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Intracellular Mechanisms Associated with  
Cocaine Induced Conditioned Place Preference

by

Stephanie K. Nygard

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2014

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This manuscript has been read and accepted by the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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## Abstract

Intracellular Mechanisms Associated with  
Cocaine Induced Conditioned Place Preference

by

Stephanie K. Nygard

Advisor: Professor Shirzad Jenab

The aim of this dissertation was to investigate the intracellular responses associated with the acquisition and expression of cocaine-context associations. ERK (extracellular regulated kinase), CREB (cAMP responsive element binding protein), FosB and  $\Delta$ FosB proteins were of particular interest due to their involvement in cocaine reward and in synaptic plasticity underlying learning and memory. We used the conditioned place preference (CPP) paradigm, which employs a Pavlovian conditioning procedure to establish an association between a drug-paired environment and the drug's rewarding effects, to study the role of these signaling pathways in cocaine-context associations. *N*-methyl-D-aspartate receptor (NMDAR) antagonism prior to cocaine administration during conditioning blocked the acquisition of cocaine CPP and reduced Nucleus Accumbens (NAc) phosphorylated-ERK (pERK) levels following the CPP test (drug-free). NAc pERK levels increased after re-exposure to the cocaine-paired environment regardless of CPP expression. Conversely, Caudate Putamen (CPu) pERK and FosB protein levels only increased after CPP expression and re-exposure to the cocaine chamber. These results suggest that NAc ERK phosphorylation may be involved with retrieving the contextual information of a cocaine-association, without the expression of the behavior. Finally, we investigated whether ERK/CREB intracellular responses underlying cocaine environmental associations are sexually dimorphic. Following CPP expression, cocaine treated rats showed increased NAc pERK and pCREB and CPu  $\Delta$ FosB levels. Cocaine females had a larger increase in CPu  $\Delta$ FosB levels than cocaine males; partly due to lower protein levels in saline female rats when compared to saline males. CPP scores were positively correlated to NAc pERK and CPu FosB protein levels, suggesting that similar to males, the ERK/CREB intracellular pathway undergoes cocaine induced neuroplasticity in female rats. However, there seem to be intrinsic

sexual dimorphisms that may contribute to responses expressed after cocaine-CPP. Taken together, the different patterns of intracellular responses in the NAc and CPu likely indicate region specific roles for pERK/pCREB/FosB signaling in the acquisition and retrieval of cocaine-context associations. These results will aid in the advancement of general knowledge about the molecular formation and retrieval of cocaine-associated memories that can be used in the future when designing treatments for cocaine addiction that target both prevention and relapse.

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## Chapter 1: Introduction

### I. Background

Cocaine, an alkaloid extracted from the leaves of the *Erythroxylon coca* plant, is an illicit, frequently abused psychoactive stimulant that enhances sensorimotor and locomotor behaviors. Cocaine addiction is a major clinical problem and has a high rate of relapse (McHugh et al., 2013). It is the second most commonly found drug in the systems of persons treated in emergency rooms (D'Onofrio et al., 2006). Cocaine can be inhaled, snorted, smoked, or injected. In low quantities, cocaine produces feelings of psychological well-being, increased confidence, and alertness. During a typical cocaine binge, users will administer the drug every 10-30 minutes when it is available. Cocaine withdrawal is characterized by agitation, depression, fatigue, insomnia, cravings for cocaine, and exhaustion (McKim & Hancock, 2013).

### A. Drug Addiction Theory

Drug addiction is characterized by compulsive drug intake, craving and drug-seeking behaviors that exist regardless of the knowledge of the negative consequences associated with drugs of abuse (Belin et al., 2008). Traditionally, theories of drug addiction emphasized the physical dependence produced by chronic drug use illustrated by adverse physical and psychological withdrawal symptoms and high tendency of relapse experienced by drug addicts. It is now commonly accepted that physical dependence is not the sole cause of drug addiction due to the fact that recovered drug addicts often relapse after abstaining from drug use for long periods of time and well after physical withdrawal symptoms are no longer present (Di Chiara, 1999). Relapse is particularly common when preceded by drug-associated stimuli; stimuli

(including but not limited to, paraphernalia, persons and/or environments) that have been associated with the rewarding effects produced by drugs of abuse (Hyman, 2005; Nestler, 2002). Recent advances regarding memory and reward-associated learning have led to the development of a new model of drug addiction suggesting that addiction may actually be a severe pathological disorder involving abnormal usurpation of the brain systems and mechanisms that under normal conditions mediate learning and memory (Brenhouse & Andersen, 2008; Hyman, 2005). More recent theories of addiction place a much greater emphasis on dependence caused by an associative learning mechanism in which the rewarding effects of drugs become associated with various stimuli in one's environment (Di Chiara, 1999).

In the natural environment, organisms require many resources essential to survival and obtaining these resources such as food or shelter for example, is naturally rewarding. Organisms become motivated to obtain these goals and the behaviors involved in obtaining them are reinforced and the behavior increases (Hyman, 2005). The increase in behaviors that produce rewarding outcomes occurs through instrumental conditioning where a contingency is formed between the behavior and the desired outcome (Cardinal & Everitt, 2004). Addictive drugs act on the same regions of the brain that produce these rewarding effects and increase the incentive value of taking the drug (Robbins, et al., 2008; Cardinal & Everitt, 2004). The contingency that is developed between taking the drug and reward, usually in the form of some internal euphoric state produced by taking the drug may initially serve in promoting repeated use of a drug (Di Chiara, 1999). Feelings of withdrawal after drug use may be negatively reinforcing, also increasing the motivational value of taking the drug (Isokawa, 2012). Thus, it has been hypothesized that the rewarding effects of drugs lead to the formation of drug-related memories through instrumental goal-directed response-reward associations (Robbins & Everitt, 2002; Robinson & Berridge, 2000).

Although the rewarding properties of drugs are necessary for addiction to develop, alone they are insufficient in explaining the transition from drug use to drug addiction. The transition occurs because drug reward increases the motivational valence of stimuli in the environment that

through learned associations can predict drug accessibility (Di Chiara, 1999). In the natural environment external cues come to be associated with reinforced behaviors through Pavlovian conditioning mechanisms, acting as conditioned stimuli that directly motivates behavior in the absence of the original unconditioned stimulus (Cardinal & Everitt, 2004). The same is true for environmental stimuli that become associated with the rewarding properties of addictive drugs. Drug-related stimuli can act as conditioned (or incentive) reinforcers which motivate drug-seeking behaviors by inducing the retrieval of drug related memories which occurs even without the presence of the drug (Robbins, et al., 2008; Volkow et al., 2003). Environmental stimuli associated with natural reinforcers such as food and sex become predictive of complex behavioral sequences involved with obtaining rewards and due to the habitual nature of these behaviors, they are accomplished efficiently and almost automatically regardless of their complexity (Hyman, 2005). Habit-learning may occur in the development of drug addiction where complex sequences of behavior are learned and produced automatically when in the presence of drug-associated stimuli (Volkow et al., 2003). These learned associations between drugs and environmental stimuli may lead to the uncontrollable drug use, cravings and relapse involved with drug addiction (Kalivas & Volkow, 2005; Robbins & Everitt, 2002).

Experience stimulates learning and memory formation which induces stable changes in brain structure and in turn produce persistent behavioral changes (Nestler, 2002). Disorders of memory or learning are usually thought to involve deficits in these cognitive brain functions. It is becoming clear that drug addiction may be just the opposite, acting through the same brain pathways involved with normal reward-learning and memory to create pathological associations (Hyman, 2005). Most areas of the brain that undergo cocaine-induced plasticity are associated with the drug reward-cues that may trigger the maladaptive behavioral responses seen in drug addicts (Hamilton & Kolb, 2005).

It is important to understand that the pharmacological effects produced by drugs of abuse, although usually harmful, cannot fully explain drug addiction (Everitt, 1997). Different types of drugs or classes of drugs have diverse effects on the brain and produce vastly differing

physiological, behavioral and psychological outcomes depending on the individual (Hyman, 2005). One thing that most drugs of abuse have in common is that they all seem to act on the brain's Dopamine (DA) system, the major brain neurotransmitter system involved with reward and motivation for everyday activities (Volkow et al., 2003). For example, in humans, dialysis studies show that cocaine increases synaptic concentrations of DA in the nucleus accumbens (NAc) (Di Chiara & Imperato, 1988). After repeated use of drugs of abuse, individuals may begin to lose control over drug craving and compulsive drug seeking behaviors due to the neural adaptations on the natural reward pathways that occur due to the associations formed between environmental stimuli and drugs of abuse (Belin et al., 2008). Recent human neuroimaging studies have shown that many of the same areas of the brain are activated both when presented with a drug and when presented with drug-associated stimuli in the absence of the drug itself. Again these were the same areas of the brain thought to be associated with reward processing and associative learning, suggesting that drug-associated memories are a key element in the development of drug addiction and relapse (Belin et al., 2013; Robbins et al., 2008).

#### B. Conditioned Place Preference (CPP)

The conditioned place preference (CPP) paradigm is commonly used with animal models in the study of drug addiction. CPP experiments use a simple Pavlovian conditioning procedure to test the importance of environmental stimuli in cueing drug reward. The CPP procedure involves the repeated pairing of a drug unconditioned stimulus (US) with a specific environment that acts as a conditioned stimulus (CS), producing a learned association between the environment in which the drug was administered and the rewarding effects of the drug (Bardo & Bevins, 2000). Testing is conducted without drug treatment, and it is assumed that if the animal spends more time in the context that was previously paired with drug administration, that the drug was in fact rewarding and an association was learned. Since the preference for the drug-paired environment is observed while animals are in a drug-free state, a memory of this association presumably must be present (Hsu, Schroeder, & Packard, 2002; Koob et al., 2004).



To date, there seems to be a clear understanding of the neural circuitry, brain regions, and neurotransmitters involved in learning, memory, and addiction (McHugh et al., 2013). Convergent glutamatergic circuitry is embedded within the mesolimbic reward system and overlap with the circuitry associated with learning and memory processes as well as goal directed behaviors and executive functioning (Kelley, 2004; See Figure 1). Drugs of abuse, including cocaine produce changes in this circuitry that may lead to addiction. CPP behavioral models exploit this circuitry by using a Pavlovian conditioning procedure to determine the importance of environmental stimuli in cueing drug reward (Bardo & Bevins, 2000; Domjan, 2005). Specifically, the CPP model allows for exploration of the underlying maladaptive changes in the signal transduction pathways that occur in response to cocaine when paired with a specific environment (Taylor, Olausson, Quinn, & Torregrossa, 2008). CPP studies have revealed that the mechanisms underlying cocaine-induced CPP differ depending on what aspect of CPP is measured. These processes include the initial acquisition and expression of cocaine-induced CPP (memory consolidation), CPP reinstatement, extinction, and reconsolidation of reward-environment associated memories (Aguilar, Rodriguez-Arias & Minarro, 2008).

### C. Neural circuitry of addiction, learning and memory

Drugs of abuse are thought to produce their rewarding effects primarily through the mesolimbic DAergic pathway, the primary reward/motivation circuitry of the brain. DAergic neurons of the ventral tegmental area (VTA) project to the NAc and form connections with the Hippocampus (HIP), striatum (including the NAc and Caudate Putamen (CPu)) and Prefrontal Cortex (PFC) (Hyman & Malenka, 2001). Embedded within this system is the glutamatergic corticolimbic circuitry which appears to be mediated by this DAergic pathway that underlies addiction (Thomas, Kalivas, & Shaham, 2008). Medium spiny neurons in the NAc receive both DAergic and glutamatergic afferents and the outputs of these neurons are mediated by both DA and glutamate transmission (Suto, Ecker, & Wise, 2009). Direct glutamatergic connections between the HIP and NAc, and from the cortex and thalamus to the CPu, facilitate the reciprocal activation of DAergic neurons in the other brain regions of this pathway (Thompson et al., 2002).

Inhibitory  $\gamma$ -amino butyric acid (GABA) and peptidergic neurons also project from the NAc to the VTA and thalamus (Groenewegen, Wright, & Beijer 1996) (See Figure 1).

Increases in DA in the NAc have been implicated in mediating drug-reward, and it is not surprising that evidence suggests a role for DA in the NAc in reward-related learning. Inputs to medium spiny neurons in the NAc consist of converging DAergic and glutamatergic afferents, which may produce an increase in strength of glutamatergic outputs from this area (Gerdjikov et al., 2004). Specifically, DAergic neurons of the VTA can be activated by direct excitatory connections between the HIP and NAc (Kelley & Domesick, 1982; Sesack & Pickel 1990). Glutamatergic axons extending from the thalamus and cortex containing both DA and glutamate receptors converge onto the spines of medium spiny DA neurons in the striatum as well as the PFC (Reviewed in Murray et al., 2013). Extensive glutamatergic inputs to the NAc from the PFC and limbic areas are thought to carry the contextual information necessary for the development of goal-directed behaviors (Grace, 2000). Connections to the thalamus and cortex both from and to all of these areas have been shown to be essential to integrating sensory information in order for learning/behavior changes to occur. Finally, connections between motor areas and the PFC and striatum serve to aid in habit and reward learning and the control of voluntary movement in the actual production of new behavior (Kelley, 2004; Wise, 1998) The transition from drug use/abuse to compulsive drug seeking behaviors is thought to rely on a (McHugh et al., 2013; Murray et al., 2013)

Figure 1

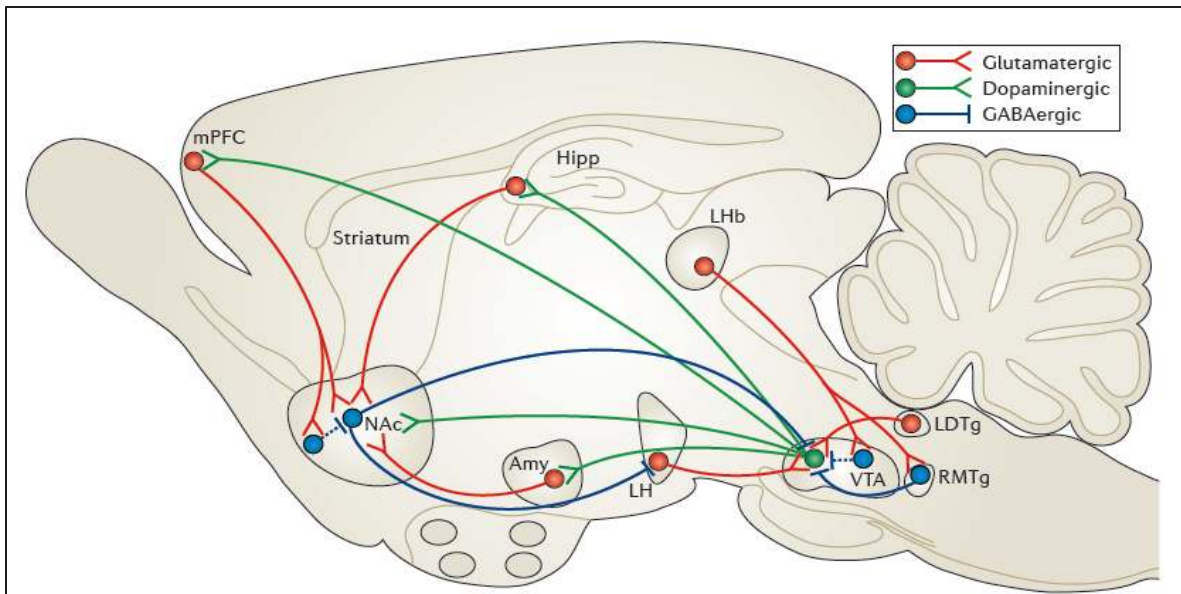


Figure 1. Convergent neural circuitry associated with cocaine addiction, learning and memory. DAergic projections from VTA terminate in the Pfc, NAc, and CPU (striatum). The HIP maintains glutamatergic connections with the VTA, NAc, and Pfc (Adapted from Russo & Nestler, 2013).

## Neurocircuitry of learning and memory

The HIP, amygdala and cerebral cortex are the key areas of the brain that have been associated with the neuroadaptations underlying most learning and memory processes. For example, the HIP functions in mediating both declarative and spatial memory, as well as associative learning and the consolidation/reconsolidation of new memories (Nestler, 2002). The amygdala has been associated with emotional memory and reward-related learning (Koob, 2009). Increases in DA in the NAc have been implicated in mediating drug-reward, and therefore it is not surprising that there is evidence suggesting a role for DA in the NAc in reward-related learning. As stated previously, inputs to medium spiny neurons in the NAc consist of converging DAergic and glutamatergic afferents which may produce an increase in strength of glutamatergic outputs from this area (Gerdjikov et al., 2004). Specifically, DAergic neurons of the VTA can be activated by direct excitatory connections between the hippocampus and NAc (Kelley & Domesick, 1982; Sesack and Pickel 1990). Glutamatergic axons extending from the thalamus and cortex containing both DA and glutamate receptors converge onto the spines of medium spiny dopamine neurons in the striatum as well as the PFC (Russo & Nestler, 2013).

Extensive glutamatergic inputs to the NAc from the prefrontal cortex (which receives inputs from DAergic neurons in the VTA) and limbic areas are thought to carry the contextual information necessary for the development of goal-directed behaviors (Grace, 2000). Connections to the thalamus and cortex both from and to all of these areas have been shown to be essential to integrating sensory information in order for learning/behavior changes to occur. And finally, connections between motor areas and the PFC and striatum serve to aid in habit and reward learning and the control of voluntary movement in the actual production of new behavior (Kelley, 2004). It therefore seems obvious that both DA and glutamate transmission are essential to initiating the intracellular processes that underlie the synaptic and neuronal plasticity mediating learning and memory. Since so much of this circuitry overlaps with the mesolimbic circuitry that is thought to underlie addiction (discussed above), and recent evidence suggesting the

convergence of these two neurotransmitter (DA and glutamate) systems, focus has been brought to studying the potential roles of cellular molecules in contributing to the synaptic plasticity involved with learning, memory and cocaine addiction (Kelley, 2004).

#### D. Cocaine: neural mechanisms

Cocaine affects several monoamine systems by blocking re-uptake via binding to the neuronal plasma membrane transporters for DA (DAT), norepinephrine and serotonin resulting in increased extracellular and synaptic monoamine concentrations (Koob, 1992; Ritz et al., 1987; Uhl, Hall, & Sora, 2002). Specifically, cocaine acts as a non-competitive DAT inhibitor, binding to the transporter at a different site than DA allowing for an increase in postsynaptic DA receptor (DAR) binding (See Figure 2) (Povlock & Schenk, 1997; Wayment, Meiergard, & Schenk, 1998). Increased postsynaptic D1 binding induces the activation of both adenylyl cyclase and phospholipase C Beta that causes calcium ( $Ca^{2+}$ ) release from the endoplasmic reticulum (of the postsynaptic cell), providing the  $Ca^{2+}$  necessary to activate the kinases and transcription factors underlying the long and short term neural and behavioral changes associated with cocaine exposure (Su & Hayashi, 2001). DAT-knockout studies have shown that cocaine-induced increases in locomotor behavior are DAT dependent. Likewise, other DA reuptake inhibitors have reinforcing effects and are readily self-administered both by rodents and non-human primates (Uhl, et al., 2002).

Five types of DA receptors (all of which are 7 transmembrane G protein-coupled) have been identified and separated into two distinct subfamilies based on pharmacologic and intracellular signaling similarities: D1-like (D1 & D5) and D2-like (D2, D3, & D4). The D1 receptor, which is found in abundance in the NAc, HIP, amygdala and CPu, is the most numerous and widely distributed DA receptor subtype (Karasinska, George, Cheng, & O'Dowd, 2005; Missale et al., 1998). The increase in activation of postsynaptic DA receptors that occurs with repeated cocaine exposure results in the activation of a series of molecular events that lead to long lasting neural adaptations and changes in gene expression (Dudman et al, 2003). D1 and D2 DARs

Figure 2

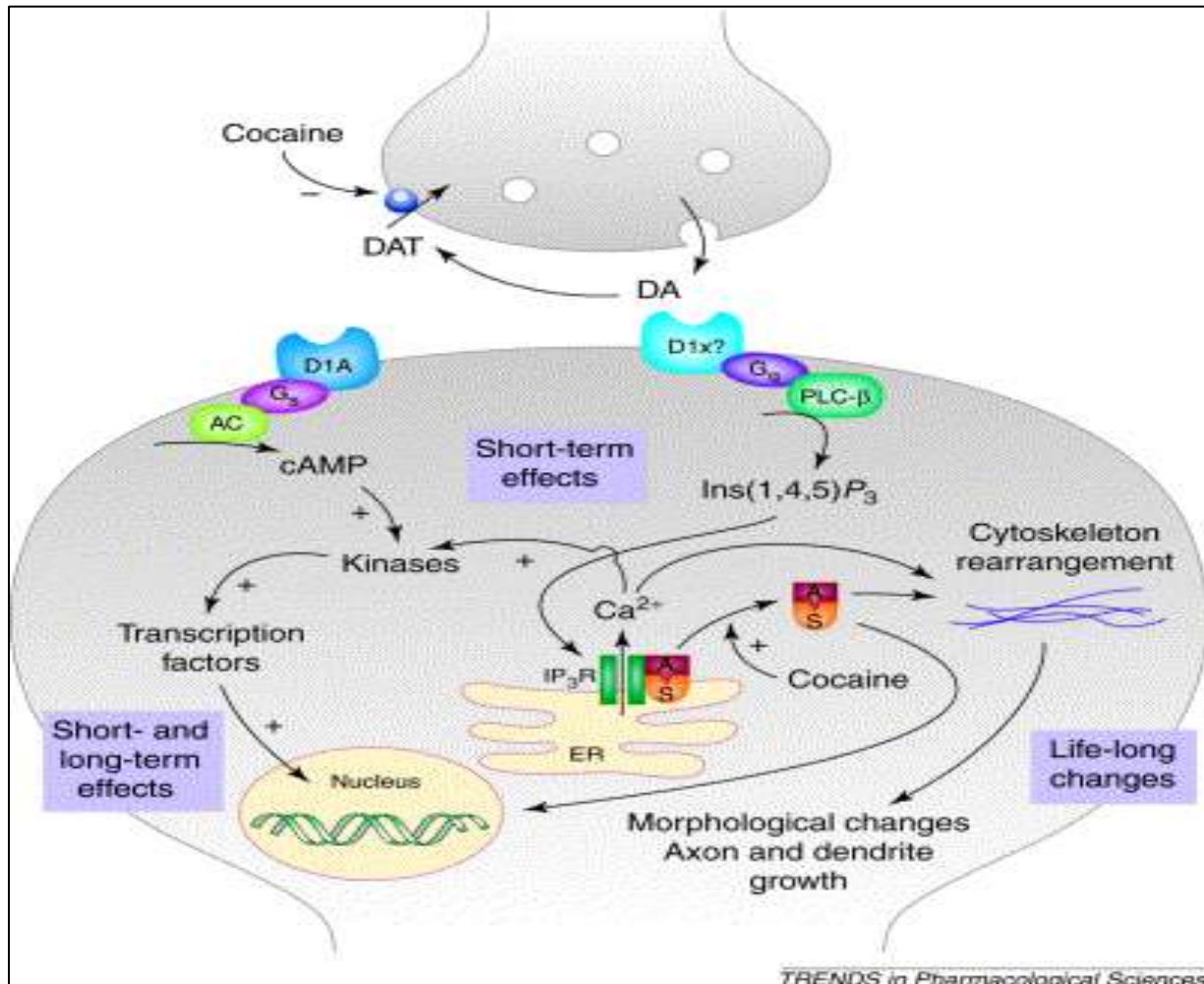


Figure 2. Short and long term synaptic and cellular effects of cocaine exposure. Cocaine binds to DAT preventing DA reuptake and produces increases synaptic DA concentrations leading to cAMP and protein kinase activation resulting in changes in transcription factor and gene expression (Adapted from Su & Hayashi, 2001).

however, differ in this respect as to the molecular events produced by their stimulation by cocaine. For example, Lee et al. (2006) observed an increase in  $\Delta$ FosB expression and dendritic spine densities on the medium spiny DA neurons transgenically expressing either D1 or D2 receptors in the NAc 2 days after chronic cocaine treatment. These increases in spine density and gene expression were only stable (lasted at least 30 days after last cocaine treatment) in D1 expressing neurons suggesting that D1 and not D2 receptors may be responsible for the long term neural alterations underlying the long term changes in behavior seen in addiction. Acute cocaine administration has been shown to induce c-Fos expression in striatum, and D1, but not D2 receptor antagonism blocks the induction of c-Fos as well as the phosphorylation of signaling molecules essential to its expression including CREB and Elk-1 (Guan, Tao, & Li, 2009; Zhuang, Belluscio, & Hen, 2000).

Increases in synaptic DA levels in the NAc mediate cocaine reward most likely through the activation of DA D1 receptors (Lee et al., 2006). CPP for cocaine is blocked by systematic administration of D1 receptor antagonists but not D2 antagonists (Cervo & Samanin, 1995). Also, D1 and D3 DAR activation by cocaine has been shown to produce oppositely regulated effects. For example, D1R knockout mice do not develop CPP to cocaine, whereas D3 knockouts do express cocaine-induced CPP behavior (Chen & Xu, 2010). However, a single injection of a D1 receptor antagonist just prior to CPP testing (instead of during the acquisition phase) does not block the expression of cocaine-CPP (Cervo & Samanin, 1995). This suggests a potential role for D1 receptors in the initial rewarding properties of cocaine, and the acquisition of cocaine-associated memories, but not in the retrieval of the reward-environment association.

#### Cocaine effects on glutamate receptors

In addition to increasing synaptic DA levels, extracellular synaptic concentrations of glutamate are also modulated in response to cocaine exposure. Not surprisingly, glutamate receptors (including NMDA, AMPA/Kainate, and metabotropic receptors) are found in high

abundances in the brain areas discussed above in regards to the circuitry underlying neuronal plasticity and addiction (Kelley, 2004). Reid and Berger (1996) found that synaptic glutamate concentrations were increased in rats after chronic cocaine treatment and remained altered up to ten days after cocaine treatment when challenged with cocaine. McFarland, et al., (2003) found increases in extracellular glutamate levels in the NAc after cocaine-primed reinstatement of self administration which was abolished when glutamatergic afferents from the PFC were blocked. Reinstatement of cocaine-seeking behavior was blocked by intra-NAc AMPAR antagonism but not DAR antagonism (Cornish & Kalivas, 2000).

