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Learning to Be a Cocaine Addict: Behavioral, Pharmacological, and Molecular Characterization of Individual Differences in Initial Learning of Cocaine-Environment Associations

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Learning to Be a Cocaine Addict: Behavioral, Pharmacological, and Molecular Characterization in Initial Learning of Cocaine-Environment Associations

by

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Dedication

To my husband for his love and support

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Learning to Be a Cocaine Addict: Behavioral, Pharmacological, and Molecular Characterization of Individual Differences in Initial Learning of Cocaine-Environment Associations

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Cocaine addiction is a chronic, relapsing disease affecting millions of Americans, and differences between individuals modulate the progression from cocaine use to addiction. Learned associations between cocaine and environmental stimuli develop in the subset of patients who become addicted, and exposure to these stimuli facilitates relapse to cocaine-taking. Classical conditioning underlies the development and expression of these learned associations, and several systems implicated in both the behavioral response to cocaine and in learning and memory—e.g., serotonin$_2$ receptors (5-HT$_2$R), ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate receptor subunit 1 (GluR1), and a signaling system associated with these receptors (i.e., the mitogen-activated protein kinase extracellular-signal regulated kinase; ERK) in the cortical-limbic regions--may modulate the acquisition and expression of cocaine-environment associations. Individual

differences play a major role in the development of addiction, and behavioral models are needed study these implications in the learning of cocaine-environment associations. We utilized the conditioned place preference (CPP) paradigm in laboratory rats to model cocaine-environment associations and developed a new method for the analysis of CPP data that allows for identification of factors that modulate individual sensitivity to the development of cocaine-environment associations, pharmacological treatments that are effective only in subpopulations of subjects, and molecular neuroadaptations that differ among subjects susceptible to the development of cocaine-environment associations and non-susceptible individuals. We uncovered roles for 5-HT_2R in the acquisition and expression of cocaine-environment associations formed after a single pairing of cocaine and environment, suggesting a role for these receptors in modulating the development and retrieval of initial cocaine-environment associations. We observed an increase in the phosphorylation of GluR1 and enhanced expression of total ERK protein in the prefrontal cortex upon retrieval of cocaine-environment associations. These studies suggest that the ability to learn strong cocaine-environment associations is associated with a unique set of neuroadaptations and is a predictor of those who will initiate development of a cocaine addiction.

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Abbreviations

5,7-DHT	5,7-dihydroxytryptamine
5-HT	serotonin
5-HT$_{2A}$R	serotonin 2A receptor
5-HT$_{2C}$R	serotonin 2C receptor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	analysis of variance
CREB	cyclic AMP response element binding
CPP	conditioned place preference
CS	conditioned stimulus
CS^{+}	paired conditioned stimulus
CS^{-}	unpaired conditioned stimulus
DA	dopamine
DSM-IV	Diagnostic and Statistical Manual, 4th Edition
ERK	extracellular-signal regulated kinase
GABA	γ-aminobutyric acid
GluR	glutamate receptor
GluR1	AMPA glutamate receptor subunit type 1
IP	intraperitoneal
MAPK	mitogen associated protein kinase
NAc	nucleus accumbens
NIDA	National Institute on Drug Abuse
NMDA	*N*-methyl-*D*-aspartate
NR1	NMDA receptor subunit 1

NR2A	NMDA receptor subunit 2A
NR2B	NMDA receptor subunit 2B
pERK	phosphorylated ERK
pGluR1	phosphorylated GluR1
PKA	protein kinase A
PFC	prefrontal cortex
UCR	unconditioned response
US	unconditioned stimulus
VTA	ventral tegmental area

Chapter 1: Introduction

The abuse of psychoactive drugs continues to be a serious medical and social problem in the United States. The 2006 National Survey on Drug Use and Health estimated that 20.4 million Americans were current illicit drug users (2006). Of the illicit drugs, cocaine remains one of the most commonly abused substances, as 2.4 million Americans are current cocaine users (2006). Some sources suggest that the rate of cocaine use is increasing (2007). Humans administer cocaine hydrochloride intranasally or via intravenous injection (Gawin & Kleber 1985), while cocaine freebase is inhaled (Gawin & Kleber 1985). Cocaine is commonly used in "runs" (Gawin & Kleber 1985) or "binges" (Siegel 1985) in which multiple doses of the drug are taken over a 4 to 48 hour period (Siegel 1985). A recent report suggested that cocaine users spend an average of $500 per week on cocaine (Lexau *et al.* 1998), though others have reported use of up to a $100,000/year (Gawin & Kleber 1988).

Cocaine use leads to feelings of euphoria, extreme alertness and enhanced self-confidence, leading to a magnification of pleasure (Gawin 1991). Social anxiety and the need for sleep are decreased (Gawin 1991; Gawin & Kleber 1988). Extreme euphoria is achieved during binges, and this heightened emotional state prompts the formation of strong associations between environmental cues and the effects of cocaine (Gawin & Kleber 1988). Acute cocaine intoxication also leads to increases in blood pressure and heart rate (Boghdadi & Henning 1997). Both the psychoactive and physiological effects of cocaine result from the ability of cocaine to block the reuptake transporters of three monoamine neurotransmitter--dopamine (DA), norepinephrine, and

serotonin (5-HT) (Koe 1976)--and thus increase the concentrations of these neurotransmitters in the synapse. The inhibition of norepinephrine reuptake is implicated in the peripheral effects (Boghdadi & Henning 1997). Acute and chronic cocaine use are associated with several serious physiological side effects, including hypertension, myocardial infarction, cardiac dysrhythmias, seizures, and sudden death (Boghdadi & Henning 1997).

COCAINE ADDICTION

Repeated cocaine use induces further negative physiological effects and can lead to the psychological consequence of addiction, a chronic, relapsing disease (McLellan *et al.* 2000). In most patients, addiction develops over several years; during this period, control over cocaine use (typified by daily use) is lost and use of cocaine in binges becomes common (Gawin & Ellinwood 1988;Gawin & Kleber 1988). The Diagnostic and Statistical Manual, 4th Edition (DSM-IV) of the American Psychiatric Association defines two categories of cocaine use disorders: cocaine abuse and cocaine dependence, the latter of which is synonymous with cocaine addiction. Cocaine abuse, the less severe condition, is defined by the presence of one of the four following symptoms during a twelve month period: *use resulting in failure to fulfill a major role obligation at work, school, or home; recurrent use in situations in which it is physically hazardous; recurrent substance-related legal problems;* and *continued use despite having persistent or recurrent social or interpersonal problems exacerbated by the effects of the substance*. The diagnosis of cocaine dependence requires that at least three of the following symptoms be present within a twelve month period: *tolerance to drug effects; withdrawal symptoms; cocaine use in larger amounts*

or over a longer period of time than intended; persistent desire or unsuccessful attempts to decrease cocaine use; great deal of time spent in activities necessary to obtain cocaine; important social, occupational, or recreational activities given up or reduced; and *cocaine use continues despite knowledge of a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by cocaine*. Symptoms of tolerance or withdrawal are not necessary for a diagnosis of cocaine dependence, which recognizes that these symptoms may not be pronounced with cocaine addiction (O'Brien 2008).

Two comprehensive disease models have been suggested for addiction: the first conceives of addiction as a chronic, relapsing disease similar to diabetes (McLellan *et al.* 2000), and the second utilizes an infectious disease model (O'Brien 2003). The chronic disease model suggests that addiction is similar to several other chronic diseases, including type 2 diabetes mellitus, hypertension, and asthma in terms of genetic heritability, role of personal responsibility, and response to treatment (McLellan *et al.* 2000). The infectious disease model, which shows significant overlap with the chronic disease model, suggests that the development of addiction is controlled by several factors that can be characterized as agent/drug-specific (i.e., rapidity of onset of drug effects), host specific (i.e., traits that predispose to addiction), and environmental (i.e, peer pressure; O'Brien 2003). Both of these theories seek to put addiction into a larger disease context to highlight common features with other diseases and to guide conceptualizations of treatments for addiction.

These theories of addiction emphasize that differences between individuals affect many aspects of the development and maintenance of cocaine addiction. For example, individual differences play a role in the choice of

substance that is used (Lejuez *et al.* 2007) and the initial motivation for using cocaine (Gawin & Kleber 1985; Gunnarsdottir *et al.* 2000). The most striking role of individual differences may be in the development of addiction, as only a small percentage (<20%) of cocaine users become addicted (Wagner & Anthony 2002 ; Kendler *et al.* 2003; O'Brien & Anthony 2005). Thus, differences between individuals exist for multiple aspects of drug use, including the propensity for drug use to lead to drug addiction.

The precise factors that lead some to transition from casual cocaine use to addiction are unknown, although both genetic and environmental factors play a role (Kendler *et al.* 2003; Kreek *et al.* 2005; Bierut *et al.* 2008). Epidemiologic studies have suggested that a younger age at initial drug use (first use in early adolescence) predicts the likelihood of development of an addiction (Anthony & Petronis 1995), although a later age of onset of drug use is associated with a faster transition to addiction (Behrendt *et al.* 2009). The co-occurrence of a mental illness, including anxiety, depression, or conduct disorder, increases the risk for the development of addiction in adolescents (Sung *et al.* 2004). These differences between individuals pose a challenge for the development and application of treatments for cocaine addiction.

Several behavioral therapies, including contingency management, cognitive behavioral therapy, relapse prevention, and the combination of contingency management and cognitive behavioral therapy, are effective treatments for cocaine addiction (Dutra *et al.* 2008). Each of these methods seeks to decrease the ability of cues to prompt relapse, and contingency management specifically rewards abstinence. However, only a third of patients who meet the criterion for substance dependence seek treatment (Compton *et al.*

2007), and access to treatment in the United States is severely limited secondary to a wide variety of structural problems that inhibit treatment delivery (McLellan & Meyers 2004). To expand treatment options, the National Institute on Drug Abuse (NIDA) has encouraged the development of pharmacotherapies for cocaine addiction, and several candidate medications have been identified (Vocci *et al.* 2005). Although clinical trials have been conducted with a wide variety of compounds (Mello & Negus 1996; Haney & Spealman 2008), no medications for the treatment of cocaine addiction are currently available (Grabowski *et al.* 2004; O'Brien 2008). Thus, identification of the mechanisms by which cocaine produces addiction as well as the factors that modulate this condition is critically necessary.

COCAINE-CONTEXT ASSOCIATIONS

Role in Relapse

Environmental cues associated with the effects of cocaine also play a major role in the establishment and maintenance of cocaine addiction. Through classical conditioning, previously neutral elements in the environment (stimuli) that are present during cocaine-taking become associated with the effect of the drug (O'Brien *et al.* 1992), and these learned associations between cocaine and cocaine-associated stimuli are powerful forces that can induce a strong desire for cocaine (Childress *et al.* 1999). This effect can be studied in the human laboratory, as the presentation of cocaine-associated stimuli to cocaine users in this setting induced a strong desire for cocaine (Childress *et al.* 1999; Volkow *et al.* 2006) and evoked drug-seeking behavior (Panlilio *et al.* 2005). This phenomenon occurred in both detoxified, treatment seeking-patients (Childress

et al. 1999) and those who were not treatment-seeking (Volkow *et al.* 2006). During cue presentation sessions, patients showed activation (assessed by regional cerebral blood flow) in limbic brain areas (Childress *et al.* 1999)—areas that animal studies suggest are critical to the rewarding properties of cocaine (see below)—even when cue images were presented too rapidly to be processed consciously (Childress *et al.* 2008). Thus, cocaine-associated cues play a significant role in the maintenance of addiction, and research into the biological factors that control these associations is necessary for the development of effective treatments for cocaine addiction.

Animal Models: Conditioned Place Preference and Cue Reinstatement of Self-Administration

The theory that classical conditioning plays a major role in the development of learned associations between the effects of cocaine and environmental cues suggests that certain properties of cocaine become associated with the environment in which the drug is experienced leading to the ability of environmental cues alone to trigger relapse in abstinent users. Cocaine serves as the unconditioned stimulus (US), the rewarding and discriminative stimulus properties experienced upon exposure to cocaine are the unconditioned response (UCR), and the paired environment is a conditioned stimulus (CS). These learned associations between cocaine and the environment in which it is experienced can be modeled in animals using the conditioned place preference paradigm (CPP) (Carr *et al.* 1989; Bardo & Bevins 2000; Tzschentke 2007). During the acquisition phase of the CPP paradigm, animals are treated with cocaine before placement in one environment and with saline before placement

in a distinct environment. The amount of time spent in each environment during a cocaine-free test session is then measured (expression test). An increase in time spent in the environment in which cocaine was experienced is taken as evidence that an association between cocaine and the environment has been learned by the animal and is referred to as a CPP. The temporal separation between the conditioning sessions and the expression test enables the acquisition of the cocaine-environment associations to be examined separately from the expression of the learned association between the effects of cocaine and environmental cues (Tzschentke 1998). For example, the ability of a pharmacological agent to disrupt the learning of the cocaine-environment association (acquisition) can be tested by injecting animals with the agent prior to each cocaine conditioning session; a lack of a CPP upon expression test is then interpreted as a blockade by the pharmacological agent of the acquisition of the cocaine-environment association. In a separate group of animals, the ability of the agent to alter the expression of the memory can be tested by giving the agent selectively prior to the expression test session. A lack of CPP expression in this case is interpreted as a blockade by the pharmacological agent of the retrieval of the cocaine-environment association. Thus, the CPP paradigm offers the ability to study phases of learning in the formation and retrieval of cocaine-environment associations under conditions in which the dose of cocaine and the number of pairings is under control of the investigator (Bardo & Bevins 2000). An additional advantage of the CPP paradigm is the ability to condition a cocaine-environment association following only a single pairing of cocaine and the environment (Bardo *et al.* 1986), which allows for the study of learned associations free from

neuroadaptations associated with repeated cocaine treatment (Bardo & Bevins 2000).

The ability of cues to drive behavior can also be modeled with the cue-induced reinstatement of self-administration paradigm (Shaham *et al.* 2003). Rats are implanted with in-dwelling subcutaneous jugular vein catheters, and placement of the rats in a specially designed apparatus allows the rat to perform an operant (i.e., nose poke or lever press) to receive an infusion of a cocaine solution, which can be paired with a novel stimulus ("cue," i.e., light illumination) or with the self-administration environment ("context") (Shaham *et al.* 2003). Rats will rapidly learn to self-administer cocaine (for example, see Nic Dhonnchadha *et al.* 2008). Non-contingent presentation of the cue alone will engender a high rate of operant performance, which is interpreted as evidence for a learned association between the cue and drug delivery (Shaham *et al.* 2003). This phenomenon is thought to mimic the ability of cocaine-associated cues to prompt relapse to drug-taking in humans.

Neuroanatomy

An early theory of the neurocircuits involved in addiction emphasized a role for increased synaptic dopamine in the terminal fields of the mesolimbic and mesocorticortical pathways as critically important to the addictive properties of cocaine (Wise & Bozarth 1987; Wise 1996). The mesolimbic pathway is traditionally defined as a collection of dopaminergic neurons that originate in the ventral tegmental area (VTA) and project rostrally to several areas including the nucleus accumbens (NAc), hippocampus, and amygdala, although the term can be used to describe either a more restricted or broader set of connections

(Ikemoto 2007). The mesocortical pathway originates in the VTA and projects to the cerebral cortex, including the prefrontal cortex (PFC). Several lines of evidence have implicated the NAc as critical to both the rewarding properties of cocaine (Di Chiara & Imperato 1988; Hurd *et al.* 1989; Rodd-Henricks *et al.* 2002; Navailles *et al.* 2007) and learned associations between environmental stimuli and the effects of cocaine (Phillips *et al.* 2003). Several of the terminal regions—the PFC, hippocampus, amygdala--have all been implicated as subserving critical roles in the learning of and memory for cocaine-context associations (Brown *et al.* 1992; Franklin & Druhan 2000; Zavala *et al.* 2008).

Prefrontal Cortex

The PFC plays a role in signaling the unconditioned rewarding properties of cocaine (reviewed in Tzschentke 2000). Neurotoxin-induced lesion of the PFC inhibited the acquisition of a cocaine CPP (Tzschentke & Schmidt 1999; Pum *et al.* 2008). This region is also involved in the retrieval of cocaine-cue (McLaughlin & See 2003) or context associations (Fuchs *et al.* 2005). *In vivo* electrophysiological recording in rats previously trained in cocaine self-administration has demonstrated a sub-population of PFC neurons that increase firing in response to presentation of cocaine-paired cues (Rebec & Sun 2005). Thus, the PFC is critical to the formation and retrieval of cocaine-environment associations.

The PFC, as opposed to the hippocampus and amygdala (see below), may be particularly important for signaling related to cocaine reward. Lesion of the PFC did not affect acquisition of an amphetamine CPP (Tzschentke & Schmidt 1999) nor the acquisition of instrumental transfer learning which was

reinforced by presentation of a cue previously paired with sucrose delivery (Burns *et al.* 1993). Re-exposure to a cocaine-paired (Brown & Fibiger 1993; Franklin & Druhan 2000), but not a food-paired (Zombeck *et al.* 2008), environment has been associated with PFC activation as measured by the expression of c-Fos (a marker of neuronal activity; Sagar *et al.* 1988) and a related protein (Fos related antigen). Thus, the PFC is critical to the neuronal circuit involved in the formation and expression of cocaine-context associations.

Hippocampus

Several major divisions of the hippocampus and related cortex have been implicated in this form of learning, including the dorsal hippocampus, ventral hippocampus, dentate gyrus, and the entorhinal cortex. Lesion (Meyers *et al.* 2003) or temporary inactivation (Meyers *et al.* 2006) of the dorsal hippocampus blocked the acquisition of a cocaine CPP, and temporary inactivation of this area prevented the expression of a cocaine CPP (Meyers *et al.* 2006) and context-induced reinstatement of self-administration (Fuchs *et al.* 2005). Acquisition studies thus suggest a role for the dorsal hippocampus in the learning of cocaine-context associations, and expression studies suggest a role for the dorsal hippocampus in the retrieval of these memories. The dorsal hippocampus has also been implicated in the learning of stimulus-environment associations for both non-drug appetitive (Ferbinteanu & McDonald 2001) and aversive stimuli (for review, see Sanders *et al.* 2003). Thus, the dorsal hippocampus is critical for the acquisition and retrieval of several types of stimulus-context associations, including associations between cocaine and environmental contexts.

Other subdivisions of the hippocampus—the ventral hippocampus, dentate gyrus, and entorhinal cortex—have also been implicated in the acquisition of cocaine-context associations. Although the ventral hippocampus may play a role in the acquisition and expression of cocaine-cue associations when drug delivery is controlled by the animal (Rogers & See 2007; Atkins *et al.* 2008), a lesion of this area had no effect on the acquisition of a cocaine CPP (Meyers *et al.* 2003). Lesion of the dentate gyrus following colchicine infusion blocked the acquisition of a cocaine CPP (Hernandez-Rabaza *et al.* 2008), as did lesion of the serotonergic fibers in the entorhinal cortex with the toxin 5,7-dihydroxytryptamine (5,7-DHT; Pum *et al*. 2008). Thus, several parts of the hippocampus and related cortex are involved in the formation of cocaine-context associations.

Amygdala

The amygdala nuclei are also involved in the formation and expression of cocaine-context associations. Exposure to an environment previously paired with cocaine was associated with expression of the protein c-Fos in the amygdala (Brown *et al.* 1992). Various subdivisions of the amygdala have been implicated in the acquisiton (Hiroi & White 1991; Brown & Fibiger 1993; Fuchs *et al.* 2002) and expression of psychostimulant-environment associations, including cocaine CPP (Hiroi & White 1991; McLaughlin & See 2003; Peters *et al.* 2008). The basolateral subdivision of the amygdala appears critical to the formation of context associations, both for an appetitive non-drug reinforcer (Cador *et al.* 1989; Burns *et al.* 1993) and non-drug aversive punishers (for review, see

Fanselow & LeDoux 1999; Maren 2003). Thus, the amygdala is an important structure for the formation and expression of cocaine-cue associations.

Neurochemistry

Several lines of evidence have implicated dopamine signaling as critical to the rewarding properties of cocaine (Di Chiara & Imperato 1988; Hurd *et al.* 1989; Rodd-Henricks *et al.* 2002; Navailles *et al.* 2007) and learned associations between environmental stimuli and the effects of cocaine (Phillips *et al.* 2003). Recent work has focused attention on the roles of other neurotransmitters, including 5-HT and glutamate, in signaling cocaine-environment associations.

Serotonin

The 5-HT receptor family consists of sixteen different receptors (Hoyer *et al.* 2002). Of these, the 5-HT_{2A} receptor (5-HT_{2A}R) and the 5-HT_{2C} receptor (5-HT_{2C}R) have been implicated in mediating the behavioral effects of cocaine (Bubar & Cunningham 2006). The 5-HT_{2A}R and 5-HT_{2C}R are seven transmembrane domain G-protein coupled receptors encoded by different genes, although these two receptors share a high degree sequence homology (Hoyer *et al.* 2002). Despite these similarities, work from our laboratory and others has consistently shown an opposing action of these two receptors on the acute effects of cocaine. For example, both a selective antagonist at the 5-HT_{2A}R (McMahon & Cunningham 2001) and a preferential 5-HT_{2C}R agonist (Filip *et al.* 2004) blocked cocaine-induced increases in locomotor activity in rats.

Signaling through these 5-HT receptors has also been implicated in mediating cocaine-environment associations. Non-selective *antagonists* at the 5-HT_{2A}R block the acquisition of a cocaine CPP (Kosten & Nestler 1994; Jones &

McMillen 1995; Meil & Schechter 1997; Arolfo & McMillen 2000). A selective 5-HT_{2A}R antagonist inhibited the expression of cue-induced reinstatement of cocaine seeking (Nic Dhonnchadha *et al*. 2008). These results suggest that the 5-HT_{2A}R plays a critical facilitatory role in the acquisition and expression of cocaine-context associations.

The 5-HT_{2C}R also plays a role in modulating cocaine-environment associations. *d*-Fenfluramine, a drug that enhances 5-HT release, attenuated cue-induced reinstatement of cocaine-seeking (Burmeister *et al.* 2004), an effect that was blocked by pretreatment with the selective 5-HT_{2C}R antagonist SB 242084 (Burmeister *et al.* 2004). Treatment with either of two agonists that are preferential for the 5-HT_{2C}R, MK 212 and Ro 60-0175, prevented the expression of the cue- (Neisewander & Acosta 2007; Burbassi & Cervo 2008) or context-induced reinstatement of self-administration (Fletcher *et al.* 2008), although interpretation of the blockade of cue-induced reinstatement is complicated by a suppression of basal locomotor activity by the doses of agonist that inhibited reinstatement (Neisewander & Acosta 2007). MK 212 treatment also prevented the expression of a classically conditioned response to a cocaine-paired environment, and this behavior was enhanced by treatment with the 5-HT_{2C}R antagonist SB 242084 (Liu & Cunningham 2006). The 5-HT_{2C}R modulation of cocaine-environment associations may be specific to cocaine, as Ro 60-0175 treatment had no effect on the reinstatement induced by sucrose-paired cues (Burbassi & Cervo 2008). These results suggest that the 5-HT_{2C}R plays a critical inhibitory role in the expression of cocaine-context associations.

Glutamate

Glutamatergic signaling has been implicated in several aspects of addiction (Kalivas *et al.* 2009). The glutamate receptor (GluR) family consists of four major classes: metabotropic receptors, ionotropic kainate receptors, ionotropic α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors, and ionotropic n-methyl-d-aspartate (NMDA) receptors. Both AMPA and NMDA receptors have been heavily implicated in learning (Klann & Sweatt 2008) and drug addiction (Kelley 2004). These receptors are heteromeric ion channels formed by a combination of subunits; for AMPA receptors, these subunits are termed GluR1-4, while NMDA receptors are composed of the constitutively expressed NR1 and the modulatory NR2A-D and NR3A subunits (Dingledine *et al.* 1999; Cull-Candy *et al.* 2001). The binding of glutamate to AMPA receptors induces the opening of these channels and the influx of sodium ions; the calcium permeability of AMPA receptors is dependent on the subunit composition (Dingledine *et al.* 1999). NMDA receptors require the binding of glutamate and glycine and simultaneous membrane depolarization in order to allow influx of calcium ions.

