (with a 20 s time-out) in the absence of cocaine reinforcement. Rats continued for six daily extinction sessions in which lever presses had no programmed consequences, and were then tested during a 1 h extinction phase followed immediately by a 1 h cue-induced reinstatement test. During the cue reinstatement test, active lever presses resulted in light + tone CS presentations in the absence of cocaine reinforcement. Following 6 additional days of extinction, rats underwent a 1 h extinction session followed by a 30 min cocaine-primed reinstatement test for which each rat was injected with cocaine (10 mg / kg, i.p.) at the beginning of the test. During the cocaine-primed reinstatement test, lever presses were recorded, but had no programmed consequences. At the end of this final test, the brains were harvested, after decapitation, for histological verification of cannulae sites, and phospho-protein and BDNF protein analysis. Experiment 3 was conducted to determine if BDNF would suppress cocaine seeking in abstinent rats if it was infused into the mPFC 22 h before the test instead of immediately following the last self-administration session. In this experiment, rats remained in the home cage (abstinence) for 6 days following the last self-administration session. On the 6th day of abstinence, rats received an intra-PFC infusion of BDNF or vehicle, but did not return to the self-administration environment.

On abstinence day 7, rats underwent a 2 h test in the self-administration chambers, in which each active lever press resulted in a contingent 5 s presentation of the light + tone CS (with a 20 s time-out) in the absence of cocaine reinforcement. At the end of the experiment, the rats were decapitated and the brains were harvested for histological verification of cannulae sites.

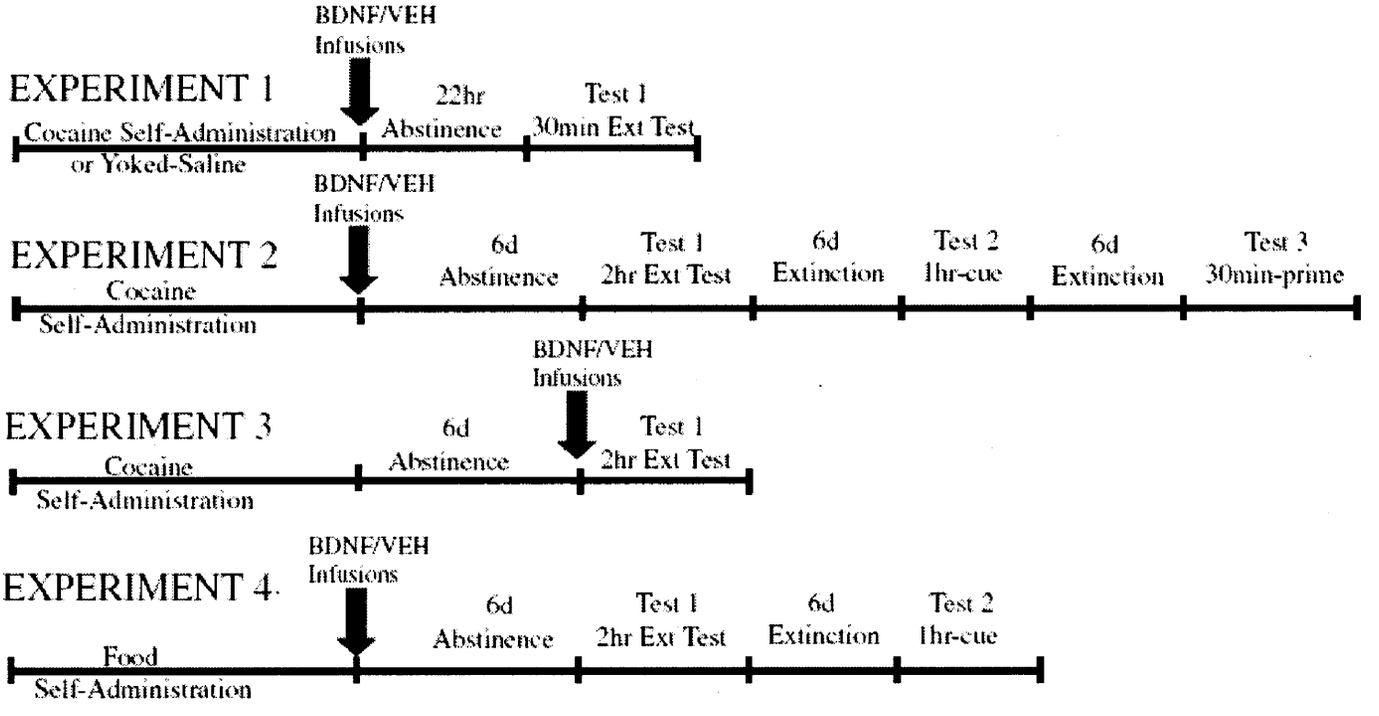

Fig. 3-1. Experimental Paradigm. Schematic diagram of the experimental paradigms used in experiments 1-4.

Food self-administration and reinstatement

Experiment 4 was designed to be as similar as possible to the design of the abstinence and cue-induced reinstatement phases of Experiment 2, except that the rats learned to self-administer food instead of cocaine. Rats initially lever-pressed for food reinforcement (45 mg Noyes food pellets) on a fixed ratio 1 schedule of reinforcement, during daily 2 h sessions on 10 consecutive days. Subjects then remained in the home cage for a 6 day abstinence period, during which they were maintained at 90% ad libitum

weight. Rats then underwent a 2 h post-abstinence test, during which active lever pressing resulted in the presentation of the previously paired light + tone but no food. Following 6 days of extinction training, during which lever presses were recorded but never resulted in food pellet or CS delivery, rats underwent a cue-induced reinstatement test for food-seeking behavior. During the cue-induced reinstatement test, active lever presses resulted in the presentation of the CS previously paired with food during self-administration. The 1 h test was preceded by 1 h of extinction training.

Tissue processing and histology

Rats in Experiments 1–4 were killed by rapid decapitation following the completion of the final extinction or reinstatement test. Brains were immediately removed and frozen in isopentane, then stored at 80° C until they were sectioned on a cryostat. Sections through the PFC were sectioned at 12 μm for cannulae placement verification. The remaining forebrain from rats in Experiments 1 and 2 was sectioned from rostral to caudal up to AP 1.7 from bregma. It was then removed from the chuck and sectioned caudal to rostral up to AP 0.7 from bregma. The remaining slab of tissue contained the dorsal and ventral striatal target areas that were extracted using a 13-gauge tissue punch. Frozen punches were stored at -80° C prior to being processed for immunoblotting.

Immunoblotting

The method described by Toda et al. (2003) was used. Protein was extracted from both the NAc and caudate putamen, and the tissue was sonicated in Trizol (Invitrogen,

Carlsbad, CA, USA). Samples were analyzed for protein, diluted to equalize for the protein concentrations, and aliquoted. Samples were stored in the sample buffer [Tris–HCl, 0.5 mm, pH 6.8; glycerol 10%; sodium dodecyl sulfate (SDS) 10%; b-mercaptoethanol, 5%; bromophenol blue, 0.05% w/v], boiled for 2 min and loaded onto gels (12% SDS). Proteins were separated by application of 30 mA constant current for 25–30 min, transferred onto polyvinylidene fluoride (PVDF) membrane strips (200 mA for 60 min; Millipore, Billerica, MA, USA), and immunoblotted with anti-phospho-ERK 1 / 2 (1 : 500-phospho-p44 / 42 MAP kinase, Thr202 / Tyr204, Cell Signaling Technologies, Danvers, MA, USA) for 60 min at room temperature. PVDF strips were washed and incubated for 60 min at room temperature with secondary antibody [horseradish peroxidase (HRP)-linked anti-rabbit antibody 1 : 1000; Amersham Biosciences, Piscataway, NJ, USA] and protein complexes were visualized by enhanced chemiluminescence ECL detection (Amersham Biosciences). Immunoblots were exposed to Hyperfilm (Amersham Biosciences). Following imaging, membranes were incubated for 10 min in stripping agent (re-blot plus Western blot mild antibody stripping solution; Chemicon International, Temecula, CA, USA) and then re-exposed to primary antibody specific for non-phosphorylated ERK 1 / 2 (1:1000) overnight. Protein bands were quantified by densitometry using Image J software. Analyses were performed by evaluating the ratio of phospho-ERK / total ERK expression of each sample within a single membrane. The ratio was normalized across trials (a minimum of three comprehensive, individual trials was performed for each experiment) by expressing each ratio value as a percentage of the control within each individual trial.

BDNF enzyme-linked immunosorbent assay (ELISA)

BDNF levels were assessed using ELISA-E_{max} Immunoassay System (Promega, Madison, WI, USA) kits, according to the manufacturer's protocol. Flat-bottom plates were coated with an anti-BDNF monoclonal antibody (1:1000) overnight. After blocking non-specific binding, immobilized anti-BDNF monoclonal antibody was incubated with brain tissue samples containing BDNF protein or BDNF standards serially diluted to prepare a standard curve, followed by anti-human BDNF polyclonal antibody (1 : 500). The complex was bound using an IgY antibody conjugated to HRP as a tertiary reactant. After repeatedly washing unbound conjugate, plates were incubated with tetramethylbenzidine chromagenic substrate, and color change was measured in an ELISA plate reader at 450 nm. Using this kit, BDNF can be quantified in the range 7.8–500 pg / mL. Measurements given by the plate reader were calibrated against a standard curve prepared with human BDNF protein from Promega plotted in serial dilution with a correlation coefficient of $r_2 > 0.95$ accepted. Adjusted concentrations of BDNF expression were normalized to the average concentration of the control condition.

Statistical analysis

Responding on both the active and inactive levers across groups during relapse to cocaine seeking was compared with responding during the last 3 days of self-administration or responding during the extinction session that preceded the test as appropriate in each of the studies. Due to the experimental design (multiple observations per rat) the data are correlated, so a statistical model capable of accounting for this correlation was used for the behavioral data to avoid biased P-values (Fitzmaurice et al.,

2004). Further, the response variables (number of active and inactive lever presses) are 'count' data, so an approach that does not assume normality was selected. To address both of these issues in the statistical analysis, a generalized estimating equation approach using a Poisson distribution (Litrell et al., 2002) was implemented using SAS v9.1 (SAS Institute, Cary, NC, USA), with treatment (e.g. BDNF or vehicle) and condition (e.g. extinction or cocaine challenge) as explanatory covariates (factors). Generalized estimating equation methods were first proposed by Liang & Zeger (1986), and details of their statistical properties are further described by Fitzmaurice et al. 2004. These methods are similar to mixed model analyses of variance (ANOVAs), as both explain a correlated response variable with explanatory covariates) ANOVA is used for response data that are normally distributed, whereas generalized estimating equations are used for binary or count data. Generalized estimating equations use a chi-square because it is effectively doing two analyses: one with no covariates and another with all of the covariates of interest. It then does a goodness of fit test comparing the improvement in the model with covariates over the model without them. For data that were approximately normally distributed (immunoblotting and ELISA), a mixed model ANOVA was performed and multiple comparisons were adjusted via the Tukey-Kramer test (for unequal n per group) when a significant F-value was obtained. P-values < 0.05 were considered statistically significant.

Results

Histology

Figure 2 illustrates the placement of infusion cannula tips in the mPFC (anterior cingulate or prelimbic cortex) of rats that received bilateral BDNF or vehicle infusions in

all experiments. Subjects with cannula tracts that did not meet the criteria of bilateral placement in anterior cingulate or prelimbic cortex were not included in the schematic, and the data from these animals (n = 4 from Experiment 1, n= 3 from Experiment 2, n=1 from Experiment 3, n=1 from Experiment 4) were excluded from analysis. After histological verification, the experimental groups were as follows: Experiment 1: cocaine self-administering (no cannulae) (n = 8), yoked saline (no cannulae) (n = 10); intra-PFC BDNF (n = 9), intra-PFC vehicle (n = 7); Experiment 2 (cocaine self-administering): intra-PFC BDNF (n = 12) and intra-PFC vehicle (n = 12); Experiment 3: intra-PFC BDNF (n = 12) and intra-PFC vehicle (n = 11); Experiment 4 (food self-administering) intra-PFC BDNF (n = 5) and intra-PFC vehicle (n = 7).

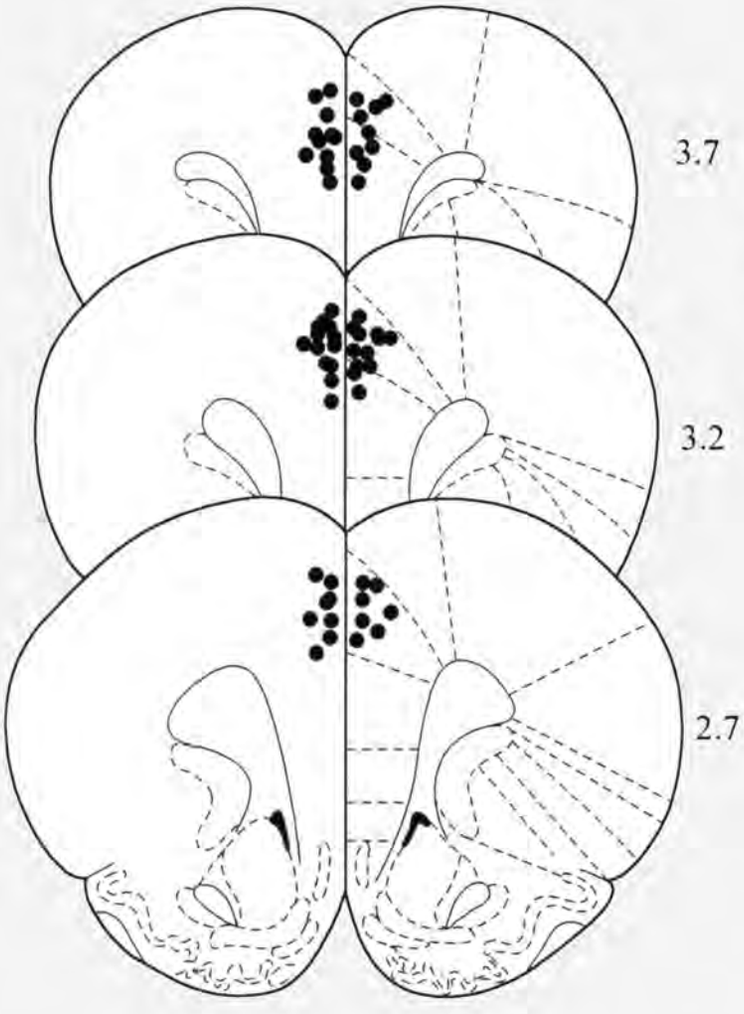

Fig. 3-2. Cannula Placement within the PFC. Representation of the most ventral point of the infusion cannulae within the mPFC. Measurements correspond to distance from bregma in millimeters (Paxinos and Watson, 1998).

Experiment 1: Intra-PFC BDNF infusion suppressed extinction responding, increased BDNF protein in NAc, and prevented a cocaine-induced decrease in phospho-ERK in NAc

All rats exhibited stable active (right) lever responding over the last 3 days of self-administration that did not differ between treatment groups (mean ± SEM for vehicle 52.19 ± 5.74; for BDNF 51.60 ± 4.40; $\chi^2 = 0.01$; df_1; $P > 0.9$). Twenty-two hours after the 10th self-administration session, a 30 min extinction test was conducted after which the brains were harvested for phospho-protein measurements. Rats with a cocaine history exhibited significantly greater active lever pressing during the 30 min test than those with a yoked-saline history (Fig. 3A, left$\chi 2 = 52.3$; df_1; $P < 0.001$). Rats with a cocaine history that had been infused with BDNF responded significantly less on the active lever than the vehicle-infused rats with a cocaine history (Fig. 3A, right $\chi^2 = 4.3$; df_1; $P < 0.05$). There was no significant difference in inactive lever pressing between groups during the 30 min extinction test ($\chi^2 = 1.1$; df_1; $P = 0.3$). BDNF protein in the NAc of rats that received an intra-PFC BDNF infusion 22 h before the extinction test was significantly greater than in the NAc of rats in the other three groups (ANOVA $F_{3,28} = 6.0$; $P < 0.01$, Fig. 3B). In contrast, there was no difference between groups in BDNF protein expression in the caudate putamen (data not shown). Interestingly, there was a significant decrease in phospho- ERK expression within the NAc of uncannulated and cannulated (vehicle-treated) rats with a cocaine history compared with yoked saline control rats (ANOVA $F_{2,51} = 15.5$, $P < 0.0001$, Fig. 3C). In contrast, phospho-ERK expression within the NAc was greater in rats that received intra-PFC BDNF infusions than in intra-PFC vehicle infused rats (Tukey Kramer, $P < 0.005$) or in uncannulated cocaine self-administering rats ($P < 0.0001$). There was, however, a significant effect of cocaine self-administration on

phospho-ERK / total ERK expression within the caudate putamen in the presence or absence of BDNF infusion ($F_{3,15} = 3.2$, $P < 0.05$, Fig. 3D). Pair-wise comparisons (Tukey Kramer) revealed a significant decrease in phospho- ERK / total ERK expression in uncannulated rats that self-administered cocaine ($P < 0.05$), and rats that self-administered cocaine and received intra-PFC vehicle ($P < 0.01$) or BDNF ($P < 0.05$) compared with yoked-saline controls.