Increases in the GluR1, GluR2/3 and NMDAR1 subunits of glutamate receptors are found in the VTA and NAc of behaviorally cocaine-sensitized rats after 2 days of withdrawal from cocaine (Churchill, Swanson, Urbina, & Kalivas, 1999). Schumann and Yaka (2009) also observed an increase in NMDAR subunit expression in the NAc of cocaine-sensitized rats 21 days after cocaine treatment and this increase was correlated with an increase in intracellular ERK activity and expression of AMPAR subunit GluR1. Both NMDAR antagonism and intra-NAc injections of U0126 (a MEK inhibitor) blocked the increase in ERK activation and GluR1 expression suggesting that the increase in GluR1 expression is dependent on ERK phosphorylation and increased NMDAR expression (Miller and Marshall 2005).

Cocaine effects on interactions between DA and glutamate receptors

One of the first studies to elucidate an interaction between DA and glutamate transmission in response to cocaine used single cell recordings of DA neurons in the VTA of cocaine-treated rats (White, Hu, Zhang, & Wolf, 1995). They revealed that these neurons fire at a faster rate in response to glutamate after five days of cocaine treatment compared to those of saline controls indicating an interaction between DA and glutamate transmission and more importantly that excitatory cells projecting onto the DA neurons of the mesolimbic pathway were experiencing synaptic plasticity in response to cocaine (White et al., 1995). Since then, Ungless et al., (2001) went on to show that this increase in LTP lasts for at least 5 days (but not 10 days) and was associated with an increased expression of the GluR1 AMPA and NMDAR1 glutamate



receptor subunits (Fitzgerald et al 1996). The LTP of these excitatory synapses was inhibited by NMDAR antagonism at the time of cocaine administration which suggests a role for NMDARs in the development of cocaine addiction (Ungless et al., 2001).

Studies are now more focused on the intracellular signaling, transcriptional and epigenetic regulation of mechanisms underlying the activation and recruitment of these receptors and resulting potentiation of the synapses involved with cocaine addiction and relapse (Nestler, 2014; Robison & Nestler, 2011). Stimulation of D1 receptors in the NAc and CPu results in PKA activation and the phosphorylation of the NMDAR R1 subunit at Ser 897, allowing for both direct and indirect protein-protein interactions (Jiao, Zhang, Gao, Lou, Zhang, & Xu, 2007). Just as enhanced glutamatergic activity seen in the HIP which is associated with LTP is the result of postsynaptically enhanced glutamate transmission, cocaine produces an increase in the synaptic transmission of glutamate, not only by increasing glutamate in the synapse, but also by increasing the excitatory postsynaptic effect that glutamate has on DAergic neurons in the VTA and NAc (Nestler, 2001). This may increase NMDAR sensitivity to glutamate and although glutamate levels do not change in response to D1 or PKA activation,  $Ca^{2+}$  influx through NMDARs is markedly increased (after D1/PKA activation) resulting in increased intracellular levels of  $Ca^{2+}$  which is blocked by MK-801, an NMDAR antagonist (Dudman, et al., 2003). Antagonizing NMDARs in the VTA blocks the induction of behavioral sensitization to cocaine and both D1 and NMDAR antagonists prevent cocaine induced gene expression in rats (Torres & Rivier, 1993). For example, MK-801 has also been shown to substantially reduce cocaine-induced increases in Fos and preprodynorphin mRNA levels (Jenab, Festa, Russo, Wu, Inturrisi, & Quinones-Jenab, 2003).

It has been well established that D1 receptor activation is required for the glutamatergic induction of LTP at synapses in the HIP, striatum, and cortex. A direct interaction between D1Rs and the NR1 subunit of the NMDAR forms a complex at striatal synapses and serves to maintain and strengthen synaptic strength in response to changes in synaptic concentrations of DA (Fiorentini, Gardoni, Spano, Lucas, & Missale, 2003). Both D1 and NMDAR antagonists have

been shown to block the acquisition and expression of cocaine CPP (Cervo & Salamin, 1995). Zweifel, Argilli, Bonci, and Palmiter (2008) used DA neuron specific NMDAR knockout mice to examine the role of NMDAR signaling in DA neurons in the production of cocaine induced behaviors and found that locomotor sensitization to cocaine was unaffected in these mice. However, the acquisition of CPP for cocaine was blocked, suggesting an important role for NMDAR dependent signaling and plasticity in DA neurons for the formation of cocaine-associated memories (Thomas et al., 2008). Cocaine-induced DA and glutamate receptor activation therefore leads to the activation of molecular signaling cascades causing functional changes in the expression of signaling proteins and genes associated with the addictive state (See Figure 3).

Figure 3

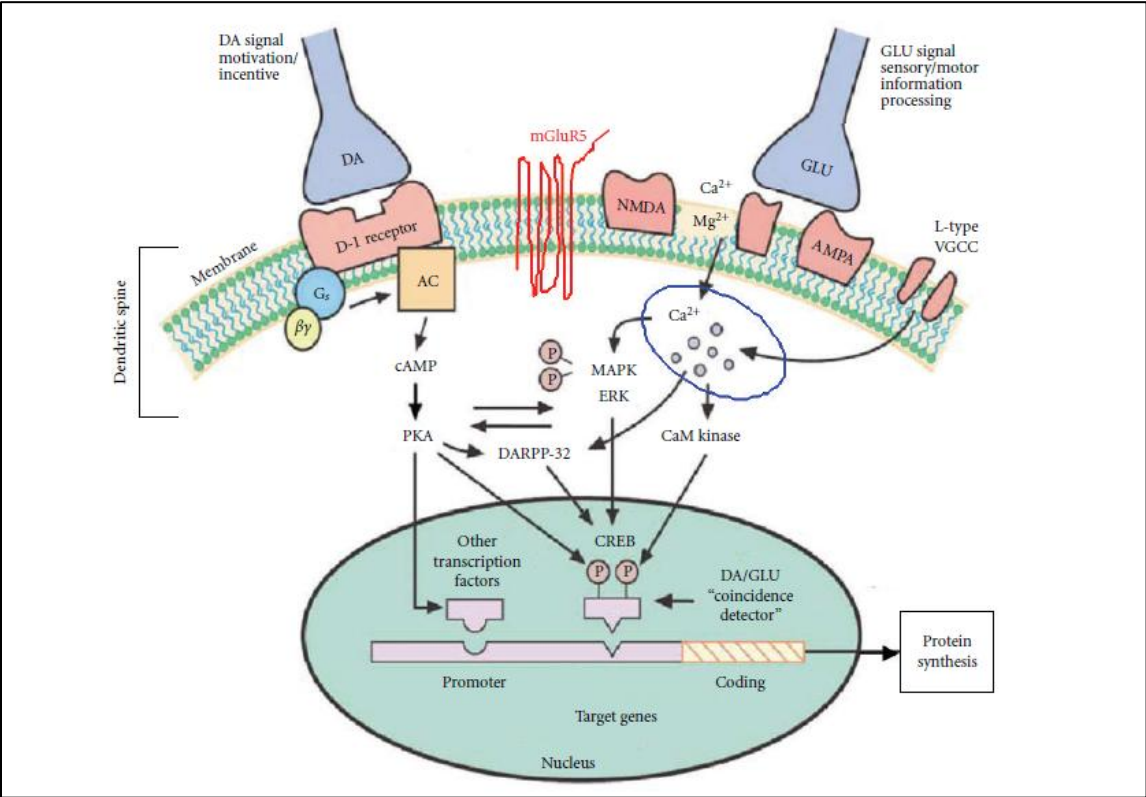


Figure 3. ERK signaling pathways. The ERK pathway is activated by the simultaneous activation of D1 and NMDA type glutamate receptors. ERK activation also results in an increase of AMPARs to the cell membrane (Isowaka, 2012).

## II: Downstream cocaine-induced intracellular signaling

Acute and chronic cocaine administration induces the phosphorylation of ERK, a signaling molecule of the mitogen activated protein kinase (MAPK) signal transduction family, in a DA dependent manner (Berhow & Nestler, 1996; Jenab et al., 2005; Sun et al., 2008). In rats, ERK has been repeatedly implicated in the acquisition of psychostimulant-induced CPP and retrieval of cocaine-environment memories (Gerdjikov et al., 2004; Liu et al., 2011; Miller & Marshall, 2005; Pan et al., 2011; Valjent et al., 2006). Cocaine-induced ERK phosphorylation produces rapid increases in membrane excitability that after repeated exposure lead to long term changes in protein and gene expression associated with signaling reward (Lu et al., 2006; Nestler, 2001). Downstream of ERK, cAMP response element binding protein (CREB), FosB and  $\Delta$ FosB are transcription factors associated with experience-dependent synaptic plasticity and long-term molecular neuroadaptations in response to cocaine (Carlezon et al., 2005; Larson et al., 2010; Marazziti et al., 2011; Zhang et al., 2006). In male rats, acute cocaine exposure increases striatal CREB phosphorylation and FosB protein levels after cocaine CPP expression and chronic cocaine exposure produces a persistent accumulation of NAc  $\Delta$ FosB levels (Harris et al., 2007; McClung & Nestler, 2003; Nestler et al., 2001; Rawas et al., 2012; Tropea et al., 2008).

### A. ERK

ERK, a signaling molecule of the mitogen-activated protein kinases (MAPK) signal transduction family, has been implicated in both the behavioral and rewarding effects of cocaine (Lu et al., 2006). ERK has 8 known isoforms (ERK1-8) distinguished by a Thr-Glu-Tyr motif in which phosphorylation of the Thr and Tyr residues is essential for their activation. The ERK proteins, specifically ERK 1/2, have been studied most extensively and have been implicated to be important for cell proliferation and differentiation, neuronal plasticity, and cocaine addiction (Girault et al., 2007).

Upstream, activation of NMDAR, D1R, PKA and DARPP-32 have all been shown to trigger activation of the ERK pathway (Thomas et al., 2008). Downstream, ERK activation is dependent

on MAPK/ERK kinase (MEK) activation, which occurs in a  $\text{Ca}^{2+}$  dependent manner beginning with an increase in  $\text{Ca}^{2+}$  influx which activates the small G protein Ras, activating the protein kinase Raf. Raf activation induces the phosphorylation of ERK via MEK. ERK activation is involved in the regulation of immediate early gene expression including c-Fos, Jun-B and FosB by translocating to the nucleus causing the activation of downstream transcription factors CREB, Elk-1, RSK, and MSK1 (Mattson et al., 2005; Brami-Cherrier et al., 2005; Valjent et al., 2000; Radwanska et al., 2006). ERK phosphorylation also results in the phosphorylation of the Kv4.2  $\text{K}^+$  channel subunit which reduces the outward flow of  $\text{K}^+$  thus rapidly and transiently increasing the membrane excitability (Yuan et al., 2002). In addition, ERK phosphorylation leads to an increase in AMPA receptors at the cell membrane (See Figure 4) (Qin et al., 2005).

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Figure 4

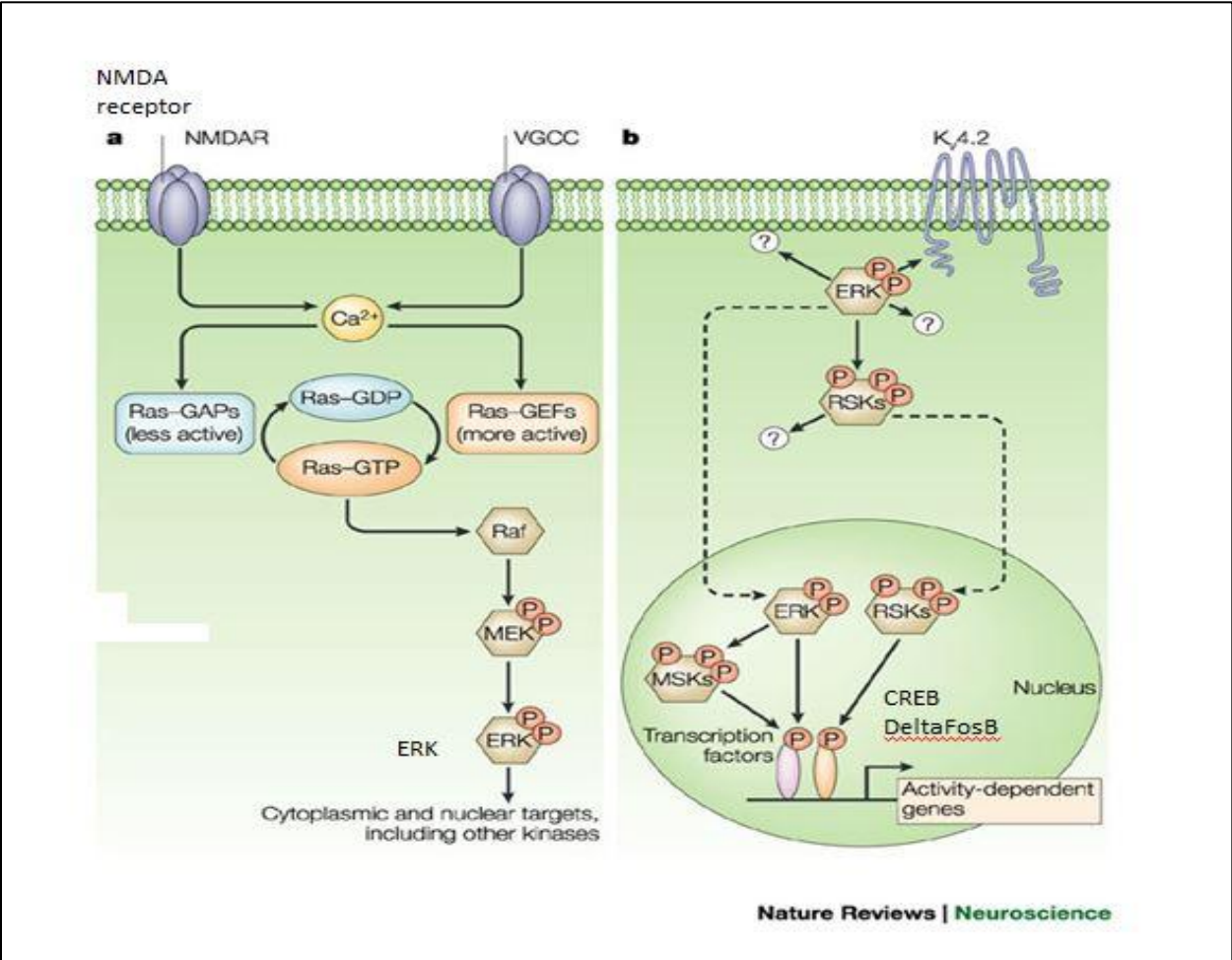


Figure 4: Cocaine-induced NMDAR dependent ERK activity. Upon phosphorylation, ERK translocates to the cell nucleus and activates transcription factors while simultaneously affecting cytoplasmic targets including AMPA receptor insertion. Adapted from Thomas & Huganir, (2004)

## Role of ERK in learning and memory

It also appears that gene transcription and de novo protein synthesis regulated by both ERK and CREB activation may be an essential requirement for learning and the consolidation of new memories (Kuo et al., 2007; Taylor, et al., 2008). ERK activation and the MAPK enzyme family is widely spread and regulated throughout the central nervous system and has been shown to be critical for a number of behavioral learning and memory tasks associated with the activation and/or control of cell membrane receptors and targets (Peng, Zhang, Zhang, Wang, & Ren, 2010). ERK activation is essential to the formation of fear associated memories, exemplified by ERK  $\frac{1}{2}$  is phosphorylated in the CA1 region of the HIP during trace fear conditioning and this learning was not observed in animals with lesions to the HIP (Atkins et al., 1998). Inhibition of ERK after fear training abolishes fear memories during retrieval tests of trace fear responses suggesting that ERK activation may also be required for the retrieval of fear memories (Huang, Chiang, Liang, Thompson, & Liu, 2010). In striatum, memory consolidation for motor skills has also been shown to critically depend on ERK activation (Bureau, Carrier, Lebel, & Cyr, 2010). ERK is also activated during training of the Morris water maze indicating a role for ERK in spatial memory as well (Blum, Moore, Adams, & Dash, 1999). Much evidence supports a critical role of the ERK signaling cascade in regulating the neuroadaptations underlying the production of synaptic plasticity through altering gene activation (Hyman, 2005; Taylor et al., 2008). For example, it has been shown that LTP in the HIP is dependent on ERK activation and HIP LTP induction is blocked by MEK inhibition (English & Sweatt, 1997). ERK has also been implicated in activating the immediate early genes necessary for the regulation NMDAR induced synaptic plasticity underlying Pavlovian fear conditioning (Ota, Monsey, Wu, Young, & Schafe, 2010).

## Role of ERK in cocaine addiction

Cocaine induced activation of the ERK pathway in the mesocorticolimbic system is mediated by the convergence of DA and glutamate transmission, specifically by its action on D1

DARs (Zhang et al., 2004). D1R activation activates PKA and thus DARP-32 activation, leads to the activation of L-type  $\text{Ca}^{2+}$  channels as well as NMDARs. Cocaine has been shown to increase ERK phosphorylation in the VTA, dorsal striatum (CPu), PFC, amygdala and NAc and ERK activity is blocked by both D1 and NMDAR antagonists (Valjent et al 2006). Also, D1 knockout mice do not show ERK activation in response to cocaine (Zhang et al., 2004). Cocaine also produces increases in the expression of BDNF which can induce ERK phosphorylation via Ras (Poo, 2001). Downstream of ERK, the activity of Elk-1 and CREB, transcription factors involved in mediating the expression of IEGs, are enhanced by cocaine induced ERK phosphorylation (Lu et al., 2006). Sun et al., (2007) showed that acute cocaine administration increases p-MEK, p-ERK, and p-ELK1 protein levels in the CPu and that these increases were associated with increased IEG expression. Cocaine activation of ERK is also associated with c-fos expression and inhibition of ERK after cocaine exposure decreases c-fos mRNA levels in the NAc (Guan, Hu, & Li, 2008). Studies using MEK inhibitors to block subsequent ERK activation have revealed a role for ERK in the behavioral effects of cocaine including sensitization, reward, and reward-associated memories. ERK activation in the VTA has been directly implicated in the development (but not expression) of psychomotor sensitization to cocaine. Specifically, the MEK inhibitor SL327 injected prior to cocaine administration prevented the development of sensitization whereas SL327 injected in previously cocaine-sensitized mice did not reverse these effects (Pierce et al., 1999; Vanderschuren & Kalivas, 2000).

## B. CREB

Another molecular candidate that has been extensively studied and shown to be involved with a number of behavioral processes including learning, memory and addiction is CREB. CREB, a member of the CREB/ATF transcription factor family, is expressed in a wide variety of cell types and brain regions (Silva et al., 1998). CREB is thought to alter gene expression beginning with its phosphorylation at SER133 which in turn causes CREs or cAMP-response elements to bind to the promoter regions of other proteins and target genes to regulate their transcription (Berke & Hyman, 2000; Hyman & Malenka, 2001).



CREB can be phosphorylated by a number of kinases depending on brain region, including both cAMP and calcium dependent protein kinases (CaMKs) providing further evidence for the role of CREB due to its function as a coincidence detector (Hyman, 2005). For example, in the NAc and HIP, cAMP activation has been shown to activate CREB directly, via the phosphorylation of PKA (Gonzalez & Montminy, 1989). In primary culture CREB has been shown to be activated by direct application of both NMDA and D1R agonists (separately) and this activation is blocked by inhibiting PKA (Das, Grunert, Milliams, & Vincent, 1997). In the NAc, CREB phosphorylation can also be controlled by DARP-32 (DA and cyclic AMP-regulated phosphoprotein 32) through the PKA and  $Ca^{2+}$  pathways. When PKA activates DARP-32 at Thr34, protein phosphatase 1 is inhibited resulting in an increase in phosphorylated CREB at Ser 133. However, if phosphorylation occurs at Thr75, DARP-32 is converted into a PKA inhibitor thereby decreasing CREB activation at Ser133 (Nairn et al., 2004; Halpain et al., 1990; Svenningsson et al., 2004).

#### Role of CREB in learning and memory

CREB has been implicated as a critical factor in behavioral memory. Increased NAc CREB phosphorylation decreases anxiety like behaviors exemplified in the elevated plus maze and open field. Stress also induces CREB phosphorylation in the NAc. Activation of CREB in the HIP and amygdala increases behavioral memory (Nestler, 2002; Pliakas et al., 2001). CREB mutant mice show intact short term memory 30 minutes after fear conditioning, but when tested again 24 hours later the mice no longer associate the context with a foot shock (possibly because they no longer care that they will be shocked) thus exhibiting no long term memory for the task (Bourtchuladze et al., 1994; Brightwell et al., 2007). CREB phosphorylation increases the excitability of the medium spiny neurons in the NAc, producing an increase in NMDARs at the cell surface, thus increasing NMDAR glutamate transmission (Dong et al., 2006; Huang et al., 2008). Taken together, CREB phosphorylation in the NAc seems to be a necessary element to experience-dependent learning

## Role of CREB in cocaine addiction

Drugs of abuse alter CREB function and activity. Although it may seem as if CREB activation occurs in response to a large number of stimuli (everything) and produces a wide variety of effects, it is likely that it works in combination of transcription factors that underlie post activity-dependent plasticity. For example, Fos, which contains a binding site for CREB in its promoter region (CREB has been implicated in a critical regulator of Fos), also contains binding sites for other transcription factors including ELK-1 (Herdegen & Leah, 1998).

In order to understand the role that CREB plays mediating cocaine addiction it is important to understand how CREB is altered differently in different brain regions in response to cocaine. Within the mesolimbic pathway, CREB is found in higher abundance in the NAc as compared to the VTA (Walters, Kuo, & Blendy, 2003). Increases in the activated form of CREB (phosphorylated CREB) in the NAc occur in response to both chronic and acute exposure to cocaine and pCREB in the HIP is observed after acute cocaine administration (Kuo, et al., 2007; Nestler, 2002). Liu et al. (2006) found that cocaine induced CREB activation in the CPu was blocked after both D1 receptor antagonism and MEK inhibition (not simultaneously) suggesting that cocaine activates CREB indirectly through the activation of D1 receptors which initiates the molecular events leading to ERK activation. CREB activation and the resulting gene expression and neuroadaptations that occur in response to acute cocaine exposure seem to be dependent on the phosphorylation of ERK via the MEK pathway (See Figure 4). Chronic cocaine treatment reduces CREB and DARP-32 Thr-34 phosphorylation in the CPu which suggests that alterations in p-CREB levels in response to chronic cocaine administration are mediated by the activation of PKA/DARP-32 (Di Benedetto et al., 2006). McClung and Nestler (2003) also found that acute cocaine exposure induces CREB dependent gene expression and decreases the rewarding effects of cocaine whereas chronic cocaine treatment induced gene expression dependent on the transcription factor  $\Delta$ FosB, which correspondingly increased the rewarding and motivational effects of cocaine.

Even though cocaine induces CREB activation, the rewarding effects of cocaine have been shown to be decreased by using viral mediated gene transfer to locally overexpress CREB in the NAc (Carlezon et al., 1998). Also, local expression of a dominant negative mutant of CREB, mCREB, which binds to SER133 thereby preventing its phosphorylation, in the NAc have been shown to produce increases in cocaine reward (Barrot et al., 2002). mCREB mutants also show a reduction in depressive like states. For example, these mice do not exhibit a threshold increase for intracranial self-stimulation in response to k-opioid receptor agonism, which in wild type mice produces the opposite effect (DiNieri et al., 2009). CREB activation in the NAc may be involved in other aspects of cocaine addiction such as functioning in the process of creating persistent long term neural changes that lead to and facilitate the maintenance of addictive behaviors.

### C. Sirtuins

Sirtuins are a family of genes made up of seven NAD<sup>+</sup>-dependent enzymes, shown to play a role in age-related diseases and neurodegeneration (Chopra et al. 2012). SIRT (Silent Information Regulator of Transcription) are class III histone deacetylases (HDACs). SIRT1 and SIRT2 have recently been implicated in drug abuse (Renthal et al., 2009). SIRT1 is found mainly in the nucleus and cytoplasm and SIRT2 is found mainly in the cytoplasm and regulates cytoskeletal proteins and plasticity mechanisms (Domnez 2012). Recent evidence suggests an increase in SIRT1/2 subtypes enhance the rewarding effect of abused drugs including cocaine (Hating, 2012; Ferguson et al., 2013), and mediate drug-induced neuroplasticity (Gao et al., 2010). NAc SIRT 1 and 2 protein levels increase following cocaine administration and Resveratrol (a SIRT1 and 2 agonist) increased the rewarding effects of cocaine (Renthal et al., 2009). Local inhibition of SIRT1 and SIRT2 in the NAc decrease cocaine reward exemplified by attenuated CPP (Renthal et al., 2009). Inhibition of SIRT1 has been found to decrease ERK phosphorylation (Domnez 2012; Li 2008; Ota 2006; Zhao et al., 2012) and resveratrol increased ERK phosphorylation (Huang, 2008), suggesting that Sirtuins may play a role in regulating ERK activity. Sirtuin activity may also regulate CREB phosphorylation and  $\Delta$ FosB overexpression after

cocaine exposure (See Figure 5; Gao et al., 2010; Renthal et al., 2009; Robison & Nestler, 2011).

### III: Cocaine-associated memories: Role of p-CREB/p-ERK/FosB

Most likely through its activity on the D1 DA receptor, both chronic and acute doses of cocaine produce changes in CREB phosphorylation in the NAc and HIP which suggests a role for CREB in the establishment of reward-associated memories (Hyman, 2005). After repeated cocaine exposure, Marin et al., (2009) found an increase in cocaine-induced NAc CREB phosphorylation that was context specific, occurring only in animals that were in the environment paired with cocaine. Kuo et al., (2007) used the CPP paradigm to examine p-CREB levels in the HIP, nucleus accumbens, and PFC of rats at 10, 30 and 60 minutes after one cocaine-place pairing. Increases in hippocampal p-CREB were observed at all three time intervals, while increases in p-CREB in the NAc were only seen after 10 minutes, indicating a possible role for pCREB in the NAc in the initial memory consolidation process. To further test the role of pCREB in memory formation in the NAc, Kuo et al., (2007) infused CREB antisense oligodeoxynucleotides directly into the NAc prior to cocaine administration. After a 50 percent decline in accumbal CREB levels, CPP memory consolidation appeared to be disrupted because CPP behavior was not expressed (Kuo et al., 2007).