Several lines of evidence have implicated AMPA receptors in mediating cocaine-environment associations, although the literature contains several conflicting studies. Systemic pharmacological inhibition of AMPA receptors (including receptors that contain GluR1) with CNQX blocked acquisition and expression of a cocaine CPP (Maldonado *et al.* 2007) and expression of cue-induced reinstatement (Backstrom & Hyytia 2006). Others, however, have found that pharmacological inhibition of AMPA receptors selectively blocked the expression but not the acquisition of a cocaine CPP (Cervo & Samanin 1995).

This discrepancy between pharmacological studies is paralleled in studies utilizing genetic manipulations to identify the specific role of the GluR1 subunit in modulating responses to cocaine-environment associations, as germline GluR1 knockout mice acquire a cocaine CPP under some (Mead *et al.* 2005) but not all conditions (Dong *et al.* 2004). Thus, the role of AMPA glutamate receptors in the acquisition and expression of cocaine-environment associations remains to be determined.

Intracellular Signaling

Several alterations in molecular signaling have been identified in animals that exhibit learning of cocaine-context associations (Valjent *et al.* 2000; Beninger & Gerdjikov 2004; Miller & Marshall 2005; Lai *et al.* 2008; Tropea *et al.* 2008). These studies have identified the mitogen-activated protein kinase (MAPK) extracellular-signal regulated kinase (ERK) as a key molecular substrate for this behavior. ERK is a mitogen-associated protein kinase (MAPK) that regulates a wide variety of cellular mechanisms by phosphorylating serine and/or threonine residues of an array of proteins that represent diverse protein classes, including transcription factors, cytoskeletal proteins, membrane-associated proteins, and other kinases (Grewal *et al.* 1999). The activation of ERK, itself, requires dual phosphorylation by the MAPK/ERK kinase (MEK) (Lu *et al.* 2006), which is stimulated via several different upstream signaling cascades (Werry *et al.* 2005b). A role for ERK in the learning of stimulus-environment associations was first identified in classical fear conditioning, and these early studies documented an activation of ERK (assessed as phosphorylation) in the hippocampus of animals one hour after the conditioning session (Atkins *et al.*

1998; Selcher *et al.* 2002). This increase in the ERK phosphorylation (pERK) occurred in the absence of a change in total ERK protein expression and was necessary for learning, as pharmacological inhibition of ERK activation prevented acquisition of fear conditioning (Atkins *et al.* 1998).

Activation of ERK appears to be similarly involved in the acquisition and expression of cocaine CPP. Expression of a cocaine CPP (Miller & Marshall 2005) and re-exposure of drug-free animals to a cocaine-paired environment (Tropea *et al.* 2008) are associated with activation of ERK; this activation occurred in the absence of changes in total ERK protein expression. Systemic administration of an ERK inhibitor blocked the acquisition of a cocaine CPP (Valjent *et al.* 2000). Intra-NAc blockade of ERK activation blocked the acquisition of an amphetamine CPP (Gerdjikov *et al.* 2004) and the expression of a cocaine CPP (Miller & Marshall 2005). Together, these studies suggest a vital role for ERK activation in the acquisition and expression of cocaine CPP.

The series of studies that suggested a role for ERK in mediating cocaine-environment associations has also implicated several modulators upstream of ERK activation as well as downstream targets. Upstream modulators of ERK activation include the 5-HT_{2A}R and 5-HT_{2C}R (Quinn *et al.* 2002; Werry *et al.* 2005a) as well as the NMDA receptor (Lu *et al.* 2006; Zhai *et al.* 2008). An NMDA receptor antagonist inhibited cocaine-induced ERK activation (Valjent *et al.* 2000). One consequence of ERK activation is activation of the transcription factor CREB (Carlezon *et al.* 2005), and CREB activation is increased in rats that express a cocaine CPP (Miller & Marshall 2005). The possible roles of these upstream and downstream targets of ERK activation in the acquisition and

expression of cocaine-environment associations, however, are not yet fully defined.

The following set of studies were designed to create a behavioral, pharmacological, and molecular characterization of the CPP paradigm to model the individual differences involved in the transition from casual to compulsive drug use. The first set of studies (**Chapter 2**) examined the behavioral characteristics of CPP expression and identified a role for the 5-HT_{2C}R in modulating the expression of the CPP formed after a single pairing of cocaine and environment. The second set of studies (**Chapter 3**) utilized pharmacological techniques to examine the role of the 5-HT_{2A}R in the modulation of the acquisition and expression of both a single- and four-trial cocaine CPP. A third set of studies (**Chapter 4**) examined the signaling events that are associated with the expression of a single trial cocaine CPP compared to re-exposure to cocaine-paired cues. The overall goal of the studies presented in this dissertation was to model in rats the initial period of human cocaine taking to better understand the differences among groups of subjects that change with and modulate this period.

Chapter 2: Novel Approach to Data Analysis in Cocaine Conditioned Place Preference

ABSTRACT

Only a sub-group of human drug users progress from initial drug-taking to drug addiction. An important aspect of this progression is the influence of the learned association between the effects of the drug and the environment in which it is experienced on continued drug-taking and -seeking. These associations can be modeled using the conditioned place preference (CPP) paradigm, although no current method of CPP analysis allows for identification of within group varability among subjects. In the present study, we adapted a “criterion” method of analysis to separate “CPP expressing” from “non-CPP expressing” rats to more directly study within group variability in the CPP paradigm. Male Sprague-Dawley rats were conditioned with cocaine (5, 10, 20 mg/kg) or saline in an unbiased three chamber CPP apparatus in either a single or four trial CPP procedure. A classification and regression tree analysis of time spent in the cocaine-paired chamber established a time of 324 sec spent in the cocaine-paired chamber as the criterion for cocaine CPP expression. This criterion effectively discriminated control from cocaine conditioned rats and was reliable for rats trained in both a single and four trial CPP procedure. The criterion method showed an enhanced ability to detect effective doses of cocaine in the single trial procedure and a blockade of CPP expression by MK 212 (0.125 mg/kg) treatment in a sub-group of rats. These data support the utility of the criterion analysis as an adjunct to traditional methods that compare group averages in CPP.

INTRODUCTION

Variability in the response to cocaine plays a major role in the development of cocaine abuse and addiction. One estimate predicts that five to six percent of cocaine users will become addicted within the first two years of cocaine use (O'Brien & Anthony 2005). The biological substrates that allow a sub-group of cocaine users to remain in control of their drug use while a different sub-group becomes addicted are largely unknown. Achievement of sustained abstinence in the sub-group of users who do become addicted to cocaine is challenging as stimuli commonly associated with cocaine use (i.e., drug paraphernalia) elicit craving and relapse to drug-taking (Childress *et al.* 1999), a process likely supported by the development of learned, classically-conditioned associations between the effects of cocaine (unconditioned response) and the environment in which cocaine is experienced (conditioned stimulus; Childress *et al.* 1992). These observations suggest a critical need for a method to analyze differences between population sub-groups in the response to cocaine-associated cue environments.

The conditioned place preference (CPP) paradigm is a very attractive animal model of these conditioned cocaine-environment associations. In the acquisition phase of this paradigm, cocaine administration is temporally paired with placement in a distinct environment, and treatment with saline is paired with an alternate environment. Although the exact properties of cocaine that become associated with the environment during the conditioning sessions are unknown, both the rewarding and discriminative stimulus properties of cocaine are thought to comprise (in part) the unconditioned stimulus and thus are important for the

formation of the CPP. Following conditioning sessions, subjects are allowed to freely roam both environments and the time spent in each environment is assessed. During this expression test, increased time spent in the environment formerly paired with cocaine is defined as a CPP and suggests that an association between cocaine and the environment has been learned (Bevins & Cunningham 2006).

Determination of CPP expression typically involves computation of the average performance on a specific metric (i.e., time in the cocaine-paired environment; Cunningham *et al.* 2003). Previous reports suggest, however, that calculating a group average may obscure differences between population sub-groups in the acquisition or expression of CPP. For example, Adams and colleagues (Adams *et al.* 2001) observed variability in the ability of the dopamine D_1 receptor antagonist SCH 23390 (0.03 mg/kg) to block the expression of cocaine CPP; specifically, rats treated with SCH 23390 before the test session "could be divided into three separate and equal-sized groups" based upon differences in the amount of time spent in the chamber during the test session that had been paired with cocaine compared to that paired with saline. Population sub-groups have also been observed in CPP when 3,4-methylenedioxymethamphetamine (MDMA) was used as the conditioning drug; in this research, some animals showed a strong CPP, while others showed an aversion to the MDMA-paired chamber (Daza-Losada *et al.* 2007). These reports suggest the need for an additional method for the analysis of CPP data that allows for separation of subjects within a treatment group. A method that allows for the dichotomous categorization of individual subjects within a treatment group

as “CPP expressing” or “non-CPP expressing” would provide a complementary approach to consider differences among subjects within a treatment population.

We thus sought to develop a criterion for CPP analysis that would allow individual subjects to be categorized into two sub-groups: “CPP expressing” or “non-CPP expressing.” To establish this criterion, data from rats conditioned with saline in both environments (control) were compared with data from rats conditioned with cocaine to identify a numerical cut-off based on the number of seconds spent in the cocaine-paired chamber that appropriately separated control from cocaine groups. The criterion was confirmed by comparing the dose-effect relationship for cocaine to elicit a CPP generated by the criterion analysis to that generated by a traditional analysis of time spent in the cocaine-paired chamber (Martin-Iverson & Reimer 1996; Belzung & Barreau 2000). Following establishment, this criterion method was used to analyze the effects of several doses of MK 212, a preferential agonist at the serotonin 2C receptor (5-HT_{2C}R), on the expression of single-trial CPP. MK 212 was investigated given previous evidence suggesting involvement of the 5-HT_{2C}R in the behavioral response to cocaine-associated cues in rats (Liu & Cunningham 2006). Employing the criterion analysis, we demonstrate an enhanced ability to detect a cocaine CPP in the single trial procedure as well as pharmacological blockade of CPP in a sub-group of rats, suggesting this new method of analysis for CPP data may be useful as an adjunct to traditional methods for the study of differences between sub-groups in the CPP paradigm.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighed 225-275 g at the beginning of the study. The rats were housed four per cage in standard, clear plastic rodent cages with food and water available *ad libitum* in a temperature (21-23°C) and humidity (55-65%) controlled environment under a 12-h light/dark cycle (lights on 0700 h). Animals were acclimated to the colony for at least one week and were handled prior to the start of experimental sessions. All experiments were conducted during the light phase of the light-dark cycle (0800-1800 h) and were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and with approval from the Institutional Animal Care and Use Committee.

Drugs

Cocaine HCl salt (National Institute on Drug Abuse, Research Triangle, NC) and MK 212 [6-chloro-2-(1-piperazinyl)pyrazine hydrocholoride; Tocris, Ellisville, MO] were dissolved in 0.9% NaCl. All injections were given IP in a volume of 1 ml/kg. Doses of all drugs refer to the weight of the salt. Doses of MK 212 utilized in this study were based on previous work from our laboratory (Liu & Cunningham 2006).

Conditioned Place Preference Apparatus

The CPP apparatuses were housed within sound- and light-attenuating cabinets and contained three sensory environments distinguished by wall colors and floor textures (n=8, ENV-013, Med Associates, Georgia, VT). The two side conditioning chambers (interior dimensions: 25.5 cm L x 21.0 cm W x 20.9 cm H) were separated by a smaller chamber (13.2 cm L X 21.0 cm W x 20.9 cm H). One conditioning chamber had white walls and a stainless steel mesh (1.3 x 1.3 cm) floor and the other conditioning chamber had black walls and a floor of stainless steel rods (4.8 mm placed on 1.6 cm centers). The center chamber had gray walls and a floor of sheet metal. Guillotine doors separated each conditioning compartment from the center compartment. The intensity of ambient illumination was adjusted to 7 lux in each conditioning chamber and 30 lux in the center chamber to balance side preference for each conditioning chamber (Roma & Riley 2005). Automated data collection was accomplished through photobeam detectors. There were 15 infrared photobeam detectors that were 4.5 cm above the chamber floor. Of these, six photobeams were arrayed along the length of each conditioning compartment 1.25 cm from the end wall with 5 cm between beams. Three photobeams were arrayed along the length of the central compartment spaced 4.75 cm apart. The photobeams were connected to a computer interface, and MED-PC software (Med Associates) recorded the time spent in each chamber based on the recorded activity counts (any beam break within the current chamber) between disruption of (1) the entrance beam of that chamber (beam break beyond the first beam of a chamber) and (2) the entrance beam of a different chamber.

Acquisition and Expression of Cocaine Conditioned Place Preference

Each experiment used separate groups of naïve animals. The CPP training consisted of three phases (preconditioning, conditioning, and test) which occurred over consecutive days. In all phases, the animals were transferred in their home cages from the animal colony to the test room 5 min before the animals were placed in the CPP apparatus. To determine baseline preference (preconditioning), rats were placed in the center chamber with the guillotine doors raised and allowed to freely roam the apparatus for 30 min. The time spent in each conditioning chamber was recorded, and the conditioning chamber in which an animal spent the least amount of time was designated the initially least-preferred chamber for that animal. Conditioning was performed using a biased protocol in which cocaine was paired with the initially least-preferred chamber (Spyraki *et al.* 1982; Blander *et al.* 1984; Isaac *et al.* 1989).

Cocaine CPP conditioning sessions consisted of alternating sessions of cocaine and saline injections with the guillotine doors in place. Two sessions were conducted each day separated by at least six hours (Shippenberg & Heidbreder 1995), thus rats experienced both a morning and an afternoon conditioning session. For cocaine CPP training, one daily conditioning session began with an injection of cocaine and immediate confinement to the initially least-preferred chamber for 45 min. The other daily conditioning session began with a saline (1 mL/kg, IP) injection and immediate confinement to the alternate conditioning chamber for 45 min. Thus, some rats received cocaine during the morning session, and others received cocaine during the afternoon session. Control rats were injected with saline prior to placement into each conditioning chamber for both sessions on each day. At the termination of the session, rats

were returned to their home cages. Rats experienced one conditioning session with cocaine and one with saline for studies in the single-trial conditioning procedure. In the four-trial conditioning procedure, rats experienced eight alternating sessions of cocaine and saline injections over four consecutive days.

The expression test for CPP was conducted 16-24 hrs after the final conditioning session. The time of the test session (morning vs. afternoon) was counterbalanced across rats relative to the time of cocaine conditioning. Rats were placed in the center chamber with the guillotine doors raised and allowed to freely roam the apparatus for 15 min while the time spent in each chamber was recorded. Data are presented as mean time (sec $\pm$ S.E.M.) spent in the initially least-preferred chamber.

Establishment of Criterion for CPP Expression

To develop a criterion that would allow the identification of a sub-group of rats that express a CPP versus a sub-group that does not express a cocaine CPP, we rationalized that control rats (conditioned with saline) should not express a CPP, while animals conditioned with cocaine should express a CPP. We thus set out the goal of identifying a numerical cut-off (i.e., number of seconds spent during the test session in the initially least-preferred chamber) that would separate the control from cocaine groups. To establish this value, we compiled CPP data from the expression test in animals (n=279) conditioned with saline (control) or cocaine (one pairing with 20 mg/kg of cocaine or four pairings with 10 mg/kg of cocaine without any other drug treatments); these data were collected in the course of several studies run in our laboratory over a 3-year period (dela Cruz and Cunningham, in preparation). The amount of time spent by each rat in the initially least-preferred chamber during the expression test session

was organized into 25 sec time bins (i.e., 200-224 sec, 225-249 sec, etc). The resulting curves for control and cocaine groups were compared, and exact P values for the chi square test for independence were estimated using Cytel Studio Software (version 6.3, Cambridge, MA; Kupperman 1960) using the Monte Carlo option. The mean and median for each group (control and cocaine) were calculated and a t-test was used to compare the group means.

To establish the numerical cut-off that could serve as a criterion for CPP expression, we subjected the compiled CPP data to classification and regression tree analysis (CART; Salford Systems, San Diego, CA). The CART analysis is a form of binary partitioning (Lewis 2000) used to identify population subgroups as well as the factor (i.e., number of seconds spent during the test session in the initially least-preferred chamber) upon which the groups differ (Lemon *et al.* 2003). The data from all subjects (for our analysis, the amount of time each rat spent during the test session in the initially least-preferred chamber) were input into a "parent node" which is then split into two "child nodes" based on a "splitting" criterion. This splitting criterion seeks to minimize the average impurity in each child node by achieving the largest difference between the impurity of the parent node and the weighted average impurity of the two child nodes (Lemon *et al.* 2003). The minimum value for impurity is zero, which implies no variability in the dependent variable; the maximum value for impurity is 0.5 when subjects from the underlying subpopulations are equally distributed in the child nodes (Zhang *et al.* 1996; Lemon *et al.* 2003). Thus, the splitting criterion established by the CART analysis was deemed the criterion used to categorically identify rats that express or do not express a cocaine CPP.

Assessment of Cocaine CPP Criterion Reliability

To assess the ability of the criterion to identify rats that express cocaine CPP, we first analyzed the expression of CPP following conditioning with different doses of cocaine (0, 5, 10, or 20 mg/kg) in separate groups of rats. Preconditioning was conducted as described, and rats were then randomly assigned to one of eight groups: single trial control (0 mg/kg), single trial 5 mg/kg, single trial 10 mg/kg, single trial 20 mg/kg, four trial control, four trial 5 mg/kg, four trial 10 mg/kg, and four trial 20 mg/kg. Conditioning was conducted as described. Testing was conducted 16-24 hrs after the final conditioning session, as described. The percentage of subjects that met the criterion for positive expression of CPP (and thus categorized as "CPP expressing") was compared in the control and cocaine groups using χ^2 analysis and simple comparisons between the control and each cocaine group were conducted using Fisher's exact test (Sheskin 2004). For purposes of comparison, one-way ANOVA followed by Student-Newman-Keuls' test was used to compare the amount of time spent during the test session in the initially least-preferred chamber between each conditioning group.

Application of Cocaine CPP Criterion

Following assessment of the reliability of the criterion, the cutoff was applied to analyze a data set in which rats showed variability within a given conditioning group in the amount of time spent in the initially least-preferred chamber during the CPP expression test. The effects of the preferential 5-HT_{2C}R agonist MK 212 (0, 0.125, 0.25 mg/kg) on the expression of a single-trial cocaine (20 mg/kg) CPP were determined. Preconditioning was conducted as described above and rats were randomly divided into six groups. The next day, two 45 min

conditioning sessions took place 6 hours apart. In one session, cocaine rats received a cocaine (20 mg/kg) injection immediately prior to confinement to the chamber determined to be least-preferred during preconditioning; in the other conditioning session, cocaine animals received saline (1 ml/kg) immediately prior to confinement to the opposite conditioning chamber. Control animals received saline immediately prior to both conditioning sessions. The expression test for CPP was conducted 16-24 hours following the final conditioning session. Rats (n=21-23/group) received an injection of MK 212 or saline 10 min before the expression test session.

Two analyses were performed to assess the ability of MK 212 to block the expression of cocaine CPP. The binary outcome of "CPP expression" (yes ≥ 324 sec versus no < 324 sec) was used in a logistic regression with predictor variables conditioning (saline versus cocaine) and MK 212 treatment (0, 0.125, 0.25 mg/kg) and on the conditioning x treatment interaction. *A priori* individual comparisons were performed using categorical modeling via the CATMOD procedure in SAS. For comparison, a traditional analysis using the continuous variable mean time spent in the initially least-preferred chamber during the test session was analyzed using a two-way ANOVA with factors conditioning (saline versus cocaine) and MK 212 treatment (0, 0.125, 0.25 mg/kg) and the conditioning x treatment interaction. *A priori* individual contrasts were conducted for pairwise comparisons. For all comparisons, α was set at 0.05.

RESULTS

Establishment of Criterion for CPP Expression

Data from preconditioning sessions were compiled for all rats (n=342) in this study. Out of the 1800 sec session, rats spent a mean (± SEM) of 700.06 sec (± 8.68) on the black side (39.0% of total time) and 680.01 sec (± 8.95) on the white side (38.0% of total time); the remaining time (420.00 ± 7.17 sec) was logged in the center grey chamber. The white chamber was the initially least preferred chamber for 51% of rats, and the black chamber was initially least preferred for 49% of rats. A $\chi 2$ analysis indicated that these percentages did not significantly differ than those due to chance, thus the CPP apparatus with the conditions employed is considered unbiased.

The time spent during the test session in the initially least-preferred chamber for rats in either the control (n=121) or cocaine (n=158) groups was divided into 25 sec bins and plotted. We observed a clear distinction between the amount of time spent in the initially least-preferred chamber during the test session in cocaine versus control groups, as expected, and a $\chi 2$ analysis revealed a significant difference between the distribution of control and cocaine group members across time bins ($p<0.0001$, **Fig. 2.1**). For the control group, the bin that contained the most rats (n=23) was 275-299 sec, with an average time (±SEM) spent in the initially least-preferred chamber equal to 275 ± 8 sec (Fig. 2.1A). Rats in the control group spent a median of 276 sec (interquartile range 225-319 sec, Fig. 2.1B) in the initially least-preferred chamber during the test session. The control group spent 338 ± 8 sec in the initially more-preferred chamber during the test session.

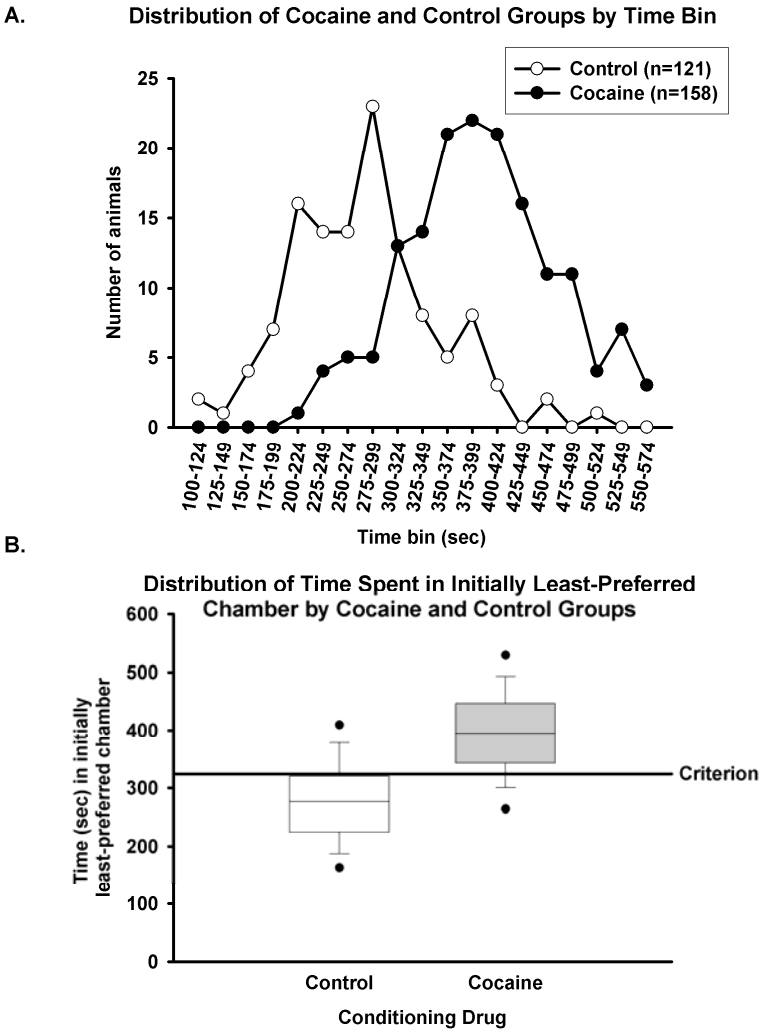


Figure 2.1: Distribution of time spent in the initially least-preferred chamber by control and cocaine-conditioned groups. A, Rats in control (n=121, open circles) and cocaine (n=158, black circles) groups were tested for the expression of CPP in a 15 min session. The amount of time spent by each rat during the test session in the initially least-preferred chamber was categorized according to 25 sec bins. $\chi 2$ analysis revealed a significant difference between the two distributions ($p<0.0001$). B, Rats in control (open box) and cocaine groups (grey box) were tested for the expression of CPP in a 15 min session. The line in the center of the box represents the median time, and the top and bottom of the box represent the 75th and 25th percentiles, respectively. The black circles represent the 95th and 5th percentiles. The solid line ("criterion") represents the 324 sec criterion for the expression of cocaine CPP.