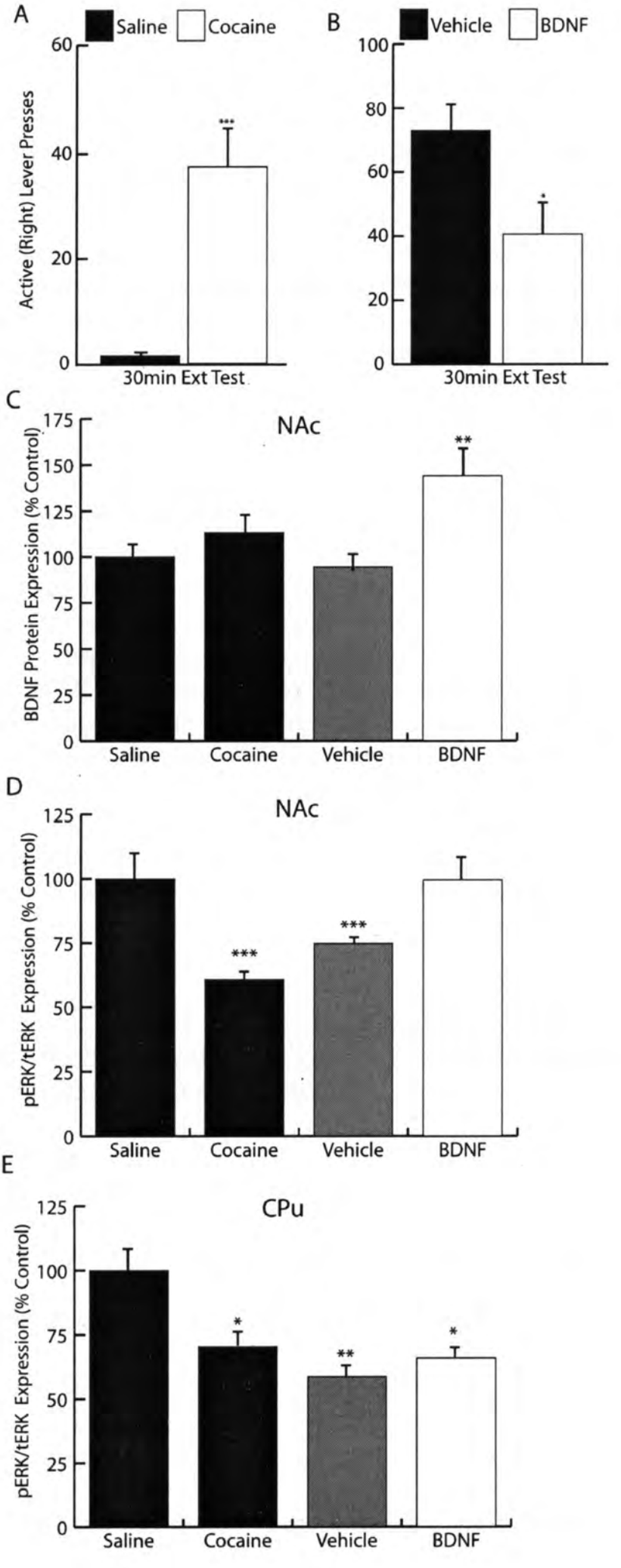
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Fig. 3-3. Intra-PFC brain-derived neurotrophic factor (BDNF) suppressed cocaine-seeking behavior during a 30 min extinction test after 22 h of abstinence, increased BDNF protein, and normalized phospho-extracellular regulated kinase (pERK) levels in the NAc. Data are expressed as the mean number of active lever presses ± SEM. (A) Rats with a cocaine self-administration history (n = 8) press the active lever significantly more during a 30 min extinction test than yoked-saline rats (n = 10, ***P < 0.001). (B) A bilateral intra-PFC infusion of BDNF attenuated cocaine-seeking behavior during the 30 min extinction test in rats with a cocaine history. The BDNF-treated rats (n = 9) exhibited significantly fewer active lever presses during the 30 min extinction test than the vehicle-treated rats (n = 7, *P < 0.05). (C) Rats with a cocaine history (n = 9) that received a bilateral intra-PFC infusion of BDNF 22 h prior to a 30 min extinction test exhibited significantly greater BDNF protein expression within the NAc than cannulated, vehicle-infused rats (n = 9), uncannulated rats (n = 6) with a cocaine history, or saline-yoked controls (n = 8, **P < 0.01). (D) Cocaine-self-administration significantly decreased the phospho-ERK / total ERK ratio within the NAc of both uncannulated rats (***P < 0.001) and rats that received a bilateral infusion of vehicle (PBS) into the PFC (***P < 0.001), as compared with uncannulated saline controls. In contrast, intra-PFC BDNF infusions following cocaine self-administration prevented the decrease in phospho- ERK / total ERK ratio induced by cocaine self-administration. n = 7–8 per group. (E) Cocaine self-administration significantly decreased the phospho- ERK / total ERK ratio within the caudate putamen (CPu) of both uncannulated rats (*P < 0.05) and rats that received a bilateral infusion of vehicle (PBS) into the PFC (**P < 0.01), as compared with saline controls. However, intra-PFC BDNF infusions following cocaine self-administration did not prevent the decrease in phospho-ERK / total ERK ratio induced by cocaine self-administration (*P < 0.05). n = 4 per group.

Experiment 2: Intra-PFC BDNF infusion, immediately after the last cocaine exposure, suppressed responding following abstinence and cue- and cocaine-induced reinstatement following extinction

Test 1: post-abstinence test

The effect of an intra-PFC BDNF infusion on cocaine seeking was evaluated after 6 days of abstinence (Fig. 4A). During the 2 h test, lever presses resulted in the response-contingent presentation of the light + tone CS only. The vehicle-treated group ($\chi 2 = 7.99$; df_1; P = 0.005), but not the BDNF-treated group ($\chi 2 = 0.27$; df_1; P = 0.6), responded significantly more on the active lever during the post-abstinence test than during the last 3 days of self-administration. Furthermore, the intra-PFC BDNF-infused rats responded

significantly less on the active lever than the vehicle-infused rats during the extinction test ($\chi 2 = 11.43$; df_1; $P < 0.001$). Additionally, vehicle-treated ($\chi 2 = 665.5$; df_1; $P < 0.001$) and BDNF-treated ($\chi 2 = 19.2$; df_1; $P < 0.001$) rats responded significantly more on the inactive lever during the post-abstinence test compared with the last 3 days of cocaine self-administration, as reported previously (Fuchs et al., 2006).

Test 2: cue-induced reinstatement

Following Test 1 and 6 days of extinction training, during which there were no differences in the extinction rate or amount of lever pressing between groups (data not shown), the rats underwent a cue-induced reinstatement test that consisted of a 1 h extinction phase and a 1 h cue phase (Fig. 4B). During the cue phase, active lever pressing resulted in contingent presentation of the light + tone CS. Vehicle-treated rats responded significantly more on the active lever during the cue test than in the preceding hour of extinction ($\chi 2 = 8.01$; df_1; $P = 0.005$). In contrast, the intra-PFC BDNF-infused rats responded less than the vehicle-treated rats on the active ($\chi 2 = 17.8$; df_1; $P < 0.001$) and inactive ($\chi 2 = 4.05$; df_1; $P = 0.04$) lever during the cue test.

Test 3: cocaine-induced reinstatement

Following 6 additional days of extinction training, during which there were no differences in the extinction rate or amount of lever pressing between groups (data not shown), the rats underwent a cocaine-primed reinstatement test (Fig. 4C). This consisted of a 1 h extinction phase and a 30 min cocaine test phase (timed in order to harvest brains for phospho-ERK measurements that decline rapidly). At the end of the extinction phase, rats received an injection of cocaine (10 mg/kg, i.p.). Because the extinction phase was

longer than the cocaine prime test phase, only the average number of lever presses during the last 30 min of extinction was compared with that during the 30 min cocaine prime test. Vehicle-treated rats responded significantly more on the active lever during the 30 min cocaine prime test than during the preceding 30 min of extinction ($\chi 2 = 24.82$; df1; $P < 0.001$), demonstrating reinstatement of cocaine seeking. Once again, the intra- PFC BDNF-infused rats pressed the active lever significantly less than the intra-PFC vehicle-infused rats during the cocaine-primed reinstatement test ($\chi 2 = 4.8$; df1; $P < 0.05$). There was no significant difference in inactive lever pressing during the cocaine-induced reinstatement session between groups ($\chi 2 = 2.96$; df1; $P > 0.05$) or during extinction ($\chi 2 = 0.06$; df1; $P > 0.5$). There was no significant difference in phospho-ERK expression in the NAc ($F1,20 = 0.12$; $P = 0.735$) or the caudate putamen ($F1,18 = 0.00$; $P = 0.996$) of intra-PFC BDNF-treated rats as compared with intra-PFC vehicle-treated rats 30 min after the cocaine infusion.

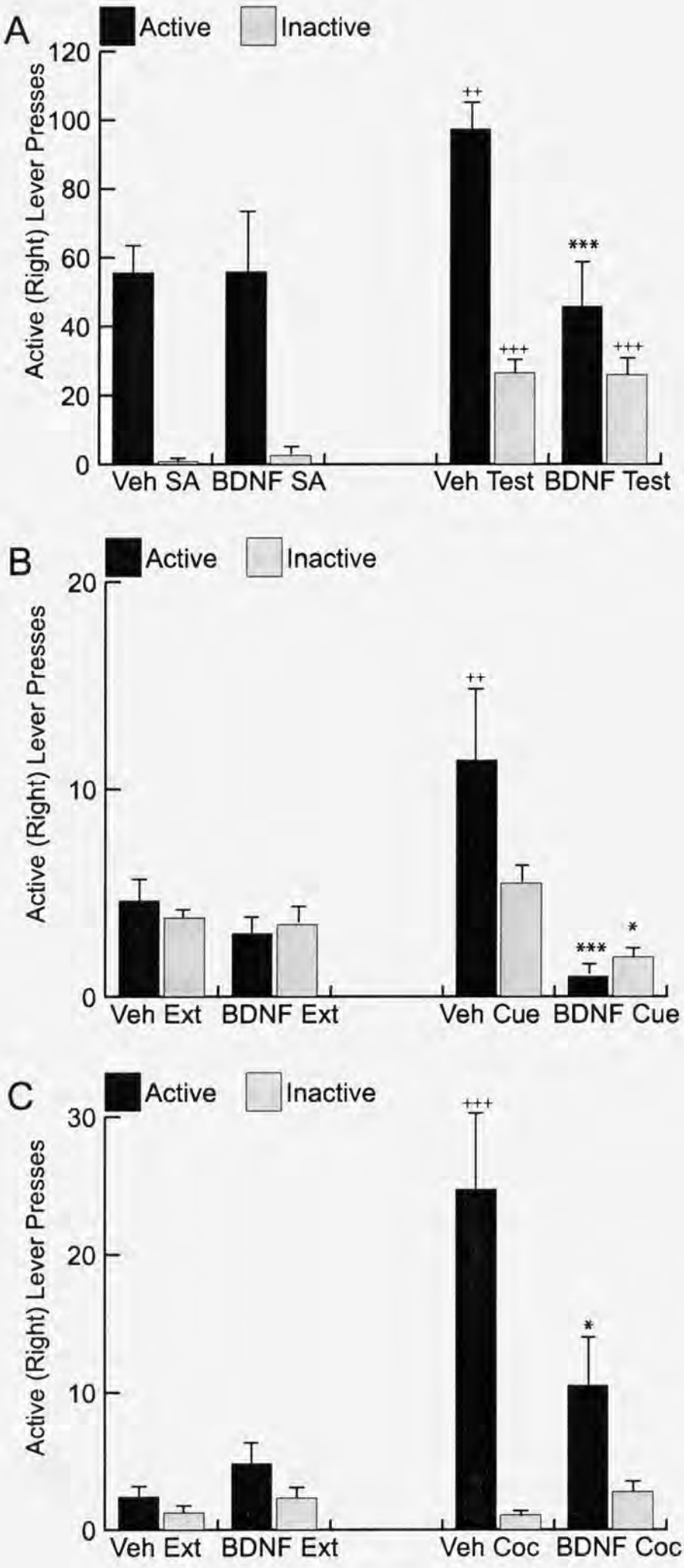
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Figure 3-4. A bilateral intra-PFC infusion of brain-derived neurotrophic factor attenuated cocaine-seeking behavior. Data for vehicle- and BDNF treated rats are expressed as mean ± SEM. n = 12 per group. (A) Left: there was no difference in the number of active or inactive lever presses by rats subsequently treated with intra-PFC BDNF or vehicle during the last 3 days of cocaine self-administration (Veh SA vs BDNF SA). Right: after 6 days of abstinence, vehicle-treated rats exhibited a significant increase in responding during the post-abstinence test when compared with cocaine self-administration (++P < 0.01). Both BDNF- and vehicle-treated rats exhibited a significant increase in inactive lever pressing during the post-abstinence test compared with cocaine self-administration (+++P < 0.001). Furthermore, the BDNF treated rats exhibited significantly fewer active lever presses during the post abstinence test than the vehicle-treated rats (***P = 0.001). (B) Left: after 6 days of extinction training, the number of active or inactive lever presses during the hour of extinction that preceded the cue-induced reinstatement test was not significantly different between groups. Right: the intra-PFC vehicle treated rats responded more on the active lever during the cue test than during the preceding hour of extinction (first ++P < 0.01). The BDNF-treated rats exhibited significantly fewer active (***P < 0.001) and inactive (*P < 0.05) lever presses during the cue-induced reinstatement test than vehicle-treated rats. (C) Left: the number of active or inactive lever presses during the 30 min of the extinction phase that preceded the 30 min cocaine-primed reinstatement test was not different between treatment groups. Right: vehicle-treated rats exhibited a significant increase in responding during the cocaine-prime test compared with the extinction phase (+++P < 0.001). The BDNF-treated rats exhibited significantly fewer active lever presses during the cocaine-induced reinstatement test than the vehicle-treated rats (*P < 0.05).

Experiment 3: An intra-PFC BDNF infusion on the 6th day of abstinence does not alter cocaine-seeking behavior

Post-abstinence test

The effect of a BDNF infusion into the PFC on the 6th day of abstinence was evaluated during a 2 h post-abstinence test. During the 2 h test, rats were allowed to lever press in the self-administration environment in the absence of drug reinforcement, but in the response contingent presence of the light + tone CS (Fig. 5). Both vehicle treated ($\chi 2$ = 78.8; df_1; P < 0.001) and BDNF-treated ($\chi 2$ = 13.8; df_1; P < 0.001) rats exhibited significantly more active lever pressing during the 2 h test than during the last 3 days of cocaine self-administration. Additionally, both vehicle-treated ($\chi 2$ = 11.8; df_1; P < 0.001) and BDNF-treated ($\chi 2$ = 9.39; df_1; P = 0.002) rats exhibited significantly more inactive

lever pressing during the 2 h test than during the last 3 days of cocaine self-administration. However, there was no difference between groups in active ($\chi 2 = 0.47$; df_1; $P = 0.5$) or inactive ($\chi 2 = 0.35$; df1; $P = 0.6$) lever pressing.