Figure 5

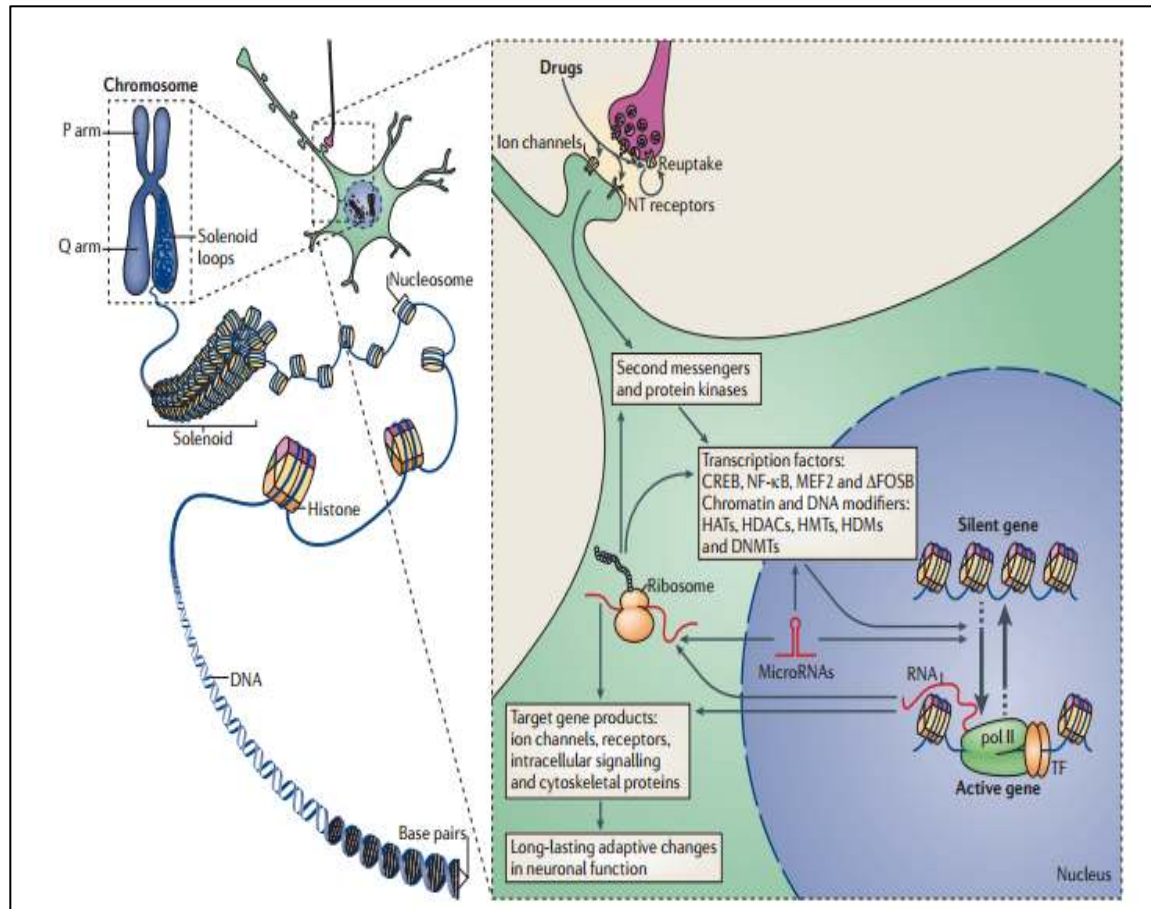


Figure 5: Transcriptional and epigenetic effects of cocaine and other abused drugs. ERK signaling affects downstream transcription and may produce long-term epigenetic modifications (Robison & Nestler, 2011)

Tropea et al., (2008) found an increase in CREB phosphorylation at SER133 as well as phosphorylated DARPP-32 at Thr34 and decreases in p-DARPP-32 at Thr75 in the NAc of mice re-exposed to the cocaine-paired chamber two days after exhibiting a preference for that environment. Zachariou et al., (2006) found that DARPP-32 mutant mice lacking the phosphorylation site at Thr34 do not exhibit cocaine conditioned place preference. Miller and Marshall (2005) found that cocaine induced conditioned place preference produced increases in the activation of ERK, CREB, Elk-1, and Fos in the NAc core (not shell) of male rats. They were also able to block retrieval of CPP and the activation of these proteins by infusing U0126, an inhibitor of MEK directly into the NAc core both two and 15 days after the initial preference was tested. This provides evidence contradictory to that of Tropea et al., (2008) in which an increase in p-ERK was not seen in the NAc after cocaine-paired environment re-exposure. The differences in results could be explained through the differences in cocaine dose (5mg/kg versus 15 mg/kg respectively), time points examined and method of CPP testing. Miller and Marshall (2005) examined protein levels activated during the actual chamber preference test whereas Tropea et al. examined protein levels during re-exposure to the cocaine-paired chamber two days after preference testing.

The ERK pathway has been shown to be an essential requirement for the conversion of short term memory to long term memory (Atkins et al., 1998, Valjent et al., 2000; Brambilla et al., 1997, Mazzucchelli et al., 2002, Ferguson et al., 2006). Paradoxically, since ERK is an important regulator of CREB phosphorylation and transcription (Xing et al., 1996), and because pCREB increases after cocaine-CPP (Kuo et al., 2007; Tropea et al., 2008), mutant mice overexpressing a dominant negative version of CREB in striatum (inhibiting CREB activation selectively in the striatum) exhibit increased sensitivity to cocaine induced CPP (Fasano, et al., 2009). Genetically inhibiting CREB activation may produce unique adaptations at the cellular level and lead to the selective activation of genes that normally suppress responses to drugs of abuse. Chen and Xu (2010) found that D1 knockout mice do not develop cocaine CPP and that pERK levels in the NAc and PFC were correspondingly reduced compared to mice with a preference for the cocaine-paired environment, suggesting that ERK activation via D1 receptors is involved with initial

association learning. Furthermore, Cocaine-CPP expressing rats also show increases in c-fos and other IEGs in the Pfc, basolateral amygdala and NAc core (Miller & Marshall, 2004). Valjent et al., (2006) administered SL327 just prior to a cocaine-place pairing but after an initial preference for the cocaine paired-environment had been established. It was found that MEK inhibition alone, without cocaine treatment eliminated the preference seen the day before, whereas if SL327 was given along with cocaine, the preference remained intact, indicating that reconsolidation for cocaine-association memories is dependent on ERK activation. It is possible that ERK is responsible for mediating the rewarding effects of cocaine along with the motivational salience of the environment that comes to be associated with them (Lu et al., 2006). Taken together, the results of these studies provide strong evidence for the role of ERK/CREB intracellular signaling pathways in mediating the structural neuronal plasticity involved with cocaine associated memory formation (See Figure 6).

Figure 6

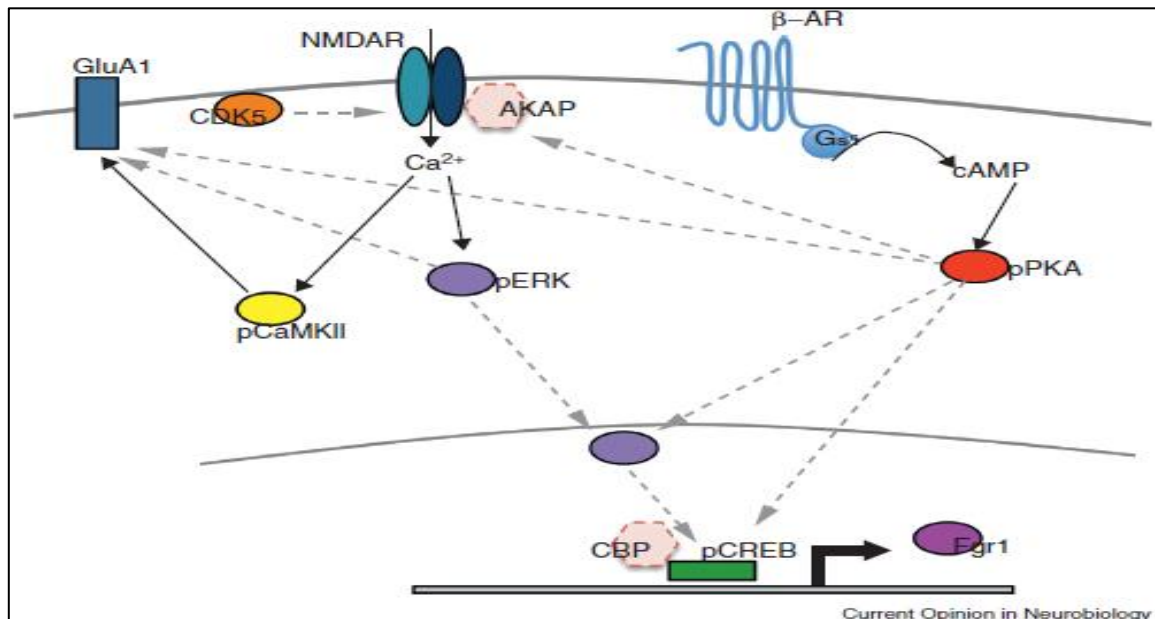


Figure 6: Reward related memory acquisition and retrieval mechanisms. Cocaine-contextual memories may be acquired through NMDAR dependent downstream signaling including the ERK and PKA signaling cascades (Tronson & Taylor, 2013)



#### IV: Significance of work and working hypotheses

The intracellular mechanisms discussed above are due to the activation by cocaine of DA and glutamate receptors simultaneously; promoting neural plasticity while cocaine is present allowing for the encoding and consolidation of drug-related memories (Girault et al., 2007). After the formation of the environmental association due to repeated pairings of the drug in the same environment, just being presented with the drug paired environment is sufficient to produce DA receptor activation which leads to increased recruitment of glutamate receptor activity based on the fact that the given environment was often a strong predictor for reward in the past, and thus the ERK pathway becomes activated in absence of the drug (Girault et al., 2007). It is probable that the DA D1 receptor acts as an error signal for predicting reward, while the NMDARs may carry the contextual information that comes to be associated with the rewarding properties of the drug. Currently, a complete understanding of the relationship between intracellular signaling pathways activated during the formation of cocaine-reward associations and those activated upon retrieval of the association is lacking (See Figure 7). Completion of this proposal will elucidate the role of ERK/CREB signaling pathways in both the acquisition and retrieval of cocaine-associated memories. First, by antagonizing NMDARs prior to cocaine treatment, during the acquisition phase of CPP, mechanisms downstream of ERK phosphorylation during CPP expression will be elucidated. Pharmacological inhibition of SIRT1 and SIRT2 proteins during CPP acquisition will provide insight to a novel mechanism by which cocaine-associations are acquired. Second, by using two doses of cocaine to explore the molecular substrates activated upon re-exposure to a cocaine-paired environment, we will show that ERK is primarily responsible for mediating the rewarding aspects of cocaine and is thus necessary for CPP expression. Finally, we will assess cocaine-CPP induced changes in intracellular signaling molecules in females to determine if these responses are sexually dimorphic. Results of this dissertation provide advances in our knowledge of the molecular formation and retrieval of cocaine-associated memories that can be used in the future when designing treatments for cocaine addiction, to aid in both the cessation of addiction and prevention of relapse.

Figure 7

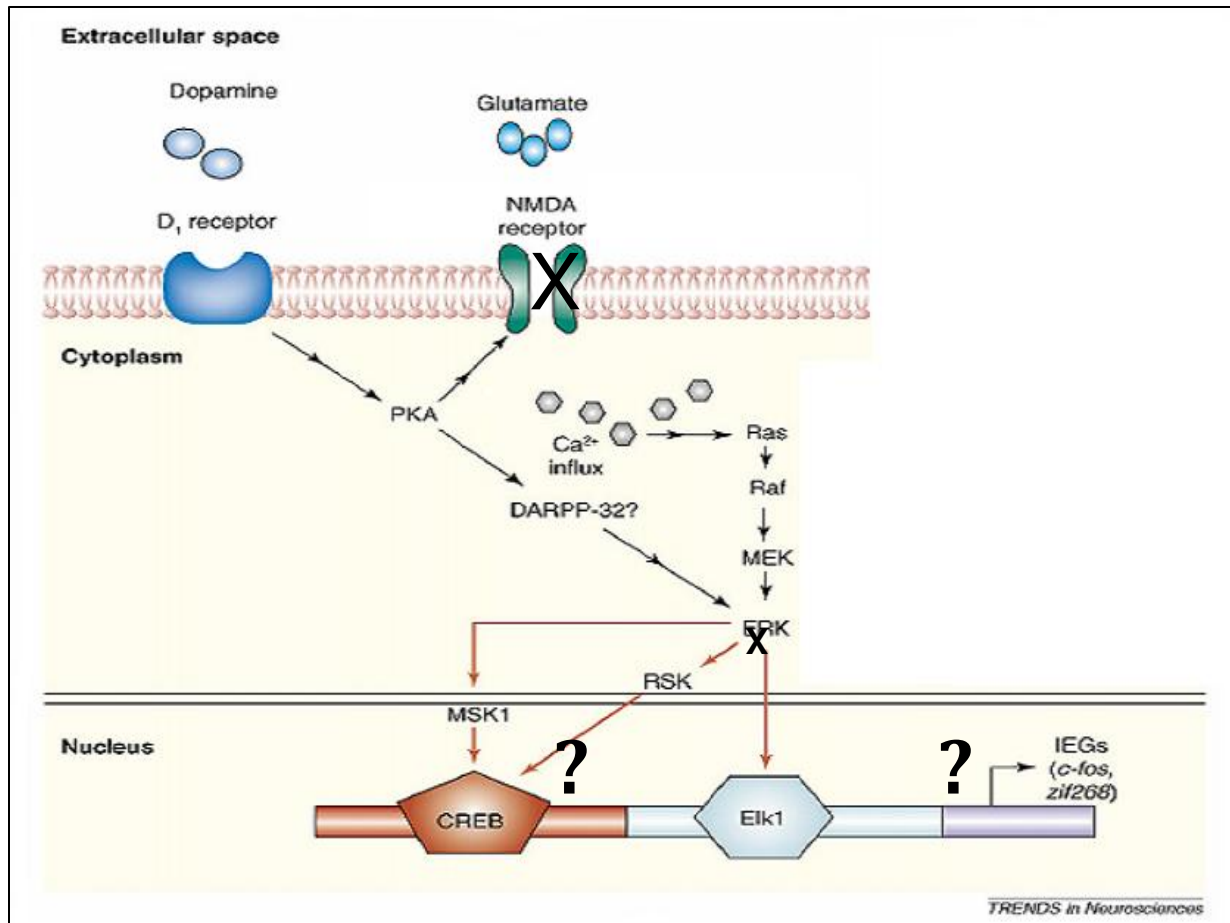


Figure 7: Hypothetical model of mechanisms regulating the expression of a cocaine-context association. It is expected that blocking NMDARs during conditioning will prevent the acquisition of cocaine CPP and the ERK pathway will subsequently be inhibited during the CPP retrieval test.

## V. Specific Aims and hypotheses

Aim I: To test the hypothesis that NMDAR and SIRT inhibition will block cocaine-CPP, ERK phosphorylation and subsequent immediate early gene expression observed after CPP expression, we will pharmacologically inhibit NMDARs, SIRT1, and SIRT2 (in separate experiments) before cocaine administration. To this end, we will administer the non-competitive NMDAR antagonist MK-801, the SIRT1 antagonist EX-527, or the SIRT2 antagonist AK-7 prior to cocaine treatment during CPP conditioning sessions. It is expected that these pharmacological manipulations will prevent the acquisition of cocaine CPP and ERK phosphorylation in the striatum (CPu and NAc). Specifically, cocaine-treated rats will express CPP behavior and corresponding increase in striatal pERK, whereas antagonist pre-treated rats are not expected to develop cocaine CPP and are hypothesized to have pERK levels similar to saline-control rats.

Aim II: To test the hypothesis that increased pERK, pCREB, and FosB observed after cocaine-CPP expression are conditioned responses to the cocaine-associated context, rather than cocaine itself or withdrawal, we re-exposed cocaine-treated rats to an environment previously paired with cocaine or saline, drug-free after 8 days of cocaine-CPP training. Rats were conditioned with one of two doses of cocaine (5 or 20mg/kg) and reintroduced to either the cocaine-paired or saline-paired environment 24 hours after CPP testing and protein levels will be assessed through western blotting. We hypothesized that after CPP acquisition, rats expressing cocaine-CPP and reintroduced to the cocaine-paired environment would have increased phosphorylated ERK, CREB, and FosB protein levels compared to rats re-exposed to the context previously paired with saline. We also hypothesized that conditioned locomotor behavior will be observed in animals re-exposed to the cocaine paired chamber compared to cocaine-treated animals re-exposed to the saline paired chamber and that increases in protein levels would correlate to CPP behavior and protein levels.

Aim III: It is hypothesized that females will show enhanced cocaine CPP magnitude, locomotor responses to cocaine, and associated increases in intracellular signaling in reward, learning and memory brain areas after CPP expression, compared to males. To this end, male and female rats will be conditioned with the same dose of cocaine (20mg/kg) and protein expression will be measured immediately following the CPP test. It is hypothesized that males and females will both show increases in pERK and pCREB levels after cocaine CPP expression, but the cocaine-induced increases in protein levels will be greater in females.

## Chapter 2: Effects of NMDAR, SIRT1 and SIRT2 inhibition on the acquisition and expression cocaine-CPP

### I. Introduction

Learned cocaine-environment associations play a major role in cocaine addiction and relapse. Cocaine increases synaptic concentrations of DA and glutamate, leading to the activation of molecular signaling cascades that cause functional changes in protein and gene expression and drug induced behavior (Pierce et al., 1996; Reid & Berger 1996; Ritz et al., 1987; Smith et al., 1995; Uhl, et al., 2002). The striatum, made up of the NAc and CPu are key brain regions important for the regulation of associative reward learning and habitual responses. These brain regions undergo cocaine-induced neuroplastic changes in intracellular signaling similar to those underlying long-term memory processes For example, ERK, CREB and Fos proteins are signaling molecules that have been implicated in cocaine-reward and learning/memory processes

DAR activation is required for the glutamatergic induction of LTP at synapses in the striatum. The interaction between D<sub>1</sub>DARs and the NR1 subunit of the NMDDAR forms a complex at striatal synapses and serves to maintain and strengthen synaptic activity in response to changes in synaptic DA concentrations (Fiorentini et al.,2003). Downstream, ERK, a signaling molecule of the MAPK signal transduction family, is phosphorylated after acute cocaine administration (Corbille et al., 2007; Jenab et al., 2005; Sun et al., 2007; Valjent et al., 2000; 2005; Zhang et al., 2004). The ERK pathway is an important regulator of the transcription factor CREB phosphorylation and subsequent transcription (Xing et al., 1996) and much evidence supports a critical role of neuroadaptations produced by CREB and ERK signaling cascade in regulating synaptic plasticity through the alteration of gene activation (Berk et al., 1998; Hyman, 2005; Taylor et al., 2008; Wolf et al., 2002). CREB is thought to alter gene expression beginning with its phosphorylation at SER133 which causes CREs to bind to the promoter regions of other proteins and target genes thereby regulating synaptic plasticity and potentially learning and memory formation (Lonze & Ginty, 2002; Silva et al., 1998). Therefore, gene transcription and de novo protein synthesis regulated by both ERK and CREB activation may be an essential requirement for learning and the consolidation of new memories, including those associated with cocaine (Kuo et al., 2007; Taylor, et al., 2008).

For example, blockade of ERK activation (pharmacologically or genetically) prevents CPP to cocaine indicating that the ERK pathway may be an essential requirement for the development of cocaine-associated memories (Miller & Marshall 2005; Atkins et al., 1998; Valjent et al., 2000; Brambilla et al., 1997; Mazzucchelli et al., 2002; Ferguson et al., 2006). Increases in pCREB are observed in the NAc 10 minutes after one cocaine-place pairing, indicating a possible role for NAc pCREB in the initial memory consolidation process (Kuo et al., 2007). Miller and Marshall (2005) found that cocaine induced CPP produces increases in activation of ERK, CREB, Elk-1, and Fos in the NAc of male rats. Increases in pERK coincide with the synaptic plasticity that is observed through the increased functioning and sensitivity to glutamate in both AMPAR and NMDARs in DA neurons due to D<sub>1</sub>DAR activation by cocaine (Gao et al., 2006; Liu et al., 2006; Yang, 2000). NMDAR antagonism has been shown to block the acquisition of cocaine CPP (Cervo & Samanin, 1995).

Given the role of NMDARs in memory formation, we aimed to investigate changes in NMDAR-dependent intracellular signaling cascades associated with cocaine-context associations. Specifically, we used the non-competitive NMDAR antagonist MK-801 to block the acquisition of a cocaine-environment association using a CPP model. We hypothesized that ERK phosphorylation is dependent on glutamate signaling through NMDARs and that MK-801 administration prior to cocaine administration would block ERK phosphorylation. We also expected to see similar NMDAR/ERK-dependent changes in pCREB, FosB, and  $\Delta$ FosB protein levels.

## II. Methods

### Animals

Eight week old male Fischer rats (Charles River, Kingston, NY, USA) were individually housed in standard cages and maintained on a 12 hour light/dark cycle with ad libitum access to food and water. Rats were given 7 days before any experimental procedures began, and were handled once per day 4 days prior to any testing. Animal care and use was in accordance with

the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, Bethesda, MD, USA) and approved by the Hunter College, CUNY, Institutional Animal Care and Use Committee.

#### Drugs and Antibodies

Cocaine hydrochloride, MK-801, EX-527 (E7034), and AK-7 (SML0152) were purchased from Sigma Chemical Co. (St. Louis, MO). DMSO was the vehicle for the SIRT inhibitors and saline was the vehicle for cocaine and mk-801. Primary antibodies for pERK (9101), ERK (9102), SIRT1 (2493), SIRT2 (12672), FosB (5G4) and CREB (9197) were purchased from Cell Signaling Technologies (Beverly, MA). The primary antibody against pCREB (06-519) was purchased from Millipore (Billerica, MA, USA) and  $\alpha$ -tubulin (sc-8035) was purchased from Santa Cruz Technologies (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit (NA-934) and anti-mouse (NA-931) IgG were purchased from Amersham Pharmacia (Piscataway, NJ).

#### CPP Apparatus

The place preference apparatus (purchased from Med Associates, Georgia, VT) consisted of a rectangular cage with three chambers: 2 square conditioning chambers (28cm in length) separated by a neutral rectangular chamber (12cm long and 4cm wide). The two conditioning chambers were differentiated by tactile and visual cues; in one, the floor was a stainless steel mesh and the walls were white, and in the other, the floor was made up of a grid of stainless steel rods and the walls were black. The middle chamber had grey walls and a smooth PVC floor. The chambers were separated by computer-automated guillotine doors, allowing free access among all three chambers during pre-test and testing phases. Locomotion was measured using a computerized photo-beam system and MED-PC software, which recorded time spent in each chamber, total locomotor behavior (as defined as the sum of all horizontal counts), entrances into each chamber (defined as multiple beams broken between two chambers) and exploratory behavior (defined as a single broken beam between two chambers without entrance).

### CPP Procedure Experiment 1

During the preconditioning test, the guillotine doors were open and rats were placed into the neutral middle chamber and allowed to freely explore all three chambers for 15 minutes. Rats were randomly assigned to one of three treatment groups as follows: saline/saline, saline/cocaine, or mk-801/cocaine treatment group. Conditioning occurred over the next four days consisting of alternating drug/saline treatments on alternate days (2 cocaine/mk-801 treatments and 2 saline treatments on alternating days). On the first day of conditioning rats were pretreated with an i.p. injection of saline and received another saline injection 30 minutes later and were immediately confined to one of the conditioning chambers for 30 minutes. On the second day, rats (n = 9-10 animals/group) were pretreated with i.p. injections of saline (0.9%) or MK-801 (.25 mg/kg) followed 30 minutes later by an i.p. injection of saline or cocaine (20 mg/kg) and immediately confined for 30 minutes to the chamber opposite from conditioning day one (counterbalanced so that half of the rats received cocaine in black and saline and white and vice versa). Control rats received both saline pretreatment and saline again 30 minutes later and were confined to alternating chambers on alternating days. CPP testing was conducted in a drug-free state the day after the last conditioning session and followed the same procedure as the preconditioning test.

### CPP procedure Experiment 2

Following the pre-test, rats were randomly assigned to a DMSO/ saline control group (n = 6), or a DMSO/cocaine, EX-527/cocaine, or AK-7/cocaine treatment group (n = 8 per group). Conditioning followed the same procedures as described for experiment one, but instead of saline or MK-801 pre-treatment on days 2 and 4 of conditioning, rats were pre-treated with DMSO, EX-527 (10mg/kg) or AK-7 (10mg/kg) one hour before cocaine treatment and 30 minute confinement to one of the conditioning chambers.



## Protein preparation

Immediately after CPP testing (experiment 1) or 24 hours after the CPP test following drug-free forced re-exposure to the cocaine-chamber (experiment 2) rats were euthanized by rapid decapitation after a short CO<sub>2</sub> exposure. Brains were removed and flash frozen in 2methylbutane (-40 °C) and stored at -80 °C until used. Tissue punches of the NAc and CPu were dissected out of each brain on a cold glass plate. Tissue was homogenized with a Polytron handheld homogenizer (Kinematica, Luzern, Switzerland) in lysis buffer (50mM Tris-HCl, 150 mMNaCl, 2 mM EDTA, 10% Glycerol, 1% Triton X-100, 1% sodium deoxycholic acid) containing a phosphatase inhibitor cocktail. Homogenates were incubated for 30 minutes and centrifuged for 15 minutes (13,000 rpm, 4°C). Supernatants were collected and stored at -80°C until used for western blot analysis.

## Protein measurement and Western Blot analysis

The total protein content was found using a Bradford kit from Bio-Rad laboratories (Hercules, CA). 30-50ug of protein extracts were boiled for 5 minutes in Lammeli buffer with 1% Beta-mercapthoethanol, followed by electrophoresis onto 10% Tris-HCl SDS-PAGE gels and transfer onto PVDF membranes. Membranes were first blocked at room temperature with a solution of 5% nonfat dry milk in Tris-buffered saline with Tween-20 (TBST; pH = 7.4) for 1 hour. After 3 washes with TBST, membranes were incubated overnight at 4°C with the primary antibody for p-ERK, FosB, SIRT1, SIRT2, or p-CREB, (1:3000). Membranes were then washed with TBST three more times and incubated for 1 hour at room temperature with the appropriate secondary antibody (1:1000). After three more washes with TBST, a chemiluminescence kit (ECL; Amersham Pharmacia, Piscataway, NJ) was used and the membranes were exposed to x-ray film to detect antibody binding. All membranes were re-probed with the antibody for  $\alpha$ -tubulin. Phosphorylated proteins were also re-probed for their respective total protein in order to normalize the protein levels. Films were scanned and analyzed with ImageJ (NIH).