To further demonstrate the unbiased nature of the apparatus, the amount of time that control (n=121) animals spent in each chamber during the 15 min test session was analyzed. On test day, control animals spent an average (±SEM) of 306.84 sec (±7.17) in the black chamber (34.1% of total time) and 305.89 sec (±7.889) in the white chamber (34.0% of total time); the remaining time (287.43 ± 7.45 sec, 31.9% of total time) was logged in the center grey chamber. Thus, non-drug conditioned animals do not develop a natural preference for one conditioning chamber, and the apparatus conditions employed are considered unbiased.

For the cocaine group, the bin that contained the most rats (n=22) was 375-399 sec, with an average time (±SEM) spent in the initially least-preferred chamber equal to 394 ± 6 sec (**Fig. 2.1A**). In comparison, the cocaine group spent an average time (±SEM) of 273 ± 5 sec in the initially more-preferred (saline paired) chamber, significantly less time than was spent in the initially least-preferred chamber ($p<0.0001$). This group spent 234 ± 5 sec in the center grey chamber. Rats in the cocaine group spent a median of 394 sec (interquartile range 343-446 sec, **Fig. 2.1B**) in the initially least-preferred chamber during the test session. A significant difference between the average amount of time spent in the initially least-preferred chamber during the CPP expression test was observed between the control and cocaine groups (**Fig. 2.1A**, $T=13.05$, $p<0.0001$).

To develop a criterion for the expression of CPP, an assessment of the amount of time spent in the initially least-preferred chamber during the expression test by rats in the cocaine group compared to control group was performed using CART analysis (see Methods). This analysis revealed that a

criterion of 324 sec yielded the greatest separation between cocaine and control groups. Thus, any rat that spent at least 324 sec in the initially least-preferred chamber during the test session was categorized as a “CPP expressing” subject.

Binary classification based upon a specific criterion provides a means to discriminate subjects within a population as either “CPP expressing” or “non-CPP expressing.” In a perfect separation, all control rats would be classified as “non-CPP expressing” and all cocaine rats would be classified as “CPP expressing.” However, a perfect separation based upon a binary classification is rarely seen, and, indeed, the distributions of data collected in control and cocaine groups during the test session overlap (**Fig. 2.1A**). Binary classification schemes are commonly used in the clinical literature to assess the likelihood of disease presence given a positive test result, and this field has developed language to describe the four possible outcomes from this type of categorization: true positive, true negative, false positive, or false negative. “Positive” describes a positive test result, while “negative” describes a negative test result. “True” describes a test result consistent with disease state (“true positive” is a positive test result when disease is present while a “true negative” is a negative test result when disease is absent) and “false” describes a test result inconsistent with disease state (“false positive” is a positive test result in the absence of disease while “false negative” is a negative test result in the presence of disease). We have utilized this language to assess how well a classification provided by the criterion (“expressing” or “not expressing”) matched the actual conditioning protocol (cocaine vs control). Based on the CPP criterion (324 sec), rats defined as not expressing (<324 sec) are classified as “negative,” and those defined as expressing (≥ 324 sec) are classified as “positive.” Rats in the *cocaine* group

correctly classified as “CPP expressing” are classified as “true positives,” and rats in the cocaine group defined as “non-CPP expressing” are “false negatives.” Rats in the *control* group correctly classified as “non-CPP expressing” are “true negatives,” while those defined as “CPP expressing” are considered “false positives.” With these designations in place, we then determined the true positive and true negative probabilities of the 324 sec criterion. The true positive probability (Sheskin 2004)--equal to the number of true positives divided by the total number of rats in the cocaine group--is a measure of the ability of the criterion to identify animals that express a CPP. A criterion of 324 sec gives a true positive probability equal to 0.83. The true negative probability, which is equal to the number of true negatives divided by the total number of rats in the control group, is a measure of the ability of the criterion to exclude animals that do not express a CPP (Sheskin 2004). The 324 sec criterion gives a true negative probability equal to 0.78. True positive and true negative probabilities are inversely related; thus, choosing a criterion requires balancing these probabilities. The 324 sec criterion classifies 83% of animals conditioned with cocaine as expressing a CPP and classifies 78% of animals in the control group as not expressing a CPP. As this criterion gives appropriately high values for both the true positive and true negative probabilities, this analysis supports the decision to use a criterion of 324 sec.

Examination of the time spent during the test session in each chamber by the subjects classified as “CPP expressing” compared to those classified as “non-CPP expressing” provides additional verification of the criterion. During the test session, the “CPP expressing” subjects (n=131) spent an average (±SEM) of 417 ± 5 sec in the initially least-preferred chamber and 259 ± 5 in the initially

more-preferred chamber, clearly demonstrating a cocaine CPP. For comparison, the "non-CPP expressing" subjects (n=27) spent an average (±SEM) of 283 ± 6 sec in the initially least-preferred chamber and average (±SEM) of 337 ± 14 sec in the initially more-preferred chamber; thus, these animals do not express a CPP. Based on this comparison, the criterion can be said to appropriately separate animals that express a CPP from those that do not.

Two groups of subjects trained on a cocaine CPP were utilized to generate the criterion: one group of rats (n=71) experienced a single pairing of the conditioning environment and cocaine (20 mg/kg), while a second, separate group of rats experienced four pairings of the conditioning environment and cocaine (10 mg/kg). Our next analysis tested the hypothesis that the criterion of 324 sec for the expression of CPP was applicable to both cocaine groups by separately analyzing the data from rats that experienced one cocaine conditioning session with 20 mg/kg from those that experienced four conditioning sessions with 10 mg/kg of cocaine (**Fig. 2.2**). The time each animal spent in the initially least-preferred chamber during the test session was grouped into 25 sec bins and plotted (**Fig. 2.2A**). The distributions of time spent in the initially least-preferred chamber during the test session for the rats that experienced one (20 mg/kg) or four (10 mg/kg) cocaine conditioning sessions overlapped neatly. A χ^2 analysis revealed no difference between the frequency distributions of single- and four-trial group members across time bins. The average time (±SEM) in the initially least-preferred chamber spent during the test session for the single (397 ± 9 sec) and four trial (392 ± 8 sec) groups did not differ (**Fig. 2.2A**, T=154, p = 0.51). The 324 sec criterion classified 82% of rats conditioned with the single-trial procedure and 84% of rats conditioned with the four-trial procedure as

expressing a CPP. Rats in the single-trial conditioning group spent a median of 397 sec (interquartile range 343-448 sec, **Fig. 2.2B**) in the initially least-preferred chamber during the test session. Rats in the four-trial conditioning group spent a median of 392 sec (interquartile range 341-435 sec, **Fig. 2.2B**) in the initially least preferred chamber during the test session. These overlapping distributions emphasize a similarity in CPP expression between the group of rats conditioned with a single pairing of 20 mg/kg of cocaine and environment and those rats conditioned with four pairings of 10 mg/kg of cocaine and environment. We thus conclude that the criterion of 324 sec for the expression of CPP is reliable regardless of the two protocols chosen for conditioning.

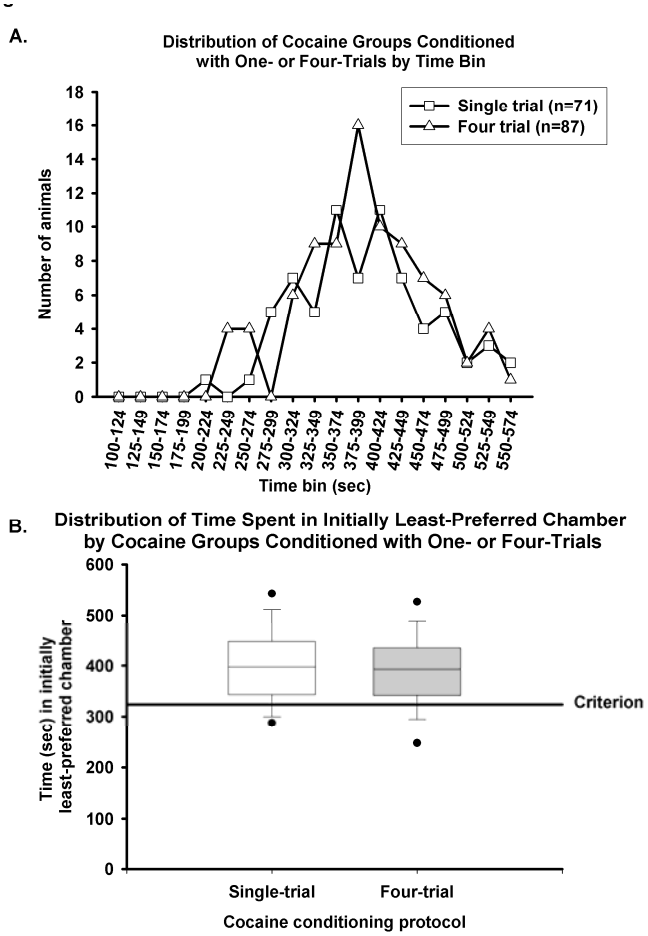


Figure 2.2: The distribution of time spent in the initially least-preferred chamber does not depend on number of conditioning sessions. A, Following conditioning with either a single pairing of 20 mg/kg of cocaine (open square, n=71) or four pairings of 10 mg/kg of cocaine (open triangle, n=87) with the environment, animals were tested for the expression of CPP in a 15 min session. The amount of time spent by each rat in the initially least-preferred chamber during the test session was categorized according to 25 sec bins. No differences in the percentage of animals meeting the CPP expression criterion were observed between the two conditioning procedures. B, Rats in single- (open box) and four-trial groups (grey box) were tested for the expression of CPP in a 15 min session. The line in the center of the box represents the median, and the top and bottom of the box represent the 75th and 25th percentiles, respectively. The filled circles represent the 95th and 5th percentiles. The solid line represents the 324 sec criterion for the expression of cocaine CPP.

Assessment of Cocaine CPP Criterion Reliability

To assess the reliability of the CPP criterion, we compared the dose-effect function for cocaine CPP acquisition generated by the criterion analysis (which compares the percentage of group members that meet or exceed the CPP criterion) to that generated by a traditional analysis (which compares the average group time spent during the test session in the initially least-preferred chamber). The dose effect function was examined for three doses of cocaine (5, 10, 20 mg/kg) in both the single- and four-pairing paradigms. An analysis of the proportion of rats in each group that spent at least 324 sec in the initially least-preferred chamber during the test session (and thus met the criterion for CPP expression) revealed a significant effect of a single cocaine conditioning trial on the percentage of rats meeting the CPP criterion (**Fig. 2.3A**, left; Fisher's exact test, $p<0.05$). A priori comparisons revealed that both 10 and 20 mg/kg evoked a single-trial CPP upon expression test (Fisher's exact test, $p<0.05$). Eighty-three percent of rats conditioned with either 10 or 20 mg/kg of cocaine, respectively, met the criterion compared to 33% of rats in the control group (0 dose; **Fig. 2.3A**). These data were separately examined using a more-widely employed ("traditional") analysis that compared the mean time spent in the initially least least-preferred chamber during the test session between control and cocaine groups. In this analysis, an ANOVA revealed a main effect of cocaine conditioning on the mean time spent in the initially least-preferred chamber during the test session (**Fig. 2.3A**, right; $F_{3,47}=3.75$, $p<0.05$). *A priori* comparisons revealed that a single conditioning session with 20 mg/kg of cocaine significantly

increased the time spent in the initially least-preferred chamber compared to control ($p<0.05$).

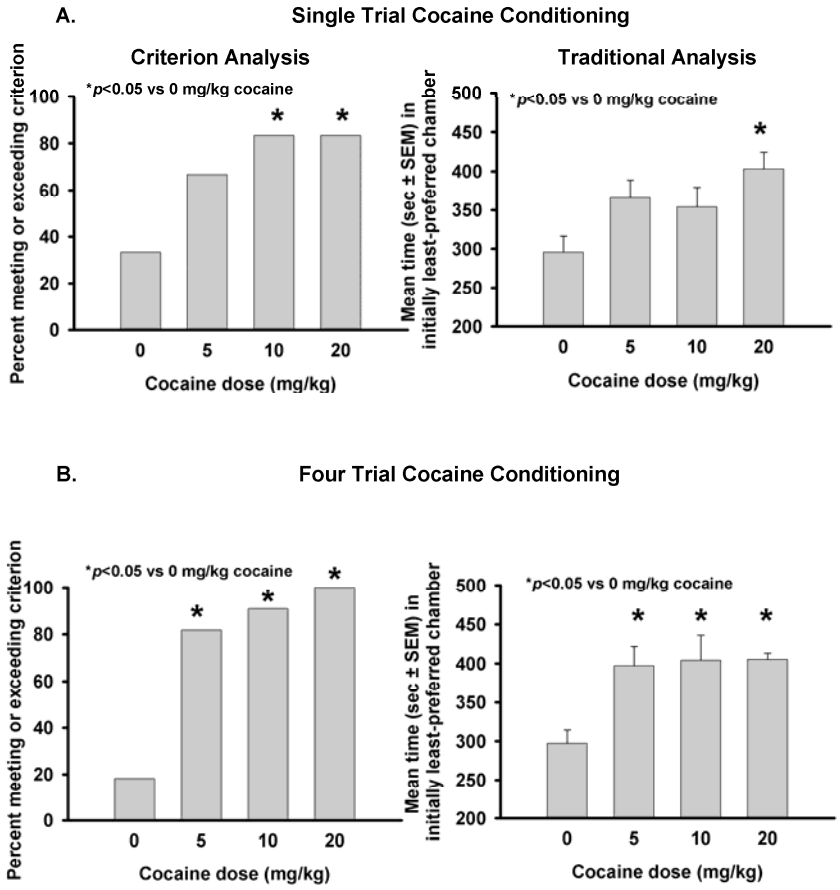


Figure 2.3: Cocaine produces a CPP following a single or four pairings of cocaine and environment. Following either a single (A) or four (B) conditioning sessions in which the environment was paired with cocaine (0, 5, 10, 20 mg/kg), the amount of time rats (n=11-12/group) spent during a 15 minute drug-free test session in the initially least-preferred chamber was observed. A, left, Criterion analysis comparing the proportion of animals spending at least 324 sec during the test session in the initially least-preferred chamber revealed a significant effect of cocaine (10 or 20 mg/kg) conditioning. A, right, Traditional analysis comparing the mean time spent in the initially least-preferred chamber during the test session by each conditioning group revealed a significant effect of cocaine (20 mg/kg) conditioning. B, left, Criterion analysis demonstrated a significant effect of cocaine (5, 10, 20 mg/kg) conditioning following four pairings. B, right, Traditional analysis also demonstrated a significant effect of cocaine (5, 10, 20 mg/kg) after four pairings. * $p < 0.05$ vs 0 mg/kg cocaine (control)

An analysis of the proportion of rats in each group that spent at least 324 sec in the initially least-preferred chamber during the test session revealed a significant effect of four cocaine conditioning trials on the percentage of rats meeting the CPP criterion (**Fig. 2.3B**, left, Fisher's exact test, $p<0.0001$). A priori comparisons revealed that four pairings of each dose of cocaine (5, 10, or 20 mg/kg, $p<0.01$) with the environment resulted in the expression of a CPP (Fig. 2.3B, left). These data were separately examined using a traditional analysis that compared the group mean time spent in the initially least-preferred chamber during the test session. In this analysis, an ANOVA revealed a main effect of cocaine conditioning (**Fig. 2.3B**, right, $F_{3,43}=5.68$, $p<0.01$) on the mean time spent in the initially least-preferred chamber during the test session. *A priori* comparisons revealed a significant effect of each cocaine dose tested (**Fig. 2.3B**, right, $p<0.05$). These results demonstrate a somewhat enhanced ability of the criterion analysis for detecting conditioning of a cocaine CPP in the single-trial procedure, but not a four-trial procedure. The 324 sec criterion has also been verified on an independent dataset in which the control group consisted of 16 rats and the cocaine group consisted of 40 rats (Chapter 4). The 324 sec criterion classified 75% of animals conditioned with cocaine as expressing a CPP and 63% of animals in the control group as not expressing a CPP (Chapter 4). Thus, we consider the criterion of 324 sec to appropriately differentiate control from cocaine conditioned rats, consistent with the true positive and true negative probabilities of the original data set.

Application of Cocaine CPP Criterion

The ability of MK 212 treatment to alter the expression of a single-trial cocaine CPP (20 mg/kg) was assessed as an application of the 324 sec criterion. Criterion analysis of the percentage of rats that met the 324 sec criterion for the expression of cocaine CPP revealed a main effect of cocaine conditioning (**Fig. 2.4A**; Wald chi-square=10.43, p=0.001), and an interaction between conditioning and MK 212 treatment (Wald chi-square=9.80, p=0.007) in the absence of a main effect of MK 212 treatment (Wald chi-square=1.47, p=0.48). An *a priori* comparison revealed a significant difference between the control and cocaine groups administered vehicle before the expression test (p<0.001). The cocaine group expressed a CPP, as assessed by the percentage of rats meeting or exceeding the 324 sec criterion. Fewer subjects met or exceeded the criterion in the cocaine group treated with the lower dose of MK 212 (0.125 mg/kg) before the expression test (**Fig. 2.4A**, p=0.02), indicating a blockade of CPP expression by the MK 212 treatment in a distinct sub-group of the rats tested. For comparison, a two-way ANOVA comparing the mean time spent in the originally least-preferred chamber during the test session revealed a main effect of conditioning (**Fig. 2.4B**, $F_{1,129}$=33.69, p<0.001). There was no main effect of MK 212 treatment ($F_{2,129}$=1.43, p=0.24), although a cocaine conditioning x MK 212 treatment interaction ($F_{2,129}$=3.13, p=0.05) was observed. An *a priori* comparison conducted with linear contrasts revealed a significant difference between the control and cocaine groups administered vehicle before the expression test (p<0.01). The cocaine group expressed a CPP, as assessed by the average time spent during the test session in the initially least-preferred chamber. In contrast to the results obtained with the criterion analysis, no differences were observed

between the cocaine-conditioned groups, indicating no effect of MK 212 treatment on the expression of single-trial cocaine CPP in this analysis. A composite analysis (**Fig. 2.4C**) that overlays the amount of time during the test session each rat spent in the initially least-preferred chamber with the group means for this value allows for direct comparison of the criterion and traditional analyses. This analysis highlights the differences between groups in the variability of time spent during the test session in the initially least-preferred chamber. For example, the values for individuals (open circles) in the cocaine group administered vehicle prior to the test session are tightly clustered around the average value (filled square). In comparison, the time spent during the test session in the initially least-preferred chamber by the individual subjects in the cocaine group treated with MK 212 (0.125 mg/kg) are widely spaced, demonstrating sub-groups within the population in the response to MK 212 treatment in rats conditioned with cocaine. The criterion method of analysis allows for quantification of two separate sub-groups.

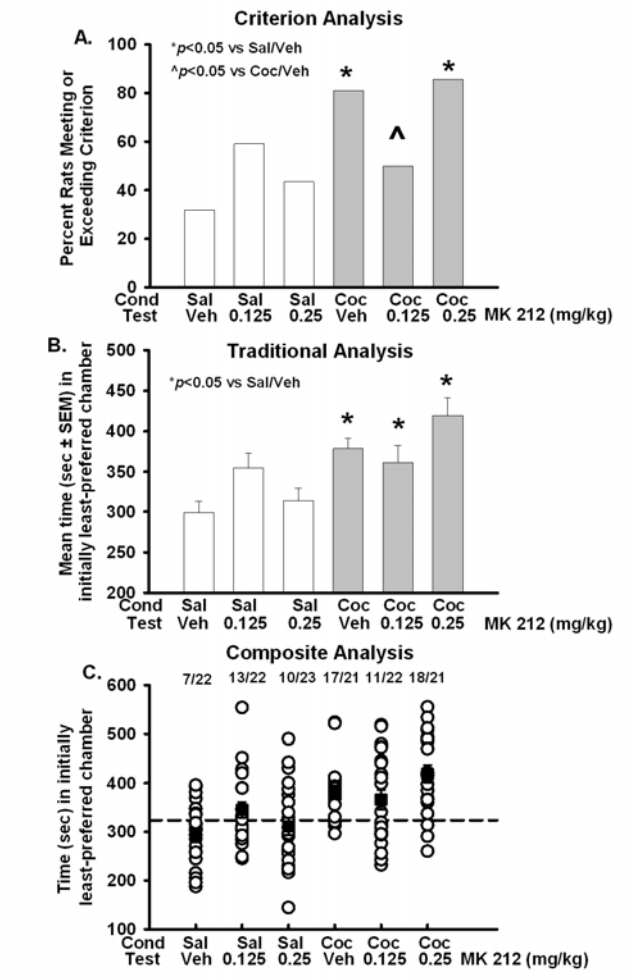


Figure 2.4: MK 212 treatment blocks expression of a single trial cocaine (20 mg/kg) CPP in the criterion but not traditional analysis. Following preconditioning, animals were conditioned with saline (open bars) or cocaine (20 mg/kg, grey bars) to the initially least-preferred side of the chamber. Ten minutes before the test session, animals were treated with MK 212 (0, 0.125, 0.25 mg/kg). A, Criterion analysis of the percentage of animals meeting or exceeding the 324 sec criterion for CPP expression revealed a blockade of expression of a single trial cocaine CPP by MK 212 (0.125 mg/kg). B, Traditional analysis comparing the mean group time during the test session in the initially least-preferred chamber demonstrated that MK 212 treatment did not alter the expression of a single trial cocaine CPP. C, Composite analysis demonstrated overlap of criterion and traditional analyses; the amount of time spent during the test session in the initially least-preferred chamber by individual animals (open circles); proportion at top gives the number of animals meeting the criterion/number in treatment group. Dashed line (- - -) represents 324 sec criterion. Black boxes represent group mean ± SEM. *p<0.05 vs vehicle-saline; ^p<0.05 vs vehicle-cocaine; cond=conditioning drug, test=injection before test session.

DISCUSSION

We established a criterion value (324 sec) for the expression of a cocaine CPP based on the time spent in the initially least-preferred chamber during the test session that effectively discriminated control from cocaine-conditioned rats. This criterion was reliable for two different groups of rats: those conditioned with either one pairing of 20 mg/kg of cocaine or those conditioned with four pairings of 10 mg/kg of cocaine with the CPP environment. The criterion method revealed an additional effective dose of cocaine in the single-trial procedure and a pharmacological blockade of CPP expression by MK 212 (0.125 mg/kg) in a sub-group of rats. The utility of the criterion method is the ability to identify and quantify the presence of two distinct sub-groups within a given conditioning group. In current methods for assessing CPP expression, the presence of a sub-group within a treatment group may be obscured by the need to compare averaged results to that of a control group (Adams *et al.* 2001; Daza-Losada *et al.* 2007). The proposed criterion method, by classifying the behavior of each subject as "CPP expressing" or "non-CPP expressing," allows for the detection of such sub-groups. We propose that the criterion method is complementary to other methods of analysis of CPP data currently in use and may be a particularly useful adjunct in analyses of pharmacological manipulations in CPP paradigms as well as in analysis of molecular mechanisms that underlie CPP.

The criterion analysis creates a binary, "all-or-none" classification for each subject, in that each rat in the study is classified as either "expressing" or "not expressing" a CPP. This method of analysis thus transforms continuous time recordings of seconds spent in a chamber into a discrete, all-or-none classification. Several groups have suggested that the dose-effect function for

expression of cocaine CPP is not graded in that different doses of cocaine tend to produce the same level of CPP expression (Bardo & Bevins 2000). For example, in the present research (**Fig. 2.3B**, right), the time spent in the initially least-preferred chamber during the test session did not differ between rats conditioned with 5, 10, or 20 mg/kg of cocaine in the four-trial procedure. Similar results have been observed in other analyses of the dose-effect function for cocaine CPP in rats (Nomikos & Spyraki 1988; Durazzo *et al.* 1994; O'Dell *et al.* 1996) as well as in mice conditioned with cocaine (Brabant *et al.* 2005) or amphetamine (Mead *et al.* 1999), although exceptions to the all-or-none dose-effect function have been observed (Allan *et al.* 2001). Thus, the criterion method of data analysis, which classifies individual subjects as "CPP expressing" or "non-CPP expressing" parallels previous analyses that suggest a CPP can be observed following conditioning only with certain doses of cocaine (CPP is expressed) and not with other doses (CPP is not expressed). The criterion method may serve as an adjunct to traditional methods of analysis to help detect dose-effect functions for cocaine CPP, as the criterion detected an additional effective dose of cocaine (**Fig. 2.3A**, left). By converting the continuous variable of time into the discrete, "CPP expressing" vs "non-CPP expressing" classification, the ability to correlate time with another variable (i.e., locomotor activity) is lost. Thus, the criterion method of analysis is more valuable in some applications (presence of discrete groups) than others.