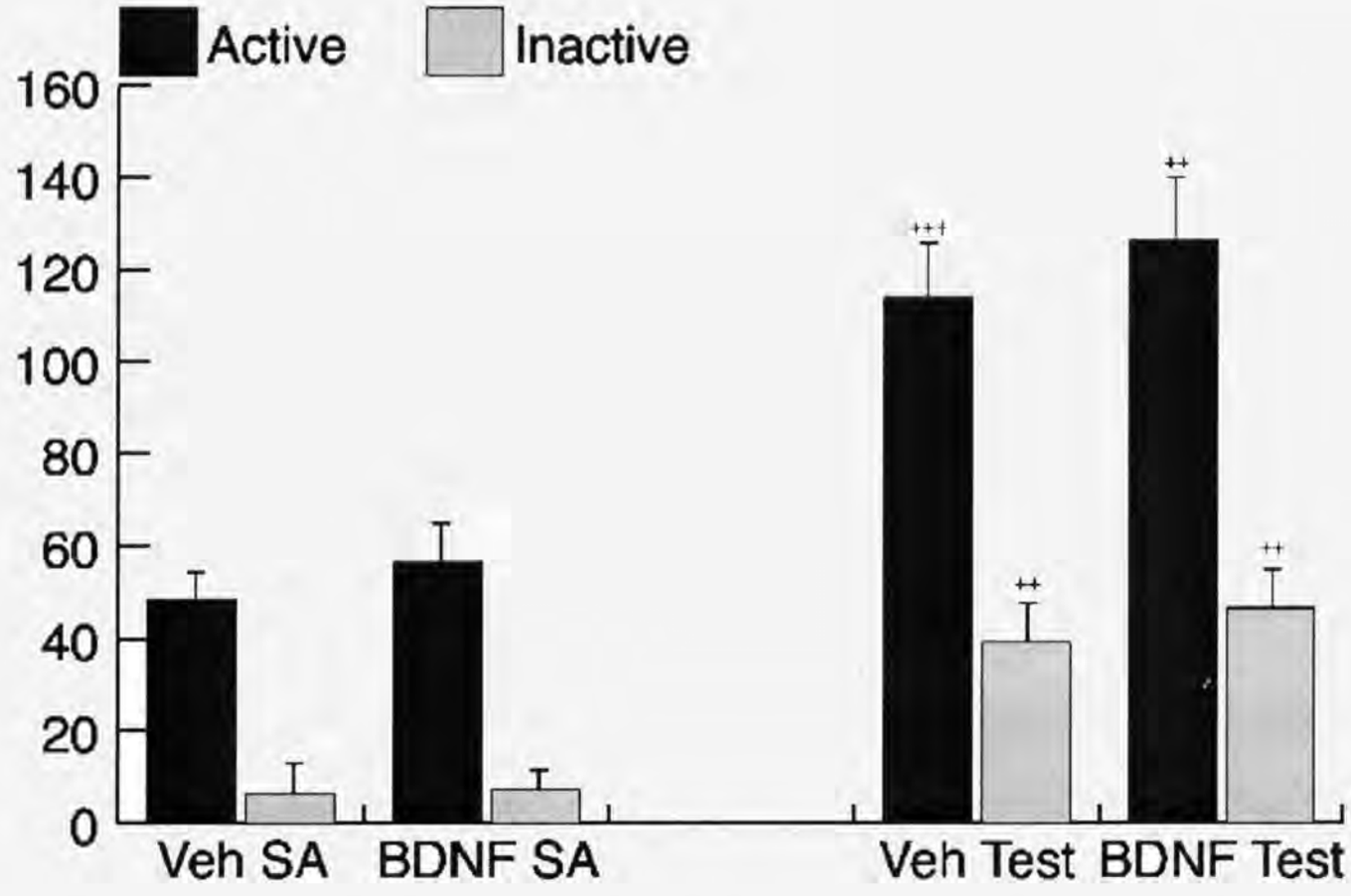

Fig. 3-5. An intra-PFC infusion of BDNF after 6 days of abstinence failed to significantly alter cue/contextual reinstatement to cocaine-seeking behavior. Data for vehicle (N=11) and BDNF-treated (N=12) rats are expressed as mean ± SEM. Left: The number of active and inactive lever presses over the last three days of self-administration did not differ between treatment groups. Right: BDNF-treated rats showed a significant increase in both active (++P<0.01) and inactive (++P=0.01) lever presses during the 2-hour post-abstinence test compared with self-administration. Vehicle-treated rats also showed significant increase in both active (+++P<0.001) and inactive (++P=0.01) lever presses during the 2-hour post-abstinence test compared with self-administration.

Experiment 4: Intra-PFC BDNF infusion does not alter food self-administration or cue-induced reinstatement of food-seeking

Test 1: post-abstinence test

The effect of a BDNF infusion into the PFC on food-seeking behavior was evaluated after 6 days of abstinence. During the 2 h test, rats were allowed to lever press in the self-administration environment in the absence of food reinforcement, but in the response-contingent presence of the light + tone CS (Fig. 6A). Statistical analysis

revealed that vehicle-treated ($\chi 2 = 36.9$; df_1; $P < 0.001$) and BDNF-treated ($\chi 2 = 13.0$; df_1; $P < 0.001$) rats showed significantly lower active lever responding during the first extinction session compared with the last 3 days of food self-administration. However, active lever responding during the post-abstinence test did not differ between BDNF- and vehicle-treated rats. In addition, both vehicle-treated ($\chi 2 = 6.4$; df_1; $P = 0.002$) and BDNF-treated ($\chi 2 = 4.29$; df_1; $P = 0.04$) rats showed significantly higher inactive lever responding during the 2 h test than during the last 3 days of food self-administration.

Test 2: cue-induced reinstatement

Following 6 days of extinction training, rats underwent a cue-induced reinstatement test that consisted of a 1 h extinction phase and a 1 h cue phase (Fig. 6B). During the cue phase, active lever presses resulted in contingent presentation of the light + tone CS. Statistical analysis revealed that both BDNF-treated ($\chi 2 = 2.83$; df_1; $P = 0.04$) and vehicle-treated ($\chi 2 = 40.07$; df_1; $P < 0.001$) rats exhibited a significant increase in active lever responding during the cue-induced reinstatement test relative to extinction. However, there was no difference in active lever presses between the vehicle-treated and BDNF-treated groups ($\chi 2 = 0.94$; df_1; $P = 0.33$). In addition, there were no differences in inactive lever presses between groups during either the extinction phase or the cue test.

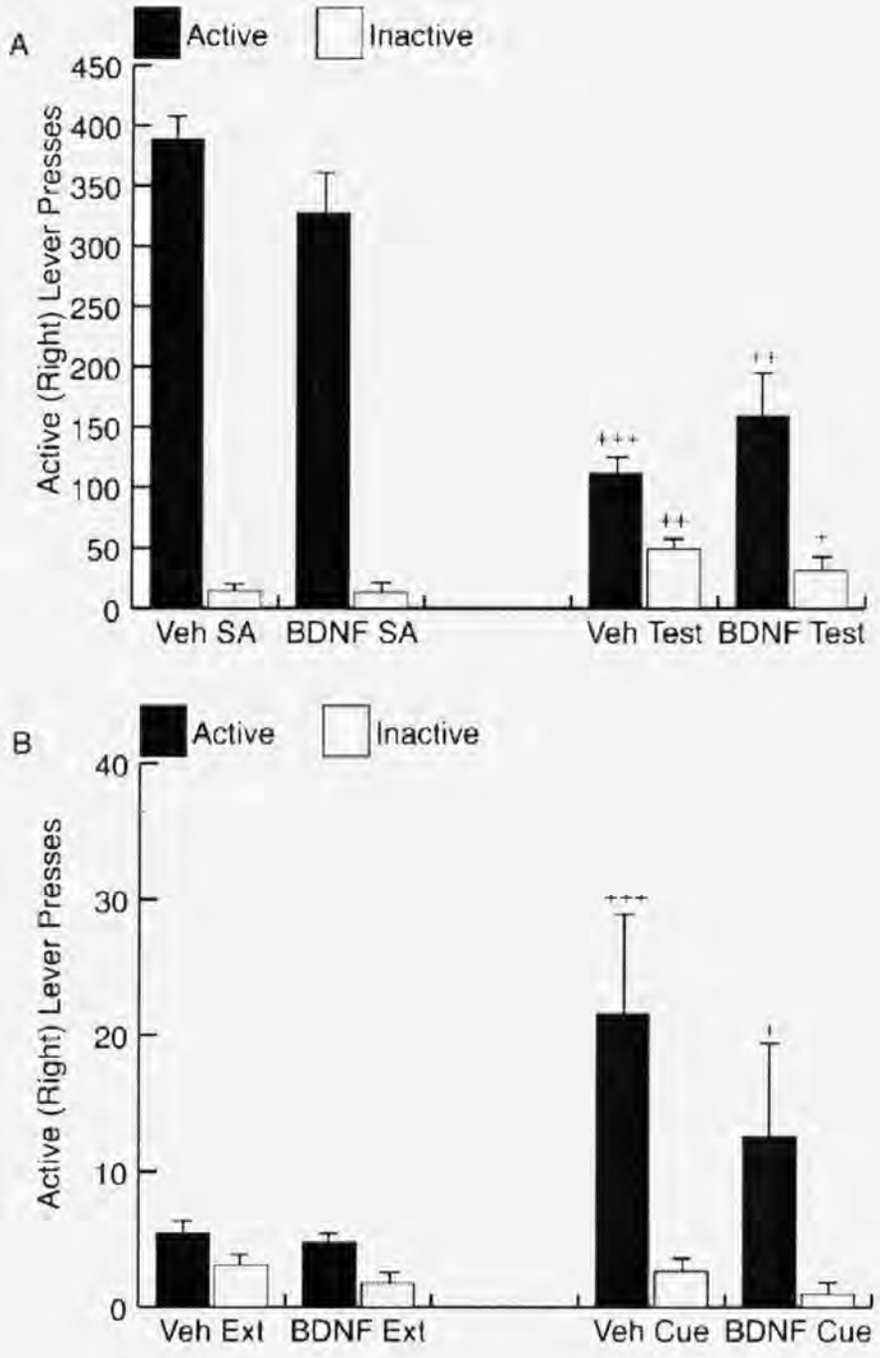

Fig. 3-6. An intra-PFC infusion of BDNF failed to significantly alter cue/contextual reinstatement of food-seeking behavior. Data for vehicle (N=7) and BDNF-treated (N=5) rats are expressed as mean ± SEM. (A) Left: Active or inactive lever presses during the last three days of food self-administration (Veh SA vs. BDNF SA) did not differ between treatment groups. Right: Rats in both the vehicle-treated ($^{+++}P<0.001$) and BDNF-treated ($^{++}P<0.01$) groups exhibited significantly less active lever responding during the post-abstinence test as compared to responding during the last three days of self-administration. However, rats in both the vehicle-treated (++P<0.01) and BDNF-treated (+P<0.05) groups exhibited significant increases in inactive lever pressing during the post-abstinence test compared to responding during the last three days of self-administration. (B) Left: The number of active or inactive lever presses during the hour of extinction that preceded the cue test did not significantly different between treatment groups. Right: Both vehicle- treated ($^{+++}P<0.001$) and BDNF-treated (+P<0.05) groups exhibited a similar increase in active lever responding during the cue-induced reinstatement test relative to extinction.

Discussion

The present study represents the first report to examine the role of PFC BDNF in cocaine-motivated behavior. A BDNF infusion into the PFC immediately after the last cocaine self-administration session attenuated: (a) cocaine seeking upon re-exposure to the self-administration environment after 22 h or 6 days of abstinence; and (b) the reinstatement of extinguished cocaine seeking produced by either CS presentation or a cocaine-priming injection. In contrast, when BDNF was infused 6 days after the end of chronic cocaine self-administration, it had no effect on cocaine seeking. These effects were specific to cocaine-motivated behavior, as intra-PFC BDNF treatment failed to alter food-seeking behavior in a fixed ratio 1 paradigm that directly paralleled the cocaine-seeking experimental design in session length, contingent presentation of the CS and reward availability. Furthermore, intra-PFC BDNF infusion blocked the suppressive effect of cocaine self-administration on phospho-ERK and raised BDNF protein levels in NAc, but not the caudate putamen, 22 h later. Together, these findings suggest that BDNF infusion into the PFC has a selective suppressive effect on cocaine-seeking behavior, possibly by increasing BDNF and normalizing phospho-ERK expression in the NAc during early abstinence.

TrkB receptors are expressed by interneurons and pyramidal neurons in the cerebral cortex, but endogenous BDNF is expressed only by pyramidal neurons (Kokaia et al., 1993). However, BDNF and TrkB receptors are both found in postsynaptic densities at asymmetric (excitatory) synapses in the cerebral cortex (Aoki et al., 2000). BDNF is anterogradely transported to excitatory terminals in the cerebral cortex and to subcortical target structures including the striatum (Altar et al., 1997), where it is released

in a calcium-dependent manner (Hartmann et al., 2001; Balkowiec & Katz, 2002; Pang et al., 2004). Thus, it is likely that after an intra-PFC infusion, targeted in the dorsal prelimbic cortex that has dense projections to the NAc (Vertes, 2004; Gabbott et al., 2005), exogenous BDNF binds to TrkB receptors, becomes internalized and is then transported to the NAc, leading to elevated levels at 22 h. In support of this possibility, exogenous BDNF is transported to remote sites in the CNS after intraventricular or intracerebral infusion in monkeys (Mufson et al., 1996). Additional evidence that exogenous BDNF is internalized, transported and becomes available for activity-dependent secretion has been demonstrated in primary hippocampal cultures (Santi et al., 2006). Furthermore, 22 h after infusion of BDNF into the mPFC of naive rats, BDNF immunoreactivity is elevated in the NAc and amygdala, but not in the PFC or caudate putamen, as compared with vehicle-infused rats (T.W. Whitfield, W.J. Berglind and J.F. McGinty, Appendix Figure 3). Thus, BDNF may have local effects at the site of infusion (PFC) and distal effects in PFC-target areas like NAc. The possibility that exogenous BDNF is transported to targets of PFC projection neurons will be directly investigated in future studies by infusing a fluorescently tagged BDNF (Stroh et al., 2004) into the PFC and searching for its presence in remote targets at different time points after infusion, including the lateral hypothalamus and mediodorsal nucleus of the thalamus, which receive the heaviest projections from the anterior cingulate and prelimbic cortex (Gabbott et al., 2005). BDNF has opposite effects on cortical pyramidal and interneuron excitatory synapses in cortical cultures: it decreases the a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid quantal amplitude of pyramidal neurons, whereas it increases the AMPA quantal amplitude of interneurons (Rutherford et al., 1998). This phenomenon

requires several hours to develop in the presence of synaptic blockade and is known as homeostatic plasticity or synaptic scaling (Turrigiano & Nelson, 2004). Thus, when cortical activity is high and BDNF release is increased, synaptic strengths are scaled to promote decreased pyramidal neuronal activity and increased inhibitory interneuron activity. Cocaine increases activity in cortico-accumbens projection neurons in animals with a history of cocaine self-administration, resulting in acute increased extracellular glutamate in NAc, even though animals with a cocaine history exhibit lower basal glutamate levels (McFarland et al., 2003). Thus, it is possible that elevated BDNF in the cortico-accumbens pathway attenuates cocaine seeking by preventing this cocaine-induced glutamate overshoot or decreasing a cocaine withdrawal-induced increase in the surface expression of AMPA receptors in the NAc (Boudreau et al., 2007). However, other mechanisms are possible, particularly because it is not known whether an increase in glutamatergic transmission occurs in response to re-exposure of animals to a cocaine-paired environment or in response to discrete CS presentation, similar to exposure of animals to a cocaine challenge injection. Intra-PFC BDNF infusion immediately after the last cocaine exposure, but not when delayed for 6 days, was effective in decreasing cocaine seeking in abstinent rats and in rats that received explicit extinction training.

These effects of exogenous BDNF are different than its effects following infusion into subcortical brain structures. For example, BDNF infused into the VTA or NAc augments cocaine-induced sensitization (Horger et al., 1999) and cocaine seeking (Graham et al., 2007; Lu et al., 2004). Recent evidence indicates that synaptic sensitization in midbrain dopamine neurons during protracted cocaine withdrawal is BDNF- and N-methyl-d-aspartate (NMDA) receptor dependent (Pu et al., 2006),

implying that elevated BDNF in the VTA may contribute to synaptic potentiation that may trigger drug seeking. Furthermore, infusion of BDNF in the NAc may augment the effects of cocaine by directly stimulating TrkB receptors on medium spiny neurons (Freeman et al., 2003). In contrast, potentiation of glutamate signaling in the PFC, as discussed above, may contribute to suppression of drug seeking by restoring homeostasis to glutamatergic signaling, a hypothesis we propose to investigate. Interestingly, it is not unprecedented that BDNF may have opposite effects in different brain regions: BDNF infusion in the hippocampus has an antidepressant-like effect (Shirayama et al., 2002), whereas its infusion in the VTA has a depression-inducing-like effect (Eisch, 2003). Therefore, as noted by Berton & Nestler (2006) with regard to antidepressant therapies, proposing BDNF ligands as novel therapeutics for drug addiction may be dependent on developing BDNF signaling targets that differ in competing brain regions.