## Data analysis

CPP scores (time spent in the cocaine paired chamber minus the time spent in the saline paired chamber) and total locomotor responses during the CPP test were analyzed with one way ANOVAs. Paired samples t-tests were used to test for differences in time spent, explorations, and entrances into the cocaine and saline paired chambers during the CPP test. Locomotor behavior during conditioning sessions was analyzed with a mixed two way ANOVA (conditioning day x treatment). Western blot data were converted to a ratio of specific protein levels to total protein levels or  $\alpha$ -tubulin using arbitrary densitometric units, expressed as a percentage of saline controls, and analyzed using one-way ANOVAs. Statistical significance was determined at  $p < 0.05$ . LSD post hoc analysis was used following all significant one-way ANOVAs.

## III. Results

### CPP and cocaine induced locomotor behavior after cocaine and MK801 treatments

No differences were seen in time spent in either chamber or total locomotor activity during the preconditioning test (not shown). Cocaine-treated rats spent significantly more time in the cocaine paired chamber than the saline paired chamber during CPP testing [ $t(8) = 3.52$ ,  $p < 0.01$ ; Figure 8A]. No difference in time spent in either chamber in saline controls or rats pre-treated with MK-801 [ $t(9) = 0.55$ ,  $p = 0.96$  and  $t(8) = 0.16$ ,  $p = 0.88$ , respectively; Figure 8A]. Likewise, cocaine-treated rats had significantly higher CPP scores than MK-801 pretreated or saline treated rats [ $F(2,27) = 3.59$ ,  $p < 0.05$ ; Figure 8B]. Cocaine-treated rats explored the cocaine-paired chamber significantly more than the saline chamber, while no differences were observed between any other experimental group [ $F(2,27) = 8.00$ ,  $p < 0.05$ ; Table 1].

A significant interaction effect of treatment and conditioning day showed that although cocaine increased locomotor responses during conditioning regardless of pre-treatment compared to saline controls, MK-801 pretreatment significantly increased cocaine-induced

locomotor responses [ $F(6,81) = 9.53$ ,  $p < 0.01$ ; Figure 9A]. Total locomotor behavior during the CPP test did not differ based on treatment [Figure 9B].

#### pERK 1/2, pCREB and FosB/ $\Delta$ FosB protein levels after cocaine and MK801 pre-treatments

No changes were seen in total ERK or CREB protein levels in any brain area examined [not shown]. A one-way ANOVA revealed that cocaine only treated rats (saline pretreatment) had significantly higher NAc pERK levels after CPP expression than saline controls and MK-801 pretreated rats [ $F(2,15) = 12.58$ ,  $p < 0.01$ ]. MK-801 pretreatment before cocaine injections during conditioning significantly reduced the cocaine-induced increase in NAc pERK levels after CPP expression [ $p < 0.05$  for all comparisons; Figure 10A]. NAc pERK levels were significantly correlated to CPP scores, [ $r = 0.64$ ,  $p < 0.05$ ; Figure 10A]. A one-way ANOVA revealed a significant effect of treatment on NAc pCREB levels [ $F(2,15) = 4.02$ ,  $p < 0.05$ ; Figure 10B]. Cocaine treated rats had significantly higher NAc pCREB levels than saline controls and MK-801 pretreated rats [ $p < 0.05$  for all comparisons]. No changes were seen in CPu pERK or pCREB levels (Figure 11A and 11B respectively). Neither CPu pERK or pCREB were correlated to CPP scores (Figure 11A and B).

Cocaine did not significantly increase NAc FosB levels compared to saline control rats [FosB:  $F(2,13) = 3.40$ ,  $p = 0.07$ ; Figure 12A]. NAc  $\Delta$ FosB were significantly increased after cocaine treatment regardless of pre-treatment [ $F(2,15) = 16.40$ ,  $p < 0.05$ ; Figure 12A]. Correlations between NAc FosB and  $\Delta$ FosB levels and CPP scores failed to reach significance, [ $r = 0.53$ ,  $p = 0.07$ ;  $r = 0.50$ ,  $p = 0.08$ , Figure 12B and C]. However, cocaine increased CPu FosB and  $\Delta$ FosB levels and MK-801 pretreatment significantly reduced these increases [FosB:  $F(2,15) = 11.10$ ,  $p < 0.01$ ;  $\Delta$ FosB:  $F(2,15) = 15.11$ ,  $p < 0.01$ ; Figure 13A]. CPu FosB and  $\Delta$ FosB were significantly correlated to CPP scores, [ $r = 0.64$ ,  $p < 0.05$ ;  $r = 0.73$ ,  $p < 0.05$ , Figure 13B and C].

## SIRT1 and SIRT2 protein levels after cocaine and MK-801 pre-treatments

A significant one-way ANOVA revealed that NAc SIRT1 levels were higher in MK-801 pretreated rats compared to saline controls [ $F(2,14) = 5.40$ ,  $p < 0.05$ ; Figure 14A]. NAc SIRT2 levels remained unchanged based on treatment (Figure 14B). No changes were seen in CPU SIRT1 (Figure 15A). Whereas cocaine induced increases in CPU SIRT2 levels and the increase was reduced by MK-801 pretreatment [ $F(2,15) = 10.71$ ,  $p < 0.01$ ; Figure 15A]. CPU SIRT2 protein levels were also significantly correlated to CPP scores [ $r = 0.60$ ,  $p < 0.05$ ; Figure 15B].

## CPP and cocaine induced locomotor behavior after cocaine and EX-527 and AK-7 treatments

No differences were seen in time spent in either chamber or total locomotor activity during the preconditioning test (not shown). Cocaine-treated rats spent significantly more time in the cocaine paired chamber than the saline paired chamber during CPP testing [ $t(7) = 2.70$ ,  $p < 0.05$ ; Figure 16A]. No difference in time spent in either chamber in saline controls [ $t(5) = 0.03$ ,  $p = 0.98$ ], rats pre-treated with EX-527 [ $t(7) = 1.20$ ,  $p = 0.30$ ] or AK-7, [ $t(7) = 1.50$ ,  $p = 0.20$ ; Figure 16A]. DMSO/Cocaine-treated rats had significantly higher CPP scores than saline treated rats [ $F(3,24) = 3.21$ ,  $p < 0.05$ ; Figure 16B).

A significant interaction effect of treatment and conditioning day showed that although cocaine increased locomotor responses during conditioning regardless of pre-treatment compared to saline controls, EX-527 pretreatment significantly increased cocaine-induced locomotor responses on conditioning day four (the second cocaine treatment) [ $F(9,78) = 3.05$ ,  $p < 0.01$ ; Figure 17A]. Total locomotor responses during the CPP test did not differ based on treatment (Figure 17B).

pERK 1/2, pCREB and FosB/ $\Delta$ FosB protein levels after cocaine and EX-527 or AK-7 pre-treatments

We found a significant effect of treatment on NAc pERK levels [ $F(3) = 8.01, p < 0.05$ ], where NAc pERK protein levels were significantly lower in rats pre-treated with EX-527 compared to DMSO/Cocaine ( $p < 0.05$  for all comparisons; Figure 18A). CPU pERK levels did not change based on treatment [ $F(3) = 1.50, p = 0.09$ ; Figure 18B]. NAc FosB and  $\Delta$ FosB levels were increased after cocaine-treatment [FosB:  $F(3, 13) = 5.20, p < 0.05$ ;  $\Delta$ FosB:  $F(3, 13) = 4.82, p < 0.05$ ]. AK-7 pre-treatment attenuated this effect; AK-7/cocaine rats had lower FosB and  $\Delta$ FosB levels than DMSO/cocaine and EX-527/cocaine rats ( $p < 0.05$ ; Figure 19A and B). CPU FosB levels were increased after cocaine-treatment [ $F(3, 13) = 5.90, p < 0.05$ ] and pre-treatment with EX-527 and AK-7 potentiated this effect [ $p < 0.05$ ; Figure 20A]. CPU  $\Delta$ FosB levels were increased after cocaine regardless of pre-treatment [ $F(3, 13) = 6.13, p < 0.05$ ; Figure 20B]. NAc SIRT1 or SIRT2 protein levels did not differ based on treatment (Figure 21A and 21B). CPU SIRT1 levels were reduced after cocaine-treatment (DMSO/cocaine) and this reduction was not observed after pre-treatment with EX-527 or AK-7 [ $F(3, 13) = 4.83, p < 0.05$ ; Figure 22A]. CPU SIRT2 levels did not differ based on treatment (Figure 22B)

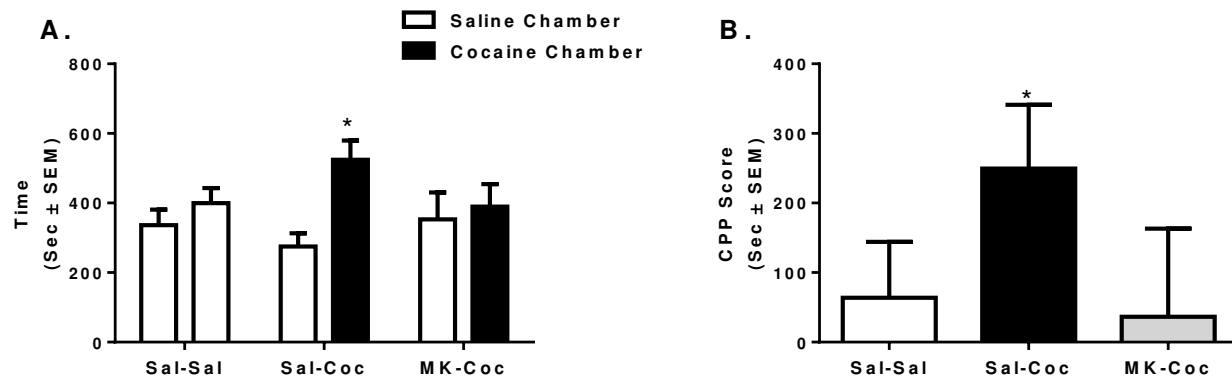


Figure 8: Effect of MK-801 pretreatment on cocaine-induced CPP acquisition. (A) Average time spent in the saline and cocaine paired chambers during the CPP test. (B) CPP scores: time spent in the cocaine paired chamber minus time spent in the saline paired chamber (in seconds  $\pm$  SEM) ( $n = 9-10$  animals per group). \* Indicates statistically significant differences at  $p < 0.05$ .

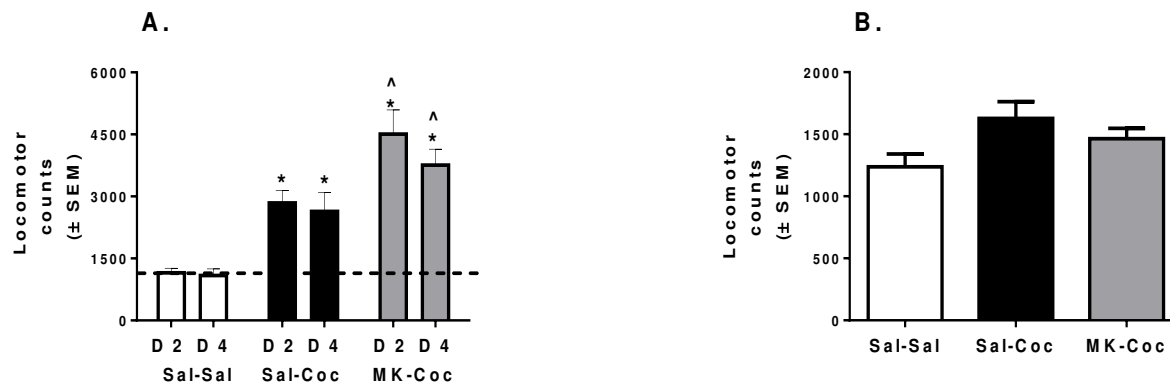


Figure 9: Effect of cocaine and MK-801 pre-treatment on locomotor responses. (A) Total locomotor responses during cocaine (days 2 and 4) or saline conditioning days did not differ across all treatment groups. Dotted line represents average of locomotor activity during saline treatment days (days 1 and 3). (B) Total locomotor responses during the CPP test (drug-free). \* Significant differences from saline controls at  $p < 0.05$ . <sup>^</sup>Significant differences from cocaine only treated rats (Sal-Coc) at  $p < 0.05$ .

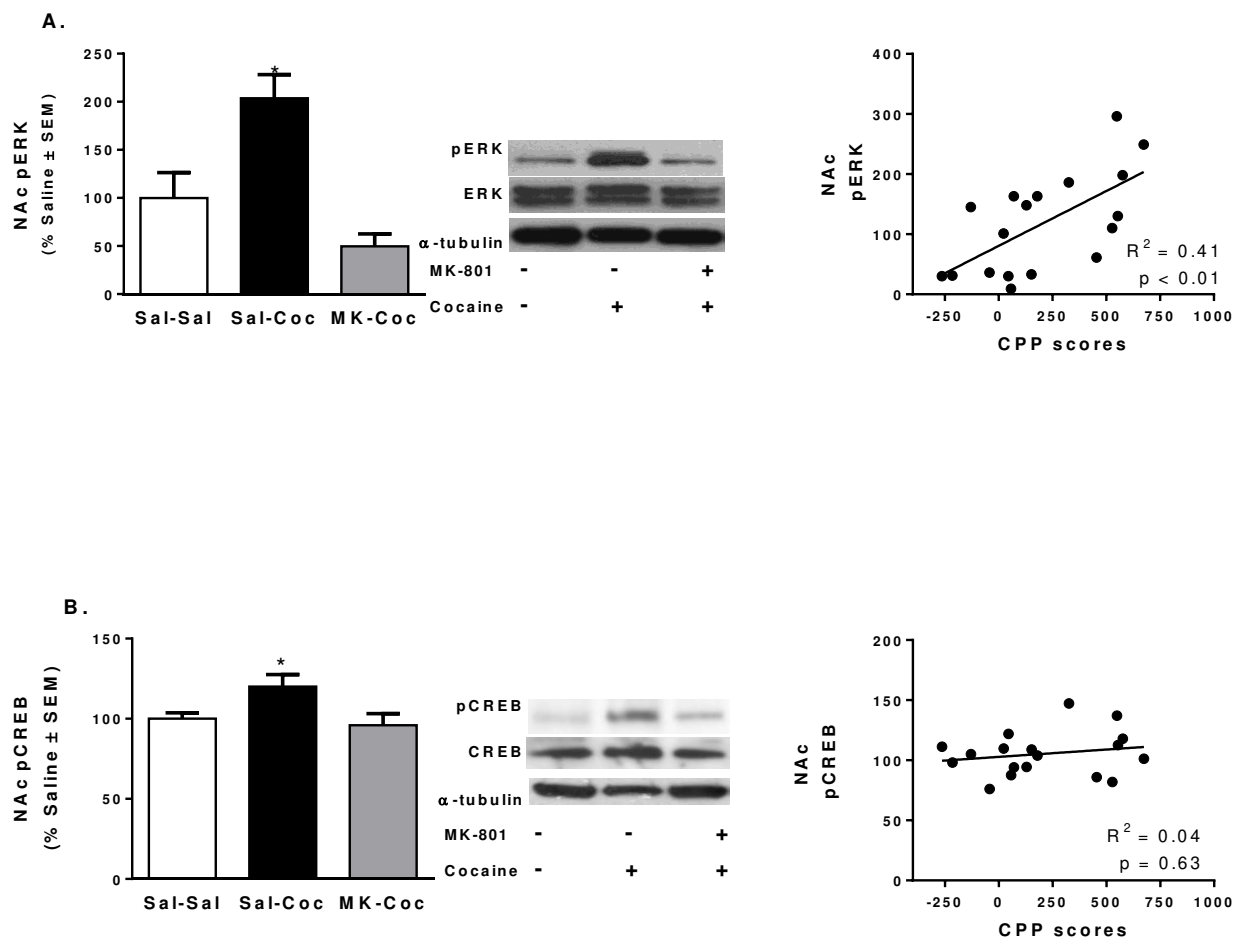


Figure 10: NAc Phosphorylated ERK 1/2 (44/42 kDa) (A: top panel) and phosphorylated CREB (B: bottom panel) protein levels and correlation to CPP scores. Phosphorylated protein levels are expressed as a ratio to their respective total protein levels and expressed as percentage of saline controls (n = 6 per group). \*Significant difference at  $p < 0.05$ .



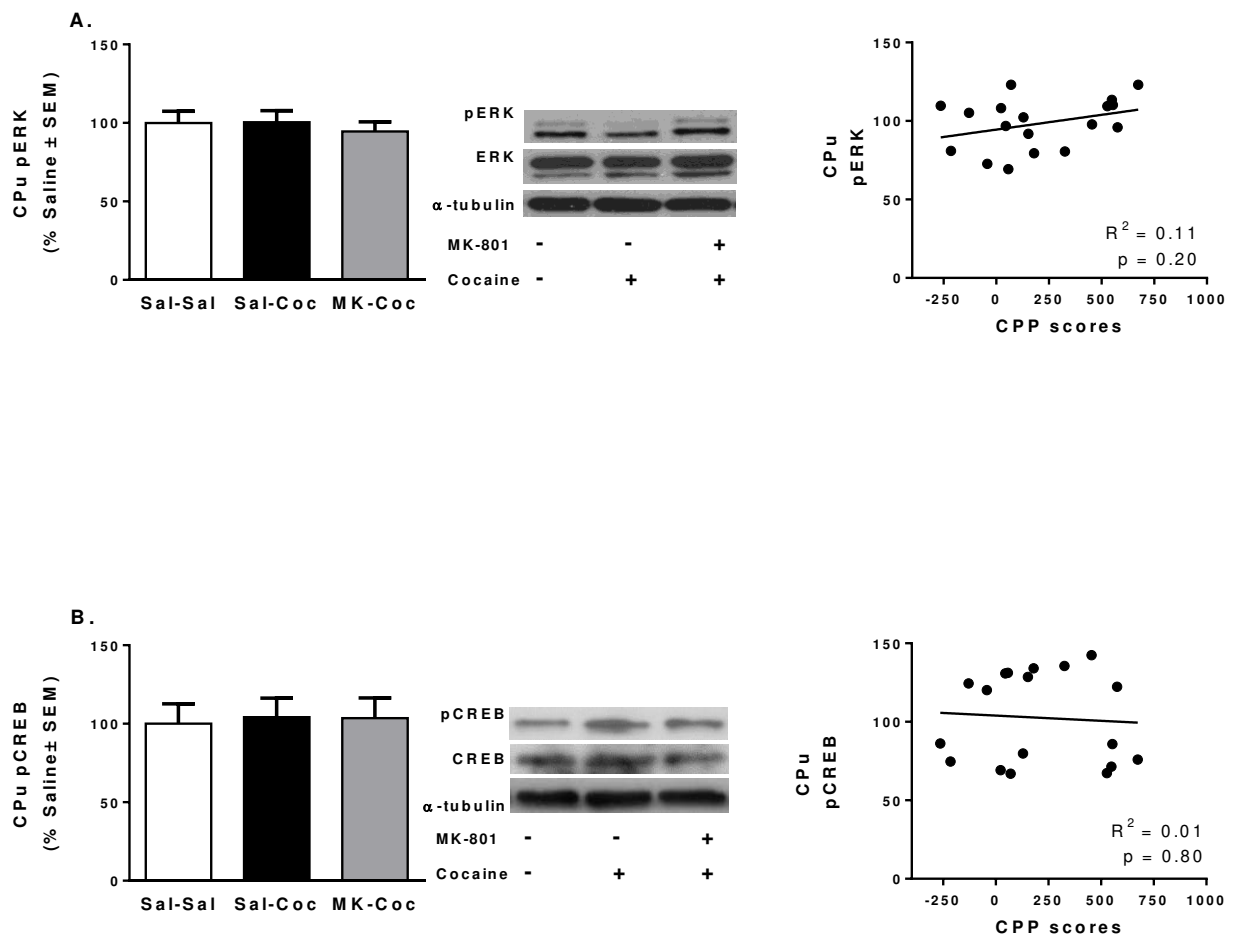


Figure 11: CPu Phosphorylated ERK 1/2 (44/42 kDa) (A: top panel) and phosphorylated CREB (B: bottom panel) protein levels and correlation to CPP scores. Phosphorylated protein levels are expressed as a ratio to their respective total protein levels and expressed as percentage of saline controls ( $n = 6$  per group). \*Significant difference at  $p < 0.05$ .

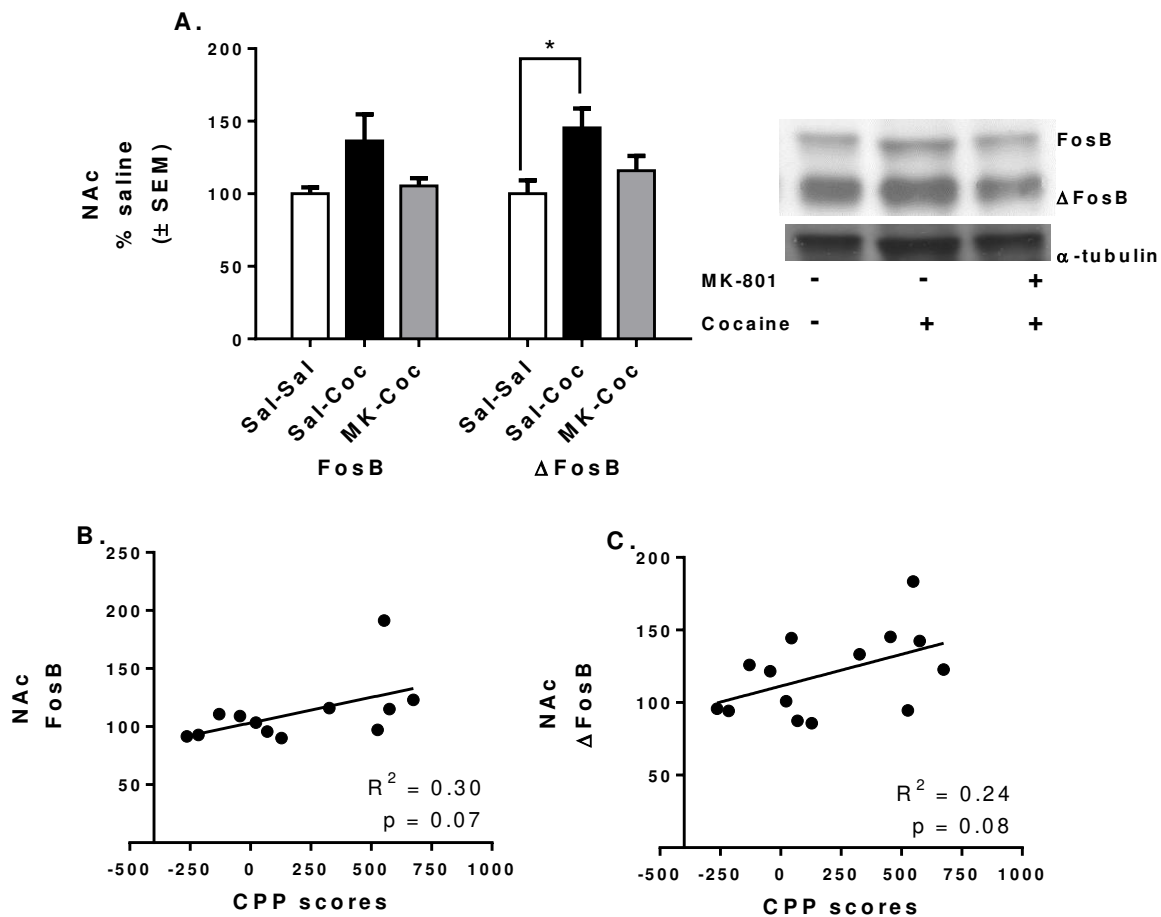


Figure 12: NAc FosB (left 3 bars, top band: 48 kDa) and  $\Delta$ FosB (right 3 bars, bottom band: 38 kDa) protein levels (A) and correlation to CPP scores (FosB:B and  $\Delta$ FosB:C).. Protein levels are expressed as a ratio to their respective  $\alpha$  tubulin levels (55 kDa) ( $\pm$ SEM) ( $n = 6$  animals per group). \*Significant difference at  $p < 0.05$ .

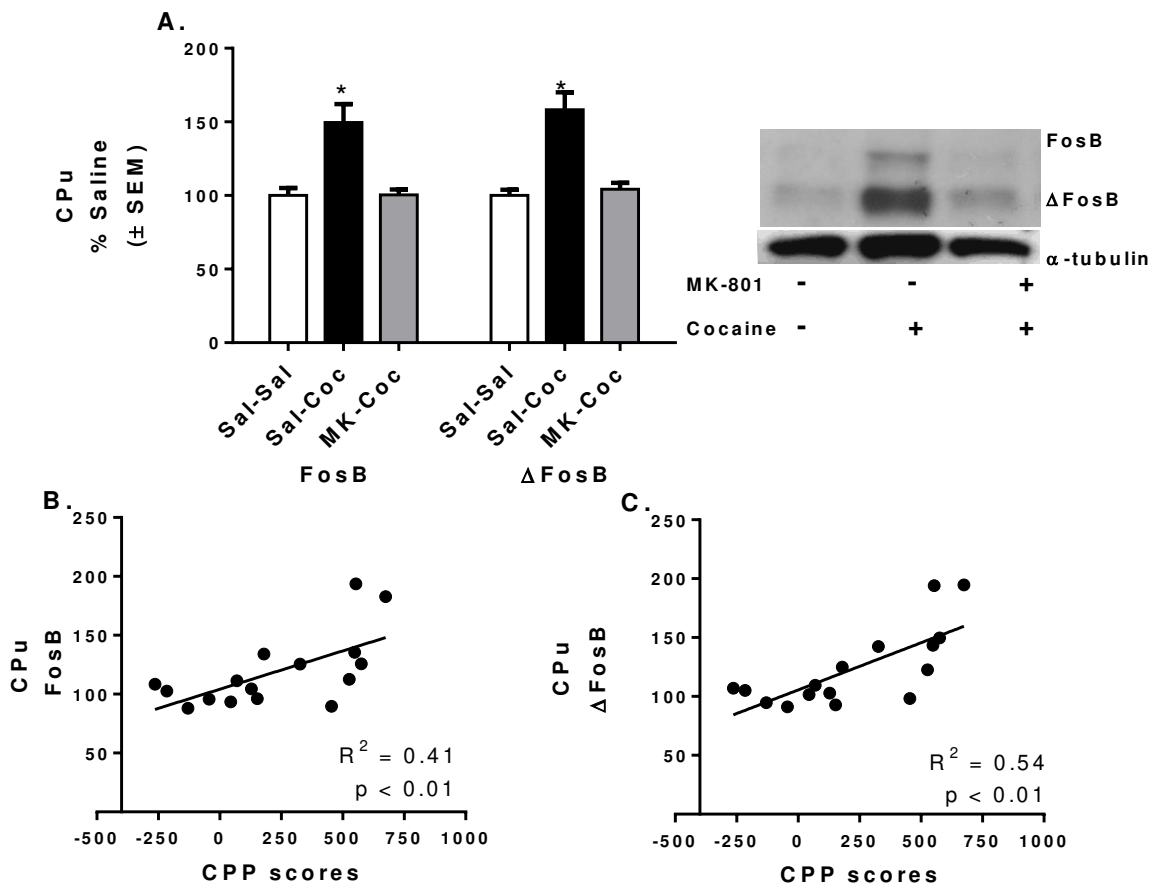


Figure 13: CPU. FosB (left 3 bars, top band: 48 kDa) and  $\Delta$ FosB (right 3 bars, bottom band: 38 kDa) protein levels (A) and correlation to CPP scores (FosB:B and  $\Delta$ FosB:C).. Protein levels are expressed as a ratio to their respective  $\alpha$  tubulin levels (55 kDa) ( $\pm$ SEM) ( $n = 6$  animals per group). \*Significant difference at  $p < 0.05$ .