Our approach to establishing a criterion was to analyze a large body of CPP data collected in our laboratory using a three-chamber apparatus. Based on these data, we have established a criterion of 324 sec spent in the initially least-preferred chamber during the test session. While the present data suggest that

the 324 sec criterion is appropriate for data collected in the commercially available three-chamber apparatus used in our laboratory, the applicability of this criterion will need further study due to the variability in the methods and equipment used in CPP studies and the inherent variability in behavioral procedures across laboratories (Crabbe *et al.* 1999). We anticipate that different labs may need to utilize different numerical values for the criterion. However, even if the exact criterion value cannot be applied in other labs, our method of compiling a large body of data from control and drug conditioned groups and subjecting the data to CART analysis to identify a criterion is widely applicable and opens the possibility for distinguishing "CPP expressing" from "non-CPP expressing" subjects within a treatment group. Because this method can be so easily applied by other laboratories, a strength of this technique may prove to be the wide flexibility and adaptability of this type of analysis.

Our paradigm employed an apparatus in which subjects showed no initial preference for a conditioning chamber (unbiased apparatus) coupled with a biased design in which cocaine administration was paired with the chamber that was determined to be least-preferred for each subject. Control animals developed no natural bias with repeated exposure to the chamber during conditioning as evidenced by equal time spent in the conditioning chambers during the test session. Although the use of a biased design has been criticized as difficult to interpret, the literature suggests that there is no difference in the magnitude of the CPP (assessed by three different dependent variables comparing control and ethanol-conditioned mice) when either a biased or unbiased design (chamber paired with conditioning drug is chosen randomly) is employed in an unbiased apparatus (Cunningham *et al.* 2003). Similarly, the

magnitude of the morphine CPP established in a biased versus unbiased design did not differ (Blander *et al.* 1984), and preliminary data collected in our laboratory also found no difference between a biased and an unbiased design on the expression of cocaine CPP (Herin and Cunningham, unpublished observation). Thus, the biased design appears to be a valid method for the conditioning of a CPP.

The possibility of separating a cocaine conditioned group into "CPP expressing" and "non-CPP expressing" subjects may be particularly useful for investigators interested in the molecular mechanisms that drive expression of CPP. The "non-CPP expressing" animals serve as an ideal control, in that they have undergone the identical drug exposure and conditioning paradigm but failed to demonstrate CPP expression. Thus, changes in molecular targets observed in the "CPP expressing," but not in the "non-CPP expressing," rats can be considered as attributable to successful CPP conditioning.

An inhibitory role for the 5-HT_{2C}R over the cellular and behavioral effects of cocaine has been well documented (Callahan & Cunningham 1995; Fletcher *et al.* 2002; Filip & Cunningham 2003; Filip *et al.* 2004), including for hyperactivity conditioned to cocaine (Liu & Cunningham 2006). Like CPP, the conditioned hyperactivity paradigm assesses the acquisition and expression of behavior that has become associated with cocaine exposure. In this assay, drug-free animals show hyperactivity in the test environment previously paired with cocaine. We previously demonstrated that treatment with the preferential 5-HT_{2C}R agonist MK 212 significantly decreased, and the 5-HT_{2C}R antagonist SB 242084 increased, cocaine-conditioned hyperactivity (Liu & Cunningham 2006), suggesting an inhibitory role for the 5-HT_{2C}R over expression of a cocaine-evoked conditioned

association. In the present study, MK 212, at a dose that did not alter basal motility (Filip *et al.* 2004) nor support acquisition of a CPP or conditioned place aversion (dela Cruz and Cunningham, unpublished observation), significantly suppressed expression of a single trial cocaine CPP as assessed by the criterion analysis. Treatment with MK 212 also suppressed cue-induced reinstatement of cocaine self-administration (Neisewander & Acosta 2007), further evidence for an inhibitory role for the 5-HT_{2C}R over the behavioral response to cocaine-associated cues. The U-shaped dose response relationship for MK 212 is in keeping with the effects of MK 212 on cocaine-induced locomotor activity (Filip *et al.* 2004). The shape of the dose response curve may be related to the partial selectively of MK 212 for the 5-HT_{2C}R relative to other 5-HT_2R (Kennett 1993), especially given that the 5-HT_{2A}R and 5-HT_{2C}R have been shown to have opposing effects on cocaine-regulated behaviors (Bubar & Cunningham 2008). This effect may also be related to the heterogeneity among populations of 5-HT_{2C}R (for review see Bubar & Cunningham 2008). Thus, the value of employing both traditional and criterion methods of analysis is demonstrated by our ability to reveal the suppressive effect of MK 212 on a subset of CPP expression using the criterion analysis.

In conclusion, we utilized classification and regression tree analysis to identify a criterion for the expression of cocaine CPP that is reliable across multiple conditioning paradigms used in our laboratory. Using this criterion to determine the percentage of subjects within a treatment group that express a CPP, we demonstrate an enhanced sensitivity of this method to identify pharmacological blockade of CPP expression in a distinct sub-group.

Chapter 3: The Selective Serotonin 2A Receptor Antagonist M100907 Blocks Acquisition and Expression of a Single Trial Cocaine Conditioned Place Preference

ABSTRACT

The ability of cues to reinstate drug-seeking remains a major hurdle in the maintenance of abstinence from cocaine use in addicted patients. Associations between cocaine and the environment in which it is experienced are formed via classical conditioning, and the acquisition and expression of these associations can be modeled in laboratory animals using the conditioned place preference (CPP) paradigm. The serotonin (5-HT) 2A receptor (5-HT_{2A}R) has been implicated in the learning of classically conditioned associations, and the present study was designed to test the hypothesis that selective blockade of 5-HT_{2A}R would block the acquisition and expression of a cocaine CPP. We compared the CPP formed by a single pairing of cocaine (20 mg/kg) plus the environment versus four pairings of cocaine (10 mg/kg) plus the environment. In the acquisition studies, male Sprague-Dawley rats received M100907 (0.2, 0.8 mg/kg) or vehicle 45 min prior to the cocaine conditioning session and were drug-free during the CPP test session. In the expression study, a separate group of male Sprague-Dawley rats received M100907 (0.2, 0.8 mg/kg) or vehicle 45 min prior to the CPP test session. Rats expressed a CPP after either one or four pairings of cocaine and environment. M100907 treatment blocked the acquisition and expression of the CPP formed by one, but not four, pairings of cocaine and environment. These results suggest a selective role for the 5-HT_{2A}R in

modulating the early development and expression of cocaine-environment associations.

INTRODUCTION

Relapse to drug-taking during abstinence is a prominent feature of cocaine addiction (McLellan *et al.* 2000), which remains a major health problem in the United States (2006). Classical conditioning processes are thought to underlie the formation and expression of cocaine-environment associations (O'Brien *et al.* 1992) that contribute to relapse, and this phenomenon can be modeled in laboratory rats using the conditioned place preference (CPP) paradigm (Bardo & Bevins 2000; Tzschentke 2007). In this paradigm, exposure to cocaine (the unconditioned stimulus, UCS) is temporally paired with placement in a distinct environment (the conditioned stimulus, CS^+), and saline treatment is paired with an alternate environment (CS^-). An increase in the time spent in the compartment formerly paired with cocaine during a subsequent cocaine-free test session is considered expression of a CPP (Bardo & Bevins 2000). This paradigm can be utilized to separately study the factors that regulate the acquisition and expression of cocaine-environment associations based on the temporal difference between the conditioning and test session (Tzschentke 1998).

Serotonin (5-HT) has been implicated in modulating the acquisition and expression of classically-conditioned associations, as well as other forms of learning and memory (Meneses 1999). Fourteen 5-HT receptors have been cloned, and all but one family member are G-protein coupled receptors (Hoyer *et al.* 2002). Of these G-protein coupled receptors, the $5\text{-}HT_{2A}$ receptor ($5\text{-}HT_{2A}R$) has been heavily implicated in mediating several aspects of learning. Stimulation

of 5-HT_{2A} receptors (5-HT_{2A}R) facilitates acquisition of a classically-conditioned eyeblink response in rabbits (reviewed in Harvey 2003), and elevated levels of 5-HT_{2A}R ligand binding is associated with a faster rate of acquisition of associative learning (Harvey *et al.* 2004). Activation of the 5-HT_{2A}R may be particularly important in the acquisition of difficult tasks, such as CS-UCS pairings with a long (> 400 msec) delay between the CS and UCS (Harvey 2003). The 5-HT_{2A}R may play a role in modulating the acquisition of cocaine-environment associations, given the involvement of the 5-HT_{2A}R in modulating the behavioral response to cocaine (Bubar & Cunningham 2008).

The involvement of the 5-HT_{2A}R in acquisition of other learned associations suggests a role for this receptor in the conditioning or expression of a cocaine CPP. This hypothesis is supported to a certain extent by observations that non-selective 5-HT_{2A}R antagonists blocked the acquisition of a cocaine CPP (Kosten & Nestler 1994; Jones & McMillen 1995; Meil & Schechter 1997; Arolfo & McMillen 2000). However, the selective role of the 5-HT_{2A}R in the expression of cocaine CPP or across various CPP conditioning paradigms has yet to be analyzed.

A CPP can be conditioned following either a single pairing (Bardo *et al.* 1986; dela Cruz *et al*. 2009) or multiple pairings of cocaine and environment (Nomikos & Spyraki 1988; Mueller & Stewart 2000; Brabant *et al.* 2005). The present study compared two learning paradigms that result in the same magnitude of learning (assessed by the amount of time spent in the cocaine-paired environment; dela Cruz *et al.* 2009) but differ in the number of cocaine-environment pairings. We chose to utilize a single-trial paradigm, in which rats received one pairing of cocaine (20 mg/kg) and environment, and a four-trial

paradigm, in which rats received four pairings of cocaine (10 mg/kg) and environment. These cocaine doses are the lowest doses that consistently generate a CPP in our laboratory in the single pairing or multiple pairing paradigms (Chapter 2, dela Cruz *et al.* 2009)). We employed the selective 5-HT_{2A}R antagonist M100907 (Kehne *et al.* 1996) to investigate the role of the 5-HT_{2A}R in the cocaine CPP paradigm using both single and four-trial paradigms. M100907 was injected either prior to cocaine conditioning sessions to investigate the role of 5-HT_{2A}R in the *acquisition* of cocaine CPP or solely prior to the cocaine-free CPP test session to investigate the role of 5-HT_{2A}R in the *expression* of cocaine CPP.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (n=284, Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighed 225-275 g at study initiation. The rats were housed four per cage in standard, clear plastic rodent cages with food and water available *ad libitum* in a temperature (21-23°C) and humidity (55-65%) controlled environment under a 12-h light/dark cycle (lights on 0700 h). Animals were acclimated to the colony for at least one week and were handled prior to the start of experimental sessions. All experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and with approval from the Institutional Animal Care and Use Committee.

Drugs

Cocaine HCl salt (National Institute on Drug Abuse, Research Triangle, NC) was dissolved in 0.9% NaCl. M100907 {[R-(+)-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol; synthesized by Thomas Ullrich and Kenner Rice, National Institutes of Health} was dissolved in a solution of 1% Tween 80 in distilled water. All drugs were injected intraperitoneally (IP) in a volume of 1 ml/kg. Doses of all drugs refer to the weight of the salt.

Conditioned Place Preference Apparatus

The CPP apparatuses were housed within sound- and light-attenuating cabinets and contained three sensory environments distinguished by wall colors and floor textures (n=8, ENV-013, Med Associates, Georgia, VT). The two side conditioning chambers (interior dimensions: 25.5 cm L x 21.0 cm W x 20.9 cm H) were separated by a smaller chamber (13.2 cm L X 21.0 cm W x 20.9 cm H). One conditioning chamber had white walls and a stainless steel mesh (1.3 x 1.3 cm) floor and the other conditioning chamber had black walls and a floor of stainless steel rods (4.8 mm placed on 1.6 cm centers). The center chamber had gray walls and a floor of sheet metal. Guillotine doors separated each conditioning compartment from the center compartment. The intensity of ambient illumination was adjusted to 7 lux in each conditioning chamber and 30 lux in the center chamber to balance side preference for each conditioning chamber (Roma & Riley 2005). Previous work in our laboratory revealed that rats do not demonstrate a consistent preference for one conditioning chamber (see **Results** and dela Cruz *et al.* 2009). Automated data collection is accomplished through photobeam detectors. There were 15 infrared photobeam detectors that were 4.5

cm above the chamber floor. Of these, six photobeams were arrayed along the length of each conditioning compartment 1.25 cm from the end wall with 5 cm between beams. Three photobeams were arrayed along the length of the central compartment spaced 4.75 cm apart. The photobeams were connected to a computer interface, and MED-PC software (Med Associates) recorded the time spent in each chamber based on the recorded activity counts (any beam break within the current chamber) between disruption of (1) the entrance beam of that chamber (beam break beyond the first beam of a chamber) and (2) the entrance beam of a different chamber.

Conditioned Place Preference Procedure

Each experiment used separate groups of naïve animals. The CPP training consisted of three phases (preconditioning, conditioning, and test) which occurred over consecutive days. In all phases, the home cages of the animals were transferred from the animal colony to the test room 5 min before the animals were placed in the CPP apparatus. To determine baseline preference (preconditioning), rats were placed in the center chamber with the guillotine doors raised and allowed to freely roam the apparatus for 30 min. The time spent in each conditioning chamber was recorded, and the conditioning chamber in which an animal spent the least amount of time was designated the initially least-preferred chamber for that animal. Conditioning was performed using a biased protocol in which cocaine was paired with the initially least-preferred chamber (Spyraki *et al.* 1982; Blander *et al.* 1984; Isaac *et al.* 1989), which was designated the "CS^+ environment."

Cocaine CPP conditioning sessions consisted of alternating sessions of cocaine and saline injections with the guillotine doors in place. Two sessions were conducted each day separated by at least six hours (Shippenberg & Heidbreder 1995), thus rats experienced both a morning and an afternoon conditioning session. For cocaine CPP training, one daily conditioning session began with an injection of cocaine and immediate confinement to the CS^+ environment for 45 min. The other daily conditioning session began with a saline (1 mL/kg, IP) injection and immediate confinement to the alternate conditioning chamber (CS^- environment) for 45 min. Thus, some rats received cocaine during the morning session, and others received cocaine during the afternoon session. Control rats were injected with saline prior to placement into each conditioning chamber for both sessions on each day. At the termination of the session, rats were returned to their home cages. Rats experienced one conditioning session with cocaine and one with saline for studies in the single-trial conditioning procedure. In the four-trial conditioning procedure, rats experienced eight alternating sessions of cocaine and saline injections over four consecutive days.

The expression test for CPP was conducted 16-24 hrs after the final conditioning session. The time of the test session (morning vs. afternoon) was counterbalanced across rats relative to the time of cocaine conditioning. Rats were placed in the center chamber with the guillotine doors raised and allowed to freely roam the apparatus for 15 min while the time spent in each chamber was recorded. Data are presented as mean time (sec $\pm$ S.E.M.) spent in the CS^+ environment

To assess the ability of M100907 to block the *acquisition* of cocaine CPP, M100907 (0, 0.2, 0.8 mg/kg) was injected 45 min before the cocaine conditioning

session for cocaine-conditioned rats and the equivalent session for control rats (i.e., conditioning with the CS^+ environment). To assess the ability of M100907 to block the *expression* of cocaine CPP, M100907 (0, 0.2, 0.8 mg/kg) was injected 45 min before the test session. Four studies were conducted to examine the effect of M100907 on cocaine CPP: single trial acquisition (n=10/group), single trial expression (n=8/group), four trial acquisition (n=10-11/group), and four trial expression (n=12-28/group). Treatment groups are described in **Table 3.1**.

Table 3.1: Description of Treatment Groups

Acquisition Studies

Paired With CS^- Environment	M100907 (mg/kg) Prior To CS^+ Environment Pairing (Pretreatment)	Paired With CS^+ Environment
Saline	0	Saline
Saline	0.2	Saline
Saline	0.8	Saline
Saline	0	Cocaine
Saline	0.2	Cocaine
Saline	0.8	Cocaine

Expresssion Studies

Paired With CS^- Environment	Paired With CS^+ Environment	M100907 (mg/kg) Prior To Test (Treatment)
Saline	Saline	0
Saline	Saline	0.2
Saline	Saline	0.8
Saline	Cocaine	0
Saline	Cocaine	0.2
Saline	Cocaine	0.8

Statistical Analysis

All statistical analyses were performed with SAS for Windows version 8.2 (SAS Institute, Cary, NC). The time spent in the conditioning and center chambers during preconditioning (Day 1) were analyzed with the nonparametric Friedman test. Two approaches were used to analyze the data from the CPP test session. The first analysis, which assessed the continuous variable mean time spent in the CS^+ environment during the test session, was conducted using a two-way ANOVA with factors (1) conditioning (saline versus cocaine) and (2) M100907 treatment (0, 0.2, 0.8 mg/kg) and the conditioning x treatment interaction. Group comparisons were specifically defined prior to the start of experiments, and thus *a priori* linear contrasts were conducted for pairwise

comparisons regardless of the *F* value. This procedure is well justified in statistical texts (Keppel 1973; Sheskin 2004) and has been utilized previously in our laboratory (Herin *et al.* 2005; Szucs *et al.* 2005).

The second approach to data analysis compared the percentage of rats in each treatment group that met a statistically-verified criterion for CPP expression previously developed in our laboratory (dela Cruz *et al.* 2009) designed to identify differential responsiveness to M100907 within a treatment group. Using classification and regression tree analysis (Zhang *et al.* 1996; Lemon *et al.* 2003), we previously determined that a criterion of 324 sec spent in the CS^+ environment during the test session provided accurate separation between saline and cocaine conditioned rats; thus, this amount of time was established as the criterion for CPP expression (dela Cruz *et al.* 2009). The binary outcome of "CPP expression" (yes ≥ 324 sec in the CS^+ environment during the test session versus no < 324 sec) was used in a logistic regression with predictor variables (1) conditioning (saline versus cocaine) and (2) M100907 treatment (0, 0.2, 0.8 mg/kg) and on the conditioning x treatment interaction. *A priori* individual comparisons were performed using categorical modeling via the CATMOD procedure in SAS. As above, group comparisons were specifically defined prior to the start of experiments; thus, *a priori* individual comparisons were performed using categorical modeling via the CATMOD procedure in SAS regardless of the overall output. For all comparisons, the experiment-wise α was set at 0.05.

RESULTS

Confirmation of Unbiased CPP Apparatus

To demonstrate the unbiased nature of the CPP apparatus, data from preconditioning sessions for the rats (n=60) used in the study of the effects of M100907 on the acquisition of a single trial cocaine CPP were analyzed. Out of the 1800 sec preconditioning session, rats spent a mean (± SEM) of 735.04 sec (± 18.17) in the black side (41% of total time) and 663.77 sec (± 18.6) in the white side (37% of total time); the remaining time (401.00 ± 16.0 sec; 22% of total time) was logged in the center chamber. No significant difference between the amount of time spent in the two conditioning chambers ($p>0.05$) was observed. Thus, the CPP apparatus is considered unbiased.

To further demonstrate the unbiased nature of the apparatus, the amount of time that control (n=10) animals spent in each chamber during the 15 min expression test session was analyzed. On the test day, control animals spent 38% of total time in the white chamber (mean ± SEM 343.89 ± 25.42 sec), 36% of total time in the black chamber (mean ± SEM 326.79 ± 24.74 sec), and 25% of total time in the grey chamber (mean ± SEM 229.38 ± 23.21 sec). These percentages are nearly identical to those observed during preconditioning (above); thus, non-drug conditioned rats did not develop a preference for one conditioning chamber, validating that the apparatus is considered unbiased.

Effect of M100907 on Single Trial Cocaine CPP

Acquisition

The ability of M100907 to suppress acquisition of the cocaine CPP formed by a single pairing of cocaine and environment was assessed in a naïve group of rats (n=10/group). In the absence of a main effect of pretreatment (**Fig. 3.1A**, $F_{2,59}$ = 2.47, p=0.094), there was a main effect of conditioning on the time spent in the CS^+ environment ($F_{1,59}$=17.05, p=0.0001) and a pretreatment x conditioning interaction ($F_{2,59}$=4.19, p=0.02). *A priori* comparisons indicated that rats pretreated with vehicle (0 mg/kg of M100907) and conditioned with cocaine exhibited a CPP (**Fig. 3.1A**). Pretreatment with either dose (0.2 or 0.8 mg/kg) of M100907 prior to the cocaine conditioning session blocked acquisition of this response (**Fig. 3.1A**, p<0.05).

Evaluation of the percentage of rats in each treatment group that met the predetermined criterion for CPP expression by spending at least 324 sec in the CS^+ environment (Chapter 2, dela Cruz *et al.* 2009) revealed a main effect of conditioning (**Fig. 3.1C**, X^2=9.30, p=0.002) in the absence of an effect of pretreatment (X^2=3.07, p=0.22) or a pretreatment x conditioning interaction (X^2=2.96, p=0.23). *A priori* comparisons revealed that the cocaine conditioned group expressed a CPP (90% of rats met the criterion) and that pretreatment with 0.8 mg/kg M100907 prior to the cocaine conditioning session blocked the acquisition of the cocaine CPP (50% of rats met the criterion, p<0.05). In this analysis, the effects of lower dose of M100907 (0.2 mg/kg) were not statistically significant (**Fig. 3.1C**, 70% of rats met the criterion). Rats that received one pairing of cocaine (20 mg/kg) plus environment expressed a CPP, and the

acquisition of this CPP was blocked by pretreatment with M100907 (0.8 mg/kg); the percent meeting the criterion uncovered a dose-related nature of the effects of M100907 pretreatment on acquisition of the single-trial cocaine CPP.

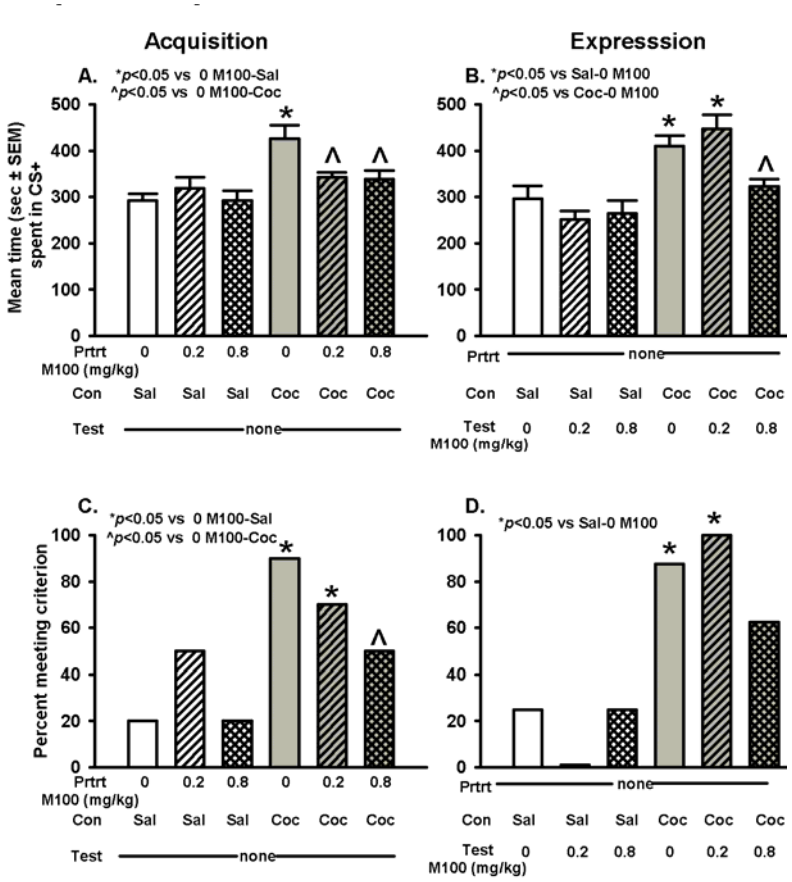


Figure 3.1: Effect of M100907 on acquisition and expression of a single trial cocaine CPP. (A, B) Data represent the average time (seconds ± SEM) spent in the CS^+ environment during the 15 minute expression test or (**C, D**) the percent of rats in each treatment group that met the criterion for CPP expression (see Methods) from two separate studies. (**A, C**) Rats (n=10/group) were pretreated with M100907 (0, 0.2, 0.8 mg/kg) 45 minutes prior a single pairing of saline or cocaine (20 mg/kg) and the CS^+ environment and tested 16-24 hrs later for the expression of CPP. (**B, D**) Rats (n=8/group) received a single pairing of either saline or cocaine (20 mg/kg) and the CS^+ environment and were treated with M100907 (0, 0.2, 0.8) 45 minutes prior to the CPP expression test. Open bar=0 mg/kg M100907, striped bar=0.2 mg/kg M100907, cross-hatched bar=0.8 mg/kg M100907. Prtrt=pretreatment dose of M100907 injected 45 min before pairing of saline or cocaine with CS+; Con=conditioning drug; Test=dose of M100907 given 45 min before CPP test session; M100=M100907; Sal=saline; Coc=cocaine; *p<0.05 vs saline conditioned-0 mg/kg M100907; ^p<0.05 vs cocaine conditioned-0 mg/kg M100907

Expression

We also investigated the ability of M100907 to block expression of single-trial cocaine CPP (n=8/group). A main effect of conditioning (**Fig. 3.1B**, $F_{1,47}$=38.61, p<0.0001), treatment ($F_{2,47}$=3.74, p=0.032), and a conditioning x treatment interaction ($F_{2,47}$=4.14, p=0.023) on the time spent in the CS^+ environment were observed. *A priori* comparisons indicated that cocaine produced a CPP and that expression of this cocaine CPP was abolished by treatment with M100907 (0.8 mg/kg) prior to the test session (**Fig. 3.1B**, p<0.05).