These data suggest that during early abstinence, BDNF interferes with cocaine-induced neuroadaptations that are critical to cocaine seeking. One adaptation reversed by BDNF is a suppression of NAc phospho-ERK expression in rats that were abstinent from cocaine self-administration for 22 h and underwent an extinction test. This suppression of phospho-ERK in the NAc of cocaine-seeking rats abstinent from cocaine self-administration contrasts with recent evidence that cocaine-induced conditioned place preference increases phospho-ERK in the NAc (Miller and Marshall, 2005). A major difference between these cocaine-seeking paradigms that may underlie differential phospho-ERK responses is that rats in the cocaine self-administration paired environment press a lever previously associated explicitly with cocaine delivery, yet they do not receive the drug reward. They are actively learning via instrumental responding in the

first extinction test that cocaine is no longer available. The withholding of reward reduces the strength of the CS and the contingent activation of midbrain dopamine and striatal neurons (Tobler et al., 2003; Schultz, 2007). As a consequence, extracellular dopamine and glutamate levels, that are required for ERK activation in the NAc (Girault et al., 2007), would be predicted to fall. In contrast, rats re-exposed to the conditioned place preference environment do not perform an instrumental task that teaches them that cocaine is no longer available. Not surprisingly, extinction of conditioned place preference is much slower to occur than extinction of self-administration behavior (Fuchs et al., 2002).

In conclusion, the prolonged effects of BDNF infusions on cocaine seeking behavior suggest that BDNF-based treatments may represent a novel approach to preventing relapse. Further study of BDNF interactions with glutamate in the cortico-accumbens pathway should shed light on the mechanisms underlying the suppressive effects of intra-PFC BDNF infusion on relapse to cocaine seeking.

CHAPTER 4

INTRA-PFC BDNF INFUSION NORMALIZES EXTRACELLULAR GLUTAMATE LEVELS IN THE NAC FOLLOWING COCAINE SELF-ADMINISTRATION

Introduction

Relapse to drug-seeking during abstinence from cocaine is marked by disturbances in glutamatergic neurotransmission in the PFC-NAc pathway in animal models of addiction. Reinstatement of drug-, cue-, or context-induced cocaine-seeking is blocked by the inactivation of the dPFC or NAc core by GABA agonists or the Na+ channel blocker, tetrodotoxin (McFarland and Kalivas 2001; Capriles et al., 2003; McFarland and See, 2003; Fuchs et al., 2004; 2005). Further, intra-NAc infusion of AMPA receptor agonists exacerbate, and antagonists block, cocaine-induced reinstatement (Cornish and Kalivas, 2000) even when cocaine is delivered directly into the PFC (Park et al., 2002). These inactivation studies support evidence from cocaine-withdrawn animals that have long-term decreases in basal, and a sharp increase in cocaine-evoked extracellular glutamate levels in NAc (Pierce et al., 1996; Baker et al., 2003). Two different mechanisms control basal and cocaine-evoked extracellular glutamate levels: basal levels are Ca^{++}-independent and cystine/glutamate antiporter (xCT)-dependent whereas cocaine-evoked levels are action potential and Ca^{++}-dependent

(Pierce et al., 1996; Baker et al., 2003). After repeated cocaine exposure, diminished xCT function can be reversed by administering *N*-acetylcysteine which normalizes basal glutamate levels, stimulates presynaptic, inhibitory metabotropic glutamate 2/3 receptors, preventing the cocaine prime-induced glutamate spike, and, consequently, suppresses reinstatement of drug seeking (Baker et al., 2003; Melendez et al., 2005). The molecular correlates that transform PFC-NAc synapses in response to cocaine remain to be elucidated; however, there is convincing preliminary evidence that BDNF is a necessary player.

BDNF activity is extensively associated with synaptic plasticity and associated changes in receptor-mediated intracellular signaling. These events occur via a number of BDNF activity-dependent mechanisms, all of which depend on the interaction of BDNF with the TrkB receptor. Initially, BDNF can behave like a fast-acting neurotransmitter and induces increased intracellular calcium release and modified ion channel activity (Kafitz et al., 1999). Neurotrophin-induced synaptic activation of Trk receptors results in phosphorylation-dependent internalization of the receptor-ligand complex that forms intracellular signaling endosomes (Grimes et al., 1996). The BDNF/TrkB dimer complex can exert its effects within the post-synaptic scaffold by inducing the phosphorylation of key intracellular signaling molecules., such as ERK, PI3K, and PLCγ (Yuen and Mobely, 1999). The induction of these signaling molecules by TrkB receptor activation is well documented and has been observed both in vivo and in vitro (Berhow et al., 1996; Cavanaugh et al., 2001; Foulstone et al., 1999; Fryer et al., 2000; Matsumoto et al., 2001; Neal et al., 2003). Furthermore, recent studies have demonstrated that infusion of anti-BDNF into NAc prevented a cocaine-induced increase in phospho-PLC□ (Graham et al.,

2007). Together, this evidence provides a strong correlation between BDNF activity, TrkB receptor activation, and/or the activation of PLC□, ERK, and PI3-kinase in cocaine-associated behaviors. Finally, BDNF is able to exert long-term adaptations by shifting transcription factor activation in favor of increased LTP and enhanced post-synaptic responses. For example, BDNF increases the phosphorylation of transcription factors, CREB and Elk-1, similar to effects observed following psychostimulants and NMDA receptor stimulation in cortical and striatal neurons (Arthur et al., 2004; Finkbeiner et al., 1997; Hardingham et al., 1999; Simpson et al. 1995; Valjent et al. 2000). Moreover, in vitro studies of mature cortical cultures have shown that BDNF induces increases in the expression and translation of mRNAs that are associated with synaptic functioning (Schratt et al., 2004). Finally, exogenous application of BDNF enhances glutamatergic transmission in the cerebral cortex by facilitating glutamate presynaptic release as well as by enhancing AMPA receptor responsiveness (Lessmann 1998).

The role of exogenous BDNF in cocaine-associated behaviors and neuro-adaptations has been demonstrated in the NAc. Intra-NAc BDNF potentiates cocaine-induced locomotor activity and lever-pressing during cocaine self-administration (Taylor and Horger, 1999). Furthermore, intra-NAc BDNF during cocaine self-administration increases cue and cocaine induced reinstatement and anti-BDNF decreases cocaine-induced reinstatement (Graham et al., 2007). In contrast, we found that conditioned cue-induced or cocaine-induced reinstatement after two weeks of extinction is significantly attenuated in rats that receive an infusion of BDNF into the PFC shortly after the last cocaine self-administration session (Chapter 3 and Berglind et al. 2007). Furthermore, an intra-PFC BDNF infusion prevented a decrease in phospho-ERK expression in the NAc

that normally occurs after 22 h of abstinence from cocaine self-administration (Chapter 3 and Berglind et al, 2007). This evidence suggests that intra-PFC BDNF suppresses reinstatement behavior by altering cocaine-induced neuroadaptations in the PFC-NAc pathway. The present study examined the effects of intra-PFC BDNF infusions on basal and cocaine-induced extracellular glutamate levels in the NAc in rats with a cocaine self-administration history.

Materials and Methods

Animals

Male Sprague-Dawley rats (N=60; Charles River), weighing 275-325 grams at the time of surgery, were housed individually on a reverse light/dark cycle. Rats were maintained on 20-25 g of rat chow per day, with water available *ad libitum*. All protocols were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996).

Lever Response Training

Rats were trained to lever press on a fixed ratio 1 schedule of food reinforcement (45 mg pellets; Noyes, Lancaster, NH) in sound-attenuated operant conditioning chambers (30 x 20 x 24 cm high; Med Associates Inc., St. Albans, VT) during a 16 h overnight training session. The chambers were equipped with two retractable levers, a stimulus light above each lever, a food pellet dispenser between the levers, a house light

on the wall opposite the levers, and a speaker connected to a tone generator (ANL-926, Med Associates Inc, St. Albans, VT). During the session, each lever press on the active lever resulted in delivery of a food pellet only. Lever presses on the inactive lever had no programmed consequences. Following food training, food pellet dispensers were removed from the chambers.

Surgery

Forty-eight h after food training, rats were anesthetized with a mixture of ketamine hydrochloride (66 mg/kg i.p.; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (1.33 mg/kg i.p.; Bayer, Shawnee Mission, KS) followed by Equithesin (0.5 ml/kg, i.p.). Catheters constructed of Silastic laboratory tubing (0.64 mm i.d., 1.19 mm o.d.; Dow Corning, Midland, Mich.) were implanted into the right jugular vein of rats and were anchored to the vein with silk suture and a hardened silicon gel ball (silicon rubber sealant; General Electric, Waterford, N.Y.) placed 33 mm from the anterior end of the catheter. The Silastic tubing ran directly under the skin to an exit point located in the mid-scapular region. The open end of the catheter was enclosed with a short piece of polyethylene tubing that had been heat-sealed and the threaded region of the catheter was covered with a threaded plastic cap when not in use. Immediately following catheter implantation, rats were mounted onto a stereotaxic device (Stoelting, Wood Dale, Ill.) and bilateral stainless steel cannulae (26 gauge, Plastics One Inc., Roanoke, VA) were implanted 1 mm above the PFC target region (AP= +3.0; ML= +0.6; DV= -1.6, relative to bregma, Paxinos and Watson, 1998). Following implantation, the mPFC guide cannulae were briefly secured to the skull with a small amount of super glue. Bilateral

stainless steel cannulae (20 gauge, Plastics One Inc.) were implanted 2 mm above the NAc target region (AP=2.5; ML=0.6 6°, relative to bregma, Paxinos and Watson, 1998). Following implantation of the dialysis guide cannulae, cranioplastic cement anchored by three steel machine screws was applied to permanently secure the mPFC and NAc cannulae to the scull. Stylets (Plastics One, Inc.) were placed into the guide cannulae to prevent blockage. Following surgery, rats were infused i.v. with 0.1 ml each of cefazolin (100 mg/ml) and heparinized saline (70 U/ml) once daily during a 5 day recovery period. Catheter patency was verified by infusing 0.1 ml of methohexital sodium (20 mg/ml, i.v.; Eli Lilly and Co., Indianapolis, IN), which produces a rapid loss of muscle tone only when administered intravenously.

Cocaine Self-administration

Cocaine self-administration was conducted in standard self-administration operant chambers (30 x 20 x 24 cm; Med Associates, St Albans, VT). Each chamber contained two retractable levers (7 cm above the chamber floor) on each side of the front wall. A white circular stimulus light (2.5 W, 24-V bulb) was located on the panel 7 cm above the active (right) lever, and a red house light (2.5 W, 24-V bulb) was located on the wall on the opposite end of the chamber. The infusion line was tethered to a liquid swivel (Instech, Plymouth Meeting, PA) mounted on a suspended counterbalance. The self-administration apparatus was enclosed in a sound-attenuating chamber (Med Associates). Cocaine hydrochloride (4 mg/ml; National Institute on Drug Abuse/ RTI International, Research Triangle Park, NC) was delivered using a computer-controlled infusion pump

located outside the chamber. The entire system was computer controlled using Med PC for Windows.

Rats self-administered cocaine on 10 consecutive days during 2 h daily sessions. Figure 1 illustrates the experimental design of each of the experiments. Rats received 0.1 ml of heparinized saline (10 U/ml, i.v.) prior to each self-administration session. Animals were then connected to the infusion tether and each session began immediately, signaled by the movement of both levers into the chamber. Cocaine reinforcement was available on a FR1 schedule of reinforcement explicitly paired with a compound conditioned stimulus (CS) consisting of illumination of a white stimulus light located above the active lever and a tone (2 kHz, 15 dB above ambient noise). Responses on the active (right) lever resulted in the delivery of cocaine (0.2 mg/0.05 ml bolus) over 2 sec, and each infusion was followed by a 20 sec 'time-out' period. During the time-out, active lever presses were recorded, but had no programmed consequences. Responses on the inactive (left) lever were also recorded, but had no programmed consequences. Yoked-saline rats (experiment 1-see below) received an infusion of saline (0.05 ml bolus) over 2 sec, contingent upon the cocaine infusion received by the self-administering rat in the adjacent self-administration chamber. Rats underwent 10 days of cocaine self-administration prior to a single intracranial infusion.

Abstinence, extinction, and reinstatement

Figure 4-1 illustrates the experimental design for experiments one and two during which rats self-administered cocaine for 10 days. Immediately following the final self-administration session, rats in both experiments received an intra-PFC infusion of either

BDNF (0.75 μg/μl/side) or vehicle (1 μl). Following self-administration rats in experiment 1 underwent 10 days of extinction training, during which lever presses had no programmed consequences. Following extinction, rats in experiment one underwent a single 2 h cocaine prime-induced reinstatement test for which each rat was injected with cocaine (10 mg/kg, i.p.) at the beginning of the test. During the cocaine-primed reinstatement test, lever presses were recorded, but had no programmed consequences. Following the cocaine prime-induced reinstatement test, rats in experiment one underwent 7-10 further days of extinction training before undergoing glutamate micro-dialysis, during which glutamate extracellular levels within the NAc were evaluated before and after a cocaine prime (10 mg/kg, i.p.). Rats in experiment two differed from those in experiment one in that they underwent 14 days of extinction training before their first dialysis day, and a further 7 days of extinction training before their second day of dialysis performed in the contralateral hemisphere. No-net flux micro-dialysis was performed during both dialysis days in experiment two.

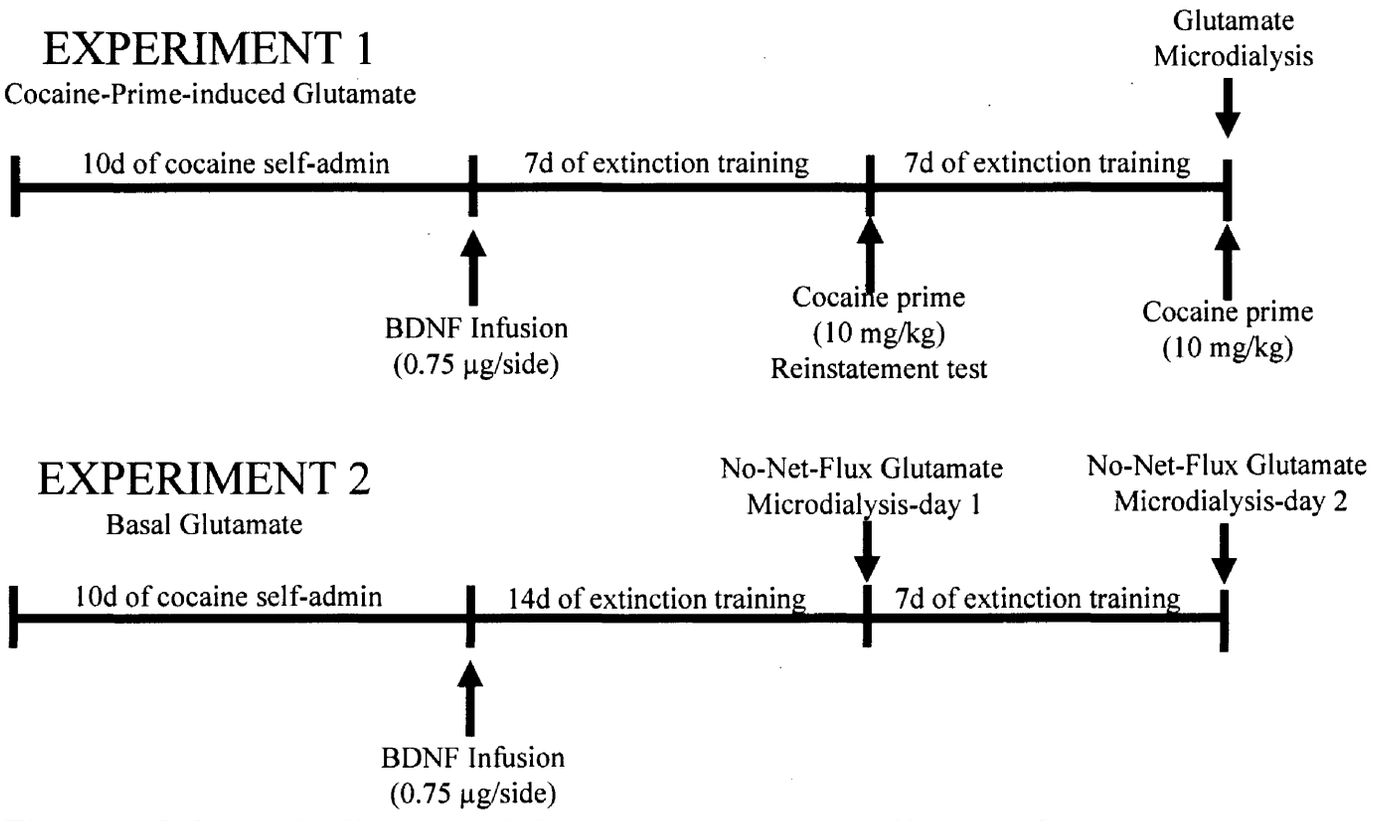

Fig. 4-1. Schematic diagram of the experimental paradigm used.

mPFC Intracranial Infusions

We adopted a single infusion protocol and chose a dose of BDNF (0.75 μg/side) previously used *in vivo* (Lu et al., 2004; Berglind et al., 2007).. For BDNF or vehicle (10 mM PBS) infusions, infusion cannulae (33 gauge; Plastics One Inc.) were inserted bilaterally into the guide cannulae such that 1 mm of the infusion cannulae extended past the end of the guide cannulae. Human recombinant BDNF (R&D Systems, Inc, Minneapolis, MN) or vehicle (sterile phosphate buffered saline) was infused using gas-tight Hamilton syringes (10 μl) and an infusion pump (Harvard Apparatus, Holliston, MA). A volume of 0.5 μl was infused over 2 min and the infusion cannulae remained in the guide cannulae for one min before and after the infusion.