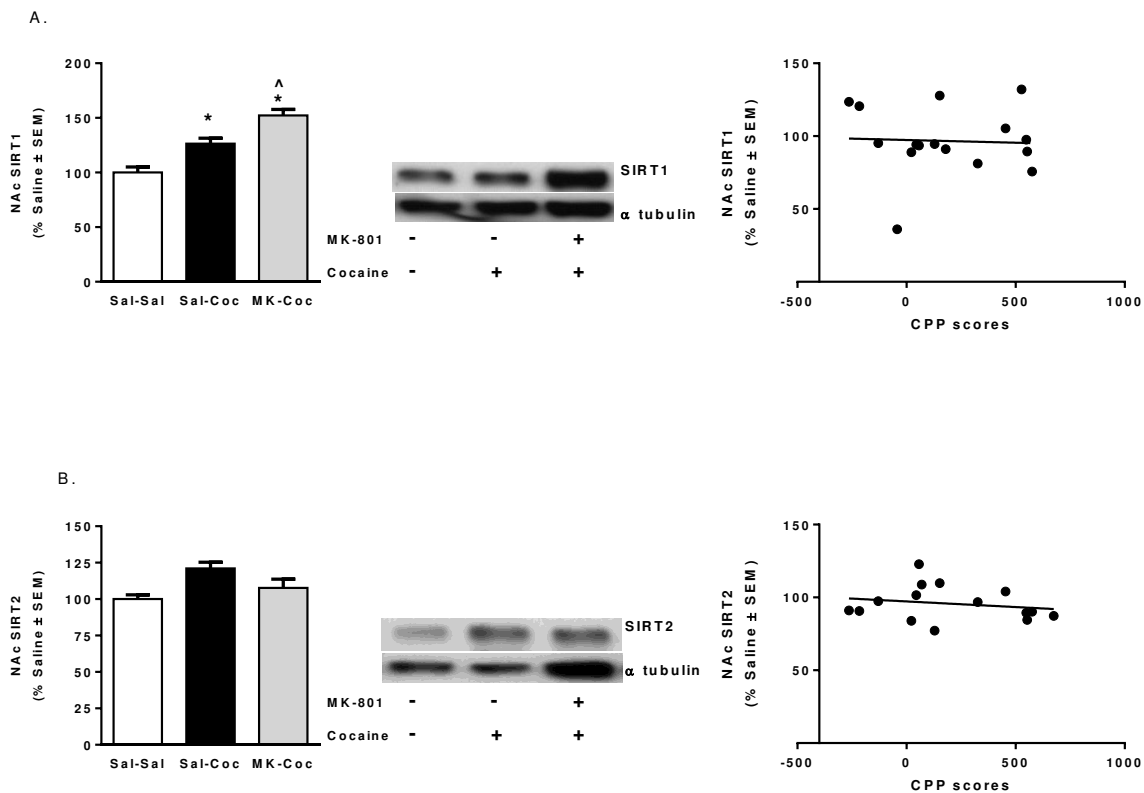


Figure 14: NAc SIRT1 (120kDa) (A) and SIRT2 (43kDa) (B) protein levels and correlation to CPP scores (B & D). Protein levels are expressed as a ratio to their respective  $\alpha$  tubulin levels (55 kDa) ( $\pm$ SEM) (n = 4-5 animals per group). \*Significant difference at p < 0.05. ^Significant differences from cocaine only treated rats (Sal-Coc) at p < 0.05.

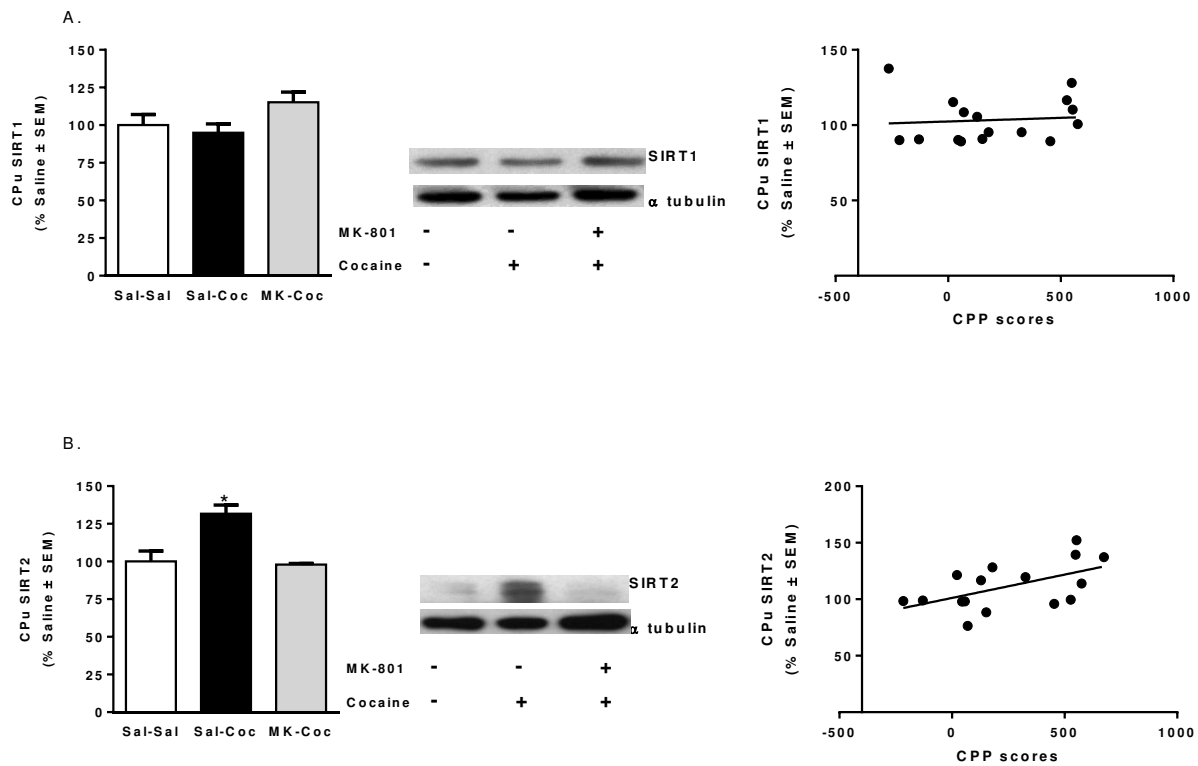


Figure 15: CPu SIRT1 (120kda) (A) and SIRT2 (43kda) (B) protein levels and correlation to CPP scores. Protein levels are expressed as a ratio to their respective  $\alpha$  tubulin levels (55 kDa) ( $\pm$ SEM) (n = 4-5 animals per group). \*Significant difference at p < 0.05. ^Significant differences from cocaine only treated rats (Sal-Coc) at p < 0.05.

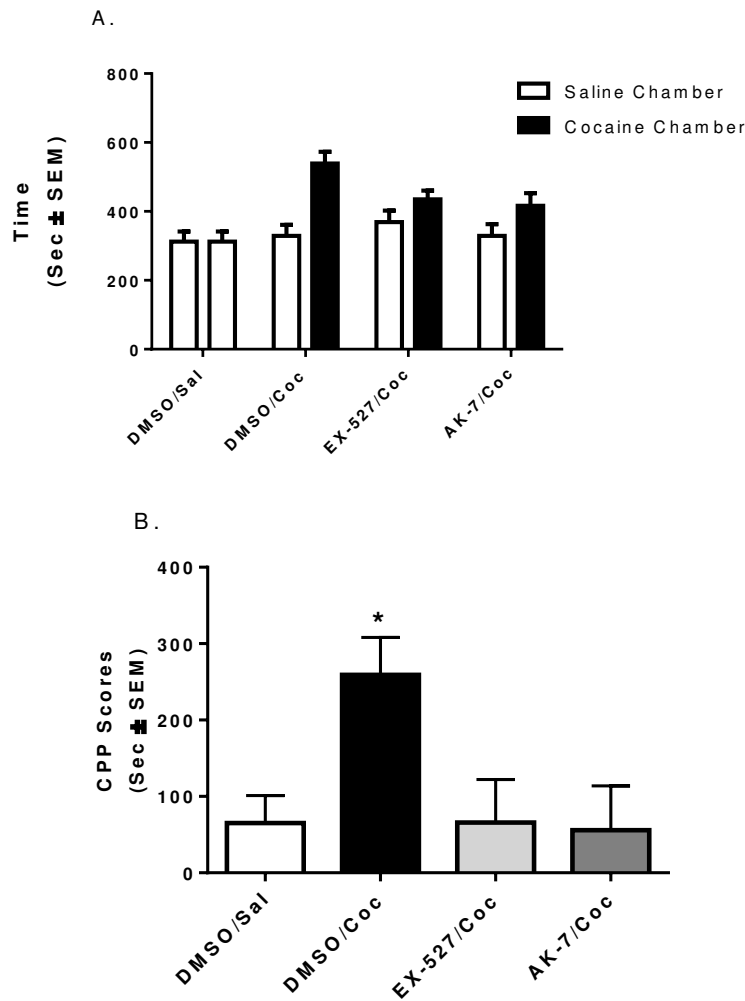


Figure 16: Effect of EX-527 and AK-7 pre-treatment on cocaine-induced CPP acquisition. (A) Average time spent in the saline and cocaine paired chambers during the CPP test. (B) CPP scores: time spent in the cocaine paired chamber minus time spent in the saline paired chamber (in seconds  $\pm$  SEM) ( $n = 9-10$  animals per group). \*Significant difference at  $p < 0.05$ .

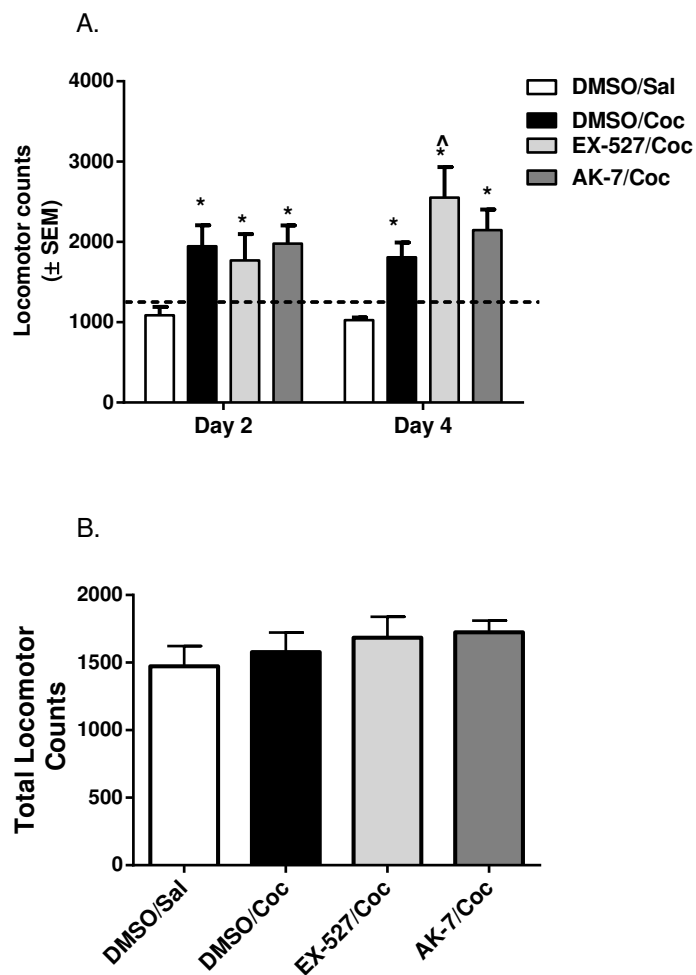


Figure 17: Effect of cocaine and EX-527 or AK-7 pre-treatment on locomotor responses. (A) Total locomotor responses during cocaine (days 2 and 4) conditioning days did not differ across all treatment groups. Dotted line represents average of locomotor activity during saline treatment days (days 1 and 3). (B) Total locomotor responses during the CPP test (drug-free). \*Significant difference at  $p < 0.05$ . <sup>^</sup>Significant differences from cocaine only treated rats (DMSO- Coc) at  $p < 0.05$ .

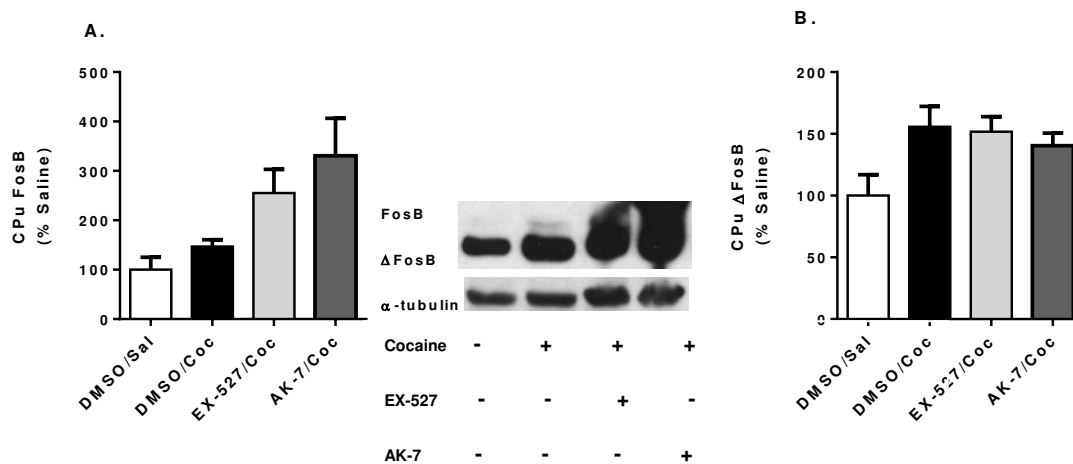


Figure 18. Phosphorylated ERK 1/2 (44/42 kDa) protein levels in the NAc (A), and CPu (B) after CPP testing. Phosphorylated protein levels are expressed as a ratio to their respective total protein levels and expressed as percentage of saline controls (n = 6 per group). \*Indicates statistically significant differences from saline controls at  $p < 0.05$ . ^Indicates statistically significant difference from DMSO/Cocaine treatment group at  $p < 0.05$ .



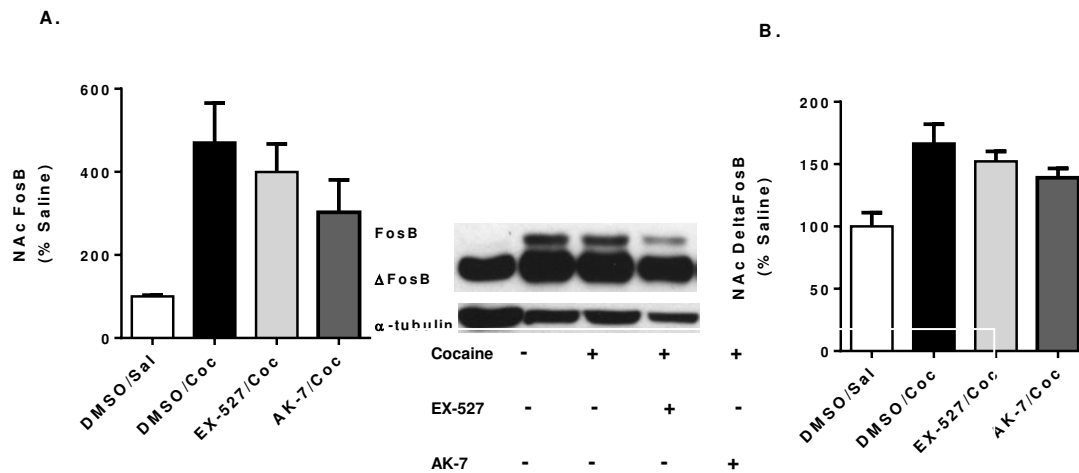


Figure 19: NAc FosB (A: top band: 48 kDa) and ΔFosB (B: bottom band: 38 kDa) protein levels after CPP testing.. Protein levels are expressed as a ratio to their respective α tubulin levels (55 kDa) ( $\pm$ SEM) ( $n = 4$  animals per group). \*Indicates statistically significant differences at  $p < 0.05$ . ^Indicates statistically significant difference from DMSO/Cocaine treatment group at  $p < 0.05$ .

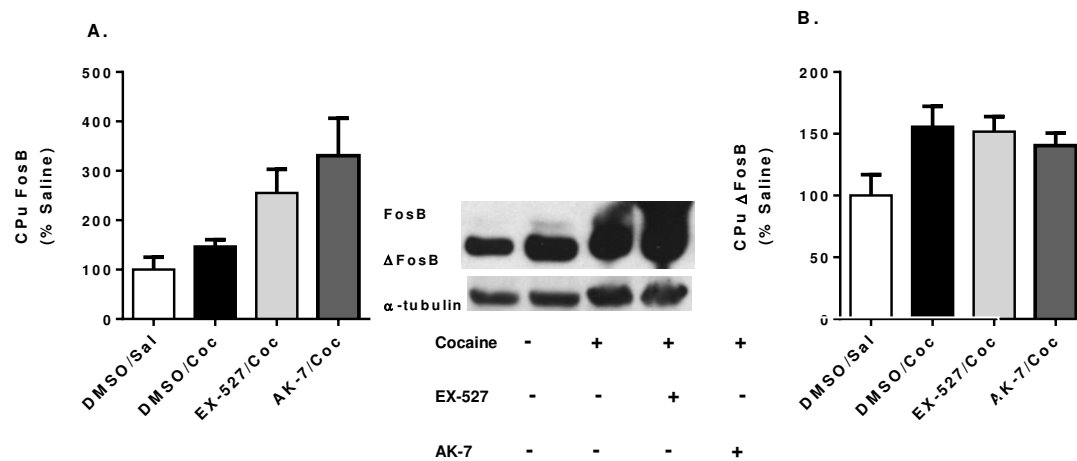


Figure 20: CPU FosB (A: top band: 48 kDa) and  $\Delta$ FosB (B: bottom band: 38 kDa) protein levels, of rats after CPP testing. Protein levels are expressed as a ratio to their respective  $\alpha$  tubulin levels (55 kDa) ( $\pm$ SEM) ( $n = 4$  animals per group). \*Indicates statistically significant differences at  $p < 0.05$ . ^Indicates statistically significant difference from DMSO/Cocaine treatment group at  $p < 0.05$ .

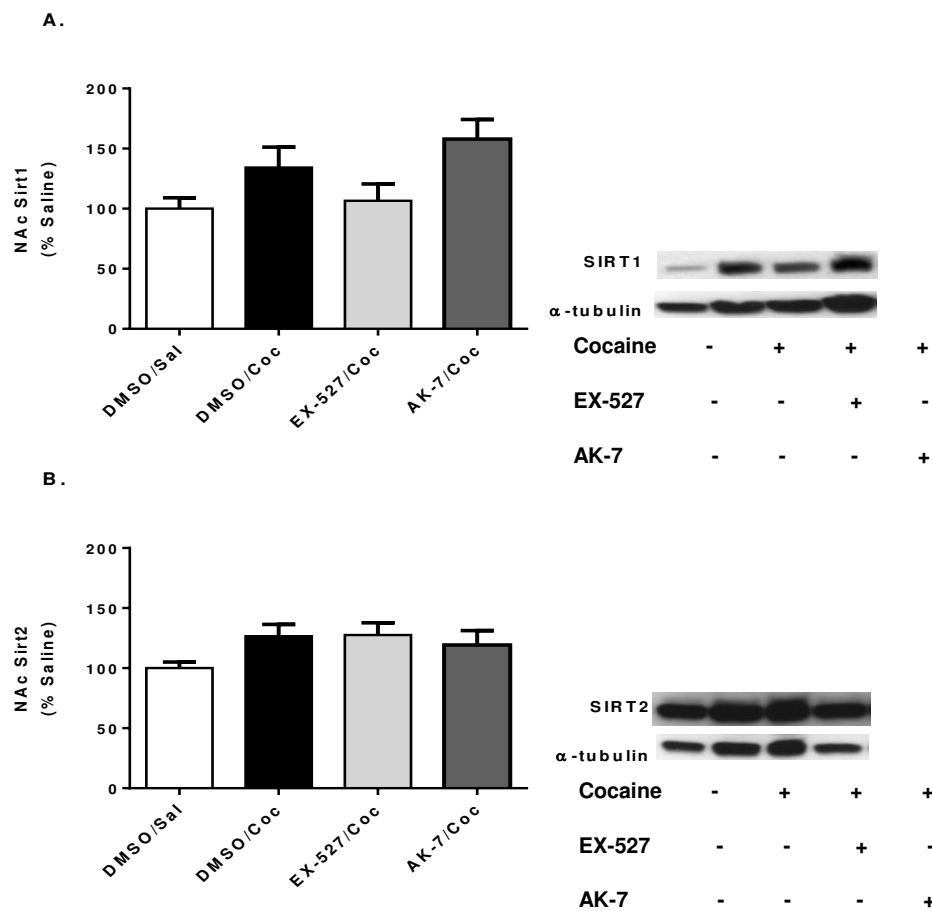


Figure 21: NAc SIRT1 (120kda) (A) and SIRT2 (43kda) (B) protein levels after CPP testing. Protein levels are expressed as a ratio to their respective  $\alpha$  tubulin levels (55 kDa) ( $\pm$ SEM) (n = 4-5 animals per group). \*Indicates statistically significant differences at  $p < 0.05$ . ^Indicates significant differences from DMSO/Cocaine group at  $p < 0.05$ .

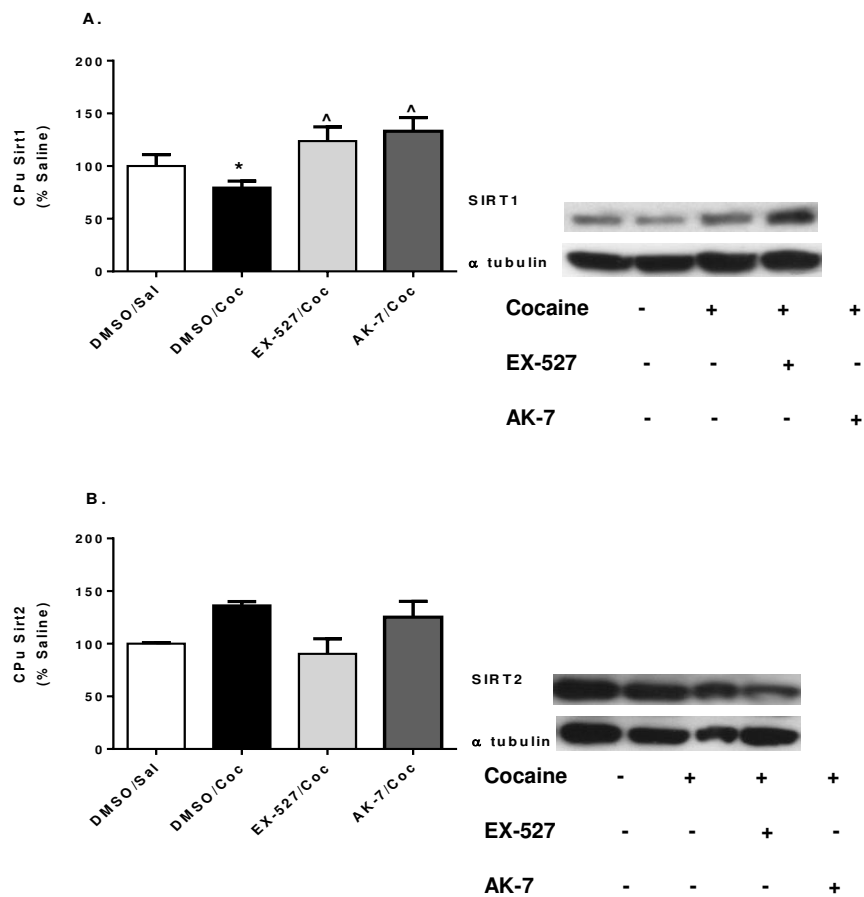


Figure 22: CPU SIRT1 (120kDa) and SIRT2 (43kDa) protein levels in the NAc (A), and CPU (B) of rats after CPP testing. Protein levels are expressed as a ratio to their respective  $\alpha$  tubulin levels (55 kDa) ( $\pm$ SEM) ( $n = 4-5$  animals per group). \*Indicates statistically significant differences at  $p < 0.05$ . ^Indicates significant differences from DMSO/Cocaine group at  $p < 0.05$ .

Table 1

## Cocaine effects on entrances and explorations during CPP test

	Explorations		Entrances	
	Sal-chamber	Coc-chamber	Sal-chamber	Coc-chamber
Sal-Sal	55.88 ± 26.23	45.88 ± 19.71	58.77 ± 27.83	58.77 ± 22.02
Sal-Coc	58.10 ± 23.90	83.40 ± 23.40*	64.40 ± 31.28	70.90 ± 23.90
MK-Coc	61.88 ± 27.03	62.00 ± 17.60	65.00 ± 30.39	49.00 ± 11.92

Data are shown as mean ( $\pm$ SEM) number of entrances and explorations while in the saline-paired and cocaine-paired chambers during CPP testing (n = 9-10 animals per group). \*Significant difference from saline-paired chamber at  $p < .05$ .

#### IV. Discussion

As predicted, MK-801 blocked both the acquisition of cocaine CPP and the increase in NAc pERK and pCREB levels. Consistent with previous research, these data indicate that NAc ERK phosphorylation via NMDARs may be necessary for the acquisition and expression of cocaine-induced CPP and the retrieval of cocaine-associated memories. Since pERK levels were significantly correlated to CPP scores and pCREB levels were not, may indicate differential roles for NMDAR dependent NAc pERK and pCREB signaling. NAc CREB phosphorylation may be involved with the retrieval of cocaine-associated memories and NAc ERK phosphorylation may be involved in the rewarding aspect and thus motivational properties leading to the formation of cocaine-environment associations.