Evaluation of the percentage of rats in each treatment group that met the predetermined criterion for CPP expression by spending at least 324 sec in the CS^+ environment (Chapter 2, dela Cruz *et al.* 2009) revealed a main effect of conditioning (**Fig. 3.1D**, X^2=14.33, p=0.0002), but not treatment (X^2=0.68, p=0.71) or a conditioning x treatment interaction (X^2=2.96, p=0.23). *A priori* comparisons revealed that the groups conditioned with cocaine and treated with either 0 mg/kg of M100907 prior to the test session expressed a CPP (**Fig. 3.1D**, 87.5% of rats met criterion, p<0.05 vs control). Treatment with the higher dose of M100907 (0.8 mg/kg), but not the lower (0.2 mg/kg), prevented the expression of a cocaine CPP (62.5% and 100% of rats met criterion, respectively). A CPP was expressed by rats that received a single pairing of cocaine (20 mg/kg) and environment and the expression of this CPP was prevented by M100907 (0.8 mg/kg) treatment prior to the test session.

Effect of M100907 on Four Trial Cocaine CPP

Acquisition

We examined the ability of M100907 (0.2, 0.8 mg/kg) to block the acquisition of a cocaine (10 mg/kg) CPP after four pairings of cocaine and environment in a separate group of rats (n=10-11/group). Analysis of the amount of time spent in the CS^+ environment demonstrated a main effect of conditioning (**Fig. 3.2A**, $F_{1,63}$=70.39, p<0.0001) but no main effect of pretreatment ($F_{2,63}$=1.33, p=0.27) or pretreatment x conditioning interaction ($F_{2,63}$=0.44, p=0.65). *A priori* comparisons indicated that animals conditioned with cocaine exhibited a CPP regardless of pretreatment with M100907 prior to each cocaine conditioning session (**Fig. 3.2A**).

Evaluation of the percentage of rats in each treatment group that met the predetermined criterion for CPP expression by spending at least 324 sec in the CS^+ environment (Chapter 2, dela Cruz *et al.* 2009) revealed a main effect of conditioning (**Fig. 3.2C**, X^2=22.76, p<0.0001) in the absence of an effect of pretreatment (X^2=0.88, p=0.64) or a pretreatment x conditioning interaction (X^2=0.77, p=0.68). *A priori* comparisons indicated that animals conditioned with cocaine exhibited a CPP regardless of pretreatment with M100907 prior to each cocaine conditioning session (**Fig. 3.2C**). Rats that received four pairings of cocaine (10 mg/kg) and environment expressed a CPP and the acquisition of this CPP was unaffected by M100907 (0.2, 0.8 mg/kg) pretreatment.

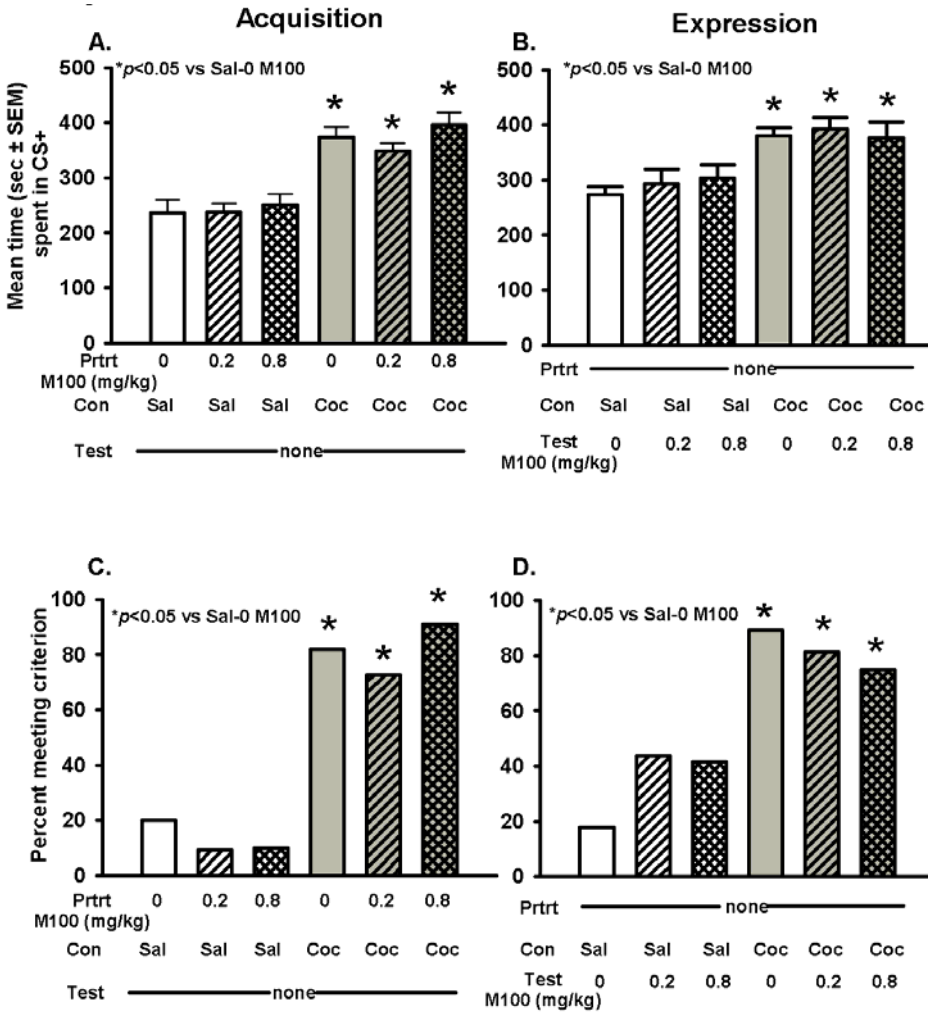


Figure 3.2: Effect of M100907 on acquisition and expression of a four trial cocaine CPP. (**A**, **B**) Data represent the average time (seconds ± SEM) spent in the CS+ environment during the 15 minute expression test or (**C**, **D**) the percent of rats in each treatment group that met the criterion for CPP expression (see Methods) from two separate studies. (**A**, **C**) Rats (n=10/group) were pretreated with M100907 (0, 0.2, 0.8 mg/kg) 45 minutes prior each of four pairings of saline or cocaine (20 mg/kg) and the CS+ environment and tested 16-24 hrs later for the expression of CPP. (**B**, **D**) Rats (n=8/group) received a four pairing of either saline or cocaine (20 mg/kg) and the CS+ environment and were treated with M100907 (0, 0.2, 0.8) 45 minutes prior to the CPP expression test. Open bar=0 mg/kg M100907, striped bar=0.2 mg/kg M100907, cross-hatched bar=0.8 mg/kg M100907. Prtrt=pretreatment dose of M100907 injected 45 min before pairing of saline or cocaine with CS+; Con=conditioning drug; Test=dose of M100907 given 45 min before CPP test session; M100=M100907; Sal=saline; Coc=cocaine; *p<0.05 vs saline conditioned-0 mg/kg M100907

Expression

Additionally, we examined the ability of M100907 (0.2, 0.8 mg/kg) to block expression of a four-trial CPP in a separate group of animals (n=12-28/group). Analysis of the amount of time spent in the CS^+ environment demonstrated a main effect of conditioning (**Fig. 3.2B**, $F_{1,111}$=38.56, p<0.0001), no main effect of treatment ($F_{2,111}$=0.43, p=0.65), and no conditioning x treatment interaction ($F_{2,111}$=0.34, p=0.71). *A priori* comparisons indicated that cocaine produced a CPP and that expression of this response was not altered by M100907 treatment prior to the test session (**Fig. 3.2B**).

Evaluation of the percentage of rats in each treatment group that met the predetermined criterion for CPP expression by spending at least 324 sec in the CS^+ environment (Chapter 2, dela Cruz *et al.* 2009) revealed a main effect of conditioning (**Fig. 3.2D**, X^2=22.35, p<0.0001) in the absence of an effect of treatment (X^2=0.31, p=0.85) or a conditioning x treatment interaction (X^2=4.39, p=0.11). *A priori* comparisons revealed that all groups conditioned with cocaine expressed a CPP (**Fig. 3.2D**), indicating no effect of M100907 treatment prior to the CPP test session on expression of a four-trial cocaine CPP.

DISCUSSION

The studies presented here are the first to systematically analyze the ability of a selective 5-HT_{2A}R antagonist to modulate the acquisition and expression of a cocaine CPP. The results of these studies indicate that selective blockade of the 5-HT_{2A}R suppresses the acquisition and expression of a cocaine CPP after a single pairing, but not four pairings, of cocaine and environment.

Further assessment of individual differences in the response to blockade of the 5-HT_{2A}R by M100907 revealed a dose-related effect of M100907 to block the acquisition of single-trial cocaine CPP. These results suggest that the 5-HT_{2A}R may be a key component in the initial development and expression of cocaine-environment conditioned associations. However, this receptor appears to lose the ability to modulate the acquisition and retrieval of cocaine-environment associations upon repeated exposure to cocaine.

These studies reveal a seemingly paradoxical role for the 5-HT_{2A}R in the development and expression of a learned cocaine-environment associations dependent on the number of pairings. With the evidence that activation of 5-HT_{2A}R promotes learning in difficult tasks (Harvey 2003), the current studies suggest that the single trial procedure may be a more difficult task for animals to learn and thus more susceptible to disruption by the selective 5-HT_{2A}R antagonist. Our study design attempted to minimize the role of task "difficulty" by pairing a higher dose of cocaine (20 mg/kg) with the CS^+ environment in the single trial procedure compared to the four trial procedure (environment paired with 10 mg/kg). Although the "strength" of the conditioned response, assessed by time spent in the CS^+ environment during the test session or the percentage of rats in the cocaine-conditioned group that met the CPP criterion, appears similar across the single and four trial paradigms (compare rats conditioned with cocaine that received vehicle is **Fig. 3.1** versus **Fig. 3.2** and see Chapter 2 and dela Cruz *et al.* 2009), it remains possible that a different, unidentified dependent variable might reveal differences in the strength of the conditioned cocaine-environment association between the single and four trial paradigms. Our studies raise the interesting possibility that pretreatment with a 5-HT_{2A}R agonist may allow for the

acquisition of a single-trial CPP conditioned with a subthreshold dose (i.e., 5 or 10 mg/kg) of cocaine in this paradigm.

Our identification of a role for the 5-HT_{2A}R in the acquisition of a cocaine CPP fits well with earlier data suggesting a role for this receptor in the acquisition of a cocaine CPP (Kosten & Nestler 1994; Jones & McMillen 1995; Meil & Schechter 1997; Arolfo & McMillen 2000) and in other classically-conditioned behaviors (Harvey 2003); these studies are the first to identify a role for the 5-HT_{2A}R in the expression of cocaine CPP. Our studies thus expand a growing literature on the role of the 5-HT_{2A}R in mediating acquisition of classically-conditioned associations in rabbits (Harvey 2003). In addition, recent evidence has suggested a role for the 5-HT_{2A}R in the consolidation of an autoshaping task in rodents (Meneses *et al.* 1997). "Consolidation" describes the process by which memories are transferred from short-term to long-term memory (Schafe *et al.* 2001), and a pharmacological compound is said to disrupt consolidation if post-training treatment with the compound disrupts the later expression of CPP (for example, Cervo *et al.* 1997). A preliminary study in our laboratory found no effect of M100907 treatment immediately following conditioning on the consolidation of a single trial cocaine CPP (dela Cruz and Cunningham, unpublished observation); these negative results should be interpreted with caution, however, as blockade of consolidation is highly dependent of the timing of pharmacological interventions (Hsu *et al.* 2002; Schroeder & Packard 2002; Blaiss & Janak 2006), and it remains possible that M100907 injections at a different time or that repeated M100907 treatments might disrupt the consolidation of cocaine-environment associations. The current studies thus add

to a growing body of literature that suggests multiple roles for the 5-HT_{2A}R in the acquisition and expression of cocaine-environment associations.

An alternative explanation for the differential ability of M100907 treatment to disrupt single versus four trial cocaine CPP is that repeated cocaine treatment may alter 5-HT_{2A}R-mediated signaling. Studies examining the cellular and molecular regulation of the 5-HT_{2A}R by acute versus repeated treatments of cocaine have produced complex results that appear to be dependent on the pattern of cocaine treatment. A single injection of cocaine does not alter the cortical density of 5-HT_{2A}R (Carrasco & Battaglia 2007) but may increase 5-HT_{2A}R-mediated phospholipase C activity (Carrasco & Battaglia 2007) that would promote 5-HT_{2A}R-mediated signaling. Repeated cocaine treatment (15 mg/kg twice per day for 7 days) increased head shakes and prolactin release induced by challenge with the nonselective 5-HT_2R agonist (±)-1-(2,5-dimethoxy-4-iodo-phenyl)-2-aminopropane (DOI; Baumann & Rothman 1996), suggesting a functional supersensitivity 5-HT_{2A}R. The same treatment regimen increased the expression of the 5-HT_{2A}R signaling partner $G\alpha_{11}$ in the hypothalamic paraventricular nucleus (Carrasco *et al.* 2004) without altering expression of this protein in the frontal cortex (Carrasco & Battaglia 2007). Although this increase in expression of the 5-HT_{2A}R signaling partner might suggest a resultant supersensitivity of the 5-HT_{2A}R, recent data from cell culture studies suggested that increased phosphorylation of $G\alpha_{11}$ promotes internalization of the receptor (Shi *et al.* 2007). Thus, the upregulation of $G\alpha_{11}$ observed by Carrasco and colleagues could lead to a decrease in the surface expression of the 5-HT_{2A}R. In summary, while neither a single or repeated injections of cocaine alter the overall cellular expression of 5-HT_{2A}R, the effects of repeated cocaine injections on 5-

HT_{2A}R-related signaling components may lead to a functional supersensitivity or internalization and resultant functional downregulation of the 5-HT_{2A}R. Our present results demonstrating a loss of inhibition by M100907 following repeated pairings of cocaine and environment are consistent with the hypothesis that repeated cocaine treatment promotes functional downregulation of 5-HT_{2A}R. Because we observed no blockade of CPP in both the acquisition study (in which rats received four injections of M100907 and four injections of cocaine) and the expression study (in which rats received one injection of M100907 and four injections of cocaine), we propose that the repeated cocaine treatment is the major factor that contributes to regulation of the 5-HT_{2A}R.

The doses of M100907 utilized in the current studies were based on previous results from our laboratory in which 0.2 mg/kg of M100907 had the highest effectiveness for blocking cocaine-induced locomotor activity and 0.8 mg/kg of M100907 had the highest effectiveness for attenuating the discriminative stimulus effects of cocaine (McMahon & Cunningham 2001). While its selectivity has been called into question (Dekeyne *et al.* 2002), M100907 demonstrated nanomolar affinity for the 5-HT_{2A}R (K_i=0.85 nM) with 100-fold selectivity for the 5-HT_{2A}R over the closely related 5-HT_{2C}R (K_i=88 nM) and α_1 adrenergic receptor (K_i=128 nM) (Kehne *et al.* 1996). In an *in vitro* assessment of the selectively of M100907, this compound demonstrated an IC_{50} equal to 0.6 nM at 5-HT_{2A}R and 770 nM at 5-HT_{2C}R (Kehne *et al.* 1996). The ED_{50} for inhibiting an α_1 receptor-mediated behavior was greater than 16 mg/kg, well above the doses used in the present study (Kehne *et al.* 1996). Thus, we are confident that the ability of M100907 to inhibit the acquisition and expression of a single trial

cocaine CPP stems from the ability of this compound to act as an antagonist at 5-HT_{2A}R.

In conclusion, the studies presented here represent the most complete examination to date of the role of the 5-HT_{2A}R in regulating cocaine CPP. We demonstrated that treatment with a selective 5-HT_{2A}R antagonist blocked the acquisition and expression of a single, but not four, trial cocaine CPP. These data suggest that the 5-HT_{2A}R is a key component of the initial development and expression of cocaine-environment conditioned associations. However, repeated treatment with cocaine, M100907, or the combination promotes a functional desensitization of the 5-HT_{2A}R, thereby eliminating this receptor from further regulation of the conditioned associations over time.

Chapter 4: Protein Expression and Activation in Prefrontal Cortex Associated With Expression of a Cocaine Conditioned Place Preference

ABSTRACT

Identification of molecular substrates that underlie the long-lasting ability of cocaine-associated environments to prompt relapse is critical to development of new pharmaceutical treatments for cocaine addictioin. Altered phosphorylation and expression of the MAP kinase extracellular-signal regulated kinase (ERK) and the AMPA glutamate receptor subunit 1 (GluR1) in corticolimbic circuits has been suggested as critical to formation and retrieval of cocaine-environment associations. To test the hypothesis that these neuroadaptations are critical to the learning events that occur early in the development of cocaine-environment associations, we investigated the expression and phosphorylation of ERK and GluR1 in corticolimbic circuits isolated from rats behaviorally described to express a cocaine CPP after a single pairing of cocaine (20 mg/kg) and environment. To separately identify neuroadaptations associated with expression of CPP from those associated with passive exposure to the cocaine-paired environment, rats were sacrificed either immediately following the CPP expression test or after confinement to only the cocaine-paired environment, respectively. We found that GluR1 protein expression was decreased in the synaptosome-enriched fraction isolated from the prefrontal cortex (PFC) of all rats conditioned with cocaine and sacrificed immediately after the test session (regardless of CPP expression). This observation suggests that GluR1 expression in the PFC is particularly sensitive to cocaine treatment regardless of

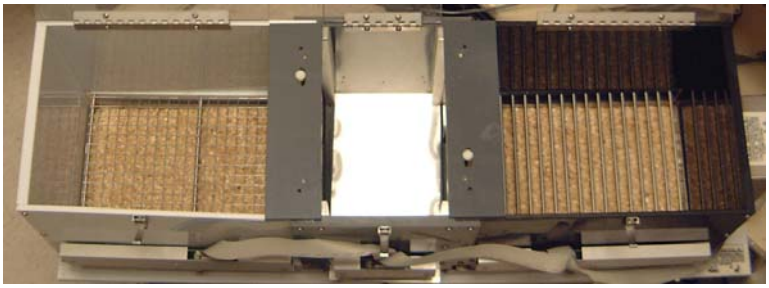
the associative learning situation. On the other hand, increased expression of ERK and activation of GluR1 in the synaptic fraction isolated from the PFC was observed immediately after passive re-exposure to the cocaine-paired environment selectively in rats that expressed a CPP, suggesting that these neuroadaptations are associated with retrieval of this learned cocaine-environment association. Protein expression and activation of ERK, GluR1 or other proteins examined were unchanged in the hippocampus or amygdala under the present experimental conditions. These data suggest that very specific molecular neuroadaptations in the PFC associated with cocaine-environment associations occur early and highlight a critical role for this region in the retrieval of cocaine-environment associations.

Introduction

Classically conditioned associations made between the effects of cocaine and environmental cues present during drug-taking are a major feature of cocaine addiction that contributes to the chronic nature of this condition (Ehrman *et al.* 1992; Childress *et al.* 1999; McLellan *et al.* 2000). The cocaine conditioned place preference (CPP) paradigm is an animal model that is widely used to study the acquisition and expression of classically conditioned cocaine-environment associations (Bardo & Bevins 2000; Tzschentke 2007). The goal of the present study was to identify cell signaling events critical to the expression of a single-trial cocaine CPP to better understand the neuroplasticity that occurs during the initial development of cocaine-environment associations (Bardo & Bevins 2000). We tested the hypothesis that the neuroadaptations that are associated with the expression of a cocaine CPP are distinct from those that occur with passive re-

exposure to the cocaine-paired (CS+) environment (**Fig 4.1**). The CPP expression test consists of a free choice test in which the rats spend time in all parts of the apparatus: the CS+ environment, the CS- environment, and the neutral intermediate chamber; a preference for the CS+ environment is described as a CPP and interpreted as a learned association between cocaine and the environment in which the drug was experienced. Thus, during the CPP expression test, the animals behaviorally demonstrate retrieval of the cocaine-environment association based on the amount of time spent in the CS+ environment. This expression test session can be contrasted with a passive re-exposure session, in which the rats are placed by the experimenter into the apparatus and only allowed access to the CS^+ environment. Thus, identification of neuroadaptations with passive re-exposure to the CS^+ environment controls for potential effects that expression of behavior and dual exposure to the CS^+ and CS^- environments may have on protein expression or activation. We also hypothesized that the neuroadaptations associated with retrieval of cocaine-environment associations would be localized to the synapse, the predominant site of plasticity thought to be critical to learning and memory (Maren 2005; Lee 2006). We focused on a molecular signaling pathways involving glutamate receptor subunit 1 (GluR1)-containing AMPA glutamate receptors and extracellular-signal regulated kinase (ERK), as studies utilizing multiple-pairing CPP paradigms with cocaine (Valjent *et al.* 2000; Miller & Marshall 2005; Tropea *et al.* 2008), opioids (Li *et al.* 2008; Liu *et al.* 2009) or amphetamine (Gerdjikov *et al.* 2004) have indicated that these molecules may be important in mediating the acquisition and/or expression of CPP.

A. Expression Test: Access to All Environments



B. Re-Exposure Session: Access to Only CS^+ Environment

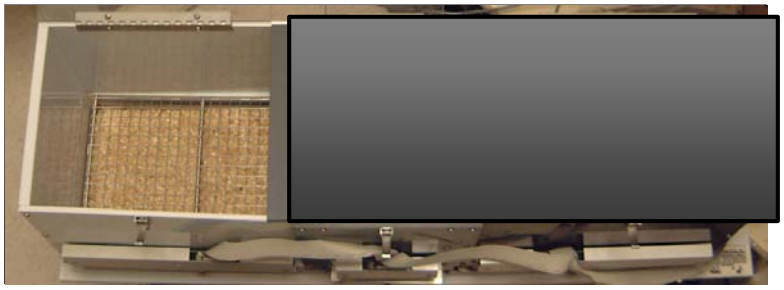


Figure 4.1: Diagram of Expression Test and Re-Exposure Sessions. Pictures represent the behavioral apparatus used to assess cocaine conditioned place preference that contained two separate conditioning environments (white side and black side) separated by the intermediary grey compartment. Guillotine doors could be raised to allow access to all environments or lowered to separate environments. (**A**) During the test session, guillotine doors were raised and the amount of time spent in each compartment was measured. (**B**) During the re-exposure session, rats were allowed access only to the environment that had previously been paired with cocaine (CS^+ environment).

The functional properties and synaptic insertion of ionotropic AMPA receptors depends on the composition and phosphorylation of the GluR subunits (GluR1-4) that unite to form each functional heteromeric AMPA receptor (Dingledine *et al.* 1999; Zhu *et al.* 2002). For example, phosphorylation at serine 845 by protein kinase A (PKA) enhances the function of GluR1-containing receptors by increasing channel open probability (Roche *et al.* 1996; Banke *et al.* 2000); thus, GluR1 subunits that are phosphorylated at serine 845 are described

as “activated.” Changes in expression and phosphorylation of GluR1 receptors have been observed after expression of CPP (Sakurai *et al.* 2007) and passive re-exposure to the CS+ environment (Tropea *et al.* 2008), respectively, suggesting that this subunit is critical to mediating cocaine-environment associations.