Glutamate microdialysis

Micro-dialysis probes were constructed utilizing fused silica for inlet and outlet tubing into 26-gauge cannulae topped with a semi-permeable membrane with 2 mm of active length. On the evening before the sample collection, subjects were housed in the operant chamber and probes were inserted into the NAc via previously implanted 20-gauge cannulae. Dialysis buffer (5 mM glucose, 2.5 mM Kcl, 140 mM NaCl, 1.4 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 0.15 % PBS, pH 7.4) was perfused through the probe overnight at 0.2µl/min. In the morning, the flow rate was increased to 2.0 µl/min for 2 h. Each rat then received an i.p. injection of cocaine (10 mg/kg), following which, samples were collected every ten minutes for an additional 2 h. All samples were collected into 10 µl of 0.05 M HCl. Samples were then stored at –80°C for future analysis of extracellular glutamate (see below).

No-net-flux microdialysis

No-net-flux micro-dialysis was performed in the same manner as described above. However, following collection of baseline samples for 2 h, the no-net-flux procedure began by changing the perfusate to dialysis buffer containing 2.5, 5, or 10 µM glutamate. Liquid switches were used to minimize the pressure fluctuations while changing dialysis buffers with varying concentrations of glutamate. Six samples were collected at 10 min intervals for each concentration.

Analalysis and quantification of extracellular glutamate

We analyzed glutamate levels from the NAc using high performance liquid chromatography (HPLC) as described previously (Bell et al., 2000). Samples were loaded on an autosampler (Gilson Medical Electric, Middleton, WI) that derivatized the sample with *o*-pthalaldehyde prior to injecting it into the column via circulating mobile phase (100 mM sodium dihydrogen phosphate monobasic, 90 M EDTA-Na_4, and 15% acetonitrile v/v, pH 6.04). Amino acid separation was done using a reversed-phase C18 (15 cm) column. A florescence spectrophotometer (RF-10A-Shimadzu) detected glutamate concentrations using an excitation wavelength of 340 nm and an emission wavelength of 455 nm. A chart recorder recorded the peaks and peak heights were measured manually and quantified using external glutamate standard curves as described (Melendez et al., 2005).

Results

Histology

Subjects with cannulae tracts that failed to fall within the selected criteria for mPFC for infusion cannulae or NAc for dialysis cannulae placement were excluded from the schematic and the subjects' data was excluded from the data analysis (n=4).

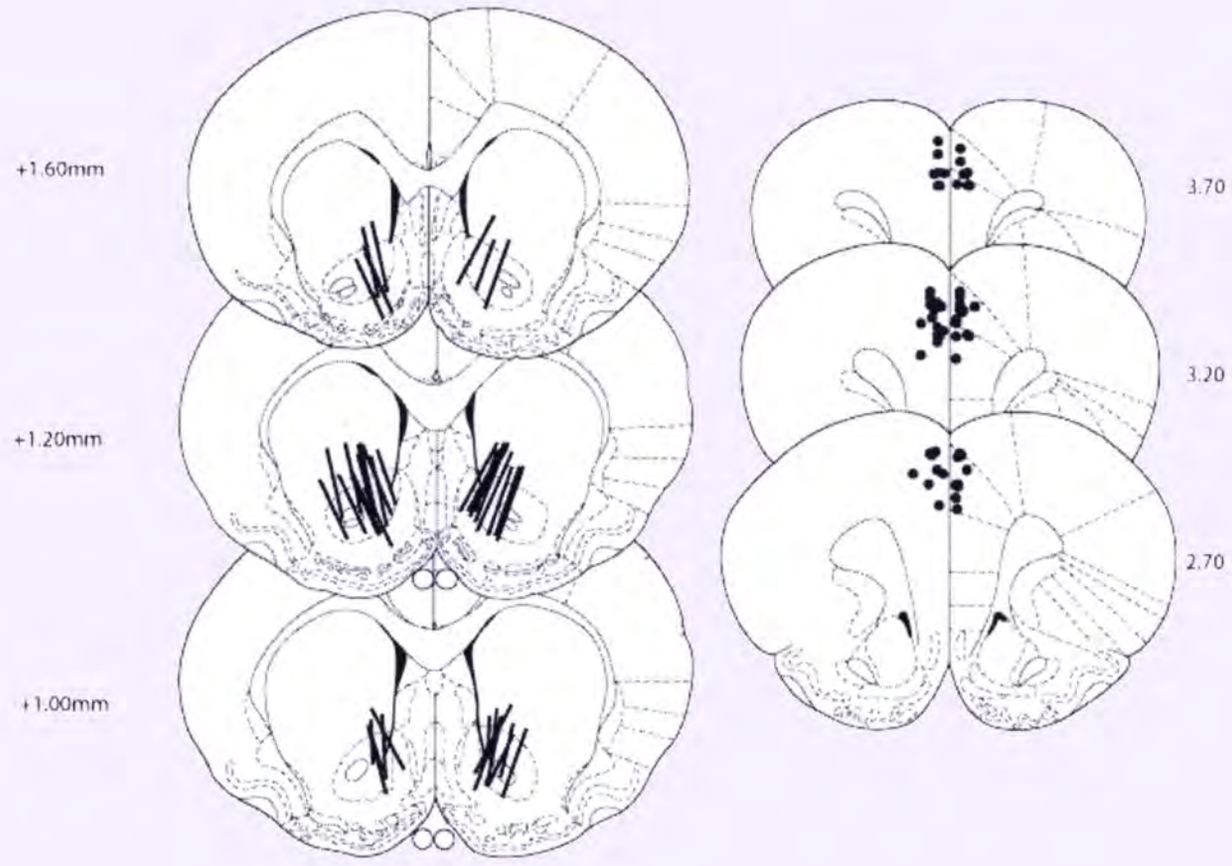

Figure 4-2: Cannula Placement. A schematic representation of the active length of the dialysis membrane within the NAc (A) and the tip of the infusion cannulae within the PFC (B)

Self-administration, extinction, and reinstatement

All rats exhibited stable active (right) lever responding over the last three days of self-administration that did not differ between groups (Fig 4-3). An ANOVA revealed no effect of treatment on active lever pressing over the last three days of self-administration ($F_{1,18}$=0.034; $p>0.05$). Furthermore there was no significant effect of treatment on inactive lever-pressing over the last three days of self-administration ($F_{1,18}$=1.03; $p>0.05$). All rats underwent extinction training until criterion (less than 25 active lever presses per day). There was a significant effect of intra-PFC BDNF treatment on active lever responding during the first day of extinction (($F_{1,18}$=3.434; $p<0.05$), but not inactive lever-pressing (($F_{1,18}$=0.001; $p>0.05$). However, there was no effect of intra-PFC BDNF

treatment on active lever-pressing ($F_{1,18}$=0.009; p>0.05), or inactive lever-pressing ($F_{1,18}$=0.056; p>0.05) during the last two days of extinction training prior to reinstatement testing .

All rats in experiment one underwent a single cocaine-induced reinstatement session. An ANOVA revealed a significant effect of intra-PFC BDNF treatment on active lever-pressing during the 2 h reinstatement test (($F_{1,12}$=10.66; p<0.05), but not on inactive lever-pressing ($F_{1,12}$=2.995; p>0.05).

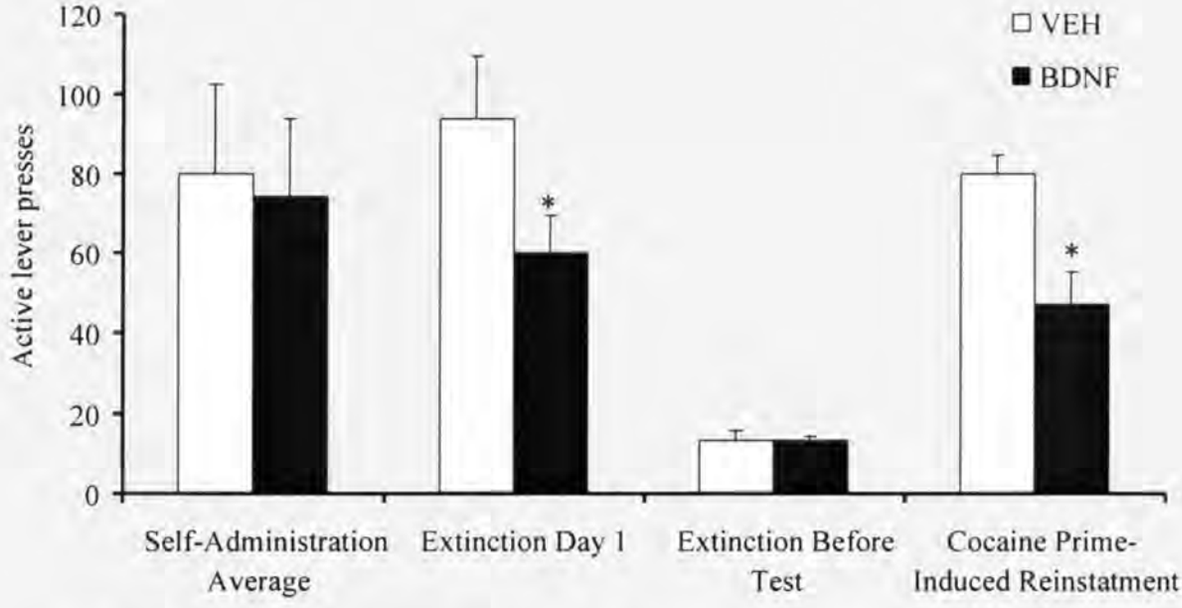

Figure 4-3: Behavior Effects of Intra-PFC BDNF. Effects of intra-PFC BDNF infusion on self-administration, extinction, and cocaine-prime induced reinstatement.

Experiment 1

Intra-PFC BNDF blocked a cocaine prime-induced increase in extracellular levels of glutamate within the NAc

Following extinction training, each rat in experiment one underwent micro-dialysis procedures to evaluate extracellular glutamate levels within the NAc before and after a single cocaine prime (10 mg/kg, i.p.). Basal and cocaine-induced glutamate levels

were measured for two h at 10 min intervals. Statistical analysis was determined by evaluating the area under the curve (AUC) for each group during the 2 h basal samples and for the 2 h following the cocaine prime. An ANOVA revealed a significant interaction of group X time ($F_{1,18}$=4.798; $p<0.01$). A Tukey's HSD test revealed a significant effect of intra-PFC BDNF treatment on the AUC following the cocaine prime ($p=0.013$), but not during baseline collection ($p=0.77$). Furthermore, a Tukey's HSD test revealed a significant effect of the cocaine prime itself as compared to baseline in the vehicle-treated group ($p=0.011$) but not in the BDNF-treated group ($p=0.969$).

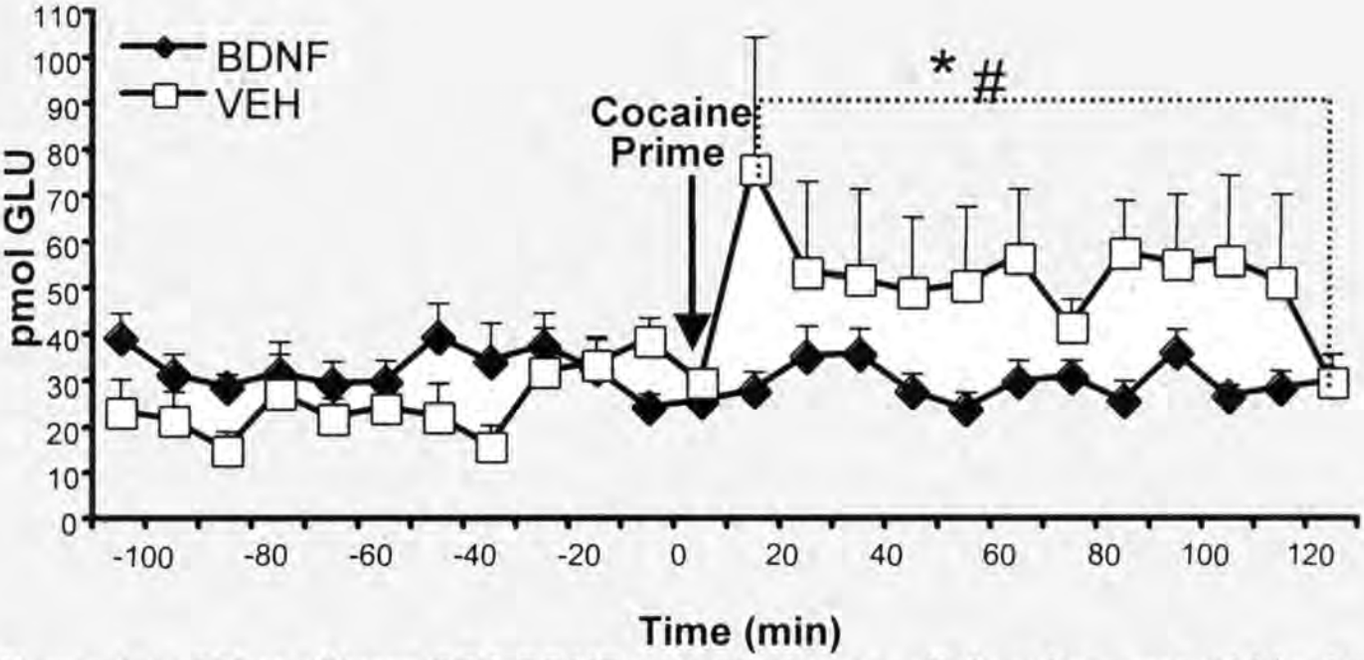

Figure 4-4: Effect of intra-PFC BDNF on evoked extracellular glutamate within the NAc Effect on intra-PFC BDNF, administered after the last cocaine self-administration session, on extracellular glutamate levels before and after a single cocaine challenge (10 mg/kg, i.p.) in rats with a cocaine self-administration history. Rats that received intra-PFC infusions of BDNF exhibited a suppression of the increase in glutamate observed in the rats that received intra-PFC vehicle infusions. *, $p<0.05$ AUC of BDNF animals following cocaine prime vs. AUC of vehicle-treated animals following cocaine prime ; #, $p<0.05$, vehicle-treated baseline vs. vehicle-treated following cocaine-prime.