ERK phosphorylation is associated with the rewarding effects of cocaine and inhibition of the ERK pathway prevents CPP to cocaine indicating that this pathway may be an essential requirement for the conversion of short term memory to long term memory (Atkins et al, 1998; Brambilla et al 1997; Ferguson et al 2006; Valjent et al 2000). Similar to our results, Miller and Marshall (2005) blocked both cocaine-CPP retrieval and ERK activation by infusing a MEK inhibitor directly into the NAc two and 15 days after an initial preference test. NAc ERK phosphorylation seems to be involved in mediating cocaine-reward as well as reward-associated memory formation and retrieval. It is also evident that NAc ERK activation in response to cocaine-environment exposure may be dependent on the activation of NMDARs that occurs due to the associations that were made between the environment and rewarding properties of cocaine.

NAc pCREB levels were not correlated to CPP scores. Consistent with our results, mutant mice overexpressing a dominant negative version of CREB in striatum (thereby inhibiting CREB activation selectively in the striatum) exhibit increased sensitivity to cocaine induced CPP suggesting that NAc ERK-dependent CREB phosphorylation may not be associated with CPP behaviors (Fasano, et al 2009). As stated previously, the activation of D1 DARs activates PKA which in turn activates DARP-32 at Thr34. PP1 is then activated, releasing its inhibition of CREB, thus allowing for CREB phosphorylation at Ser133 (Hemmings et al., 1984a; Hemmings, et al, 1984b). In the NAc, pCREB achieved through the activation of D1Rs may be associated with both

the formation and retrieval of cocaine associated memories and can explain why CREB activity in the NAc was not changed in response to NMDAR antagonism. Inhibiting PKA also blocks the expression of cocaine induced CPP if administered prior to, but not after each cocaine-place pairing suggesting that PKA may be transiently activated during the CPP acquisition phase, and may aid in the initiation of the molecular cascade underlying the environment-cocaine reward memory consolidation process (Cervo, et al 1997). Future research should aim to use D1R and PKA antagonism during CPP training and testing in order to more clearly elucidate the role of CREB in mediating cocaine-CPP. Inhibition of D1Rs and/or PKA should result in the blockade of both CPP behaviors and NAc CREB phosphorylation, whereas NAc ERK activity should remain unaffected.

CPu pERK and pCREB levels were unaffected by NMDAR antagonism which may be a temporal issue. Exposure to the cocaine-context during the CPP test may induce the phosphorylation of these proteins at a time point prior to that measured in this study. Whether or not the protein levels measured here are working as part of the same pathway is still unclear. However, it seems that NAc ERK and CREB phosphorylation and CPu FosB accumulation after exposure to a cocaine-associated environment is dependent on NMDAR signaling during cocaine exposure. NAc and CPu appear to differentially regulate  $\Delta$ FosB accumulation, where in the CPu  $\Delta$ FosB protein level increase after cocaine was blocked by NMDAR antagonism while NAc cocaine-induced  $\Delta$ FosB levels were not NMDAR dependent. This agrees with recent research suggesting that  $\Delta$ FosB differentially affects the inducibility of FosB gene expression in the NAc and CPu (Damez-Werno et al., 2012).

### Chapter 3: Differential intracellular signaling in the Nucleus Accumbens and Caudate Putamen during retrieval of a cocaine context association.

#### I. Introduction

Cocaine-cue induced “cravings” are a major cause of relapse in cocaine addicts. Relapse is particularly common when preceded by drug-associated stimuli; stimuli (including but not limited to, paraphernalia, persons and/or environments) associated with the rewarding effects produced by drugs of abuse (Hyman, 2005; Nestler, 2002). Drug reward increases the motivational valence of stimuli in the environment that through Pavlovian learning mechanisms, become conditioned stimuli that directly motivate behavior in the absence of the original unconditioned stimulus (Cardinal & Everitt, 2004; Kelley, 2004). Understanding the role of drug-induced neuroplastic changes in intracellular signaling underlying these associations has important implications for relapse prevention and treatment (Milton, 2012). Recent research has shown that the motivational and associative components of cocaine-environment associations may be dissociable and are mediated by different brain regions (Theberge et al., 2010; Wells et al., 2013). Disrupting reconsolidation of the motivational component may be helpful for the development of new treatments (Tronson & Taylor, 2013).

The striatum, made up of the NAc and CPU is a key brain region important for the regulation of reward learning and habitual responses (Wickens et al., 2007). Cocaine produces neuroplastic changes in intracellular signaling in these regions known to underlie long-term memory processes (Madsen et al., 2012). Acute cocaine increases NAc and CPU Fos protein expression, ERK and CREB phosphorylation and chronic cocaine induces persistent increases in NAc and CPU  $\Delta$ FosB (Jenab et al., 2005; Sun et al., 2007; Nestler, 2005). pERK is required for associative learning and ERK inhibition (pharmacologically or genetically) prevents cocaine CPP, indicating that the ERK pathway may be an essential requirement for the development of cocaine-associated memories (Atkins et al., 1998; Brambilla et al., 1997; Ferguson et al., 2006; Miller & Marshall, 2005; Valjent et al., 2000).



CPP models allow for direct exploration of the underlying maladaptive changes in the signal transduction pathways that occur after exposure to an environment previously paired with cocaine (Taylor et al., 2008). To test whether the intracellular responses measured after cocaine-CPP expression are conditioned responses to the cocaine-associated context, rather than cocaine itself or withdrawal, we re-exposed cocaine-treated rats to an environment previously paired with cocaine or saline, drug-free after 8 days of cocaine-CPP training. Rats were conditioned with one of two doses of cocaine (5 or 20mg/kg) and reintroduced to either the cocaine-paired or saline-paired environment 24 hours after CPP testing. We hypothesized that after CPP acquisition, rats expressing cocaine-CPP and reintroduced to the cocaine-paired environment would have increased phosphorylated ERK, CREB, and FosB protein levels compared to rats re-exposed to the context previously paired with saline.

## II. Methods

### Animals

Individually housed eight week old male Fischer rats (Charles River, Kingston, NY, USA) with free access to food and water were kept on a 12 hour light/dark cycle. Animal care was in adherence with the guide for the care and use of laboratory animals and approval was obtained by the Institutional Animal Care and Use committee at Hunter College.

### CPP Procedure

An unbiased and counterbalanced CPP procedure using a 3-chamber conditioning apparatus as previously described was used. During the preconditioning test, the guillotine doors were open and rats were placed into the neutral middle chamber and allowed to freely explore all three chambers for 20 minutes. Rats were then randomly assigned to one of two groups to be conditioned with 5mg/kg or 20mg/kg cocaine. Conditioning consisted of alternating saline/cocaine treatments over the next eight days. On days 1, 3, 5 and 7 of conditioning, rats were administered an intra-peritoneal (i.p.) injection of saline and immediately confined to one of the conditioning chambers for 30 minutes. On days 2, 4, 6, and 8, rats were treated with cocaine and immediately

confined to the opposite chamber for 30 minutes. Conditioning was counterbalanced so that half of the animals received black side cocaine pairings and the other half received white side cocaine pairings. CPP testing was conducted with rats in a drug-free state the day after the last conditioning session and followed the same procedure as the preconditioning test (24 hours after the last cocaine treatment rats were given free access to all three chambers for 20 minutes). All rats in this experiment were conditioned with one of the two cocaine doses. We did not have a saline-only control group treated with saline in both chambers on alternate days. The unbiased design of our CPP protocol make the comparison of time spent in the cocaine-paired chamber to time spent in the saline-paired chamber during the CPP test sufficient to establish CPP behavior, (Cunningham, Ferree, and Howard, 2003).

The day after the CPP test, (48 hours after the last cocaine treatment), each group of rats was split into two subgroups (four groups total) and re-introduced to one of the conditioning chambers. Rats were then confined to either the cocaine-paired or saline-paired chamber for 30 minutes without cocaine treatment (n = 4 to 5 per group). Subgroups were as follows: 5mg/kg cocaine-paired: conditioned with 5mg/kg cocaine and re-exposed to the cocaine-chamber; 5mg/kg saline-paired: conditioned with 5mg/kg cocaine and re-exposed to the saline-paired chamber; 20mg/kg cocaine-paired: conditioned with 20mg/kg cocaine and re-exposed to the cocaine-chamber; 20mg/kg saline-paired: conditioned with 20mg/kg cocaine and re-exposed to the saline-paired chamber. After 30 minute re-exposure to one of the conditioning chambers (drug-free), rats were briefly exposed to CO<sub>2</sub> and rapidly decapitated.

#### Protein preparation and Western Blot analysis

Brains were removed and flash frozen in 2methylbutane (-40 °C) and stored at -80 °C until used. Tissue punches of NAc and CPu were dissected on a cold glass plate and homogenized as previously described. A chemiluminescence kit (Clarity ECL, BioRad) was used and the membranes were exposed to x-ray film to detect antibody binding. All membranes were re-probed with the antibody for  $\alpha$ -tubulin and their respective total protein (phosphorylated

proteins) to normalize the protein levels. Films were scanned and analyzed using ImageJ (NIH) to quantify the intensity of the protein bands.

#### Data analysis

CPP scores are defined as the time spent in the cocaine-paired chamber minus the time spent in the saline-paired chamber during testing. Main effects of the “Conditioning-chamber” variable refers to differences between rats re-exposed to the saline-paired and cocaine-paired chamber the day after the CPP test, regardless of cocaine-conditioning dose. Main effects of the “dose” variable refer to differences between rats conditioned with 5mg/kg and 20mg/kg, regardless of chamber re-exposure subgroup. CPP scores and other CPP induced responses were analyzed with paired samples t-tests for each cocaine dose. Comparisons of behavioral and western blot data made between the four subgroups were done with 2 x 2 (conditioning -chamber x dose) ANOVAs. Significant main effects were followed by independent samples t-tests with Bonferonni corrections and significant interactions were followed by pairwise comparisons of simple main effects.

Locomotor responses were measured at two time points: (1) during the CPP test and (2) during re-exposure to the cocaine-paired or saline-paired chamber 24 hours after the CPP test. A 2 x 2 (dose x conditioning-chamber) ANOVA was used to compare locomotor responses during re-exposure to either the cocaine-paired or saline-paired chamber. Pearson correlation analysis was used to assess the relationship between CPP scores and locomotor responses recorded during both time points. Western blot data were converted to a ratio of phosphorylated protein levels to total protein levels which were normalized to  $\alpha$ -tubulin as a loading control, using arbitrary densitometric units and analyzed with 2-way (dose x conditioning-chamber) ANOVAs. Statistical significance was determined at the  $p < 0.05$  level.

### III. Results

#### Cocaine effects on CPP behaviors are dose dependent

CPP was not expressed in rats conditioned with 5mg/kg cocaine—no differences were seen in time spent in the cocaine-paired chamber or the saline-paired chamber [ $t(8) = 1.77$ ,  $p = 0.20$ ; Figure 23]. Conversely, rats conditioned with 20mg/kg cocaine spent significantly more time in the cocaine-paired than in the saline-paired chamber during the CPP test day [ $t(8) = 2.72$ ,  $p < 0.01$ ; Figure 23]. No significant differences were found in total locomotor responses during the CPP test, explorations or entrances into the cocaine-paired or saline paired-chamber after conditioning with either dose (Table 2).

A significant main effect of conditioning-chamber indicated that rats re-exposed to the chamber previously paired with cocaine were more active than those re-exposed to the chamber previously paired with saline [ $F(1, 14) = 13.52$ ,  $p < 0.01$ ]; locomotor activity of 20mg/kg cocaine-paired rats was significantly higher than 20mg/kg saline-paired rats [ $p < 0.05$ ; Figure 24A]. Regardless of cocaine conditioning dose, locomotor responses in rats re-exposed to the cocaine-paired chamber were significantly correlated to CPP scores [5mg/kg:  $r = 0.9$ ,  $p < 0.05$ ; 20mg/kg:  $r = 0.8$ ,  $p = 0.05$ ; Figure 24B and 24C, respectively].

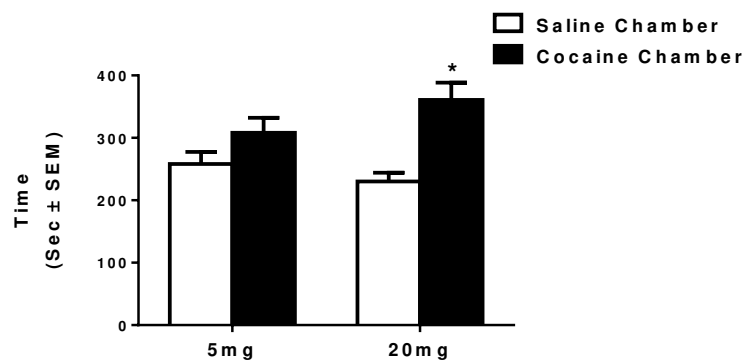
#### NAc pERK, pCREB and FosB/ $\Delta$ FosB protein levels

No changes were seen in total ERK or CREB protein levels in any brain area examined (Table 2). A significant main effect of conditioning-chamber on NAc pERK levels was found [ $F(1, 14) = 9.72$ ,  $p < 0.05$ ; Figure 25A]. NAc pERK levels increased after re-exposure to the cocaine-paired chamber regardless of cocaine conditioning dose [ $p < 0.05$  for all comparisons; Figure 25A]. Conversely, we found significant main effects of dose on NAc pCREB, FosB, and  $\Delta$ FosB protein levels [pCREB:  $F(1, 14) = 7.33$ ,  $p < 0.05$ ; Figure 25B; FosB:  $F(1, 12) = 15.24$ ,  $p < 0.01$ ; Figure 25C;  $\Delta$ FosB:  $F(1, 12) = 5.98$ ,  $p < 0.05$ ; Figure 3C]. NAc pCREB, FosB, and  $\Delta$ FosB levels were higher after conditioning with 20mg/kg cocaine compared to 5mg/kg cocaine

regardless of re-exposure to the cocaine-paired or saline-paired chamber [ $p < 0.05$  for all comparisons; Figure 25].

#### CPu pERK, pCREB and FosB/ $\Delta$ FosB protein levels

CPu pERK was increased in rats re-exposed to the cocaine-paired chamber only after conditioning with 20mg/kg cocaine [ $F(1,14) = 11.30, p < 0.01$ ; Figure 26A]. CPu pCREB levels did not change based on cocaine dose or chamber re-exposure (Figure 26B). A significant dose by conditioning-chamber interaction effect on CPu FosB levels was also observed [ $F(1,12) = 5.10, p < 0.05$ ]. CPu FosB levels in rats re exposed cocaine-paired chamber after conditioning with 20mg/kg were increased compared to 20mg/kg saline-paired and rats conditioned with 5mg/kg cocaine and re-exposed to the saline-paired chamber or the cocaine-paired chamber [ $F(1,12) = 14.82, p < 0.01$ ; Figure 26C].



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Figure 23: Average time spent (seconds  $\pm$  SEM) in the saline-paired chamber and cocaine-paired chamber during the CPP test after conditioning with 5 or 20mg/kg cocaine. \*Significant difference at the  $p < 0.05$  level ( $n = 9$  animals per group).

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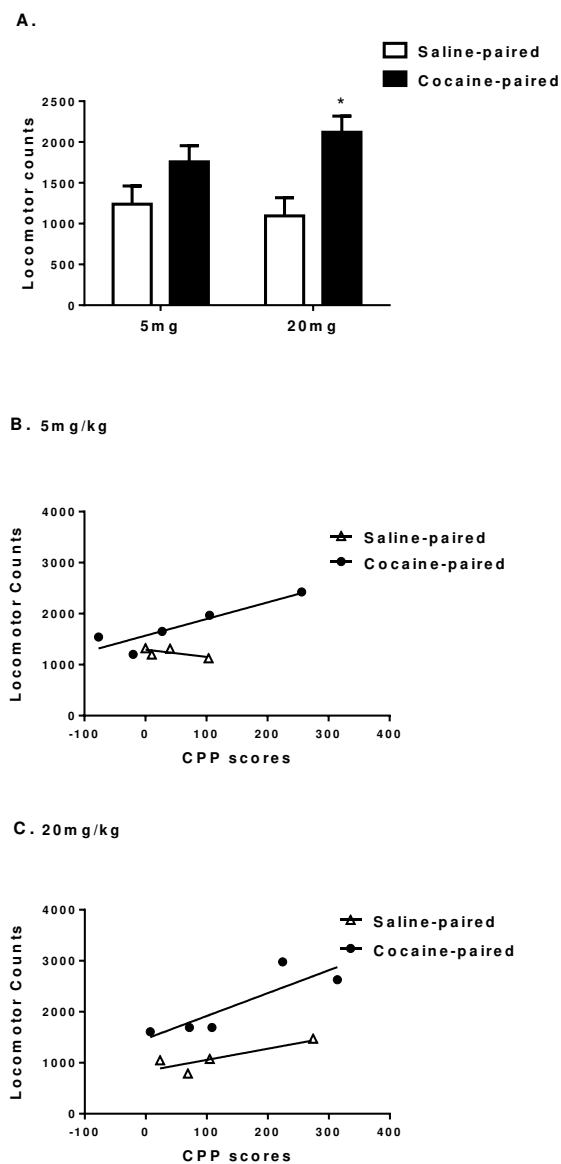


Figure 24. Locomotor responses during re-exposure to the cocaine-paired or saline-paired chamber 24 hours after the CPP test after conditioning with 5 or 20mg/kg cocaine. (A) Total locomotor counts (mean  $\pm$  SEM) in rats re-exposed to the saline-paired (white bars) or cocaine-paired (black bars) chamber. \* Significant difference at the  $p < 0.05$  level ( $n = 4$  to 5 animals per group). (B & C) Correlations between CPP scores and locomotor counts during saline-chamber (triangles) or cocaine-chamber (circles) re-exposure after conditioning with (B) 5mg/kg or (C) 20mg/kg.

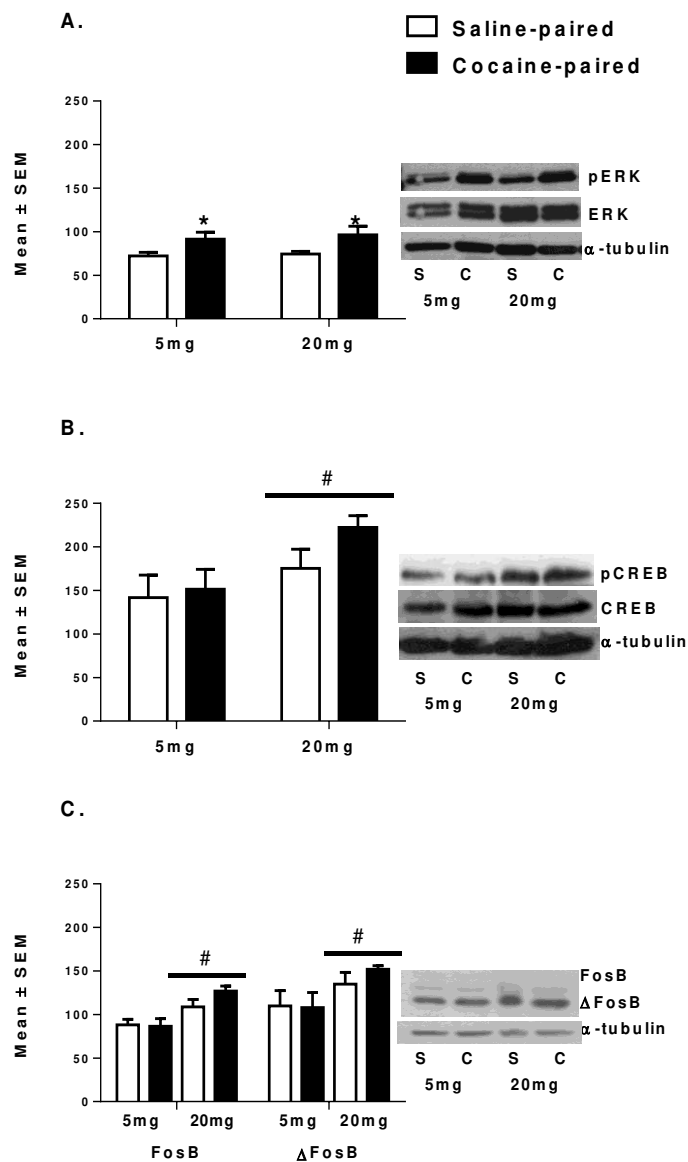


Figure 25. NAc (A) pERK1/2, (B) pCREB and (C) FosB/ $\Delta$ FosB protein levels after re-exposure to the cocaine paired or saline paired chamber after conditioning with 5mg/kg (left) or 20mg/kg (right) cocaine (24 hours after initial CPP test). Phosphorylated protein levels are expressed as a ratio to total protein levels (normalized to  $\alpha$  tubulin). \* Significant difference from rats re-exposed to the saline-paired chamber of the same dose at the  $p < 0.05$  level. # Significant difference based on conditioning dose ( $n = 4$  to 5 animals per group).



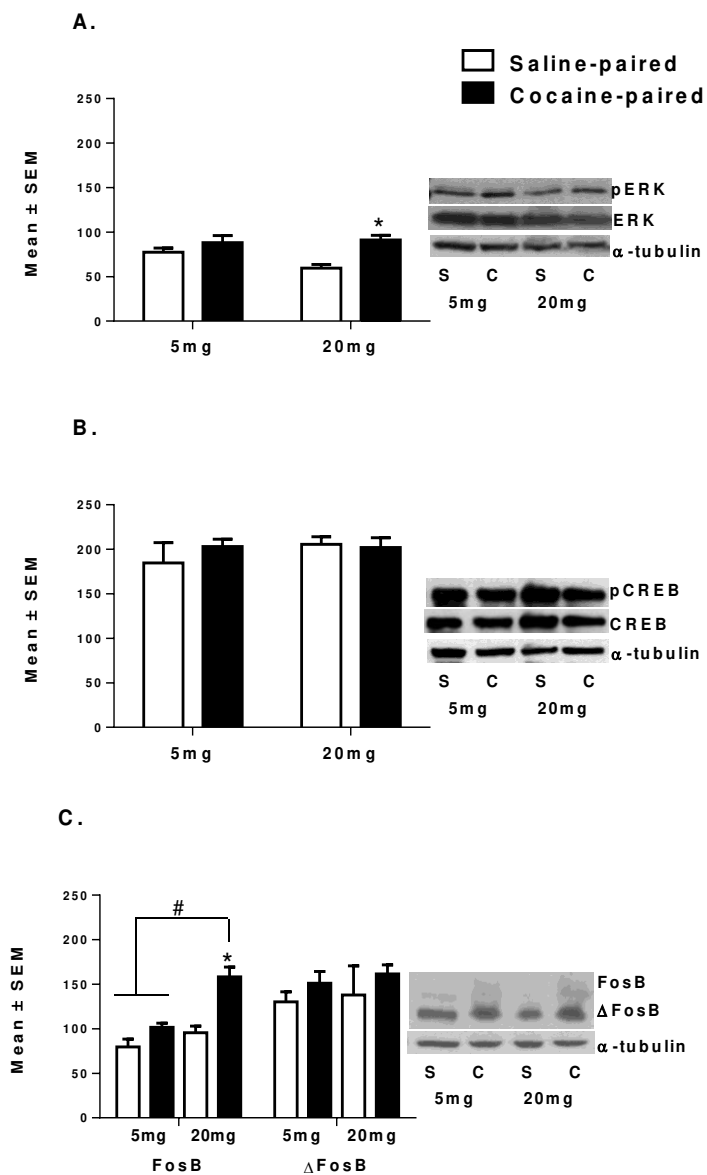


Figure 26. CPu (A) pERK1/2, (B) pCREB and (C) FosB/ $\Delta$ FosB protein levels after re-exposure to the cocaine paired or saline paired chamber after conditioning with 5mg/kg (left) or 20mg/kg (right) cocaine (24 hours after initial CPP test). Phosphorylated protein levels are expressed as a ratio to total protein levels (normalized to  $\alpha$ -tubulin). \* Significant difference from rats re-exposed to the saline-paired chamber of the same dose at the  $p < 0.05$  level. # Significant difference based on conditioning dose ( $n = 4$  to 5 animals per group).

Table 2

Cocaine effects on entrances and explorations during CPP test after 5mg/kg and 20mg/kg cocaine

Dose	Explorations		Entrances	
	Sal-paired	Coc-paired	Sal-paired	Coc-paired
5mg/kg	69.30 ± 20.26	64.40 ± 17.20	89.20 ± 6.8	104.03 ± 15.5
20mg/kg	52.60 ± 15.21	59.33 ± 7.01	75.22 ± 7.92	77.44 ± 13.50

Data are shown as mean ( $\pm$ SEM) number of entrances and explorations while in the saline-paired and cocaine-paired chambers during CPP testing (n = 9 animals per group). \*Significant difference from saline-paired chamber at  $p < 0.05$ .

Table 3

Total ERK and CREB protein levels after CPP expression

Region	5 mg/kg		20 mg/kg	
	Saline-paired	Cocaine-paired	Saline-paired	Cocaine-paired
ERK				
NAc	89.92 ± 6.10	80.54 ± 8.30	88.24 ± 7.90	96.12 ± 7.63
Cpu	103.90 ± 6.40	102.14 ± 4.40	102.01 ± 2.72	90.30 ± 8.60
CREB				
Nac	218.30 ± 7.10	221.71 ± 5.53	224.30 ± 4.63	225.10 ± 4.30
Cpu	204.70 ± 14.10	204.43 ± 4.00	199.02 ± 2.90	197.50 ± 9.10

Data are shown as mean arbitrary densitometric units ( $\pm$ SEM) normalized to  $\alpha$ -tubulin (n = 4 to 5 animals per group). No significant differences were observed based on treatment.

#### IV. Discussion

Here we show that exposure to an environment previously paired with cocaine during CPP training increases NAc pERK levels compared to animals re-exposed to the control environment- regardless of whether or not CPP behavior was expressed. These data are difficult to associate with cocaine-CPP due to the lack of behavioral change. We only saw CPP behavior after conditioning with 20mg/kg cocaine- but not 5mg/kg cocaine. However, we saw some evidence of conditioned locomotion after conditioning with both doses. Conditioning with either cocaine dose increased locomotor responses in the cocaine-paired chamber during the CPP test. Additionally, CPP scores were positively correlated to locomotor counts in the cocaine chamber during the CPP test and during forced re-exposure to the cocaine-paired context. Our results suggest region specific intracellular responses may be associated with different aspects of a drug-environment association. Similar intracellular responses with or without the expression of a behavior suggest that these may not be regulating the motivational properties that control the behavior expression.