Activation of ERK, which can occur downstream of NMDA receptor activation (Lu *et al.* 2006), has also been linked to the acquisition and expression of psychostimulant CPP. For example, pharmacological inhibition of ERK activity blocks the acquisition of a cocaine CPP (Valjent *et al.* 2000), and an increase in phosphorylated ERK has been observed in animals that express a cocaine CPP (Miller & Marshall 2005). Activation of a downstream target of ERK (e.g., the transcription factor CREB) is also associated with the expression of CPP (Miller & Marshall 2005) or passive re-exposure to the CS+ environment (Tropea *et al.* 2008). Activated (phosphorylated) CREB binds to cAMP-response element (CRE) consensus sites located in the regulatory region of numerous genes, including GluR1, to enhance transcription (Carlezon *et al.* 2005). Thus, ERK, CREB, and GluR1-containing AMPA receptors and NMDA receptors are well poised to play critical roles in the retrieval of cocaine-environment associations. We initiated our studies by examining the activation and expression of ERK and GluR1 as markers of learning-induced neuroadaptations in key brain nodes for learning and memory (e.g, prefrontal cortex, amygdala and hippocampus).

The present study tested the hypothesis that increased activation and expression of ERK and GluR1 are associated with the retrieval and expression of classically conditioned cocaine-environment associations. Western blot analyses were conducted to assess the activation and expression of these proteins in the

prefrontal cortex (PFC), amygdala, and hippocampus in a single-pairing cocaine CPP paradigm. Our goal was to identify neuroadaptations that occur early in the development of cocaine-environment associations in the absence of neuroadaptations secondary to repeated exposure to cocaine (Bardo & Bevins 2000). Use of a novel analysis of CPP behavior that distinguishes animals in the cocaine-conditioned group that expressed a CPP from animals in this group that did not express a cocaine CPP (dela Cruz *et al.* 2009) enabled identification of the molecular adaptations critical to expression of the cocaine CPP separately from those attributable to cocaine exposure (Boudreau *et al.* 2007). Using this unique study design, we identified changes in PFC ERK expression and GluR1 activation upon exposure to CS^+ environment selectively in animals that had previously demonstrated a cocaine CPP. These results prompted subsequent analyses of NMDA receptor subunits as an additional type of ionotropic glutamate receptor and an upstream trigger for ERK signaling, as well as downstream target of the ERK signaling pathway (CREB expression and phosphorylation) to determine if a cascade of events related to passive re-exposure to the CS^+ environment could be identified.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (n=56; Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighed 225-275 g at the beginning of the study. The rats were housed two to four per cage in standard plastic rodent cages with food and water available *ad libitum* in a temperature (21-23°C) and humidity (55-65%) controlled

environment under a 12-h light/dark cycle (lights on 0700 h). Animals were acclimated to the colony for at least one week and were handled four times prior to the start of experimental sessions. All experiments were conducted during the light phase of the light-dark cycle (0800-1800 h) and were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and with approval from the Institutional Animal Care and Use Committee.

Drugs

Cocaine HCl salt (National Institute on Drug Abuse, Research Triangle, NC) and chloral hydrate salt (Sigma-Aldrich Co., St. Louis, MO) were dissolved in 0.9% NaCl and injected IP in a volume of 1 ml/kg and 2 ml/kg, respectively. All doses refer to the weight of the salt.

Conditioned Place Preference Apparatus

The CPP chambers were housed within sound- and light-attenuating cabinets and contained three sensory environments distinguished by wall colors and floor textures (n=8, ENV-013, MedAssociates, Georgia, VT). The two side (conditioning) chambers (interior dimensions: 25.5 cm L x 21.0 cm W x 20.9 cm H) were separated by a smaller chamber (13.2 cm L X 21.0 cm W x 20.9 cm H). One side chamber had white walls and a stainless steel mesh (1.3 x 1.3 cm) floor and the other side chamber had black walls and a floor of stainless steel rods (4.8 mm placed on 1.6 cm centers). The center chamber had gray walls and a floor of sheet metal. Guillotine doors separated each conditioning compartment

from the center compartment. The intensity of ambient illumination was adjusted to 7 lux in each side chamber and 30 lux in the center chamber to balance side preference for each large side chamber (Roma & Riley 2005). Automated data collection was accomplished through 15 infrared photobeam detectors placed 4.5 cm above the chamber floor with six photobeams across arrayed along the length of each side compartment. These photobeams were located 1.25 cm from the end wall with 5 cm between beams. Three photobeams were arrayed along the length of the central compartment spaced 4.75 cm apart. The photobeams were connected to a computer interface, and MED-PC software (MedAssociates) recorded the time spent in each chamber based on the recorded activity counts (any beam break within the current chamber) between disruption of (1) the entrance beam of that chamber (beam break beyond the first beam of a chamber) and (2) the entrance beam of a different chamber. Previous research in our laboratory demonstrates the unbiased nature of the apparatus (Chapters 2 and 3, dela Cruz *et al.* 2009).

Conditioned Place Preference Paradigm

In the present study, one cohort of rats experienced a three–phase CPP paradigm (preconditioning, conditioning, and expression test), while a second cohort experienced a four-phase paradigm (preconditioning, conditioning, expression test, and re-exposure) that occurred over consecutive days. In all phases, the animals were transferred in their home cages from the animal colony to the test room 5 min before the animals were placed in the CPP apparatus. The

preconditioning phase was utilized to determine baseline preference. Rats were placed in the center chamber with the guillotine doors raised and allowed to freely roam the apparatus for 30 min. The time spent in each conditioning chamber was recorded, and the conditioning chamber in which a rat spent the least amount of time was designated the "initially least-preferred chamber" for that rat.

The *conditioning* phase consisted of two sessions with the guillotine doors in place. Conditioning was performed using a biased protocol in which cocaine was paired with the initially least-preferred chamber (Spyraki *et al.* 1982; Blander *et al.* 1984; Isaac *et al.* 1989; Meyers *et al.* 2003), designated the CS^+ environment. For cocaine-treated rats, one of the conditioning sessions began with an injection of cocaine and immediate confinement to the CS^+ environment for 45 min. The other conditioning session began with a saline (1 mL/kg, IP) injection and immediate confinement to the alternate conditioning chamber (CS^- environment) for 45 min. Control rats were injected with saline prior to placement into each conditioning chamber for both sessions. For control animals, the initially least-preferred chamber was also designated as the CS^+ environment and the alternate conditioning chamber was designated the CS^- environment. In both cocaine and control groups, the two conditioning sessions were separated by at least six hours (Shippenberg & Heidbreder 1995) and the order of conditioning sessions was counterbalanced across rats. At the termination of each session, rats were returned to their home cages.

The *expression test* for CPP was conducted 16-24 hrs after the final conditioning session. Drug-free rats were placed in the center chamber with the guillotine doors raised and allowed to freely roam the apparatus for 15 min while the time spent in each chamber was recorded. Data are presented as mean time (sec ± S.E.M.) spent in the CS^+ environment. One cohort of rats (Expression Test Cohort; n=24) was sacrificed immediately following the test session.

In the second cohort (Re-Exposure Cohort; n=32) all rats were returned to the conditioning chambers for a re-exposure session on the day after the test session. In this *re-exposure* session, drug-free rats were confined to the CS^+ environment (with the guillotine doors down) for 30 min. No behavioral data were collected during this session. These rats were sacrificed immediately following this re-exposure session (Tropea *et al.* 2008).

Western Blot Analysis

Tissue dissection. At the time of sacrifice, rats were anesthetized with chloral hydrate (800 mg/kg, i.p.) and sacrificed via decapitation. Brain tissue was rapidly removed and dissected by hand on ice (Heffner *et al.* 1980). Briefly, 2 mm thick coronal sections were cut with the aid of a brain matrix (Harvard Apparatus, Holliston, MA). Selected nuclei were dissected with a scalpel using visual anatomical markers as guides. Approximate sections used in dissections were PFC +3.2 mm and amygdala and hippocampus -3.3 mm relative to bregma (**Fig. 4.2**) (Paxinos & Watson 1998). Tissue sections were flash frozen in liquid nitrogen and stored at -80°C until further processing.

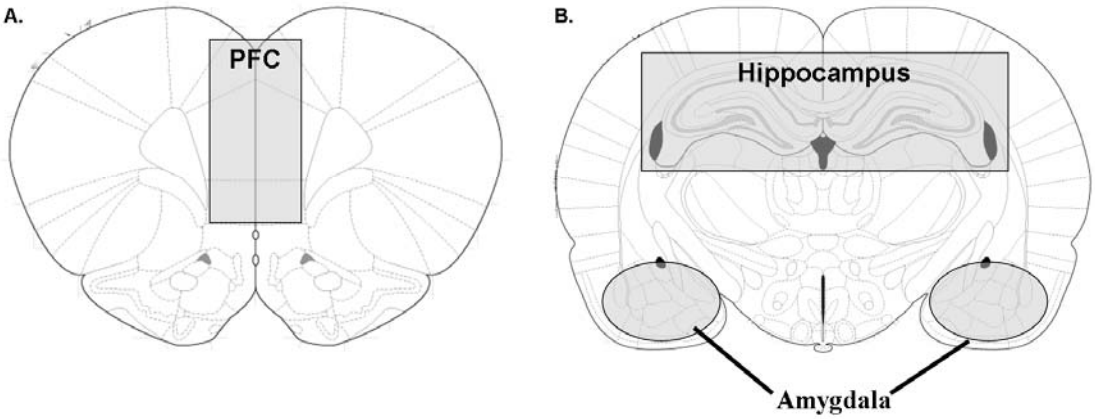


Figure 4.2: Diagram of representative sections for tissue dissection. Following sacrifice of each rat, brain tissue from the (**A**) prefrontal cortex (PFC) and (**B**) hippocampus and amygdala was rapidly dissected by hand on ice. Shaded boxes demonstrate the dissected regions.

Isolation of synaptosome-enriched fraction. Differential centrifugation was used to isolate a synaptosome-enriched fraction from each tissue sample. Prefrontal cortex, amygdala and hippocampus tissue from each rat were separately homogenized by hand on ice using a glass Teflon homogenizer in Krebs-glucose buffer (125 mM NaCl, 1.2 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 22 mM Na_2CO_3, 1 mM NaH_2PO_4, 10 mM glucose, 320 mM sucrose) with protease inhibitor cocktail (Sigma; 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin hydrochloride, n-(trans-epoxysuccinyl)-l-leucine 4-guanidinobutylamide, leupeptin hemisulfate salt, and pepstatin A) and phosphatase inhbitor cocktails (Sigma; cocktail 1:cantharidin, bomotetramisole,

and microcystin LR; cocktail 2: sodium orthovanadate, sodium molybdate, sodium tartrate, and imidazole). An aliquot of the total homogenate was saved for subsequent Western blot analysis. The rest of the homogenized tissue was centrifuged at 1,000g for 10 min to remove cell nuclei and debris (P1). The resulting supernatant (S1) was spun at 16,000 g for 20 min to isolate a synaptosome-enriched pellet (P2). The supernatant (S2) was discarded. Both the P1 and P2 pellets were resuspended in resuspension buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 0.5% NP-40) with protease and phosphatase inhibitor cocktails as above. Protein concentration of each sample was determined using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, Il).

Gels for Western blots. Fifteen to thirty micrograms of protein from each sample were prepared in standard SDS loading buffer for Western blot analysis; a standard amount was loaded onto each gel. The amount of protein loaded for each gel depended on the brain area and target protein. For example, 20 ug/lane was loaded for all hippocampus and amygdala samples. For PFC nuclear samples (for CREB staining), 30 ug were loaded in each lane. PFC total homogenate samples were loaded at 20 ug/lane, and PFC synaptosome-enriched samples were loaded at 15 ug/lane. Samples were heated at 70ºC for 10 min and loaded into 4-12% NuPage Bis-Tris precast gels (Invitrogen, Carlsbad, CA). Gels were run in NuPage MES SDS running buffer (Invitrogen) according to manufacturer's instructions and then prepared for wet transfer.

Transfer was performed in NuPage transfer buffer (Invitrogen) with 20% methanol on ice for 3 hrs at 100 V or overnight at 60 V. Membranes were stained with Ponceau S and gels were stained with Coomassie Blue to assure equal efficiency of transfer.

Western blotting. Membranes were blocked for 1 hour in Li-Cor blocking buffer (Li-Cor Biosciences, Lincoln, NE) diluted 1:1 in Tris-buffered saline, pH 7.4 (TBS) before incubation in primary antibody. All antibodies were diluted in Li-Cor blocking buffer diluted 1:1 with TBS with 0.1% Tween-20 (TBST). Overnight incubation at 4ºC was performed with the following primary antibodies: mouse anti-phospho ERK1/2 (1:1000, #9106, Cell Signaling Technology, Danvers, MA), rabbit anti-ERK1/2 (1:1000, #9102, Cell Signaling), rabbit anti-phosphoSer845 GluR1 (1:1000, Ab5849, Chemicon, Billerica, MA), rabbit anti-GluR1 (1:1000, Ab1504, Chemicon), mouse anti-NR2A (1:1000, #612286 BD Biosciences, San Jose, CA), and mouse anti-NR2B (1:1000, #610416 BD Biosciences). Two hour incubation at 25°C was performed with the mouse anti-NR1 (1:1000, #556308 BD Biosciences) antibody. One hour incubation at 25°C was performed with a mouse anti-actin (1:5000, Mab 1501, Chemicon) antibody. Following incubation with primary antibody, membranes were rinsed with TBS and washed 4 X 10 min in TBST. Membranes were then incubated for 1 hour at 25°C in secondary antibody (goat anti-mouse or goat anti-rabbit, 1:10,000, Li-Cor Biosciences). Following incubation, membranes were rinsed with TBS and washed 4 X 10 min in TBST. Antibody binding was detected using the Odyssey Infrared Imaging

System (Li-Cor Biosciences). Following staining for the phosphorylated protein, membranes were stripped via incubation in re-blotting solution (Chemicon) for 10-20 min at 25°C prior to staining for total proteins. Membranes were stripped again prior to staining for actin for normalization of protein loading. All Western blots were replicated 2-3 times.

Data Analysis

In the behavior experiments, expression of cocaine CPP was determined by the amount of time spent in the CS^+ environment during the test session. Previous work from our laboratory (Chapter 2, dela Cruz *et al.* 2009) has established a statistically-verified criterion for the expression of cocaine CPP based on the amount of time spent in the CS^+ environment during the test session. Using this criterion, rats conditioned with cocaine were divided into 2 groups: rats that met the criterion for cocaine CPP expression ("CPP expressing") and rats that did not meet the criterion ("non-CPP expressing"). A one-way analysis of variance (ANOVA) with pre-planned linear contrasts was used to compare time spent in the CS^+ environment between CPP expressing, non-CPP expressing, and control groups. The alpha level was set at p=0.05.

For protein analyses, expression of each protein of interest was normalized to actin and expressed relative to control, with control set at 100% expression. Differences in the expression of proteins of interest among the three groups defined by the behavior analyses were conducted with a one-way ANOVA with linear contrasts. Group comparisons were specifically defined prior

to the start of experiments, and thus *a priori* linear contrasts were conducted for pairwise comparisons regardless of the *F* value. This procedure is well justified in statistical texts (Keppel 1973; Sheskin 2004) and has been utilized previously in our laboratory (Herin *et al.* 2005; Szucs *et al.* 2005). The alpha level was set at p=0.05.

RESULTS

Cocaine Place Preference Conditioning

Previous research in our laboratory has demonstrated that rats conditioned with cocaine in the CPP paradigm can subdivided into two populations based on the amount of time spent in the CS^+ environment. Rats that spend at least 324 sec in the CS^+ environment can be classified as "expressing" a CPP, while those that spend less than 324 sec are categorized as "not expressing" a CPP (Chapter 2; dela Cruz *et al.* 2009). Applying this criterion to the behavioral data collected from the Expression Test Cohort, we revealed a "CPP expressing" group that met the criterion for CPP expression (n=10) and a "non-CPP expressing" group that did not meet this criterion (n=6). The "CPP expressing" group spent an average (±SEM) of 416 ± 29 sec in the CS^+ environment and 296 ± 24 sec in the CS^- environment, while the "non-CPP expressing" group spent 268 ± 14 sec in the CS^+ environment and 348 ± 20 sec in the CS^- environment. A one-way ANOVA with linear contrasts comparing the amount of time spent in the CS^+ environment revealed a significant effect of group (F=11.94, p<0.001); this effect was driven by the "CPP expressing" group, which spent significantly more time in the CS^+ environment than did the control

group (**Fig. 4.3A**, F=19.96, $p<0.001$) or the "non-CPP expressing" group (**Fig. 4.3A**, F=13.19, $p<0.01$). No difference in the amount of time spent in CS^+ environment between the control and "non-CPP expressing" groups was revealed (**Fig. 4.3A**, F=0.20, $p=0.66$).

Analysis of the time spent in the CS^+ environment during the test session for the Re-exposure Cohort revealed a "CPP expressing" group that met the criterion for CPP expression (n=20) and a "non-CPP expressing" group that did not meet this criterion (n=4). The "CPP expressing" group spent an average (±SEM) of 407 ± 10 sec in the CS^+ and 277 ± 13 sec in the CS^- environment, while the "non-CPP expressing" group spent 291 ± 10 sec in the CS^+ and 362 ± 22 sec in the CS^- environment. These values are similar to those observed in the Expression Test Cohort. A one-way ANOVA with linear contrasts comparing the amount of time spent in the CS^+ environment during the test session revealed a significant effect of group (F=10.92, $p<0.001$); this effect was driven by the "CPP expressing" group, as this group differed significantly from the control group (**Fig. 4.3B**, F=10.08, $p<0.01$) and the "non-CPP expressing" group (**Fig. 4.3B**, F=16.32, $p<0.001$). No difference in the amount of time spent in CS^+ environment during the test session was revealed between the control and "non-CPP expressing" groups (**Fig. 4.3B**, F=2.09, $p=0.15$).

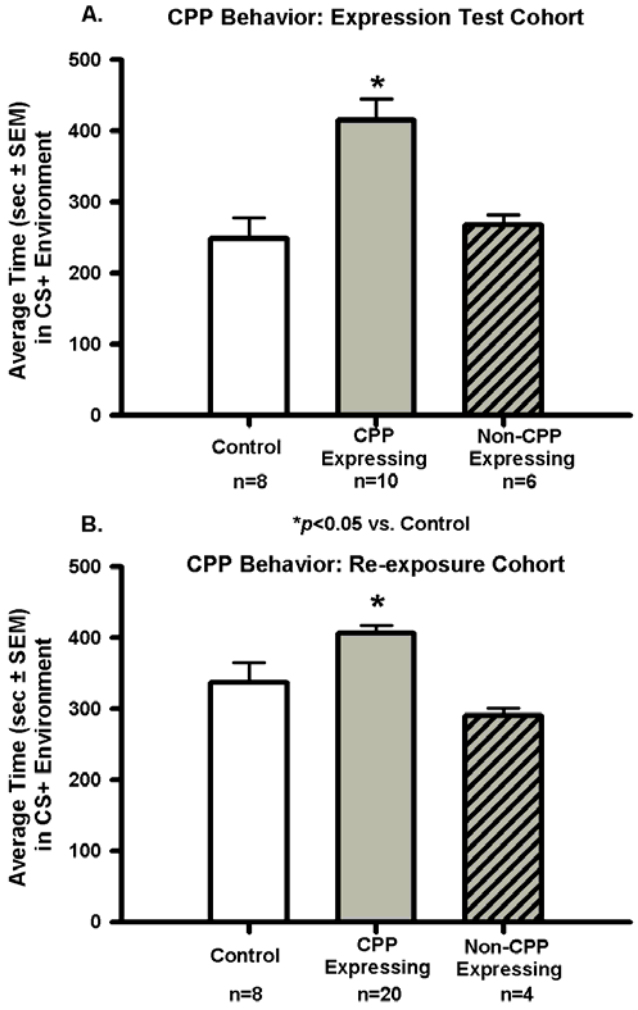


Figure 4.3: Behavioral data from CPP expression tests. Data represent the average time (seconds±SEM) spent in the CS^+ environment during the 15 min expression test from two cohorts of rats: **(A)** Expression Test cohort, sacrificed immediately after the expression test, and **(B)** Re-exposure Cohort, sacrificed immediately after re-exposure to the CS^+ environment on the day following the expression test session. The CPP expression test was conducted following a single pairing of cocaine (20 mg/kg) and environment. Rats in each cohort were sub-divided in to three groups: control group (white bar), who were conditioned with saline in the CS^+ environment; "CPP expressing" group (grey bar), a sub-group of the cocaine-conditioned rats that displayed a strong preference (>324 seconds) for the CS^+ environment; and "non-CPP expressing" group (striped grey bar), a sub-group of cocaine-conditioned rats that showed no preference for the CS^+ environment. The number of rats included in each sub-group is displayed on the x-axis below the group name. *p<0.05 vs. Control

Protein Expression: Expression Test Cohort

PFC Total Homogenate. No differences in the activation or expression of ERK (**Fig. 4.4A,B**) or in the activation (phosphorylation of serine 845) of GluR1 (**Fig. 4.5A**) between groups were seen in the total homogenate of tissue isolated from the PFC. A one-way ANOVA with pre-planned linear contrasts comparing the expression of GluR1 protein in the total homogenate isolated from the PFC revealed a trend towards an effect of group ($F=2.70$, $p=0.09$); this trend was driven by a previous history of cocaine exposure. The expression of GluR1 protein was significantly lower in PFC tissue isolated from the "CPP expressing" group compared to the control group (**Fig. 4.5B**, $F=4.90$, $p=0.04$) while the "non-CPP expressing" group tended to have decreased GluR1 expression compared to the control group (**Fig. 4.5B**, $F=2.81$, $p=0.11$). No difference between the "CPP expressing" and "non-CPP expressing" groups was observed (**Fig. 4.5B**, $F=0.10$, $p=0.75$). Thus, GluR1 protein expression tended to be decreased in the total homogenate of tissue isolated from the PFC of all rats that received cocaine, regardless of the results of the CPP expression test.

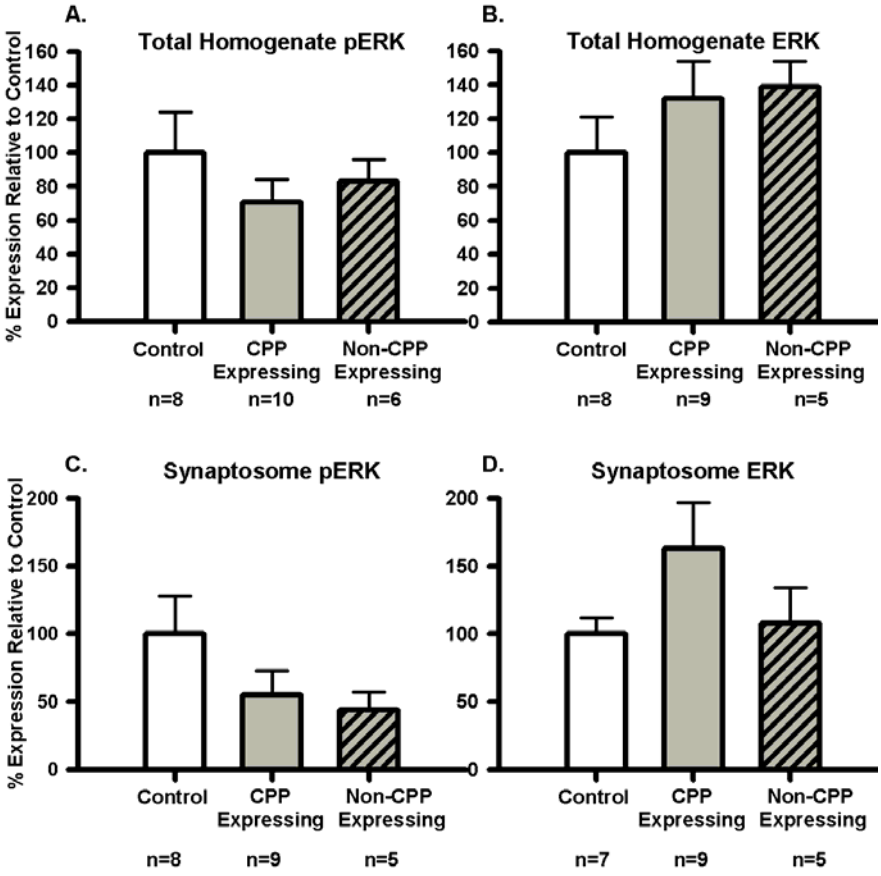


Figure 4.4: Expression Test Cohort: activation and expression of ERK in the PFC. Data presented are the results of densitometric analysis of Western blots to detect (**A**,**C**) dual ERK phosphorylation at threonine 202 and tyrosine 204 (pERK) and (**B**,**D**) total ERK expression in the PFC of rats sacrificed immediately following the 15 minute CPP expression test session in a single-trial cocaine (20 mg/kg) CPP paradigm. Samples of total homogenate (**A**, **B**) or a synaptosome-enriched fraction (Synaptosome; **C**,**D**) were analyzed. Pre-planned comparisons were conducted to compare ERK activation and expression among rats subdivided into three groups: Control (white bar), "CPP expressing" (grey bar) and "non-CPP expressing" (grey striped bar), based on the results of the CPP expression test conducted prior to sacrifice (see **Figure 4.3A** for details). No differences in ERK activation or expression in the total homogenate or synaptosome-enriched fraction of PFC tissue were observed.