Experiment 2

Intra-PFC BDNF normalized basal levels of extracellular glutamate within the NAc

Figure 4-5 illustrates the gain or loss of glutamate as a function of the concentration of glutamate added to the perfusate (Lonnroth et al., 1987). A positive number on the y-axis is indicative of a net diffusion of glutamate into the brain and a negative number indicates a net diffusion from the brain to the collected perfusate. The y-axis intercept is indicative of the basal extracellular glutamate concentration within the NAc for each group. The slope of each line indicates the *in vivo* recovery in each group. Using linear regression analysis, the slope was not significantly different between groups ($F_{1,14}$=1.34, $p>0.05$). However, rats that received intra-PFC BDNF treatment demonstrated a significant difference in the y-intercept ($F_{1,14}$=5.693, $p<0.05$), indicating that BDNF-treated rats had significantly higher basal extracellular glutamate levels within the NAc as compared to vehicle-treated rats. Also, the value of glutamate measured coming out was significantly different for each concentration of glutamate added to the perfusate in BDNF-treated rats as compared to vehicle-treated rats (0 μM- $F_{1,14}$=5.44, $p<0.05$; 2.5 μM- $F_{1,14}$=7.711, $p<0.05$; 5.0 μM, $p<0.05$; 10 μM- $F_{1,14}$=13.78, $p<0.05$).

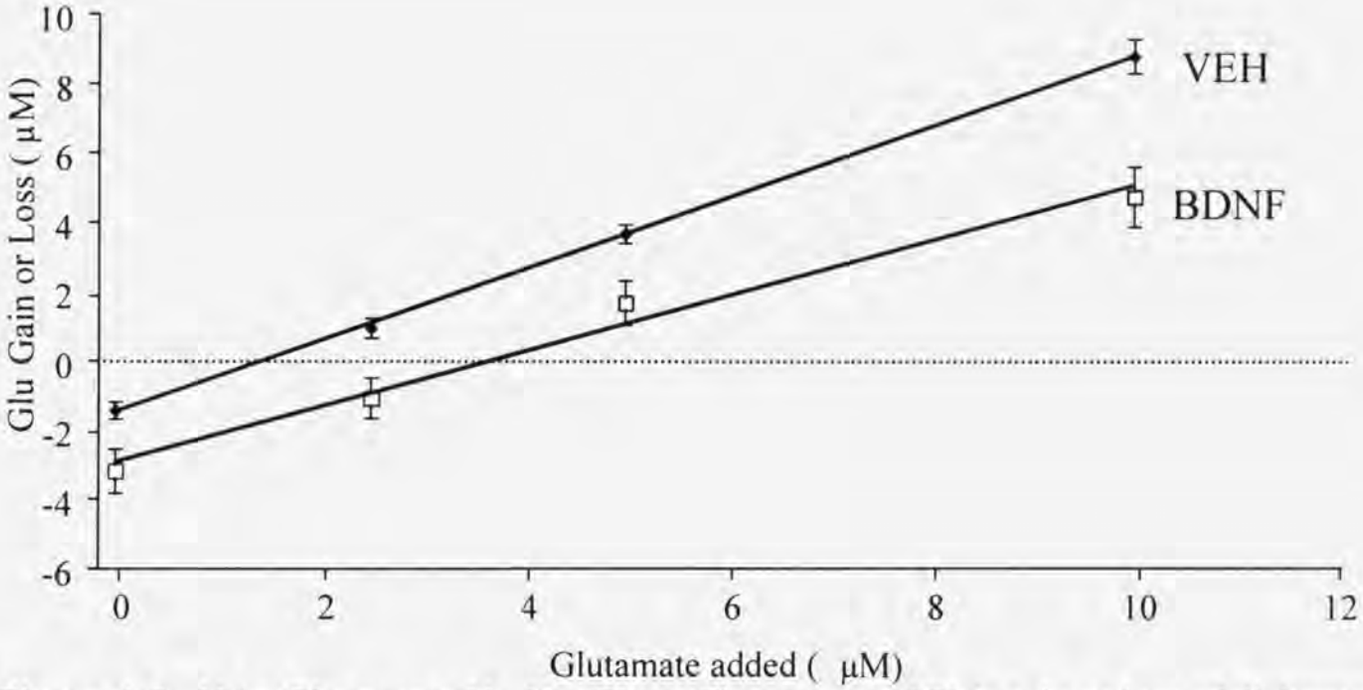

Figure 4-5. NAc Glutamate No-Net Flux. The mean ± (SEM) gain or loss of dialysate glutamate concentrations to and from the brain as a function of the perfusate glutamate concentration in the NAc. A positive number on the ordinate indicates diffusion of glutamate from the brain to the dialysate.

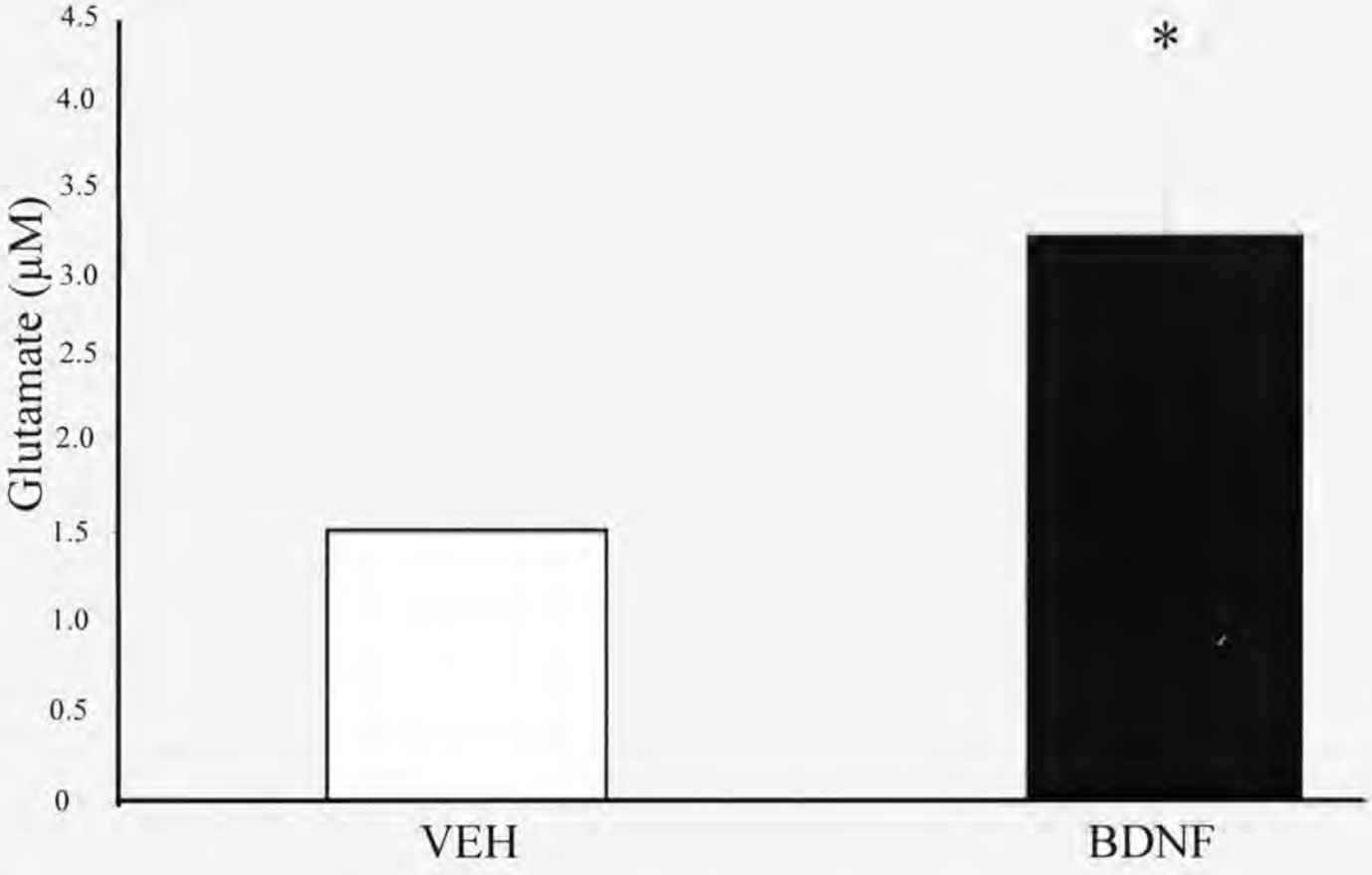

Figure 4-6: Point of No-Net Flux. The zero points on the y-axis of figure 4-5 indicate the steady state at which there is no net flux of glutamate across the dialysis membrane and provides a measure of the basal extracellular glutamate concentration. Intra-PFC BDNF treatment significantly increased the zero point, indicating an increase in the basal extracellular glutamate concentrations in the rats compared with those that received intra-PFC vehicle infusions (*$p<0.05$).

Discussion

In the present study, an intra-PFC infusion of BDNF produced a significant alteration in both the basal and cocaine prime-evoked levels of extracellular glutamate in the NAc of rats with a cocaine self-administration history. Specifically, when BDNF was infused into the mPFC immediately after the final cocaine self-administration session, basal levels of glutamate within the NAc were greater after 2-3 weeks of extinction

training than in rats that received intra-PFC vehicle infusions. Furthermore, there was a significant increase in extracellular glutamate in the NAc of vehicle-infused rats that underwent cocaine self-administration and received a cocaine-priming infusion 2-3 weeks later, and this increase in glutamate was completely prevented by intra-PFC BDNF treatment.

There is substantial evidence that repeated cocaine self-administration induces neuroadaptations within the PFC that lead to decreases in PFC activity. This hypoactivity has been demonstrated by decreases in glucose utilization, proportional to the duration of self-administration (Macey et al., 2004) and a significant decrease in firing rates and membrane oscillations in PFC pyramidal neurons after repeated cocaine in rats (Trantham et al., 2002). Additionally, this quiescent state during withdrawal from chronic cocaine is contrasted by an exaggerated elevation in activity upon re-exposure to cocaine-related stimuli or by cocaine itself. An elegant study by Sun and Rebec (2006) utilized an electrophysiological approach to elucidate this cocaine-induced hypofrontality. They found that PFC basal activity was significantly decreased after repeated cocaine self-administration; however, there was a significant increase in burst firing rates in response to cocaine. This bi-phasic effect of chronic cocaine on PFC activity that affects glutamate release within the NAc is illustrated in a pivotal study by McFarland and colleagues (2003). They demonstrated significant a decrease in basal extracellular glutamate following withdrawal from cocaine self-administration contrasted by increases in extracellular glutamate, following a cocaine prime, within the NAc. Further, Miguens and colleagues (2007) have recently reported that the cocaine self administration-induced decrease in basal NAc glutamate levels is present immediately before daily self

administration sessions and after the *first* day of extinction responding. They also demonstrated a cocaine prime-induced increase in extracellular glutamate levels in NAc. Taken together, these studies clearly illustrate a decrease in basal activity and an increase in cocaine-evoked activity in the PFC and suppressed extracellular glutamate tone and hyper-responsiveness to a cocaine challenge in the NAc. These alterations in the homeostatic balance of PFC-NAc neurons are likely caused by compensatory changes within the PFC in response to repeated cocaine, shifting PFC-NAc output toward the impulsive behavior associated with relapse. Intra-PFC BDNF appears to be able to stabilize the cocaine-induced depression in PFC activity and restore the balance in PFC-NAc glutamatergic tone.

There are several possible explanations for the actions of intra-PFC administered BDNF on NAc extracellular glutamate levels. First, it is possible that exogenous BDNF binds to TrkB receptors and is taken up by, and transported throughout, neurons in the PFC where it can affect several intracellular compartments in the, dendrites, soma, and axons (Kohara et al., 2001). Evidence exists that supports the ability of exogenous BDNF to elicit action potentials in cortical neurons by activating a TrkB-dependent sodium ion conductance (Kafitz et al., 1999). In this way, BDNF may increase glutamate exocytosis from PFC-NAc neurons and restore extracellular glutamate tone by stimulating mGluR 2/3 receptors, bypassing dysfunctional cystine /glutamate exchange (Moran et al., 2005). Mechanistically, exogenous BDNF activates presynaptic TrkB receptors and enhances depolarization-induced glutamate release from cortical synaptosomes via ERK-dependent phosphorylation of synapsin (Jovanovic et al., 2000) and enhances depolarization-evoked, glutamate exocytosis by activation of PLC, which induces the release of intracellular

Ca^{++} stores, in cultured cortical neurons (Matsumoto et al., 2001). Alternatively, BDNF may interact with xCT in an unknown manner to restore non-synaptically derived extracellular glutamate. However, a direct interaction between anterogradely transported BDNF in PFC-NAc neurons and xCT expressed by local glia in the NAc is somewhat implausible. Further studies in which Ca^{++} is inhibited or xCT and mGluR2/3 receptors are blocked (Moran et al., 2005) or glutamate uptake is measured may help determine the mechanism by which BDNF restores basal extracellular glutamate levels and prevents a cocaine-prime-induced increase in glutamate in the NAc.

CHAPTER 5

SUMMARY, DISCUSSION, AND FUTURE DIRECTIONS

Summary and Significance

In the present body of work, BDNF mRNA expression within the PFC was significantly decreased following 22 h of abstinence from repeated cocaine self-administration. This decrease in BDNF mRNA expression was not present following 21 d of abstinence from repeated cocaine self-administration but a significant increase in BDNF protein was observed at this time-point. These data indicate that BDNF expression within the PFC shifts from a relatively modest mRNA reduction in early abstinence to a more pronounced elevation following extended abstinence. This increase in endogenous BDNF that occurs gradually over the duration of abstinence may contribute to the propensity to relapse into drug-seeking behavior. However, exogenous BDNF administered into the PFC immediately following the final cocaine self-administration session attenuated relapse to cocaine-seeking following re-exposure to cocaine-associated contexts, cues, and cocaine itself up to 3 weeks later. Furthermore, 22 h following repeated cocaine self-administration, a decrease in pERK expression within the NAc was prevented and extinction responding was attenuated by intra-PFC BDNF

treatment. After more extensive withdrawal in rats whose active lever pressing behavior was extinguished, intra-PFC BDNF treatment prevented a cocaine-induced increase in extracellular glutamate within the NAc. Moreover, a decrease in basal extracellular glutamate levels induced by repeated cocaine self-administration was normalized by intra-PFC BDNF administration.

The findings of this study are novel and significant for several reasons. First, the enduring effects of a single intra-PFC BNDF infusion on drug-seeking behavior following extended abstinence are unique and have therapeutic significance. The fact that a single infusion of BDNF is able to alter cocaine-seeking behavior in a consistent manner over different durations of withdrawal is remarkable considering the possible therapeutic value of such an extended duration of activity following a single treatment. Second, intra-PFC BDNF infusions significantly reduced lever-pressing previously associated with cocaine availability following re-exposure to multiple stimuli, including context, cues, and a cocaine prime, at every time-point of withdrawal tested. Third, the critical timing of the intra-PFC BNDF infusion (immediately following the last cocaine exposure) represents a "therapeutic window" during which BDNF is able to reduce drug-seeking behavior. Fourth, the ability of intra-PFC BDNF to restore phospho-protein expression and glutamatergic transmission in the NAc suggests that exogenous BDNF is taken up and transported throughout PFC-NAc neurons, in contrast to drugs that act only in the local extracellular environment in which they are infused. Finally, the effects of intra-PFC BDNF infusions on drug-seeking behavior are the opposite of those described in previous reports in which BDNF was infused into the VTA or NAc respectively (See Table 5-1 for summary; Lu et al., 2004; Graham et al., 2007). This striking difference in

behavioral effects demonstrates the diverse actions of exogenous BDNF in different brain regions and its ability to restore glutamatergic transmission in the PFC-NAc pathway that is distorted by chronic cocaine.

			PFC (PRESENT STUDY)	***NAc***	***VTA***
Effect of Cocaine SA On Endogenous BDNF	**Early withdrawal**	Protein Expression	No change	No change (Grimm et al.,2003)	No change (Grimm et al.,2003)
		mRNA Expression	Decreased	N/A	N/A
	Late withdrawal	Protein Expression	Increased	Increased (Grimm et al.,2003)	Increased (Grimm et al.,2003
		mRNA Expression	No change	N/A	N/A
Effect of Exogenous BDNF Following Cocaine SA	**Early withdrawal**		Attenuates extinction responding Increases pERK expression in NAc	Increases extinction responding (Graham et al., 2007)	N/A
	Late withdrawal		Attenuates reinstatement responding Prevents cocaine-induced changes in NAc extracellular glutamate	Increases cue and cocaine-induced reinstatement (Graham et al., 2007)	Increases Cue/Contextual Reinstatement (Lu et al., 2004)

Table 5-1: Summary of studies examining the changes in BDNF expression induced by cocaine self-administration and the effect of exogenous BDNF.