We also saw that CPu pERK and FosB were increased after CPP expression- in rats re-introduced to the cocaine-chamber compared to those re-introduced to the saline-chamber and rats that did not express CPP behavior. These results suggest that the CPu and NAc may have differential roles in the expression of cocaine-CPP behavior and the dissociable aspects of the learned association. NAc pERK may be more involved with the associative component due to the increase after cocaine-context re-exposure regardless of CPP expression. Wells et al., (2013) recently found that NAc ERK inhibition during reconsolidation of an instrumental cocaine-context association had no effect on subsequent cocaine-seeking behavior. Based on our results, the lack of impairment makes sense because NAc ERK phosphorylation was increased even if cocaine-seeking behaviors are not exhibited. Therefore, inhibiting ERK during reconsolidation (exposure to the cocaine-context) would not alter subsequent cocaine-seeking because the motivational components of the association are still intact. pERK in NAc involved with cocaine-memory consolidation and retrieval, but possibly not reconsolidation (Ding et al., 2013; Miller &

Marshall, 2005; Theberge et al., 2010) Our results suggest a region specific role for ERK signaling based on memory stage and component of memory.

The increase in NAc pERK observed in the present study is contradictory to the results of Tropea et al., (2008) in which an increase in NAc pERK was not seen after cocaine-paired environment re-exposure. The differences in results can potentially be explained through the differences in cocaine dose, time points examined and method of CPP testing. However, the role of NAc pERK has been extensively studied and our results are consistent with most previous research. Marin et al., (2009) found a pattern of context specific cocaine-induced NAc ERK and CREB phosphorylation suggesting that ERK phosphorylation of CREB. However, these results were obtained in response to a drug challenge, indicating that cocaine-induced ERK activation may regulate CREB phosphorylation when in the presence of cocaine. In the present study, the lack of the presence of cocaine during testing indicates however, that the increase in CPu pERK and FosB was not due to the presence of cocaine. Rather, the increase was most likely due to the presence of the environment that had been associated with cocaine and therefore may be necessary for the retrieval of a cocaine-associated memory. Overall, the changes we observed in CPu and NAc pERK suggest a multi-functional role for pERK in the retrieval of cocaine-context associations.

Alternatively, the increased CPu pERK and FosB levels may be related to the increased locomotor counts in rats re-exposed to the cocaine-chamber and may therefore play a role in the expression of a conditioned locomotor response. Therefore, the CPu may be more important to CPP expression and the motivational aspects of the cocaine-contextual association- probably those driving the motivation to engage in motor behaviors (Wickens et al., 2007). pERK is necessary for the development of conditioned locomotor behavior in a cocaine-paired context (Valjent et al., 2006). Based on our results, it is still unclear whether

Our observation of increased NAc FosB and  $\Delta$ FosB in CPP expressing rats compared to non-CPP expressing rats confirms previous reports that CPP expression involves immediate early gene expression (Miller & Marshall, 2004). Our data also suggest that IEG expression

associated with CPP behavior is increased after exposure to a cocaine-associated context and may play a role in motivation and persistence of drug-seeking behaviors. Recently, the NAc and CPu were shown to differ in regards to the epigenetic mechanisms underlying  $\Delta$ FosB accumulation following chronic cocaine exposure (Damez-Werno et al., 2012). Future research should look at connections between these brain regions and others known to be involved in associative learning, attention and emotional processing. For example, a role for the amygdala in the attentional processes necessary for motivational associative learning has recently been shown in humans (Li et al., 2011) and rodents (Wells et al., 2013).

#### Chapter4: Sexually dimorphic intracellular responses after cocaine–induced conditioned place preference expression

(Adapted from Nygard et al., 2013)

##### I. Introduction

Addiction studies consistently show greater responses among females than males in various cocaine-related outcomes. As more attention is paid to sex-specific and hormonal effects on cocaine abuse, it is increasingly apparent that sex differences are present at all phases of drug abuse, from initiation through escalation of use and progression to addiction. For example, human females report a slower onset of the subjective effects of cocaine, undergo shorter periods of abstinence between cocaine use and experience cravings after cocaine use when presented with cocaine-associated cues more frequently than males (Anker & Carroll, 2011; Elman et al., 2001; Lynch, 2006; Quinones-Jenab & Jenab, 2010). Female rats learn to self-administer cocaine faster (Lynch & Carroll, 1999) and exhibit enhanced locomotor activity and behavioral sensitization to both acute and chronic cocaine administration compared to males (Chin et al., 2001; Craft & Stratmann, 1996; Festa et al., 2004; Sell et al., 2000; Van Haaren & Meyer, 1991). Still to be determined is the contribution of sex differences in central nervous system plasticity and cellular responses for the development of learned drug-environment associations that play an important role in addiction.

Sex differences in the mesocorticolimbic reward circuitry, including DAR distribution, DA binding properties and intracellular signaling has been postulated to underlie sexually dimorphic responses to cocaine (Becker and Hu, 2008; Festa et al. 2006; Walker et al. 2006). Female rats express cocaine-induced CPP in response to lower cocaine doses (Russo et al., 2003a; Zakharova et al., 2009) and after fewer cocaine-place pairings than males (Russo et al., 2003a). Differences in DAR sensitivity may underlie sex differences in cocaine CPP and the formation of cocaine environment associations. For example, Nazarian et al., (2004) found that regardless of sex, lower doses of a D<sub>1</sub>DAR antagonist blocked cocaine CPP acquisition, whereas higher doses of the antagonist only blocked CPP in males. However, the extent to which downstream

molecular signaling in response to sexually dimorphic cocaine induced changes in DA activity contribute cocaine CPP remains to be determined.

Sex differences in DA response to cocaine combined with the substantial evidence for the important role of ERK, CREB, and Fos proteins in cocaine CPP in males suggest that sex differences may underlie the formation of drug associated memories. However, to our knowledge, studies that have used the CPP paradigm to investigate molecular alterations involved with cocaine-context associations have only used male rats. We aimed to investigate the potential sex differences in the intracellular signaling molecules underlying the expression of cocaine environment associations. Specifically, we examined pERK, pCREB, FosB, and  $\Delta$ FosB protein levels in mesocorticolimbic regions associated with reward, learning, and memory (NAc, CPu, PFC, and HIP), after cocaine CPP expression in male and female rats. We hypothesized that cocaine CPP expression would be associated with similar changes in protein levels in male and female cocaine treated rats. We also expected any sex differences in protein levels to be correlated with sex differences in the magnitude of CPP behavior.

## II. Methods

### Animals

8 week old male and female Fischer rats (Charles River, Raleigh, NC) were maintained on a 12 hour light/dark cycle and individually housed with ad libitum access to food and water. Rats were randomly assigned to a saline only control group or a saline/cocaine treatment group for conditioning (n = 8-10 per group). Estrous cycle stage was not monitored because vaginal lavages attenuate cocaine induced locomotor behavior, produce CPP (Walker et al. 2002) and increase NAc  $\Delta$ FosB (Reviewed in Robison and Nestler, 2011). Animal care and use was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, Bethesda, MD, USA) and approved by the Hunter College, CUNY, Institutional Animal care and Use Committee.



## CPP procedure

Rats were conditioned with 20 mg/kg cocaine, i.p., using a standard unbiased and counterbalanced 4-day CPP procedure as previously described. The day before conditioning, rats were placed into the neutral middle chamber and allowed to explore all three chambers for 20 minutes. On odd numbered days, rats were administered saline and confined to one of the conditioning chambers for 30 minutes. On even numbered days, rats were treated with cocaine and confined to the opposite chamber for 30 minutes. Control rats were administered saline in both chambers on alternate days. CPP testing was conducted 24 hours after the last conditioning session (drug-free), following the same procedure as the preconditioning test. Preliminary testing in our lab has shown that this paradigm produces equivalent CPP scores between sexes as compared to Russo et al. (2003a). The 2 major differences between our paradigm and Russo et al. (2003a) are: (1) animals were not handled daily for 30 minutes for a week prior to our testing; and (2) we did not begin the CPP test with a 5 minute acclimation period in the test apparatus before a 15 minute test. Pre-handling of rats has been shown to affect overall CPP scores in male rats (Cunningham et al., 2006; 2011).

## Western Blot and Data analysis

Paired-samples t-tests were used to assess differences in entrances, explorations, and the time spent in the cocaine and saline paired chambers for each group during CPP testing. Significant CPP behavior was defined as spending significantly more time in the cocaine paired chamber than the saline paired chamber during preference testing. To measure differences in the magnitude of CPP, preference scores were calculated by subtracting the time spent in the saline paired chamber from the time spent in the cocaine paired chamber during testing. Due to the unbiased conditioning procedures used in this study, we did not compare the time spent in the cocaine paired chamber to the time spent in that chamber during the pretest (Cunningham et al. 2006). Changes in the magnitude of CPP scores and total locomotor activity were assessed with 2 x 2 (sex x treatment) ANOVAs. To assess the relationship between CPP scores and locomotor

activity during the CPP test, we conducted a Bivariate Pearson correlation analysis between CPP scores and locomotor activity in cocaine males and females (separately).

Films were scanned on an Epson Perfection v700 desktop scanner and Western blot band intensities were quantified with imageJ molecular quantifying software (NIH). Levels of pERK1/2 were quantified together. Total protein levels were normalized to their respective  $\alpha$ -tubulin levels (as a loading control), and total protein levels were used for the normalization of phosphorylated protein levels (where appropriate) and expressed as arbitrary densitometric units. Changes in protein levels were assessed with 2 x 2 (sex by treatment) ANOVAs. Significant main effects were compared using unpaired t-tests with Bonferonni corrections (where appropriate) and significant interactions were followed by pairwise comparisons of simple main effects. To account for differences in protein phosphorylation between saline treated males and females, in a separate analysis, we assessed sex differences in the magnitude of change in protein phosphorylation from saline in cocaine treated rats by normalizing raw data to saline controls of the respective sex (arbitrarily set to 100%). In cases where a cocaine-induced increase in protein levels was observed in male or female cocaine treated rats compared to their respective saline controls, independent samples t-tests were then conducted on percentage control data between male and female cocaine treated rats. Additionally, Bivariate Pearson Correlations were run on normalized data for male and female cocaine treated animals (separately) to identify relationships between CPP scores, locomotor activity and phosphorylated protein levels. Correlation analysis between CPP scores and protein levels were performed using data obtained from the animals used for molecular analysis.

### III. Results

#### CPP behavior

No differences in any treatment group were observed during the initial preconditioning test in the time spent in each chamber or in total locomotor behavior, confirming the unbiased nature of the CPP apparatus and testing protocol (data not shown). During CPP testing, cocaine treated rats spent significantly more time in the cocaine paired chamber than the saline paired

chamber [male:  $t(8) = 2.55$ ,  $p < 0.05$ ; female:  $t(9) = 3.4$ ,  $p < 0.05$ ; Figure 27A]. Only cocaine treated female rats showed significant increases in explorations and entrances into the cocaine paired chamber compared to the saline paired chamber [explorations:  $t(9) = 3.7$ ,  $p < 0.01$ ; entrances:  $t(9) = 2.6$ ,  $p < 0.05$ ; Table 4]. A significant main effect of treatment was seen on the magnitude of CPP scores [ $F(1,35) = 30.15$ ,  $p < 0.01$ ; Figure 27B]. However, no sex differences in the magnitude of CPP scores were seen [ $F(1,35) = 1.49$ ,  $p = 0.23$ ].

A significant main effect of sex on total locomotor responses was observed [ $F(1,35) = 40.20$ ,  $p < .01$ ; Figure 27C]. Regardless of treatment, females were more active than males [ $p < 0.05$  for all comparisons]. A significant interaction between sex and treatment was also seen [ $F(1,35) = 4.81$ ,  $p < 0.05$ ]. Cocaine treated females displayed more locomotor counts than males [ $F(1,35) = 34.58$ ,  $p < 0.001$ ] and female saline controls [ $F(1,35) = 11.01$ ,  $p < 0.01$ ; Figure 27C]. Additionally, total locomotor responses were significantly correlated to CPP scores in female rats [ $r = 0.70$ ,  $p < 0.01$ ] but not in males [ $r = 0.07$ ,  $p = 0.80$ ; Figure 27D].

#### ERK phosphorylation in the NAc, CPu, PfC, and HIP in male and female rats

In the NAc, CPu, PfC and HIP no differences in total ERK or CREB protein levels were seen (Table 5). However, a main effect of treatment on NAc pERK protein levels was observed [ $F(1,12) = 15.83$ ,  $p < 0.01$ ; Figure 28A]. Regardless of sex, NAc pERK protein levels increased in cocaine treated rats [males:  $t(6) = 4.3$ ,  $p < .01$ ; females  $t(6) = 3.04$ ,  $p < 0.05$ ]. CPu pERK levels did not change based on sex or treatment (Figure 28B).

In the PfC, a significant main effect of sex on pERK levels was observed [ $F(1,12) = 8.2$ ,  $p < 0.05$ ; Figure 28C]. However, this was partly due to sex differences in saline controls; pERK levels in saline males were significantly higher than saline females [ $t(6) = 3.1$ ,  $p < 0.05$ ]. On PfC pERK levels, a significant main effect of treatment was also observed [ $F(1,12) = 20.2$ ,  $p < 0.01$ ; Figure 28C]. PfC pERK levels were higher in cocaine females than saline females [ $t(6) = 4.2$ ,  $p < 0.01$ ]. Likewise, cocaine females displayed a greater magnitude of change in PfC pERK levels than cocaine males [ $t(6) = 4.3$ ,  $p < 0.01$ ; Table 6].

In the HIP, a significant interaction between sex and treatment on pERK protein levels was obtained [ $F(1,11) = 16.1, p < 0.01$ ; Figure 28D]. HIP pERK levels were significantly higher in cocaine treated females and cocaine treated males than saline controls of their respective sex [males:  $F(1,11) = 5.16, p < 0.05$ ; females:  $F(1,11) = 28.96, p < 0.01$ ]. Furthermore, HIP pERK levels were significantly higher in saline treated males than saline treated females [ $F(1,11) = 23.03, p < 0.01$ ; Figure 28D]. After cocaine CPP expression, sex differences were seen in the magnitude of change in HIP pERK levels; cocaine treated females had a larger magnitude of change from saline than cocaine males [ $t(6) = 5.04, p < 0.01$ ; Table 6].

CREB phosphorylation in the NAc, CPu, PFC, and HIP of male and female rats

In the NAc, a significant main effect of treatment on pCREB levels was observed [ $F(1,12) = 20.44, p < 0.01$ ; Figure 29A]; pCREB levels were higher in cocaine animals than saline controls [males:  $t(6) = 4.7, p < 0.01$ ; females:  $t(6) = 2.7, p < 0.05$ ]. Similarly, a significant main effect of treatment on PFC pCREB levels was observed [ $F(1,12) = 5.8, p < 0.05$ ; Figure 29C]. However, only cocaine treated males had significantly higher PFC pCREB levels than saline males [ $t(6) = 2.6, p < 0.05$ ]. The magnitude of change in PFC pCREB levels was greater in cocaine treated males than cocaine females, [ $t(6) = 2.5, p < 0.05$ ; Table 6]. No changes were seen in CPu or HIP pCREB levels (Figure 29B and 29D).

Fos/ $\Delta$ FosB levels in the NAc, CPu, PFC, and HIP in male and female rats

In the NAc, a significant main effect of treatment on  $\Delta$ FosB levels was observed [ $F(1,11) = 22.8, p < 0.01$ ; Figure 30A]. In both male and female cocaine treated rats, NAc  $\Delta$ FosB levels were higher than their respective saline controls [males:  $t(6) = 4.9, p < 0.01$ ; females:  $t(5) = 2.9, p < 0.05$ ; Figure 4A]. NAc FosB levels did not significantly differ (Figure 30A).

In the CPu, a significant main effect of treatment was seen for FosB levels [ $F(1,12) = 46.44, p < 0.01$ ; Figure 30B]. FosB levels were significantly increased in both cocaine males and females compared to saline control animals [males:  $t(6) = 3.4, p < 0.01$ ; females:  $t(6) = 6.7, p < 0.01$ ; Figure 30B]. The magnitude of change in CPu FosB levels in cocaine treated rats was

significantly greater in females than males [ $t(6) = -2.6, p < 0.05$ ; Table 6]. A significant main effect of treatment on CPu  $\Delta$ FosB protein levels was observed [ $F(1,12) = 41.01, p < 0.01$ ]. In both sexes,  $\Delta$ FosB protein levels were increased compared to saline animals [males:  $t(6) = 2.5, p < 0.05$ ; females:  $t(6) = 8.6, p < 0.01$ ]. A significant main effect of sex on CPu  $\Delta$ FosB levels was also observed [ $F(1,12) = 13.6, p < 0.01$ ; Figure 30B]. Saline treated males had higher  $\Delta$ FosB levels than saline females [ $t(6) = 3.6, p < 0.05$ ]. The magnitude of change in  $\Delta$ FosB protein levels in cocaine females was significantly greater than in cocaine treated males [ $t(6) = -5.9, p < 0.01$ ; Table 6]. No changes were seen in Pfc or HIP FosB/ $\Delta$ FosB levels [Figure 30C and 30D].

#### Correlations between CPP behavior and phosphorylated protein levels

As shown in Table 7 (See Figure 31 for sample plot), CPP scores were significantly correlated to NAc pERK, Pfc pERK and CPu FosB in male rats [NAc pERK:  $r = 0.79$ ; Pfc pERK:  $r = 0.86$ ; CPu FosB:  $r = 0.76$ ;  $p < 0.05$  for all comparisons]. Similarly, CPP scores were significantly correlated to NAc pERK, Pfc pERK and CPu FosB in female rats [NAc pERK:  $r = 0.81$ ; Pfc pERK:  $r = 0.76$ ; CPu FosB:  $r = 0.76$ ;  $p < 0.05$  for all comparisons]. In female rats, significant correlations were also seen between CPP scores and levels of HIP pERK, NAc pCREB and CPu  $\Delta$ FosB [HIP pERK:  $r = 0.70$ ; NAc pCREB:  $r = 0.78$ ; CPu  $\Delta$ FosB:  $r = 0.77$ ;  $p < 0.05$  for all comparisons]. In males, CPP scores were significantly correlated with NAc  $\Delta$ FosB levels [ $r = 0.76, p < 0.05$ ]. No significant correlations were found between locomotor responses and phospho-protein levels changed by cocaine (See Table 8).

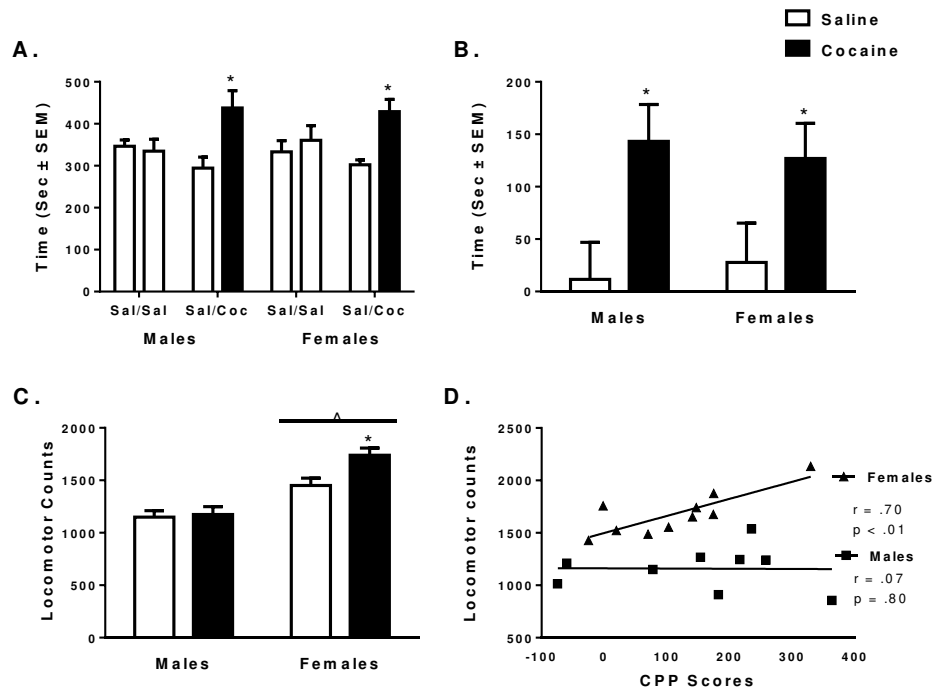


Figure 27: CPP and locomotor responses in male and female rats during testing after conditioning with 20mg/kg cocaine. (A) Average time spent (in seconds  $\pm$  SEM) in the saline paired chamber compared to the cocaine paired chamber (B) CPP scores and (C) total locomotor activity in saline (white bars) and cocaine (black bars) treated males and females ( $n = 8-10$  rats per group). \*Indicates significant difference from saline controls of the same sex at  $p < 0.05$ . ^Indicates significant main effect of sex at  $p < 0.05$ . (D) Correlation between CPP scores and total locomotor activity during the CPP test in cocaine treated male (squares) and female (triangles) rats. The Pearson Correlation coefficients and  $p$ -values are displayed within the plot.

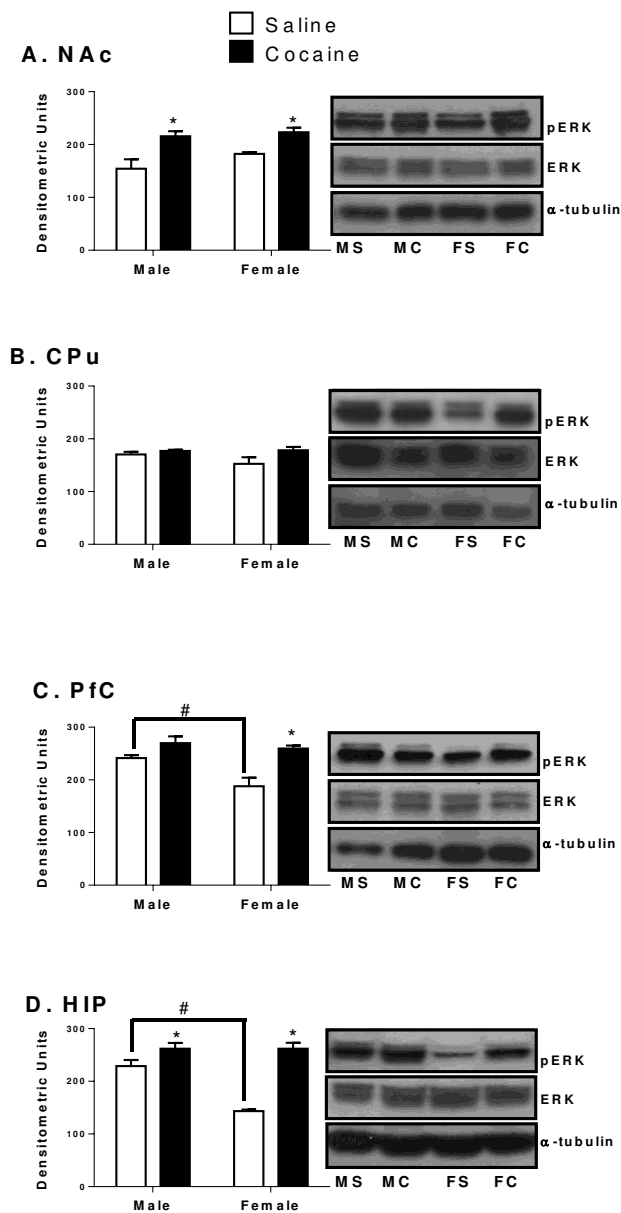


Figure 28: Phosphorylated ERK1/2 protein levels (measured at 44/42 kDa) in the NAc (A), CPu (B), PfC (C), and HIP (D) of male and female rats after CPP testing. Phosphorylated protein levels are expressed as a ratio to their respective total protein levels (normalized to  $\alpha$  tubulin, 55 kDa)( $\pm$ SEM) ( $n = 4$  animals per group). \*Significant difference from saline controls of the same sex at  $p < 0.05$ . #Significant difference between male and female saline animals at  $p < 0.05$ .

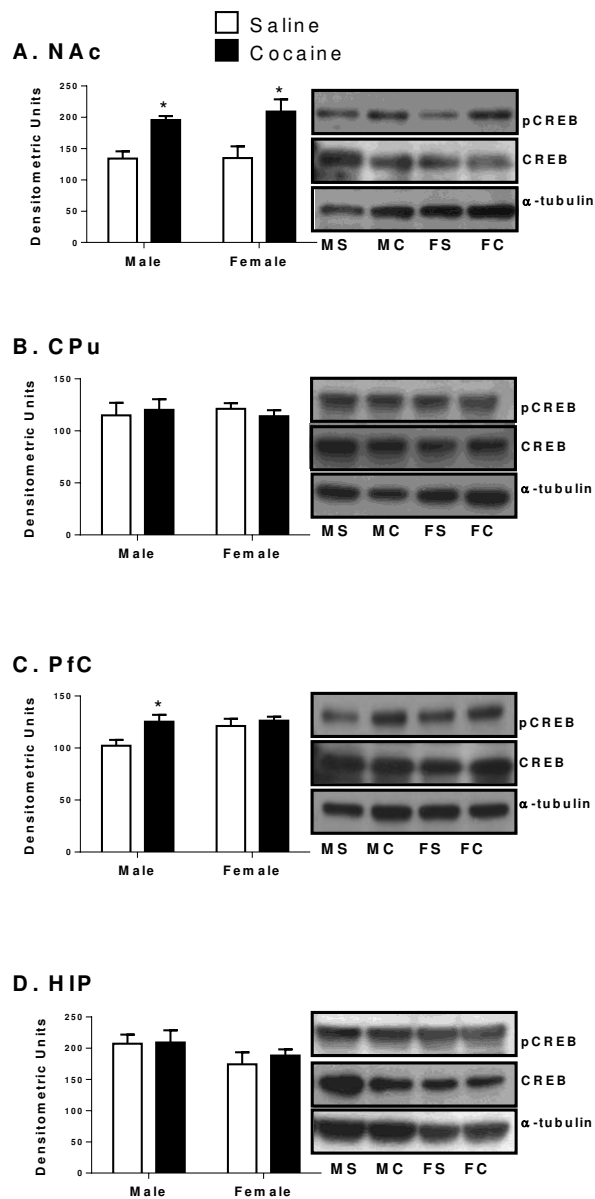


Figure 29: Phosphorylated CREB protein levels (measured at 46 kDa) in the NAc (A), CPu (B), PfC (C), and HIP (D) of male and female rats after CPP testing. Phosphorylated protein levels are expressed as a ratio to their respective total protein levels (normalized to  $\alpha$  tubulin, 55 kDa)( $\pm$ SEM) ( $n = 4$  animals per group). \*Significant difference from saline controls of the same sex at  $p < 0.05$ .