PFC Synaptosome. The protein expression in the synaptosome-enriched fraction of the PFC followed a similar pattern to the total homogenate, suggesting that changes at the synapse drove the changes observed in the total homogenate. No differences in the activation or expression of ERK (**Fig. 4.4C**,**D**) or in the activation (phosphorylation of serine 845) of GluR1 (**Fig. 4.5C**) between groups were seen in the synaptosome-enriched fraction isolated from the PFC. A one-way ANOVA with pre-planned linear contrasts comparing the expression of GluR1 protein in the synaptosome-enriched fraction isolated from the PFC suggested a downregulation of GluR1 expression. This analysis revealed a trend toward an effect of group ($F=2.71$, $p=0.09$), with the trend driven by a previous history of cocaine exposure. Expression of GluR1 protein tended to be decreased in the synaptosome-enriched fraction isolated from the "CPP expressing" group compared to the control group (**Fig. 4.5D**, $F=3.40$, $p=0.08$), and GluR1 expression was significantly decreased in "non-CPP expressing" group compared to the control group (**Fig. 4.5D**, $F=4.54$, $p=0.05$). No difference in GluR1 expression was observed between "CPP expressing" and "non-CPP expressing" groups (**Fig. 4.5D**, $F=0.24$, $p=0.63$), suggesting that GluR1 expression was decreased in all rats that received cocaine conditioning without regard to the results of the CPP expression test.

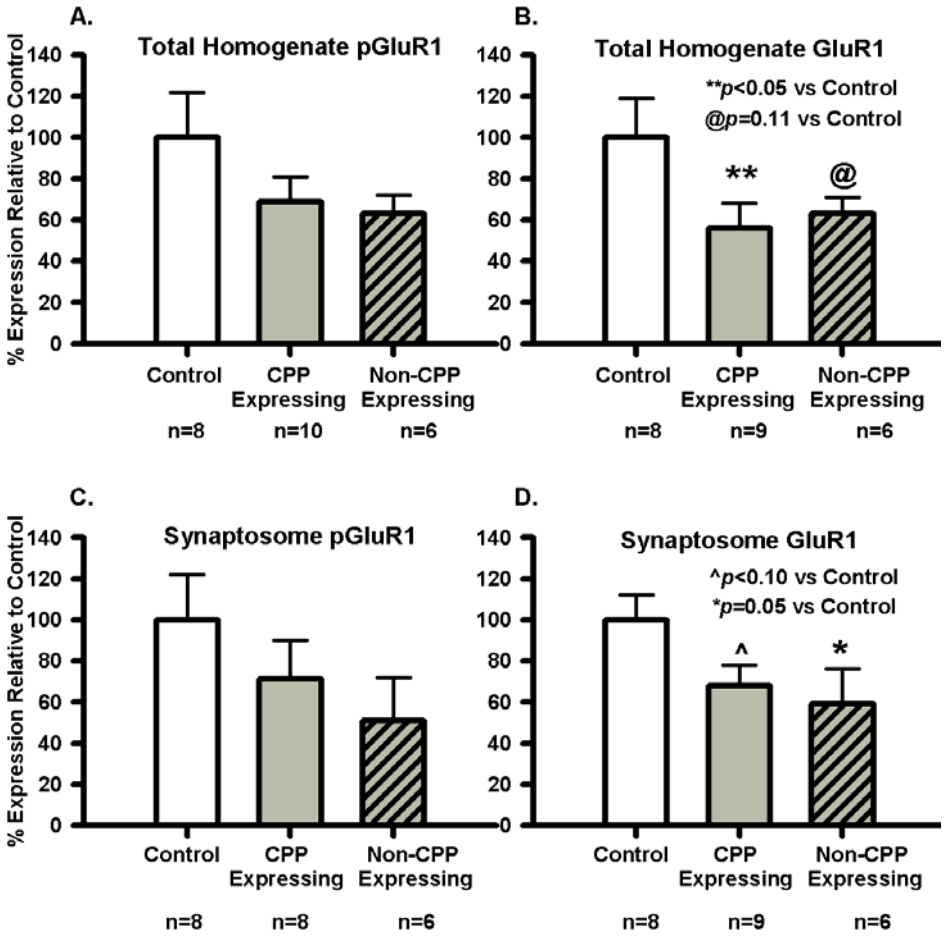


Figure 4.5: Expression Test Cohort: activation and expression of GluR1 in the PFC. Data presented are the results of densitometric analysis of Western blots to detect (**A,C**) GluR1 phosphorylation at serine 845 (pGluR1) and (**B,D**) total GluR1 expression in the PFC of rats sacrificed immediately following the 15 minute CPP expression test session in a single-trial cocaine (20 mg/kg) CPP paradigm. Samples of total homogenate (**A, B**) or a synaptosome-enriched fraction (Synaptosome; **C,D**) were analyzed. Pre-planned comparisons were conducted to compare GluR1 activation and expression among rats subdivided into three groups: Control (white bar), "CPP expressing" (grey bar) and "non-CPP expressing" (grey striped bar), based on the results of the CPP expression test conducted prior to sacrifice (see **Figure 4.3A** for details). Total GluR1 expression was decreased in both the total homogenate and synaptosome-enriched fraction in all rats that received cocaine. $^{**}p<0.05$, $^{*}p=0.05$, $^{\wedge}p<0.10$, $@p=0.11$ versus Control.

Other Brain Areas. No differences in the activation or expression of ERK or GluR1 between groups were observed in the total homogenate or synaptosome-enriched fractions isolated from the amygdala or hippocampus (**Table 4.1**).

Table 4.1: Expression Test Cohort: Amygdala and Hippocampus Protein Expression

Western Blot Densitometry Expressed as Percent Relative to Control							
Brain Area	Fraction	**pERK**			**ERK**		
		Control	**CPP Expressing**	**Non-CPP Expressing**	**Control**	**CPP Expressing**	**Non-CPP Expressing**
Amygdala	Total	100±5	97±6	115±4	100±6	103±5	105±10
	Synaptosome	100±11	106±12	114±12	100±15	87±10	110±25
Hippocampus	Total	100±11	98±22	133±48	100±6	94±9	90±11
	Synaptosome	100±21	51±12	170±101	100±12	105±17	147±52
		pGluR1			**GluR1**		
		Control	**CPP Expressing**	**Non-CPP Expressing**	**Control**	**CPP Expressing**	**Non-CPP Expressing**
Amygdala	Total	100±5	91±6	99±11	100±9	101±7	111±14
	Synaptosome	100±13	104±12	133±3	100±15	124±14	132±11
Hippocampus	Total	100±5	111±19	91±20	100±14	92±13	81±21
	Synaptosome	100±12	117±28	132±49	100±12	80±16	157±61

Protein Expression: Re-Exposure Cohort

PFC Total Homogenate. No differences in the activation or expression of ERK (**Fig. 4.6A,B**) or GluR1 (**Fig. 4.7A,B**) between groups were seen in the total homogenate of tissue isolated from the PFC.

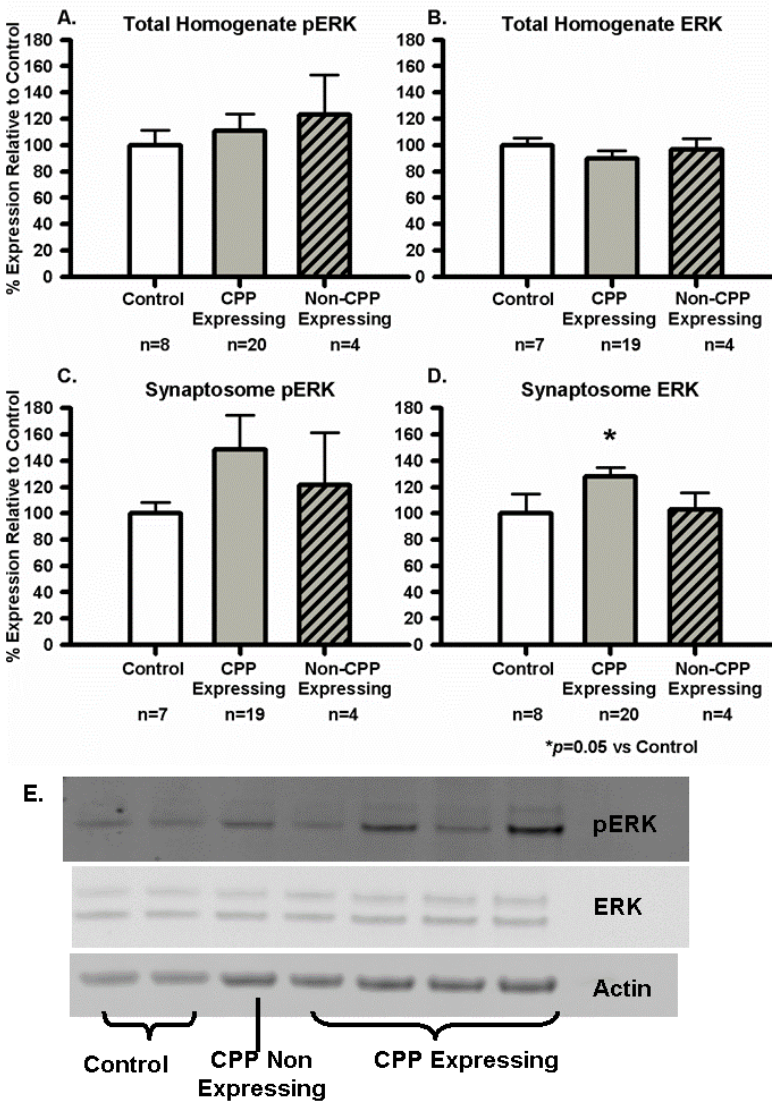


Figure 4.6: Re-exposure Cohort: activation and expression of ERK in the PFC. Data presented are the results of densitometric analysis of Western blots to detect (**A,C**) dual ERK phosphorylation at threonine 202 and tyrosine 204 (pERK) and (**B,D**) total ERK expression in the PFC of rats sacrificed immediately following the 30 minute re-exposure to the CS^+ environment in a single-trial cocaine (20 mg/kg) CPP paradigm. Samples of total homogenate (**A, B**) or a synaptosome-enriched fraction (Synaptosome; **C,D**) were analyzed. Pre-planned comparisons were conducted to compare ERK activation and expression among rats subdivided into three groups: Control (white bar), "CPP expressing" (grey bar) and "non-CPP expressing" (grey striped bar), based on the results of the CPP expression test conducted prior to sacrifice (see **Figure 4.3B** for details). (**E**) Representative Western blot of total homogenate. Total ERK expression was increased in the synaptosome-enriched fraction isolated from the "CPP expressing" group. *p=0.05 versus Control

PFC Synaptosome. Although no differences in the phosphorylation of ERK in the synaptosome-enriched fraction isolated from the PFC were observed (**Fig. 4.6C**), a significant increase in ERK protein was observed selectively in the "CPP expressing" group (**Fig. 4.6D**). A one-way ANOVA with pre-planned linear contrasts comparing the expression of ERK protein in the synaptosome-enriched fraction isolated from the PFC revealed a trend toward an effect of group ($F=2.46$, $p=0.10$); this trend was driven by the "CPP expressing" group, as this group differed significantly from the control group (**Fig. 4.6D**, $F=4.00$, $p=0.05$). No difference in ERK protein expression between the control and "non-CPP expressing" groups was revealed (**Fig. 4.6D**, $F=0.02$, $p=0.89$). Thus, enhanced expression of ERK protein in the PFC synaptosome was seen upon re-exposure to the cocaine-paired environment selectively in rats that expressed a cocaine CPP.

We observed a trend towards an increase in the phosphorylation of serine 845 of GluR1 in the synaptosome-enriched fraction isolated from the PFC (**Fig. 4.7C**), without a change in GluR1 protein expression in this fraction (**Fig. 4.7D**). A one-way ANOVA comparing the phosphorylation of GluR1 in the synaptosome-enriched fraction isolated from the PFC revealed no main effect of group ($F=2.21$, $p=0.13$). Pre-planned linear contrasts revealed a trend toward an increase in the "CPP expressing" group compared to the control group (**Fig. 4.7C**, $F=3.18$, $p=0.09$). No difference in the phosphorylation of GluR1 between the control and "non-CPP expressing" groups was revealed (**Fig. 4.7C**, $F=0.01$, $p=0.92$). Thus, a trend towards enhanced phosphorylation of GluR1 in the synaptosome was seen upon re-exposure to the cocaine-paired environment selectively in rats that expressed a cocaine CPP.

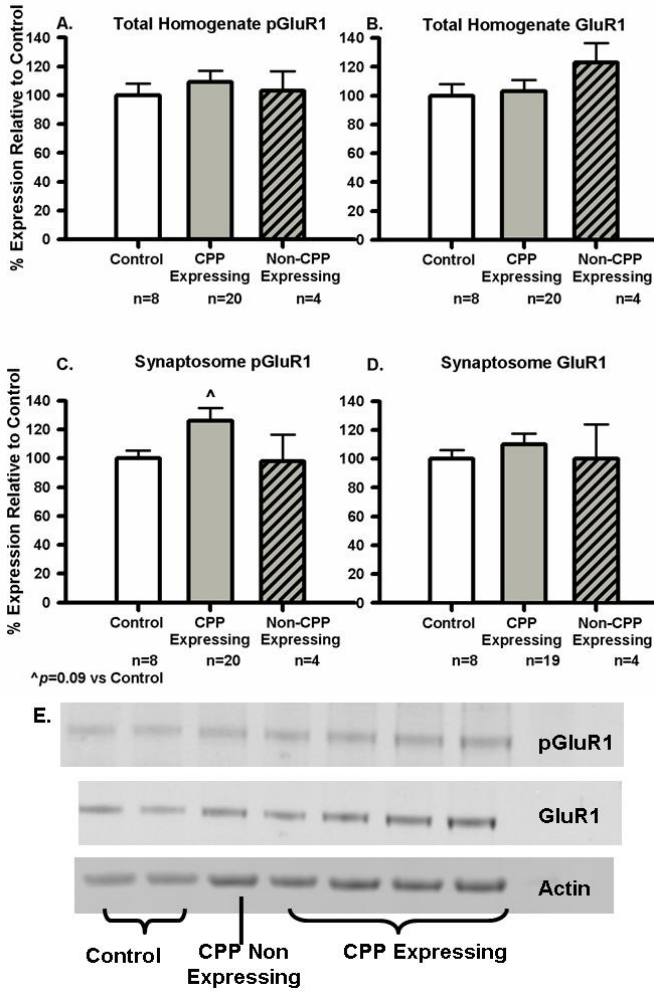


Figure 4.7: Re-exposure Cohort: Activation and expression of GluR1 in the PFC. Data presented are the results of densitometric analysis of Western blots to detect (**A,C**) GluR1 phosphorylation at serine 845 (pGluR1) and (**B,D**) total GluR1 expression in the PFC of rats sacrificed immediately following the 30 minute re-exposure to the CS^+ environment in a single-trial cocaine (20 mg/kg) CPP paradigm. Samples of total homogenate (**A, B**) or a synaptosome-enriched fraction (Synaptosome; **C,D**) were analyzed. Pre-planned comparisons were conducted to compare GluR1 activation and expression among rats subdivided into three groups: Control (white bar), "CPP expressing" (grey bar) and "non-CPP expressing" (grey striped bar), based on the results of the CPP expression test conducted prior to sacrifice (see **Figure 4.3B** for details). (**E**) Representative Western blot of total homogenate. Phosphorylation of GluR1 tended towards an increase in the synaptosome-enriched fraction isolated from the "CPP expressing" group. ^*p*=0.09 versus Control

Other brain areas. No differences in the activation or expression of ERK or GluR1 in the total homogenate or synaptosome-enriched fractions isolated from the amygdala or hippocampus between control, "CPP expressing," and "non-CPP expressing" groups were observed (**Table 4.2**).

Table 4.2: Re-Exposure Cohort: Amygdala and Hippocampus Protein Expression

Western Blot Densitometry Expressed as Percent Relative to Control							
Brain Area	Fraction	**pERK**			**ERK**		
		Control	**CPP Expressing**	**Non-CPP Expressing**	**Control**	**CPP Expressing**	**Non-CPP Expressing**
Amygdala	Total	100±26	82±12	103±33	100±24	89±12	95±25
	Synaptosome	100±10	106±12	125±18	100±6	111±9	102±17
Hippocampus	Total	100±13	98±8	89±13	100±16	89±8	92±19
	Synaptosome	100±13	138±15	110±32	100±16	118±14	132±44
		pGluR1			**GluR1**		
		Control	**CPP Expressing**	**Non-CPP Expressing**	**Control**	**CPP Expressing**	**Non-CPP Expressing**
Amygdala	Total	100±27	88±8	79±19	100±25	89±9	97±29
	Synaptosome	100±10	124±13	115±36	100±17	105±10	97±29
Hippocampus	Total	100±6	95±6	101±11	100±4	98±6	95±7
	Synaptosome	100±6	94±6	99±8	100±5	103±5	96±9

Additional Protein Analyses. Building upon our findings suggesting that ERK expression and GluR1 phosphorylation were selectively altered by re-exposure to only the CS^+ environment in the synaptosome-enriched fraction isolated from rats that expressed a cocaine CPP, we investigated the expression of several additional proteins in the Re-Exposure Cohort. To test the hypothesis that enhanced ERK expression in the synaptosome would lead to activation of the transcription factor cAMP response element binding (CREB), we measured

the activation (phosphorylation) and expression of this protein in a nuclear-enriched fraction. CREB activation and expression were determined only in the nuclear-enriched fraction, as preliminary data determined that this protein could not be consistently detected in the total homogenate. No differences in the activation or expression of CREB between groups were seen in the nuclear-enriched fraction isolated from PFC tissue (**Table 4.3**).

Table 4.3: Re-Exposure Cohort: Additional Proteins

Western Blot Densitometry Expressed as Percent Relative to Control			
Protein	**Control**	**CPP Expressing**	**Non-CPP Expressing**
pCREB (Nuclear)	100±16	86±12	85±29
CREB (Nuclear)	100±16	107±8	98±8
NR1 (Synaptosome)	100±11	102±10	73±8
NR2A (Synaptosome)	100±14	90±7	92±11
NR2B (Synaptosome)	100±8	98±8	117±11

To test the hypothesis that enhanced expression of ERK occurred downstream of alterations in NMDA receptor-mediated signaling, we assessed the expression of the NMDA receptor subunits NR1, NR2A, and NR2B in the synaptosome-enriched fraction isolated from the PFC. NMDA receptors, like GluR1-containing AMPA receptors, are ionotropic glutamate receptors heavily implicated in learning; thus, assessment of NMDA receptor subunit expression served as additional assessment of synaptic adaptations associated with learning. No differences in the expression of any NMDA receptor subunit between control, “CPP expressing”, and “non-CPP expressing” groups were seen (**Table 4.3**).

Discussion

Utilizing a novel experimental design in rats, we identified changes in ERK expression and GluR1 activation in the PFC associated with passive re-exposure to a cocaine-paired environment. At the timepoint assessed (immediately post-exposure), synaptosomal ERK expression was increased upon passive re-exposure to the cocaine-associated environment exclusively in rats that had previously demonstrated a cocaine CPP following a single pairing of cocaine and the environment. In addition, these same animals showed a trend toward an enhanced activation of synaptosomal GluR1. These changes were not observed in the other brain areas examined (hippocampus and amygdala) and occurred independently of CREB activation, a downstream consequence of ERK activation, and alterations in other glutamate receptor subunits implicated in learning (e.g., the NR1, NR2A, and NR2B subunits of the NMDA receptor). These results suggest that subtle alterations in PFC protein activation and expression can be observed at early time points following conditioning and may underlie long-lasting cocaine-environment associations.

The molecular adaptations detected in the present study were localized to the PFC, lending further support to the hypothesis that the PFC is a key site for signaling the conditioned rewarding properties of cocaine-paired stimuli and consistent with prior a report implicating the PFC in the acquisition of a cocaine-environment association (Tzschentke & Schmidt 1999). In rats previously trained in a cocaine self-administration paradigm, the firing rate of a sub-population of PFC neurons was enhanced upon exposure to cocaine-paired cues (Rebec & Sun 2005), and temporary inactivation of the dorsomedial PFC with GABA

agonists prevented discrete cue- (McLaughlin & See 2003) or context- (Fuchs *et al.* 2005) induced reinstatement of cocaine-seeking behavior. The present results extend this earlier research by implicating proteins localized to the PFC as important mediators of the response to cocaine-environment associations developed after a single pairing of cocaine and the environment. These data suggest that the PFC is critical to the development and expression of cocaine-environment associations early in the learning process.

Our studies identified changes in ERK and GluR1 within the PFC triggered upon passive exposure to a cocaine-associated environment. A selective increase in synaptosomal ERK protein expression and activation (phosphorylation) of GluR1 was detected upon re-exposure to the CS^+ environment exclusively in animals that had previously expressed a cocaine CPP; animals that underwent the single-trial conditioning with cocaine but did not express the CPP displayed levels of ERK expression and GluR1 activation similar to rats in the control group that did not receive cocaine. These results suggest adaptations to ERK and GluR1 in the PFC occur upon recognition of a cocaine-associated environment and therefore may be important components of the molecular adaptations that underlie relapse to drug use.

Previous work has implicated ERK as a key signaling component associated with multi-trial cocaine CPP (Valjent *et al.* 2000; Miller & Marshall 2005; Tropea *et al.* 2008). These studies, which utilized three pairings of cocaine and environment, demonstrated enhanced ERK phosphorylation in the NAc following the CPP test session (Miller & Marshall 2005) and in the hippocampus upon passive re-exposure to the CS^+ environment (Tropea *et al.* 2008) in the absence of enhanced ERK expression. To our knowledge, the activation or

expression of ERK in the PFC following multi-trial cocaine CPP conditioning has not been examined, although a recent report suggests that ERK expression is decreased in frontal association cortex in mice sacrificed immediately following the test session for a morphine (5 mg/kg, 8 pairings) CPP (Li *et al.* 2008). We have thus identified a novel molecular adaptation that occurs with passive re-exposure to a cocaine-paired environment following a single pairing of cocaine and environment.

A trend towards increased GluR1 phosphorylation at serine 845 was also observed upon passive re-exposure to the cocaine-paired environment only in rats that had previously expressed a cocaine CPP. This observation suggests that activation of the GluR1 receptor, which occurs downstream of PKA activation (Roche *et al.* 1996), is enhanced upon recognition of a cocaine-associated environment. Indeed, previous studies have shown that systemic pharmacological inhibition of AMPA receptors (a mixed population that includes GluR1-containing receptors) blocked expression of a cocaine CPP (Cervo & Samanin 1995; Maldonado *et al.* 2007). Further, germline genetic deletion of the GluR1 subunit in mice appears to prevent acquisition of a cocaine CPP under certain conditions (Dong *et al.* 2004), but see (Mead *et al.* 2005). Thus, our findings lend additional support to the hypothesis that activation of AMPA glutamate receptors, particularly those containing the GluR1 subunit, occurs with re-expsoure to cocaine-paired stimuli.