Together these findings stress the distinctiveness of the effect of intra-PFC BDNF on cocaine-induced drug-seeking and on the cocaine-induced neuroadaptations in the PFC-NAc pathway. Considering the scope of this body of work, the variety of protocols employed, and the novelty of the findings there is little corroborating evidence to guide the mechanistic interpretation of the findings. However, integrating what is known about the properties of the PFC-NAc pathway following chronic cocaine, the role of BDNF within the NAc-PFC pathway, and the neuroadaptations driving the propensity to relapse,

a plausible explanation of the observed findings can be offered. Two central questions frame the discussion of the mechanisms underlying the behavioral effects of intra-PFC BDNF:

1. What effect does exogenous BDNF have on the local cortical circuitry?
2. How does intra-PFC BDNF prevent cocaine-induced neuroadaptations in the NAc?

BDNF Regulates Homeostasis via Synaptic-Scaling Mechanisms in the PFC

Figure 6-1 illustrates the local circuitry within the prefrontal cortex that is characterized by recurrent connections between GABAergic interneurons and glutamatergic pyramidal neurons. Endogenous BDNF is uniquely suited to regulate PFC activity for several reasons. First, BDNF is produced by cortical pyramidal neurons and BDNF mRNA expression is present in significant amounts throughout the cortex with the exception of the agranular layer (Altar et al., 1997). Activity-dependent release of BDNF stimulates TrkB receptors located both pre-and post-synaptically on cortical pyramidal neurons and interneurons (Cabelli et al., 1996). Additionally, BDNF has been shown to induce glutamate release from cortical astrocytes and pyramidal cells (Pascual et al., 2001). Finally, BDNF signaling is able to alter long-term neuroadaptations by altering gene expression within post-synaptic neurons (Watson et al., 1999).

In addition to these cellular mechanisms of action, BDNF also regulates global PFC activity and modulates PFC homeostasis. For example, in response to major increases in input by decreasing the quantal amplitude of AMPA-mediated excitation on pyramidal neurons and enhancing the GABAergic inhibitory amplitude at excitatory synapses, contributing to the homeostatic regulation of inhibitory vs. excitatory synaptic

strength within this circuitry (Rutherford et al., 1998; Turrigiano and Nelson, 2004). However, when cortical activity is depressed over extended periods of time and BDNF expression is reduced, increased sensitivity of pyramidal cells to excitatory input leads to abnormal hyperactivity. This scenario is extremely provocative considering that repeated cocaine induces a disruption in the homeostatic imbalance within the PFC (Trantham et al., 2002; Sun and Rebec, 2006). Specifically, in the absence of cocaine, during withdrawal, there are increases in inhibitory neurotransmission within the PFC and decreases in basal pyramidal cell activity (Jayaram and Steketee, 2005; Nasif et al., 2005; Jayaram and Steketee, 2006). However, this is contrasted by the fact that the responsiveness of the PFC to excitatory inputs is greatly enhanced by increases in GluR sensitivity and increased propensity for LTP (Dong et al., 2005; Hu, 2007; Huang et al., 2007).

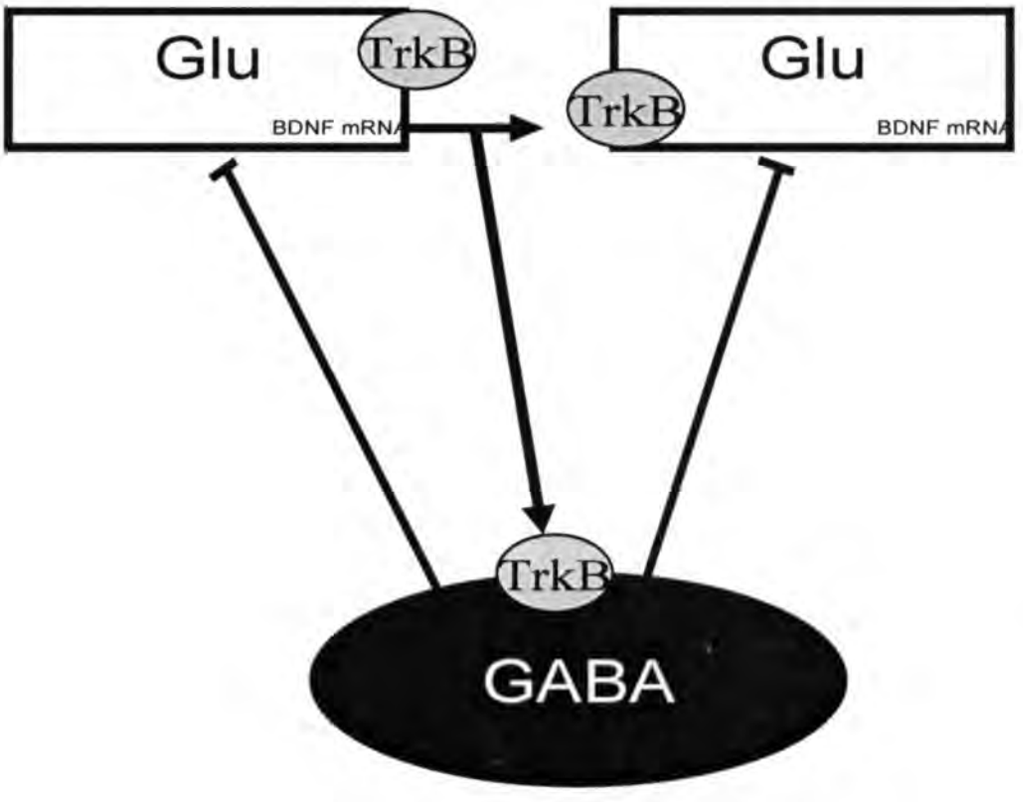

Figure 6-1: Recurrent connections between glutamatergic pyramidal neurons and GABAergic interneurons that express TrkB receptors within the cerebral cortex. BDNF regulates the synaptic strength of pyramidal neurons and interneurons in response to strong stimuli to maintain homeostatic control of PFC activity. The level of excitation

and resultant firing is carefully stabilized by the interaction of these two cortical neuronal subtypes. At the synaptic level, increases and decreases in quantal release, known as quantal scaling, have been hypothesized to underlie synaptic plasticity in the cerebral cortex. BDNF signaling within the cortex is thought to drive synaptic scaling (Adapted from Rutherford et al., 1998).

Exogenous BDNF restores PFC homeostasis withdrawal from cocaine self-administration

In the present study, a significant decrease in BDNF mRNA expression within the PFC was observed during early withdrawal, which is evidence of suppressed PFC activity. Furthermore, a decrease in basal, and an increase in evoked, glutamate within the NAc was demonstrated similar to the findings of McFarland et al (2003), re-emphasizing that cocaine decreases basal, and enhances cocaine-induced, glutamatergic transmission. However, rats that received intra-PFC BDNF treatment did not exhibit either the decrease in basal glutamate levels or an increase in glutamate levels following a cocaine priming injection. This evidence suggests that intra-PFC BDNF is able to stabilize the cocaine-induced depression in PFC activity and restore the imbalance in PFC homeostasis. The mechanisms that drive the restoration of PFC-NAc neurotransmission by BDNF may involve the unique homeostatic role that BDNF has demonstrated within the cerebral cortex.

In the present study exogenous BDNF was infused within the PFC within 20 minutes of the end of the last cocaine self-administration session. It is likely that a permissive state of the PFC existed at the time BDNF was infused that led to enduring behavioral and molecular changes. There is some evidence that there is a transient increase in extracellular glutamate within the PFC following repeated cocaine (Williams and Steketee, 2004). Also, a recent study demonstrates that extracellular levels of

glutamate within the NAc are increased during the self-administration session; however, the greatest increases were observed during the 40 minutes immediately following the end of the self-administration session (Miguéns et al., 2007). This report suggests that cocaine-induced glutamatergic transmission in the PFC activity peaks at the same time that BDNF is infused in our study. The synergistic effect of BDNF infusion and cocaine-induced pyramidal cell firing may be sufficient to restore the homeostatic balance within the cortical networks. Therefore the effect of exogenous BDNF to prevent reinstatement behavior may endure long after the acute effects of the injection by initiating changes in the PFC homeostatic set point, an effect that could persist and account for the long-term effects of an intra-PFC BDNF infusion. As BDNF is restoring regulatory control over PFC activity, it may also have trans-synaptic effects within the NAc that are also important in reversing the cocaine-induced neuroadaptations that drive drug-seeking.

Effects of intra-PFC BDNF within the Striatum

Summary

Many of the long-term neuroadaptations induced by chronic exposure to cocaine have been observed within the NAc. These included multiple alterations in presynaptic and postsynaptic dopamine and glutamate signaling (Vanderschuren and Kalivas, 2000; Cornish and Kalivas, 2001; Hemby et al., 2005; Boudreau et al., 2007). Modifications of dopaminergic synapses have a common thread in that most are associated with increased sensitivity of molecules associated with dopamine signaling. For example, D1 dopamine receptors remain supersensitive in response to electrophysiological stimulation throughout withdrawal (Henry and White, 1991). Additionally, there is an increase in the releasability of pre-synaptic dopamine stores, dependent upon calcium/calmodulin-

dependent protein kinase II activation (Pierce and Kalivas, 1997). These and other long-lasting changes in dopamine signaling may contribute to the overall role of the NAc on reinstatement to cocaine-seeking behavior but presently there is no clear evidence of a essential association between reinstatement behavior and alterations in dopamine neuro-transmission. This may be due to the compulsory nature of the dopamine response to cocaine, which remains unchanged regardless of prior drug history or duration of withdrawal (McFarland et al., 2003).

The role of glutamate signaling within the NAc on cocaine-induced neuroadaptations and drug-seeking is critical. Many of the enduring changes in glutamate transmission generally follow a common pattern of basal depression contrasted by an exaggerated response to cocaine-related stimulation. This includes decreased basal extracellular glutamate and enhancement of glutamate levels following a cocaine challenge (Pierce et al., 1996; McFarland et al., 2003; Miguens et al., 2007). The decreased basal glutamate neurotransmission has been associated with reduced activity of the cystine-glutamate exchanger, a passive exchanger driven by concentration gradient and located on glial cells within the NAc (Baker et al., 2003). Furthermore, it has been suggested that the enduring decreases in basal glutamate decrease the tone on presynaptic mGluRs, inhibitory autoreceptors, which allow for increased glutamate release following re-exposure to cocaine (Xi et al., 2004; Melendez et Al, 2005; Moran et al., 2005).

Little is known about the role of BDNF in the long-term effects of cocaine within the NAc. It is known that NAc BDNF expression is largely derived from glutamatergic pyramidal cells (Altar et al., 1997). Additionally, BDNF protein expression is increased progressively following extended durations of abstinence and this has lead to suggestions

that BDNF plays a role in the incubation of craving phenomenon (Grimm et al., 2003). However, it remains unknown if the increase in BDNF protein expression is presynaptic and/or postsynaptic and what neuronal sub-type(s) within the NAc is harboring it. While the role of endogenous BDNF activity on cocaine-induced NAc neuroadapatations remains unclear, exogenous BDNF within the NAc consistently produces increases in cocaine-associated behaviors (Horger et al., 1999; Graham et al., 2007).

In the present study, intra-PFC BDNF prevented cocaine-induced reductions in phospho-ERK within the NAc during early withdrawal. Further, intra-PFC BDNF prevented cocaine-induced reductions in basal glutamate and the enhanced release of glutamate upon re-exposure to cocaine after extended withdrawal. Additionally, considering that increased BDNF protein expression was observed within the NAc 22 h after an intra-PFC BNDF infusion in preliminary studies (see Appendix Figure 3), it is very likely that BDNF infused into the PFC is anterogradely transported to the NAc where it is expressed within 22 h. BDNF is anterogradely transported in large dense core vesicles via microtubule-based motor proteins, likely kinesin-1 (Butowt and Bartheld, 2007). , and released in an activity-dependent manner (Matsumoto et al., 2008). Studies have demonstrated that this type of transport operates at approximately 250mm/day (Hirokawa, 1998). This would allow ample time for BDNF that has been infused into the PFC to be transported and have local effects within the NAc. This assumption, once confirmed, can be exploited in future studies to delineate the precise mechanisms that are underlying cocaine-seeking behavior. Either intra-PFC BDNF is fundamentally altering local PFC activity as discussed above and this is sufficient to drive all of the behavioral effects and trans-synaptic molecular changes or BDNF is anterogradely transported in

PFC-NAc neurons and is synaptically released in an activity-dependent manner into the NAc milieu. The possible effects of synaptic BDNF release within the NAc after intra-PFC infusion on cocaine-associated phospho-ERK and glutamate transmission are discussed below.

PFC-NAc BDNF Transmission: Pre-Synaptic Activity

Extracellular glutamate levels within the NAc were normalized in rats that received intra-PFC BDNF immediately following the final cocaine self-administration session. Although this effect is may involve increased pyramidal cell activity from the PFC, the effect of BDNF on pre-synaptic glutamate release at the level of the NAc should not be overlooked. BDNF has been shown to induce increases in both glutamate and dopamine release from pre-synaptic stores, an effect that was diminished by TTX and low calcium and was dependent upon TrB signaling (Paredes et al., 2007). TrkB receptors, located pre-synaptically within the NAc, may be activated by released BDNF and alter the pre-synaptic machinery associated with neurotransmitter release. In particular, BDNF-induced stimulation of pre-synaptic TrkB receptors induces activation of ERK MAPK that leads to the enhanced phosphorylation of synapsin in cortical synaptosomes (Jovanovic et al., 2000). An increase in activated synapsin is responsible for trafficking synaptic vesicles from a reserve pool to a stand-by-ready pool that is released by exocytosis. In this scenario, BDNF would be anterogradely transported to the NAc, released from pyramidal cell terminals, bind and activate pre-synaptic TrkB receptors, initiate the cascade that leads to the phosphorylation of ERK, that in turn activates synapsin, allowing the phosphorylated synapsin to increase vesicular release by

upgrading the vesicle from "soon" to "next", and finally the ready pool is released in an activity-dependent manner. Considering that the activity of synapsin is ERK-dependent and a decrease in phospho-ERK expression occurred after cocaine self-administration, there is a positive correlation with a reduction in glutamate release from the pre-synaptic terminal of cocaine self-administering rats. In rats that received intra-PFC BDNF treatment, the decrease in phospho-ERK and extracellular glutamate changes within the NAc were prevented. Therefore, it is likely that BDNF exerts its effects within the NAc by altering presynaptic glutamate release in early abstinence, a hypothesis that needs to be tested. Additionally, it is unlikely that BDNF anterogradely transported to the NAc from the PFC would affect DA release within the NAc considering pyramidal neurons do not directly synapse with dopaminergic neurons. Furthermore, considering the proximal association of the DA efferents with MSNs within the striatum, it is unlikely that BDNF arising from the cortex would ever interact with presynaptic DA terminals or illicit DA release within the NAc. Interestingly, this may contribute to the behavioral differences observed when BDNF is infused into the cortex and is transported via a glutamatergic pathway to the NAc rather than infused directly into the NAc or via the VTA dopaminergic pathway, both of which could potentially increase DA release in the NAc. Therefore, consideration that BDNF is released from PFC-NAc terminals after infusion and activates postsynaptic TrkB receptors on medium spiny neurons (Freeman et al., 2003) to promote the restoration of glutamatergic-driven plasticity is discussed below.

Trans-synaptic BDNF can alter post-synaptic activity

BDNF has been shown to evoke rapid post-synaptic excitation by increased ion channel activation in several brain areas including the cortex (Kafitz et al. 1999, Rose et

al., 2000). BDNF also binds to post-synaptic TrkB receptors and initiates several kinase cascades that have multiple post-synaptic effects, such as CREB phosphorylation (Marini et al., 2004; Wu et al., 2004). Also, BDNF induces alterations in the post-synaptic neuron that increase the synaptic efficacy of AMPA receptors (Lessmann 1998). Therefore, it is possible that the cocaine-induced decrease in phospho-ERK expression within the NAc following 22 h of withdrawal was postsynaptic and prevented by synaptic release of BDNF and stimulation of TrkB receptors in the NAc after intra-PFC BDNF treatment. However, a post-synaptic action of BDNF in the NAc is made less likely by the opposite (enhanced) cocaine-seeking effects of BDNF infused directly into the NAc in cocaine self-administering rats (Graham et al., 2007).