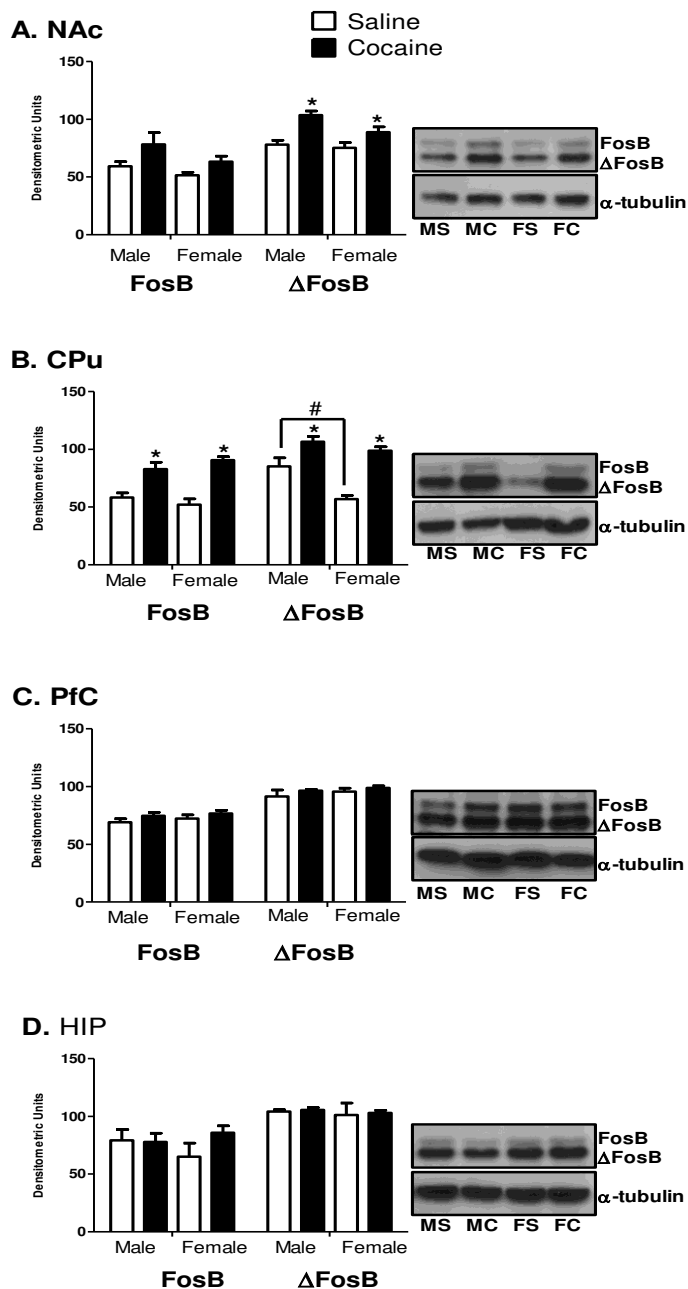
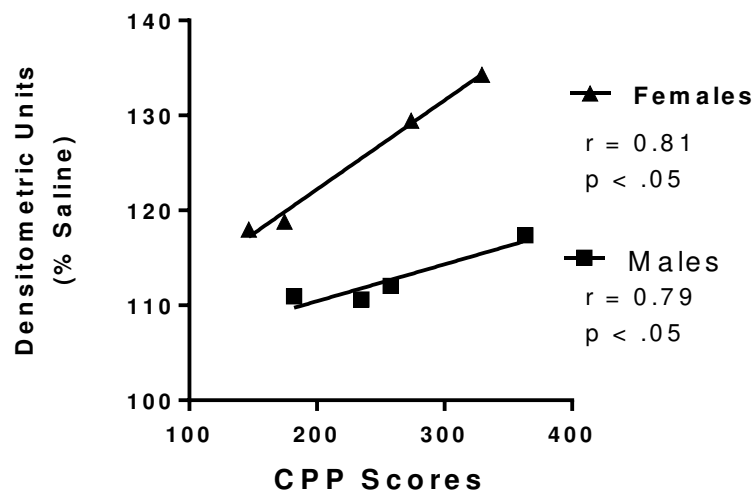


Figure 30: FosB (left 4 bars, top band: 48 kDa) and  $\Delta$ FosB (right 4 bars, bottom band: 38 kDa) protein levels in the NAc (A), CPu (B), PfC (C), and HIP (D) of male and female saline and cocaine rats after CPP testing. Protein levels are expressed as a ratio to their respective  $\alpha$  tubulin levels (55 kDa) ( $\pm$ SEM) ( $n = 4$  animals per group). \*Significant difference from saline controls of the same sex at  $p < 0.05$ . #Significant difference between male and female saline animals at  $p < 0.05$ .



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Figure 31: Representative scatter plot of correlations between CPP scores and phospho-protein levels. NAc pERK protein levels (expressed as a percentage of saline controls) were significantly correlated to CPP scores in both male (squares) and female (triangles) cocaine treated rats ( $n = 4$  animals per group). The Pearson correlation coefficients and p-values are displayed within the plot.

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Table 4

## Cocaine effects on entrances and explorations during CPP test

		Entrances		Explorations	
		Saline-paired	Cocaine-paired	Saline-paired	Cocaine-paired
Male	Saline	94.2±7.8	83.3±6.8	69.9±7.3	71.2±2.9
	Cocaine	73.2±8.2	83.1±8.4	69.9±3.3	73.2±5.5
Female	Saline	93.0±11	113±11.7	72.8±5.5	64.5±3.7
	Cocaine	98.8±5.4	124.5±12.2*	61.9±3.6	84.6±3.5*

Data are shown as mean ( $\pm$ SEM) number of entrances and explorations while in the saline-paired and cocaine-paired chambers during CPP testing (n = 8-10 animals per group).

\*Significant difference from saline-paired chamber at  $p < .05$ .

Table 5

Total ERK and CREB protein levels after CPP expression in male and female rats

Protein	Male		Female	
	Saline	Cocaine	Saline	Cocaine
<b>ERK</b>				
NAc	87.2±2.9	89.4±4.7	88.0±3.6	84.8±1.9
CPu	111.8±3.0	109.8±1.8	109.7±1.9	109.3± 3.4
PfC	74.1±3.9	71.9±3.5	66.3±1.9	72.4±2.1
HIP	75.4±2.2	82.9±6.3	81.4±3.5	77.5±3.1
<b>CREB</b>				
NAc	94.8±8.4	85.2±5.1	77.8±4.9	76.4±5.7
CPu	119.9±10	118.3±8.5	117.0±6	119.7±6.5
PfC	112.2±5.9	104.2±3.4	100.8±2.3	109.1±3.3
HIP	107.6±8.8	116.9±8.5	131.2±12.8	120.3±11.3

Data are shown as mean arbitrary densitometric units ( $\pm$ SEM) normalized to  $\alpha$ -tubulin (n = 4 animals per group). No significant differences were observed based on treatment or sex.

Table 6

Percentage change from saline of pERK, pCREB, FosB and  $\Delta$ FosB protein levels after CPP expression in cocaine-treated rats

	pERK		pCREB		FosB		$\Delta$ FosB	
	Male	Female	Male	Female	Male	Female	Male	Female
NAc	112.8±1.6	122.9±3.8	118.1±3.7	118.4±5.3	112.8±7.4	110.9±13.7	138.2±11.1	117.8±4.2
CPu	103.9±2.8	124.3±3.1	104.6±8.8	94.2±4.7	142.0±10.3	173.3±6.1 <sup>#</sup>	124.9±5.4	173.8±6.2 <sup>#</sup>
PfC	111.7±5.4	138.1±3.0	122.6±6.6	104.3±3.1	108.1±4.2	106.0±3.8	105.6±1.3	103.2±1.9
HIP	122.0±3.2	166.9±8.3 <sup>#</sup>	100.8±8.5	108.1±8.6	98.4±9.5	118.6±17.1	101.2±2.1	101.7±2.3

Data are presented as densitometric units expressed as a mean percentage of saline controls of the respective sex which were set to 100% ( $\pm$  SEM) (n = 4 animals per group). Boldface indicates a significant difference between cocaine treated males and females at  $p < 0.05$ . Boldface<sup>#</sup> indicates cases in which a significant ( $p < .05$ ) difference between saline and cocaine animals.

Table 7

Pearson correlation coefficients (r) for CPP scores and phospho-protein levels

	pERK		pCREB		FosB		$\Delta$ FosB	
	Male	Female	Male	Female	Male	Female	Male	Female
NAc	0.79	0.81	0.51	0.78	0.29	0.23	0.76	0.34
CPu	0.39	0.08	-0.04	-0.22	0.76	0.76	0.37	0.77
PfC	0.86	0.76	0.35	0.01	-0.03	0.35	-0.08	0.22
HIP	0.67	0.70	-0.02	0.65	-0.15	-0.17	0.15	0.03

Correlations were assessed between CPP scores and protein levels normalized to saline controls of the respective sex (n = 4 animals per group). Bold values indicate a significant correlation at  $p < 0.05$ . See Figure 5 for a representative plot.

Table 8

Pearson correlation coefficients (r) for locomotor responses and phospho-protein levels

	pERK		pCREB		FosB		ΔFosB	
	Male	Female	Male	Female	Male	Female	Male	Female
NAc	0.22	-0.09	0.34	0.26	-0.43	-0.69	0.42	-0.05
CPu	0.82	0.09	-0.76	-0.10	0.20	-0.12	0.09	-0.09
PfC	0.32	0.35	-0.23	0.52	-0.38	-0.07	-0.35	-0.38
HIP	-0.02	-0.30	-0.06	0.54	-0.52	0.03	-0.34	-0.58

Correlations were assessed between total locomotor counts during the CPP test and protein levels normalized to saline controls of the respective sex (n = 4 animals per group). Bold values indicate a significant correlation at  $p < 0.05$ .

#### IV. Discussion

Although both male and female rats displayed similar levels of CPP, some components of these behavioral responses were sexually dimorphic. Females displayed greater total locomotor responses during the CPP test than males, regardless of treatment. However, cocaine females exhibited greater motor responses, including entrances and explorations of the cocaine-paired chamber in addition to total locomotor activity, when compared to male rats and saline controls. We also show that similar to males, females undergo changes in ERK/CREB molecular signaling after cocaine CPP expression and that some response patterns on this pathway are sexually dimorphic. Specifically, we showed that sex affects: (1) the location of these changes and (2) the magnitude of the changes in this pathway. The degree to which alterations in protein levels are due to CPP and/or locomotor responses is still unclear. However, we observed positive correlations between protein levels and CPP scores, but not between protein levels and locomotor activity. Additionally, in regions where cocaine induced increases in protein levels in both males and females compared to saline controls, we saw no sex differences in phospho-protein levels in cocaine rats. Instead, we observed sex differences in phospho-protein levels between saline treated males and females, resulting in sex differences in the magnitude of change from saline in cocaine rats. Therefore, we postulate that these changes are in part mediated by sex differences in basal levels in the ERK/ $\Delta$ FosB cascade.

Studies that report cocaine induced CPP and increased locomotor responses during CPP testing have been shown with various cocaine doses (Bobzean et al., 2010; Russo et al., 2003a,b; Zakharova et al., 2009). In the present study, CPP scores were positively correlated to total locomotor responses in females, but not males. Previous research in male rats has reported conflicting evidence for a correlation between locomotor responses and cocaine CPP (Allen et al. 2007; Kosten and Miserendino, 1998), and others suggest that individual differences may account for differences in psychostimulant locomotor activity and CPP behavior (Mathews et al. 2010; Seymour and Wagner, 2008). Associations between activity and CPP may be more pronounced in females possibly due to individual differences and/or gonadal hormone levels, but



this postulate requires further study. Although numerous studies report the effects of gonadal hormones on CPP (Reviewed in Anker and Carroll, 2011; Festa and Quinones-Jenab, 2004), it is unknown whether estrous cycle stage affects CPP behavior. Because we did not observe differences across groups in behavioral and locomotor responses prior to conditioning and our female behavioral and molecular data are no more variable than our male data, it is likely not the case that differences in estrous cycle stage are hiding or increasing the changes in behavior or protein phosphorylation reported here. However, some cocaine-induced phospho-protein levels have been shown to vary based on estrous cycle stage. For example, previous research shows that NAc pCREB increased 15 minutes after cocaine exposure in estrus females, but no basal differences in NAc or CPu pCREB or  $\Delta$ FosB were seen based on cycle stage (Weiner et al. 2009). Therefore, our results are likely not due to estrous cycle effects, but more likely due to sex differences in neural organization utilizing sex specific intracellular responses.

Our results are consistent with Sato et al. (2011), in which chronic cocaine treatment resulted in more locomotor responses and a greater magnitude change in CPu FosB/ $\Delta$ FosB in females than males. Contrary to Sato et al. (2011), we saw no sex difference in cocaine induced NAc  $\Delta$ FosB increase, which may be because of the difference in cocaine-treatment regimens used. Sato et al. (2011) report these differences in NAc and CPu  $\Delta$ FosB levels after chronic cocaine administration, whereas animals in our study were only treated with cocaine twice. These results suggest region specific sexually dimorphic responses to cocaine after CPP expression. In males, cocaine induced ERK phosphorylation regulates  $\Delta$ FosB accumulation underlying long-term changes in synaptic plasticity associated with addiction (Radwanska et al. 2006; Valjent et al. 2000). We report that although cocaine females' displayed a greater increase in CPu  $\Delta$ FosB levels than males, the resulting protein levels were similar in cocaine males and females. Although we did not demonstrate a direct correlation between locomotor behavior and CPu  $\Delta$ FosB levels, locomotor responses and CPu  $\Delta$ FosB levels were significantly correlated to CPP scores in cocaine females, but not in males. In addition to cocaine reward, increased  $\Delta$ FosB has been associated with cocaine seeking behaviors and cocaine induced locomotor responses (Kelz et al. 1999). It is therefore reasonable to postulate that our observation of females enhanced

responding during the CPP test may be related to their larger increase in CPu  $\Delta$ FosB levels. To what extent differences in locomotor behavior during CPP testing are a component of the conditioned response to cocaine and thus a sex difference in cocaine-induced conditioned activity is yet to be determined.

In addition to enhanced locomotor activity, Nazarian et al. (2009) found that in female rats, cocaine-induced CREB phosphorylation was greater, but shorter lasting than in males, providing evidence that females' enhanced locomotor response to cocaine may be due to a rapid increase and drop in pCREB. Our observed sex differences in protein levels could also reflect temporal sex differences in protein phosphorylation. Similarly, we found that PfC pERK levels were only increased in cocaine females and not in cocaine males, whereas PfC pCREB levels were increased in cocaine males, but not females. It is therefore possible that ERK phosphorylation in response to a cocaine-associated context may be faster acting in males than females, leading to a more rapid increase in pCREB levels, indicating potential sex differences in the time course of protein phosphorylation that underlie cocaine-cue-induced behaviors (Radwanska et al. 2006). PfC pERK and pCREB levels were positively correlated to CPP scores in males and females, an indication that in the PfC ERK/CREB responses induced after cocaine CPP expression are sexually dimorphic. Likewise, sexually dimorphic changes in DA activity and downstream molecular events have previously been seen in PfC responses to cocaine (Sun et al. 2010). Additionally, when HIP pERK and PfC pERK levels were lower in saline females than saline males, cocaine-induced increases in FosB/ $\Delta$ FosB were not observed. Thus, males and females show region specific variations in ERK/ $\Delta$ FosB signaling related to cocaine/cocaine-cue induced behaviors associated with addiction (Becker et al. 2012).

## Chapter 5: Discussion and overall conclusions

The findings in this dissertation are consistent with most of the previous literature on cocaine induced CPP behaviors. Our molecular results are also consistent with most of the previous literature associating changes in protein phosphorylation with cocaine CPP. Here we show that after CPP training, different patterns of intracellular signaling responses in the NAc and CPu. After CPP expression, NAc pERK, pCREB, and  $\Delta$ fosB were consistently increased. We also observed positive correlations between CPP scores and NAc pERK levels across all studies. The increased NAc pERK levels and positive correlation to CPP scores in cocaine treated males and females was expected and is consistent with previous literature showing that NAc ERK phosphorylation is associated with the expression of cocaine CPP (Miller & Marshall 2004, 2005; Valjent et al., 2000; 2004). In the CPu, increased pERK was only observed after forced re-exposure to the cocaine-chamber, 24 hours after the CPP test. No differences in CPu pERK and pCREB were seen at any other time point, indicating temporal differences in protein expression in these regions. However, we saw robust increases in CPu FosB/  $\Delta$ FosB that were positively correlated to CPP scores. These data indicate a region specific role for pERK/pCREB/FosB intracellular signaling in the acquisition and subsequent expression/retrieval of cocaine-context associations. Pretreatment of MK-801 (0.25 mg/kg. i.p.), 30 minutes prior to cocaine administration during conditioning blocked the acquisition of cocaine CPP and attenuated NAc pERK and pCREB and CPu FosB/  $\Delta$ FosB. This suggests that NAc ERK and CREB phosphorylation after exposure to a cocaine-associated context and CPu  $\Delta$ FosB accumulation seems to be dependent on NMDAR activation during cocaine exposure. It also suggests that the NAc and CPu differentially regulate  $\Delta$ FosB accumulation after repeated cocaine treatment and provide evidence for region specific regulation of motivation and reward processing and responses to cocaine-paired cues.

We found that CPP behavior was expressed only after conditioning with the higher dose of cocaine (20mg/kg). Locomotor responses during the CPP test and re-exposure session were correlated to CPP scores after conditioning with either cocaine dose. NAc pERK levels were

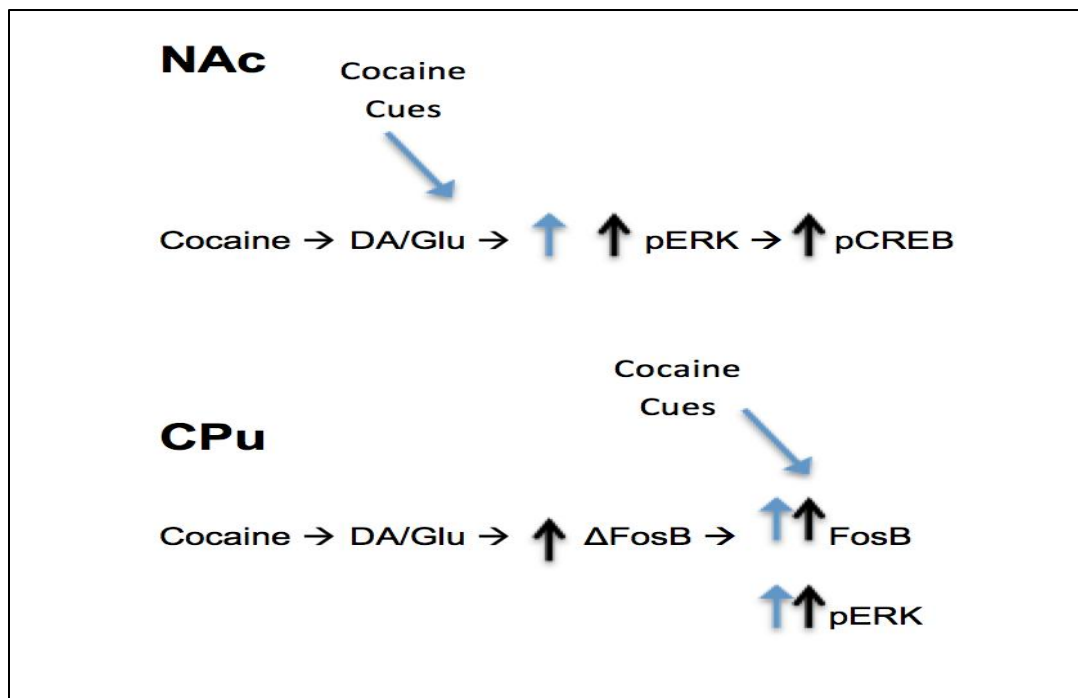
increased after re-exposure to the cocaine-paired, but not the saline-paired environment 24 hours after the CPP test, regardless of whether or not CPP behavior was expressed. CPU pERK and FosB protein levels increased after re-exposure to the cocaine chamber only after conditioning with the higher cocaine dose. NAc pCREB, FosB/ $\Delta$ FosB dose dependently increased, regardless of re-exposure context. In addition to temporal differences in protein expression in the NAc and CPU, these proteins may be regulating different aspects of a cocaine-contextual memory. It is necessary to explore the precise mechanisms regulating these differences. Future studies need to be done in order to clarify the increased NAc pERK we observed after cocaine-CPP training and re-exposure to the cocaine-paired environment (but not the control environment) regardless of whether or not CPP behavior was expressed. However, given the role of ERK phosphorylation in associative learning, our data seem to suggest that NAc pERK is necessary to form the association, but is not sufficient to motivate drug-seeking behavior. Again, these data are difficult to interpret and test due to the lack of behavioral readout. Our results suggest that NAc ERK phosphorylation may be involved with retrieving the contextual information of a cocaine-association, without the expression of the behavior. The higher cocaine dose, independent of environment, resulted in increased NAc FosB,  $\Delta$ FosB and phosphorylated CREB (pCREB) protein levels compared to those conditioned with 5mg/kg cocaine. A dose-dependent increase in  $\Delta$ FosB protein levels further implicates NAc  $\Delta$ FosB accumulation in contributing to reward and motivational properties involved with cocaine-associated memories (Lobo et al., 2013). These dose dependent effects may represent downstream regulators of drug-reward processing, and thus work in combination with NAc pERK to establish the contextual motivation and subsequently respond with drug-seeking behaviors when faced with a previously drug-paired context.

Increases in NAc pERK corresponded with increased NAc pCREB and  $\Delta$ FosB protein levels. Increased  $\Delta$ FosB is consistent with previous research that shows  $\Delta$ FosB accumulates in the NAc after cocaine exposure and enhances cocaine reward (Nestler, 2005; Rawas et al. 2012). It remains to be elucidated whether the observed increases in  $\Delta$ FosB protein levels were in response to the cocaine associated context or were a lingering result of cocaine exposure. Due to the short time course of acute cocaine-induced phosphorylation of ERK, CREB, and FosB, we

postulate that increases in pERK, pCREB, and FosB levels probably reflect conditioned responses to the cocaine paired environment in males and females (Girault et al., 2007; Harris et al., 2007; Kuo et al., 2007; Rawas et al., 2012; Sun et al., 2008). Since we did not test a cocaine treated control group in an unpaired context, we cannot rule out the possibility that these changes were due to cocaine alone and not to cocaine-environment associations. However, an overall limitation of the CPP paradigm is that even with the use of an un-paired control group, stimuli in the testing environment may potentially become indirectly associated with cocaine effects and therefore mask differences between paired and unpaired groups (Cunningham et al., 2011). Transcription factors mediate different aspects of addiction.  $\Delta$ FosB increases sensitivity to natural and drug reward. Our results suggest that exposure to cocaine increases NAc pERK and pCREB, which are subsequently increased by drug-free exposure to drug-associated cues. In the CPu, cocaine-induced  $\Delta$ FosB accumulation may be regulating increases in FosB and pERK after drug-free exposure to drug-associated cues (See Figure 32).

Increases in pCREB are seen after chronic cocaine use and may explain why Tropea et al. (2008) observed an increase in p-CREB upon re-exposure to the cocaine-paired environment suggesting that CREB activation (independent of the ERK pathway) may be necessary for the retrieval of cocaine CPP. Furthermore, because of the acute increase in NAc p-CREB observed by Kuo et al. (2007) after a single cocaine-place pairing, it could be that cocaine-induced PKA/DARP-32 activation of CREB may be associated with the initial learning and formation of cocaine associated memories. Marin et al. (2009) found the same pattern increased of context specific cocaine-induced ERK activation in the NAc as CREB phosphorylation suggesting that ERK activation of CREB is responsible for mediating the cocaine-associated memory. However, these results were obtained in response to a drug challenge, indicating that cocaine-induced ERK activation may regulate CREB phosphorylation when in the presence of cocaine and may be involved with the retrieval of the rewarding aspect of these memories.

Figure 32



**Figure 32.** Proposed mechanisms of the observed differential regulation of ERK/CREB/FosB signaling after cocaine CPP expression. Exposure to cocaine increases NAc pERK and pCREB, which are subsequently increased by drug-free exposure to drug-associated cues. In the CPu, cocaine-induced  $\Delta$ FosB accumulation may be regulating increases in FosB and pERK after drug-free exposure to drug-associated cues.

Sexual dimorphisms outside the NAc in ERK/CREB/ $\Delta$ FosB levels were region specific. Similar to males, we demonstrate that in female rats the ERK/CREB signaling cascade is modified after the expression of cocaine induced CPP. However, the NAc was the only brain region in which we observed corresponding increases in pERK, pCREB, and  $\Delta$ FosB, regardless of sex. Results of studies that associate CPP behaviors in males with molecular or synaptic changes outside of the NAc should be cautious when extending these results to females. The changes in ERK/CREB/ $\Delta$ FosB protein levels reported here reflect changes in signaling molecules associated with neuroplastic changes in mesolimbic circuitry. Our observation of the lack of sex differences in the NAc are in accordance with human fMRI studies showing similar patterns of NAc activation in cocaine dependent males and females in response to cocaine conditioned cues, while cocaine-cue induced activation of frontal regions differed based on sex (Kilts et al., 2004). Overall, our results suggest novel sexual dimorphisms in molecular alterations observed after cocaine CPP expression, likely due to sex differences present prior to cocaine exposure.

#### Novel Results Reported

The results presented in this dissertation provide evidence for the role of ERK and CREB intracellular signaling pathways in mediating the neuronal plasticity involved with cocaine associated memories. Specifically, we show for the first time that the NAc and CPu regulate cocaine-induced  $\Delta$ FosB accumulation using different mechanisms and may play different roles in cocaine-induced behaviors. We also show that ERK phosphorylation after cocaine-CPP expression is context-specific and plays a role in the reward-association rather than cocaine-reward alone. Additionally, we show that changes in NAc intracellular signaling were similar in males and females, but sex differences were observed in other brain regions. This result is important due to the large amount of research implicating the NAc in cocaine-reward in males can likely be generalized to include females. Our results will aid in the advancement of the general knowledge about the molecular formation and retrieval of cocaine-associated memories that can be used in the future when designing treatments for cocaine addiction to assist in both the

cessation of addiction and prevention of relapse. The long-term goal of addiction research is to develop treatment strategies that target relapse prevention and diagnostic approaches based on neurobiological markers. Our results will aid in the advancement of more efficient and affective pharmacological treatment strategies. By targeting the intracellular responses we have shown to be associated with motivating drug-seeking behavior, it may be possible for future treatments that can specifically eliminate the motivational components of addiction.



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