Alterations in the GluR1 receptor expression were also detected in the total homogenate and synaptosome-enriched fractions of the PFC in the cohort of rats sacrificed immediately following the expression test session. Examination of GluR1 protein expression following the CPP test session revealed a decrease

in PFC GluR1 protein expression in rats that expressed a cocaine CPP ("CPP expressing") and in animals that were conditioned with cocaine but did not express a cocaine CPP ("non-CPP expressing"). Further, the GluR1 expression levels in the "non-CPP expressing" group were similar to the "CPP expressing" cohort. These results indicate that the alterations in GluR1 expression detected following the test session in the present paradigm are most likely due to exposure to cocaine, rather than being related to the expression of a cocaine CPP. Separation of cocaine-conditioned rats by the criterion into "CPP expressing" and "non-CPP expressing" groups for protein analysis was critical to forming this conclusion, as, in the absence of this important analysis of each rat, we would have inappropriately concluded that the decrease in GluR1 expression was related to the acquisition or expression of a cocaine CPP .

A limited number of studies have revealed that a single cocaine injection can alter GluR1 activation and mRNA expression, although most studies measuring cocaine-induced changes in GluR1 expression involved repeated administration of cocaine. Acute cocaine treatment enhanced phosphorylation of GluR1 at serine 845 in the neostriatum (Snyder *et al.* 2000) and increased GluR1 mRNA in the NAc and ventral tegmental area (VTA) 1, 24, or 48 hours after treatment (Grignaschi *et al.* 2004). However, GluR1 protein expression was not altered in VTA tissue isolated from rats sacrificed 16-18 hours after injection (Fitzgerald *et al.* 1996), nor were changes in GluR1 expression detected in the hippocampus or amygdala at 24 hours following cocaine injection in the present study. Taken together, results from previous studies and those conducted here suggest that GluR1 expression and activation can be altered by a single injection of cocaine, although the effects appear to be dependent upon brain area, and

little is known regarding the timecourse of these alterations. These neuroadaptations, however, may be a critical component of the behavioral alterations that occur upon a single administration of cocaine.

We did not detect differences in the activation or expression of ERK or GluR1 in the amygdala or hippocampus following either the CPP test session or passive re-exposure to the CS^+ environment in the present study. These results were rather surprising given that a recent study by Tropea and colleagues (Tropea *et al.* 2008) that demonstrated increased activation of ERK and GluR1 in the dorsal hippocampus following re-exposure to a CS^+ environment that had been paired three times with cocaine (10 mg/kg). This discrepancy suggests that initial learning modeled in the single-trial paradigm differs from the learning that occurs with multiple pairings of cocaine and environment. A growing body of literature suggests differential neuronal responses following single versus repeated cocaine treatment (present results; Thomas *et al.* 2001; Filip *et al.* 2004; Mattson *et al.* 2005; Huang *et al.* 2007; Kourrich *et al.* 2007). Additional research examining differences between single- and multi-trial CPP paradigms will be necessary to delineate the precise roles for ERK and GluR1 activation in the hippocampus in the retrieval of cocaine-environment associations.

We chose to analyze the activation of CREB in attempt to provide a broader picture of how ERK and GluR1 signaling are involved in the recognition of cocaine-associated environmental cues, as activation of this transcription factor has been previously demonstrated to occur downstream of ERK activation in rats that express a cocaine CPP (Miller & Marshall 2005) and to regulate GluR1 expression (Carlezon *et al.* 2005). Further, we examined expression levels of the NR1, NR2A, and NR2B subunits of the NMDA receptor, activation of

which is critical to the ability of cocaine to induce phosphorylation of ERK (Valjent *et al.* 2000); activation of this receptor is also critical to learning and memory (Klann & Sweatt 2008). However, under the parameters of the present study, no differences in nuclear CREB activation or expression of NMDA receptor subunit expression in synaptosomes were detected in the PFC. These results may suggest that these proteins are not involved in the retrieval of cocaine-environment associations, although alterations in expression/activation of the proteins may occur under different conditions (e.g., if measured at a different timepoint).

An important feature of the present study that must be taken into account when interpreting the results is the timeframe in which the animals were sacrificed and tissue was collected for analysis. In the current work, animals were sacrificed immediately following a 15 min CPP test or a 30 min context re-exposure session that occurred 24 hours after the CPP test. This "snapshot" approach is advantageous to simultaneously analyze the expression and activation of several proteins across a variety of brain areas. The drawback to this approach, however, is that the timeframe chosen may not represent the peak window in which adaptations occur for all the proteins examined. The timepoints for the present study were chosen based on previous studies demonstrating changes in ERK phosphorylation in animals sacrificed 15 min after a 15 min CPP test session (Miller & Marshall 2005) or immediately following a 20 min re-exposure session that occurred 48 hours after the CPP test (Tropea *et al.* 2008). Despite similarity to timepoints at which alterations in ERK phosphorylation were detected, the present study did not detect changes in ERK phosphorylation following the CPP test or upon re-exposure to the CS^+ environment, suggesting

that the differential influence upon ERK phosphorylation is more likely due to the single- vs. multiple-trial CPP paradigms utilized in the present and prior studies, respectively. However, we cannot exclude the possibility that changes in ERK phosphorylation or expression and activation of the other proteins examined in the present study did occur outside of the time period analyzed. A more high-throughput method for analyzing protein expression than traditional Western blot analyses would be necessary, however, to provide a more complete picture of how activation and expression of multiple proteins is altered among various brain areas over time.

Our research highlights the role of the PFC in mediating cocaine-context associations, as all observed changes in protein expression and activation occurred in this area. Human neuroimaging studies have heavily implicated the PFC in mediating cue-induced craving, as presentation of cocaine-associated cues increases activity in multiple PFC subdivisions (Childress *et al.* 1999; Kosten *et al.* 2006). Thus, additional research to investigate the functional role of the PFC in both single- and multi-trial cocaine conditioning paradigms is needed to better understand the control this area exerts over the ability of cocaine-associated cues to induce relapse to drug-seeking.

Chapter 5: Conclusions

The studies in this dissertation were inspired by a vexing conundrum of research into the syndrome of cocaine addiction: why does this disease develop in only a subset of those who use cocaine? We propose that the ability to learn strong associations between the effects of cocaine and the environment in which the drug is experienced is a fundamental predictor of individuals who will initiate the progression to addiction. We further propose that those who develop the learned associations do so because they are susceptible to the induction of neuroplasticity within the prefrontal cortex (PFC); this neuroplasticity represents the molecular substrate of learning that then supports the retrieval and reconsolidation of cocaine-environment associations. However, even the population that develops learned cocaine-environment associations is not homogeneous as a further subset is susceptible to blockade of retrieval by pharmacological manipulations.

We began our studies with the knowledge that stimuli associated with cocaine induced both a strong desire to use the drug and increased blood flow in several brain areas implicated in reward in abstinent cocaine users (Childress *et al.* 1999). We utilized the conditioned place preference (CPP) paradigm to model in laboratory rats the ability of cocaine-associated cues to drive behavior. The CPP paradigm assesses the expression of learned cocaine-environment associations during a free choice test in which rats are given the opportunity to spend time in either an environment that has previously been paired with cocaine treatment or in an environment that has been paired with saline. Rats that spend more time in contact with the cocaine-paired environment are described as

"expressing a CPP." Our first study (Chapter 2) demonstrated that approximately 80% of rats that experienced the cocaine conditioning procedure demonstrated a CPP. In other words, only a subset of rats that are exposed to cocaine demonstrate an ability of a cocaine-associated environment to drive behavior. It is thus tempting to suggest that this subpopulation of rats that successfully learns the cocaine-environment association models the subset of human users that develop addiction. Although this topic remains to be explored in cocaine-dependent subjects, reactivity to cigarette-associated cues is associated with the level of nicotine dependence (Payne *et al.* 1996) and treatment outcome in subjects attempting to quit smoking (Payne *et al.* 2006, but see Avants *et al.* 1995) suggesting a relationship between the severity of addiction and the ability of cue-associations to drive behavior.

The subpopulation of rats that learned the cocaine-environment association, however, may model the population of humans *resistant* to the initiation of consistent cocaine-taking and subsequent development of addiction. Although 80% of rats learn the CPP, less than 20% of humans that use cocaine develop an addiction (Wagner & Anthony 2002) . We also observed a CPP in rats that experienced only a single pairing of cocaine and environment, while in humans the vast majority of addictions are believed to develop with repeated incidents of cocaine use (O'Brien *et al.* 1992). By developing a criterion method of analysis, however, we were able to separate rats that expressed a CPP (learned the cocaine-environment association) from those who did not and thus identify individual differences within the CPP paradigm. Prior to these studies, no method for quantification of individual differences in CPP was available despite the importance of individual differences in the clinical syndrome. The proposal

that rats that learn the cocaine-environment association model a population prone to initiate addiction could be tested by comparing cocaine self-administration among “CPP expressing” and “non-CPP expressing” rats. We hypothesize that the “CPP expressing” rats will acquire self-administration at a lower cocaine dose than will “non-CPP expressing” rats and that “CPP expressing” rats will acquire self-administration at a faster rate. Each of these properties would be consistent with our proposal that susceptibility to the development of learned cocaine-environment association predicts the likelihood to initiate consistent cocaine-taking. We propose that use of the criterion method will allow for a better understanding of the factors that separate the population of subjects that is susceptible to the development and expression of learned cocaine-environment associations, which may translate to a better understanding of the factors that control the development and maintenance of cocaine addiction in humans.

We then asked whether subjects that learned the cocaine-environment association were differentially susceptible to pharmacological manipulation of CPP acquisition and expression by ligands for the two members of the 5-HT_2 receptor family expressed most abundantly in brain (e.g., 5-HT_{2A}R and 5-HT_{2C}R; Hoyer *et al.* 2002), and we observed two different types of differential sensitivity to these manipulations. The first type of differential sensitivity appears to be based on the relative strength of learning, as the selective 5-HT_{2A}R antagonist M100907 blocked the acquisition and expression of the CPP formed after one pairing, but not four pairings, of cocaine and environment. The second type of differential sensitivity suggests differences between individuals in the modulation of learned cocaine-environment associations by stimulation of the 5-HT_{2C}R, as

we observed a block of the expression of the CPP formed after a single pairing of cocaine and environment by treatment with the 5-HT_{2C}R agonist MK 212 in only a subgroup of rats; some rats conditioned with cocaine and treated with MK 212 displayed a strong CPP. These two types of differential sensitivity will now be considered separately.

The observed blockade of single, but not four, trial CPP by M100907 suggests the ability of the 5-HT_{2A}R to modulate the acquisition and expression of CPP is lost with multiple pairings of cocaine and environment. It is intriguing that M100907 treatment blocked both acquisition and expression, as these two phases represent fundamentally different learning processes. The acquisition of cocaine CPP requires detection of the environmental cues, detection of the interoceptive cue caused by cocaine (i.e., the cocaine discriminative stimulus), interpretation of this cue as rewarding, association of the reward with the environmental cue, and commitment of the association to long-term memory. Treatment with M100907 could block any of these steps and successfully block the acquisition of a cocaine CPP. Previous work from our laboratory suggests that M100907 blocks the interoceptive cocaine cue and the rewarding properties of cocaine, as M100907 blocks the discriminative stimulus effects of cocaine in the drug discrimination paradigm and the locomotor activity effects of cocaine in a modified open field locomotor assay (McMahon & Cunningham 2001). The suggestion that M100907 treatment blocks the rewarding effects of cocaine has been questioned, however, as this treatment does not block the reinforcing properties of this drug in the self-administration paradigm (Nic Dhonnchadha *et al.* 2008). Treatment with M100907 may also block the formation of the association, as stimulation of 5-HT_{2A}R has long been associated with the

development of other classically conditioned associations (Harvey 1996) and with the transfer of information from short-term to long-term memory storage (Meneses *et al.* 1997). Repeated cocaine treatment, by altering the expression or signaling partners of the 5-HT_{2A}R, could alter each of these steps, prompting the loss of control by the 5-HT_{2A}R antagonist in the four trial paradigm. Thus, pharmacological blockade of 5-HT_{2A} receptors could act at many levels to prevent acquisition of a cocaine CPP.

Separate processes are involved in the expression of CPP, including perception of the environmental cues, retrieval of the cocaine-context association, and an ability of the association to drive behavior. We are not aware of previous studies that have specifically examined the role of the 5-HT_{2A}R in the retrieval of classically conditioned associations, making it difficult to differentiate between these possibilities. Blocking the ability of the association to drive behavior would certainly be beneficial to humans seeking to remain abstinent from cocaine use. One major theory of addiction suggests that drug-seeking in the face of cues is a habit, meaning that this behavior occurs in a stereotyped fashion without regard to reward value (Everitt & Robbins 2005), and anecdotal reports from patients who have relapsed support this notion (O'Brien *et al.* 1992). It is clear, however, that the aspect of expression modulated by 5-HT_{2A}R changes with repeated cocaine administration. It may be that the 5-HT_{2A}R antagonist can prevent the ability of cues to drive behaviors in situations with weaker (one pairing) but not stronger (four pairings) cocaine-environment associations. This lack of effectiveness in a situation with a strong cocaine-environment association may limit the clinical utility of M100907 as a treatment for addiction, as most patients experience countless pairings of cocaine and

environmental cues before seeking treatment. Thus, our studies uncovered a differential sensitivity to the blockade of cocaine CPP expression by a selective 5-HT_{2A}R antagonist based on the strength of the cocaine-environment association.

Two types of alterations may underlie the differential sensitivity among rats to blockade of CPP expression by a 5-HT_{2C}R agonist with implications for the development of novel pharmaceutical adjunct treatments for addiction: editing of the mRNA for the 5-HT_{2C}R and single nucleotide polymorphism (SNP) in the 5-HT_{2C}R gene. The 5-HT_{2C}R is a seven transmembrane domain protein coupled to several G-proteins (Berg *et al.* 2008). mRNA editing of the 5-HT_{2C}R results in single amino acid substitutions at up to 3 positions within the second intracellular domain (Burns *et al.* 1997), and the degree of editing controlled the constitutive activity and the signaling response to ligand stimulation of this receptor (Herrick-Davis *et al.* 1999). The degree of editing differs among subjects and is associated with the locomotor response to novelty in animals (Dracheva *et al.* 2009) and suicide completion in humans (Gurevich *et al.* 2002). Several SNPs in both the promoter and coding regions of the 5-HT_{2C}R gene have been identified, although the implications of these SNPs on expression and function of the 5-HT_{2C}R are unclear (Reynolds *et al.* 2005). Differential expression of 5-HT_{2C}R gene SNPs has been associated with both the therapeutic effects of antipsychotic medications as well as negative side effects of these compounds (Reynolds *et al.* 2005). These examples suggest that individual differences in 5-HT_{2C}R properties result in phenotypic differences in several diseases and the response to treatment. These studies suggest that individual differences in the response to 5-HT_{2C}R agonists may play a role in the effectiveness of new

treatments for cocaine addiction and opens one avenue for personalized medicine in addiction treatment, as patients who express the more highly edited 5-HT_{2C}R isoforms (which display less constitutive activity) may be good candidates for treatment with a 5-HT_{2C}R agonist to block the ability of cocaine-associated cues to induce relapse.

Neuroplasticity within the PFC appears to be another key factor for the development of cocaine-environment associations that drive behavior, as we observed increased expression of ERK and activation of GluR1 upon re-exposure to the cocaine-paired environment selectively in rats that expressed a CPP. The PFC plays major roles in working memory and executive function, suggesting that information relayed in this region is well poised to regulate the ability of a learned association to drive behavior.

The PFC receives both information about the sensory environment and the effects of cocaine and regulates other nuclei in the "reward circuit," making this region especially susceptible to the formation and maintenance of cocaine-environment associations and the ability of these associations to drive drug-seeking behavior. The PFC receives glutamatergic input from the thalamus (Pirot *et al.* 1994) that carries sensory information and dopaminergic input from the ventral tegmental area carrying reward information (Wise & Bozarth 1987; **Fig 5.1**). The PFC sends projections to both the VTA and the NAc and thus further regulates circuits involved in mediating the effects of cocaine and learned associations between cocaine and environmental cues. Signaling within the VTA is critical to the acquisition of a cocaine CPP (Harris & Aston-Jones 2003). The nucleus accumbens also plays a critical role in signaling the rewarding properties of cocaine (Di Chiara & Imperato 1988; Hurd *et al.* 1989; Rodd-Henricks *et al.*

2002; Navailles *et al.* 2007). Presentation of a cocaine-paired cue evoked increases in extracellular DA in the NAc (Phillips *et al.* 2003), and together these studies demonstrated that this nucleus responded to both cocaine infusion and cocaine-paired cues. By receiving input concerning reward and environmental stimuli and by regulating signaling within other parts of the "reward pathway," the PFC regulates the formation and expression of cocaine-environment associations.

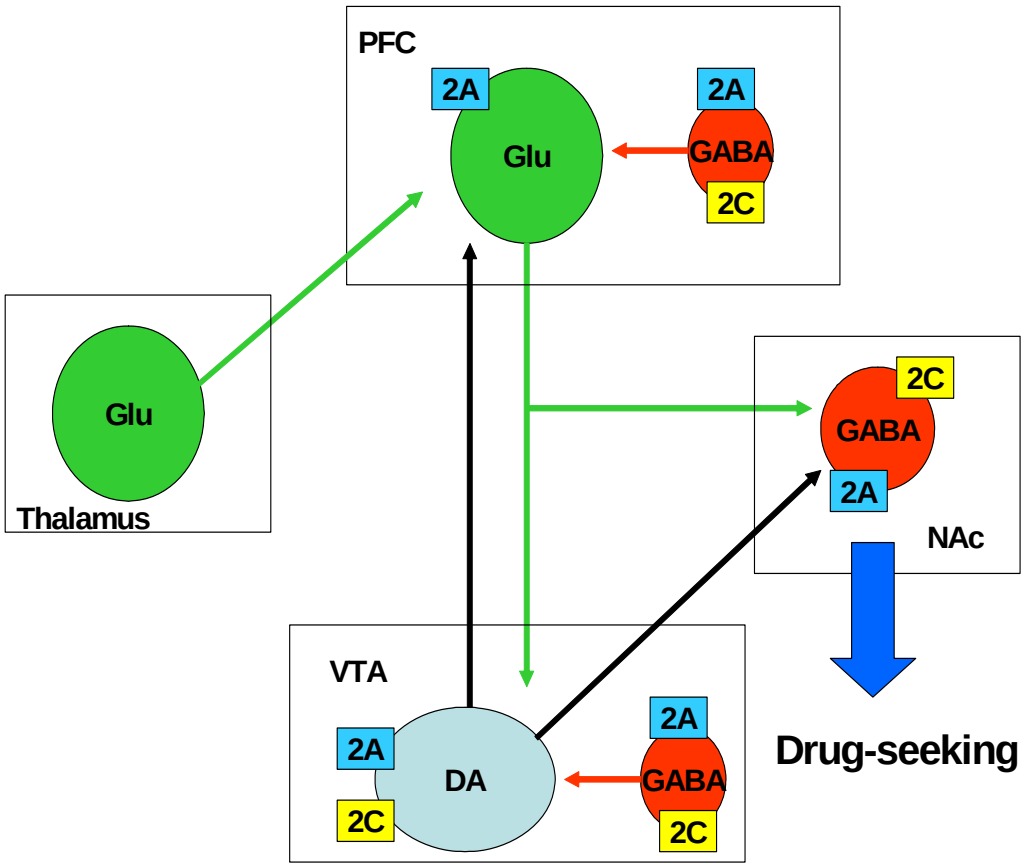


Figure 5.1: Diagram of a subset of PFC connections. The prefrontal cortex (PFC) receives sensory input from the thalamus and reward information from the VTA, making the PFC susceptible to the formation of cocaine-environment associations. In turn, the PFC regulates signaling in the both the ventral tegmental area (VTA) and nucleus accumbens (NAc), key regions implicated in signaling drug reward and the drug-environment associations. The PFC thus receives input from and regulates the output of the "reward pathway," and modulates the ability of cocaine-associated environmental cues to drive drug-seeking. Additionally, both 5-HT_{2A}R (turquoise square) and 5-HT_{2C}R (yellow square) mRNA and protein are co-expressed by glutamatergic projection neurons (Glu, green circle), GABAergic interneurons (GABA, red circle), and DAergic projection neurons (DA, blue circle) in the PFC and ventral tegmental area (VTA), respectively. Both receptors are also expressed by neurons localized to the nucleus accumbens (NAc).

The localization of 5-HT_{2A}R and 5-HT_{2C}R within the PFC suggests that this receptor population is responsible for the ability of a 5-HT_{2A}R antagonist or a 5-HT_{2C}R agonist to block the acquisition and expression of a cocaine CPP. The expression of 5-HT_{2A}R by glutamatergic neurons in the PFC that project to the VTA (**Fig. 5.1**) implies that stimulation of these receptors will lead to excitation of VTA DA cells and thus increased release of DA in the NAc and PFC, a key feature of the neuronal signal for reward (Wise & Bozarth 1987). The presence of a 5-HT_{2A}R antagonist, however, would prevent cocaine-induced increases in 5-HT binding to 5-HT_{2A}R and thus prevent the ability of cocaine to induce reward. The expression of 5-HT_{2C}R by GABAergic interneurons within the PFC suggests that treatment with a 5-HT_{2C}R agonist would increase GABA release in the PFC and thus inhibit the projections from the PFC to the VTA (**Fig. 5.1**), leading to a dampening of the cocaine-induced reward signal. The precise function of each receptor population in modulating the expression of cocaine-environment associations is unknown, although previous research from our laboratory suggests that infusion of a 5-HT_{2C}R agonist into the PFC blocks the expression of a classically conditioned cocaine-environment association (Liu and Cunningham, unpublished observations). The role of the PFC population of 5-HT_{2A}R in modulating cocaine-environment associations awaits investigation, although our current studies suggest that intra-PFC infusion of a 5-HT_{2A}R antagonist will block the acquisition and expression of a cocaine CPP.

Combination therapies that disrupt the ability of the PFC to drive cue-induced cocaine seeking behavior are likely to be important in the treatment of addiction. These studies suggest that a medication (or combination of medications) that targets AMPA glutamate receptors along with the 5-HT_{2A}R and

5-HT_{2C}R may effectively modulate PFC signaling, and clinical trials utilizing ligands directed at these receptors are currently underway. For example, multiple studies are currently testing the hypothesis that 5-HT_{2A}R antagonists or 5-HT_{2C}R agonists may be effective treatments for cocaine addiction and the ability of cocaine-paired cues to drive a return to drug-seeking (www.clinicaltrials.gov). An additional trial utilizing the drug n-acetylcysteine is currently testing the hypothesis that this compound, which plays a role in the regulation of intracellular glutamate concentrations, may reduce the behavioral response to cues in patients with cocaine addiction (www.clinicaltrials.gov). Our findings suggests that the combination of therapies that regulate 5-HT signaling via the 5-HT_{2A}R and 5-HT_{2C}R and glutamate signaling via AMPA receptors is uniquely poised to regulate signaling in the PFC critical to cocaine-environment associations and serve as a medical adjunct therapy for the prevention of relapse in patients working to gain control of their addiction to cocaine.

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Vita

Adriane M. dela Cruz was born Adriane M. Sandler on February 27, 1980 in Houston, Texas to Dr. Carl M. Sandler and Susan J. Sandler. After graduating from St. John's School in Houston, Ms. dela Cruz attended Amherst College in Amherst, Massachusetts. She graduated *magna cum laude* in May, 2002 with a Bachelor of Arts in Neuroscience, and she earned departmental honors for completing an undergraduate thesis. Her early research experiences included a summer fellowship to study properties of cellular membrane lipids in apoptosis at Amherst College and participating in the Summer Undergraduate Research Program at UTMB, where she worked in laboratory of Dr. Kathryn Cunningham. Ms. dela Cruz enrolled in the MD-PhD Combined Degree Program at UTMB in the summer of 2002. After completing the first two years of medical school, Ms. dela Cruz joined the laboratory of Dr. Kathryn A. Cunningham to complete her dissertation research. During her graduate school career, Ms. dela Cruz has earned several honors and awards. She was awarded a predoctoral National Research Service Award from the National Institute on Drug Abuse to fund her research. Ms. dela Cruz has been awarded the Jason E. Perlman Research Award (2006), the Betty Williams Scholarship (2007), UTMB Faculty Women's Club Scholarship Award (2007), and the Kay and Cary Cooper, PhD Scholarship Award (2008) by the UTMB Graduate School of Biological Sciences. Ms. dela Cruz has been active in teaching by tutoring medical students in neuroscience and nephrology and serving as a mentor to two participants in the Summer Undergraduate Research Program. She has served the UTMB community by serving on several MD-PhD program committees, including a two-year term as a

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