Future Directions

Because the mechanisms that underlie the suppressive effect of intra-PFC BDNF infusions on cocaine-seeking behavior are not yet known, future directions should focus on identifying the site and mechanisms of action of BDNF's effects. It may be that BDNF in the PFC acts locally on TrkB receptors and alters the activation state of plasticity-related intracellular signaling cascades, including PI3K PLC, and/or MAPK, driving intra-PFC BDNF effects on attenuation of drug-seeking behavior. Using selective inhibitors of Trk receptors, MEK and PI3K in the PFC, the experiments that will attempt to answer these questions would be executed as follows:

1) Infuse the selective Trk inhibitor, K252a, the MEK inhibitor (U0126) or the selective PI3K inhibitor (LY294002) into either the PFC or NAc along with BDNF to determine

the site of action of BDNF and the role of those individual BDNF-associated intracellular signaling cascades on the intra-PFC BDNF-induced suppression of cocaine-seeking behavior.

2) Utilize fluorescently-tagged exogenous BDNF infused into the PFC and measure fluorescence levels in afferent targets of the PFC after different time-points. This would help to determine the spread of BDNF and help to elucidate the site of action of BDNF and its effects on reinstatement behavior.

3) Determine the effect of intra-PFC BDNF infusions on the electrophysiological characteristics of pyramidal cells that project to the NAc. This would include determining what changes if any exist in firing rates or changes in burst firing.

4) Determine whether the ability of intra-PFC BDNF to normalize extracellular glutamate levels in the NAc occurs during early withdrawal and is Ca^{++}-dependent or not.

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APPENDIX 1: EFFECTS OF INTRA-VTA BDNF ON REINSTATEMENT BEHAVIOR; AN INTRA-VTA INFUSION OF BDNF FAILED TO ALTER REINSTATEMENT BEHAVIOR

Materials and Methods

All procedures used are identical to those described in detail in chapter 3. The only difference was the stereotaxic coordinates used during the intra-cranial implantation of cannula aimed at the VTA: (A/P=-5.2, M/L=1.2, D/V=-8.0; at an angle of 8°).

Results

Histology

A schematic indicating the placement of the infusion cannulae tips of animals that received bilateral BDNF or vehicle into the VTA (experiment 2) is shown in Fig. 1. Subjects with cannulae tracts that did not meet the criteria of bilateral VTA placement are not included in the schematic and the data from these animals was discarded.

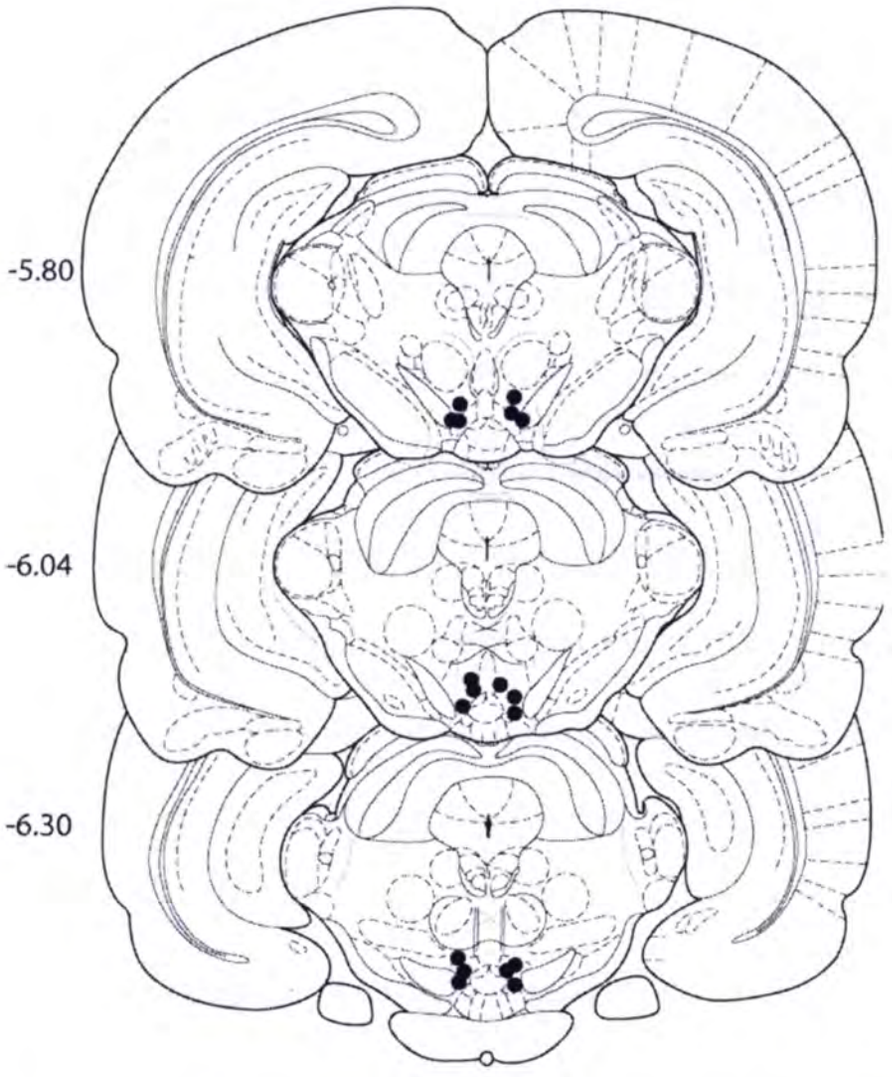

Figure A1. Schematic representing the cannula placement within the VTA.

Test 1: Post-abstinence extinction Test.

The effect of a BDNF infusion into the VTA on extinction responding after 6 d of abstinence was evaluated (Fig A2a). A two-way repeated measures mixed effects ANOVA revealed a main effect of time ($F_{1,21}$=15.39, P<0.01) but no effect of treatment ($F_{1,21}$=1.64, P>0.05) or a treatment*time interaction($F_{1,21}$=1.23, P>0.05) on active lever pressing between groups. There was no difference between groups on inactive lever pressing during the extinction test (P>0.05). Rats in both treatment groups responded significantly more on the active lever during the extinction test than during the last three days of self-administration (P=0.008), demonstrating reinstatement of drug seeking.

There was no difference in responding on the active lever between BDNF-infused rats and vehicle-infused rats during the extinction test (P=0.18 -Fig A2a right).

Test 2: Cue-induced reinstatement. : Responding on either the active ($F_{1,23}$=0.45, P>0.05) or inactive (F_{123}=0.58, P>0.05) lever was not significantly different between groups during six d of extinction training (data not shown). A two-way repeated measures mixed effects ANOVA revealed a main effect of time ($F_{1,21}$=6.48, P<0.05) but no effect of treatment ($F_{1,21}$=0.00, P>0.05) or a treatment*time interaction ($F_{1,21}$=0.08, P>0.05) on active lever pressing between groups and no effect on inactive lever pressing during the cue test (P>0.05). Vehicle-treated rats responded significantly more on the active lever during the cue test than in the preceding hour of extinction (P=0.05).

Test 3: Cocaine-induced reinstatement.

A two-way repeated measures mixed effects ANOVA revealed that there was a group main effect of time ($F_{1,21}$=6.09, P=0.02) but no group main effect of treatment ($F_{1,21}$=1.35, P=0.26) or treatment*time interaction ($F_{1,21}$0.04, P=0.85)), on active lever pressing in test 3. (Fig A2c). Also there was no effect on inactive lever pressing during the cocaine prime test ($F_{1,22}$=4.70, P>0.05). There was no significant difference in active lever responding during the cocaine prime test in the BDNF-treated group from the 30 min extinction session that preceded it (P>0.05) but there was a significant increase in active lever responding during the cocaine prime test in the vehicle-treated group as compared with the 30 min extinction session that preceded it (P<0.05).

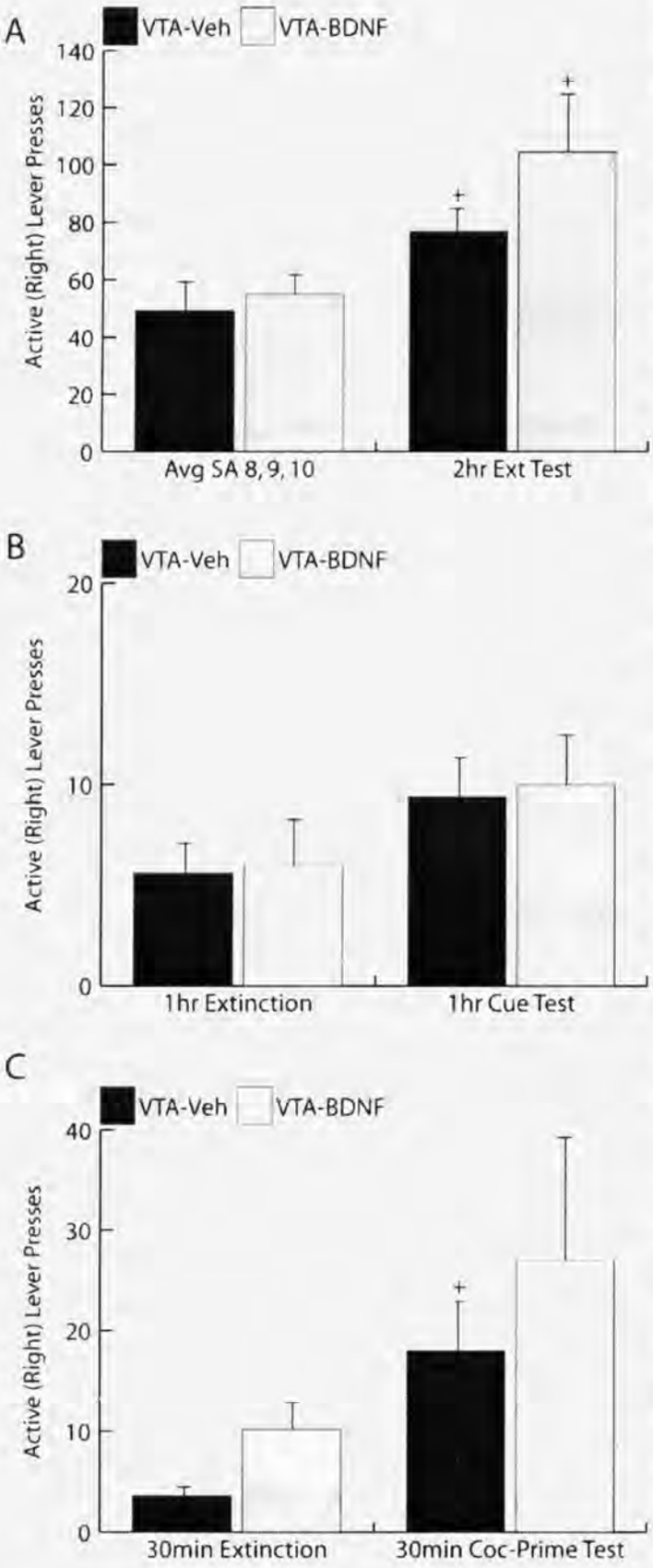

Figure A2. A bilateral intra-VTA infusion of BDNF did not attenuate cocaine-seeking behavior. (A) Left- There was no difference between the mean number of active lever presses in vehicle- or BDNF-infused rats over the last three days (8,9,10) of cocaine self-administration (Avg SA +/- SEM). Right- After 6 days of abstinence, the number of active lever presses during the two-hour extinction test was not significantly different in rats that received an intra-VTA infusion of BDNF (n=10) as compared to rats that received intra-VTA vehicle (n=13). Rats in both treatment groups exhibited significantly increased active lever responding during the extinction test as compared to the average active lever responding over the last three days of self-administration (+p=0.01) (B) After 6 days of extinction training, the mean (+/- SEM) number of active lever presses during the hour of extinction that preceded the cue test was not significantly different between groups. Right- The mean (+/- SEM) number of active lever presses during the one-hour cue-induced reinstatement test was not significantly different in rats that received an intra-VTA infusion of BDNF (n=10) as compared to rats that received intra-VTA vehicle (n=13). (C) Left- The mean number (+/- SEM) of active lever presses during the last 30 min of extinction that preceded the 30 min cocaine-prime test was not different between treatment groups. Right-The number of active lever presses during the 30-min coc-induced reinstatement test was not significantly different in BDNF-infused rats (n=10) as compared to rats that received intra-VTA vehicle (n=13). Rats in both treatment groups exhibited a significant increase in responding during the cocaine-prime test as compared to the preceding 30 min extinction session (+p<0.01).

APPENDIX 2: EFFECTS OF INTRA-PFC BDNF INFUSION IN NAÏVE RATS ON BDNF PROTEIN EXPRESSION 24 HOURS FOLLOWING THE INFUSION

Materials and Methods

Naïve rats underwent intracranial surgery as described in chapter 3. Intra-PFC infusions of either BDNF (0.75µg/side;N=6) or vehicle (PBS; N=6) were given following 7 days of recovery from surgery. BDNF protein expression was evaluated 24 hours following the infusion from tissue dissected from the medial prefrontal cortex (mPFC), NAc, lateral caudate putamen (L CPu), medial caudate putamen (M CPu), basolateral amygdale (BLA), and central amygdala (CeA). ELISA assays, as described in chapter 3, were used to quantify changes in BDNF protein expression between groups.

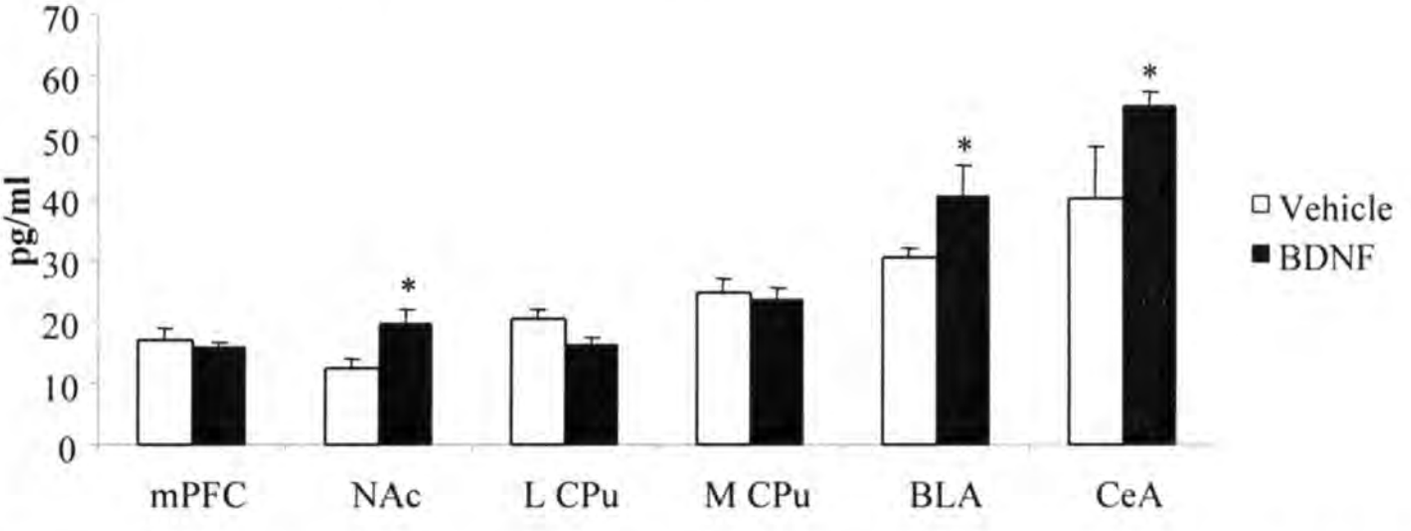

Figure A3. BDNF Protein Expression 24 hours following an intra-PFC BDNF infusions. 24 hours following an intra-PFC BDNF infusion there was a significant increase in BDNF protein expression within the NAc, BLA, and CeA (* $p<0.005$). No significant difference in BDNF protein expression was observed in the mPFC, L CPu, or M CPu.